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OAE Publishing Inc.
245 E Main Street ste122, Alhambra, CA 91801, USA
Website: www.oaepublish.com

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E-mail: editorialoffice@hrjournal.net
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Hepatoma Res 2018;4:75 <http://dx.doi.org/10.20517/2394-5079.2018.98>

Original Article

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Clinical and molecular sub-classification of hepatocellular carcinoma relative to alpha-fetoprotein level in an Asia-Pacific island cohort

Scott T. Nishioka¹, Miles M. Sato¹, Linda L. Wong², Maarit Tiirikainen², Sandi A. Kwee^{1,2,3}

¹The Queen's Medical Center, Honolulu, HI 96813, USA.

²Cancer Biology Program and Genomics Shared Resource, University of Hawaii Cancer Center, University of Hawaii, Honolulu, HI 96822, USA.

³Hamamatsu/Queen's PET Imaging Center, The Queen's Medical Center, Honolulu, HI 96813, USA.

Correspondence to: Dr. Sandi A. Kwee, The Queen's Medical Center, Honolulu, HI 96813, USA. E-mail: kwee@hawaii.edu

How to cite this article: Nishioka ST, Sato MM, Wong LL, Tiirikainen M, Kwee SA. Clinical and molecular sub-classification of hepatocellular carcinoma relative to alpha-fetoprotein level in an Asia-Pacific island cohort. *Hepatoma Res* 2018;4:1. <http://dx.doi.org/10.20517/2394-5079.2017.46>

Received: 20 Oct 2017 **First Decision:** 21 Nov 2017 **Revised:** 5 Dec 2017 **Accepted:** 5 Dec 2017 **Published:** 12 Jan 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Lu Liu **Production Editor:** Huan-Liang Wu

Abstract

Aim: Increased serum alpha-fetoprotein (AFP) levels are associated with specific molecular sub-classes of hepatocellular carcinoma (HCC), supporting AFP as a predictive or therapeutic biomarker for precision treatment of this disease. Considering recent efforts to validate HCC molecular classification systems across different populations, we applied existing signature-based classification templates to Hawaii cohorts and examined whether associations between HCC molecular sub-class, AFP levels, and clinical features found elsewhere can also be found in Hawaii, a region with a unique demographic and risk factor profile for HCC.

Methods: Whole-genome expression profiling was performed on HCC tumors collected from 40 patients following partial hepatectomy. Tumors underwent transcriptome-based categorization into 3 molecular sub-classes (S1, S2, and S3). Patient groups based on molecular sub-class and AFP level were then compared with regards to clinical features and survival. Differences associated with AFP level and other clinical parameters were also examined at the gene signature level by gene set enrichment analysis.

Results: Statistically confident (false discovery rate < 0.05) sub-classifications were made in 98% (39/40) of tumors. Patient sub-groups differed significantly with regards to serum AFP level, with significantly lower levels in the S3 sub-group as compared to S1 ($P = 0.048$) and S2 ($P = 0.010$). Serum AFP > 400 ng/mL predicted significant tumor enrichment for genes corresponding to *MYC* target activation, high cell proliferation, poor clinical prognosis, and the S2 sub-class. AFP > 400 ng/mL and non-S3 tumor classification were found to be significant predictors of overall survival.



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Conclusion: Distinct sub-classes of HCC associated with different molecular features and survival outcomes can be detected with statistical confidence in a Pacific Island cohort. Molecular classification signatures and other predictive markers for HCC that are valid for all patient populations are needed to support multi-center efforts to develop targeted therapies for HCC.

Keywords: Hepatocellular carcinoma, alpha-fetoprotein, survival, gene expression, enrichment analysis, molecular signature, Asia-Pacific, Hawaii

INTRODUCTION

Hepatocellular carcinoma (HCC) remains a leading cause of cancer-related mortality worldwide despite current extensive knowledge about its preventable risk factors^[1,2]. The highest incidence rates of HCC are in areas with endemic hepatitis B virus (HBV) infection such as China and Sub-Saharan Africa, however HCC incidence has been increasing in Oceania, Europe, and the United States (US) owing to the rising prevalence of other HCC risk factors such as hepatitis C virus (HCV) infection and steatohepatitis^[1,3]. In the US alone, HCC diagnoses have tripled since 1975, and with a 5-year survival rate as low as 12%, HCC is fast becoming a leading cause of cancer-related mortality in this region^[3]. Reflecting its predominantly Asian and Pacific Islander demographic, Hawaii has one of the highest incidence rates of liver cancer in the US, with an age-adjusted incidence rate of 11.0 per 100,000 that is considerably higher than the overall US rate of 7.6 per 100,000^[4]. Given that the distribution of HCC risk factors in Hawaii differs from that of both Asia and the continental US^[5-7], studies involving cohorts from Hawaii may provide additional insights into the disease.

HCC is renowned for its genomic and molecular diversity. Recent attempts at HCC molecular sub-classification have produced multiple, sometimes orthogonal, classification systems that associate with various clinical, histological, and molecular features^[8-12]. The molecular diversity of HCC makes targeted therapy challenging, since it dilutes any individual therapeutic target within the patient population, leading to weaker overall benefit in conventional clinical trials^[13-15]. Consequently, it is not surprising that among HCC clinical trials to date, all molecularly-specific agents have failed, and only multi-targeting agents such as sorafenib have shown efficacy^[16]. A robust molecular sub-classification system for HCC could enable clinical trials to enrich study cohorts according to tumoral expression of targeted molecular pathways^[11,15-17]. In fact, this may be the most important next step in advancing patient-individualized treatment of HCC. It would therefore be prudent to validate HCC sub-classification systems across many different patient populations worldwide.

Serum alpha-fetoprotein (AFP) measurement has been used extensively for HCC screening and diagnosis, despite being associated with a limited diagnostic sensitivity of approximately 66%^[18,19]. Possibly explaining this limited sensitivity, different molecular sub-classes of HCC have been associated with different degrees of AFP production^[10-12]. Clinically, differences in AFP production have also been associated with gross and histopathologic tumor differences, including differences in tumor size, multinodular appearance, and vascular invasion^[20]. AFP may also be directly involved in tumor pathogenesis through its involvement in several mitogen and anti-apoptotic pathways, as well as potentially by exerting paracrine effects on immune and other non-tumor cells^[21,22]. Given these associations, AFP could have significant value beyond that of a diagnostic marker. While several molecular classification systems for HCC have been associated with differences in AFP levels across their respective sub-classes^[10,11], these associations along with the classification systems themselves are in need of further validation across many different population cohorts. Since Hawaii has a unique and diverse patient population, we assessed the feasibility of applying HCC molecular classification systems derived from other patient populations to those in Hawaii and examined the relationship of AFP and other clinical parameters to the transcriptomic features of HCC.

METHODS

Tissue samples

Forty patients diagnosed with BCLC stage A HCC who were referred to a single medical center for primary treatment of HCC by partial hepatectomy were prospectively recruited to participate in an institutional review-board approved clinical research study with written informed consent. All patients were deemed clinical candidates for hepatic resection by an attending surgeon, and a separate informed consent process for surgery was completed before study recruitment.

Whole transcriptome analysis

At the time of surgery, tumor and adjacent non-tumor samples were taken from the resection specimen and conserved in separate containers with RNA Later medium (Thermo Fisher, Waltham, MA). RNAs were subsequently extracted from homogenized frozen liver tissue lysates in RLT Plus buffer with the All Prep DNA/RNA Mini kit (Qiagen, Valencia, CA). The isolated RNAs were then stored at -80 °C until analysis.

The analytical quality of the total RNAs was assayed using a Bioanalyzer with RNA 6000 Nano chips (Agilent, Santa Clara, CA) prior to use for this study. Isolated RNAs were then processed following the WG-DASL assay protocol (Illumina Inc., Sunnyvale, California). Resulting PCR products were hybridized onto the Illumina HumanHT-12 v4 Expression Bead Chips covering over 24,000 transcripts with genome-wide coverage of well-characterized genes, gene candidates, and splice variants. Arrays were scanned using the iScan™ instrument and expression levels were quantified using Genome Studio software (Illumina Inc., Sunnyvale, CA). The resulting expression data matrix contained 40 columns representing individual tumor samples and 20,818 rows corresponding to gene expression data.

This gene expression dataset was pre-processed by generalized log₂ transformation with background subtraction, quantile normalization, and row centering. Each sample was annotated with corresponding clinical data such as age, gender, FIB-4 score, AFP level, and HCC risk factor data, as obtained from clinical records. All tumor samples, gene expression data, and clinical parameters were de-identified and assigned a serial number to maintain patient confidentiality.

Tumor classification based on gene expression signature

Tumor molecular classification was based on the Hoshida system, using sub-classification signatures previously subjected to meta-analysis in 6 different patient cohorts collected from 3 continents (Asia, Europe, and North America)^[10]. Based on this classification system, samples were categorized by nearest template into 3 distinct HCC sub-classes (labeled S1, S2, and S3)^[23]. A false discovery rate (FDR) < 0.05 was used as the statistical criterion for confident sub-class label assignments.

Clinical classification

The histologic diagnosis of HCC was established for each patient by clinical pathology. These diagnoses were further confirmed in all tumor samples by a single board-certified hepatobiliary pathologist. Tumor samples were then sub-categorized based on several clinical parameters to be later used as classes for gene set enrichment analysis (GSEA). These categorizations were based on the distribution of each clinical parameter for all tumor samples. The clinical parameters to be used as class phenotype labels were selected a priori. They were age, gender, FIB-4 score, AFP level, and presence of HBV infection. Except for gender and HBV infection, which are binary, parameters were dichotomized for GSEA based on analysis of dispersion. AFP levels displayed a bimodal distribution so that a cut-off point between “high” and “low” AFP values could be made at the histogram trough corresponding to 400 ng/mL. Coincidentally, an AFP cut-off point of 400 ng/mL is frequently used clinically as a highly specific cut-off for confirming HCC diagnosis^[24], and also frequently serves as a cut-off point for determining eligibility in clinical trials involving agents with potential selectivity for AFP-producing tumors (e.g. NCT02435433). In contrast, the distribution

of FIB-4 scores was highly skewed and did not fit a normal or bimodal distribution to provide a logical location for the cut-off point. A FIB-4 cut-off was therefore prospectively chosen based on review of previous literature regarding FIB-4 scores and their prognostic value. A study conducted by Chan *et al.*^[16], which aimed to determine an optimal cut-off point for diagnosing and prognosticating advanced liver fibrosis after curative liver resection in HCC patients found that a FIB-4 index of 2.87 optimized both sensitivity and specificity. As a result, samples were dichotomized based on a FIB-4 score of 2.87.

GSEA

GSEA was used to test the hypothesis that gene expression profiles corresponding to a priori defined gene sets differ between samples belonging to 2 distinct phenotype classes^[25]. Using a Java-based implementation of the GSEA algorithm (GSEA v3.0, Broad Institute, Boston, MA), the enrichment of gene sets of interest within tumors corresponding to a given clinical phenotype were sought. To perform significance testing against a null-hypothesis, permutation testing was performed to compute enrichment scores for 1000 random phenotype assignments. A FDR of less than 0.25 was used to indicate significant enrichment and prompt further inquiry about tumor biology using biomedical literature referenced in the GSEA output.

Current versions (v6.0) of curated collections of gene sets were downloaded from an online database MSigDB (MSigDB, Broad Institute, Boston, MA) from within the GSEA Java application. The Hallmarks collection (comprised of 50 gene sets composed of coherently expressed genes reflecting well-defined biological states or processes) and the chemical and genetic perturbations (CGP) collection (comprised of 2675 gene sets reflecting gene signatures derived from published biomedical literature) were used for this study. The CGP collection includes gene signatures reflecting genetic and chemical perturbations from a broad variety of diseases. To estimate the number of HCC-related gene sets in the CGP collection, a query for “hepatocellular carcinoma” was performed using the search mechanism of the mSigDB online portal (<http://software.broadinstitute.org/gsea/msigdb/index.jsp>). This revealed 107 gene sets within the CGP collection related to HCC that were supported by literature from Medline-indexed journals. These gene sets included multiple published gene signatures for HCC molecular classification^[8,10,11,26] and prognostication^[12,27].

Statistical methods

Differences involving normally distributed variables were assessed by *t*-test or analysis of variance. *Post hoc* multiple pair wise comparisons were performed by the Steel-Dwass test. Comparisons among categorical or dichotomized variables were assessed using Fisher’s exact test. Kaplan-Meier analysis was used to compare overall survival rates post-surgical resection in patients stratified by AFP > 400 ng/mL and AFP ≤ 400 ng/mL, and by combined S1 and S2 tumor subclasses vs. S3 subclass. Differences in survival curves were assessed using the Log-Rank tests. Cox proportional hazard ratios were also computed for the effects of AFP level differences and tumor class differences on overall survival post-surgical resection. Adjustments to proportional hazards regression models were made only if multiple significant univariate predictors of overall survival were identified. All statistical analyses were carried out using SAS version 9.3 (SAS Institute, Cary, NC).

RESULTS

Patient clinical characteristics and demographics (*n* = 40) are summarized in Table 1. There were no significant differences in various clinical parameters including age, gender, HBV infection, HCV infection, significant alcohol use, Edmondson-Steiner grade, or proportion of high FIB4 scores between the AFP > 400 ng/mL and AFP ≤ 400 ng/mL groups of patients [Table 2].

Tumor classification

The number of tumors mapped into tumor sub-class S1, S2, and S3, were 12, 4, and 23 respectively. Only one tumor could not be classified based on a FDR < 0.05. The remaining sub-class assignments were also

Table 1. Clinical characteristics and demographics of the patient cohort

Characteristics	Data
No. of patients	40
Mean age, years	64.0
Gender, male/female	30/10
HBV-infected, <i>n</i> (%)	5 (12.5%)
HCV-infected, <i>n</i> (%)	9 (22.5%)
Alcohol abuse, <i>n</i> (%)	3 (7.5%)
Combination HBV/alcohol, <i>n</i> (%)	5 (12.5%)
Combination HCV/alcohol, <i>n</i> (%)	8 (20.0%)
Racial category	
Asian	21
Native Hawaiian/Pacific Islander	9
White	8
Black/African American	2

HBV: hepatitis B virus; HCV: hepatitis C virus

Table 2. Comparison of clinical characteristics between patients with serum AFP > 400 ng/mL and lower AFP values

Characteristics	AFP (ng/mL)		P-value
	> 400	≤ 400	
No. of patients	9	31	
Mean age, years	67.1	62.9	0.314
Gender			
Male	6 (66.7%)	24 (77.4%)	0.665
Female	3 (33.3%)	7 (22.6%)	
FIB4 score			
≥ 2.87	3 (33.3%)	15 (48.4%)	0.476
< 2.87	6 (66.7%)	16 (51.6%)	
Edmondson - Steiner Grade			
ES 1	0 (0.0%)	3 (9.7%)	0.351
ES 2	3 (33.3%)	18 (58.1%)	
ES 3	5 (55.6%)	8 (25.8%)	
ES 4	1 (11.1%)	2 (6.5%)	
Risk factors			
HBV	0 (0.0%)	5 (16.1%)	0.522
HCV	3 (33.3%)	6 (19.4%)	
Alcohol	0 (0.0%)	3 (9.7%)	
HBV/alcohol	1 (11.1%)	4 (12.9%)	
HCV/alcohol	1 (11.1%)	7 (22.6%)	
None	4 (44.4%)	6 (19.4%)	

AFP: alpha-fetoprotein; HBV: hepatitis B virus; HCV: hepatitis C virus

statistically significant based on Bonferroni-corrected *P*-values < 0.05. A heat map depicting classification signature expression patterns and a Venn diagram summarizing the number of differentially expressed signature genes between sub-classes are shown in [Figure 1](#). Corresponding serum AFP levels differed significantly across tumor sub-classes (Wilcoxon *P* = 0.002). Post hoc pair wise testing adjusted for multiple comparisons revealed significant differences in AFP levels between sub-classes S3 and S1 (72 vs. 2332 ng/mL, *P* = 0.048) and between S3 and S2 (72 vs. 4277 ng/mL, *P* = 0.010). Functional annotation of the sub-classification results by Gene Ontology Biological Processes is shown in [Supplementary Table 1](#).

GSEA results

In comparing HCC tumors associated with serum AFP > 400 ng/mL (high AFP class) with those associated with lower AFP levels, multiple gene sets from the Hallmarks and CGP collections were significant based on FDR < 0.25. From the Hallmarks collection, 7/50 gene sets were identified as significantly enriching the elevated AFP class of tumors. These gene sets are summarized in [Supplementary Table 2](#). Two of the top scoring gene sets from this collection, MYC_TARGETS_V1 and MYC_TARGETS_V2 (with FDR 0.057 and 0.077, respectively), are comprised of genes known to be upregulated in response to MYC oncogene

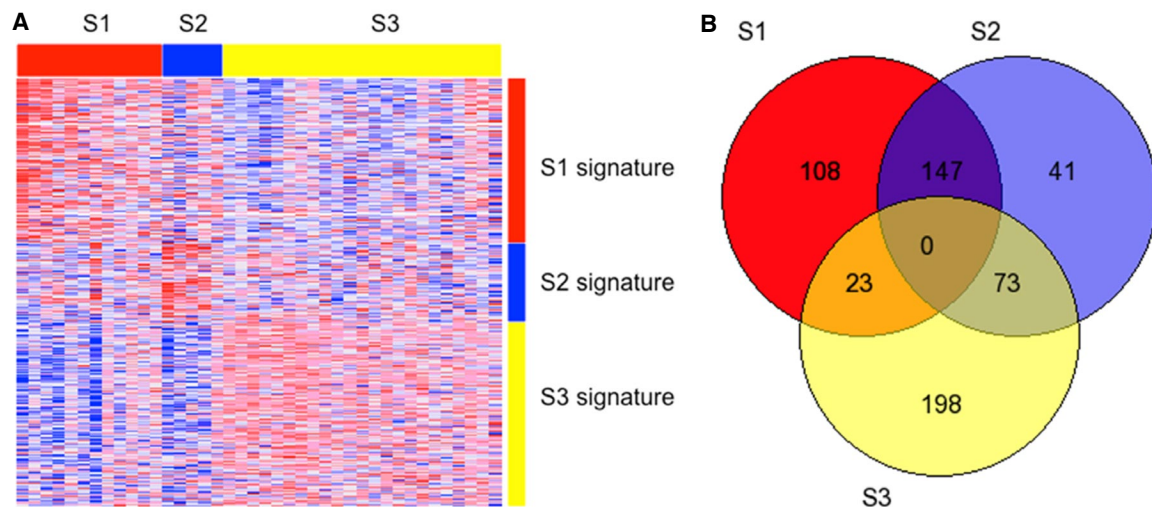


Figure 1. (A) Clustered heat map showing expression pattern of the HCC subclassification signature among 39 patients. Columns represent tumor samples clustered into S1 (red), S2 (blue), and S3 (yellow) HCC sub-classes. Rows represent genes comprising the S1 (red), S2 (blue), and S3 (yellow) classification signatures. Confident prediction (FDR < 0.05) occurred in 98% (39/40) of cases; (B) Venn diagram showing the number of HCC sub-classification genes that are common and differentially expressed among the S1, S2, and S3 sub-classes. HCC: hepatocellular carcinoma; FDR: false discovery rate

activation. Their enrichment score plots are shown in [Figure 2A and B](#), respectively. These gene sets are relatively independent of each other since they hold only 18 genes in common and are comprised of 200 and 58 genes respectively. Thus, their mutual significance compounds support that the tumors in the high AFP class are enriched for genes controlled by MYC, a finding that is also consistent with previous literature implicating MYC oncogene activation in the pathogenesis of HCC tumors associated with high serum AFP levels^[10].

Another top ranked gene set from the Hallmarks collection was UNFOLDED_PROTEIN_RESPONSE (FDR 0.069 and family-wise error rate $P = 0.044$). The enrichment score plot for this gene set is shown in [Figure 2C](#). This gene set is comprised of genes associated with unfolded protein response (UPR). There is recent evidence to suggest that AFP production is proteostatically regulated in part by UPR, although all exact mechanisms have not yet been clarified^[28]. In one study, exposure of HCC cells to sorafenib led to changes in UPR that affected AFP production independent of an effect on cell viability, a finding that suggests that AFP could potentially serve as a biomarker of tumor proteostatic response^[29]. The remaining significant gene sets from the Hallmarks collection (E2F_TARGETS, G2M_CHECKPOINT, DNA_REPAIR, and MITOTIC_SPINDLE) were all found to relate to cell proliferation, as the E2F transcription factory family is known to integrate cell cycle progression with DNA repair, replication, and G2/M checkpoints^[30]. A heat map based on the list of ranked genes from GSEA using the Hallmarks gene set collection is shown in [Supplementary Figure 1](#).

GSEA using the CGP collection identified 351 gene sets as being significantly enriched in the high AFP class of tumors. These gene sets and their corresponding significance and enrichment scores are summarized in [Supplementary Table 3](#). Although this collection (comprised of 2675 signatures derived from a broad variety of diseases and conditions) included relatively few HCC-related gene sets, a disproportionate number of them were found to be significant [\[Table 3\]](#).

Several of the gene signatures found to be significant have previously been associated with high AFP levels, including HOSHIDA_LIVER_CANCER_SUBCLASS_S2^[10], CHIANG_LIVER_CANCER_SUBCLASS_PROLIFERATION_UP^[8], and YAMASHITA_LIVER_CANCER_WITH_EPCAM_UP^[12]. In addition to

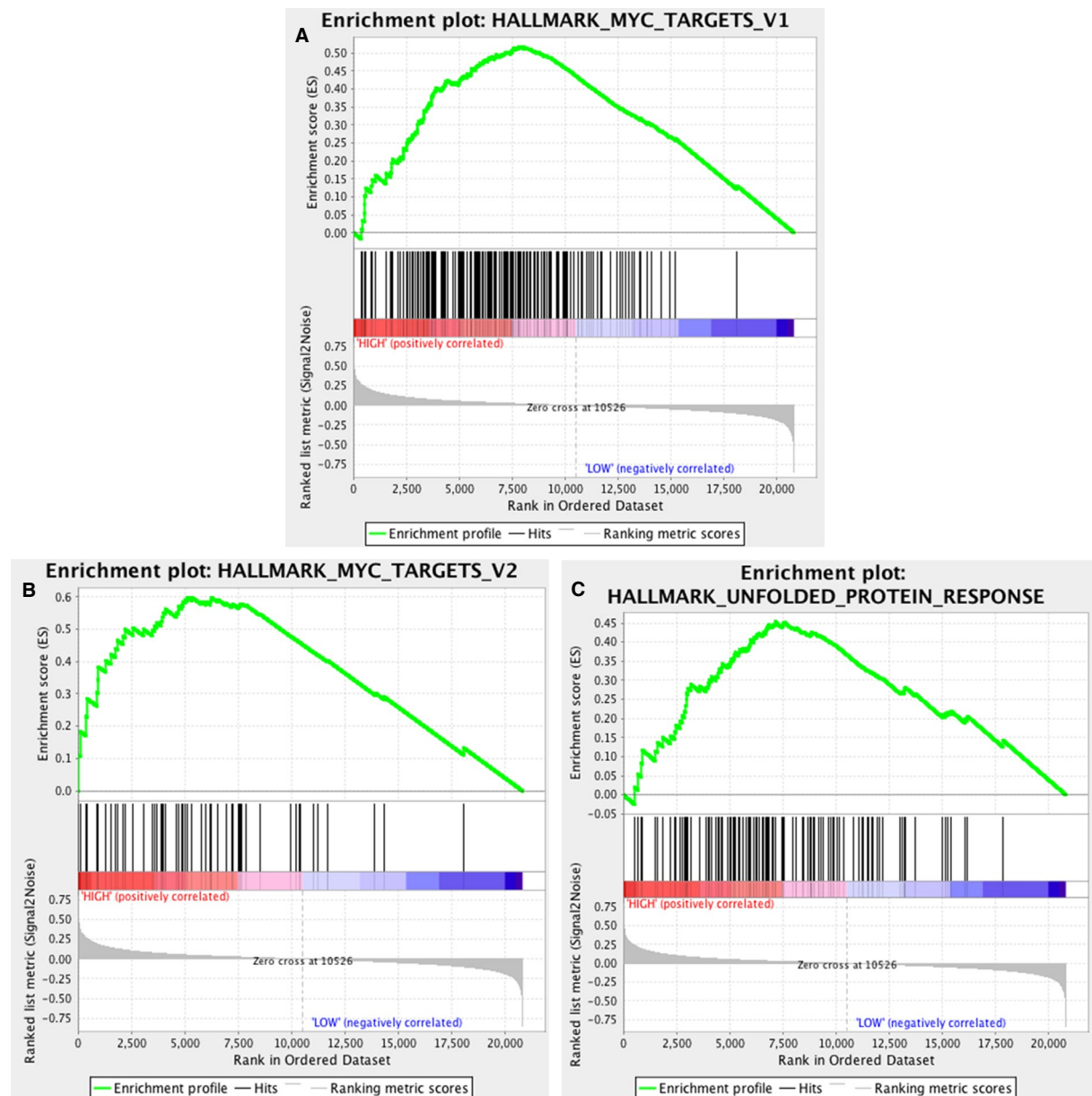


Figure 2. Profile of the running enrichment score and position of gene set members on the rank ordered list for (A) MYC_TARGETS_V1; (B) MYC_TARGETS_V2; and (C) UNFOLDED_PROTEIN_RESPONSE from the HALLMARKS gene set collection. The two MYC_TARGETS gene sets (comprised of 200 and 58 genes, respectively) share only 18 genes in common

these HCC-specific gene sets, 14 gene sets from the CGP collection representing MYC target genes were also found to be significantly enriched in the high AFP class, which falls in agreement with the enrichment analysis results obtained using the Hallmarks collection. A heat map of the top ranked genes from this analysis is shown in [Supplementary Figure 2](#). No significant differences in gene set enrichment were found between phenotype classes determined by age, gender, FIB4 or HBV-status using either the Hallmarks or CGP collections.

Survival analysis

A Kaplan-Meier plot comparing the survival of patients with serum AFP level > 400 ng/mL versus patients with lower serum AFP levels is shown in [Figure 3A](#). Serum AFP level > 400 ng/mL was significantly associated with poorer overall survival (Log-Rank $P = 0.040$) and a hazard ratio for mortality of 3.1 (95%CI 1.0-9.7, $P = 0.050$).

Table 3. HCC-related gene sets from the chemical and genetic perturbations collection enriched in the high AFP tumor class

Name	FDR
BOYALT_LIVER_CANCER_SUBCLASS_G3_UP	0.086
HOSHIDA_LIVER_CANCER_SUBCLASS_S2	0.088
BOYALT_LIVER_CANCER_SUBCLASS_G123_UP	0.099
CHIANG_LIVER_CANCER_SUBCLASS_PROLIFERATION_UP	0.101
YAMASHITA_LIVER_CANCER_WITH_EPCAM_UP	0.104
SAKAI_CHRONIC_HEPATITIS_VS_LIVER_CANCER_UP	0.118
LEE_LIVER_CANCER_SURVIVAL_DN	0.147
CHIANG_LIVER_CANCER_SUBCLASS_UNANNOTATED_DN	0.186
BOYALT_LIVER_CANCER_SUBCLASS_G23_UP	0.228
BOYALT_LIVER_CANCER_SUBCLASS_G12_UP	0.252

HCC: hepatocellular carcinoma; FDR: false discovery rate

For survival analysis based on the molecular classification of tumors, patients whose tumors were assigned to sub-classes S1 and S2 were grouped together because of the similarity in gene expression between sub-classes S1 and S2 relative to S3 [Figure 1] and the significant associations of sub-classes S1 and S2 with higher AFP levels (mean 2819 ng/mL) as compared to S3 (mean 72 ng/mL). A Kaplan-Meier plot comparing the survival rates of patients with non-S3 tumors *vs.* patients with S3 tumors is shown in Figure 3B. Non-S3 tumors were significantly associated with poorer overall survival (Log-Rank $P = 0.024$) and a mortality hazard ratio of 3.6 (95% confidence interval 1.1-11.6, $P = 0.035$). Age, gender, FIB4 > 2.87, and HBV infection were not found to be significant predictors of overall survival following liver resection.

DISCUSSION

In this study, tumoral differences were examined at the gene signature level between HCC sub-groups categorized on the basis of AFP and other clinical parameters. Using GSEA, we found no significant differences in gene set enrichment between tumors categorized by patient age, gender, clinical severity of liver fibrosis, and HBV infection. However, we did find significant differences between tumors categorized by patient serum AFP level. These differences proved to be biologically coherent across analyses involving two distinct gene set collections from the mSigDB molecular signature repository. Specifically, using the mSigDB Hallmarks collection of gene sets, we found serum AFP levels > 400 ng/mL to be associated with gene set enrichments corresponding to MYC oncogene activation, enhanced DNA replication/repair, and cell cycle progression, all of which are defining properties of highly proliferating tumors. In addition, we found tumors from patients with high serum AFP levels to be significantly enriched for genes associated with proteostasis, a potential mechanism for the release of AFP by tumor cells^[22].

Using the larger CGP gene set collection comprised of 2675 gene signatures, we found that tumors associated with high serum AFP levels were also significantly enriched for genes belonging to several existing molecular classification signatures for HCC. Three of these signatures have already been associated with high AFP levels by previous studies. The first signature corresponds to the S2 tumor sub-class defined by Hoshida *et al.*^[10]. In addition to being associated with high serum AFP levels, this sub-class of HCC tumors is characterized by MYC oncogene activation and enhanced cellular proliferation. Thus, these results are concordant with the results obtained by GSEA using the Hallmarks gene set collection. Another HCC sub-classification signature found significantly enriched in the high AFP class of tumors corresponds to a “proliferation” sub-class of HCC described by Chiang *et al.*^[8]. In addition to being associated with high serum AFP levels, this sub-class is associated with chromosomal instability and overexpression of proliferation-related genes. The third HCC classification signature that was significant in our analysis corresponds to an EpCam signature defined by Yamashita *et al.*^[12]. In their study, this signature, when combined with AFP expression, identifies four patient sub-groups, each with their own unique sub-signature and survival pattern. Notably, the AFP-positive sub-groups were associated with higher TNM stage and worse clinical prognosis^[12]. Altogether, these distinct

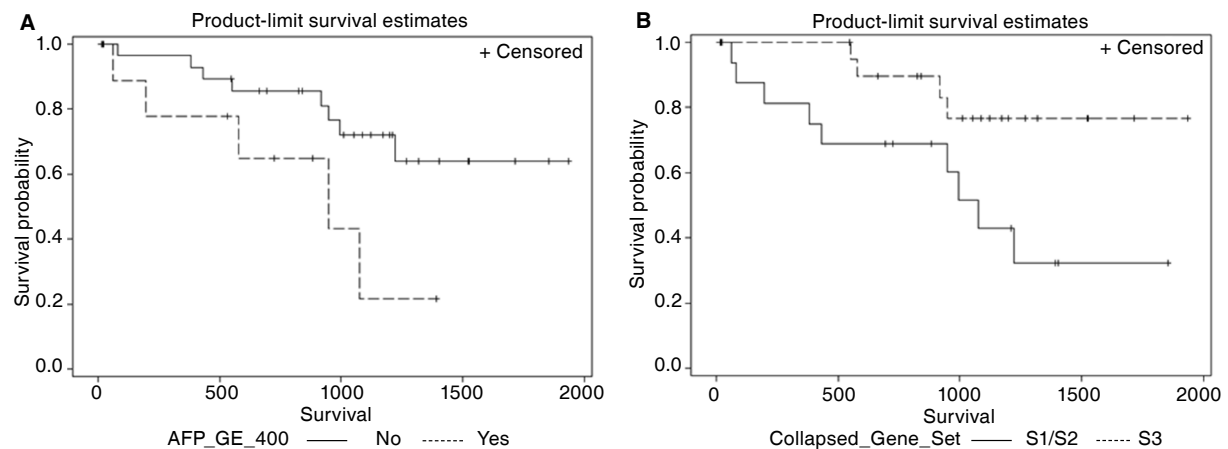


Figure 3. Kaplan-Meier survival rate curves showing significant survival differences among (A) patients with AFP > 400 ng/mL (AFP_GE_400) vs. M 400 ng/mL (Log-Rank $P = 0.040$), and among (B) patients with tumor subclass S1 & S2 vs. tumor subclass S3 (Log-Rank $P = 0.024$)

signatures (from three different molecular classification systems) were consistent in ascribing aggressive biological traits to the high AFP class of tumors in our study.

Supporting the premise that aggressive tumor biology leads to worse clinical outcomes, we also found the LEE_LIVER_CANCER_SURVIVAL_DN gene signature (comprised of genes highly expressed in HCC associated with poor survival^[27]), to be significantly enriched in the high AFP class of tumors from our study. Confirming this prognostic result, we found overall survival rates of patients with AFP levels > 400 ng/mL to be significantly lower than those with AFP level ≤ 400 ng/mL. Furthermore, survival analysis based on molecular tumor classification revealed significantly lower survival rates in patients with tumor sub-classes associated with high AFP levels.

Of course, clinical associations between high AFP levels and poor prognosis in HCC are not unique to our study. Elevations in AFP have been widely shown to predict poorer prognosis, especially when used in combination with other clinical markers^[31]. We also previously found an association between increased AFP levels (> 400 ng/mL) and HCC recurrence following liver transplantation^[24]. Our study contributes to this existing knowledge by showing the potential of functional genomics to link clinical measurements of serum AFP to the molecular mechanisms of AFP production, other tumor biological traits, and molecular tumor classification to provide clues on therapeutic target enrichment and treatment outcome in an understudied patient population.

Because many contemporary clinical guidelines do not require a histopathological diagnosis before treatment of HCC, tumor tissue is often not available for molecular profiling in the clinical setting. This inadvertently poses a barrier to routine molecular classification of HCC based on tumor tissue. While liquid biopsy techniques are being developed to profile HCC circulating tumor cells and associated cell-free DNA, serum AFP remains the most readily available hematogenous biomarker for HCC. Because of associations between AFP expression and the expression of other potential cellular targets^[11,12,32], serum AFP may have value as a surrogate predictive biomarker for molecularly-targeted therapy. In pre-clinical studies, differences in AFP expression have already been correlated with differences in therapeutic response. For example, differences in response to the Src/Abl kinase inhibitor, dasatinib have been observed between AFP-positive and AFP-negative HCC cell lines^[33]. AFP expression status has also shown high correlation with specific HCC molecular subtypes in different cell lines^[34]. Thus, AFP expression status is an important variable for interpreting the results of both pre-clinical studies and clinical trials of HCC.

Several limitations of the present study should be recognized. First, the tumor samples analyzed in this study were collected from patients recruited from a single medical center in the state of Hawaii. This raises the possibility of selection bias. However, the likelihood of such bias is reduced given the prospective nature of this study and the fact that our center is responsible for treating most of the HCC patients in Hawaii. Because Hawaii is a small territory, the number of patients presenting annually with HCC is also relatively small despite the high incidence, and thus the statistical power of this study is limited. However, unlike unsupervised methods, GSEA has been found to be fairly robust with sample size in the range of the present study^[35]. Another potential limitation relates to the fact that gene expression analysis was performed by sampling only a small peripheral portion of the tumor. Because of this, our results cannot account for the possibility of intra-tumoral heterogeneity. Notwithstanding this methodologic limitation, the results of this study did produce a biologically-coherent depiction of HCC tumors associated with high serum AFP levels.

Our study provides additional data supporting the clinical relevance of gene signatures for HCC derived from many different cohorts, including those from Asia, Europe, and North America. Because there are studies suggesting that ancestry and genetics may influence HCC genomes^[36], it is prudent to validate predictive gene signatures for HCC in a broad spectrum of patients before accepting them into mainstream application. While some gene signatures for HCC have already been subject to further validation^[10], none have been thoroughly validated to a global extent. Our study, conducted in a racially and ethnically diverse HCC cohort, provides further evidence to support the generalizability of gene signatures for clinical molecular classification of HCC. Specifically, we confirmed that several externally derived molecular sub-classes of HCC associated with distinct molecular features and survival outcomes could be detected with statistical confidence in a cohort of patients from Hawaii. The generalization of these signatures will support their use in multi-center efforts aimed at developing targeted therapies for HCC.

In conclusion, herein we provide supporting evidence that a molecular classification system for HCC developed using cohorts from North America, Europe, and Asia is applicable to patients in Hawaii. Similar to other cohorts, the findings in the present study also indicate that elevated AFP is significantly associated with more aggressive tumor characteristics and poor clinical outcome, as well as gene expression related to cell cycle progression, DNA damage response, and MYC oncogene pathways. Confirming the ability to apply the same molecular classification system to tumors from different populations is a crucial step to broadening the use of genomic enrichment strategies in global multi-center clinical trials. Establishing that similar distributions of tumor sub-classes exist in different populations will also increase confidence that molecularly-targeted therapies found to be beneficial in one cohort can be similarly effective in cohorts from other populations.

DECLARATIONS

Authors' contributions

Conception: Wong LL, Kwee SA

Study design: Nishioka ST, Wong LL, Kwee SA

Data collection: Sato MM, Wong LL, Tiirikainen M, Kwee SA

Data analysis: Nishioka ST, Sato MM, Tiirikainen M, Kwee SA

Interpretation of results and manuscript writing: Nishioka ST, Sato MM, Wong LL, Tiirikainen M, Kwee SA

Data source and availability

Corresponding author may be contacted for any data inquiries.

Financial support and sponsorship

This work was supported by U.S. National Institutes of Health grants R01CA161209-06 and U54MD007584-07.

Conflicts of interest

No conflicts of interest were reported by the co-authors.

Patient consent

Written informed consent was completed before study recruitment.

Ethics approval

This clinical research study was approved by the institutional review board of the study site.

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Original Article

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Comparison of outcomes between laparoscopic vs. open liver resection for intermediate stage hepatocellular carcinoma

Avril David^{1,2}, YoungRok Choi¹, Ho-Seong Han¹, Yoo-Seok Yoon¹, Jai Young Cho¹

¹Department of Surgery, Seoul National University College of Medicine, Seoul National University Bundang Hospital, Seongnam-si, Gyeonggi-do 13620, South Korea.

²Department of Surgery, Rizal Medical Center, Pasig City 1600, Philippines.

Correspondence to: Dr. YoungRok Choi, Department of Surgery, Seoul National University Bundang Hospital 82, Gumi-ro 173 Beon-gil, Bundang-gu, Seongnam-si, Gyeonggi-do 13620, South Korea. E-mail: choiyoungrokr@gmail.com

How to cite this article: David A, Choi YR, Han HS, Yoon YS, Cho JY. Comparison of outcomes between laparoscopic vs. open liver resection for intermediate stage hepatocellular carcinoma. *Hepatoma Res* 2018;4:2. <http://dx.doi.org/10.20517/2394-5079.2017.51>

Received: 8 Dec 2017 **First Decision:** 11 Dec 2017 **Revised:** 18 Dec 2017 **Accepted:** 19 Dec 2017 **Published:** 18 Jan 2018

Science Editor: Kenneth S.H. Chok **Copy Editor:** Lu Liu **Production Editor:** Huan-Liang Wu

Abstract

Aim: The Barcelona Clinic Liver Score (BCLC) currently limits hepatic resection only for small, solitary tumors measuring 2-3 cm with no signs of portal hypertension (PHT) or macrovascular invasion. The aim of this study was to show the benefit of surgical resection, and to compare the peri-operative and long-term outcomes between laparoscopic liver resection (LLR) and open liver resection (OLR) for hepatocellular carcinoma (HCC) classified as intermediate stage (B) under BCLC.

Methods: From 2004 to 2013, 49 patients staged as intermediate (BCLC B) and who underwent hepatic resection was included. These patients were divided into LLR or OLR. Demographics, tumor characteristics, recurrence rates and overall survival (OS) were compared between the 2 groups.

Results: Forty-nine patients were included and grouped into LLR ($n = 28$) and OLR ($n = 21$). The average tumor number was 2 ± 1 for both groups, while the mean tumor size was 4.4 ± 1.7 cm and 5.3 ± 2.6 cm for the LLR and OLR group, respectively. When compared with OLR, LLR had lower post-operative complications (14.3% vs. 33.3%, $P = 0.114$), and a statistically significant shorter hospital stay than the OLR group (9 vs. 21 days, $P = 0.023$). The LLR group also achieved a statistically significant difference in complete R0 resection as compared with the OLR group ($P = 0.016$). The OS and disease-free survival (DFS) at 1, 3 and 5 years were comparable between LLR and OLR (OS: 89.1% vs. 76.2%; 70.4% vs. 55.9%; 58.6% vs. 43.5%, $P = 0.583$; DFS: 59.3% vs. 51.0%; 20.2% vs. 44.6%; 16.2% vs. 37.2%, $P = 0.947$, respectively).

Conclusion: LLR showed comparable outcomes compared to OLR in the treatment of HCC staged BCLC B. Therefore, LLR as well as OLR can be considered in selective patients in the BCLC B group.



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Keywords: Hepatocellular carcinoma, minimally invasive surgical procedures, laparoscopy

INTRODUCTION

Although incidence and mortality rates for cancer overall are declining, based on the 2017 National Comprehensive Cancer Network (NCCN) guidelines for hepatobiliary cancers, the incidence and mortality rates for liver cancer are increasing^[1]. In particular, hepatocellular carcinoma (HCC) is the most common primary solid tumor in the world, and is ranked as the third leading cause of cancer-related death worldwide^[2]. Several staging systems have been developed in order to guide the management decisions in patients with HCC; among those, the Barcelona Clinic Liver Cancer (BCLC) Classification system has been approved and widely accepted in clinical practice^[3]. According to the BCLC algorithm, curative treatment [including surgical resection, radiofrequency ablation (RFA) and transplantation] is recommended only for very early or early-stage (stage 0-A) HCC, whereas palliative treatment is recommended for intermediate and advanced stage HCC (stage B-C)^[3]. In this regard, BCLC indication for curative resection is markedly limited. However, recent studies have shown that surgical resection can provide good outcomes for both short and long-term, despite the presence of portal hypertension, multi-nodular disease, large nodules (> 3 cm), and those with macrovascular infiltration^[4]. Particularly in the last decade, laparoscopic liver resection (LLR) may particularly be beneficial since it is less invasive, with significantly less post-operative complications, but with comparable oncologic outcomes to the open approach^[5].

The aim of this study is to show the benefit of surgical resection, and to compare the peri-operative and long-term outcomes, particularly in terms of recurrence and overall survival (OS), between LLR and open liver resection (OLR) for HCC classified as intermediate stage (B) under BCLC.

METHODS

Study design and patient selection

A retrospective review of the Electronic Medical Record database was done to identify all patients who underwent primary liver resection for HCC. From January 2004 to December 2013, a total of 1287 hepatectomies was performed at Seoul National University Bundang Hospital. Of these hepatectomies, 389 patients underwent liver resection for HCC. Among these patients, 49 patients staged as intermediate stage (BCLC B) were identified and selected. The study population included 2 comparable groups: OLR group composed of 21 patients, and the LLR group comprised of 28 patients.

Preoperative evaluation

A complete evaluation and surgical treatment for patients with HCC were discussed during multi-disciplinary meetings. Liver function was evaluated using complete biochemical profiles and liver function tests, including indocyanine green (ICG)₁₅ tests. Results were expressed as percentage of ICG retained 15 min (ICG₁₅) after injection. Resectability was decided by a multidisciplinary team approach.

Liver volume measurement

Triple-phase computed tomography (CT) was routinely used for preoperative imaging evaluation. The axial images were then loaded to a computer workstation where the semimanual Rapidia software (Infinit Co., Ltd., Seoul, Korea) was installed. Cross-sectional areas of the liver on each transverse-slice image were obtained by manual tracing of the liver contour using a cursor, with free-curves drawn by experienced surgeons. Liver parenchymal volume was then generated automatically by summation of all the manually calculated areas of successive transverse-slice images^[6]. The minimal amount of sufficient future liver remnant of > 30% was mandatory before surgery^[7].

Surgical technique

All liver resections were performed by a specialist hepatobiliary surgeon proficient in laparoscopic and open liver surgery. In general, the approach to both OLR and LLR was similar. Major anatomical resection

was preferred for larger tumors or where tumor was in proximity to major vascular structures, requiring formal anatomical resection, otherwise non-anatomical resection was performed when adequate margins could be achieved. Standard vascular stapling devices were used for both OLR and LLR when required, and combination of ultrasonic dissection using cavitron ultrasonic surgical aspirator and harmonic scalpel was used for parenchymal transection. Glissonian approach and individual approach were used to isolate and resect the hilar structures.

Clinical outcomes

Patient demographics, including age, gender, body mass index (BMI), previous abdominal surgery, RFA or transcatheter arterial chemoembolization (TACE), and Child-Turcotte-Pugh classification were recorded. Peri-operative outcomes included intraoperative and post-operative complication rate, severity of complications based on Clavien-Dindo classification, type of hepatic resection, resection margin status, estimated blood loss, length of stay, total operative time (incision to closure time), and amount of transfusion. Histological analysis of resected HCC specimens was also assessed, including the Edmondson histological grade, PT staging, microvascular invasion, tumor number, and maximal tumor diameter.

Follow-up, survival and recurrence

After resection, patients were followed up 1 month after surgery, then every 3 months in the first 2 post-operative years and then at 6-month intervals for post-operative years 3 to 5, using serum α -feto-protein (AFP), with multi-phase contrast enhanced CT or magnetic resonance imaging (MRI), or Gadoterate disodium (Gd-EOB-DTPA) enhanced MRI of the liver.

The OS was calculated from the day of surgery until the day of death or last contact. The recurrence-free survival of patients who recurred was defined as the time from the day of surgery to the day of imaging study that confirmed tumor recurrence.

Statistical analysis

Descriptive statistics were reported as a mean with standard deviation for continuous variables, and as a number and percentage for discrete variables. OS and recurrence-free survival were calculated by the Kaplan-Meier method and differences were compared by the log-rank test. Statistical significance was defined as $P < 0.05$. Statistical analysis was carried out using SPSS software (version 20; SPSS, Chicago, IL, USA).

RESULTS

Clinicopathologic characteristics of patients with BCLC stage B according to treatment group

Demographics and peri-operative outcomes according to treatment group are shown in Table 1. Six out of 28 patients required conversion from a laparoscopic to open resection. There was no statistical difference between these groups in terms of age, gender, BMI, history of previous abdominal surgery, biochemistry profiles (albumin, bilirubin, prothrombin, AST and ALT levels, platelet), and preoperative AFP levels.

Majority of resections in the LLR group were minor resections (75.0% vs. 38.1%, $P = 0.009$). The LLR group had longer mean operation time (350 vs. 339 min, $P = 0.066$) and higher estimated blood loss (1707 vs. 1055 mL, $P = 0.039$). However, LLR group was able to achieve R0 resection in all resections, compared to 81.0% in the OLR group.

Histological findings

There were a greater proportion of cirrhotic patients in the LLR and OLR groups (67.9% vs. 42.9%, respectively, $P = 0.080$) [Table 2]. Although the OLR group had significantly greater mean tumor diameter ($P = 0.048$), there was no significant difference between the number of tumors removed in both treatment groups ($P = 0.074$). There was also no statistically significant difference of resection margin, presence of microvascular invasion and Edmondson-Steiner grading between LLR and OLR groups ($P = 0.649, 0.740$ and

Table 1. Basic characteristics of patients with BCLC stage B according to treatment group

	OLR (n = 21)	LLR (n = 28)	P
Age (years)	63 ± 12	58 ± 11	0.080
Sex (% male)	17 (81%)	18 (64%)	0.201
BMI (Kg/m ²)	23.0 ± 3.2	24.8 ± 3.7	0.094
Previous abdominal surgery	4 (19.0%)	1 (3.6%)	0.077
Previous TACE	11 (52.4%)	6 (21.4%)	0.024
Previous RFA	3 (14.3%)	0 (0%)	0.039
Child-Turcott-Pugh classification			0.122
A	21 (100%)	25 (89.3%)	
B	0	3 (10.7%)	
Albumin (g/dL)	4.0 ± 0.4	4.1 ± 0.4	0.918
Total bilirubin (mg/dL)	0.7 ± 0.2	0.8 ± 0.4	0.457
Prothrombin time (INR)	1.1 ± 0.1	1.1 ± 0.1	0.110
AST (U/L)	37.1 ± 22.9	39.2 ± 19.7	0.742
ALT (U/L)	32.1 ± 17.7	38.9 ± 19.8	0.218
Platelet (x 1000/mcL)	162.1 ± 82.5	139.4 ± 57.3	0.289
Viral disease			0.727
HCV	14 (66.7%)	20 (71.4%)	
HBV	2 (9.5%)	4 (14.3%)	
Both positive	0	0	
Both negative	5 (23.8%)	4 (14.3%)	
ICGR15 (%)	10.0 ± 4.2	9.9 ± 7.9	0.937
AFP (ng/mL)	1191.4 ± 4750.7	539.2 ± 1491.3	0.550
Type of hepatic resection			0.009
Minor	8 (38.1%)	21 (75.0%)	
Major	13 (61.9%)	7 (25.0%)	
Resection margin (cm)	1.7 ± 2.4	1.4 ± 1.5	0.610
R0	17 (81.0%)	28 (100.0%)	0.017
R1	4 (19.0%)	0	
Operation time (min)	339.0 ± 90.9	350.3 ± 171.2	0.066
Estimated blood loss (mL)	1055.3 ± 889.6	1707.5 ± 3294.7	0.039
Transfusion done	9 (42.9%)	12 (42.9%)	1.000
RBC	2.7 ± 1.8	6.3 ± 5.8	0.022
FFP	0.8 ± 1.6	2.1 ± 2.4	0.056

LLR: laparoscopic liver resection; OLR: open liver resection; BMI: body mass index; TACE: transarterial chemoembolization; RFA: radiofrequency ablation; AST: aspartate aminotransferase; ALT: alanine aminotransferase; HBV: hepatitis B virus; HCV: hepatitis C virus; ICGR15: indocyanine green retention test at 15 min; AFP: alpha-fetoprotein; PRBC: packed RBC; red blood cell, FFP: fresh frozen plasma. Values are expressed as mean ± standard deviation or number (percent).

0.425 respectively). In the pathologic TNM stage, the T stage was different between two groups ($P = 0.031$).

Postoperative complications, hospital stay and recurrence

As seen in Table 3, the LLR group had a higher rate of intraoperative complications than the OLR group (17.9% vs. 14.3%; $P = 0.738$), but a lower rate of postoperative complications (14.3% vs. 33.3%; $P = 0.114$). There was no significant difference in overall serious postoperative complications between the LLR and OLR groups.

Patients in the LLR group had a significantly shorter postoperative hospital stay (median = 9 days; range: 4-30) than the OLR group (median = 21 days; range: 7-147, $P = 0.023$).

In terms of recurrence, LLR had similar recurrence rates with OLR (78.6% vs. 71.4%, $P = 0.565$) during the follow-up of 63 ± 40 months and 48 ± 43 months in LLR and OLR respectively. For both LLR and OLR, majority of the recurrences were intrahepatic recurrences (77.3% vs. 93.3%, respectively).

Overall survival and disease-free survival

The OS at 1, 3 and 5 years for LLR was 89.1%, 70.4% and 58.6%, respectively; and 76.2%, 55.9% and 43.5%, respectively for OLR. OS for both groups was similar ($P = 0.583$) and there was also no statistically

Table 2. Pathologic findings in patients according to treatment group

	OLR (n = 21)	LLR (n = 28)	P
Greatest tumor size (cm; mean \pm 4 SD)	5.3 \pm 2.6	4.4 \pm 1.7	0.048
Tumor number	2.0 \pm 1.2	1.5 \pm 0.7	0.074
Margin distance (cm; mean \pm 4 SD)	1.7 \pm 2.4	1.4 \pm 1.5	0.649
ES tumor grade (I/II/III/IV)	0:8:12:1:0	1:12:10:4:1	0.425
pT stage			0.031
T1	2	9	
T2	9	15	
T3	9	2	
T4	1	1	
Cirrhosis present	9 (42.9%)	19 (67.9%)	0.080
Microvascular invasion	10 (47.6%)	12 (42.9%)	0.740

LLR: laparoscopic liver resection; OLR: open liver resection; ES: Edmondson-Steiner. Values are expressed as mean \pm standard deviation or number (percent).

Table 3. Complications according to treatment group

	OLR (n = 21)	LLR (n = 28)	P
Intraoperative	3 (14.3%)	5 (17.9%)	0.738
Postoperative	7 (33.3%)	4 (14.3%)	0.114
Clavien-Dindo classification			0.256
Grade I	2	0	
Grade II	0	0	
Grade IIIa	3	3	
Grade IIIb	2	1	
Grade IVa/IVb	0	0	
Postoperative serious complications (Clavien-Dindo Grade III or higher)	5 (23.8%)	4 (14.3%)	0.237
Hospital stay (days; mean \pm SD: range)	20.6 \pm 30.1 (7-147)	9.2 \pm 4.9 (4-30)	0.023

LLR: laparoscopic liver resection; OLR: open liver resection

significant difference in terms of DFS between the groups ($P = 0.947$). The 1-, 3- and 5-year DFS was 59.3%, 20.2% and 16.2% for the LLR group and 51.0%, 44.6% and 37.2% for OLR groups, respectively [Figure 1].

DISCUSSION

HCC is widely endemic, with 80% of new cases worldwide expected to develop in Asia alone^[8]. The management of HCC is complex, due to the presence of two disease processes: the primary malignancy and the underlying liver pathology that accompanies HCC. Thus a reliable management algorithm to guide therapeutic decisions in these patients is needed.

Currently, the BCLC classification system is one of the most widely recognized and approved staging systems for HCC, since it considers multiple factors such as tumor stage and function, patient's performance status, as well as cancer-related symptoms^[9]. According to BCLC, intermediate stage (BCLC-B) patients are asymptomatic (PS score 0), with large multi-nodular tumors but without macrovascular invasion or extrahepatic spread^[8]. The estimated 3-year survival for patients with untreated stage B HCC ranges from 8% to 50%; given the invasiveness of surgery, palliation with TACE is therefore recommended for these subset of patients^[9]. However, TACE cannot induce complete tumor necrosis especially in large tumors, with reported response rates in literature as low as 2%^[8,10].

Liver resection, based on the BCLC system, is usually reserved for patients with small, single tumors, with absence of portal hypertension or hyperbilirubinemia^[9]. Recent data has however, supported the benefit of surgical resection in terms of short- and long-term oncologic outcomes despite the presence of large, multinodular nodules and macrovascular invasion^[4]. In a recent study done by Kim *et al.*^[11], overall median

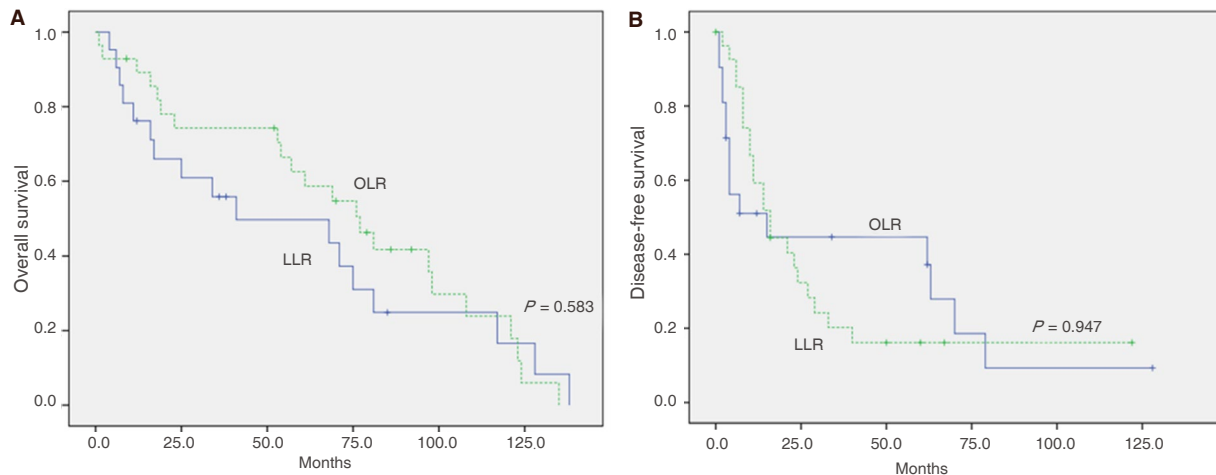


Figure 1. Overall survival and recurrence-free survival for laparoscopic and open liver resection for intermediate stage BCLC-B hepatocellular carcinoma. A: Overall survival. B: Recurrence-free survival for laparoscopic and open liver resection for intermediate stage BCLC-B hepatocellular carcinoma. Solid line: open liver resection (blue line); dot line: lap liver resection (green line). The OS at 1, 3 and 5 years for LLR was 89.1%, 70.4% and 58.6%, respectively; and 76.2%, 55.9% and 43.5%, respectively for OLR ($P = 0.583$). The 1-, 3- and 5-year DFS was 59.3%, 20.2% and 16.2% for the LLR group and 51.0%, 44.6% and 37.2% for OLR groups, respectively ($P = 0.947$). LLR: laparoscopic liver resection; OLR: open liver resection; BCLC: Barcelona Clinic Liver Score

survival was higher in patients who received surgical resection compared to those who received non-surgical therapy (50.9 vs. 22.1 months), with an overall 5-year survival of 63% in the resection group. This is in part due to recent advances in surgical devices and techniques and improved perioperative care, with some high-volume centers reporting surgical mortality rates as low as 0.8%^[12].

However, because of the underlying liver disease concomitant with HCC, patients undergoing open liver resection are still at a high risk of developing significant postoperative complications^[2]. LLR, since its inception in 1993, is currently being considered as a feasible, safe and less invasive alternative to open surgery in the case of malignant hepatic tumors^[13]. Particularly, the benefit of LLR is more pronounced in this population of cirrhotic patients, despite initial studies that considered it a contraindication. The minimally invasiveness of LLR may decrease the risk of peri-operative complications and mortality^[9], as evidenced by our present study wherein the LLR group was associated with a lower rate of post-operative complications. In terms of mortality, there was no observed 90-day post-operative mortality in both the LLR and OLR groups. Previous studies have shown longer operative times for laparoscopic surgery^[13], and this is also consistent with our study because most of the cases included were done during the early phase of LLR.

Adequacy of resection margin, which was initially a limitation for laparoscopic surgery due to the inability of direct palpation of tumor, has been overcome by the use of intraoperative Doppler ultrasonography, and has greatly facilitated the achievement of good oncologic outcomes. Indeed as shown in our present study, tumor-free margins on pathological examination were similar between the LLR and OLR group, and this is consistent with results of previous studies^[13].

The high propensity of HCC for recurrence after resection is well-documented, with reported recurrence rates ranging from 70% to 100%; among several factors, number of tumors was noted to be the most significant predictor. Indeed, previous data have shown that the 5-year DFS rates after surgical resection for multiple HCCs ranges from 0 to 26%^[12]. This is consistent with our present study, which revealed comparable 5-year DFS rates for both the LLR and OLR group at 16.2% and 37.2%, respectively, despite the presence of multiple tumors for both treatment groups. Also, our study showed that liver resection was associated with 5-year OS rates of almost 60% for the LLR group and 44% for the OLR group, despite the presence of multiple, large tumors.

One of the major limitations of this study is its retrospective nature and small sample size with difference in basic characteristics in both groups. However, our 5-year OS rate of 60% and 44% for LLR and OLR, respectively, is satisfactory, and may justify the expansion of indications for surgical resection in the case of multiple, large HCC, if the liver functions remains at Child-Pugh class A.

In conclusion, this retrospective study demonstrates that LLR and OLR have comparable OS and DFS rates for BCLC-B HCC patients with multiple or large tumors. Particularly, decreased postoperative complications and shorter hospital stay, with successful achievement of adequate resection margins, was observed in the LLR group. It was demonstrated that good oncologic and perioperative outcomes can be achieved with LLR for HCC.

DECLARATIONS

Authors' contributions

Collecting and analyzing data, writing the manuscript: David A

Design this study, analyzing data, finalizing the manuscript: Choi YR

Analyzing data and drafting the manuscript: Han HS, Yoon YS, Cho JY

Data source and availability

Data and survey materials are available upon request from the corresponding author.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Agreement exemption allowed by the Institutional Review Board (IRB) of Seoul National University Bundang Hospital (SNUBH).

Ethics approval

This retrospective, observational study was approved by the Institutional Review Board (IRB) of Seoul National University BundangHospital (SNUBH, B-1801-442-108).

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Original Article

Open Access



Hepatocellular carcinoma in the setting of interferon-free treatment for chronic HCV hepatitis - experience of a single center

Elena Laura Iliescu¹, Adriana Mercan-Stanciu¹, Letitia Toma¹, Elena Simona Ioanutescu¹, Radu Dumitru², Daniel Rusie³

¹Department of Internal Medicine II, Fundeni Clinical Institute, Bucharest 022328, Romania.

²Department of Interventional Radiology, Fundeni Clinical Institute, Bucharest 022328, Romania.

³Department of General Surgery, Clinical Emergency Hospital, Bucharest 014461, Romania.

Correspondence to: Dr. Elena Laura Iliescu, Department of Internal Medicine II, Fundeni Clinical Institute, Bucharest 022328, Romania. E-mail: laura_ate@yahoo.com

How to cite this article: Iliescu EL, Mercan-Stanciu A, Toma L, Ioanutescu ES, Dumitru R, Rusie D. Hepatocellular carcinoma in the setting of interferon-free treatment for chronic HCV hepatitis - experience of a single center. *Hepatoma Res* 2018;4:3. <http://dx.doi.org/10.20517/2394-5079.2017.48>

Received: 7 Nov 2017 **First Decision:** 26 Dec 2017 **Revised:** 9 Jan 2018 **Accepted:** 10 Jan 2018 **Published:** 18 Jan 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Aim: This study aims to analyze the particularities of hepatitis C induced hepatocellular carcinoma (HCC), developed during or after treatment with direct-acting antivirals.

Methods: We conducted an observational prospective study on 278 patients, who underwent treatment for hepatitis C related liver cirrhosis and respectively for F3 chronic hepatitis C. Liver status was assessed using biological parameters and imagistic evaluation (ultrasonography, computed tomography scan, magnetic resonance imaging).

Results: The follow-up time was 14 months. Before therapy, 69.3% of the cirrhotic patients and 26.7% of those with F3 degree of liver fibrosis had high levels of alpha-fetoprotein, with no imagistic evidence of HCC. During treatment, HCC was confirmed in 5 patients, 2 of them presenting portal vein thrombosis (PVT). Antiviral therapy was not interrupted. Two patients developed HCC at the end of treatment, while 4 of them were diagnosed with HCC after three months of ending the treatment. Excepting the ones with PVT, all patients underwent trans-arterial chemoembolization.

Conclusion: All patients acquired sustained virological response. The screening for HCC should not be stopped after achievement of sustained virological response. Patients who develop HCC after antiviral treatment often need to be



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evaluated by magnetic resonance imaging in order to detect the extension of the disease.

Keywords: Hepatitis C virus, direct-acting antiviral therapy, hepatocellular carcinoma, trans-arterial chemoembolization

INTRODUCTION

Nowadays, hepatitis C virus (HCV) infection represents a global health problem, affecting over 160 million people worldwide^[1]. The progression of hepatitis C induced liver disease can be insidious, gradual, during several decades, leading to liver cirrhosis (LC) and hepatocellular carcinoma (HCC). It is estimated that, within 20 years of viral infection, about 20%-30% of subjects will develop LC^[2]. There is evidence that 2.8%-11.7% of the patients with compensated LC will develop hepatic decompensation sooner or later; as for the incidence of HCC, a percentage of 1.8%-8.3% has been reported^[3]. The use of pegylated interferon (IFN) in combination with ribavirin, in treating the HCV infection, leads to a sustained virological response (SVR) in about 50% of patients and it is known to have significant side effects^[4].

Fortunately, recently there has been a much better understanding of the HCV particularities and structure. The efforts to improve the hepatitis C management resulted in the discovery and development of direct-acting antivirals (DAAs), which are meant to interfere with specific steps in HCV replication; in other words, they directly interact with HCV encoded proteins, resulting in the disruption of viral replication^[5].

Therefore, DAAs have shown promising effects, increasing the rates of SVR to more than 90% with notably fewer side effects^[6]. Although the number of HCV-infected subjects is high, the access to the IFN-free therapy is still limited, due to increased costs and due to the fact that the HCV infection remains highly underdiagnosed^[7-9]. The use of nucleoside and protease inhibitors has relative contraindications in subjects diagnosed with end-stage renal disease.

It is also worth mentioning that the IFN-free treatments are designed to cure the viral infection, but not the liver disease itself, once the HCV has led to LC or HCC. Furthermore, the risk of complications persists even after achieving SVR, although there is evidence demonstrating improvement in liver function tests after using DAAs^[10,11].

Also, even after achieving SVR, re-infection is a possibility that can occur in 10%-15% of patients, especially in individuals at risk, such as intravenous drug users^[12].

This study aims to assess the effect of DAAs on liver function and to analyze the particularities of HCC diagnosed during or after treatment with Paritaprevir/Ombitasvir/Ritonavir and Dasabuvir with or without ribavirin (a treatment that is not recommended for patients with decompensated cirrhosis or liver cancer).

METHODS

In this study, we included a number of 278 patients, all of them infected with HCV genotype 1b, who received IFN-free treatment with Paritaprevir/Ombitasvir/Ritonavir and Dasabuvir, with or without Ribavirin, for 12 weeks.

An informed written consent was taken from all the participants and all their records were confidential. The scientific purpose of the study, as well as the implications of the therapy itself were presented in detail to each subject, as well as any unexpected research-related risk that may appear.

Viral infection was assessed in each patient by quantitative HCV ribonucleic acid (RNA) tests, describing a high viral load in all patients, with more than 800,000 IU/L. In order to estimate the degree of fibrosis, each

of the 278 patients underwent a Fibromax evaluation, resulting in 173 patients with F4 degree of fibrosis (cirrhotic patients) and 105 patients with F3 degree of fibrosis. None of the patients underwent liver biopsy for liver evaluation, due to its invasive character and susceptibility of associated complications.

The follow-up time was 14 months (since January 2016 until March 2017). Screening for liver cancer in the subjects included in the study was periodically performed by assessment of alpha-fetoprotein (AFP) levels and abdominal ultrasonography; both parameters were determined before starting the IFN-free therapy, monthly (during therapy), and also every three months afterwards.

According to the protocol of evaluation, the liver assessment was started by performing an AFP determination as well as an abdominal ultrasonography at the beginning of the therapy. If AFP levels were found high (twice the normal value) or ultrasonography abnormalities were spotted, a computed tomography (CT) scan or contrast-enhanced ultrasonography (CEUS) would be performed, in order to obtain more information. CT was not routinely performed as a first-hand screening method for HCC on all patients, at the beginning of the study, due to the potential risk related to ionizing radiation exposure and contrast-induced injury, and also because the use of CT-scan in all the patients was not considered to be cost-effective. Moreover, the AASLD and EASL-EORTC surveillance and diagnostic algorithms in HCC state that only cirrhotic patients are considered candidates for surveillance, and surveillance should be performed with ultrasound every 6 months. It is also worth mentioning that ultrasound examination of all the individuals included in the study was done by highly experienced personnel, with extensive experience in the field of hepatic imaging. Patients with HCC diagnosed in this stage were not included in the study and did not receive any form of IFN-free treatment, as the presence of HCC represents a contraindication for the therapy. AFP and liver enzymes were also determined at week 4 and week 8 of therapy. At the end of treatment (EOT) (week 12), the evaluation protocol included AFP and abdominal ultrasonography. Any abnormality in these parameters would impose a CT scan for further investigation, as well as a magnetic resonance imaging (MRI) (if the CT scan turned out to be inconclusive).

RESULTS

We prospectively collected and analyzed data from 278 patients infected with HCV genotype 1b, 37.76% of which (105 individuals) had F3 degree of fibrosis, while 173 of the subjects (meaning approximately 62.24%) were already in the F4 cirrhosis stage. They were all treated with DAAs (paritaprevir/ombitasvir/ritonavir and dasabuvir, with or without ribavirin) for 12 weeks. Most of the participants (53.24%) were females; the percent of liver cirrhosis among women was 60.8%. The male population included 46.76% of the subjects, with a similar percent of 63.8% cirrhotic participants among the male population. The numeric distribution of patients by gender and degree of fibrosis are shown in [Figure 1](#).

The mean age was 60.29 ± 11.9 years. The follow up time was 14 months (from January 2016 to March 2017). Before starting the therapy, 69.3% of the cirrhotic patients and 26.7% of the patients with F3 degree of liver fibrosis presented higher than normal levels of AFP, with blood values up to 70 ng/mL. The mean AFP level, at the initiation of therapy, was 13.39 ± 11.18 ng/mL. Contrast-enhanced ultrasonography was performed for 79% of patients. Neither CEUS nor the CT scan revealed any HCC nodules at the beginning of therapy. The distribution of patients with high AFP levels, according to gender, fibrosis and means of further evaluation are shown in [Figure 2](#).

During the 4th week of treatment, 5 participants presented elevated levels of AFP. All of them had previously been classified as cirrhotic (F4 on Fibromax evaluation). Abdominal CT scan was therefore performed on all 5 patients, showing single HCC nodules in 2 subjects and multiple HCC nodules in the other three. Furthermore, the CT scan identified signs of portal vein thrombosis (PVT) in 2 of the 5 patients (1 patient

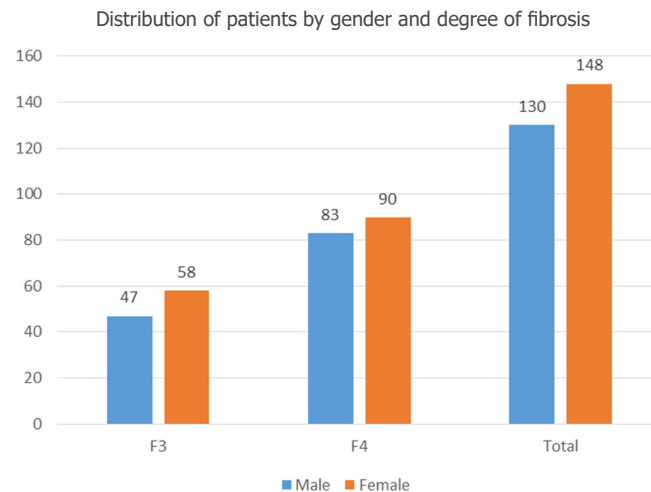


Figure 1. Distribution of patients by gender and degree of fibrosis

Distribution of patients with high AFP levels according to gender, fibrosis, and means of further evaluation

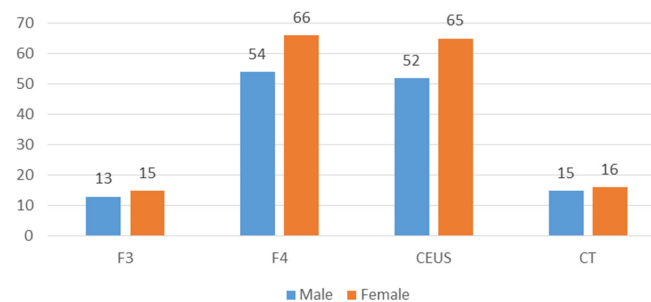


Figure 2. The distribution of patients with high AFP levels, according to gender, fibrosis and means of further evaluation. AFP: alpha-fetoprotein; CT: computed tomography; CEUS: contrast-enhanced ultrasonography

Evolution of patients diagnosed with HCC on treatment

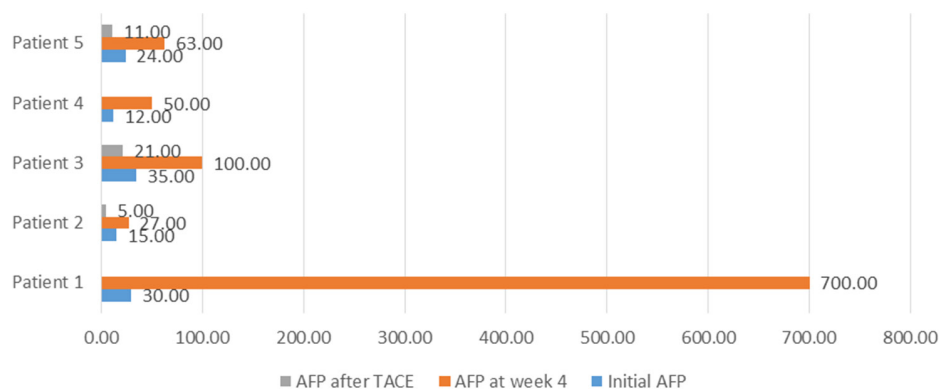


Figure 3. Evolution of patients diagnosed with HCC on treatment. HCC: hepatocellular carcinoma; AFP: alpha-fetoprotein

with single HCC nodule and PVT, and 1 patient with multiple HCC nodules and PVT). The direct antiviral therapy was not interrupted in any of the cases. The 3 subjects diagnosed with HCC and no signs of PVT underwent trans-arterial chemoembolization (TACE) with doxorubicin, while on antiviral therapy, with good outcome. [Figure 3](#) reveals the evolution of the patients who were diagnosed with HCC during treatment.

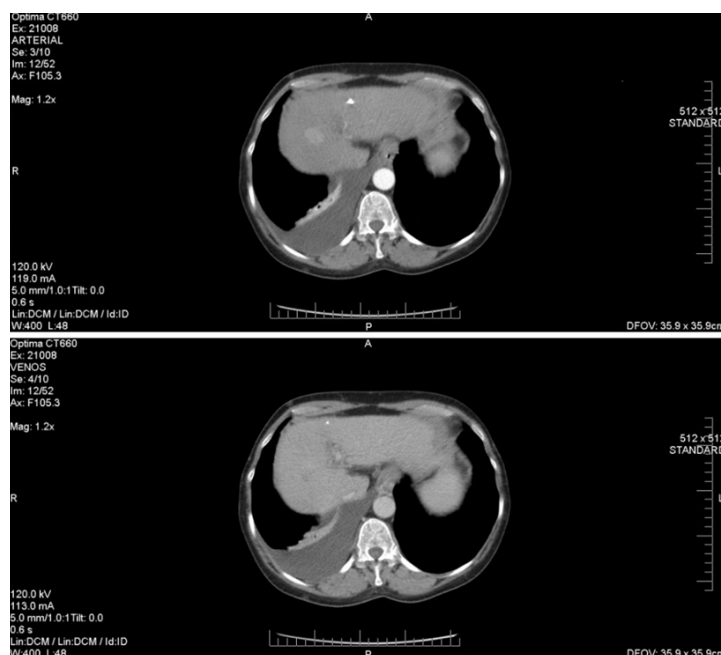


Figure 4. Hepatocellular carcinoma associated with portal vein thrombosis (diagnosed during DAAs treatment). DAAs: direct-acting antivirals

Figure 4 describes the CT image of a HCC diagnosed while the patient was undergoing DAA treatment. We note the presence of a hepatic mass (of about 28/23 mm), characterized by a typical hyper-enhancement during arterial phase, while, in the portal venous phase, the lesion is rapidly becoming indistinct or hypo-attenuating, by comparison to the rest of the liver tissue (venous phase washout). Also, in this patient's case, there are signs of thrombosis of the portal vein trunk, which may be a common phenomenon in individuals with HCC. Compared to HCC without PVT, the association of the two pathological entities is known to be more aggressive and to have a significantly higher chance of complications, as it represents a contraindication for both surgery and TACE. The two patients that were discovered to have HCC and PVC co-occurrence started receiving Sorafenib only after they completed the IFN-free therapy.

At the EOT (week 12), 2 more F4 participants presented higher levels of AFP (twice the values recorded before starting the IFN-free therapy). As they both had previously normal results of the CT scan performed at the beginning of treatment, they were investigated by abdominal MRI, which revealed single HCC nodules, in both cases. These patients refused surgical resection of the nodules and also underwent TACE, with a significant decrease in AFP levels, as it is shown in Figure 5.

At 12 weeks after the EOT, the blood tests performed on the participants revealed increased levels of AFP (up to 10 times the initial value) in other 3 F4 patients; also, there was one cirrhotic patient who had normal AFP, but in whose case the abdominal ultrasonography showed a single HCC nodule. In the patients with high AFP levels, abdominal CT scan was not able to determine the exact sizes and extension of the nodules, thus an MRI evaluation was required. In all 3 cases, the lesions identified by the MRI were larger and more extensive than the CT scan had anticipated.

One of the three patients diagnosed with HCC at SVR was a 64-year-old female; in her case, the abdominal CT revealed three nodular lesions in segment 8 (27/30 mm, 16/18 mm and respectively 10/6 mm), that displayed slight hyper-vascularization in the arterial phase, followed by a late washout, as seen in Figure 6.

As the patient underwent an MRI investigation, the lesions appeared to be in hypo-signal on T1 and also, it was revealed that the hepatic masses had bigger dimensions than they initially appeared on the CT [Figure 7].

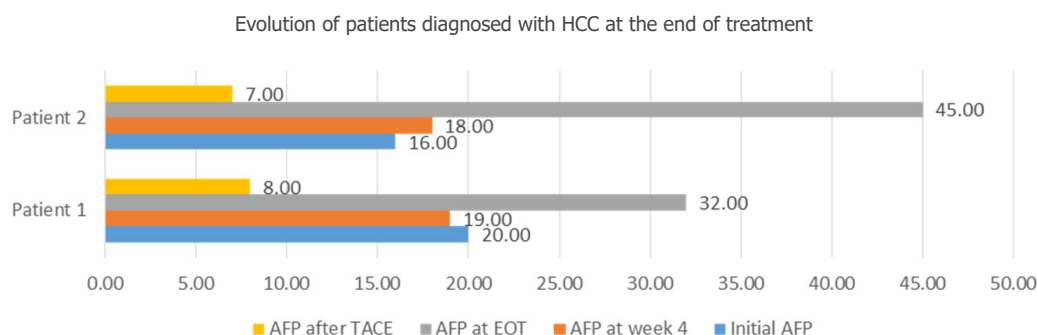


Figure 5. Evolution of patients diagnosed with HCC at the EOT. HCC: hepatocellular carcinoma; AFP: alpha-fetoprotein; TACE: trans-arterial chemoembolization; EOT: end of treatment



Figure 6. CT image showing three nodular lesions, in the arterial phase. CT: computed tomography

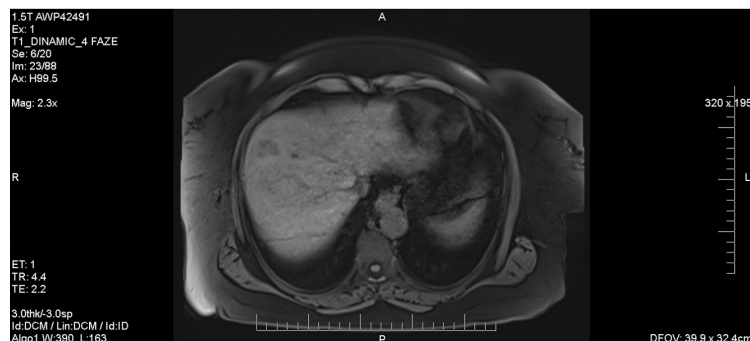


Figure 7. Abdominal MRI showing liver lesions in hypo-signal on T1 sequences. MRI: magnetic resonance imaging

The MRI exploration also revealed a lesion located in the hepatic dome, also in hypo-signal on T1, which had not been visible on the CT images [Figure 8]; given the presence of the hepatic dome mass, surgical resection was not indicated in this case. This imagistic pattern is consistent with an infiltrative type HCC.

All the patients that presented high levels of AFP at SVR (12 weeks after the EOT), also underwent chemoembolization, with one month follow-up showing no tumor progression and decreased AFP levels. The one patient with normal AFP levels was also evaluated by MRI due to iodine allergy and underwent one session of chemoembolization, with excellent results - no tumor rebound 9 months after the procedure.

Figure 9 shows the evolution of all three patients with elevated levels of AFP at 3 months after ending the antiviral treatment.

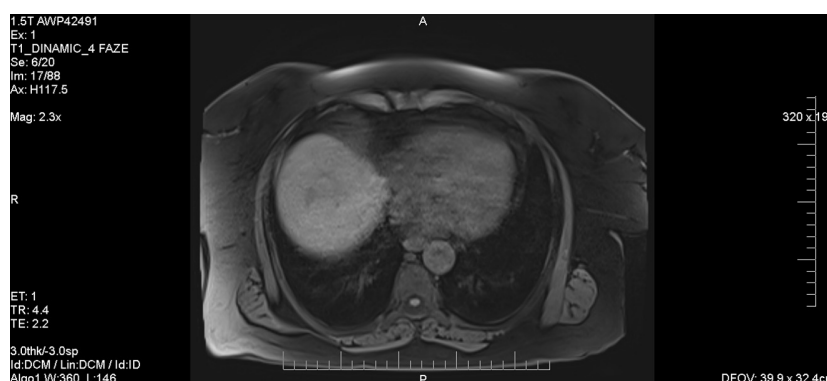


Figure 8. Abdominal MRI showing liver mass in the hepatic dome. MRI: magnetic resonance imaging

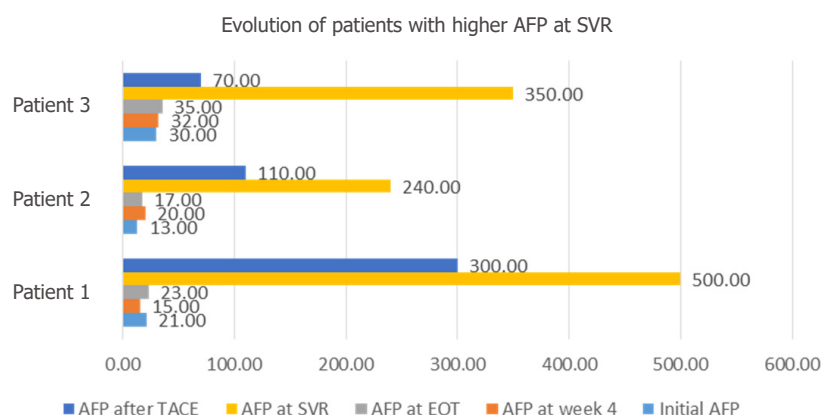


Figure 9. Evolution of patients with higher AFP at SVR. AFP: alpha-fetoprotein; SVR: sustained virological response; TACE: trans-arterial chemoembolization; EOT: end of treatment

Notably, all patients acquired SVR. Overall, the risk of HCC in our study group was 1.51%. When reported to F4 patients, the risk of HCC increases to 6.35%.

DISCUSSION

It is acknowledged that patients with HCV compensated cirrhosis who achieved SVR due to IFN-based therapy, were shown to be at a lower risk of developing HCC^[13]. It was also demonstrated that patients with HCV-induced chronic liver disease had a decrease of the fibrosis level, under DAA treatment^[14]. For the past several decades, pegylated interferon and ribavirin therapies were used to treat most of the patients with HCV associated liver disease, but they showed various side effects and toxicities.

The use of DAA treatment regimens has opened a new era in the approach of HCV-induced liver disease, reducing the need for liver transplantation^[15,16]. However, there is evidence of the occurrence or recurrence of HCC in patients with chronic HCV infection, who received DAA therapy, achieving SVR, as shown in a recent publication^[17]. Therefore, we consider that the association between IFN-free treatment and the development of HCC in patients with chronic HCV infection, should be further investigated and discussed, as it represents a highly important issue in hepatology.

There are several variables associated with an increased risk of developing HCC after SVR, such as advanced liver fibrosis, older age, alcohol abuse, metabolic diseases (especially diabetes mellitus) and the persistence of hepatic inflammation^[18,19]. A variant in genotype 1b HCV core protein Gln70 (His 70) may also be incriminated in the increase of HCC incidence^[20].

In our study, the occurrence of HCC in patients treated with DAAs was noted during therapy, as well as at the end and also three months after completing it. Older age, comorbidities, advanced fibrosis are all factors that should be taken into consideration when analyzing the increased risk of developing HCC. The mean age of the participants included in our study was 60.29 ± 11.19 years, and more than 60% of them had an F4 degree of fibrosis, which may justify the occurrence of HCC in these cases. The risk of HCC development in patients with compensated cirrhosis in our study group is consistent with literature data^[3]. However, in spite of the HCC occurrence, SVR was achieved in all the patients that continued antiviral therapy.

It is worth mentioning that, while the alpha interferon based regimens activate natural killer (NK) cells, the DAAs rapidly decrease the levels of HCV RNA, thus leading to a blockage in NK cells activation^[21]. This way, the protective effect of the inflammatory mechanisms is lost and liver regeneration as well as carcinogenesis may appear^[22]. There is also evidence showing an elevated level of vascular endothelial growth factor after the initiation of IFN-free treatment^[23].

Given the fact that most of the HCC cases found in our study were detected in the first month of therapy, the hypothesis that the tumors were already there, before starting the DAA treatment, simply becoming radiologically detectable after initiating the therapy, should be taken into consideration.

The imaging of HCC is complicated and may have limitations, especially in the early stages, as the tumor has a variety of radiologic appearances and may coexist with regeneration and dysplastic nodules in the cirrhotic liver. In patients with HCC diagnosed during or at the end of DAA therapy, it is most likely that the tumors were already there, mainly due to their dimensions at the time of diagnosis. However, this only emphasizes the importance of ultrasound and AFP follow-up, even during antiviral therapy. In this case, we do not consider that the initial evaluation of these patients should have included a mandatory CT scan, as it has not been proven to be cost-effective; also, a hypothetical mandatory imagistic evaluation would not be CT scan, but liver MRI, with further increases of the imbalance cost-effectiveness. In patients diagnosed with HCC after achieving SVR, it is most likely that the tumors developed during or after antiviral therapy. They were of small dimensions and all presented the infiltrative pattern previously discussed.

Therefore, we recommend the usage of a combination of ultrasound and serum AFP as a primary surveillance method for HCC, especially in cirrhotic patients. If abnormalities are detected by these methods, further exploration by CT and MRI is required.

In conclusion, the use of DAAs is not associated with a decrease in the development of HCC. Therefore, the screening for HCC should not be stopped after achievement of SVR, as IFN-free treatments cure the viral infection, not the liver disease itself. Patients who develop HCC after antiviral treatment need to be evaluated by MRI in order to detect the extension of the disease as these tumors are more often infiltrative. More studies should be undoubtedly performed, before determining the association between DAA therapy and HCC development.

DECLARATIONS

Authors' contributions

Concept and design: Iliescu EL, Mercan-Stanciu A, Toma L

Data acquisition: Mercan-Stanciu A, Toma L, Rusie D

Data analysis: Iliescu EL, Ioanitescu ES, Dumitru R

Manuscript preparation: Iliescu EL, Mercan-Stanciu A, Toma L

Critical revision and finalizing of the manuscript: Iliescu EL

Data source and availability

The data obtained through the medical record strictly respected the privacy policy and ethics code of our institute.

Financial support and sponsorship

None.

Conflicts of interest

All the authors declare that they do not have anything to disclose with respect to this manuscript.

Patient consent

An informed written consent was taken from all the participants and all their records were confidential.

Ethics approval

The study did not require Institutional Review Board approval.

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Cohort Profile

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Qidong hepatitis B virus infection cohort: a 25-year prospective study in high risk area of primary liver cancer

Tao-Yang Chen^{1#}, Geng-Sun Qian^{2#}, Chun-Sun Fan^{1,2}, Yan Sun¹, Jin-Bing Wang¹, Pei-Xin Lu¹, Xue-Feng Xue¹, Yan Wu¹, Qi-Nan Zhang¹, Yan Jin², Yi-Qian Wu², Yu Gan², Jian-Quan Lu¹, Thomas W. Kensler³, John D. Groopman³, Hong Tu²

¹Department of Etiology, Qidong Liver Cancer Institute, Qidong People's Hospital, Qidong 226200, Jiangsu, China.

²State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200032, China.

³Environmental Health and Engineering, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, USA.

#Contribute equally to this work.

Correspondence to: Dr. Hong Tu, State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200032, China. E-mail: tuhong@shsci.org

How to cite this article: Chen TY, Qian GS, Fan CS, Sun Y, Wang JB, Lu PX, Xue XF, Wu Y, Zhang QN, Jin Y, Wu YQ, Gan Y, Lu JQ, Kensler TW, Groopman JD, Tu H. Qidong hepatitis B virus infection cohort: a 25-year prospective study in high risk area of primary liver cancer. *Hepatoma Res* 2018;4:4. <http://dx.doi.org/10.20517/2394-5079.2017.50>

Received: 30 Nov 2017 **First Decision:** 21 Dec 2017 **Revised:** 18 Jan 2018 **Accepted:** 19 Jan 2018 **Published:** 26 Jan 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Qidong hepatitis B virus (HBV) infection cohort (QBC) is a prospective community-based study designed to investigate causative factors of primary liver cancer (PLC) in Qidong, China, where both PLC and HBV infection are highly endemic. Residents aged 20-65 years, living in seven townships of Qidong, were surveyed using hepatitis B surface antigen (HBsAg) serum test and invited to participate in QBC from June 1991 to December 1991. A total of 852 and 786 participants were enrolled in HBsAg-positive and HBsAg-negative sub-cohorts in May 1992, respectively. All participants were actively followed up in person, received HBsAg, alanine aminotransferase, alpha-fetoprotein tests and upper abdominal ultrasonic examination, and donated blood and urine samples once or twice a year. The total response rate was 99.6%, and the number of incident PLC was 201 till the end of February 2017. The ratio of incidence rates was 12.32 [95% confidence interval (CI): 7.16-21.21, $P < 0.0001$] in HBsAg-positive arm compared with HBsAg-negative arm. The relative risk of PLC was 13.25 (95% CI: 6.67-26.33, $P < 0.0001$) and 28.05 (95% CI: 13.87-56.73, $P < 0.0001$) in the HBsAg⁺/HBeAg⁻ group and the HBsAg⁺/HBeAg⁺ group, respectively, as compared to the HBsAg⁻/HBeAg⁻ group. A series of novel PLC-related mutations including A2159G, A2189C and G2203W at the C gene, A799G, A987G and T1055A at the P gene of HBV genome were identified by using samples from the cohort. The mutation in HBV basal core promoter region of HBV genome has an accumulative effect on the occurrence of PLC. In addition, the tripartite relationship of aflatoxin exposure, P53 mutation and PLC was also



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investigated. QBC will be used to develop dynamic prediction model for PLC risk by using its long-term follow-up information and serial blood samples. This model is expected to improve the efficiency of PLC screening in HBV infection individuals.

Keywords: Prospective cohort, hepatitis B virus, primary liver cancer

HOW WAS THE STUDY INITIATED?

Qidong City, named Qidong County before 1989, is located on the north shore of the Yangtze River and has a population of approximately 1.1 million. In the Early 1970s, a population-based retrospective survey on cancer mortality revealed that the mortality rate ascribed to primary liver cancer (PLC) in Qidong was $49.04/10^5$, placing PLC as the leading cause of cancer mortality in Qidong. This also exceeded the rates of all other areas in eastern China^[1]. Subsequently, a national population-based incidence survey conducted during 1983 to 1987 showed that the PLC incidence rate in Qidong was $85.1/10^5$ in males and $23.3/10^5$ in females, respectively^[2], both being in the top rank across mainland China. “Qidong high incidence area of liver cancer” became known worldwide subsequently. Two retrospective cohort studies in Qidong indicated that hepatitis B virus (HBV) was a major risk factor contributing to PLC risk with relative risk of 17.4^[3] and 5.93^[4]. Other etiological factors had also been suggested to explain the endemic of PLC in Qidong, including dietary aflatoxin contamination^[5], selenium deficiency^[6], and drinking water polluted by blue green algal toxins^[7]. However, the magnitude of the contribution of each etiologic factor to the endemic of PLC and the role of potential synergistic interactions among these factors were uncertain. In order to extensively investigate the relationship between HBV infection and PLC endemic, and collect serial bio-samples of cohort members which were not available from the previous cohorts in Qidong, investigators from Shanghai Cancer Institute and Qidong Liver Cancer Institute initiated a prospective cohort study named “Qidong Hepatitis B Virus Infection Cohort (QBC)” in 1991. Later on, a research team from John Hopkins University joined in the beginning of 1994. The QBC aimed to recruit participants positive with serum hepatitis B surface antigen (HBsAg) as the exposure group and those who were HBsAg negative as the non-exposure group, and then to observe prospectively PLC occurrence as the primary outcome. Bio-samples were collected periodically for analysis of the kinetic changes of viral and host factors during the natural history of HBV infection. The study protocol and informed consent were approved by the human subjects review committees at the Qidong Liver Cancer Institute, Shanghai Cancer Institute and John Hopkins University.

WHAT DOES THE STUDY COVER?

The overarching goal of the QBC was to elucidate the causative factors of PLC and to identify effective measures to prevent this lethal malignancy. Initially, the QBC focused on understanding the proportion of HBV infection contributing to the endemic of PLC in Qidong. Later, taking advantage of serial plasma samples, the QBC was expanded to explore the interactions between HBV and aflatoxin exposure as well as to probe associations of aflatoxin metabolism or metabolizing enzymes with PLC. Additionally, several molecular epidemiologic studies were carried out to understand the relationship between HBV variations and PLC occurrence in order to identify new molecular biomarkers for early detection or prediction of PLC utilizing stored pre-diagnostic plasma samples. Meanwhile, a bio-specimen bank containing longitudinally collected blood, urine, liver tissues was established successfully.

WHO WAS IN THE STUDY?

Residents living in the Haidong district of Qidong City, which included 7 towns named “Haifu**”, “Jinhai”, “Xiangyang”, “Juyang”, “Shaozhi”, “Dongyuan”, and “Hefeng*”, were considered as potential participants [Figure 1]. In the 1980s-1990s, the total number of residents in each of these towns was approximately 15,000, representing the PLC endemic population of Qidong^[8]. From June 1991 to December 1991, local physicians carried out door to door visits, asking questions about medical history of viral hepatitis. Residents aged 20-65 years who claimed to have a history of acute or chronic hepatitis, or who were HBsAg positive in past



Figure 1. Location of the participants in the Qidong hepatitis B virus infection cohort

screenings during physical examination were regarded as potential candidates. A total of 1157 potential candidates were identified and invited for HBsAg testing by the ELISA kit from Shanghai Kehua Bio-engineering Co., Ltd (KHB) within the following 6 months to confirm their HBsAg carrier status. Only those who were confirmed to be HBsAg positive in the second-round test and who signed the informed consent were enrolled into the study as participants in the HBV exposed sub-cohort. Meanwhile, local residents who claimed no history of hepatitis and who tested negative for HBsAg with a similar distribution of age, gender, living habits (type of drinking water and staple food), and living places were invited to participate in the HBV unexposed sub-cohort upon receiving their signed consent. Exclusion criteria were the same for both sub-cohorts, including those who had been diagnosed with cancer of any site, or who subsequently died within the first 12 months after enrollment into the cohort [Figure 2]. The final number of the participants in HBsAg positive and HBsAg negative sub-cohorts in May 1992 were 852 and 786, respectively. The mean age of HBsAg-positive participants was 37.06 ± 11.24 years (251 in below 30 years group (group I), 301 in between 30-40 years group (group II), 300 in above 40 years group (group III); while the mean age of HBsAg-negative participants was 41.20 ± 12.12 years (158 in group I, 237 in group II, 391 in group III). The male to female ratios in HBsAg-positive group was 5.45:1, and in HBsAg-negative group was 6.08:1.

HOW OFTEN WAS THE FOLLOW-UP?

All participants were followed up at least once every year. From 2009 to 2017, those who were HBsAg positive and had one of the following conditions: α -fetoprotein (AFP, tested by KHB ELISA Kit) and alanine aminotransferase (ALT) (tested by dinitrophenylhydrazine method using KHB reagent) higher than normal value, or abnormal liver ultrasound (GE Healthcare) findings such as liver nodule and liver cirrhosis, were followed every 6 months. The annual active follow-ups were usually conducted in April and October, while non-respondents were tracked during the traditional Chinese Lunar New Year to guarantee a high response rate. Subjects who presented symptoms such as indigestion, jaundice, or discomfort in hepatic zones were immediately arranged to receive upper abdomen ultrasonic screening and recheck of serum AFP levels. Each participant continues to have free access to clinics affiliated with the Qidong Liver Cancer Institute to receive a free physical examination if he/she felt any discomfort or experienced any indisposed symptoms.

The occurrence of PLC was found not only by the routine active follow-up, but also by annual data linkage with the Qidong Cancer Registry, a well-maintained population-based cancer registry^[9]. For deceased individuals, death certificates were requested from the Qidong Death Registry, another population-based registry in Qidong. Non-responders were regularly contacted by both staff members of the QBC and local physicians until participants were confirmed to have withdrawn. With such active and passive follow-up, loss to follow-up only occurred when participants migrated out of Qidong and failed to respond. Since the

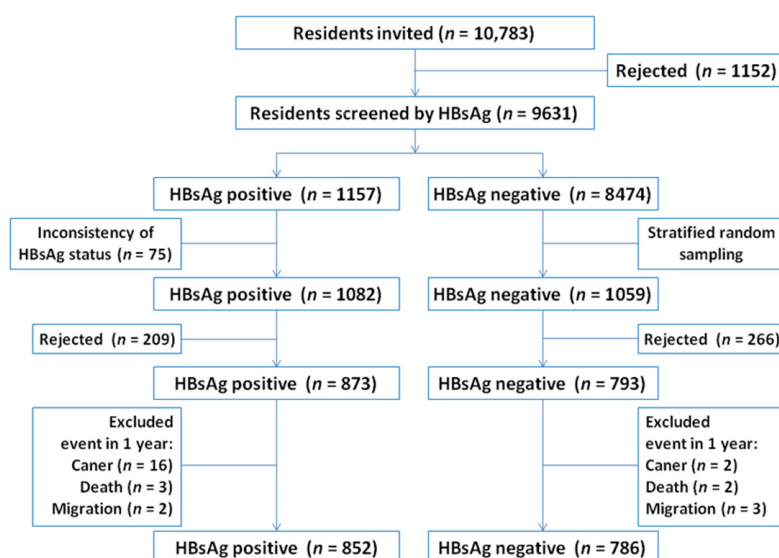


Figure 2. Flow chart for enrollment of participants into Qidong hepatitis B virus infection cohort. HBsAg: hepatitis B surface antigen

migration rate of the local population older than their 30s is low, attrition from the QBC was rare. The total response rate as of the end of February 2017 was 99.6%.

To construct a bio-bank to facilitate future research, blood samples were collected both at baseline and during every follow-up. Plasma and white blood cells were divided into aliquots and stored under appropriate conditions at the Shanghai Cancer Institute, the Qidong Liver Cancer Institute, and the John Hopkins University. Their coding system was the same as preserved in the Qidong Liver Cancer Institute. As of February 2017, a total of 23,815 plasma samples, 17,581 urine samples and 17,581 white blood cells from cohort members were acquired and properly stored. There were 1453, 1163, 815 and 144 participants donating serial plasma samples of more than 5, 10, 15 and 20 years' duration, respectively. Apart from body fluid and blood samples, 35 tumor tissue samples and adjacent non-tumor liver tissues from PLC incident cases within the QBC were well preserved in liquid nitrogen as well.

WHAT HAS BEEN MEASURED?

Questionnaires

At the baseline survey between 1991 and 1992 certified doctors and nurses with the aid of trained local physicians conducted the personal interviews by asking information on socio-demographic characteristics and past medical history. A standardized, structured questionnaire completed through face to face interviews was administrated in 1998 and covered all participants. The questions included socio-demographic data, dietary habits, type of drinking water, consumption of alcohol, tea, and tobacco, past medical history and present medical condition, family history of cancer, menstrual and reproductive history (females only), and vaccination history. In 2012, an updated questionnaire was implemented, and some new variables such as history of diabetes and use of antiviral therapy in members of the HBV exposed sub-cohort were also documented. Brief items and variables of both structured questionnaires were illustrated in [Table 1](#).

Physical examination and blood tests

During each follow-up, height, weight, and blood pressure were measured and recorded. Laboratory tests for HBsAg, ALT, and AFP and upper abdominal ultrasonic exam were also performed at least once a year between 1992 and 2017. Antibody to hepatitis C virus (HCV) was measured in 2009 to determine the rate of co-infection of HBV and HCV. In the 2009 and 2012 follow-ups, HBV serum markers, including HBsAg,

Table 1. Key variables of structure questionnaires

Baseline questionnaires (1998)	Updated questionnaires (2012)
Unique code	Occupation
Name	Family size
Gender	Family income
ID code (containing birth date and gender)	Temporary place
Occupation	Staple food
Home address	Drinking water
Employer	Alcohol drinking
Phone call	Tea drinking
Birth place	Smoking
Education	Medical history
Marital status	Family history of cancer
Financial condition	Family history of HCC
Staple food	Diabetes history
Drinking water	High blood pressure history
Tobacco consumption	
Alcohol drinking	
Tea drinking	
Medical history	
Menstrual and reproductive history (if female)	
Vaccination history	
Prophylactic intervention	

HCC: hepatocellular carcinoma

antibody to hepatitis B surface antigen, hepatitis B e antigen, antibody to hepatitis B e antigen, and antibody to hepatitis B core antigen, as well as urine glucose, and fasting blood glucose were also measured. In particular, baseline HBV DNA load, a well acknowledged viral parameter, was surveyed by using cryopreserved plasma samples between 2014 and 2015.

Other parameters based on nested case-control design

Several nested case-control studies have been carried out by using samples collected in the QBC. Exposure biomarkers and genetic variation markers measured were determined for some of the study participants, including aflatoxin metabolism^[10], aflatoxin-albumin adducts^[11], polymorphism of Glutathione S-Transferase T1 and M1^[12], epoxide hydrolase^[13], xeroderma pigmentosum group D^[14], codon 249 mutation of p53, loss of heterozygosity at chromosome 4q^[15], HBV genotype and versatile HBV mutations in X gene^[16-20], S gene^[21], C gene^[22,23], and P gene^[24-27] of HBV genome.

WHAT HAS BEEN FOUND?

HBV infection and PLC endemic in Qidong

By the end of February 2017, after a median follow-up duration of 24.83 years, a total of 201 incident PLC cases were identified in the QBC. PLC was the most common cancer type, comprising more than 65% (201/304) of all cancer cases. PLC incidence in the HBV exposed sub-cohort was significantly higher than that of the HBV non-exposed sub-cohort with an incidence rate ratio (IRR) of 12.32 (95% CI: 7.16-21.21, $P < 0.0001$). No other statistically significant IRR were observed on any other cancers including lung, gastric, colorectal *etc.* [Table 2]. These findings, in addition to our previous publications on the QBC, define HBV infection as the most important etiologic factor for explaining the PLC epidemic in Qidong^[28-30].

Furthermore, we have explored the association between HBeAg status, HBV DNA load and PLC risk in the HBV exposed sub-cohort. We found that the relative risk of PLC was 13.25 (95% CI: 6.67-26.33, $P < 0.0001$) and 28.05 (95% CI: 13.87-56.73, $P < 0.0001$) in the HBsAg⁺/HBeAg⁻ group and the HBsAg⁺/HBeAg⁺ group, respectively, as compared to the HBsAg⁻/HBeAg⁻ group^[31,32]. Those with levels of HBV DNA more than 250 copies/mL had a 4.78-fold risk of PLC compared to those without detectable HBV DNA. The HBsAg carriers with serum HBV DNA between 10^5 and 10^6 copies/mL had the greatest PLC risk, that is to say, greater than

Table 2. Incidence rates of PLC and other main incident cancer types in HBsAg positive and HBsAg negative sub-cohort with calculation of incidence rate ratios and hazard ratios

Cancer site	HBsAg positive (n = 852)			HBsAg negative (n = 786)			IR ratios	95% CI	P value	HR	95% CI	P value
	n	PY	IR (n/10 ⁵)	n	PY	IR (n/10 ⁵)						
Liver	187	16,853	1110	14	17,680	79	12.32	7.16-21.21	< 0.0001	14.00	8.13-24.1	< 0.0001
Lung	11	17,270	64	22	17,679	124	0.46	0.22-0.95	0.0361	0.53	0.26-1.08	0.0815
Gastric	12	17,242	70	11	17,649	62	1.01	0.44-2.28	0.9878	1.10	0.49-2.49	0.8214
Colorectal	2	17,230	12	7	17,673	40	0.26	0.05-1.27	0.0963	0.15	0.02-1.20	0.0736
Pancreatic	5	17,261	29	3	17,695	17	1.54	0.37-6.43	0.5558	1.80	0.43-7.51	0.4231
Esophagus	1	17,276	6	6	17,690	34	0.15	0.02-1.28	0.0830	0.17	0.02-1.42	0.1022
Bladder	2	17,272	12	3	17,692	17	0.62	0.10-3.78	0.5944	0.71	0.12-4.27	0.7099
Others	11	17,251	64	9	17,685	51	1.13	0.47-2.72	0.7894	1.29	0.54-3.12	0.5696

PLC: primary liver cancer; HBsAg: hepatitis B surface antigen; PY: person years; IR: incidence rate; CI: confidence interval; HR: hazard ratio

those with serum HBV DNA more than 10⁶ copies/mL^[33]. This observation was discrepant with results from Taiwan^[34], but consistent with the results from another cohort study in Qidong^[35].

HBV variations and hepatocellular carcinoma

HBV DNA mutation has been considered to be linked with hepatocellular carcinoma (HCC)^[36]. However, this relationship had never been evaluated in Qidong before we initiated a series of studies concerning HBV variation and the sequelae of HBV infection. By using the plasma samples from the members of the QBC, we found the A1762T/G1764A double mutation of the HBV basal core promoter (BCP) was frequently detected in HBV infected participants^[16]. However, the A1762T/G1764A double mutation alone was not sufficient to produce a statistically significant association with PLC. We reported, for the first time, that it was the triple or quadruple mutation occurring at nucleotide positions 1762, 1764, 1766 and 1768 that played roles in the development of PLC. While the odd ratio of PLC patients with the A1762T/G1764A double mutation alone was 0.393 (95% CI: 0.234-0.660), it increased to 1.861 (95% CI: 1.161-2.984) with the triple mutation and to 4.434 (95% CI: 1.630-12.063) with the quadruple mutation in BCP region^[18]. Functional studies revealed that the triple mutation could largely abrogate the colony inhibitory activity of HBx, suggesting that the enhanced risk of HCC caused by BCP variants could be attributable to the aberrant activity of HBx. These results highlight the importance of the cumulative effects of BCP mutations on PLC risk^[19].

By sequencing the HBV genome, we identified and validated a series of novel PLC-related mutations. These mutations include A2159G, A2189C and G2203W at C gene^[23], A799G, A987G and T1055A at P gene^[24], and A1479T at X gene^[18]. By using capillary gel electrophoresis, we found that it was the short fragment, rather than larger fragment, contributing to the association of Pre-S deletion with HCC^[26,27]. In addition to the above novel findings, we also verified the association of some known HBV mutations, such as HBV pre-S2 start codon mutation^[21], C1653T and T1753C^[19], with HCC in Qidong.

Taking advantage of serial plasma samples collected from patients between chronic hepatitis B and manifestation of PLC, we were able to report the temporal order of HBV mutation during the course of PLC development. While A1762T/G1764A, C1653T, A799G, A987G, T1055A, pre-S deletion could be detected in the plasma long before PLC diagnosis, T1753C, C1766T and T1768A mutations appeared only one or two years before PLC diagnosis^[18,20,23]. These observations provide valuable information for HCC prediction and screening when using HBV mutations as the marker.

Aflatoxin exposure, P53 mutation and PLC

Aflatoxin's role in PLC epidemic were also evaluated in Qidong, after an important cohort study in Shanghai^[37], by both nested case-control and cohort analysis in the QBC^[38]. P53 G249T mutation is an indicator of aflatoxin exposure. The high prevalence of this mutation suggests aflatoxin as an important etiological factor of HCC in Qidong^[39]. P53 mutations were determined initially in surgical resection tissues

from PLC cases^[40]. It was found that around 50% of PLC cases in Qidong had a G to T transversion at the third position of codon 249 in the P53 gene. Consistent with the results in PLC tissues, the codon 249 mutation of P53 was also detected in 46.7% of the plasma samples from PLC patients^[41]. Moreover, this mutation was detected at least 1 year prior to diagnosis in the plasma samples of 4 of 8 cases, suggesting P53 mutation could be an early biomarker for PLC^[42]. We also have found that PLC risk increased with the elevated concentration of serum AFB₁-albumin adducts, which is a direct biomarker for aflatoxin exposure. Lastly, a sharp decline in the age-standardized rate of PLC documented by the QCR has occurred subsequent to a population-scale change in dietary food stuff from maize to corn in the 80s and 90s. The concomitant more than 1000-fold decline in aflatoxin exposures has occurred well before the implementation of a universal vaccination program against HBV in this region^[43].

WHAT ARE THE MAIN STRENGTHS AND WEAKNESS OF THE STUDY?

The main strengths of the QBC are: (1) The QBC is a cohort study with long-term and continuous follow-up, as well as a very low rate of attrition. To our knowledge, this is a community-based HBV infected cohort with the longest period of observation worldwide. During the past two decades, the participants of the cohort have been followed up once or twice each year, which has produced continuous data for research on PLC etiology. The high quality data from cancer registry and vital statistics of Qidong lend confidence and perspective to the results. (2) The QBC database comprises a large amount of clinical and laboratory information. Structured questionnaires were implemented first in 1998 and updated in 2012, which alleviates concerns that the exposure status of related factors such as smoking and drinking could have changed during the past two decades. Serum viral and biochemical indicators such as HBsAg, AFP, and ALT at each round of follow up have been measured by the consistent kits from KHB Company to make longitudinal analysis possible as is the case with other examination such as abdominal ultrasonography. Although HBV DNA load, HBeAg, HBV genotype and HBV common mutations were not tested at baseline, they were examined using archived plasma collected at baseline and from the year when PLC was diagnosed. (3) The bio-sample bank based on this cohort now has serial plasma, white cell and urine samples. Such valuable samples collected before and after diagnosis of PLC provide a superior opportunity for evaluation of novel diagnostic markers of PLC. Indeed, key findings mentioned above were facilitated by availability of longitudinal collection of plasma samples. To our knowledge, such community-based HBV infected cohorts usually have only baseline blood samples for each participant. The characteristic of serial samples is exceptional. (4) Although the QBC is not a large scale cohort, it has already generated 201 PLC cases. This number has surpassed any others of its kind and will meet the needs of any sophisticated statistical analysis related to the study of PLC etiology and prognosis.

CAN I GET HOLD OF THE DATA? WHERE CAN I FIND OUT MORE?

The QBC study offers a unique opportunity to further research. Data collection documents and bio-samples are stored at QDLICI and SCI. We encourage interested research teams to make contact with our current leader and chief investigator of this cohort, Dr. Tao-Yang Chen, at E-mail: ty110@263.net, and Dr. Hong Tu, at E-mail: tuhong@shsci.org.

DECLARATIONS

Acknowledgments

We thank Dr. Yawei Zhang from Yale University School of Public Health for her help in the manuscript preparation. The contribution to data collection made by local physicians, nurses, ultrasound doctors, interviewers and laboratory staff is greatly and sincerely acknowledged. The authors are also appreciative of all the senior researchers who have ever worked in the “Qidong high incidence scene of liver cancer”. Only with their outstanding efforts contributed in the earlier years, could it be realistic for a following generation of

investigators to continue. Last but not least, we thank all the study participants whose longstanding personal dedication and commitment have been paramount to make this study possible.

Authors' contributions

Current co-principle investigators of QBC: Chen TY, Tu H

Former co-principle investigators of QBC: Qian GS, Lu PX

Cohort maintenance and data collection: Sun Y, Wang JB, Lu PX, Xue XF, Wu Y, Zhang QN, Lu JQ

Molecular and biochemistry experiments of QBC: Jin Y, Wu YQ, Gan Y

Collaborative researches on aflatoxin and HBV: Kensler TW, Groopman JD

Cleaned raw data, performed statistical analysis and wrote the preliminary manuscript: Fan CS

Revised the manuscript and added important intellectual contents into the manuscript: Chen TY, Qian GS, Tu H

Data source and availability

Data collection documents and bio-samples are stored at QDLCI and SCI. Interested research teams can contact with Dr. Tao-Yang Chen at E-mail: ty110@263.net, and Dr. Hong Tu at E-mail: tuhong@shsci.org.

Financial support and sponsorship

This work was supported by Chinese National Key Project of Eighth Five-year (grant number 859140303, GQ); Chinese National Key Project of Ninth Five-year (grant number 969060107, GQ); Chinese National Key Project Specialized for Infectious Diseases (grant numbers 2008ZX10002-015, HT; 2008ZX10002-018, TC; 2012ZX10002008-003, TC; 2012ZX10002-008-002, HT; 2017ZX10201201-006-002, TC; 2017ZX10201201-008-003, HT); National Institute of Environmental Health Sciences grant PO1 (grant number ES06052 USA, JDG and TWK); Chinese National Key Research Projects for Precision Medicine (grant number 2017YFC0908103, JL); Research Funding for Young Medical Talents of Nantong Municipal Commission of Health and Family Planning (grant number WQZ2015007, CF).

Conflict of interest

There are no conflicts of interest.

Patient consent

All participants signed the informed consent before study.

Ethics approval

The study protocol and informed consent were approved by the human subjects review committees at the Qidong Liver Cancer Institute, Shanghai Cancer Institute and John Hopkins University.

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Review

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Treatment of high-burden hepatocellular carcinoma: an oncologist perspective

Landon L. Chan¹, Stephen L. Chan^{1,2,3,4}

¹Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China.

²State Key Laboratory of Oncology in South China, Hong Kong, China.

³Sir YK Pao Center for Cancer, Department of Clinical Oncology, Hong Kong Cancer Institute, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, China.

⁴Institute of Digestive Disease, The Chinese University of Hong Kong, Hong Kong, China.

Correspondence to: Dr. Stephen L. Chan, Department of Clinical Oncology, Prince of Wales Hospital, Shatin, New Territories, Hong Kong, China. E-mail: chanlam_stephen@cuhk.edu.hk

How to cite this article: Chan LL, Chan SL. Treatment of high-burden hepatocellular carcinoma: an oncologist perspective. *Hepatoma Res* 2018;4:5. <http://dx.doi.org/10.20517/2394-5079.2017.49>

Received: 29 Nov 2017 **First Decision:** 17 Jan 2018 **Revised:** 22 Jan 2018 **Accepted:** 22 Jan 2018 **Published:** 7 Feb 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Hepatocellular carcinoma (HCC) is recognized as a major global healthcare burden. Although there have been tremendous improvements in cancer screening and treatment, HCC mortality rate remains high. Many patients with HCC present late to medical attention and thus are not candidates for curative treatment. They typically have high tumor burden at presentation showing heterogeneity in anatomical factors and biochemical profile. Despite the relatively poor prognosis for these patients, significant improvements can still be made in survival if the optimal treatment modality is chosen. Currently, there is no international consensus on how to manage this group of heterogeneous, high-burden HCC. In this article, we will address this question by reviewing the latest available evidences. Our definition of “high-burden HCC” will be based on three factors: size, number of tumors and the presence of macrovascular invasion. The different treatment modalities, namely surgery, intra-arterial therapy, radiotherapy and systemic therapy, and their respective supportive evidences, will be discussed. In the end, we will summarize with our views on the future direction of research priorities for the management of high-burden HCC.

Keywords: Cancer, hepatocellular carcinoma, liver

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major healthcare burden in the world. It represents 6% and 9% of the global cancer incidence and mortality respectively^[1]. It is the second most common cause of cancer-related death worldwide^[1]. Although major advancements have been made in cancer screening, diagnosis



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and treatment, prognosis of liver cancer remains poor. In 2012, World Health Organization estimated the incidence-to-mortality ratio of liver cancer to be as high as 95%^[1].

One of the major challenges in treating HCC is its heterogeneity and complexity. In contrast to other cancers, the prognosis of HCC not only depends on the tumor load, but also on the underlying etiology as well as the remaining liver reserve. Multiple staging systems have been proposed in the management of HCC. Many of them classify the patients into three groups. The first group of patients are those with the best prognosis, with little tumor burden and good liver reserve. They are often offered treatment with curative intent. The second group represents those patients with advanced disease of which tumor load is high and liver reserve is poor. These patients have very few treatment options and are offered systemic therapy, enrollment into clinical trials or supportive treatment.

The third group is the intermediate group which includes patients who do not fulfill the criteria of the first and second group. They have high tumor burden yet with relatively good liver reserve, and are potential candidates for multiple or combination of therapies, some of which can be with curative intent. This is the group which is made up of the most heterogeneous patient population, and hence it remains a challenge to devise the best therapeutic strategy for them.

In this review, the latest therapeutic options for this heterogeneous, high-tumor burden group of HCC patients will be discussed. Firstly, we will define our target population of high-burden HCC based on the size, the number of tumors, and the presence of portal vein invasion. Secondly, we will outline the various therapeutic options available and evaluate their impact on survival. Thirdly, we will briefly discuss the etiological adjunctive treatment for high-burden HCC. Finally, we will summarize the future directions in the management of high-burden HCC.

DEFINITION

Multiple factors have been identified to affect the survival rates of patients with HCC. While many of them are surrogate markers of liver reserve, a few anatomical factors have also been found to persistently affect prognosis^[2-4], including the size, the number of tumors and the presence of portal vein invasion. The application of these anatomical factors is important because it affects the choice of optimal treatment modalities.

Historically, large HCC is defined as tumors of size ≥ 5 cm, owing to the poor efficacy of radiofrequency ablation in managing HCC beyond that size. This is also the cutoff used in the Barcelona Clinic Liver Cancer (BCLC) staging system to classify tumors which are not amenable to curative treatment. Multiplicity of tumor is usually defined as number of tumors ≥ 3 , and the higher number of tumors means curative treatment would unlikely be successful. Portal vein invasion is another important poor prognostic indicator, not only because it indicates an advanced disease, it would also limit the number of feasible treatment options. According to BCLC, portal vein invasion is a contraindication for transarterial chemoembolization (TACE). As a result, only systemic therapy and best supportive care are feasible options for this group of patients.

The focus of our discussion will be on treatment options available to high-burden HCC, which we define as HCC satisfying the following criteria: (1) presence of any tumor of size ≥ 5 cm; (2) number of tumors ≥ 3 ; (3) presence of portal vein invasion; and (4) without extrahepatic metastasis. This group of patients were traditionally considered to carry a grim outlook but recent treatment advancements have improved their prognosis.

TREATMENT OPTIONS FOR HIGH-BURDEN HCC

In the literature, a plethora of therapeutic options are available for high-burden HCC. These include surgery, TACE, transarterial radioembolization (TARE), radiotherapy (RT) and systemic therapy. The choice of

therapy depends on the extent of the disease, the liver function and the patient's performance status. Each treatment option will be discussed individually here.

Surgery

Previously thought only to have a role in early HCC, advancement in surgical techniques have enabled hepatic resection to become a therapeutic option for high-burden HCC. Although high quality evidence is still lacking, many retrospective studies have provided support for hepatic resection to be a safe and effective method in managing high-burden HCC. In fact, many Asian liver centers prefer hepatic resection, as long as it is feasible, to other local treatment options. We will now review the recent studies published between 2007 and 2017 to give the most updated picture of the efficacy of hepatic resection in the management of high-burden HCC^[5-39] [Table 1]. Of note, few studies have examined the effect of tumor size and number of tumors independently on survival, so we would group them together in the following discussion, with large (≥ 5 cm) and multifocal tumor as one single population (large/multifocal HCC).

For patients with large/multifocal high-burden HCC treated with surgery, the median survival rate was 27.6 months, and the median 1-, 3-, and 5-year overall survival rates were 74.3%, 51.2%, and 39.2% respectively. Among patients treated with surgery, survival was particularly favorable among those with solitary large tumor (≥ 5 cm), with median 1-, 3-, and 5-year survival rates of 87.2%, 63.2%, and 56.1% respectively. Large tumor size has been repeatedly reported as a poor prognostic factor for HCC. This is consistent with the results we found in high-burden HCC treated with surgery [Table 2]: the median 1-, 3-, and 5-year overall survival rates for huge/multifocal tumor (≥ 10 cm) were 70.0%, 45.0%, and 36.0%, whereas those for moderately-large/multifocal tumors (≥ 5 and < 10 cm) were 73.0%, 55.1%, and 50.8% respectively. However, it is worth noting that larger tumors do not appear to be associated with higher post-operative mortality. The median postoperative mortality for huge/multifocal (≥ 10 cm) tumors was 2.6%, compared with 4.3% for large/multifocal tumors.

Portal vein invasion remains to be another poor prognostic factor for HCC patients despite advancements in treatment modalities, especially for tumors invading into the main or contralateral portal vein^[40]. Surgery has been considered contraindicated by many institutions, including the BCLC system^[41]. However, many studies, particularly those from the Asian centers, have reported hepatic resection to be safe and effective for patients with portal vein invasion^[28,42-58] [Table 3]. The median 1-, 3- and 5-year overall survival rates for patients with all forms of portal vein invasion treated with surgery were 61.0%, 32.9% and 27.0% respectively. The prognosis worsens with the degree of portal vein involvement [Table 4]. For Vp1 and Vp2 involvement, the median 1-, 3- and 5-year overall survival rates after surgery were 69.1%, 42.2% and 38.7%, whereas for those with main portals or the 1st branch involvement (Vp3 and Vp4), the median 1-, 3- and 5-year overall survival rates after surgery were 52.8%, 23.4% and 14.6% respectively [Table 5].

Transarterial chemoembolization

Before the advent of intra-arterial therapy, surgery has been the mainstay of treatment for HCC. However, less than 30% of patients were eligible for liver resection due to advanced staging of the disease^[59,60]. TACE revolutionized the treatment for high-burden HCC when it was first introduced in the early 90's^[61-65]. It takes advantage of the differential portal and arterial contributions to the blood supply of the tumor and the normal liver parenchyma. Normal liver parenchyma receives majority of the blood supply from the portal vein while the tumor feeds itself mainly from the hepatic arteries. The effects of TACE are two-fold. First, it delivers cytotoxic drugs to kill tumor cells. At the same time, by embolization of the arterial supply to the tumor, it creates an ischemic environment while keeping the cytotoxic agents within the tumor. The overall effect is to induce tumor necrosis via both direct poisoning and starvation.

Nowadays, TACE is the treatment of choice for unresectable high-burden HCC. The positive efficacy of TACE has been reported in numerous case reports and retrospective studies since its introduction in

Table 1. Recent studies on the efficacy of surgical resection in the management of large/multifocal high-burden hepatocellular carcinoma

Year	Place	Authors	Type (S/M/A)	Size: ≥ 5 cm	Size: 5-10 cm	Size: ≥ 10 cm	Number of patients (n)	1-year survival (%)	3-year survival (%)	5-year survival (%)	Median survival (months)	Post-operative mortality (%)	Recruit-ment year
2007	South Korea	Cho <i>et al.</i> ^[5]	S	-	61	-	61	85.0	59.0	52.9	-	1.6	1998-2001
2007	South Korea	Lee <i>et al.</i> ^[6]	A	-	-	100	100	66.0	44.0	31.0	-	2.0	1997-2003
2007	Singapore	Pandey <i>et al.</i> ^[7]	A	-	-	166	166	-	-	28.6	20.0	3.0	1995-2006
2007	Canada	Shah <i>et al.</i> ^[8]	A	-	-	24	24	-	-	54.0	-	8.3	1993-2004
2007	UK	Young <i>et al.</i> ^[9]	A	-	42	-	42	70.0	45.0	45.0	-	7.0	1994-2006
2008	Japan	Shimada <i>et al.</i> ^[10]	A	-	-	85	85	-	-	31.5	27.6	1.2	1988-2004
2008	France	Chirica <i>et al.</i> ^[11]	A	20	-	-	20	73.0	56.0	45.0	-	-	1998-2004
2008	Japan	Taniai <i>et al.</i> ^[12]	A	-	-	29	29	-	33.6	33.6	-	6.9	1987-2006
2008	Taiwan	Wang <i>et al.</i> ^[13]	A	58	-	-	58	58.0	32.0	22.0	-	-	1990-2006
2008	Taiwan	Wang <i>et al.</i> ^[14]	A	243	-	-	243	81.5	64.4	50.5	60.4	-	1986-2002
2009	Australia	Ng <i>et al.</i> ^[15]	A	-	-	44	44	66.4	38.1	27.8	21.5	-	1990-2008
2009	China	Yang <i>et al.</i> ^[16]	A	260	-	0	260	87.0	55.5	38.2	45.5	2.3	1992-2002
2009	Korea	Choi <i>et al.</i> ^[17]	A	-	-	50	50	70.0	50.2	40.2	-	-	1996-2006
2009	Taiwan	Ho <i>et al.</i> ^[18]	A	294	-	-	294	77.4	51.9	36.6	37.9	-	1981-2000
2010	Greece	Delis <i>et al.</i> ^[19]	A	66	-	-	66	69.0	37.0	32.0	-	-	2002-2008
2010	Taiwan	Lin <i>et al.</i> ^[20]	A	93	-	-	93	83.0	49.0	-	27.6	5.4	2001-2007
2010	Italy	Ramacciato <i>et al.</i> ^[21]	M	20	-	-	20	-	-	33.6	-	-	2000-2006
2010	Italy	Ramacciato <i>et al.</i> ^[21]	S	31	-	-	31	-	-	56.1	-	-	2000-2006
2010	USA	Schiffman <i>et al.</i> ^[22]	A	78	-	-	78	-	-	20.0	-	-	1999-2005
2010	China	Wang <i>et al.</i> ^[23]	A	-	189	-	189	70.0	51.2	36.5	-	7.5	1991-2004
2011	Japan	Yamashita <i>et al.</i> ^[24]	A	0	-	53	53	74.0	43.0	35.0	-	3.8	1995-2007
2011	China	Luo <i>et al.</i> ^[26]	A	85	-	0	85	70.6	35.3	23.9	-	2.4	2004-2006
2011	China	Zhou <i>et al.</i> ^[27]	S	85	-	-	85	93.8	56.2	47.0	-	-	1995-2002
2012	Italy	Ruzzenente <i>et al.</i> ^[25]	S	0	13	-	13	76.9	68.4	68.4	-	0.0	1995-2009
2012	Taiwan	Chang <i>et al.</i> ^[28]	A	478	-	-	-	74.6	51.8	40.7	-	2.7	1991-2006
2012	Serbia	Galun <i>et al.</i> ^[29]	A	32	-	-	32	-	-	-	26.0	0.0	2001-2008
2012	Taiwan	Huang <i>et al.</i> ^[30]	A	-	-	74	74	61.9	39.4	28.9	20.4	-	2001-2005
2012	USA	Shrager <i>et al.</i> ^[31]	A	-	-	130	130	56.9	30.3	18.8	17.0	6.9 before 2002 2.3 after 2002	1992-2010
2013	Switzerland	Allemann <i>et al.</i> ^[32]	A	-	-	22	22	-	-	45.0	27.0	0.0	1997-2009
2013	Japan	Ariizumi <i>et al.</i> ^[33]	A	-	-	177	177	61.0	46.0	42.0	38.5	-	1990-2008
2014	China	Yin <i>et al.</i> ^[34]	A	88	-	-	88	76.1	51.5	-	41.0	1.1	2008-2010
2015	Taiwan	Chan <i>et al.</i> ^[35]	A	-	-	54	54	78.5	61.4	54.2	-	-	2005-2010
2016	Taiwan	Chang <i>et al.</i> ^[36]	A	-	2306	-	2306	82.1	-	50.8	-	-	2002-2010
2016	Taiwan	Chang <i>et al.</i> ^[36]	A	-	-	912	912	68.5	-	35.0	-	-	2002-2010
2016	Taiwan	Liu <i>et al.</i> ^[37]	A	224	-	-	224	88.0	76.0	63.0	-	-	-
2016	China	Zhao <i>et al.</i> ^[38]	A	82	-	-	82	77.0	56.0	43.0	-	-	2005-2011
2017	South Korea	Jin <i>et al.</i> ^[39]	S	206	-	-	206	89.3	67.4	58.0	-	-	2008-2010

A: studies consider large tumors (≥ 5 cm) with or without multifocal tumors as one single population group; S: studies only consider solitary large tumors; M: studies only consider multifocal tumors, of which size can be ≤ 5 cm

the 90's. But high-quality evidences only came in 2002, when two randomized controlled trials (RCTs) demonstrated the improvement in outcomes for patients with unresectable HCC when treated with TACE compared to conservative management^[66,67]. Subsequent meta-analysis involving 7 RCTs also demonstrated an improvement in 2-year survival rate [odds ratio 0.53; 95% confidence interval (CI): 0.32-0.89; $P = 0.017$]^[68]. Although this meta-analysis was later criticized for being small scale, using heterogeneous study population, and employing non-standardized TACE techniques and materials, many subsequent studies consistently reproduced the positive effects that TACE brought about in treating unresectable high-burden HCC^[20,26,34,37,39,56,69-71] [Table 6].

For high-burden HCC treated with TACE, the median 1-, 3- and 5-year overall survival rates were 68.4%, 42.1% and 31.1% [Table 7]. In the case of solitary large (≥ 5 cm) HCC, the median 1-, 3-, and 5-year overall

Table 2. Summary of median overall survival of large/multifocal high-burden hepatocellular carcinoma treated with surgery

	Solitary large tumor	Moderately-large/multifocal (≥ 5 cm and < 10 cm)	Huge/multifocal (≥ 10 cm)	Overall
1-year survival (%)	87.2	73.0	70.0	74.3
3-year survival (%)	63.2	55.1	45.0	51.2
5-year survival (%)	56.1	50.8	36.0	39.2

Table 3. Recent studies on the efficacy of surgical resection in the management of high-burden hepatocellular carcinoma with portal vein invasion

Year	Place	Authors	Type (S/A)	Size: ≥ 5 cm	Size: 5-10 cm	Number of patients (n)	1-year survival (%)	3-year survival (%)	5-year survival (%)	Median survival (months)	Recruitment year
2010	Taiwan	Lin et al. ^[20]	A	78	-	78	39	2	-	15.8	2001-2007
2011	China	Luo et al. ^[26]	A	-	83	83	67.2	26	18.9	19.5	2004-2006
2014	China	Yin et al. ^[34]	A	-	85	85	51.8	18.1	-	14	2008-2010
2014	China	Jianyong et al. ^[69]	S	190	-	190	87.9	76.3	57.9	-	2002-2008
2014	China	Jianyong et al. ^[69]	A	139	-	490	68.4	46	40.8	-	2002-2008
2015	South Korea	Lee et al. ^[70]	S	68	-	68	89.8	72.8	49.6	-	-
2016	Japan	Kudo et al. ^[56]	A	-	-	1576	82.2	40.2	21.1	-	1997-2006
2016	Taiwan	Liu et al. ^[37]	S	229	-	229	74	44	35	-	-
2017	South Korea	Jin et al. ^[39]	A	489	-	489	67.7	38.2	27.2	-	2003-2010
2017	Japan	Nouso et al. ^[71]	A	76	-	76	-	47.3	21.4	72	2001-2015

A: studies consider large tumors (≥ 5 cm) with or without multifocal tumors as one single population group; S: studies only consider solitary large tumors

Table 4. Classification of portal vein invasion

Degree of invasion
Vp0: no evidence of tumor thrombus invasion
Vp1: tumor thrombus distal to but not in the second-order branches
Vp2: tumor thrombus in the second-order branches
Vp3: tumor thrombus in the first-order branches
Vp4: tumor thrombus in the main trunk or contralateral or both

Table 5. Summary of median overall survival of high-burden hepatocellular carcinoma with portal vein invasion treated with surgery

	Vp1 and Vp2	Vp3 and Vp4	Overall
1-year survival (%)	69.1	52.8	61.0
3-year survival (%)	42.2	23.4	32.9
5-year survival (%)	38.7	14.6	27.0

survival rates were higher: 87.9%, 72.8%, and 49.6%. In this group of high-burden HCC, TACE appeared to be inferior to surgical resection in prolonging survival. However, if we focus on solitary large HCC (≥ 5 cm) only, TACE appeared to outperform surgical resection [Table 7]. Therefore, it appears that surgery should be the choice of treatment when the tumor is “resectable”, while TACE could be considered in the case of solitary large tumor.

TACE is commonly considered contraindicated in HCC with portal vein invasion due to the potential risk of acute liver failure resulting from post-TACE ischemia, as the normal liver parenchymal blood supply from the portal vein is already compromised. However, this contraindication has not been validated in large trials. On the contrary, a number of small retrospective studies have shown that TACE could be performed safely in patients with portal vein tumor thrombus (PVTT), provided that there was adequate liver reserve and the establishment of collateral blood circulation around the obstructed PVTT was sufficient^[72,73].

Table 6. Recent studies on the efficacy of transarterial chemoembolization in the management of high-burden hepatocellular carcinoma

Year	Place	Authors	Vascular invasion	Number of patients (n)	1-year survival (%)	3-year survival (%)	5-year survival (%)	Median survival (months)	Recruitment year
2009	Japan	Ban <i>et al.</i> ^[42]	Vp3 and Vp4	45	69.6	37.4	22.4	20	1992-2008
2010	China	Shi <i>et al.</i> ^[53]	Vp1 and Vp2	139	52.1	25.1	-	-	2001-2003
2010	China	Shi <i>et al.</i> ^[53]	Vp3	169	38.2	17.7	-	-	2001-2003
2010	China	Shi <i>et al.</i> ^[53]	Vp4	78	24.7	3.6	-	-	2001-2003
2012	Taiwan	Chang <i>et al.</i> ^[28]	-	160	57.6	33.8	29.1	-	1991-2006
2012	China	Peng <i>et al.</i> ^[43]	All types	201	42	14.1	11.1	20	2002-2007
2012	China	Chen <i>et al.</i> ^[50]	All types	88	31.1	15.2	-	9	2006-2008
2012	Japan	Matono <i>et al.</i> ^[52]	Vp3 and Vp4	29	62.1	24.1	17.2	16.6	1985-2005
2013	USA	Roayaie <i>et al.</i> ^[46]	All types	165	-	-	14	13.1	1992-2010
2013	China	Tang <i>et al.</i> ^[54]	All types	186	40.1	13.6	-	10	2006-2008
2013	France, Italy, Japan, Argentina, USA	Torzilli <i>et al.</i> ^[55]	All types	297	76	49	38	-	1990-2009
2014	Taiwan	Liu <i>et al.</i> ^[48]	Vp1 to Vp3	247	85	68	61	64	2002-2012
2014	Hong Kong	Chok <i>et al.</i> ^[57]	Vp3	71	45.8	22.7	11.2	10.9	1989-2010
2015	Japan	Kojima <i>et al.</i> ^[44]	Vp3 and Vp4	25	68	32	12	21.5	2001-2010
2016	Japan	Kokudo <i>et al.</i> ^[45]	All types	1877	74.8	49.1	39.1	34	2000-2007
2016	Korea	Lee <i>et al.</i> ^[47]	Vp1 to Vp3	40	-	-	-	19.9	2000-2011
2016	China	Zheng <i>et al.</i> ^[49]	All types	96	86.5	60.4	33.3	-	2000-2008
2016	China	Li <i>et al.</i> ^[51]	Vp4	50	35.6	0	0	-	2010-2013
2016	China	Zhang <i>et al.</i> ^[58]	Vp1 to Vp3	113	68.9	34.3	30.8	18.2	2005-2012
2016	Japan	Kudo <i>et al.</i> ^[56]	Vp3 and Vp4	852	59.8	34.3	25	-	1996-2007
2016	Japan	Kudo <i>et al.</i> ^[56]	Vp2	714	69.1	42.2	29.2	-	1996-2007
2016	Japan	Kudo <i>et al.</i> ^[56]	Vp1	1908	84.9	62.4	48.2	-	1996-2007

Table 7. Comparison of median overall survival of high-burden HCC treated with surgery and TACE

	Solitary large HCC (surgery)	Solitary large HCC (TACE)	Overall (surgery)	Overall (TACE)
1-year survival (%)	87.2	87.9	74.3	68.4
3-year survival (%)	63.2	72.8	51.2	42.1
5-year survival (%)	56.1	49.6	39.2	31.1

HCC: hepatocellular carcinoma; TACE: transarterial chemoembolization

A small number of studies have explored the possibility of TACE as a palliative treatment in high-burden HCC with portal vein invasion^[43,48,49,74-78] [Table 8]. The median 1-year overall survival rate was 50.5%. Even fewer studies have reported the median 3-year overall survival rate, likely due to the poor prognosis associated with portal vein invasion. No study thus far has compared difference in survival rate between segmental branches involvements (Vp1 and Vp2) and 1st branch or main trunk involvement (Vp3 and Vp4).

It is worth noting that many studies included in this review used conventional TACE (cTACE). However, drug-eluting bead TACE (DEB-TACE), since its introduction in 2006, was believed to be superior to cTACE. It has been demonstrated to have a lower toxicity profile compared to cTACE^[79]. However, studies so far failed to prove its ability to consistently prolong survival^[79-84]. Moreover, as a relatively new agent, only a paucity of studies has looked at its effect on high-burden HCC, particularly those with portal vein invasion. More studies are needed for this particular population of patients.

Transarterial radioembolization

Although TACE has been shown to be an effective therapy for high-burden unresectable HCC, it is associated with substantial systemic toxicities. In a Cochrane review in 2011, post-embolization syndrome, with clinical manifestations of transient fever, abdominal pain and elevated transaminases, was reported to occur in up to 80% of the patients receiving TACE^[85]. Other serious adverse events, albeit uncommon, include acute renal failure, ascites, encephalopathy and transient liver failure^[79].

Table 8. Recent studies on the efficacy of transarterial chemoembolization in the management of high-burden hepatocellular carcinoma with portal vein invasion

Year	Place	Authors	Vascular invasion	Number of patients (n)	1-year survival (%)	3-year survival (%)	5-year survival (%)	Median survival (months)	Recruitment year
2012	China	Niu <i>et al.</i> ^[78]	All types	115	27.8	-	-	8.67	2007-2010
2012	China	Peng <i>et al.</i> ^[43]	All types	402	37.8	7.3	0.5	13.1	2002-2007
2014	India	Ajit <i>et al.</i> ^[74]	All types	17	47.0	-	-	10	2011-2013
2014	Taiwan	Chern <i>et al.</i> ^[75]	Vp3 and Vp4	50.0	54.0	10.0	-	6.2	2006-2012
2014	Taiwan	Liu <i>et al.</i> ^[48]	Vp1 to Vp3	181	60	42	33	32	2002-2012
2016	China	Zheng <i>et al.</i> ^[49]	All types	134	77.6	47.6	20.9	-	2000-2008
2017	Korea	Choi <i>et al.</i> ^[76]	Vp1 and Vp2	50	-	-	-	9.4	2003-2012
2017	USA	Gorodetski <i>et al.</i> ^[77]	All types	133	-	-	-	4.53	2006-2013

In view of this, much effort has been made to devise new intra-arterial therapies with less systemic toxicities. In recent years, TARE has become an alternative to TACE in treating high-burden HCC. TARE is an intra-arterial therapy that involves the delivery of microspheres containing yttrium-90 into the hepatic arteries. TARE asserts the main effect through the internal radiotherapy delivered by Y-90, a radioactive substance, which causes necrosis of the tumor.

As data is lacking for TARE, much of the evidences came from retrospective studies of experimental intent^[86-94]. These studies either looked into the efficacy of TARE by itself, or made a comparison with TACE, the gold standard for unresectable high-burden HCC. The median survival rate for high-burden HCC treated with TARE was 15.0 (range: 11.5-20.0) months, with a response rate of 41.5% by the mRECIST criteria [Table 9]. In those studies comparing TARE and TACE retrospectively, they were not able to show any difference between survival^[88,93,94]. However, TARE was found to be associated with longer time-to-progression, less toxicity and shorter hospital stay comparing with TACE, suggesting that it may be a more favorable treatment modality for unresectable high-burden HCC. As for large solitary tumor or multifocal tumors, where TACE is known to be ineffective due to the severe adverse effects^[95], TARE could also be a preferred alternative.

Despite its better safety profile, TARE is not yet considered standard treatment by a number of clinicians. Apart from the lack of high quality evidence to support its efficacy on high-burden HCC, TARE is an expensive procedure and it requires specialized training for implementation^[96]. Given the promising results from retrospective studies, more clinical trials are needed in the coming years to formally evaluate its effectiveness and safety profile, and its potential to replace TACE's role in the treatment of unresectable high-burden HCC.

Radiotherapy

External radiation historically had limited role in the management of HCC. This is mainly due to the radiotoxicity on the non-tumorous surrounding tissue. Radiation induced liver disease (RILD) is a common side effect of radiotherapy for liver cancer. In the RTOG 84-05 dose escalation study, among the patients receiving whole liver RT of 33 Gy in 1.5 Gy, around 10% of patients experienced RILD^[97].

However, with the recent advancements in irradiation technique, treatment modalities such as 3D-conformal RT (3D-CRT) and stereotactic body radiation (SBRT) have emerged as feasible options to treat high-burden HCC. With these technologies, high dose radiation can be effectively delivered to a precise area, sparing the surrounding normal liver tissue. This is particularly important for those patients with high-burden HCC who are not eligible for surgery or local therapies due to suboptimal liver reserve, anatomical locations of the tumors or poor performance status. Therefore, radiotherapy has become an attractive alternative in those cases.

Table 9. Recent studies on the efficacy of transarterial chemoembolization in the management of high-burden hepatocellular carcinoma

Year	Place	Authors	Number of patients (n)	Evaluation criteria	Time to progression (months)	Median survival (months)	Response rate (%)	Recruitment year
2010	European	Hilgard <i>et al.</i> ^[86]	108	EASL	10	16.4	40	-
2010	USA	Salem <i>et al.</i> ^[89]	291	WHO	7.9	BCLC-B: 13.3 BCLC-C: 6.0	42	-
2010	USA	Carr <i>et al.</i> ^[90]	99	WHO	7.9	11.5	41	-
2011	European	Sangro <i>et al.</i> ^[87]	325	-	-	12.8	-	-
2011	USA	Salem <i>et al.</i> ^[88]	123	WHO	13.3	20.5	49	1999-2008
2013	Italy	Mazzaferro <i>et al.</i> ^[92]	52	RECIST/WHO/EASL	11	15	40.4	2007-2009
2013	USA	Moreno-Luna <i>et al.</i> ^[93]	61	mRECIST	-	15	51	2005-2008
2015	Korea	Kim <i>et al.</i> ^[91]	40	mRECIST	18	-	63.8	2008-2010
2015	Germany	El Fouly <i>et al.</i> ^[94]	44	mRECIST	13.3	16.4	37%	2009-2011

Multiple retrospective studies, albeit small scale, have demonstrated the efficacy and safety of 3D-CRT and SBRT in treating high-burden HCC^[54,98-109] [Table 10]. The response rates of these two techniques ranged from 22% to 76.2%, and the 1-year survival rates ranged from 16.7% to 55%. Given that this group of patients are expected to be in much poorer conditions than those amenable to surgery or intra-arterial embolization, the results achieved are encouraging. However, there has been no direct comparison between 3D-CRT and SBRT, and variability of results was wide. Therefore, larger scale studies are needed to establish the role of RT in managing high-burden HCC.

Systemic therapy

Our definition of high-burden HCC excludes patients with extrahepatic metastasis, for whom systemic therapy would be the preferred option. However, even for patients without extrahepatic metastasis, when all the other treatment modalities fail, systemic therapy would be the last resort. In this section, we will discuss the systemic therapies which are applicable to high-burden HCC [Table 11].

Targeted therapy

Traditional systemic therapy has never been favored for a long time in treating advanced HCC due to its poor efficacy and the general cytotoxicity which preclude its application in this group of frail patients. It was only since 2008, we celebrated the introduction of sorafenib, a multikinase inhibitor, which has been demonstrated to prolong survival in two large randomized controlled trials^[110,111]. In the SHARP trial, the median survival of patients with advanced disease treated with sorafenib was 10.7 months, vs. 7.9 months in those who received placebo (hazard ratio 0.69, 95%CI: 0.55-0.87; $P < 0.001$). The Asia-Pacific trial was able to replicate similar findings, suggesting sorafenib to be an effective drug across patients with advanced HCC regardless of etiology and ethnicity.

Since then, much effort has been spent on exploring newer targeted therapies. Unfortunately, none of the trials in the past decade was able to identify a better targeted agent in treating advanced HCC^[112-116]. Only recently in 2017, Bruix *et al.*^[117] in the RESORCE trial has found regorafenib, an oral multikinase inhibitor that blocks angiogenesis, oncogenesis, metastasis and tumor immunity, to be an effective second line treatment for patients who have failed sorafenib. The median survival rate for patients on regorafenib after sorafenib use was 10.6 months compared to 7.8 months in the placebo group. The side effects associated with regorafenib use are typical of multi-kinase inhibitors, including hypertension, hand-foot skin reaction and gastrointestinal disturbances. Rate of drug-related adverse events leading to discontinuation of regorafenib is similar to that of sorafenib (10% vs. 11%)^[110,117]. Regorafenib thus has become the only clinically proven second line systemic drug available in sorafenib-resistant cases thus far.

Immunotherapy

Although targeted therapy seems to have hit a roadblock, other routes of development have been ongoing. Immunotherapy is the most notable one. Ever since the introduction of immune checkpoint inhibitors

Table 10. Recent studies on the efficacy of radiotherapy in the management of high-burden hepatocellular carcinoma

Year	Place	Authors	Method	Number of patients (n)	Dose/fraction	Evaluation criteria	1-year survival (%)	3-year survival (%)	Median survival (mos)	Response rate (%)	Recruitment year
2007	Japan	Toya <i>et al.</i> ^[103]	3DCRT	38	17.5-50.4 Gy; 1.8-4 Gy/Fr	mRECIST	39.4	-	9.6	44.7	1999-2005
2009	China	Huang <i>et al.</i> ^[83]	3DCRT	326	60 Gy; 2-3 Gy/Fr	-	16.7	-	3.8	25.2	1997-2005
2010	Korea	Oh <i>et al.</i> ^[104]	TACE + 3DCRT	40	30-54 Gy; 2.5-5 Gy/Fr	-	72	-	19	62.8	2006-2007
2012	Korea	Yoon <i>et al.</i> ^[108]	TACE + 3DCRT	412	21-60 Gy; 2-5 Gy/Fr	mRECIST	42.5	-	10.6	28.1	2002-2008
2013	Canada	Bujold <i>et al.</i> ^[101]	SBRT	102	30-54 Gy; 6 Gy/Fr	mRECIST	55	-	17	44	2004-2010
2013	Korea	Bae <i>et al.</i> ^[99]	SBRT	35	30-60 Gy; 3-5 Gy/Fr	mRECIST	52	21	14	41	2003-2011
2013	China	Tang <i>et al.</i> ^[54]	TACE + 3DCRT	185	30-52 Gy; 3-4 Gy/Fr	-	42.2	17.3	12.3	-	2006-2008
2014	Canada	Culleton <i>et al.</i> ^[100]	SBRT	29	19.7-46.8 Gy; 6 Gy/Fr	mRECIST	32.3	-	7.9	-	2004-2012
2014	Korea	Cho <i>et al.</i> ^[105]	TACE + 3DCRT	67	30-45 Gy; 2-4.5 Gy/Fr	-	-	-	14.1	-	2007-2011
2016	Japan	Matsuo <i>et al.</i> ^[98]	SBRT	43	45-55 Gy; 10-15 Gy/Fr	-	49.3	-	11	67	2008-2013
2016	Japan	Matsuo <i>et al.</i> ^[98]	3DCRT	54	45-50 Gy; 15-25 Gy/Fr	-	29.3	-	6	46	2008-2013
2016	Japan	Okazaki <i>et al.</i> ^[109]	3DCRT	56	22-50 Gy; 2 Gy/Fr	mRECIST	-	-	6.4	22	2007-2013
2017	Taiwan	Lo <i>et al.</i> ^[102]	SBRT	89	25-60 Gy; 4-6 Gy/Fr	-	45.9	24.3	10.9	76.2	2007-2015

TACE: transarterial chemoembolization

to cancer treatment, results of clinical studies have far exceeded expectation. In 2013, the journal *Science* has selected cancer immunotherapy as the Breakthrough of the Year^[118]. Cancer immunotherapy has been shown to be effective in treating cancers in multiple tissue organs, most notably lung cancer, melanoma and renal-cell carcinoma^[119-121].

Latest studies have demonstrated promising results in the application of immunotherapy in treating advanced HCC^[122,123]. Nivolumab, a PD-1 inhibitor, has been shown to prolong survival in patients with advanced HCC unsuitable for surgery or other local therapies^[123]. In an international phase 1/2 trial (CheckMate040), nivolumab was demonstrated to have an objective response rate of 15%-20% in patients with advanced HCC, irrespective of line of therapy^[123]. This was a significant improvement to the first-line sorafenib therapy, with a response rate of 2%-3%^[110], and the second-line regorafenib therapy, with a response rate of 7%^[117]. The overall 9-month survival rate was 74%, which showed a marked improvement compared to the median survival of 6 months for untreated advanced HCC.

Despite the relatively promising results shown in immunotherapy on HCC, studies so far conducted were relatively small scale. Larger scales are needed to evaluate the efficacy of immunotherapy on HCC.

ETIOLOGICAL ADJUNCTIVE TREATMENT FOR HIGH-BURDEN HCC

While we have discussed above the different treatment modalities available for high-burden HCC, it is also of paramount importance to control the underlying risk factors during treatment. By far, HBV and

Table 11. Clinical trials on systemic therapy in the management of advanced HCC

Drug name	Class	Trial name	Year	Authors	Phase	Case	Control	Result
Sorafenib	Oral multikinase inhibitor	SHARP	2008	Llovet <i>et al.</i> ^[110]	Phase 3	299	303	Median survival: 10.7 (sorafenib) <i>vs.</i> 7.9 months (placebo); $P < 0.001$
Sorafenib	Oral multikinase inhibitor	Asia-Pacific	2009	Cheng <i>et al.</i> ^[111]	Phase 3	150	76	Median survival: 6.5 (sorafenib) <i>vs.</i> 4.2 months (placebo); $P = 0.014$
Cabozantinib	Oral multikinase inhibitor	CELESTIAL	2012	Verslype <i>et al.</i> ^[134]	Phase 2	41	-	Granted orphan drug status by FDA
Ramucirumab	Anti-VEGF2 monoclonal	REACH	2015	Zhu <i>et al.</i> ^[112]	Phase 3	283	282	Median survival: 9.2 (ramucirumab) <i>vs.</i> 7.6 months (placebo); $P = 0.14$
Regorafenib	Oral multikinase inhibitor	RESORCE	2017	Bruix <i>et al.</i> ^[117]	Phase 3	379	193	Median survival: 10.6 (regorafenib) <i>vs.</i> 7.8 months (placebo); $P < 0.0001$
Tivantinib	Oral multikinase inhibitor	JET-HCC	2017	Kobayashi <i>et al.</i> ^[135]	Phase 3	134	61	Press release announced that the METIV-HCC phase 3 study did not meet its primary end point of improving survival
Lenvatinib	Oral multikinase inhibitor	REFLECT	2017	Cheng <i>et al.</i> ^[116]	Non-inferior study	478	476 (sorafenib)	Median survival: 13.6 (lenvatinib) <i>vs.</i> 12.3 months (sorafenib)
Ramucirumab	Anti-VEGF2 monoclonal	REACH (subgroup analysis)	2017	Zhu <i>et al.</i> ^[112]	Phase 3	CP-A and baseline AFP > 400 ng/mL: 68 CP-B and baseline AFP > 400 ng/mL: 52	CP-A and baseline AFP > 400 ng/mL: 83 CP-B and baseline AFP > 400 ng/mL: 48	Median survival: CP-A: 8.6 (ramucirumab) <i>vs.</i> 4.8 months (placebo); $P = 0.01$ Median survival: CP-B: 5.7 (ramucirumab) <i>vs.</i> 3.6 months (placebo); $P = 0.04$
Nivolumab	Immunotherapy	CheckMate 040	2017	El-Khoueiry <i>et al.</i> ^[123]	Phase 1/2	Dose escalation phase: 48 Dose-expansion phase: 214		Response rate of 83% in 6 months; 74% in 9 month in dose expansion phase

AFP: alpha-fetoprotein; HCC: hepatocellular carcinoma

HCV infections are the most important risk factors for HCC. Together, they account for 80% of the HCC worldwide^[124]. The use of antivirals not only reduces the incidence of HCC in viral carriers, it is also effective in reducing HCC recurrence and prolonging survival. This is because viral reactivation is a major complication of HCC treatment. Patients with high-burden HCC are particularly at risk of viral reactivation due to chronic immunosuppression, higher tumor load and poorer liver reserve. Uncontrolled viral reactivation may provoke acute hepatitis, fulminant liver failure and even death.

Evidence supporting the use of antivirals as adjunctive treatment of HCC has been reviewed elsewhere^[125,126]. In general, antivirals should be administered prior to treatment of HCC once the patient is known to be a virus carrier. For HBV-related HCC, the benefit of antivirals is seen in patients treated by surgery^[127], TACE^[128] or radiotherapy^[129]. For HCV-related HCC, evidence is available for older generation interferon-based antivirals that they reduce tumor recurrence^[130,131]. On the contrary, the newer generation of antivirals, e.g. direct-acting antivirals (DAA), have been shown to increase the chance of HCC recurrence^[132,133]. However, these studies had been criticized for being small scale, short duration of observation period and lacking a proper control group. Further studies thus are needed to elucidate the effectiveness of DAAs as adjunct in the treatment of HCV-related HCC.

DISCUSSION AND CLOSING REMARKS

Our definition of high-burden HCC focuses on the “grey zone” where tumors are neither metastasized nor localized enough to have an obvious choice of treatment modality. Though they carry a worse prognosis

than the classically defined intermediate-stage HCC, if the optimal treatment can be chosen for this group of patients, the impact on their survival rates can be significant. Results from various retrospective and cohort studies in the past decade have been encouraging, providing strong support for multimodality treatment in the management of high-burden HCC.

In this review, we showed that surgical approach to high-burden HCC, if feasible, provides the highest median survival across all treatment modalities. Nonetheless, there has not been a large-scale RCT that quantified its positive effect in managing high-burden HCC in direct comparison with other treatment modalities.

In cases where surgical resection is not feasible, intra-arterial embolization is commonly adopted as an alternative treatment modality. Thus far, studies have not been able to demonstrate a significant difference in survival between the two available intra-arterial embolization options, TACE and TARE. Overall, TARE appears to be superior in terms of providing a better safety profile and associating with fewer adverse outcomes. Nonetheless, it is a novel method for HCC and expertise might only be available in selective tertiary centers.

Advancements in irradiation technique have enabled radiotherapy to emerge as another unconventional treatment option for high-burden HCC. Early results in 3D-CRT and SBRT have been promising but further evidences are needed to delineate their role in managing high-burden HCC.

Targeted therapy has been in a bottleneck for treating high-burden HCC since the introduction of sorafenib. Regorfanib, now being the second-line agent to sorafenib, is the only newer targeted agent thus far that has been proved effective in managing high-burden HCC. On the other side, breakthroughs have been made in immunotherapy in the past decade with promising results with nivolumab and other immunostimulating agents. Many RCTs are underway to further establish the role of immunotherapy in managing HCC and we expect more results to emerge in the next few years.

As majority of the HCCs are attributed from HBV or HCV infection, the use of antivirals as adjunctive treatment is also of paramount importance. It can effectively reduce HCC recurrence and prolong survival. Despite early studies regarding use of DAAs in the treatment of HCV-related HCC suggest higher tumor recurrence rate, those studies have been heavily criticized of poor design. Further studies are needed to elucidate the role of DAAs as an adjunctive treatment for HCV-related HCC.

In summary, high-burden HCC remains a difficult cancer entity to manage. Yet, multiple treatment options are available of which optimal selection can effectively prolong survival for this group of patients. Treatment modalities are evolving in the management of high-burden HCC and promising results from retrospective and cohort studies are plentiful. But high-quality studies are lacking. Larger scale controlled studies with more specific patient selection criteria are needed for various treatment modalities, to further assess and compare the benefits of these different options.

DECLARATIONS

Authors' contributions

Conceptualization: Chan LL, Chan SL

Literature search: Chan LL

First draft: Chan LL

Revision and final proofread: Chan LL, Chan SL

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Review

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Direct antiviral therapy for hepatitis C and hepatocellular carcinoma: facing the conundrum

Federica Buonfiglioli, Stefano Brillanti

Research Center for the Study of Hepatitis, Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Bologna 40138, Italy.

Correspondence to: Prof. Stefano Brillanti, Research Center for the Study of Hepatitis, Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Via Massarenti 9, Bologna 40138, Italy. E-mail: stefano.brillanti@unibo.it

How to cite this article: Buonfiglioli F, Brillanti S. Direct antiviral therapy for hepatitis C and hepatocellular carcinoma: facing the conundrum. *Hepatoma Res* 2018;4:6. <http://dx.doi.org/10.20517/2394-5079.2017.42>

Received: 11 Sep 2017 **First Decision:** 7 Feb 2018 **Revised:** 8 Feb 2018 **Accepted:** 8 Feb 2018 **Published:** 10 Feb 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Direct antiviral therapy has dramatically changed our possibility to eradicate hepatitis C virus (HCV) infection in all stages of chronic liver disease, with sustained virological response rates well above 90%. HCV eradication should lead to a better prognosis even after cirrhosis has established, including a reduced risk of developing hepatocellular carcinoma (HCC). Unfortunately, during the last two years different reports have raised the concern about a possible increased risk of developing HCC in cirrhotic patients treated with direct antivirals. In this review, we have evaluated the principal published data and have reached a few conclusions: (1) direct antiviral therapy does not seem to increase the cumulative annual rate of HCC *de novo* occurrence or recurrence; (2) direct antiviral therapy seems to accelerate the development of HCC, soon after the end of treatment, in those patients at higher risk of HCC occurrence or recurrence; and (3) preliminary reports seem to indicate that HCC developed after direct antiviral therapy has more aggressive features. These findings clearly indicate the need for aggressive and close monitoring of cirrhotic patients during and after antiviral treatment, to detect and treat HCC at their earliest occurrence.

Keywords: Direct-acting antivirals, hepatocellular carcinoma, liver cirrhosis, risk, hepatitis C

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most frequent form of cancer worldwide, and it holds the second place in malignancy-related mortality^[1,2]. Incidence and death rates of HCC are steadily rising in most parts of the world (about 2%-3% per year).

Chronic hepatitis C is a necro-inflammatory process of the liver, due to hepatitis C virus (HCV) infection, that lasts lifelong and progresses to cirrhosis in about 20% of cases^[3]. Even if liver cirrhosis *per se* is not a



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pre-malignant lesion, it represents a pre-malignant condition since almost 90% of HCV-related HCC cases emerge after cirrhosis becomes established. The annual occurrence rate of HCC has been estimated to be around 3% in HCV-related cirrhosis^[4,5]. Surgical resection, radiofrequency ablation and transarterial chemoembolization allow effective treatment of single and small HCC in a significant proportion of patients with compensated liver disease, but recurrence is common, affecting about 35% of treated patients after 2 years^[6,7].

The aim of this review was to evaluate the effect of antiviral therapy on the *de novo* occurrence and recurrence of HCC in patients with chronic hepatitis C. We searched all available publications regarding “hepatitis C”, “HCC”, “antiviral therapy”, “interferon-free”, “DAA”, “occurrence”, “recurrence” and focused our review mainly on the data reported in high-quality full-text format.

EFFECT OF INTERFERON-BASED ANTIVIRAL THERAPY ON THE DEVELOPMENT OF HCC

Until 2011, peg-interferon alfa plus ribavirin combination was the only available therapy for chronic hepatitis C. This treatment had only 40%-50% probability of curing HCV infection, and the significant side effects contraindicated its use in a significant proportion of patients. Despite these limitations, many patients with compensated liver cirrhosis had been treated during the last decade, and the effect of treatment on the development of HCC has been evaluated. In summary, achieving sustained virological response (SVR) was associated with a reduced risk of developing HCC, in comparison with patients who did not obtain an SVR after antiviral therapy^[8-10]. Despite these positive results, it remains not clear whether SVR was independently associated with the reduced risk of developing HCC. In fact, a different explanation could be that SVR occurred in those patients with a lower spontaneous probability of developing HCC, without altering the cumulative risk of HCC in the entire population of cirrhotic patients. Also, even in patients who obtain SVR, a residual annual rate of HCC is still present, as high as 2% in different groups of patients.

THE ADVENT OF DIRECT-ACTING ANTIVIRALS AGAINST HCV

Since 2013, the therapy of hepatitis C has dramatically changed. Direct-acting antivirals (DAA) are new oral drugs, with potent antiviral activity against HCV infection, highly efficacious, relatively safe and well tolerated, that can be used in all categories of patients with chronic HCV infection, including those with more advanced and even complicated liver disease^[11]. This has allowed treatment of a huge cohort of patients with liver cirrhosis, obtaining the eradication of HCV infection in the vast majority of them. Resolution of HCV infection in these patients leads great expectations about the possibility of preventing the most serious complications of liver cirrhosis, including the development of HCC. In the following paragraphs, we try to summarize the best existing evidence regarding the effects of DAA-induced HCV eradication on the development of HCC in patients with compensated liver cirrhosis.

HCC DEVELOPMENT AFTER DAA THERAPY

The story learned from the interferon era teaches us that eradication of HCV infection is not sufficient *per se* to prevent HCC development after cirrhosis has been established. Due to the possibility of treating patients with more advanced liver disease, it is not surprising to expect that a few of them may develop HCC despite HCV eradication. This topic became immediately hot after the simultaneous publication of two papers from Spain and Italy suggesting a possible increased incidence of HCC after successful DAA treatment^[12,13]. Since those publications, more than 100 papers, letters or communications have been published addressing the problem, without conclusive results. Most of the debate derives from the heterogeneity of the different studied population, the inclusion and exclusion criteria, the time points used to analyse the incidence rates, the length of follow-up, and finally the radiologic methods used for the diagnosis of HCC.

Regardless of these discrepancies, it is possible to review the published results to draw some conclusions, but a few statements need to be addressed at first: (1) the concept of incidence; (2) the characteristics of the study population; (3) the starting point and the ending point of the observation period; and (4) the distribution of events during the follow-up.

Incidence is a measure of the probability of occurrence of a given condition in a population within a specified period. The incidence rate is the number of new cases per population at risk in a given time period. From this concept derives that to analyse the incidence rate of HCC after DAA therapy it is fundamental to define both the exact starting point and the exact ending point of the observation period. Only if these time points are comparable, different study results can be compared.

The study population should be at risk of developing the medical condition. Therefore, the risk should be comparable among different study groups before performing any comparison. Since in HCV-related liver disease HCC occurs almost exclusively in patients with liver cirrhosis, the population at risk should include only patients with advanced liver fibrosis (F4 according to the METAVIR classification).

In analysing the incidence rate of HCC after DAA therapy, we must distinguish between analysing the new *de novo* occurrence of HCC and the recurrence of a new HCC in patients with prior history of successfully treated HCC. In the former situation, the starting point should be the end of DAA therapy, in the latter, we must distinguish between considering as a starting point the time of the previous HCC treatment or the end of DAA treatment. In all cases, the ending point should be defined after DAA therapy end, and the interval from the starting point must be clearly assessed.

Another important point is the distribution of events (HCC) during the follow-up. It is known that during the natural history of liver cirrhosis the development of *de novo* incident HCC is not clustered around any specific time point^[12]. Similarly, HCC recurrence is generally not clustered around specific time points, even if recurrence rate is higher during the first two years after curative treatment of the neoplastic nodule^[6]. For this reason, the median interval between DAA therapy and HCC diagnosis needs to be analysed to assess the latency period between exposure to DAA therapy and HCC development.

WHAT PUBLISHED STUDIES TELL US

In Table 1, we have summarized the results of the principal studies addressing the *de novo* occurrence and/or recurrence of HCC in HCV-infected patients, with compensated liver cirrhosis, who have been treated with DAA therapy. Due to the heterogeneity of the study populations and the different observation periods, any formal meta-analysis seems of limited utility to draw any sound conclusion. It seems more important to note some common and peculiar aspects of the results.

At first, we must differentiate between the *de novo* occurrence of new HCC in cirrhotic patients without prior history of HCC and recurrence of HCC in patients with previously treated HCC. In studies analysing the former group of patients, the observation period after DAA therapy ranged a median of 6 to 14 months, indicating a relatively short follow-up. Despite this short observation period, *de novo* HCC occurred in 1.5% to 3.9% of patients. If we consider an expected annual rate of 2% to 3% in these subjects, we can conclude that HCC occurrence is certainly not reduced after DAA treatment. On the other hand, we have not strong elements to assume that the occurrence rate is increased, without a control group. Therefore, the argument of the incidence rate of new HCC after DAA therapy remains unsettled without a definite conclusion. In any case, a real increased annual incidence rate of HCC does not seem to happen after DAA treatment.

More intriguing data come from the studies on the recurrence of HCC after DAA treatment. The analysis of the recurrence rate must take into account the interval since previous HCC treatment, due to the higher

Table 1. Principal studies reporting detailed data on the occurrence and/or recurrence of HCC after DAA therapy in patients with liver cirrhosis

References	Prior history of HCC	No. of patients	Months between HCC treatment and DAA start (median)	Months of follow-up since DAA therapy (median)	HCC cases, n (%)	Months between DAA therapy and HCC (median)
<i>De novo</i> HCC occurrence						
Conti <i>et al.</i> ^[14] (2016)	No	285	NA	6	9 (3.2)	NR
Renzulli <i>et al.</i> ^[15] (2017)	No	285	NA	14.1	11 (3.9)	2.7
Kanwal <i>et al.</i> ^[16] (2017)	No	6690	NA	9	172 (2.6)	5.6
Bielen <i>et al.</i> ^[17] (2017)	No	273	NA	6	4 (1.5)	NR
HCC recurrence						
Conti <i>et al.</i> ^[14] (2016)	Yes	59	12.5	6	17 (28.8)	NR
Kolly <i>et al.</i> ^[18] (2017)	Yes	47	21.5	9.6	19 (40.4)	NR
Reig <i>et al.</i> ^[13] (2016)	Yes	58	11.2	5.7	16 (27.6)	3.5
Renzulli <i>et al.</i> ^[15] (2017)	Yes	59	12.5	14.1	18 (30.5)	2.8
Bielen <i>et al.</i> ^[17] (2017)	Yes	29	12	6	5 (17.2)	NR
ANRS cohorts ^[19] (2016)	Yes	152	22.8	20.2	24 (15.8)	NR

HCC: hepatocellular carcinoma; DAA: direct-acting antiviral; NA: not applicable; NR: not reported

HCC recurrence rate during the first 2 years after HCC therapy. The interval since previous HCC treatment ranged from 11 to 22 months. On the other hand, the post-DAA follow up period ranged from 6 to 20 months. During this observation period, the recurrence rate was in the range from 16% to 40%. Due to the relatively short post-DAA follow-up and the relatively long pre-DAA interval since previous HCC treatment, the recurrence HCC rate does not seem negligible at all. Even in this setting, we can conclude that DAA treatment does not reduce HCC recurrence. Again, we have not strong elements to assume that the recurrence rate is increased, without a control group. Therefore, also the argument of HCC recurrence rate after DAA therapy remains unsettled without a definite conclusion.

A striking finding seems to emerge in both settings: the short median latency period between the exposure to DAA and the diagnosis of HCC. This latency period was very short both in the HCC occurrence and in the HCC recurrence cases: from a minimum of 2.7 months to a maximum of 5.6 months. As stated in the methodology of the studies, all patients had no evidence of HCC when starting DAA treatment. Why HCC developed after such a short latency period represents an important question. There is no reason to explain the clustering of HCC development soon after the end of DAA treatment in the natural history of the disease. Different hypotheses have been postulated to support rapid development of HCC after DAA therapy. They are mainly based on the possible dysregulation of the anti-tumor response, after the brutal decrease of HCV viral load induced by DAA, and/or the perturbation of the immune surveillance, caused by a swift clearance of HCV^[20,21]. Despite the absence of conclusive biological explanations, these data clearly indicate the need for close imaging evaluations to detect early HCC development after DAA therapy in cirrhotic patients.

THE CHARACTERISTICS OF HCC DEVELOPED AFTER DAA THERAPY

In addition to the accelerated development of HCC after DAA therapy, additional alarming data have been published on the characteristics of the neoplastic nodules. Two preliminary reports suggested that after DAA therapy HCC may present aggressive macroscopic patterns^[22,23]. This aspect has been recently addressed by a full paper published in *European Radiology*^[15]. The authors compared the imaging features of HCC nodules developed after DAA therapy to those not occurred after DAA, in the same population. Surprisingly, despite being similar in number and size, neoplastic nodules developed after DAA treatment showed imaging features of microvascular invasion in the majority of cases. Microvascular invasion is a well-known predictor of recurrence and poor overall survival in HCC, and a major risk factor for early HCC recurrence after curative treatment. Additional recent data suggest that HCC occurring after interferon-free treatment show a rapidly growing pattern and moderately differentiated pathologic characteristics^[24]. For these reasons, HCC developed after DAA treatment seems to have a more aggressive pattern, predictive of more severe clinical

outcomes. Even if the clinical significance of these findings needs to be confirmed in additional prospective studies, these data corroborate the hypothesis of a different biologic pathway in the neoplastic process leading to HCC after DAA treatment.

CONCLUSIONS

In this review, we have analysed the published data on the risk of developing HCC after DAA therapy. Even if definite conclusions cannot be probably drawn, there is sufficient evidence to summarize the most important findings: (1) direct antiviral therapy does not seem to increase the cumulative annual rate of HCC *de novo* occurrence or recurrence; (2) direct antiviral therapy seems to accelerate the development of HCC, soon after the end of treatment, in those patients at higher risk of HCC occurrence or recurrence; and (3) preliminary reports seem to indicate that HCC developed after direct antiviral therapy has more aggressive features. These findings clearly indicate the need for aggressive and close monitoring of cirrhotic patients during and after antiviral treatment, to detect and treat HCC at their earliest occurrence.

DECLARATIONS

Authors' contributions

Both authors equally contributed to ideation and conduction of the review.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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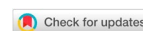
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Case Report

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Congenital absence of the portal vein complicated by hepatocellular carcinoma in the liver of an adult woman: review of imaging, literature and management

Ankit Mehta¹, Shree R. Venkat², Lorraine Portelance², Lynn G. Feun²

¹Truman Medical Center, Kansas City, MO 64108, USA.

²University of Miami Miller School of Medicine, Miami, FL 33136, USA.

Correspondence to: Dr. Shree R. Venkat, University of Miami Miller School of Medicine, 1475 NW 12th Ave, Suite C080, Miami, FL 33136, USA. E-mail: vshree@miami.edu

How to cite this article: Mehta A, Venkat SR, Portelance L, Feun LG. Congenital absence of the portal vein complicated by hepatocellular carcinoma in the liver of an adult woman: review of imaging, literature and management. *Hepatoma Res* 2018;4:7. <http://dx.doi.org/10.20517/2394-5079.2017.35>

Received: 4 Aug 2017 **First Decision:** 14 Sep 2017 **Revised:** 13 Jan 2018 **Accepted:** 18 Jan 2018 **Published:** 27 Feb 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

We present a case of absence of the portal vein and Laennec's cirrhosis in a 51-year-old female who was diagnosed with hepatocellular carcinoma (HCC). Only 101 cases of this malformation of the splanchnic vasculature have been reported of which 4 were reported to have HCC. Patient had disease progression while waiting for a liver transplant. Patient was treated with 3 separate conventional transarterial chemoembolization procedures at an outside hospital. At our institution, radioembolization of the right hepatic lobe was performed. She succumbed to liver insufficiency 8 years after being diagnosed with HCC. The features of this patient's clinical course are reviewed.

Keywords: Hepatocellular cancer, radioembolization, abernathy malformation

INTRODUCTION

The adult liver has a complex vascular architecture composed of two distinct circulatory systems. The liver is supplied by blood mostly from the portal vein (PV) and its intrahepatic branches, as well as the hepatic artery and its intrahepatic branches. The PV is responsible for carrying blood from the organs of the abdominal cavity such as the gastrointestinal tract, the spleen, pancreas, and biliary apparatus. In conventional anatomy, the splenic vein (SV) and the superior mesenteric vein (SMV) join to form the PV. The PV is then subdivided into right and left branches, which form small vessels throughout the liver that eventually drain into the sinus venosus^[1].



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During embryological development, the PV originates from the right and left vitelline veins between gestational weeks 4 to 10. There is selective involution and persistence of the peri-intestinal vitelline venous loops. The vitelline veins originally emerge from the yolk sac, cross the septum transversum, and drain into the sinus venosus. During the 3rd to 8th gestational week, abnormal patterns of involution and persistence may result in pre-duodenal, pre-biliary, or duplicated PV. Excessive involution can result in the absence of the PV as seen in type 1 portocaval shunts. Type 2 portocaval shunts may develop due to persistence of the right vitelline vein, where the shunt drains into the retrohepatic inferior vena cava (IVC), or the left vitelline vein, where the shunt drains into the suprahepatic IVC or right atrium^[2].

A London surgeon by the name of John Abernethy^[3] first described congenital absence of the PV in 1793 during a postmortem examination of a 10-month-old girl. Since, there have been 101 reported cases with 66% in women and most cases being in children. Most patients presented with encephalopathy, hepatopulmonary syndrome, or hepatorenal syndrome. Almost half of cases have liver masses at presentation such as focal nodular hyperplasia (FNH), adenomas, hepatoblastoma, or hepatocellular carcinoma (HCC)^[4].

In 1994, Morgan and Superina^[5] proposed a classification of portosystemic anomalies. Type 1 shunts are characterized by the absence of intrahepatic PV. Liver is not perfused with portal blood because of a complete shunt. A type 2 shunt is characterized as a partial shunt. The liver is perfused with portal blood in the presence of a partial shunt to systemic circulation. The type 1 shunts are subdivided into two further types, depending on the anatomy of the PV. The SV and the SMV drain separately into the IVC in a type 1a shunt. The SMV either drains into the IVC or the left renal vein. A confluence of SMV and SV is usually present in a type 1b shunt, but it does not supply the liver. While type 1 shunts are managed with liver transplant, type 2 shunts may be surgically ligated^[5].

In this report we will review a case of congenital absence of the portal vein (CAPV) in a 51-year-old woman who was diagnosed with HCC and had a history of Laennec's cirrhosis and a type 1b Abernethy malformation.

CASE REPORT

A 51-year-old female who was diagnosed in 2008 with HCC was referred to the interventional radiology clinic from the liver transplant service. She had been managed with conventional transarterial chemoembolization (c-TACE) on three separate occasions and she had signs of disease progression around the prior treated areas as marked by lipiodol. Imaging revealed PV agenesis (type 1b). Her clinical course was marked by Laennec's cirrhosis related to alcohol abuse complicated by occasional hepatic encephalopathy resulting in hospitalization. Limited pediatric history included only an episode of meningitis of unclear etiology and struggles with psychiatric illness. Histologic evaluation of liver parenchyma from a biopsy at presentation to transplant team revealed ballooning hepatocytes, mixed with collapsed hepatocytes, Mallory-Denk bodies, and glycogenated nuclei, which can be seen in the setting of alcoholic hepatitis. These were accompanied by bridging and pericellular fibrosis as seen after trichrome staining to the extent of stage 3 or severe fibrosis. A trial of sorafenib failed due to development of a rash, fatigue and weight loss. Social history was positive for prior alcohol abuse but patient stopped drinking 3 years after being diagnosed with HCC.

Interventional radiology was consulted for another TACE procedure to downstage her disease to allow for a transplant 6 years after HCC initial diagnosis. At that point her liver profile was: alkaline phosphatase 720 U/L, aspartate aminotransferase 69 U/L, total bilirubin 2.6 mg/dL, ammonia 30 μmol/L, albumin 3.0 g/dL. Her coagulation profile was normal (international normalized ratio was 1.02). Her alpha fetal protein (AFP) level was 357.1 ng/mL. Her Eastern Cooperative Oncology Group (ECOG) performance status was 0. Child Pugh score was B (8) therefore she had a expected 2-year overall survival of ~57%^[6].

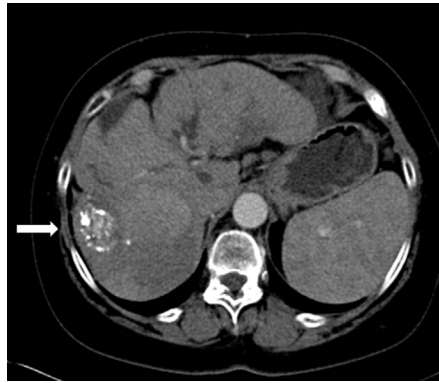


Figure 1. Hyper attenuating lesion from prior conventional transarterial chemoembolization containing lipiodol (white arrow)

Her physical examination was unremarkable notably without asterixis. She had no significant cardiac history (ejection fraction of 55% on stress test). Surgical history was non contributive. When she was presented in transplant tumor board, abdominal ultrasound showed several lesions in the liver. Computed tomography (CT) with contrast showed a mass identified in segments 6 and 7 measuring 4.6 cm × 4.0 cm × 4.5 cm with surrounding hypoattenuation of the liver parenchyma [Figure 1]. There was an additional hypodense lesion in segment 2 measuring 3.1 cm × 4.9 cm × 3.5 cm with some areas of hyperdensity. Both lesions were deemed to be changes secondary to prior TACE. Follow-up CT showed arterial enhancing lesions in the right liver lobe the dominant lesion had increased in size from 4.6 cm × 4.0 cm × 4.5 cm to 9.2 cm × 8.9 cm × 11.0 cm with washout, characteristics HCC findings [Figure 2]. Absence of the right and left PV and confluence of the SV and SMV into the IVC was also noted [Figure 3]. No collateral vessels to suggest cavernous transformation nor extrahepatic portal vein remnant can be seen. Based on the presence of multinodular disease without vascular invasion (although difficult to qualify given lack of PV), good performance status (ECOG 0), and liver function (Child Pugh B), her disease was classified as intermediate stage disease by the Barcelona Clinic Liver Classification (BCLC) system or BCLC B^[7].

At that point recommendation from the liver multidisciplinary tumor board was to repeat TACE. Yet, at the time of her evaluation in the interventional radiology clinic, TACE was not offered due to increased risk for abscess formation and progressive liver dysfunction. After referral to the oncology team, she received two intra-arterial chemoinfusions of cisplatin into the proper hepatic artery. Patient's disease continued to progress as markedly elevated AFP of 8779. Despite risk of hepatotoxicity and elevated lung shunt of 21%, she subsequently underwent radioembolization to the right lobe of the liver. She received a dose of 1.06 GBq (29.1 mCi) of Yttrium-90 (Y-90) embolic resin spheres delivered to the right lobe of the liver.

Three months follow-up CT scan showed dramatic partial response with no further enhancement in the dominant mass [Figure 4]. Incongruent to imaging findings AFP increased dramatically normalizing approximately 9 months after treatment [Table 1]. Unfortunately liver dysfunction was exacerbated due to treatment [Table 1] consistent with radiation embolization induced liver disease (REILD). Only further treatment received was octreotide and supportive care. She passed away after struggling with depression 8 years after initial diagnosis of HCC and 22 months after radioembolization therapy.

The determination of PV agenesis in this case was by imaging features only. No surgical or histopathological confirmation is available despite patient's ultimate demise. To the knowledge of the authors' no autopsy was performed.

DISCUSSION

In the case presented the mesenteric venous system and the SV joined to form a confluence of vessels yet this confluence drained directly into the suprarenal IVC. This malformation can be attributed to the embryological development of the portal venous system. This would be classified as type 1b shunt.

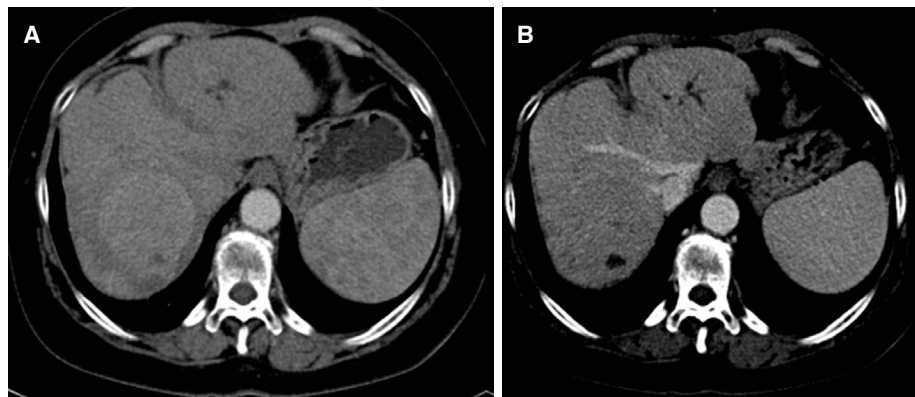


Figure 2. Contrast enhanced computed tomography revealing a 9.2 cm × 8.9 cm × 11.0 cm mass in the right lobe of the liver. (A) Arterial phase shows diffuse enhancement; (B) venous phase shows washout

Table 1. Changes of total bilirubin and AFP in reference to radioembolization

Component	Bilirubin (mg/dL)	AFP (ng/mL)
Latest reference	0.0-1.2	0.0-8.3
21 months after radioembolization	12.7	6.7
12 months after radioembolization	13.9	20.7 (H)
11 months after radioembolization	13.8	55.3 (H)
10 months after radioembolization	8.4	178.2 (H)
9 months after radioembolization	6.9	805.4 (H)
7 months after radioembolization	6.9	5450.0 (H)
6 months after radioembolization	7.4	19,394.0 (H)
4 months after radioembolization	7.4	> 60,500.0 (H)
3 months after radioembolization	4.8	55,658.0 (H)
2 weeks after radioembolization	2.9	18,662.0 (H)
1 month prior to radioembolization	2.1	8779.0 (H)
2 months prior to radioembolization	1.8	3678.0 (H)
4 months prior to radioembolization	1.6	1032.0 (H)

AFP: alpha fetal protein; H: high

There are currently 101 reported cases of CAPV. Of the reported cases, 66% of patients are females and about 70% had been diagnosed by age of 18 years; < 10% were associated with a type 2 malformation^[4]. This patient presented to our institution to undergo liver transplant evaluation. Additional associated anomalies such as congenital heart disease were absent in this case.

CAPV is associated with hepatic tumors. Hepatic changes such as FNH, HCC and hepatoblastoma were seen in 40% of cases^[1]. In this case, the patient presented with HCC. Research has shown that insulin, glucagon, and epidermal growth factor are delivered to the liver through the splanchnic venous system. These substances are vital for the hepatic regeneration. Therefore, it is suggested that absence of PV flow may result into abnormal hepatic development, function, and regenerative capacity as seen in this patient. Increased arterial hepatic flow may subsequently play a role in the development of hepatic neoplasms^[8].

To date, 4 cases of patients with CAPV have been reported to have HCC^[1]. One case was reported in a 14-year-old female, however nature of the review focused on intestinal flora compensating to result in normal ammonia levels rather than tumor description and presentation^[9]. In 2001, Lundstedt *et al.*^[10] reported a case of asymptomatic CAPV (type 1b shunt) found at time of resection of a 12-cm HCC thought to have arisen secondary to hepatitis B virus in a 51-year-old male. The patient remained disease free over 2-year follow-up period^[10]. Unlike our patient, there was no history of encephalopathy. Only the aspartate transaminase and alanine transaminase were mildly elevated^[10]. Morotti *et al.*^[11] reported a case of an 8-year-old female with Turner syndrome who was found to have CAPV at time of transplant. Liver transplantation was

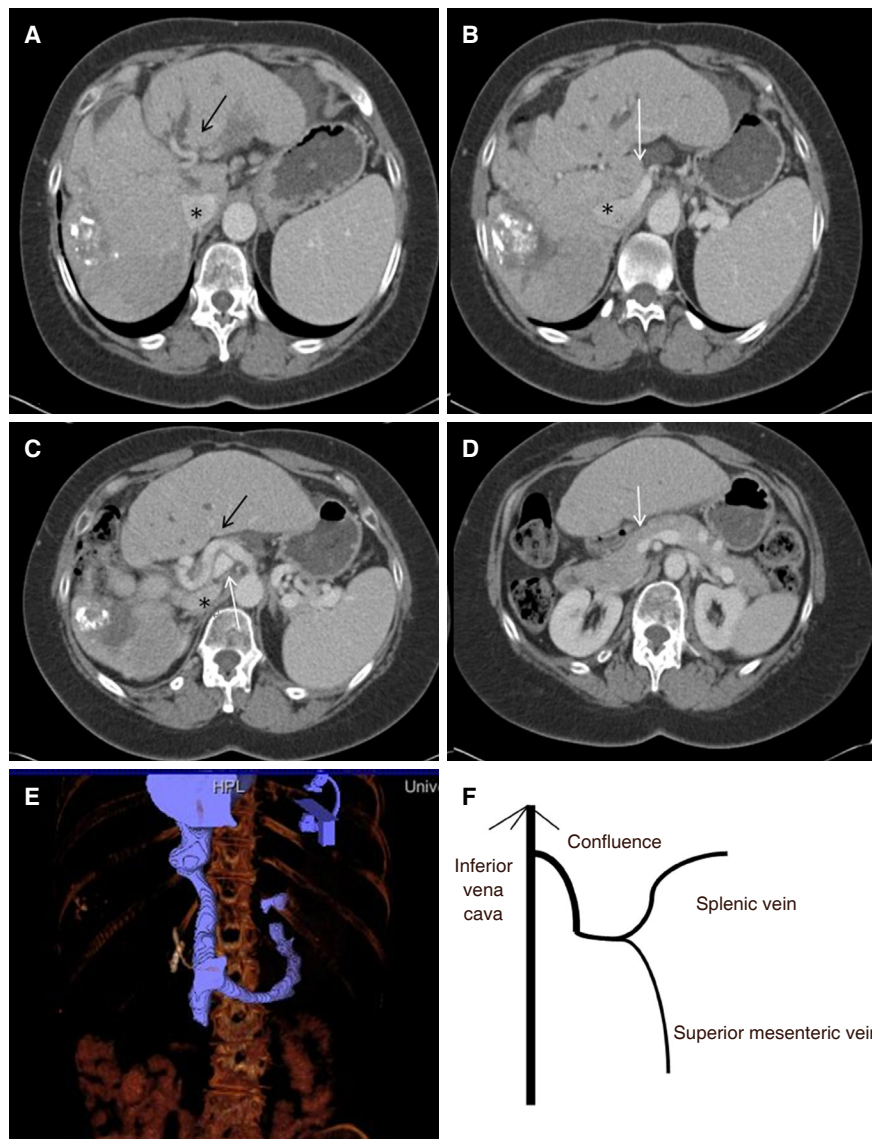


Figure 3. Type 1B Abernathy malformation. (A-D) Axial computed tomography images showing course of hepatic artery (black arrow), confluence of superior mesenteric vein and splenic vein (white arrow), inferior vena cava (asterisk), and the superior mesenteric artery (white arrow); (E) coronal reformats of findings; (F) diagram of malformation

performed due to liver dysfunction (bilirubin 13.9 mg/dL) and concern for enlarging right and left liver lesions originally shown on biopsy to be FNH^[11]. Explant specimen revealed well-differentiated HCC^[11]. It was suggested that the combination of the hormonal therapy for Turner syndrome, and vascular anomaly may have contributed to the development of HCC^[11]. Pichon *et al.*^[12] noted PV absence on an ultrasound (US) for a 36-year-old female undergoing evaluation for abdominal pain and follow-up of liver masses. The SMV and SV were found on indirect venogram at angiography and surgical evaluation to have direct but separate drainage into the IVC consistent with a type 1a shunt^[12]. A 12-cm dominant right HCC surrounded by small peripheral nodules were noted in the right hepatectomy specimen and the patient did well for the course of 2-year follow-up period^[12]. As in the case presented here, the CAPV was found incidentally while undergoing evaluation for management of HCC.

How the absence of the PV effects imaging features and resultant diagnosis of HCC is unknown. Detection of the PV abnormality can be done with US, CT or magnetic resonance imaging (MRI). The former has the benefit of no radiation, but detecting alternate shunts is difficult with US. For this reason CT or MRI

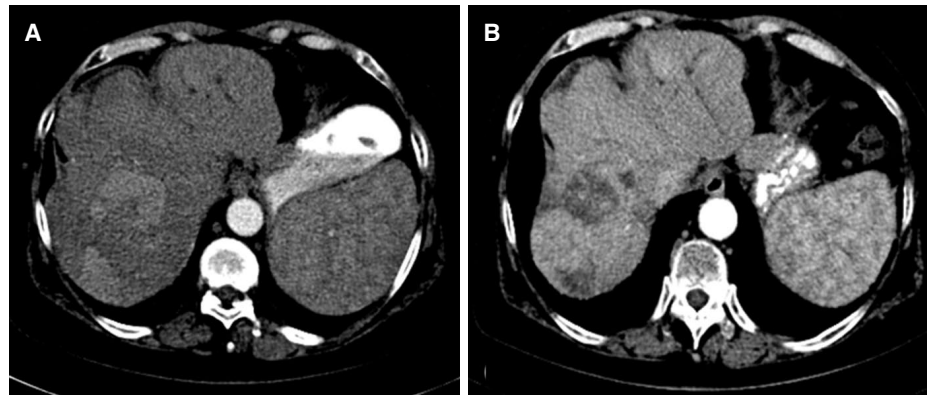


Figure 4. Follow-up images. (A) Computed tomography (CT) scan in 3 months demonstrates partial response with decreased enhancement in the central aspect of the dominant mass; (B) CT scan in 18 months shows dramatic partial response with atrophy of the treated right lobe

is preferred to trace the course of the SMV and SV^[13]. It has been shown that PV thrombosis can result in parenchyma perfusion changes readily concealing the presence of tumor on enhanced images^[14]. Hyperintensity on T2 and diffusion sequences can indicate the presence of HCC, particularly infiltrative HCC with corresponding hypointensity in comparison to liver parenchyma on T1 sequences^[14]. It has been shown in the setting of PV thrombosis, arterial hypervascularity is not well perceived likely due to increased arterial supply to background liver parenchyma^[15]. Washout however on portal venous phases was apparent in majority of these cases^[15]. Washout kinetics are poorly understood but may be related to the proportion of intravascular space to interstitial space which is greater in tumor or increased arterial pressure leading to decreased intra-tumoral portal venous blood supply^[15-18]. In our case though heterogeneous appearance on arterial phase, both arterial enhancement and washout on venous phase were apparent.

Liver dysfunction has been reported in most cases of CAPV. Our patient had a background of Laennec's cirrhosis related to alcohol abuse as well as hepatic encephalopathy. Her liver function as discussed in the case presentation fluctuated throughout her clinical course.

The patient discussed in this case also had a history of fractures and osteopenia. Osteopenia and osteoporosis are important and common complications of chronic liver disease, receiving the generic definition of hepatic osteodystrophy (HO). The development of HO may be due to both increased bone resorption and decreased bone formation. Pathogenic mechanisms are diverse and very little is known about some of them: genetic factors, alterations in calcium-vitamin D metabolism, hyperbilirubinemia, and vitamin K and insulin-like growth factor-1 deficiency^[19].

The prognosis of patients affected with CAPV generally depends on associated heart and liver anomalies in infancy. Long-term prognosis depends on the control of hepatic dysfunction and metabolic irregularities. Forty-six cases have been reported to be associated with a congenital anomaly, of which 16 were congenital cardiac disease^[4]. Congenital cardiac disease typically seen with CAPV includes: patent foramen ovale, patent ductus arteriosus, ventral septal defects, and atrial septal defects^[1].

Liver transplantation has been performed to effectively treat symptomatic patients with congenital agenesis of the PV. CAPV should not be considered a contraindication to hepatic transplantation^[1]. During an orthotopic liver transplant, the congenital portocaval shunt can be divided while repairing the caval defect and performing a PV anastomosis^[20]. Patients with hyperammonemia, portosystemic encephalopathy, hepatopulmonary syndrome, or hepatic tumors may benefit dramatically from liver transplant. Cases have been reported where transplantation has successfully reversed the hepatopulmonary syndrome caused by the Abernathy malformation as well^[20]. Other treatment modalities include balloon-occluded retrograde transvenous obliteration, embolization of shunt with coiling, and surgical modification of shunts^[4].

Unfortunately at time of consultation in our institution the patient presented here was not eligible for transplant given the extent of HCC. This prompted multidisciplinary care focused downstaging her cancer. Radioembolization in the setting of PV thrombosis has been shown to be as effective and better tolerated than TACE as PV thrombosis increases risk of necrosis^[21,22]. Given progression after c-TACE, and lack of portal supply, radioembolization was favored as a treatment. It is suggested in some cases that a background of cirrhosis can protect from such injury^[22]. In this case, c-TACE had already been performed at an outside facility. TACE has been shown to be safe and effective in patients with advanced or BCLC C disease which includes patients with varying degrees of PV thrombosis^[23,24]. However, PV thrombosis does portend a poorer prognosis^[24]. As anticipated post embolization syndrome is the most common side effect reported post TACE, while encephalopathy was found in approximately 5% of patients, and elevated liver function tests as high as 20% of cases^[23]. In a comparison, c-TACE and drug eluting bead TACE had similar safety profiles and survival rates comparable to treatment with sorafenib^[23].

The use of AFP as an oncologic marker of response to loco-regional therapy for HCC has been proven to be effective^[25]. The median time to response has been reported to be between 2 and 4 months therefore it has been suggested that AFP used to identify patients who do not respond to treatment and prompt earlier consideration of implementation of alternative strategies^[25]. Cases such as the one discussed here where there is a dramatic increase in AFP despite imaging response with delayed response in the marker (~7 months) have not widely been reported. Elevated levels of AFP have been seen in the setting of hepatic necro-inflammatory activity, which could lead to over production of AFP^[26]. This may explain the incongruent increase in AFP initially with delayed response in this case.

Despite dramatic imaging response and eventual decline of AFP, the patient developed REILD, which results from normal hepatic parenchyma exposure to radiation. The clinical course is driven by a form of sinusoidal obstruction syndrome marked by jaundice, ascites and mild increase in liver function tests. After 3 months bilirubin can rise to 3 or higher^[27]. The incidence of REILD is reported to be between 0%-4% overall^[28]. The patient was known to be at higher risk given decompensated liver function in the past therefore lobar approach was selected. However in patient with cirrhosis REILD has been noted in 0%-33% of patients who underwent whole liver treatment and 8%-15% in patients who underwent partial liver treatment^[27]. Management as in this case is supportive.

In this case the patient did derive a survival benefit from radioembolization. Patients with intermediate stage HCC are expected to have a median survival of 16 months from time of diagnosis^[29]. After radioembolization the patient survived another 22 months. The biology of her disease suggests that initial disease was less aggressive given that she survived 8 years beyond diagnosis.

In conclusion, congenital agenesis of the PV is a rare congenital anomaly due to abnormal embryologic progression. The prognosis of patients affected with CAPV can vary depending on associated heart and liver anomalies in infancy or the progression of hepatic dysfunction. Those patients are at risk of developing HCC. Liver transplantation has been effective in patients with hepatic dysfunction. When transplantation cannot be offered loco-regional therapy can offer palliative disease control and improved overall survival. However liver directed therapy in this population could be associated with increased risk of liver failure.

DECLARATIONS

Authors' contributions

Read and wrote the initial report: Mehta A

Corrected and amended areas related to imaging, intervention, and follow up: Venkat SR

Focused on the radiation aspects in terms of response and editing of clinical presentation: Portelance L

Helped review and highlighted oncology considerations: Feun LG

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Patients consent could not be obtained.

Ethics approval

While IRB approval at our institution is not required for a solitary case presentation, the data is collect as part of an IRB approved prospective database through Interventional Radiology.

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Original Article

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Prediction of post-progression survival in patients with advanced hepatocellular carcinoma treated with sorafenib by using time-dependent changes in clinical characteristics

Yoshiyuki Wada, Yuko Takami, Hajime Matsushima, Masaki Tateishi, Tomoki Ryu, Munehiro Yoshitomi, Hideki Saitsu

Department of Hepato-Biliary-Pancreatic Surgery, Clinical Research Institute, National Hospital Organization Kyushu Medical Center, Fukuoka 810-8563, Japan.

Correspondence to: Dr. Yoshiyuki Wada, Department of Hepato-Biliary-Pancreatic Surgery, Clinical Research Institute, National Hospital Organization Kyushu Medical Center, 1-8-1 Jigyohama, Chuo-ku, Fukuoka 810-8563, Japan. E-mail: yoshiwada@kyumed.jp

How to cite this article: Wada Y, Takami Y, Matsushima H, Tateishi M, Ryu T, Yoshitomi M, Saitsu H. Prediction of post-progression survival in patients with advanced hepatocellular carcinoma treated with sorafenib by using time-dependent changes in clinical characteristics. *Hepatoma Res* 2018;4:8. <http://dx.doi.org/10.20517/2394-5079.2017.39>

Received: 1 Sep 2017 **First Decision:** 20 Sep 2017 **Revised:** 19 Feb 2018 **Accepted:** 24 Feb 2018 **Published:** 6 Mar 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Aim: Sorafenib has been shown to improve time to tumor progression (TTP) and overall survival (OS) in patients with hepatocellular carcinoma (HCC); however, post-progression survival (PPS) has not been well characterized in these patients. This study aimed to evaluate the predictors of PPS by using time-dependent and dynamic changes in radiologic progression patterns, liver function, and performance status (PS) in patients with advanced HCC receiving sorafenib treatment.

Methods: We retrospectively analyzed the clinical characteristics of 128 advanced HCC patients with Child-Pugh scores M 7 at the initiation of sorafenib treatment.

Results: The median TTP, OS, and PPS were 3.8, 15.6, and 9.9 months, respectively. At the time of confirmation of radiologic progressive disease (PD), a total of 46 (35.6%) patients showed impairments in their PS of $\geq +1$ points over time. For the Child-Pugh score, 27 (21.1%) and 26 (10.9%) patients exhibited an impairment of $\geq +1$ and $\geq +2$ points, respectively. Multivariate analysis identified the following independent predictors of PPS: impairment in the PS score of $\geq +1$ point [hazard ratio (HR) 1.81, 95% confidence interval (CI) 1.16-2.82], impairment in the Child-Pugh score of $\geq +2$ points (HR 3.70, 95% CI 1.68-8.15), radiologic pattern of progression (target lesion growth and emergence of a new lesion) (HR 2.91, 95% CI 1.79-2.91), a TTP < 4 months (HR 1.87, 95% CI 1.21-2.91), second-line treatment after radiologic confirmation of PD (HR 0.16, 95% CI 0.08-0.32), and continuous sorafenib treatment after radiologic confirmation of PD (HR 1.76, 95% CI 1.06-3.00).



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Conclusion: PPS in patients with advanced HCC can be characterized by using time-dependent dynamic changes in clinical parameters.

Keywords: Contactin-associated protein-2, Isaac, neuromuscular hyperexcitability, neuromyotonia, voltage-gated potassium channel

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third common cause of cancer-related deaths worldwide. Surgical resection, liver transplantation, and ablation therapy are curative therapeutic treatments for early-stage HCC, and transcatheter arterial chemoembolization (TACE) is recommended for patients with intermediate-stage HCC who have preserved liver function^[1,2]. However, most HCC patients are diagnosed during the advanced stage of the disease; their prognosis is poor, and treatment options are limited^[1-3]. In patients who are not candidates for locoregional therapy, the oral multikinase inhibitor sorafenib has been the only systemic treatment option. Sorafenib inhibits tumor cell proliferation and tumor angiogenesis by inhibiting multiple signaling pathways. It has been shown to prolong both, progression-free survival and overall survival (OS) in patients with advanced HCC^[4,5]. Since the Sorafenib HCC Assessment Randomized Protocol (SHARP) trial showed the efficacy of sorafenib for prolonging survival in HCC patients almost 10 years ago^[4], all phase 3 trials of novel systemic drugs have failed to improve outcomes over sorafenib, both, as first-line^[6-10] and second-line treatments (following sorafenib)^[10-12]. Predicting the efficacy is difficult in sorafenib treatment, and no surrogate marker has been identified^[11-13]. Since tumor progression is a dynamic process, it may be difficult to identify predictors for survival by analyzing clinical characteristic at one static data point. Using dynamic data might help clarify the predictors of survival.

A recent regorafenib for patients with HCC who progressed on sorafenib treatment (RESORCE) study^[14] has revealed that regorafenib prolonged survival in patients with advanced HCC who were refractory to sorafenib treatment. The inclusion criteria in this study were a Child-Pugh score ≤ 6 and tolerability of sorafenib (≥ 400 mg daily for at least 20 of the 28 days before discontinuation). Based on this, the number of candidates for second-line treatment with regorafenib is likely very limited. Analyzing post-progression survival (PPS) after sorafenib treatment is desired to select candidates for second-line treatment.

In this study, we used dynamic and time-dependent data on the clinical characteristics of patients with advanced HCC, including progression patterns, impairments in liver function, and performance status (PS). Importantly, we assessed changes in these parameters by comparing them at the time of radiologic confirmation of progressive disease (PD) to baseline (the initiation of sorafenib treatment) to evaluate PPS.

METHODS

Patients

We reviewed data that were prospectively collected from 171 consecutive patients who received sorafenib (Nexavar; Bayer HealthCare Pharmaceuticals, West Haven, CT, USA) for the treatment of advanced HCC at the Department of Hepato-Biliary-Pancreatic Surgery at the National Hospital Organization Kyushu Medical Center between June 2009 and July 2016. Of these, 135 patients had radiologic PD, as assessed by the modified Response Evaluation Criteria In Solid Tumors (mRECIST)^[15]. After excluding 7 patients with a Child-Pugh score ≥ 8 , 128 patients were enrolled in the study.

HCC was diagnosed based on the results of a pathological examination or a combination of specific radiologic findings obtained via contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI) according to the criteria of the American Association for the Study of Liver Diseases^[2].

Of 128 patients, 116 were diagnosed with HCC based on the results of a pathological examination. The remaining 12 patients showed specific radiologic findings according to the criteria of the AASLD and elevated serum α -fetoprotein (AFP) levels. No patients with differentiated intracholangiocellular carcinoma and mixed-form liver cancer were included in this study.

Sorafenib was administered to patients with advanced HCC if: (1) they were not eligible for or their disease progressed after surgery, locoregional therapy, or TACE; (2) their ECOG PS was 0-1; (3) their liver function was classified as Child-Pugh A or B; and (4) they had adequate hepatic function (albumin > 2.5 g/dL, total bilirubin < 3.0 mg/dL, and alanine aminotransferase and aspartate aminotransferase levels < 5 times the upper limit of the normal range). Radiologic tumor progression was confirmed by contrast-enhanced CT or MRI. The starting dosage of sorafenib was 800 mg/day p.o. However, considering the possibility of having to discontinue sorafenib treatment at an early stage due to adverse events, the initial dosage for patients with comorbidities was reduced to 400 mg/day. Moreover, the initial dosage for patients aged ≥ 75 years, those with a body weight ≤ 40 kg, and those with a history of treatment for varices or ascites was 200-400 mg/day. The dose was increased to the standard dose according to each patient's tolerance. Treatment was continued until tumor progression, unacceptable toxicity associated with sorafenib, or withdrawal of consent. Second-line treatments after radiologic confirmation of PD included continuous sorafenib treatment, even in palliative patients upon their request. However, patients with Child-Pugh C or a PS > 2 at the time of confirmation of PD received best supportive care.

This study was approved by the Ethics Committee of the National Hospital Organization Kyushu Medical Center and performed in compliance with the Declaration of Helsinki. All patients provided written informed consent for sorafenib treatment.

Assessments

Tumor measurements were performed at baseline and every 2 months during treatment by contrast-enhanced CT or MRI. Patients visited the clinic every 2 to 4 weeks to assess treatment compliance and adverse effects. The survival status of the study participants was obtained from hospital records. Local response was determined by the mRECIST criteria^[15]. We assessed the cause of progression (patterns of progression) based on the following: 20% increase in tumor size against a known baseline lesion or the emergence of a new lesion.

Follow-up

All patients were followed-up at our outpatient clinic according to a standardized protocol that included tumor marker tests every month and MDCT or MRI every 8 weeks until the patient's death or last visit.

Statistical analysis

Statistical analyses were conducted using the JMP version 11.0 software (SAS Institute, Cary, NC, USA). Categorical variables were analyzed with the Chi-square or Fisher's exact test, as appropriate. Continuous variables were analyzed using the Student's *t*-test or the Mann-Whitney *U* test, as appropriate. Time to tumor progression (TTP), OS, and PPS were evaluated through the Kaplan-Meier method, and comparisons between groups were performed using the log-rank test. Univariate and multivariate analyses were performed using a Cox proportional hazards model and the backward elimination procedure. A *P*-value of < 0.05 was considered significant.

RESULTS

Baseline characteristics of the patients

The average age of the 128 study participants (105 men and 23 women) was 68.9 years [Table 1]. Most (*n* = 100) had a PS of 0. Regarding the preservation of liver function, 71, 43, and 14 study participants had

Table 1. Characteristics at the initiation of sorafenib treatment

Variables		n = 128
Age, year		68.9
Gender	(male/female)	105/23
Etiology	HBV/HCV/NBNC	21/86/21
ECOG PS	0/1	100/28
Child-Pugh score	5/6/7	71/43/14
Extrahepatic spread		65 (51.2%)
Macrovascular invasion		29 (23.6%)
BCLC stage	B/C	34/94
Starting dose of sorafenib	800/600/400/200	26/0/83/16
AFP (ng/mL)	(median, IQR)	55.3 (8.4-469)
DCP (mAu/mL)	(median, IQR)	92.5 (22-1877)

HBV: hepatitis B virus; HCV: hepatitis C virus; NBNC: non B non C; ECOG: Eastern Cooperative Oncology Group; PS: performance status; BCLC: Barcelona Clinical Liver Cancer; AFP: a-fetoprotein; DCP: des-g-carboxy prothrombin; IQR: interquartile range

Table 2. Change of clinical parameters at the time of confirmation of radiologic progressive disease compared with those of the initiation of sorafenib treatment

Variables		At the confirmation of radiologic PD
Impairment of PS score	≥ +1	46 (35.9%)
	≥ +2	14 (10.9%)
Impairment of Child-Pugh score	≥ +1	27 (21.1%)
	≥ +2	26 (20.3%)
Time to progression	≥ 4 months	60 (46.9%)
Radiologic progression pattern	Target lesion growth	63 (49.2%)
	New lesion	19 (14.8%)
	Target lesion growth and new lesion	46 (35.9%)

PD: progressive disease; PS: performance status

a Child-Pugh score of 5, 6, and 7, respectively. Whereas 65 patients presented with extrahepatic spread, 29 showed macrovascular invasion. At total of 34 and 94 study participants had Barcelona Clinical Liver Cancer (BCLC) stages B and C, respectively.

Second-line treatment after radiologic confirmation of PD

At the time of the radiologic confirmation of PD, 96 (75.0%) patients received subsequent second-line treatment. Of 96 patients who underwent subsequent treatment, 59 received continuous sorafenib treatment, 17 underwent TACE, 8 took part in clinical trials, 5 received hepatic arterial infusion chemotherapy, 5 underwent systemic chemotherapy, and 2 received radiotherapy.

TTP, OS, and PPS

The median TTP and OS were 3.8 months [95% confidence interval (CI), 3.2-4.4] and 15.6 months (95% CI, 12.4-18.5), respectively. The median PPS was 9.9 months (95% CI, 7.6-12.9). The TTP in this study was similar to those reported in the SHARP and AP trials.

Changes in clinical characteristics between baseline (the initiation of sorafenib treatment) and confirmation of radiologic PD

We then assessed the dynamic changes in the patients' clinical characteristics and compared them at baseline (the initiation of sorafenib treatment) to the confirmation of radiologic PD [Table 2]. A total of 46 (35.6%) and 14 (10.9%) patients showed impairments in their PS of ≥ +1 and ≥ +2 points over time, respectively. For the Child-Pugh score, 27 (21.1%) and 26 (20.3%) patients exhibited an impairment of ≥ +1 and ≥ +2 points, respectively. When we assessed the radiologic patterns of progression, 63, 19, and 46 patients showed target lesion growth only, emergence of a new lesion only, and both, target lesion growth and emergence of a new

Table 3. Predictive factors for post-progression survival

Variables		Univariate		Multivariate	
		HR (95% CI)	P value	HR (95% CI)	P value
Age, year	≥ 75	1.11 (0.72-1.66)	0.64		
Gender	Male	1.07 (0.67-1.79)	0.79		
Hepatitis B infection	Yes	0.95 (0.56-1.53)	0.84		
Hepatitis C infection	Yes	0.87 (0.58-1.32)	0.5		
Impairment of PS	≥ +1 point	2.14 (1.45-3.17)	< 0.001	1.81 (1.16-2.82)	0.01
	≥ +2 points	8.54 (4.31-16.12)	< 0.001	1.12 (0.47-2.62)	0.79
Impairment of Child-Pugh score	≥ +1 point	2.21 (1.48-3.28)	< 0.001	1.10 (0.64-1.99)	0.73
	≥ +2 points	4.82 (2.93-7.68)	< 0.001	3.70 (1.68-8.15)	< 0.01
Extrahepatic spread	Yes	1.4 (0.94-2.08)	0.15		
Macrovascular invasion	Yes	2.01 (1.25-3.13)	0.03	1.08 (0.58-1.96)	0.80
BCLC stage	C	1.83 (1.19-2.90)	< 0.01	1.33 (0.80-2.23)	0.27
Radiological progression pattern	Growth + new	3.21 (2.09-4.91)	< 0.001	2.91 (1.79-4.76)	< 0.001
Time to tumor progression	< 4 months	2.25 (1.52-3.35)	< 0.001	1.87 (1.21-2.91)	0.01
Second-line treatment post-PD	Yes	0.12 (0.07-0.20)	< 0.001	0.16 (0.08-0.32)	< 0.001
Continuous sorafenib treatment post-PD	Yes	0.67 (0.45-0.98)	0.04	1.76 (1.06-3.00)	0.03
Decline of serum AFP level 2 weeks after starting sorafenib	> 20%	1.19 (0.72-1.89)	0.48		

BCLC: Barcelona Clinical Liver Cancer; AFP: a-fetoprotein; PD: progressive disease; PS: performance status; HR: hazard ratio; CI: confidence interval

lesion, respectively. Of 34 patients with BCLC-B, 4 progressed to BCLC-C at the time of confirmation of radiologic PD based on new extrahepatic spread ($n = 3$) or occurrence of a portal tumor thrombus ($n = 1$).

Prediction of PPS

Univariate analysis revealed a significant correlation between PPS and the following parameters in patients with radiologic PD: impairments in the PS score of $\geq +1$ and $\geq +2$ points, a Child-Pugh score of 8, impairments in the Child-Pugh score of $\geq +1$ and $\geq +2$ points, macrovascular invasion, radiologic patterns of progression, a TTP of ≤ 4 months, subsequent treatment post-PD, and continuous sorafenib treatment post-PD [Table 3]. Multivariate analysis identified the following independent predictors of PPS in patients with radiologic PD: impairment in the PS score of $\geq +1$ point [hazard ratio (HR) 1.81, 95% CI 1.16-2.82], impairment in the Child-Pugh score of $\geq +2$ points (HR 3.70, 95% CI 1.68-8.15), radiologic pattern of progression (target lesion growth and emergence of a new lesion) (HR 2.91, 95% CI 1.79-4.76), a TTP < 4 months (HR 1.87, 95% CI 1.21-2.91), second-line treatment after radiologic confirmation of PD (HR 0.16, 95% CI 0.08-0.32), and continuous sorafenib treatment after radiologic confirmation of PD (HR 1.76, 95% CI 1.06-3.00) [Table 3].

DISCUSSION

In our analysis of 128 patients with advanced HCC, we found impairment in the PS score of $\geq +1$, impairment in the Child-Pugh score of $\geq +2$, a TTP < 4 months, radiologic progression pattern, second-line treatment after radiologic confirmation of PD, and continuous sorafenib treatment after radiologic confirmation of PD were predictors of PPS. Time-dependent changes in these clinical parameters played an important role in predicting PPS.

PPS has been shown to be associated with OS in patients with lung^[16], breast^[17], and colorectal cancer^[18]. Recently, a correlation between PPS and OS was also shown in patients with HCC^[19,20]. However, to the best of our knowledge, few investigations have assessed the role of dynamic and time-dependent changes in clinical characteristics in the prediction of PPS.

Based on the findings of this study, patients with advanced HCC can be referred for second-line treatment at confirmation of PD during sorafenib treatment. Our findings also imply that observing disease progression

during sorafenib treatment is very important. A decrease in liver function or a worsening in the patients' general condition during sorafenib treatment should be detected early as these patients should be referred for second-line treatment as early as possible.

Our data revealed that an impairment in the Child-Pugh score of $\geq +2$ points (and not $\geq +1$ points) but was associated with a worse PPS. In previous studies, liver function impairment was defined as Child-Pugh score B or C. However, using this definition, an impairment in the Child-Pugh score of +1 point would be only defined as liver function impairment in patients with a Child-Pugh score of A6 at baseline, but not for those with a Child-Pugh score of A5 at baseline. Furthermore, the Child-Pugh score at the confirmation of PD may not accurately represent the development of the condition. In this study, we therefore focused on the changes in Child-Pugh scores over time to evaluate the effect on PPS of a change in the score of +1 point.

PS has been shown to correlate strongly with both, tumor and cirrhotic factors, and may predict survival outcomes in patients with advanced HCC^[21,22]. In previous studies, liver function impairment was defined as a PS > 2 ^[19,20]. In contrast, this study showed that even an impairment in PS of $\geq +1$ point was associated with a worse PPS.

A recent study by Reig *et al.*^[19] showed that the radiologic progression pattern affected both, OS and PPS in HCC patients receiving sorafenib treatment. The radiologic progression pattern in previous studies included intrahepatic growth, new intrahepatic lesion, extrahepatic growth, or new extrahepatic lesion. Patients with a new extrahepatic lesion, in particular, had a worse PPS^[19,20]. When estimating a tumor response, radiologic examinations show a certain progression pattern in some patients; however, many patients have a complicated combination of progression patterns. Estimated all combination of these progression patterns, complicated combination may be difficult to comprehend. In this study, we adopted the progression pattern of target lesion growth and/or the emergence of a new lesion for a convenient and easily available approach in clinical practice.

Interestingly, a TTP of < 4 months was identified as an independent prognostic predictor in this study. A recent study reported that a TTP of < 4 months was an independent predictor of OS and PPS^[20]. Earlier PD development predicts a poorer PPS after adjusting for other survival predictors. These patients should be referred for second-line treatment. It has been reported that continuous sorafenib treatment was a useful treatment option at the time of radiologic confirmation of PD. Moreover, our previous study showed that continuing sorafenib treatment after radiologic confirmation of PD may be a useful treatment strategy, especially in patients with a TTP of ≥ 4 months^[23]. On the other hand, for patients with rapid PD, as defined by a TTP of < 4 months, alternative second-line treatments should be considered^[24].

This study had some limitations. First, this study was a retrospective study. However, all patients underwent tumor evaluation by contrast-enhanced CT or MRI every 2 months during sorafenib treatment. Furthermore, no patient was lost to follow-up. Second, the study only enrolled patients with a Child-Pugh score of ≤ 7 . Clinical trials of sorafenib showed the drug's efficacy in patients with a Child-Pugh score of ≤ 6 . However, global^[25] and Japanese^[26] observational studies revealed that sorafenib treatment was often initiated in patients with a Child-Pugh score of 7 in clinical practice. Third, the target population of this study was heterogenous and included patients with BCLC-B and -C. However, sorafenib treatment is often used for HCC patients with BCLC-B who are refractory to TACE in clinical practice. Furthermore, predictors of PPS were analyzed after adjusting for BCLC staging. Fourth, 17 patients treated with TACE as a second-line treatment after the confirmation of radiologic PD were included in this study, as it is common practice to use a combination therapy of TACE and sorafenib to control disease progression. However, the efficacy of a combination therapy of TACE and sorafenib is still controversial and should be confirmed in a randomized clinical trial^[27]. Fifth, radiologic progression pattern, as mentioned before. Finally, the size of the study

cohort was small. Therefore, further prospective studies with a larger number of subjects are required to confirm our findings.

In conclusion, we show that evaluating PPS in patients with advanced HCC by using time-dependent and dynamic changes in clinical parameters was extremely useful. Our findings may be useful for selecting second-line treatment at the time of PD. Furthermore, our data indicate that changes in liver function or worsening of a patient's general condition during sorafenib treatment should be observed carefully.

DECLARATIONS

Authors' contributions

All authors contributed equally to this work.

Data source and availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Financial support and sponsorship

None.

Conflicts of interest

The authors declare no conflicts of interest associated with this manuscript.

Patient consent

All patients provided written informed consent for sorafenib treatment.

Ethics approval

This study was approved by the Ethics Committee of the National Hospital Organization Kyushu Medical Center and performed in compliance with the Declaration of Helsinki.

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Original Article

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Lycopene treatment stalls the onset of experimentally induced hepatocellular carcinoma: a radioisotopic, physiological and biochemical analysis

Nisha Bhatia¹, Baljinder Singh², Ashwani Koul¹

¹Department of Biophysics, Panjab University, Chandigarh 160014, India.

²Department of Nuclear Medicine, Post Graduate Institute of Medical Sciences and Research (PGIMER), Chandigarh 160012, India.

Correspondence to: Prof. Ashwani Koul, Department of Biophysics, Basic Medical Sciences (BMS) Block II, South Campus, Sec-25, Panjab University, Chandigarh 160014, India. E-mail: drashwanikoul@yahoo.co.in; ashwanik@pu.ac.in

How to cite this article: Bhatia N, Singh B, Koul A. Lycopene treatment stalls the onset of experimentally induced hepatocellular carcinoma: a radioisotopic, physiological and biochemical analysis. *Hepatoma Res* 2018;4:9. <http://dx.doi.org/10.20517/2394-5079.2018.04>

Received: 24 Jan 2018 **First Decision:** 24 Feb 2018 **Revised:** 2 Mar 2018 **Accepted:** 19 Mar 2018 **Published:** 27 Mar 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Aim: The present study was aimed to determine the modulatory role of lycopene enriched tomato extract (LycT) during initiation of N-nitrosodiethylamine (NDEA) induced hepatocellular carcinoma (HCC).

Methods: Female Balb/c mice were divided into 4 groups: control, NDEA (200 mg NDEA/kg b.wt, cumulative dose), LycT (5 mg/kg b.wt, thrice a week) and LycT + NDEA. LycT administration was commenced 2 weeks prior to NDEA administration in LycT + NDEA group.

Results: NDEA treatment caused histopathological alterations in hepatic tissue and was associated with enhanced serum levels of inflammatory markers, i.e., tumor necrosis factor- α , interleukin (IL)-6 and IL- β . NDEA treatment also induced functional alterations in liver as evident by slow ^{99m}Tc-mebrofenin hepatic excretion. LycT administration to NDEA mice showed improved hepatic functional status as demonstrated by normal ^{99m}Tc-mebrofenin excretion. NDEA treatment also caused alterations in the hematological parameters such as hemoglobin, red blood cells, platelets and total leucocyte counts. A significant increase in plasma lipid peroxidation and decrease in reduced glutathione levels with alterations in various enzymatic antioxidants were observed upon NDEA treatment. LycT pre-treatment aided in boosting the antioxidant defense system and ameliorated the inflammatory and hematological alterations.



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Conclusion: As evident by improved functional, hematological and biochemical markers, it may be inferred that LycT has the potential to delay HCC initiation.

Keywords: Lycopene, ^{99m}Tc -mebrofenin, hepatocellular carcinoma, hematology, oxidative stress, inflammation

INTRODUCTION

Environmental factors including exposure to chemical pollutants due to growing industrialization and adoption of unhealthy lifestyle choices like physical inactivity, imbalanced dietary regimen and smoking play a major role in the etiology of various ailments including cancer. The existing structural embodiment of our environment and surroundings makes impossible to evade the exposure from its pernicious clutches. Among various pollutants, N-nitrosodiethylamine (NDEA) is a potent environmental carcinogen found in air, soil, water and food^[1-3]. Metabolic activation of NDEA through hepatic biotransformation enzymes renders liver as a target organ for carcinogenesis. Hepatocellular carcinoma (HCC) is figured to be the fifth most common tumors of liver across the globe and accounts for 2.5% increase of death rates every year^[4]. Extremely high incidence rate, poor prognosis and asymptomatic behavior associated with HCC makes its diagnosis restrictable at initial stages. Furthermore, angiogenesis and malignant nature of tumorous tissue may also reduce the life expectancy of HCC patients at later stages.

Early HCC diagnosis has become a priority in improving the survival among cancer patients. Despite the availability of various modalities for HCC treatment such as surgical resection, radiation and chemotherapy, the outcome of patient remains dismal. Additionally, various non-invasive modalities such as ultrasound, computed tomography, and magnetic resonance imaging are in use for early monitoring, diagnosis and stratification of HCC^[5]. But these provide only anatomical information not the functional status of the tumorous tissue. Therefore, the development of non-invasive diagnostic techniques and identification of tumor specific serological markers linked to early stages of carcinogenesis would be of great clinical significance in the early management of HCC patients. This may be accomplished by ^{99m}Tc -mebrofenin hepatobiliary functional test that mainly relies on functional perturbations in the tissue as functional alterations precede anatomical alterations^[6]. The main rationale behind the identification of candidate biomarkers relevant for cancer risk is to find a link between biological alterations in nonspecific tissues, such as blood, and the occurrence of similar events in specific tissues involved in the carcinogenesis. Moreover, blood acts as a pathological reflector of the systemic status of an animal exposed to carcinogen^[7].

Hematological parameters are surrogate markers playing a key role in diagnosing the extent of damage to blood thus acting as a prognostic indicator of HCC patients^[8]. NDEA is also known to induce chronic inflammatory responses characterized by the upregulation of pro-inflammatory cytokines and recruitment of innate immune cells to liver tissue. Chronic inflammation further predisposes hepatic tissue to the development of HCC^[9]. Free radicals and reactive oxygen species (ROS) generation during NDEA metabolism further causes the oxidation of major cellular biomolecules thus may augment an oxidative stress which may be postulated as a major contributor in the genesis of cancer^[10]. Various reports support the relationship between the hepatic antioxidant system and development of hepatocarcinogenesis^[11,12]. However, only few studies are available to evaluate the role of blood antioxidants in early diagnosis of HCC^[13,14]. These observations emphasize the need for urgent implementation of efficient strategies to curb this disease. From the past few decades, preventive control approaches using the natural products derived from common dietary sources have been the main focus of scientific research to impede the induction of carcinogenesis^[15].

Lycopene extracted from red tomatoes has found its widespread use in natural medicine because of its highest antioxidant and radical scavenging activity^[3,16-18]. Consumption of lycopene enriched tomato extract has been revealed to be effective in alleviating cancer progression due to its increased bioavailability and

synergistic effects of its multiple phytochemicals^[19,20]. The ameliorative potential of lycopene enriched extract has been found in patients of oesophageal cancer^[21]. The consistently reduced risk of chronic diseases associated with increased consumption of lycopene enriched products provides a strong foundation for its use as a potent chemopreventive agent against liver cancer.

Our earlier studies have reported the delay in progression of hepatic cancer upon lycopene enriched tomato extract (LycT) consumption which was revealed by reduced histopathological alterations, improved survival rate, reduced tumor incidence and burden^[3,18]. This was also evident through modulation in the expression of apoptosis and cell proliferation associated genes which further interferes in the progression of tumor cells^[18,22]. We have also found that LycT consumption aided in up regulating the detoxification system, reducing chromosomal aberrations and modulating physiochemical characteristics of hepatocellular membrane^[12]. Recently, in our laboratory, the role of LycT in inhibiting multiple dysregulated pathways including hypoxia, angiogenesis and metastasis has also been delineated. The study suggested that it does so by attenuating the expression of hypoxia inducible factor- α , vascular endothelial growth factor, cluster of differentiation 31, matrix metalloproteinases (MMP)-2 and MMP-9^[23]. Moreover, the modulation of hepatic tumor marker [alpha fetoprotein (AFP)] and hepatic functional markers by LycT was also demonstrated^[23].

Thus the current scientific scenario has prompted us to study HCC during its early stages of development by analyzing a panel of hematological, inflammatory and blood antioxidant markers whose dysfunction may be related to critical events in hepatic cancer progression and their intervention with LycT. The assessment of these markers in blood on a regular basis along with AFP and liver function markers may allow earlier HCC detection. In addition, the physiological perturbations occurring in the hepatic tissue during carcinogenesis was also assessed using ^{99m}Tc-mebrofenin hepatobiliary functional test.

METHODS

Animal model for development of HCC

Female Balb/c mice (25-30 g) procured from the Central Animal House facility of Panjab University, Chandigarh (India) were provided standard animal pellet diet (Ashirwad Industries, Kharar, Punjab, India) and drinking water ad libitum. The animal house was maintained at a controlled temperature of 21 °C \pm 1 °C and humidity of 50%-60% with a 12-h dark and light cycle. All the experimental studies were performed in accordance with the Indian National Science Academy Guidelines for the use and care of experimental animals and were initially approved by the Institutional Animal Ethics Committee (IAEC), Panjab University, Chandigarh (IAEC/284-295 at Sr. No. 47). The mice were acclimatized to the experimental conditions for duration of 1 week prior to the commencement of various treatments. LycT was extracted from red tomatoes using hexane/acetone/ethanol as an extraction medium as described by Gupta *et al.*^[3]. The content of lycopene in the extract was estimated using UV-VIS spectrophotometer as described earlier^[3]. LycT in the upper hexane layer showed the presence of three characteristic peaks, i.e., at 444, 470 and 503 nm. Lycopene quantification was performed at 503 nm as to avoid the interferences from other carotenoids including β -carotene, lutein, neoxanthin, *etc.*^[24,25]. The average lycopene content was approximately 14 mg/kg tomato^[3].

Female Balb/c mice were randomly segregated into 4 groups. Animals of group 1 (control) were given vehicle (olive oil) treatment orally thrice a week. Animals of group 2 (NDEA) and group 4 (LycT + NDEA) received an intraperitoneal injection of NDEA at a cumulative dose rate of 200 mg/kg body weight for a total duration of 8 weeks. Group 3 (LycT) and group 4 animals were administered LycT in olive oil orally at a dose rate of 5 mg/kg body weight thrice a week for 10 weeks. Oral administration of LycT was commenced 2 weeks prior to NDEA treatment and continued until the termination of experimental period in LycT + NDEA group.

Assessment of hepatobiliary function

At the end of 10th week, ^{99m}Tc -mebrofenin hepatobiliary functional test was performed to assess hepatocellular function, biliary obstruction and to quantify hepatic extraction fraction (HEF). 185-200 MBq of ^{99m}Tc - sodium pertechnetate prepared in normal saline was mixed with mebrofenin according to the instructions provided by the manufacturer (BRIT, India). The scintillator counter was calibrated at 140 KeV with a window setting of $\pm 20\%$ using ^{99m}Tc as a radioactive source. Mice were then positioned over the scintillation counter immediately after the intravenous administration of ^{99m}Tc -mebrofenin with liver and mediastinum in the field of view. Liver activity and blood pool activity was monitored as a function of time and then used to measure the percentage of activity retained by the hepatic tissue (hepatic retention). The time required for maximum uptake of mebrofenin (T_{peak}) as well as the time at which the activity reduces to its half ($T_{1/2 \text{ peak}}$) was also calculated for the hepatic and cardiac tissues.

Assessment of hematological parameters

Collection of whole blood samples

Blood samples from mice of different groups were collected through an ocular vein in sterilized eppendorf containing anticoagulant at the end of treatment period. Blood samples were mixed properly and processed for the estimation of various hematological parameters.

Hemoglobin

The hemoglobin (Hb) content was estimated in whole blood by cyanmethemoglobin method as given by Dacie and Lewis^[26]. The estimation is based on the oxidation of hemoglobin to cyanmethemoglobin in presence of potassium ferricyanide. The intensity of red colored complex thus formed was measured spectrophotometrically at 540 nm against a Drabkin's solution and expressed as g/dL.

Red blood cells

Total red blood cell (RBC) counts were measured in non-coagulated whole blood as per the method described by Dacie and Lewis^[26]. Hayem's fluid is an isotonic solution consisting of sodium sulphate, sodium chloride and mercuric chloride. The measurement is based on the dilution of blood samples with Hayem's fluid and then counting of the RBCs in four corners and one central square of a Neubauer's chamber. The RBC counts were further expressed as counts $\times 10^6/\text{mm}^3$.

Total leucocyte count

The counting of total leucocytes (TLC) was performed in whole blood according to the method of Dacie and Lewis^[26] using Turk's fluid. The glacial acetic acid in this fluid causes the destruction of RBCs while the gentian violet helps in the staining of white blood cells (WBCs) nuclei makes them visible under the microscope. Counting of cells was done in four corner WBC squares and expressed as counts $\times 10^3/\text{mm}^3$.

Platelet count

The counting of blood platelets was done in a hemocytometer using 1% ammonium oxalate as a platelet diluting fluid^[26]. In this, oxalate induces the complete hemolysis of RBCs and preservation of platelets. The number of platelets were counted in whole blood in all the central RBC squares under a microscope and expressed as counts $\times 10^5/\text{mm}^3$.

Differential leucocyte count

Differential leucocyte counting was performed to compute the presence and number of different type of leucocytes in blood according to the method of Dacie and Lewis^[26]. The percentage (%) counts of neutrophils and lymphocytes were determined by observing a blood smear under a microscope.

Assessment of inflammatory markers

The quantitative estimation of various inflammatory markers including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6 was carried out in serum using a commercially available kit by solid phase enzyme linked immunosorbent assay according to the instruction provided by the manufacturer (RayBiotech, Inc., USA).

Assessment of antioxidant defense system

Preparation of blood plasma

At the end of various treatments, blood was withdrawn from the retro-orbital plexus of a mouse eye in an eppendorf containing EDTA as an anticoagulant. This was followed by the centrifugation of blood samples at 3000 rpm for 15 min at 4 °C. The supernatant (plasma) thus obtained was used for the estimation of various oxidative stress markers.

Lipid peroxidation

Lipid peroxidation (LPO) levels were measured in plasma as per the method described by Trush *et al.*^[27]. It is based on the reaction of malondialdehyde (MDA) and thiobarbituric acid (TBA) to form pink colored MDA-TBA complex which has its maximum absorption intensity at 532 nm. The amount of chromophore thus obtained was measured as an index of lipid peroxidation using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ and expressed as nanomoles of MDA-TBA chromophore formed/min/mg protein.

Reduced glutathione

The plasma levels of reduced glutathione (GSH) were estimated as a total non-protein sulphydryl compound according to the method of Moron *et al.*^[28]. It involves the reduction of a 5,5'-dithiobis-2-nitrobenzoic acid by the -SH group of reduced GSH to produce a yellow colored 2-nitro-5-mercaptobenzoic acid. The optical density of the compound thus produced was measured spectrophotometrically at 412 nm and expressed as nanomoles of GSH/mg protein.

Glutathione-S-transferase

Plasma activity of glutathione-S-transferase (GST) was estimated using a method of Habig *et al.*^[29]. GST aids in the coupling of GSH with a substrate, i.e., 1-chloro-2, 4 dinitrobenzene (CDNB). The absorbance of chromophore thus formed was read at 340 nm and described as micromoles of GSH-CDNB conjugates formed/min/mg protein using an extinction coefficient of $9.6 \text{ mM}^{-1}\text{cm}^{-1}$.

GSH peroxidase

Plasma GSH-peroxidase (Px) activity was assayed as per the method given by Paglia and Valentine^[30]. It catalyzes the production of GSSG from GSH with the simultaneous oxidation of NADPH. The change in optical density was read at 340 nm based on an extinction coefficient of $6.22 \text{ mM}^{-1}\text{cm}^{-1}$ and expressed as nanomoles of NADPH consumed/min/mg protein.

Glutathione reductase

Plasma glutathione reductase (GR) activity was estimated according to the method of Williams and Arcsott^[31]. GR causes the reduction of GSSG to GSH using NADPH as a reducing agent with the simultaneous conversion of FAD to FADH $^{\cdot}$. The change in optical density was monitored at 340 nm and calculated as nanomoles of NADPH consumed/min/mg protein using an extinction coefficient of $6.22 \text{ mM}^{-1}\text{cm}^{-1}$.

Catalase

Catalase (CAT) activity was determined in plasma using the method of Luck^[32]. CAT assists in the breakdown of hydrogen peroxide to produce water and molecular oxygen. The activity was assayed at 240 nm and measured as international units (IU)/mg protein based on the extinction coefficient of $0.0394 \text{ mM}^{-1}\text{cm}^{-1}$.

Superoxide dismutase

Plasma superoxide dismutase (SOD) activity was assayed according to the method of Kono^[33] based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) mediated by superoxide radicals, which were produced by photo-oxidation of hydroxylamine hydrochloride. One unit of SOD activity is the amount of enzyme required to cause 50% inhibition in optical density. The rate of reduction of NBT complex was measured at 560 nm and described as IU/mg protein.

Assessment of histopathological alterations

After the completion of the treatment period, mice were euthanized by decapitation and liver tissues were removed carefully followed by the immediate fixation of tissue in neutral formalin. Fixed liver tissue samples were embedded, sectioned and then stained using hematoxylin and eosin staining and examined for the microscopic alterations.

Statistical analysis

Data was expressed as mean \pm standard deviation (SD). Statistical significance was analyzed using one-way analysis of variance followed by least significant difference (LSD) *post hoc* test.

RESULTS

Effect of LycT and/or NDEA on ^{99m}Tc-mebrofenin hepatobiliary functional test

After intravenous administration of ^{99m}Tc-mebrofenin, NDEA induced a significant delay in the hepatocyte uptake, retention and excretion of ^{99m}Tc-mebrofenin in comparison to control, LycT and LycT + NDEA mice [Figure 1A-D]. The hepatic extraction fraction at the end of 60 min of ^{99m}Tc-mebrofenin injection was observed to be around 8.9%, 61.9%, 11.5% and 17.8% in case of control, NDEA, LycT and LycT + NDEA group respectively [Figure 1E]. Hepatic T_{peak} value was observed to be around 5 min in both control and LycT animals [Figure 2A]. However, NDEA administration led to a significant delay in attaining maximum activity thus showing a T_{peak} value of around 10 min while animals of LycT + NDEA group showed T_{peak} value of around 7 min [Figure 2A]. The hepatic $T_{1/2 peak}$ value in case of control and LycT animals was observed to be around 7-8 min while it was around 22 min in case of LycT + NDEA group [Figure 2B]. In contrast, NDEA animals did not show any $T_{1/2 peak}$ value which may be due to the extremely slow excretion rate of ^{99m}Tc-mebrofenin.

Effect of LycT and/or NDEA on hematological parameters

Hb

A significant decline in Hb levels was observed in mice of NDEA group when compared to control and LycT ($P \leq 0.001$) groups [Table 1]. Likewise, animals of LycT + NDEA group showed a decrease in Hb levels in comparison to control and LycT ($P \leq 0.05$) mice. However, a significant elevation in Hb levels was observed in mice that received LycT along with NDEA treatment when compared to NDEA ($P \leq 0.001$) alone injected mice [Table 1]. No significant change in Hb levels was noticed between control mice and LycT administered mice.

RBC

NDEA administration caused a significant decrease in RBC counts in mice of NDEA group when compared to control and LycT ($P \leq 0.001$) groups [Table 1]. Also, a decrease in RBC counts was observed in mice of LycT + NDEA group when compared to control and LycT ($P \leq 0.01$) groups. But the increase in RBC counts was observed when NDEA mice were pre-treated with LycT when compared to NDEA ($P \leq 0.01$) intoxicated mice [Table 1]. The counts of RBC did not differ significantly between mice of control and LycT groups.

TLC

TLCs were found to be enhanced in mice of NDEA group when compared to control and LycT ($P \leq 0.001$) groups [Table 1]. In addition, mice of LycT + NDEA groups also showed elevated total leucocyte counts when

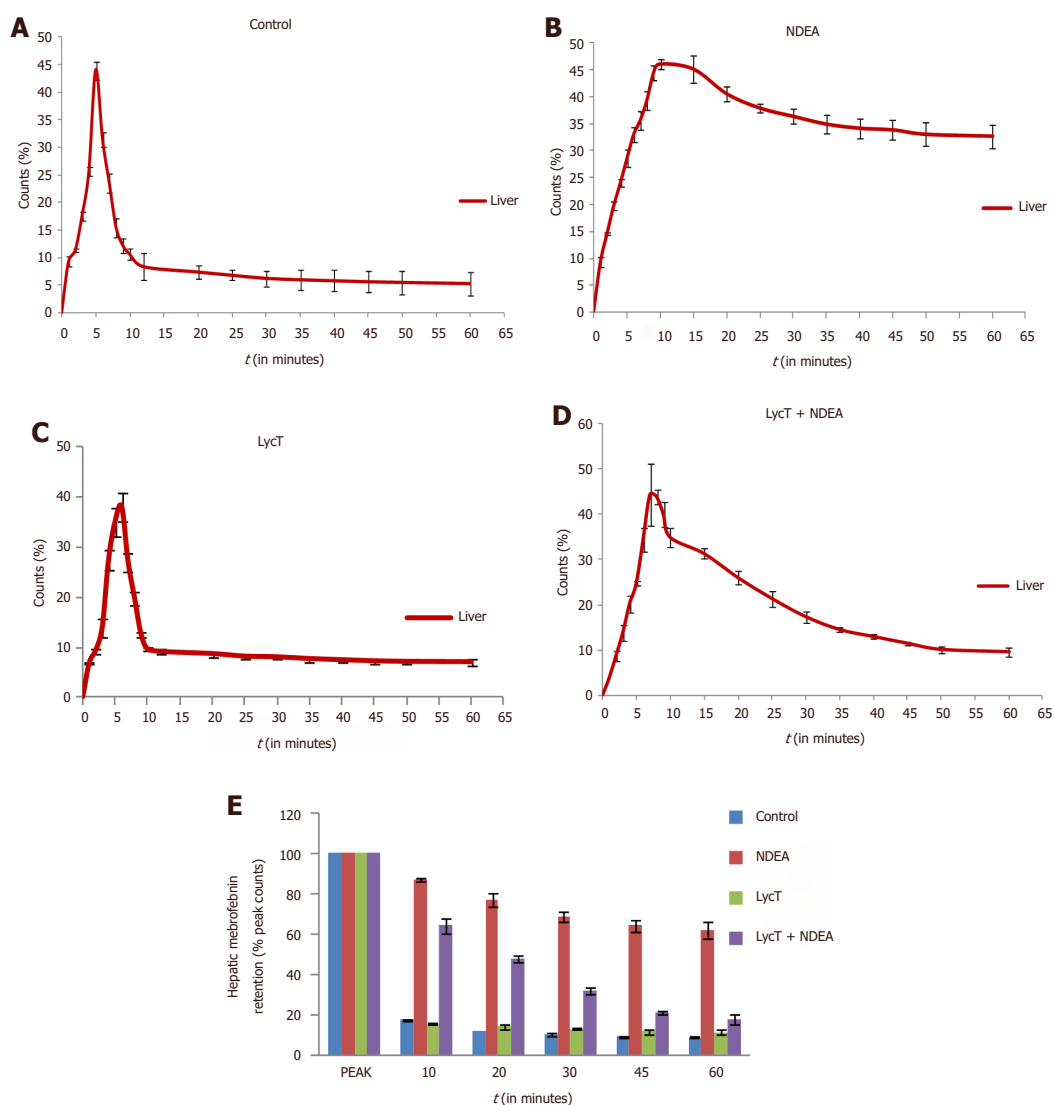


Figure 1. Effect of LycT and/or NDEA on liver time-activity curves and hepatic mebifenin retention derived from ^{99m}Tc -labeled mebifenin hepatobiliary functional test. Data is expressed as mean \pm SD ($n = 6$). NDEA: N-nitrosodiethylamine; LycT: lycopene enriched tomato extract

compared to control and LycT ($P \leq 0.01$) groups. However, the mice that received LycT in addition to NDEA treatment showed a reduction in TLC when compared to NDEA ($P \leq 0.01$) mice [Table 1]. Moreover, no statistical difference in the blood TLC was found in LycT *per se* group and control group.

Platelets

The blood platelet counts were seen to be significantly decreased in NDEA mice as compared to control and LycT ($P \leq 0.001$) mice [Table 1]. Likewise, a significant reduction in platelets counts was observed in LycT + NDEA group when compared to control ($P \leq 0.05$) and LycT ($P \leq 0.01$) group. In contrast, supplementation of NDEA mice with LycT induced a significant increase in platelet counts when compared to NDEA ($P \leq 0.001$) mice. LycT pretreatment to mice did not induce any alterations in platelets counts in comparison to normal control mice.

Neutrophils

NDEA administration led to a significant enhancement in the neutrophil counts when compared to control and LycT ($P \leq 0.001$) mice [Table 1]. Pretreatment of LycT to NDEA exposed mice also induced a marked

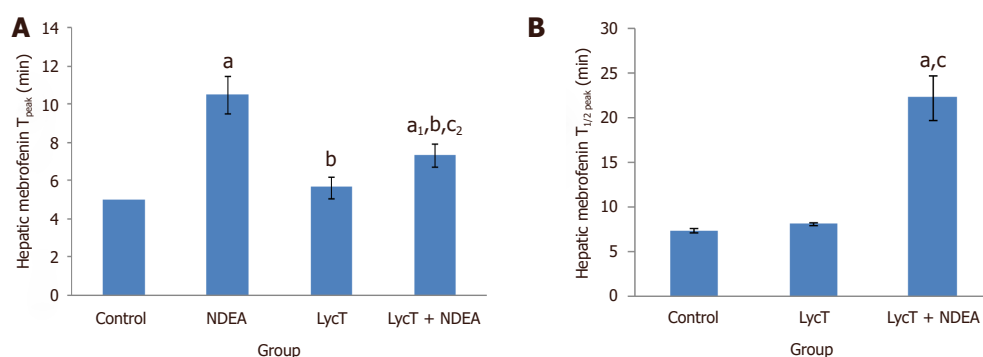


Figure 2. Effect of LycT and/or NDEA on hepatic T_{peak} (A) and $T_{1/2 peak}$ (B) of ^{99m}Tc -labeled Mebrofenin in various treatment groups. Values are expressed as mean \pm SD ($n = 5$) and analyzed by one-way analysis of variance followed by *post hoc* test. $^aP \leq 0.001$ and $^{a1}P \leq 0.01$, significant as compared to control group; $^bP \leq 0.001$, significant as compared to NDEA group; $^cP \leq 0.001$ and $^{c2}P \leq 0.05$, significant as compared to LycT group. NDEA: N-nitrosodiethylamine; LycT: lycopene enriched tomato extract

Table 1. Effect of LycT and/or NDEA on hematological parameters in different treated groups

Hematological parameters	Control	NDEA	LycT	LycT + NDEA
Hb (g/dL)	14.0 \pm 0.15	10.6 \pm 0.42 ^a	14.1 \pm 0.21 ^b	13.5 \pm 0.20 ^{a2, b, c2}
RBC (counts $\times 10^6/\text{mm}^3$)	7.90 \pm 0.10	6.53 \pm 0.35 ^a	7.97 \pm 0.25 ^b	7.17 \pm 0.15 ^{a1, b1, c1}
TLC (counts $\times 10^3/\text{mm}^3$)	7.53 \pm 0.29	9.51 \pm 0.27 ^a	7.40 \pm 0.36 ^b	8.45 \pm 0.30 ^{a1, b1, c1}
Platelets (counts $\times 10^5/\text{mm}^3$)	4.43 \pm 0.29	3.29 \pm 0.11 ^a	4.69 \pm 0.18 ^b	4.07 \pm 0.12 ^{a2, b, c1}
Neutrophils (%)	29.7 \pm 1.53	38.7 \pm 3.21 ^a	27.3 \pm 2.52 ^b	33.7 \pm 1.52 ^{b2, c1}
Lymphocytes (%)	58.7 \pm 1.53	49.7 \pm 2.08 ^a	58.3 \pm 2.52 ^b	55.7 \pm 2.51 ^{b1}

Values are expressed as mean \pm SD ($n = 5$) and analyzed by one-way analysis of variance followed by *post hoc* test. $^aP \leq 0.001$, $^{a1}P \leq 0.01$ and $^{a2}P \leq 0.05$, significant as compared to control group; $^bP \leq 0.001$, $^{b1}P \leq 0.01$ and $^{b2}P \leq 0.05$, significant as compared to NDEA group; $^{c1}P \leq 0.01$ and $^{c2}P \leq 0.05$, significant as compared to LycT group. NDEA: N-nitrosodiethylamine; LycT: lycopene enriched tomato extract; Hb: hemoglobin; RBC: red blood cell; TLC: total leucocyte count

increase in neutrophil counts in comparison to LycT ($P \leq 0.01$) mice and remained unaltered when compared to control mice. A significant decrease in these counts was observed in LycT + NDEA group when compared to NDEA ($P \leq 0.05$) group. No statistical alterations in the neutrophil counts were observed in LycT *per se* group and control group.

Lymphocytes

NDEA treatment exhibited a marked decline in blood lymphocyte counts when compared to control and LycT ($P \leq 0.001$) mice. However, no change in the lymphocyte counts was observed in LycT + NDEA group when compared to control and LycT groups [Table 1]. In contrast, LycT supplementation to NDEA exposed mice induced a significant enhancement in lymphocyte counts when compared to NDEA ($P \leq 0.01$) intoxicated mice. No change was observed in the blood lymphocyte counts in LycT group when compared to control group.

Effect of LycT and/or NDEA on serum inflammatory markers

The levels of serum TNF- α , IL-1 β and IL-6 were found to be elevated in mice exposed to NDEA when compared to control and LycT ($P \leq 0.001$) mice. LycT supplementation to NDEA animals also induced a significant enhancement in the levels of TNF- α and IL-1 β while IL-6 levels remained unaltered as compared to control and LycT ($P \leq 0.001$) mice [Figure 3A-C]. On the contrary, animals of LycT + NDEA group revealed a marked reduction in the levels of these inflammatory cytokines in comparison to NDEA ($P \leq 0.001$) afflicted group. No significant alterations in their levels were noticed between mice treated with LycT and control mice.

Effect of LycT and/or NDEA on plasma enzymatic and non-enzymatic antioxidants

LPO

NDEA treatment significantly raised the plasma LPO levels when compared to control and LycT ($P \leq 0.001$) mice [Table 2]. Further, a significant elevation in plasma LPO levels was also observed upon LycT

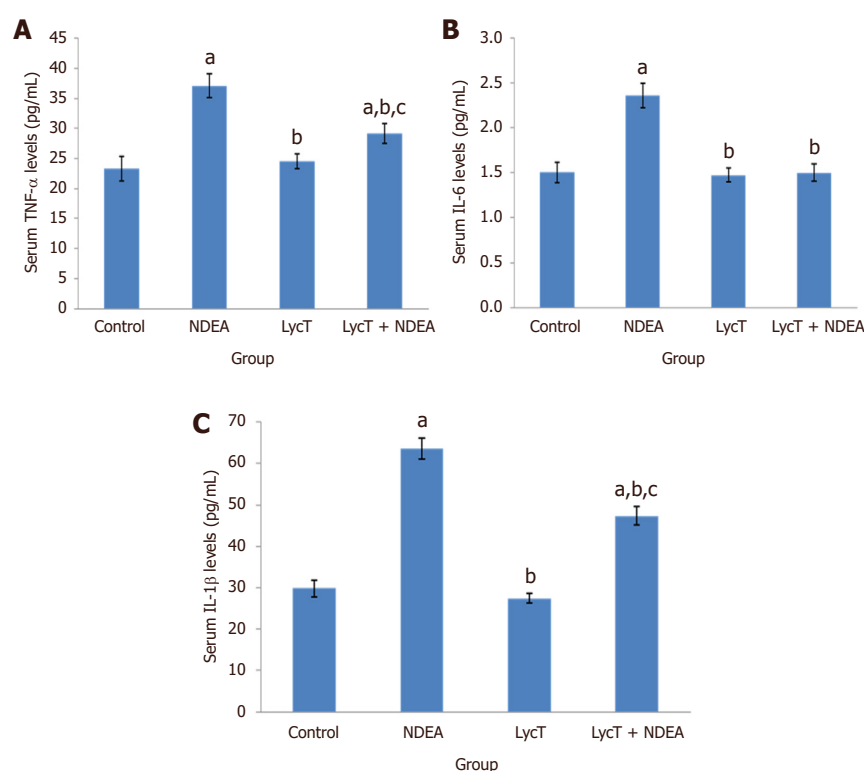


Figure 3. Effect of LycT and/or NDEA on serum inflammatory markers in different treatment groups. Values are expressed as mean \pm SD ($n = 5$) and analyzed by one-way analysis of variance followed by *post hoc* test. ^a $P \leq 0.001$, significant as compared to control group; ^b $P \leq 0.001$, significant as compared to NDEA group; ^c $P \leq 0.001$, significant as compared to LycT group. NDEA: N-nitrosodiethylamine; LycT: lycopene enriched tomato extract; TNF: tumor necrosis factor; IL: interleukin

Table 2. Effect of LycT and/or NDEA on plasma antioxidant defense system in different treated groups

	Control	NDEA	LycT	LycT + NDEA
LPO (nmol of MDA-TBA chromophore formed/mg protein)	0.02 \pm 0.002	0.08 \pm 0.003 ^a	0.03 \pm 0.002 ^b	0.05 \pm 0.005 ^{a,b,c}
GSH (nmol of GSH/mg protein)	7.60 \pm 0.23	4.11 \pm 0.45 ^a	7.29 \pm 0.20 ^b	6.25 \pm 0.59 ^{a,b,c}
GR (nmol of NADPH oxidized/min/mg protein)	1.06 \pm 0.08	0.66 \pm 0.06 ^a	1.01 \pm 0.04 ^b	0.88 \pm 0.04 ^{a,b,c}
GSH-Px (nmol of NADPH oxidized/min/mg protein)	0.67 \pm 0.08	1.06 \pm 0.09 ^a	0.68 \pm 0.06 ^b	0.81 \pm 0.05 ^{a,b,c}
GST (μ mol GSH-CDNB conjugates/min/mg protein)	0.40 \pm 0.04	0.55 \pm 0.02 ^a	0.38 \pm 0.04 ^b	0.46 \pm 0.03 ^{a,b,c}
SOD (IU/mg protein)	0.10 \pm 0.008	0.18 \pm 0.010 ^a	0.11 \pm 0.007 ^b	0.14 \pm 0.014 ^{a,b,c}
CAT (μ mol/min/mg protein)	0.61 \pm 0.01	1.10 \pm 0.10 ^a	0.58 \pm 0.03 ^b	0.84 \pm 0.02 ^{a,b,c}

Values are expressed as mean \pm SD ($n = 5$) and analyzed by one-way analysis of variance followed by *post hoc* test. ^a $P \leq 0.001$ and ^a $P \leq 0.01$, significant as compared to control group; ^b $P \leq 0.001$, significant as compared to NDEA group; ^c $P \leq 0.001$ and ^c $P \leq 0.01$, significant as compared to LycT group. NDEA: N-nitrosodiethylamine; LycT: lycopene enriched tomato extract; LPO: lipid peroxidation; GSH: glutathione; GR: glutathione reductase; GSH-Px: GSH-peroxidase; GST: glutathione-S-transferase; SOD: superoxide dismutase; CAT: catalase; MDA-TBA: malondialdehyde-thiobarbituric acid; NADPH: nicotinamide adenine dinucleotide phosphate; CDB: 1-chloro-2, 4 dinitrobenzene

supplementation to NDEA animals when compared to control and LycT ($P \leq 0.001$) animals. However, the levels of LPO came down to the baseline levels in LycT + NDEA group in comparison to NDEA ($P \leq 0.001$) group. No statistical difference in the plasma LPO levels was found in LycT *per se* group and control group.

Reduced GSH

The levels of plasma GSH were found to be declined in NDEA and LycT + NDEA mice as compared to control and LycT ($P \leq 0.001$) mice [Table 2]. However, LycT pre-treatment to tumor bearing mice caused a significant enhancement in their levels when compared to NDEA ($P \leq 0.001$) mice. No significant alterations were observed in plasma GSH levels of LycT *per se* group and control group.

GST

NDEA administration induced a significant increase in plasma GST activity when compared to control and LycT ($P \leq 0.001$) animals [Table 2]. Animals of LycT + NDEA group also showed a significant increase in GST activity in comparison to control ($P \leq 0.01$) and LycT ($P \leq 0.001$) animals. On the contrary, mice that received LycT in addition to NDEA showed a significant decrease in plasma GST activity as compared to NDEA ($P \leq 0.001$) alone administered group. No alterations were observed in plasma GST activity of LycT group when compared to control group.

GSH-Px

NDEA treated mice exhibited a significant increase in plasma GSH-Px activity when compared to control and LycT ($P \leq 0.001$) mice [Table 2]. Likewise, plasma GSH-Px activity was raised in LycT + NDEA group when compared to control and LycT ($P \leq 0.01$) group. However, administration of LycT to NDEA group of animals induced a significant reduction in the GSH-Px activity when compared to NDEA ($P \leq 0.001$) treated mice. Plasma GSH-Px activity did not differ significantly between the control and LycT group of animals.

GR

NDEA exposure exhibited a significant decline in plasma GR activity in comparison with control and LycT ($P \leq 0.001$) animals [Table 2]. Similarly, a significant decrease in GR activity was also observed in LycT + NDEA group when compared to control ($P \leq 0.001$) and LycT ($P \leq 0.01$) group. In contrast, there was a marked increase in GR activity on LycT supplementation to NDEA afflicted animals when compared to NDEA ($P \leq 0.001$) alone group. No significant alteration in the plasma activity of GR was observed between the control and LycT group.

CAT

A significant increase in plasma CAT activity was observed in NDEA and LycT + NDEA group of animals when compared to control and LycT ($P \leq 0.001$) mice [Table 2]. In contrast, pretreatment of NDEA exposed animals with LycT induced a significant decrease in CAT activity when compared to NDEA ($P \leq 0.001$) animals. Plasma CAT activity remained unaltered in mice treated with LycT as compared to control mice.

SOD

The administration of NDEA caused a significant increase in plasma SOD activity in animals of both NDEA and LycT + NDEA group when compared to control and LycT ($P \leq 0.001$) mice [Table 2]. On the contrary, a significant decrease in SOD activity was observed in LycT + NDEA group when compared to NDEA ($P \leq 0.001$) afflicted group. There was no significant alteration in SOD activity between mice treated with LycT and normal control mice.

Effect of LycT and/or NDEA on histopathological alterations

Liver sections from control and LycT mice exhibited normal histoarchitecture [Figure 4A and C]. Hexagonal hepatic lobules containing central vein in the middle and portal triad at the periphery were visible. Liver acinus was divided into three zones: zone 1, zone 2 and zone 3. Liver sections from NDEA group showed the presence of high grade dysplasia characterized by architectural and nuclear atypia, differential cytoplasmic staining with diminished sinusoidal spaces and fatty accumulation. No stromal invasion was visible in mice of NDEA group. Liver sections obtained from animals of LycT + NDEA group exhibited mild hepatocellular damage with no vascular invasion [Figure 4B and D; Table 3].

DISCUSSION

Increased oxidative stress and altered redox status during carcinogenesis accentuate the need for developing efficient strategies in curtailing the cancer development. This may be accomplished via use of LycT as an

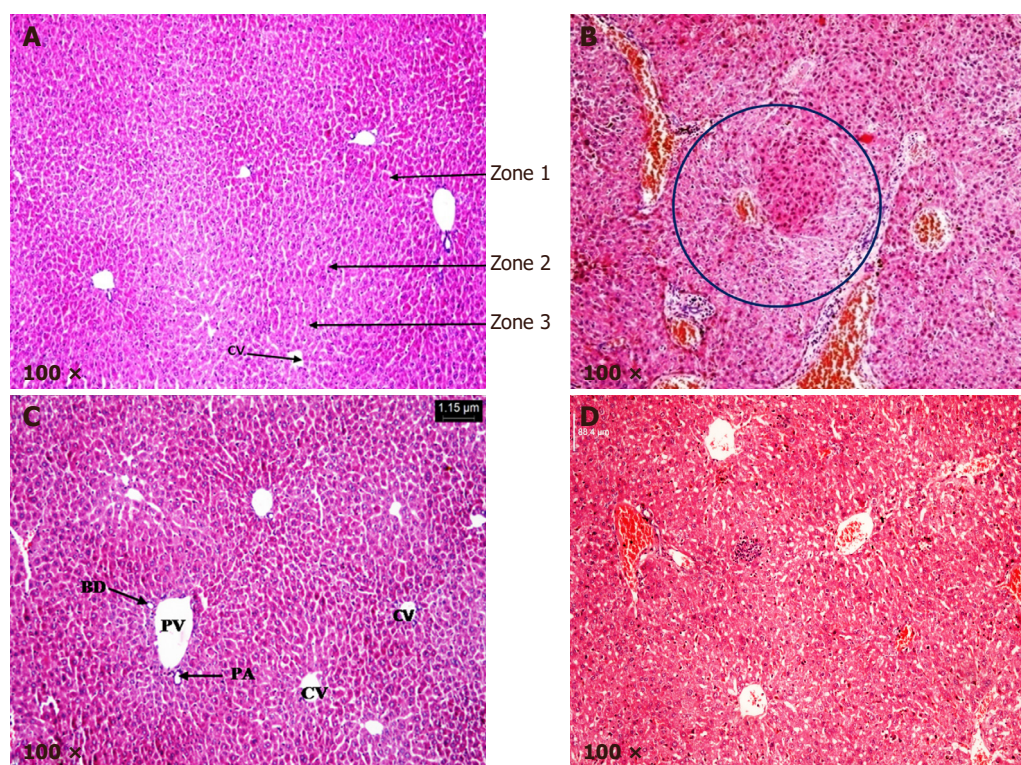


Figure 4. Histopathological analysis of hepatic tissue in various treated groups. (A) Liver sections from control group at 100× magnification revealing normal histo-architecture and the presence of three zones: zone 1, zone 2 and zone 3; (B) liver sections from NDEA group at 100× magnification revealing high grade dysplasia (encircled); (C) liver sections from LycT group at 100× magnification revealing normal histo-architecture; (D) liver sections from LycT + NDEA group at 100× magnification revealing near normal histo-architecture with infiltration of lymphocytes. CV: central vein; PA: hepatic portal artery; PV: hepatic portal vein; BD: bile duct

Table 3. Histopathological quantification of hepatic damage in NDEA and LycT + NDEA group

Groups/parameters	NDEA	LycT + NDEA
Cell density	++	+
Architectural atypia	+++	+
Nuclear density	+++	+
Differential cytoplasmic staining	++	-
Fatty accumulation	++	-

+++; extensively observed; ++: moderately observed; +: mildly observed; -: not observed. NDEA: N-nitrosodiethylamine; LycT: lycopene enriched tomato extract

exogenous antioxidant to maintain the redox balance and homeostasis. The role of LycT in delaying the process of hepatocarcinogenesis has already been studied in terms of diminished histopathological alterations, reduced mortality and induction of apoptosis^[3,18]. Moreover, the anti-angiogenic and anti-metastatic potential of lycopene was also investigated recently in our laboratory^[23]. Periodical assessment of serological markers at early stages could serve as an important parameter to evaluate the onset of hepatic pathology. Thus the present piece of work was planned to gain insight into the identification of candidate biomarkers linked to early stages of hepatocarcinogenesis and their amelioration by LycT. In addition, the physiological status of the liver was also assessed using non-invasive ^{99m}Tc-mebrofenin hepatobiliary functional test.

It helps in the evaluation of hepatocellular function, biliary obstruction thus providing the functional or physiological status of liver. In the present study, an appearance of maximum activity of ^{99m}Tc-mebrofenin in blood pool within 2-3 min in all the groups showed that NDEA exposure does not induce any effect on cardiac tissue. Being a hepatocarcinogen, NDEA induces major pathophysiological alterations in the

hepatic tissue as observed by the changes in HEF, percentage counts in the liver at different time intervals, T_{peak} and $T_{1/2 peak}$. Control and LycT animals showed the normal uptake, efficient hepatic extraction and rapid excretion from the liver. However, a significant deviation from the normal pattern of radioactivity as observed in NDEA animals confirmed the physiological alterations in hepatic tissue. The delay in hepatic uptake in NDEA animals may be due to severe hepatocellular dysfunction. Neyt *et al.*^[34] have also reported the delayed uptake due to the dysfunction of various Oatp transporters located on hepatocytes. The retention of radiotracer activity in the liver of NDEA mice up to 60 min showed marked impairment in the excretion of the activity. This may be due to the biliary obstruction induced by NDEA which was also evidenced by the inability to calculate $T_{1/2}$ value in case of NDEA animals. We have previously reported the delay in hepatic excretion in the case of DMBA induced hepatotoxicity^[35]. Joseph *et al.*^[36] observed the involvement of inflammation in delaying the hepatic excretion of ^{99m}Tc -mebrofenin. Similarly, LycT administration to NDEA insulted animals also showed delayed uptake but the clearance of the activity at 60 min showed the protective effect of LycT against hepatocarcinogenesis. Deshpande *et al.*^[37] observed the increased clearance of ^{99m}Tc -mebrofenin upon administration of dietary turmeric extract to rats exposed to D-galactosamine HCl. Our laboratory also observed the ameliorative effect of *Azadirachta indica* against DMBA induced hepatotoxicity by efficient clearance of mebrofenin^[35].

During tumor progression, cells generally demand more oxygen than is available for its growth, which results in the creation of hypoxia. Continued hypoxia leads to the adaptation of various genomic and proteomic alterations and results in the aggressive and malignant tumor phenotype. This may further causes reduced oxygen transport throughout the body due to the alterations in various hematological markers. NDEA exposed mice showed a marked decline in the levels of Hb, RBC, platelets and lymphocytes as compared to control mice. In addition to this, a significant enhancement in the neutrophils and WBC counts were observed upon NDEA exposure. The reduction in Hb and RBC suggested the occurrence of anemia in tumor bearing mice. This may be due to the increased oxidative stress induced by excessive ROS which causes the oxidative destruction of mature erythrocytes or inhibiting its production. This can also be evidenced by a decline in GSH and elevation in LPO levels on NDEA treatment^[38]. A marked enhancement in WBC counts may reflect the activation of an immune system to fight against invading particles^[39]. This was further confirmed by the release of various cytokines by activated kupffer cells and accumulation of neutrophils in hepatic cells as discussed above. Histopathological examination also supported the infiltration of leukocytes in hepatic tissue upon NDEA administration. The diminished platelets count may apparently be due to the decreased production of thrombopoietin hormone by damaged liver cells. The decrease in lymphocytes and enhanced neutrophil counts might suggest the decrease in efficiency of an immune system to cope up with the triggered inflammatory cascade^[40]. Similar observations were also noticed by Farooq *et al.*^[41] and Gangar *et al.*^[42], who also observed the alterations in various hematological parameters in patients suffering from gastric and forestomach carcinoma respectively. LycT treatment to NDEA insulted mice tends to restore the levels of these markers which might be attributed to decreased hypoxia and reduction in tumor growth by an enhancement of apoptosis^[18,23]. Several other researchers have also supported the restoration of blood parameters upon lycopene treatment thus showing its anti-inflammatory potential^[43,44].

Inflammatory cytokines also play a major role in the progression of cancer. Exposure of liver tissue to certain hepatotoxicants induces the release of pro-inflammatory cytokines by kupffer cells which can further aggravate the tumor progression by triggering of inflammatory cascade^[45]. The current findings revealed a marked increase in serum levels of various inflammatory markers, i.e., TNF- α , IL-1 β and IL-6 in tumor bearing mice. Overexpression of these cytokines may provide proliferating signals to the mutated hepatocytes through the secretion of angiogenesis and metastasis markers. These findings were in concordance with the report of Abdel-Hamid *et al.*^[46]. Supplementation of NDEA mice with LycT modulated the serum levels of these cytokines by inhibiting their production and induction of apoptosis thus showing its anti-inflammatory effect. Literature also supported the amelioration of these inflammatory markers upon administration of lycopene^[47-49].

NDEA treatment also exhibited enhanced plasma LPO and reduced GSH levels in comparison to control and LycT animals. The increase in LPO may be ascribed to the excessive formation of ROS and their diffusion into the blood by an oxidative deterioration of membrane lipids. Declined GSH levels might be due to the impairment of antioxidant defense system and increased consumption of GSH by the detoxification system. Literature also supported the alterations in these oxidative stress markers with the progression of cancer^[50,51]. Interestingly, the reversal of their levels by LycT pretreatment may probably be due to the neutralization of free radicals and enhancement of xenobiotic detoxification by LycT. The amelioration of oxidative stress by consumption of tomato enriched diet has also been reported by Dogukan *et al.*^[52] and Gupta *et al.*^[12].

The enhancement in plasma activities of SOD, CAT and GSH-Px were also observed upon NDEA administration. An elevated SOD activity causes the excessive production of deleterious H₂O₂ by the dismutation of superoxide anions which may further be counterbalanced by excess CAT and GSH-Px. However, complete neutralization of H₂O₂ may not occur due to the failure of defense system by sufficient lipid oxidation which further increases the chances of DNA damage, thus, contributing to a growth advantage to the tumorous cell^[53]. Our results are in agreement with other reports who found similar alterations in activities of these enzymes in cancer patients^[54,55]. Reduction in the activities of these enzymes by LycT supplementation to tumor bearing mice showing the antioxidant capability of LycT to scavenge the free radical formation thus mitigating intracellular oxidative damage. The present results are in harmony with the findings of Ural^[56] and Ibrahim^[44] who also reported the diminished enzymatic activities on LycT supplementation.

The increase in GST activity in NDEA intoxicated mice may lead to the excessive utilization of this enzyme in the detoxification in response to metabolic induction in the tumor cells. These results are in concordance with the observation of Sadik *et al.*^[57], who also reported the correlation between the increased GST levels and carcinogenesis. In contrast, Li *et al.*^[58] has observed the decrease in blood GST levels during the development of HCC. Suppression of GST activity upon LycT supplementation to NDEA afflicted mice indicated the protective efficacy of LycT against the induction of oxidative stress. Sadik *et al.*^[57] has also reported the restoration of GST activity upon consumption of a diet rich in fruits and vegetables. The increased activity of GSH-Px and decreased levels of GSH may lead to the accumulation of oxidized glutathione (GSSG) which further cannot be converted to GSH due to the reduction in GR activity upon NDEA treatment. Maffei *et al.*^[55] has also reported the drop in plasma GR activity in patients suffering from colorectal cancer. Pretreatment with LycT to NDEA mice attenuated the decrease in GR activity probably by radical scavenging potential and increase in GSH level thus maintaining the oxido-reductive balance. Lycopene enriched tomato extract ameliorates the oxidative stress by maintaining the integrity of cellular membrane thus preserving the antioxidants levels in the liver cells^[12,59]. The activities of various enzymatic and non-enzymatic antioxidants did not show any alterations between control and LycT mice.

It is evident that LycT supplementation modulated the inflammatory and hematological markers, boosted the antioxidant system and improved the functional status of hepatic tissue in tumor bearing mice. Data from the present study and previously published studies reiterate the potential of LycT in delaying the initiation of HCC which may have significant implications in its overall chemopreventive potential.

DECLARATIONS

Authors' contributions

Study design: Koul A, Singh B

Data analysis and manuscript preparation: Koul A, Singh B, Bhatia N

Experimental studies: Bhatia N

Literature search: Koul A, Singh B, Bhatia N

Manuscript review: Koul A, Singh B, Bhatia N

Data source and availability

The data presented is original and obtained in our laboratory. It is available with the authors and can be made available if required.

Financial support and sponsorship

This work was supported by the Department of Science and Technology (Innovation in Science Pursuit for Inspired Research-INSPIRE Program) (IF110576).

Conflicts of interest

The authors declare that they have no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

All the experimental studies were performed in accordance with the Indian National Science Academy Guidelines for the use and care of experimental animals and were initially approved by the Institutional Animal Ethics Committee (IAEC), Panjab University, Chandigarh (IAEC/284-295 at Sr. No. 47).

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Review

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Systemic therapies for hepatocellular carcinoma: a recap of the current status

Petros Giovanis¹, Manuela De Bona², Fabio Farinati³, Andrea Buda², Riccardo Berletti⁴, Fable Zustovich⁵, Simona D'Ippolito¹, Michele De Boni², Umberto Cillo³, Davide Pastorelli¹

¹Operative Unit of Medical Oncology, Santa Maria del Prato Hospital, Feltre 32032, Italy.

²Department of Gastroenterology and Digestive Endoscopy, Santa Maria del Prato Hospital, Feltre 32032, Italy.

³Department of Surgery, University of Padua, Padua 35128, Italy.

⁴Operative Unit of Radiology and Interventional Radiology, Santa Maria del Prato Hospital, Feltre 32032, Italy.

⁵Operative Unit of Medical Oncology, San Martino Hospital, Belluno 32100, Italy.

Correspondence to: Dr. Petros Giovanis, Operative Unit of Medical Oncology, Santa Maria del Prato Hospital, Via Bagnols sur Ceze 3, Azienda Ulss 1- Dolomiti, Feltre 32032, Italy. E-mail: petros.giovanis@aulss1.veneto.it

How to cite this article: Giovanis P, De Bona M, Farinati F, Buda A, Berletti R, Zustovich F, D'Ippolito S, De Boni M, Cillo U, Pastorelli D. Systemic therapies for hepatocellular carcinoma: a recap of the current status. *Hepatoma Res* 2018;4:10. <http://dx.doi.org/10.20517/2394-5079.2018.21>

Received: 18 Mar 2018 **First Decision:** 4 Apr 2018 **Revised:** 10 Apr 2018 **Accepted:** 13 Apr 2018 **Published:** 27 Apr 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Guang-Zhe Zhu **Production Editor:** Huan-Liang Wu

Abstract

After decades of frustrating nihilism due to lack of innovative therapeutic solutions, the onco-hepatological community is facing up to important novelties for the treatment of intermediate and advanced stages of liver cancer. Four new drugs have been investigated and resulted in positive data: lenvatinib resulted not inferior to the standard of care sorafenib in first line, regorafenib and cabozantinib demonstrated prolonging survival in patients progressed to sorafenib and nivolumab approved by FDA as option after first-line. Contemporary, the knowledge acquired after ten years' experience of sorafenib in patient selection and adverse events management revealed an increase of the outcomes. Physicians dedicated to treat advanced and intermediated liver cancer are close to live a new era where systemic treatments could have a huge impact on the disease. The aim of this review is to anticipate this new approach at the disease, summarizing data currently available for these therapies to identify therapeutic strategies of sequences and choosing drugs according to the patient profile.

Keywords: Hepatocellular carcinoma, liver cancer, sorafenib, regorafenib, lenvatinib, cabozantinib, nivolumab

INTRODUCTION

In the last decade the outcomes of many oncologic diseases have been dramatically transformed consequently the introduction of novel therapies; moreover, the recourse to sequential or combination strategies allowed to achieve long term benefits in overall survival (OS)^[1,2]. This aspect is particularly



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evident in areas of oncology such as breast, colon or kidney cancer where the prognosis associated with these pathologies was only of some months few years ago, conversely therapeutic strategies allow to reach survivals quantifiable in order of years at present time^[1]. Unfortunately, such therapeutic successes are still challenging for patients currently receiving diagnosis of intermediate or advanced stage of hepatocellular carcinoma (HCC), because of the restricted efficacy for systemic therapies. Such difficulty in finding an active systemic therapy is strictly related to the biology of HCC: differently than other oncological diseases, the primary malignancy of the liver occurs predominantly in patients with two diseases: the tumor and the underlying cirrhosis^[3]. Therefore, if the single drug can slow down the oncological progression, the same drug may worsen the cirrhotic component of HCC leading the patient towards to exitus^[3,4].

Systemic therapies in liver cancer achieved the first important goal in 2007 when the introduction of the target agent sorafenib provided a therapeutic option for patients with HCC in progression or evaluated not suitable for locoregional treatments^[5,6]. After this initial success, a sense of nihilism persisted in the oncology community in the subsequent decades due to failure of the new studies with molecular targeted agents to demonstrate a benefit in OS or time to tumor progression (TTP) or progression-free survival (PFS). Phase 3 trials failed to show superiority of sunitinib, erlotinib plus sorafenib, FOLFOX, doxorubicin, or non-inferiority of brivanib, linifanib to sorafenib in the first-line setting^[6]. Similarly, the drugs brivanib, everolimus, linifanib, ramucirumab and tivantinib failed pivotal studies designed in second-line settings in patients progressing or intolerant to sorafenib^[6].

In this depressing scenario a turning point was reached very recently, after the announcement of positive results of the phase 3 studies conducted with new biological therapies. The first drug showing to prolong the overall survival sequentially to sorafenib was regorafenib^[7,8]. After short time, lenvatinib resulted non-inferior to sorafenib in the first line of treatment^[7], cabozantinib showed efficacy after sorafenib progression or in patients with intolerance to this drug^[9], and nivolumab received conditioned FDA approval for patients pretreated with sorafenib^[10]. At the same time, the experience with sorafenib in HCC after many years increased outcomes in survival due to better patient selection and better adverse events management, encouraging the medical community to pursue excellence in disease management with this mature drug^[11,12].

The goal of this paper is to take stock the situation regarding systemic drugs evaluated in the intermediate and advanced HCC setting. We focused only on therapies which achieved positive results in phase 3 trials or an early approval by health authorities after phase 1/2 conducted, and we tried to position every therapy according to data nowadays available.

RADICAL THERAPIES VERSUS SYSTEMIC TREATMENTS

Radical therapies are the most frequently used treatment in HCC and are represented by surgical resection, liver transplantation and the local destruction methods known as loco-regional therapies. These are able to turn out to the radical destruction of the tumor but they have never demonstrated to prolong OS in prospective phase 3 studies conducted on HCC populations with intermediate or advanced stage^[13-15]. Therefore, current evidence indicates that potentially curative treatments result only for very early- and early-stage HCC.

The surgical treatments transplantation and resection are evaluated as potentially curative in early stages of the disease for carefully selected HCC patients, but are usually discouraged in advanced phases due to the high risk of hepatic decompensation and neoplastic progression in the frame time awaiting surgery^[15].

The BCLC algorithm reported transarterial chemoembolization (TACE) as the first-line treatment for the intermediate stage in HCC patients, however TACE may have high recurrence rates and should therefore not

Table 1. Patient unsuitable for TACE or with absolute contraindications to cTACE according to ESMO 2012 guidelines

Exclusion criteria for cTACE
Decompensated cirrhosis (Child-Pugh B \geq 8), including: -Jaundice -Clinical encephalopathy -Refractory ascites
Extensive tumour with massive replacement of both entire lobes
Severely reduced portal vein flow (e.g., portal vein occlusion or hepatofugal blood flow)
Technical contraindications to hepatic intra-arterial treatment (e.g., untreatable arteriovenous fistula)
Bilio-enteric anastomosis or biliary stents
Renal insufficiency (creatinine clearance < 30 mL/min)

cTACE: conventional transarterial chemoembolization; ESMO: European Society of Medical Oncology

Table 2. When to stop TACE? Definition of TACE failure/refractoriness

Authors	Criteria for definition of TACE failure/refractoriness
Kudo <i>et al.</i> ^[62]	Intrahepatic lesion (> 2 consecutive incomplete necrosis; > 2 consecutive appearances of a new lesion recurrence) Appearances of vascular invasion Appearance of EHS Continuous elevation of tumor markers
Yamanaka <i>et al.</i> ^[63]	TACE failure Inability to select the feeding artery of the HCC because of arterial devastation; deterioration of liver function and/or tumor thrombosis of the portal vein TACE refractory Repetitive tumor recurrence in the liver; appearance of vascular invasion; appearance of distant metastasis; continuous increase in tumor marker levels after TACE
AISF <i>et al.</i> ^[64]	The AISF expert panel considers failure of TACE the lack of objective response of the treated lesions after two procedures (consider also a multi-disciplinary decisional setting)
Sieghart <i>et al.</i> ^[21]	ART score

TACE: transhepatic arterial chemoembolization; ART: assessment for retreatment with TACE

be considered a curative therapy^[16-18]. This loco-regional treatment may not represent the only therapeutic option for this stage of patients because authors reported that TACE can deal good results in center with good experience after a careful selection of patients, but may also present technical difficulties and contraindication^[19]. Table 1 shows the main patients characteristics in which TACE is contraindicated, absolute and relative contraindications generally include features of decompensated liver disease, extensive bilobular tumor load and impaired integrity of the portal vein due to thrombosis or hepatofugal flow, as well as untreated large varices, massive tumor diameter, and severe co-morbidities.

A point of discussion is when to establish TACE failure/refractoriness and, consequently, patient should be switched to a different therapy. The lack of a clear definition of the right moment to stop the re-treatments with TACE due to failure or refractoriness may lead to unnecessary overtreatment with TACE that may worsen the liver function, precluding the opportunity to shift the patient to systemic treatments^[20]. Table 2 summarizes the criteria identified to define the failure of TACE by experts and guidelines. The assessment for retreatment with TACE (ART) score is a simple validated algorithm useful for deciding the potential benefit of undergoing a third TACE evaluating three prognostic factors: the increase in aspartate transaminase (AST) level by more than 25%, the increase in the Child-Pugh score and the absence of tumor response. TACE has a good prognostic effect on patients with ART score of 0-1.5, while patients with ART score \geq 2.5 might have minor or even no prognostic benefits^[21,22].

Similarly, to TACE, the effectiveness of the intra-hepatic radioembolization (TARE) using microspheres loaded with yttrium-90, may depend on the operator's manual skills^[23]. Table 3 reports levels of evidence associated to TARE by the principal international guidelines and recommendations; the use of TARE in HCC is supported by data based on retrospective series and uncontrolled prospective studies. Two randomized studies (SARAH and SIRVENIB), designed to show the superiority of TARE versus sorafenib,

Table 3. Primarily International Guidelines and level of evidence associated to radioembolization

Guidelines	Recommendation	Level of recommendation
ESMO 2012 ^[65]	Radioembolization may be competitive with sorafenib or TACE in subsets of patients, such as those with prior TACE failure, excellent liver function, macrovascular invasion and the absence of extra-hepatic disease	Category III, C
AASLD 2010 ^[66]	Current phase 3 studies are evaluating the place of radioembolization versus TACE in patients with intermediate stage HCC, and as single modality or combined with sorafenib in patients with advanced HCC compared with sorafenib Radioembolization with Yttrium90-labeled glass beads has been shown to induce extensive tumour necrosis with acceptable safety profile. However, there no studies demonstrating an impact on survival and hence, its value in the clinical setting has not been established and cannot be recommended as standard therapy for advanced HCC outside clinical trials	Level II

TACE: transhepatic arterial chemoembolization; HCC: hepatocellular carcinoma

Table 4. Efficacious systemic therapies for HCC

	Study	Arms	OS benefit (months)	HR	Adverse events (Grade 3-4)
1st line setting	SHARP ^[5]	Sorafenib vs. placebo	10.7 (vs. 7.9)	0.69	Hand-foot skin reaction (8%), diarrhoea (8%), fatigue (3), hypertension (3%)
	ASIA PACIFIC ^[26]	Sorafenib vs. placebo	6.5 (vs. 4.2)	0.68	Hand-foot skin reaction (11%), diarrhoea (6%), fatigue (3%)
	SELECT ^[55]	Lenvatinib vs. sorafenib	13.6 (vs. 12.3)	0.92	Hypertension (23%), increased blood bilirubin (7%), proteinuria (6%), elevate aspartate aminotransferase (5%)
2nd line setting	RESORCE ^[50]	Regorafenib vs. placebo	10.6 (vs. 7.8)	0.63	Hypertension (15%), hand-foot skin reaction (13%), fatigue (9%), diarrhoea (3%)
	CELESTIAL ^[58]	Cabozantinib vs. placebo	10.2 (vs. 8.0)	0.76	Hand-foot skin reaction (17%), hypertension (16%), increased aspartate aminotransferase (12%), fatigue (10%)
	CheckMate 040 ^[59] (expansion cohort)	Nivolumab single arm	15.6	-	Increased aspartate aminotransferase (18%), increased alanine aminotransferase (11%), increased blood bilirubin (7%), immune-mediated hepatitis (5%)

were conducted in patients with intermediate or advanced HCC no longer susceptible to TACE^[24,25]. Both trials failed to demonstrate a survival benefit from transarterial radioembolization compared with sorafenib. Moreover, the median overall survival of patients treated with TARE resulted lower than sorafenib. In addition, in both studies a significant proportion of patients randomized to TARE never received the planned therapy (26.5% and 28.6% of patients of TARE arm vs. 7% and 9.0% in the sorafenib arm respectively in SARAH and SIRVENIB). This may suggest difficulties in selecting patients and implementing TARE procedure in clinical practice.

Worth to be mentioned is that these studies demonstrated only the inferiority of TARE to systemic treatment and not the non-inferiority. In fact, in study designs the hypothesis of non-inferiority had not been prespecified in the protocol.

SYSTEMIC TREATMENTS FOR HCC

Table 4 reports the efficacious systemic therapies in intermediate and advanced stage of HCC, estimated as therapies achieving successful in phase 3 trials or early approved by health authorities accounting for phase 2 data. A summary of the main studies currently available are described in this section.

Sorafenib

Sorafenib is an oral multikinase inhibitor that has antiangiogenic, anti-proliferative, anti-metastatic and anti-immunosuppressive activities. Sorafenib inhibits the activity of targets present in the tumour cell (CRAF, BRAF, V600E BRAF, c-KIT, and FLT-3) and in the tumour vasculature (CRAF, VEGFR-2, VEGFR-3, and PDGFR-β). RAF kinases are serine/threonine kinases, whereas c-KIT, FLT-3, VEGFR-2, VEGFR-3, and

PDGFR- β are receptor tyrosine kinases. Sorafenib demonstrated a statistically significant improvement of OS in Child Pugh class A patients with intermediate or advanced HCC (BCLC stages B and C) in two large phase 3, randomized, placebo-controlled trials performed in Western countries (SHARP) and in Asia-Pacific (ASIA-PACIFIC)^[5,26].

In the SHARP trial the OS and TTP were respectively 10.7 and 5.5 months (7.9 and 2.8 months respectively for the placebo group)^[5], while in the ASIA-PACIFIC trial OS and TTP were 6.5 and 2.8 months respectively (4.2 and 1.4 months for the placebo group)^[26]. The hazard ratios for OS and TTP were nevertheless comparable between the two trials, indicating similar magnitude of clinical benefit. The observed differences in median OS and TTP in these 2 trials were probably due to poorer disease characteristics of advanced disease in the ASIA-PACIFIC trial compared to the SHARP trial^[27]. Subgroup analyses have shown that sorafenib consistently provides an OS benefit compared with placebo irrespective of baseline conditions such as disease etiology, baseline tumor burden, performance status, tumor stage, and prior therapy^[5,26,28].

A pooled global population enrolled in the two pivotal studies was analysed to identify potential predictive factors of sorafenib treatment benefit. This analysis turned out that sorafenib treatment provided a survival benefit across all categories of patients, showing a significant magnitude of benefit in patients with disease confined to the liver (without extrahepatic disease - EHS) or HCV cirrhosis or a low neutrophil-to-lymphocyte ratio status^[28].

The efficacy and the safety profile of sorafenib already observed in clinical trials were confirmed in real-world experiences assessing sorafenib in patients who are not selected by strict clinical trial criteria, including patients with comorbidities and those receiving concomitant medication^[29,30].

Both in clinical and field-practice studies, the most frequent sorafenib-associated adverse events resulted in dermatological lesions as hand-foot skin reaction, fatigue and diarrhoea, whereas treatment-related liver adverse events are overall less frequently reported^[31]. Single experiences reported that the occurrence of hypertension, diarrhoea and skin lesions are generally correlated to higher survival benefits, therefore the occurrence of some toxicities should be managed and not immediately address to discontinuation^[32-36]. At this regard, treatment strategies based on temporary suspensions followed by restart of therapy at lower doses may help in management of sorafenib tolerance in patients presenting relevant adverse events.

The results collected showed poorer outcomes in patients with Child-Pugh B cirrhosis treated with sorafenib, when compared with patients with Child-Pugh A cirrhosis. This finding could be likely attributed to a more severe liver dysfunction and more compromised conditions of Child-Pugh B patients and not to an effect of the drug itself.

With the aim to extend the benefit observed with sorafenib to patients with earlier stages of disease (BCLC-A and B), many phase 2 and 3 trials were conducted to evaluate safety and efficacy of sorafenib in surgical adjuvant setting and in combination with loco-regional therapies^[37-44]. All these studies did not achieved the primary endpoint, authors reported possible explanations due to the design of the studies. However new study of combination or in adjuvant setting are currently ongoing with different treatment schedules.

Failing results were observed also combining sorafenib with systemic therapies. Indeed, the two trials sorafenib plus erlotinib and doxorubicin plus sorafenib failed to show the superiority of the combination arms versus sorafenib alone as comparison arm^[45,46].

Currently sorafenib is the standard of care for advanced HCC as reported by many guidelines and the improvement of survival associated with this drug is supported by the highest level of evidence^[47].

Regorafenib

Regorafenib is an oral multikinase inhibitor, targeting angiogenic, stromal and oncogenic receptors VEGFR1-3, TIE-2, RAF-1, BRAF, BRAFV600, KIT, RET, PDGFR, and FGFR^[48]. Besides, studies *in vivo* reported that the drug can inhibit the factor CSF1R and to reduce levels of tumor associated macrophages, a factor implicated in the tumor-specific immune response and in tumor growth^[48]. The drug was initially approved for colorectal cancer and for gastrointestinal stromal tumor by the main health authorities.

A phase 2 study conducted on 36 patients with intermediate or advanced HCC resistant to sorafenib reported that regorafenib presented acceptable tolerability and antitumour activity^[49]. In the pivotal phase 3 study (RESORCE), more than 500 patients affected by advanced HCC with liver function Child-Pugh A and with ECOG PS 0-1, were randomised (2:1) to receive 160 mg oral regorafenib or placebo plus best supportive care once daily for 1-3 of each four week cycle^[50]. Patients were stratified for region, ECOG PS score, extrahepatic spread, vascular invasion and AFP.

Worth mentioning is that the study was designed with the aim to avoid enrolling patients potentially non-responding to tyrosine kinase inhibitor (TKI). In fact, the RESORCE study admitted only patients with a documented radiological progression to sorafenib and tolerant to the drug (defined as receiving sorafenib \geq 400 mg daily for at least 20 of the last 28 days of treatment).

The results indicated significant improvements in the primary endpoint of OS [10.6 months with regorafenib vs. 7.8 months with placebo hazard ratio (HR) 0.63, $P < 0.0001$] besides the secondary endpoints PFS (3.1 vs. 1.5 months, HR 0.46; $P < 0.001$). These benefits were maintained across all subgroups. In addition, patients treated with regorafenib had significantly better overall response and disease control rate than best supportive care. After a median 3.8 months, patients treated with regorafenib presented a reduction of 38% of the risk of death and a 54% reduction in the risk of progression or death compared to placebo^[50].

The most common AEs grade ≥ 3 in patients of the regorafenib group were hypertension (15.2% regorafenib vs. 4.7% of the placebo group), hand-foot skin reaction (12.6% vs. 0.5%), fatigue (9.1% vs. 4.7%), and diarrhea (3.2% vs. 0%)^[50].

Similarly to sorafenib, the survival subgroup analysis of RESORCE evaluating the incidence of skin lesions proposed that hand-foot skin reaction may be a marker for regorafenib activity: infactamong regorafenib-treated patients, OS was improved in patients who had HFSR at any time during the trial and who had their first HFSR event within the first cycle compared with those without HFSR during those periods (13.2 vs. 8.1 months, HR 0.66)^[50].

An additional exploratory analysis evaluating the global benefit of the sequence sorafenib-regorafenib showed that the median OS from the start of sorafenib was 26 months for the sequence sorafenib-regorafenib (whereas resulted 19.2 months in the sequence sorafenib-placebo)^[51].

Following this positive study, an indication expansion for regorafenib was approved in April 2017 in the USA, in May 2017 in Japan and in August 2017 in EU, allowing its use in second-line therapy for HCC.

Lenvatinib

Lenvatinib is a multitargeted TKI of the VEGFRs 1, 2, and 3, FGFRs 1-4, PDGFR α , RET, and KIT signaling involved in tumor angiogenesis and malignant transformation^[52]. Lenvatinib was approved at the dosage 24 mg daily to treat patients with differentiated thyroid cancer refractory to iodine-131 therapy, later the drug was approved in combination with everolimus as a treatment for the second line of renal cell carcinoma at dosage 10 mg daily^[52,53].

Favorable outcomes were achieved in a phase 2 single-arm study on 46 patients with advanced HCC treated with 12 mg of lenvatinib daily until progression or toxicity^[54]. An independent review committee referred that the median in TTP was 7.4 months, the overall response rate evaluated by modified RECIST criteria (mRECIST) was 37%, and the median OS was 18.7 months. The most common toxicities were hypertension, palmar-plantar dysesthesia, thrombocytopenia, anorexia and proteinuria. These adverse events led to dose reductions in 74% of the cases and drug discontinuation in 22%^[54].

Basing on this phase 2 data, the following phase 3 trial (REFLECT) assumed that lenvatinib exposure was influenced by body weight. Consequently, the doses used in the REFLECT trial were 8 mg for patients with weight < 60 kg and 12 mg for others. In this phase 3 study, a total of 954 patients with unresectable HCC were randomized 1:1 according to an open-label, randomized, parallel-assignment, active-controlled protocol, to compare the efficacy of lenvatinib versus sorafenib as a first-line systemic treatment according to a non-inferiority design^[55]. The primary endpoint of OS was initially evaluated for non-inferiority and then for superiority. The study excluded patients with 50% or higher liver occupation, obvious invasion of the bile duct, or invasion at the main portal vein.

The primary endpoint of non-inferiority of lenvatinib in terms of OS compared with sorafenib was met: lenvatinib resulted not-inferior in mOS (13.6 months with lenvatinib and 12.3 months with sorafenib). Differently, the OS of lenvatinib over sorafenib was not achieved. Additionally, lenvatinib achieved significant and clinically meaningful improvement in PFS (7.4 vs. 3.7 months), TTP (8.9 vs. 3.7 months) and overall response rate (24% vs. 9% by mRECIST). The median duration of treatment with lenvatinib was 5.7 vs. 3.7 months with sorafenib^[55].

Investigators detected that the treatment duration for lenvatinib arm was shorter than time to progression (5.7 vs. 8.9 months) and that this datum was not observed in the sorafenib arm (both 3.7 months). This could be related to a major incidence of lenvatinib definitive interruption before tumor progression probably due to a greater incidence of serious treatment emergent adverse events in the lenvatinib arm and not in the sorafenib arm.

In the two arms of the study, a similar level of treatment-emergent adverse events was observed for dose reductions (37% of patients in the lenvatinib arm versus 38% in the sorafenib) and drug discontinuations (9% vs. 7% respectively).

Lenvatinib showed an higher incidence of grade ≥ 3 treatment-emergent adverse events (57% vs. 49%), and the most common grade 3/4 treatment-emergent adverse events resulted hypertension (23% in lenvatinib arm vs. 14% of sorafenib), decreased weight (8% vs. 3%), decreased platelet count (6% vs. 3%), elevated aspartate aminotransferase (5% vs. 8%), decreased appetite (5% vs. 1%), diarrhea (4% vs. 4%), and palmar-plantar erythrodysesthesia (3% vs. 11%)^[55].

Cabozantinib

Cabozantinib is a tyrosine kinases inhibitor with activity directed to MET, VEGFR2, FLT3, c-KIT, and RET^[56]. This drug was initially approved by FDA on November 2012 for the treatment of metastatic medullary thyroid cancer with dosage 140 mg daily. In April 2016 Cabozantinib was approved for the second-line treatment of advanced renal cell carcinoma after prior antiangiogenic therapy while in renal cell carcinoma at dosage of 60 mg daily.

Cabozantinib was also investigated in HCC patients as part of a phase 2 randomized discontinuation trial^[57]; a cohort of 41 patients with HCC was treated with 100 mg daily of drug, the disease control rate at 12 weeks was 68% with two partial responses. The efficacy resulted independent from a prior sorafenib

therapy. Grade 3 and 4 adverse events included diarrhea (20%), palmar-plantar erythrodysesthesia (15%) and thrombocytopenia (15%). More than the half of patients (59%) required at least one dose reduction^[57].

In the randomized, double-blind, parallel-assignment, placebo-controlled phase 3 trial (CELESTIAL), 707 subjects with HCC were randomized according to a 2:1 ratio to receive cabozantinib at 60 mg daily ($n = 470$) or placebo ($n = 237$)^[58]. All the patients presented ECOG performance status of 0 or 1, Child-Pugh score of A, and resulted progressed or intolerant to at least 1 prior systemic therapy for advanced HCC. Worth to be mentioned is that 28% of subjects received two prior systemic therapies regimen, therefore CELESTIAL study enrolled also patients receiving cabozantinib as third line of treatment. The primary endpoint OS resulted significant favorable to cabozantinib: 10.2 months compared with 8.0 months with placebo, representing the 24% reduction in the risk of death (HR 0.76; 95% CI 0.63-0.92; $P = 0.0049$). The PFS with cabozantinib was 5.2 months compared with 1.9 months for placebo, corresponding to the 56% of reduction in the risk of progression or death with the targeted therapy (HR 0.44; $P < 0.0001$). In the subgroup analysis of patients receiving only prior sorafenib, the median OS was 11.3 months with cabozantinib compared with 7.2 months for placebo (HR 0.70)^[58].

In the treatment arm resulted an increased number of patients discontinuing due to treatment-related adverse events, in particular the most common grade 3/4 adverse events with cabozantinib resulted hand-foot skin reaction (17% vs. 0% in placebo arm), hypertension (16% vs. 2%), increased aspartate aminotransferase (12% vs. 7%), fatigue (10% vs. 4%), and diarrhea (10% vs. 2%). In addition, cabozantinib arm presented a higher incidence of grade 5 adverse events; in fact, 6 patients died due to hepatic failure, esophagobronchial fistula, portal vein thrombosis, upper gastrointestinal hemorrhage, pulmonary embolism, and hepatorenal syndrome. The median duration of exposure resulted in 3.8 months and 2 months in cabozantinib and placebo arms respectively^[58]. Notably, the discordance registered between PFS and DoE in the active arm versus the placebo one could be explained by the significant higher incidence of adverse events in the cabozantinib group that could have been led to an earlier treatment discontinuation, reflecting probably a not easy management of toxicities.

Nivolumab

Nivolumab in HCC was investigated in the CheckMate-040 trial, a multicenter, open label phase I/II study conducted from November 2012, to August 2016 in adults (≥ 18 years) with histologically confirmed advanced HCC with or without hepatitis C or B (HCV OR HBV) infection. A previous sorafenib treatment was allowed^[59]. The study enrolled 48 patients in a first dose-escalation phase and 214 patients in a subsequent dose-expansion phase. Primary endpoints were safety and tolerability for the escalation phase and objective response rate (evaluated by RECIST version 1.1) for the expansion phase.

In the escalation phase, patients received 0.1 to 10 mg/kg of IV nivolumab every 2 weeks. Nivolumab 3 mg/kg every 2 weeks was chosen for the dose expansion phase^[59]. The confirmed overall response rate assessed by blinded independent central review was 14.3% (22 of 154 patients), with 3 complete responses (1.9%) and 19 partial responses (12.3%). Response duration ranged from 3.2 to more than 38.2 months; 91% of those patients had responses of 6 months or longer and 55% had responses lasting 12 months or longer.

Common adverse reactions occurring in more than 20% of patients included fatigue, rash, musculoskeletal pain, pruritus, diarrhea, nausea, asthenia, cough, dyspnea, constipation, decreased appetite, back pain, arthralgia, upper respiratory tract infection, and pyrexia. Differently for the safety profile previously described in nivolumab label, patients of CheckMate-040 reported a higher incidence of elevations in transaminases and bilirubin levels: treatment-emergent grade 3 or 4 AST was observed in 27 (18%) subjects, grade 3 or 4 ALT in 16 (11%) patients, and grade 3 or 4 bilirubin in 11 (7%) patients. Immune-mediated hepatitis requiring systemic corticosteroids occurred in 8 (5%) patients^[59].

In September 2017 American Food and Drug Agency granted priority review to nivolumab for HCC and approved it for patients who have previously been treated with sorafenib, at the dosage of 240 mg every 2 weeks. As a condition of accelerated approval, larger phase 3 randomized trials of nivolumab versus sorafenib will be required to verify the clinical benefit of nivolumab for this indication. A randomized, multicenter phase 3 study of nivolumab versus sorafenib as first-line treatment in patients with advanced hepatocellular carcinoma (CheckMate 459) is ongoing with the goal to enroll 726 patients and the estimated primary completion date is October 2018^[60].

DISCUSSION

Ten years after sorafenib introduction in HCC scenario, novel knowledge about this mature drug and the availability of four new drugs are opening innovative therapeutic perspectives for patients suffering from this disease.

Firstly, new evidences coming from clinical practice allowed a renovate use of sorafenib in first line setting. In particular, an important recommendation is to remain in therapy for patients experiencing toxicity during the first weeks of therapies. In fact, at the era of the studies SHARP and ASIA PACIFIC, those patients experiencing important adverse events were considered unsuitable to sorafenib and were suggested to permanently discontinue this therapy. Differently, at the present time, prospective and retrospective data seem to indicate that most likely these patients could receive the major benefit to sorafenib. Indeed, prospective studies and retrospective experiences identified toxicities as a probable marker of response to the therapy. This suggest managing patients experiencing toxicities with an appropriate tolerable adverse event protocol, proposing momentary suspensions followed by restart of therapy at lower doses with the aim to generate drug-tolerance. Nevertheless, a small randomised clinical trial, comparing TACE plus external beam radiotherapy (RT) versus sorafenib in patients affected by hepatocellular carcinoma with macroscopic vascular invasion and Child-Pugh A liver function, demonstrated a superiority of the TACE-RT group over sorafenib: at week 12, the PFS rate was 86.7% vs. 34.3%, with a higher rate of radiologic response (33.3% vs. 2.2%), a better time of progression (31 vs. 11.7 weeks), and an overall survival of 55 vs. 43 weeks^[61].

The second innovative point of this new era is the solution to the historic unmet need due to lack of second line therapies in HCC patients progressing to sorafenib. In the past 10 years the scanty opportunity for these patients was the re-treatment with higher dose of sorafenib, or the participation in a clinical trial. Presently, regorafenib is the only second line systemic therapy available worldwide in patients progressed on sorafenib. First-line patients with a very severe intolerance to sorafenib unable to follow a tolerable adverse event protocol may will benefit from the lenvatinib, once approved by Health Authorities, because proved to be non-inferior to the standard of care sorafenib and presenting a different toxicity profile. Cabozantinib and nivolumab could be valid second-line options that allow to reach the “embarrassment of riches” also for treating intermediate and advanced stages HCC.

But these new therapeutic options open the dilemma on which second-line should be chosen. A first speculative solution could evaluate survival data observed in pivotal trials of each therapy. Survival benefit data refers that the sequence sorafenib-regorafenib is associated to 26 months, no data have been reported for the sequences sorafenib-cabozantinib and sorafenib-nivolumab. Besides, while regorafenib was evaluated only in patients with sorafenib progression, cabozantinib was studied in population generically progressed to TKIs because CELESTIAL trial enrolled also patients treated with front-line drugs different from the standard of care. Moreover, including in the trial a significant number of patients with two previous regimens, cabozantinib could be evaluated as a third line therapy too. With the goal to achieve the maximum lines of treatment, a possible sequence strategy may include the use of regorafenib in a second line after progression to sorafenib, allowing to reach a third line of therapy with cabozantinib. The role of

immunotherapy must be still understood, evaluating the efficacy and tolerability data deriving from the ongoing phase 3 studies requested by regulatory agency. These studies have concluded enrollment and final outcomes will be presented in the next months at congresses.

But a more reliable criterion that could guide the choice of such drugs is the evaluation of tolerability profile detected in clinical trials. Consequently, the choice of the second line drug should be tailored based on patients' characteristics, comorbidities and expected toxicity profile associated with each regimen. At this regard, pivotal studies indicated that regorafenib had a sorafenib-like liver toxicity profile with hepatic adverse events resulting like placebo, while cabozantinib increased liver toxicity, as evidenced by the increase in grade 3/4 transaminases of 12% suggesting attention to patients with very high transaminase values at the baseline. Similarly, nivolumab does not seem to induce the same toxicity seen for the other two drugs (hand-foot skin reaction, diarrhea, hypertension), but may have increased liver toxicity, revealed by the increase in grade 3/4 bilirubin.

An additional therapeutic opportunity for the second-line treatment could be represented also by ramucirumab. At the day of writing a press release announced that this drug demonstrated efficacy in a phase 3 study conducted on patients pretreated with sorafenib and with AFP > 400 ng/mL^[67].

CONCLUSION

Improved knowledge of the standard of care and new therapies coming up after 10 years of failure in HCC, trigger new hopes for patients and hepato-oncological community to extend the survival in a disease that remains one of the leading causes of cancer-related deaths around the world. Currently the lack of adequate predictive or potential biomarkers factors makes challenging the identification of patients who will benefit with durable responses from each therapy.

DECLARATIONS

Authors' contributions

Conception and manuscript writing, provision of study materials, collection and assembly of data: Giovanis P

Administrative support: D'Ippolito S

Data analysis and interpretation: Giovanis P, Pastorelli D

Final approval of manuscript: all authors

Financial support and sponsorship

This publication has been made possible by the Medical Writing support of dott Roberto Barsanti and dott Maria Cristina Mazzotta (Medical Department - Bayer SpA). No specific funding or grant have been provided for this publication.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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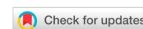
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Original Article

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A potential clinical based score in hepatitis C virus cirrhotic patients to exclude small hepatocellular carcinoma

Denise Cerqueira Paranaguá-Vezozzo^{1,2}, Celso Eduardo Lourenço Matielo¹, Daniel Ferraz de Campos Mazo^{1,3}, Lucas Souto Nacif¹, Mario Guimaraes Pessoa¹, Gleicy Luz Reinoso Pereira¹, Roque Gabriel Rezende de Lima¹, Patricia Momoyo Yoshimura Zitelli¹, Suzane Kioko Ono^{1,2}, Flair José Carrilho^{1,2}

¹Division of Gastroenterology and Hepatology, Department of Gastroenterology, University of São Paulo School of Medicine, São Paulo 05403-900, Brazil.

²São Paulo Clínicas Liver Cancer Group, São Paulo, Cancer Institute of São Paulo, University of São Paulo School of Medicine, São Paulo 01246-000, Brazil.

³Division of Gastroenterology (Gastrocentro), School of Medical Sciences, University of Campinas, Campinas 13083-878, Brazil.

Correspondence to: Dr. Denise Cerqueira Paranaguá-Vezozzo, Department of Gastroenterology, University of São Paulo School of Medicine, Av. Dr. Enéas de Carvalho Aguiar nº 255, Instituto Central, # 9159, São Paulo 05403-900, Brazil.
E-mail: denise.paranagua@gmail.com

How to cite this article: Paranaguá-Vezozzo DC, Matielo CEL, de Campos Mazo DF, Nacif LS, Pessoa MG, Pereira GLR, de Lima RGR, Zitelli PMY, Ono SK, Carrilho FJ. A potential clinical based score in hepatitis C virus cirrhotic patients to exclude small hepatocellular carcinoma. *Hepatoma Res* 2018;4:11. <http://dx.doi.org/10.20517/2394-5079.2018.17>

Received: 6 Mar 2018 **First Decision:** 12 Mar 2018 **Revised:** 20 Mar 2018 **Accepted:** 21 Mar 2018 **Published:** 27 Apr 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Aim: Hepatitis C virus (HCV) cirrhosis is an important cause of hepatocellular carcinoma (HCC). This study aimed to identify factors of HCC presence among HCV cirrhotic patients with and without small diameter HCC (≤ 3 cm).

Methods: A case control transversal study between 1998 and 2003 including 93 patients: 31 with small diameter HCC and 62 without HCC. Groups were matched by age and gender. Multiple logistic regression analysis using Akaike Information Criteria to estimate the probability of HCC was performed. A model score was generated and bootstrap analysis was performed for internal validation.

Results: Three significant laboratorial variables for HCC presence were found: alanine aminotransferase > 37 U/L [odds ratio (OR): 7.43 (1.61-34.19), $P = 0.01$], alpha-fetoprotein > 20 ng/mL [OR: 16.2 (4.17-63.01), $P < 0.001$] and platelet count $< 100,000/\text{mm}^3$ [OR: 3.62 (1.43-9.14), $P = 0.007$]. A model score with an area under curve of 0.79 (95% CI: 0.7-0.89) was built based on these variables. The negative predictive value of those classified as at low risk of HCC was 99.1%.

Conclusion: An easy and practical model score was generated. It may be an auxiliary tool for identification of HCV patients with low probability of small diameter HCC at initial evaluation composed of three serum examinations used in routine outpatient clinical practice.



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Keywords: Clinical score, hepatocellular carcinoma, hepatitis C

INTRODUCTION

Hepatocellular carcinoma (HCC) represents more than 5% of all malignant tumors, and is the fifth most common cancer in men and the eighth in women. The prevalence of this cancer is expected to increase in the coming years^[1-3]. HCC incidence varies greatly between geographical regions^[4-7]. Hepatitis C virus (HCV) infection is typically prevalent in areas with low incidence (< 3 per 100,000) of HCC, as often found in developed countries. Japan is an exception to this, with 80% of HCC patients infected with HCV^[6]. It is generally believed that the presence of cirrhosis and chronic HCV infection contribute to an increased risk of HCC^[8]. Other potential underlying risk factors include gender (male), advanced age, hepatitis B virus co-infection, alcohol abuse, a history of blood transfusion, and diabetes^[9].

Several cohort studies have shown that early HCC detection increases the potential for application of curative rather than palliative treatment. Screening strategies may allow earlier HCC diagnosis, with a potential positive impact on mortality^[10,11]. The European and American guidelines recommend abdominal ultrasonography (US) every 6 months^[12,13], but the recently updated Asia-Pacific guidelines, as well as other centers, recommend a combination of US and serum alpha-fetoprotein (AFP) measurement for HCC surveillance^[14,15].

In Brazil, HCV is the main etiology of liver cirrhosis^[16]. Among a 10-year cohort of 884 Brazilian cirrhotic patients, with almost 60% with HCV etiology, reported an incidence of HCC of 16.9% over 5 years^[16]. Improvements in diagnostic imaging and routine surveillance programs have enabled the identification of small liver nodules, meaning that the majority of our HCC cases are now diagnosed in their early stages (80%)^[17,18]. As a result, the prognosis for patients with HCC has improved considerably^[10,11,19]. However, surveillance adherence rates for HCC are far from ideal in many settings^[20]. Moreover, HCC rate detection may be lower outside specialized centers, and the diagnosis of small HCC (≤ 3 cm) can indeed be a challenge in clinical practice. Therefore, it is important to search for reliable markers for early detection or even exclusion of HCC with confidence, to assist in the management of these patients.

The aim of this study was to identify possible factors of HCC presence/absence by analyzing a set of patients with HCV-related cirrhosis, with and without small diameter HCC (≤ 3 cm).

METHODS

We performed an observational case-control study in a cohort of HCV-related cirrhosis patients with and without small diameter HCC (≤ 3 cm). The STROBE statement for reporting observational studies was followed^[21].

HCC patients

The study included 31 patients (20 male, 11 female) with HCV-related cirrhosis and HCC smaller than 3 cm, who were diagnosed and followed up at a tertiary healthcare center; the Department of Gastroenterology at the University of São Paulo School of Medicine, São Paulo, Brazil between 1998 and 2003. All patients on file eligible for inclusion in the HCC group were included. HCC diagnosis was based on one of the three following criteria: (1) biopsy and histological examination of the nodule; (2) nodules with arterial hyper vascularization and washout in at least two different dynamic imaging methods [abdominal computed tomography (CT) or magnetic resonance imaging (MRI)]; or (3) identification of a suspect growth in at least one dynamic imaging method along with serum AFP > 200 ng/mL.

All biopsies were performed with a 14G Tru-Cut® needle (Medical Technology, Gainesville, FL, USA) with ultrasound-guided puncture performed in the nodule and in the adjacent parenchyma. HCC was diagnosed

in 12 (63.1%) of the 19 biopsies performed. The remaining seven cases were included based on the progressive increase of nodule size; with consequent better definition by imaging methods (5 cases) and/or increased AFP level (2 cases).

HCC diagnosis was made with imaging in 15 patients (48.3%) and histology in 12 patients (38.8%). A combination of imaging methods and AFP levels was applied in four cases (12.9%). All 31 patients presented up to three liver nodules smaller than 3 cm in total. Some nodules were detected as part of a screening program (55%) involving abdominal US and serum AFP monitoring every 6 months, while some were referrals from other centers with diagnoses of suspected HCC. The mean nodule size was 22 mm. All patients underwent a chest computerized tomography scan and a full-body bone scan to exclude the presence of metastatic HCC.

Control group

Sixty-two patients (40 male, 22 female) with hepatitis C-related cirrhosis, but without HCC were selected from the same tertiary care center. They were paired by age and gender with the HCC group. All patients in the control group were subjected to abdominal US 6 months after data collection, to ensure that HCC had not developed. These patients were systematically screened every 6 months for HCC with US and serum AFP measurements.

The following anthropometric and clinical variables were recorded and used to categorize the control group: age (> 60 years); gender (male/female); treatment with alpha-interferon (yes/no); previous participation in a screening program (yes/no); response to antiviral treatment (yes/no); Child-Pugh score (A/B/C); esophageal varices (yes/no); upper gastrointestinal (GI) bleeding (yes/no); ascites (yes/no); hepatic encephalopathy (yes/no); spontaneous bacterial peritonitis (SBP) (yes/no); weight loss (yes/no); alcohol consumption (yes/no) and abdominal pain (yes/no).

The following serum markers were examined: AFP (≥ 20 ng/mL), total bilirubin (Bil) (> 10 ng/dL), aspartate aminotransferase (AST) (> 41 U/L), alanine aminotransferase (ALT) (> 37 U/L), alkaline phosphatase (AP) (> 129 U/L), gamma-glutamyl transpeptidase (GGT) (> 61 U/L), transferin saturation ($> 40\%$), ferritin (> 150 ng/mL), international normalized ratio (INR) (> 1.20), platelet count ($< 100,000/\text{mm}^3$), albumin (< 3.4 g/dL), fibrinogen (< 150 mg/dL), glycemia (> 110 mg/dL). We additionally recorded a descriptive analysis of the HCC histological type as well-, moderately- or poorly-differentiated. Of the 12 histologically confirmed tumors, 11 were moderately-differentiated, and only 1 was well-differentiated, while none were poorly differentiated.

This study was approved by the Institutional Review Board, fulfilling all of the requirements for retrospective studies in human subjects, according to the guidelines of the 1975 Helsinki Declaration.

Statistical analysis

Quantitative variables are presented as median, first quartile and third quartile, and qualitative variables as percentages. Differences between groups (presence/absence of HCC) regarding continuous variables were verified via the Mann-Whitney test and association between categorized variables were checked by Fisher's test. *P*-values smaller than 0.05 were considered statistically significant.

Receiver operator curve (ROC) curve was applied to all continuous variables, and cutoff values were selected to maximize the Youden index (MaxSe and MaxSp)^[22]. Simple and multivariable logistic regressions were performed to predict HCC presence. Akaike Information Criterion (AIC)^[23] was used to select the most informative variables in the backward strategy. Patients with missing data in a specific variable were excluded from the analysis of that variable.

Finally, linear predictors from multiple regressions were resized to a range from 0 to 100, and then a cutoff value was determined by a ROC curve. Performance measures given by sensitivity (Se), specificity (Sp),

Table 1. Descriptive analysis of frequencies and percentages of clinical and laboratory variables of the 93 patients HCV-related cirrhosis patients

	Control (<i>n</i> = 62)	Case (<i>n</i> = 31)	<i>P</i> value
Gender (male), <i>n</i> (%)	40 (64.52)	20 (64.52)	1
Age (year), median (min-max)	59 (52.25-66.75)	59 (52.5-66)	0.952
AFP (ng/mL)	4.95 (2.92-8.3)	10.9 (4.75-45.3)	< 0.001
Bil (mg/dL)	1.3 (0.82-2.1)	1.4 (1.05-2)	0.508
AST (U/L)	53.5 (39-84)	91 (62.5-117)	0.002
ALT (U/L)	47 (30.5-74.5)	70 (55.5-110)	0.002
GGT (U/L)	50.5 (34-113)	78 (50.5-188.5)	0.071
AP (U/L)	99.5 (80.5-131.75)	111 (79-136)	0.496
INR	1.27 (1.16-1.36)	1.24 (1.15-1.53)	0.883
Platelet count ($10^3 \times \text{mm}^3$)	118.5 (68.75-158)	83.9 (63.75-104.5)	0.02
Transferin saturation (%)	44 (28-58)	44 (30-61.25)	0.955
Ferritin (ng/mL)	78.5 (23-258.5)	325 (140.25-500.5)	0.199
Albumin (g/dL)	3.65 (3.37-4)	3.61 (3.32-3.9)	0.302
Glucose (mg/dL)	97 (88-130)	99 (87.5-108.5)	0.526
Fibrinogen (mg/dL)	214 (178-271.5)	157 (126-190)	0.04
Screening (%)	59 (95.16)	17 (54.84)	< 0.001
Ascites (%)	22 (35.48)	16 (51.61)	0.18
SBP (%)	1 (1.61)	1 (3.23)	1
Variceal bleeding (%)	7 (11.29)	4 (12.9)	1
Esophageal varices (%)	42 (67.74)	20 (64.52)	0.817
Encephalopathy (%)	7 (11.29)	4 (12.9)	1
Abdominal pain (%)	1 (2.44)	0 (0)	1
Weight loss (%)	5 (12.2)	1 (3.23)	0.227
Child-Pugh A/B/C (%)	44 (70.97)	17 (54.84)	0.139
	17 (27.42)	12 (38.71)	
	1 (1.61)	2 (6.45)	
Alcohol consumption (%)	16 (25.81)	8 (25.81)	1
Alpha-interferon therapy (%)	42 (67.74)	19 (61.29)	0.644
Treatment response (%)	10 (23.81)	0 (0)	0.056

HCV: hepatitis C virus; AFP: alpha feto protein; HCC: hepatocellular carcinoma; Bil: total bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; AP: alkaline phosphatase; INR: international normalized ratio; SBP: spontaneous bacterial peritonitis

positive (PPV) and negative (NPV) predicted values were calculated based on a HCC yearly prevalence of 3% (Brazil)^[16] and 10% (Japan)^[24] and the performance of the model was further analyzed with the bootstrap method^[25] with 1000 samples used to estimate the internal validity of performance measures. The R Project for Statistical Computing ver. 3.0.2 (R Core Team, Vienna, Austria, 2014) software package was used for statistical analyses.

RESULTS

We evaluated 93 patients with HCV-related cirrhosis, 31 of which with small HCC and 62 without HCC. Table 1 shows the frequencies and percentages of clinical and laboratory variables of the HCC and control groups. The median age in both groups was 59 years old, the majority were male, and had preserved liver function (Child-Pugh A). No differences between groups could be detected regarding liver related outcomes such as ascites ($P = 0.18$), spontaneous bacterial peritonitis ($P = 1.0$), esophageal varices ($P = 1.0$), variceal bleeding ($P = 1.0$) or hepatic encephalopathy ($P = 0.817$).

On the other hand, patients with HCC had higher levels of AFP [10.9 (4.75-45.3) vs. 4.95 (2.92-8.3) ng/mL, $P < 0.001$], AST [91 (62.5-117) vs. 53.5 (39-84) U/L, $P = 0.002$], ALT [70 (55.5-110) vs. 47 (30.5-74.5) U/L, $P = 0.002$], and were less likely to have participated in a screening program (54.84% vs. 95.16%, $P < 0.001$) than patients in the control group. Furthermore, HCC patients had a lower platelet count than their counterparts in the control group (83.9 vs. $118.5 \times 10^3 \times \text{mm}^3$, $P = 0.02$), as shown in Table 1.

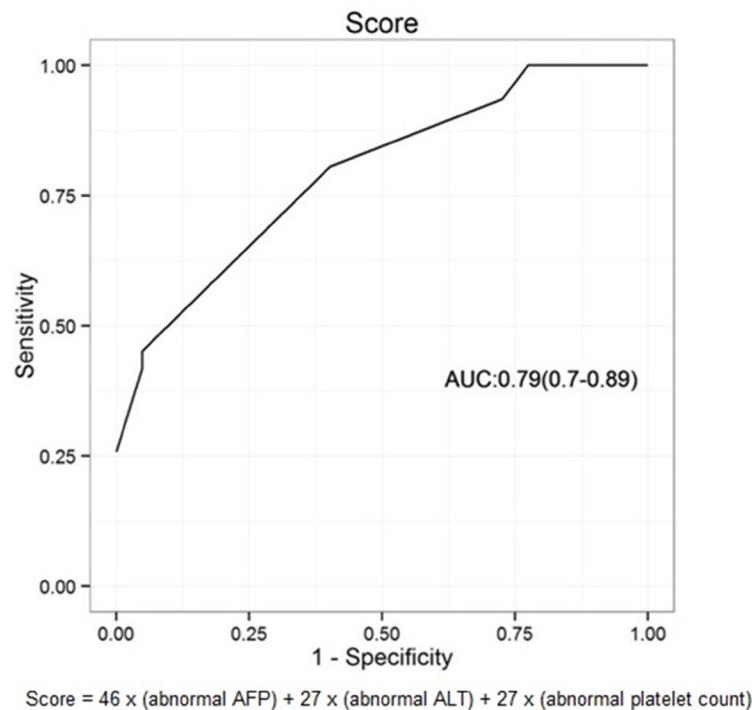


Figure 1. Receiver operator curve analysis of calculated model score for identifying hepatocellular carcinoma. ALT: alanine aminotransferase; AFP: alpha-fetoprotein; AUC: area under curve

Among HCC patients, 19 (61%) were subjected to antiviral treatment with alpha-interferon, to which none of them responded. However, among the control group, 42 (68%) were subjected to antiviral treatment, and 10 (24%) of these patients achieved sustained virological response (SVR) ($P = 0.05$).

On multivariate logistic regression [Table 2], higher AFP levels (> 20 ng/mL, $P < 0.001$), higher ALT levels (> 37 U/L, $P = 0.01$) and lower platelet count ($< 100,000/\text{mm}^3$, $P = 0.007$) were independent prediction factors of HCC presence, with odds ratios of 16.2 (4.17-63.01), 7.43 (1.61-34.19) and 3.62 (1.43-9.14), respectively.

The coefficients of the multivariable model are 3.71 ± 1 , 2.96 ± 0.77 , 1.72 ± 0.9 , 1.7 ± 0.62 for the intercept, AFP > 20 , ALT > 37 and platelet count $< 100,000$. These variables were applied to build a score capable of discriminating higher risk of HCC in HCV cirrhotic patients, with an area under curve (AUC) of 0.79 (95% CI: 0.7-0.89) [Figure 1].

Based on the findings, we propose a model score to apply to outpatients with HCV related cirrhosis, but without tumors or nodules on US or CT/MRI images undertaken during routine surveillance:

HCC Risk Score in HCV patients with cirrhosis = $46 \times (\text{abnormal AFP}) + 27 \times (\text{abnormal ALT}) + 27 \times (\text{abnormal platelet count})$

This formula requires the knowledge of the range and limits of the normal values of the aforementioned variables. For example, if AFP > 20 ng/dL, it is considered abnormal, and the score attributable to this variable is 1 (1), but if it ≤ 20 ng/dL its score is 0. Similarly, if the ALT is > 37 U/L, it is considered abnormal, and the score is 1 (1), and finally a platelet count $< 100,000/\text{mm}^3$ is considered an abnormal value, and its score is 1 (1).

Table 2. Odds ratio of risk factors for HCC presence on a multivariate logistic regression analysis

	Group	OR (95% CI)	P value
Gender	Male	1 (0.41-2.46)	1
Age (years)	> 60	1.07 (0.44-2.6)	0.88
AFP (ng/mL)	> 20	16.2 (4.17-63.01)	< 0.001
Bil (mg/dL)	> 1.0	1.58 (0.61-4.12)	0.349
AST (U/L)	> 41	3.53 (0.95-13.13)	0.06
ALT (U/L)	> 37	7.43 (1.61-34.19)	0.01
GGT (U/L)	> 61	2.65 (0.61-11.43)	0.192
AP (U/L)	> 129	-	0.995
INR	> 1.20	0.87 (0.36-2.12)	0.761
Platelet count (/mm ³)	< 100,000	3.62 (1.43-9.14)	0.007
Transferin saturation	> 40%	0.97 (0.34-2.73)	0.954
Ferritin (ng/mL)	> 150	1.8 (0.3-10.91)	0.522
Albumin (g/dL)	< 3.4	1.46 (0.58-3.67)	0.425
Glucose (mg/dL)	> 110	0.58 (0.2-1.65)	0.304
Fibrinogen (ng/mL)	< 150	1.46 (0.58-3.67)	0.425
Screening	Yes	0.06 (0.02-0.24)	< 0.001
Ascites	Yes	1.94 (0.81-4.66)	0.138
SBP	Yes	2.03 (0.12-33.67)	0.62
Variceal bleeding	Yes	1.16 (0.31-4.32)	0.821
Esophageal varices	Yes	0.87 (0.35-2.15)	0.756
Encephalopathy	Yes	1.16 (0.31-4.32)	0.821
Abdominal pain	Yes	-	0.992
Weight loss	Yes	0.24 (0.03-2.17)	0.204
Child	B	1.83 (0.72-4.62)	0.203
	C	5.18 (0.44-60.93)	0.191
Alcohol consumption	Yes	1 (0.37-2.68)	1
Alpha-Interferon therapy	Yes	0.75 (0.31-1.85)	0.538
Treatment response	No	1.02 (0.3-3.45)	0.98
	Yes	-	0.993

HCV: hepatitis C virus; AFP: alpha feto protein; HCC: hepatocellular carcinoma; Bil: total bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; AP: alkaline phosphatase; INR: international normalized ratio; SBP: spontaneous bacterial peritonitis; OD: odds ratio

Table 3. Discrimination measurements for development of the model score with different prevalent risk HCC scenario (3% and 10%) and results of its internal validation

	Estimate (95% CI)	Optimism
General cut off: 54		
Se	81% (64%-94%)	-13.1%
Sp	60% (47%-71%)	6.4%
Prevalence scenario	3%	10%
PPV	5.8%	18%
NPV	98.5%	95%
Excluding cut off: 26		
Se	100 % (89%-100%)	-7.5%
Sp	23% (14%-34%)	13%
Prevalence scenario	3%	10%
PPV	3.7%	12.2%
NPV	99.1%	96.9%
Including cut off: 100		
Se	26% (14%-43%)	-2.6%
Sp	100% (94%-100%)	0
Prevalence scenario	3%	10%
PPV	44.6%	74.2%
NPV	97.7%	92.3%

HCC: hepatocellular carcinoma; NPV: negative predictive value; PPV: positive predictive value; Se: sensitivity; Sp: specificity

Three cut-off levels of the model score were considered, and bootstrap analysis was applied to determine the optimal values for sensitivity and specificity [Table 3], since the diagnostic measures were calculated based on internal validation. The cut-off level with the best sensitivity and specificity was 54, with a sensitivity of 81% (64%-94%) and a specificity of 60% (47%-71%). In the scenario of 3% prevalence of HCC risk in HCV cirrhotic patients the best cut off value to exclude HCC is 26 [sensitivity = 100% (89%-100%); specificity = 23% (14%-34%); PPV = 3.7%; NPV = 99.1%], and the best cut off to include HCC is 100 [sensitivity = 26% (14%-43%); specificity = 100% (94%-100%); PPV = 44.6%; NPV = 97.7%]. When we changed the scenario prevalence to 10%, the results show better performances from the positive predictive values, from 5.8% to 18% at a cut off level of 54, from 44.6% to 74.2% at cut off level of 100, and from 3.7% to 12.2% at cut off level of 26.

DISCUSSION

This case control study analyzed clinical and laboratory parameters used in routine daily practice, aiming to identify patients with HCV-related cirrhosis at increased risk of HCC presence. We found that higher serum AFP and ALT levels, and lower platelet count were independent prediction factors of HCC. Such information could be used to develop more cost-effective screening strategies.

The median age in both groups was 59 years old. Velázquez *et al.*^[26] demonstrated that an age of ≥ 55 years is an independent risk factor for HCC among patients with cirrhosis and HCV. Other published data suggest a higher incidence of HCC from the age of 60^[6]. Lok *et al.*^[27] also found that older age is a predictive factor for HCC development. The HCC group (31 patients) had male:female ratio of 1.8:1; this finding is consistent with data from the literature showing that the prevalence of HCC is 2 to 4 times higher in male patients^[24].

We found no differences in liver related outcomes, such as ascites, spontaneous bacterial peritonitis, esophageal varices, variceal bleeding or hepatic encephalopathy between groups. This suggests that HCC does not alter the pathogenesis of the early clinical stages of HCV-related cirrhosis in more advanced stages. A previous study showed that hepatic encephalopathy and ascites were not related to the development of HCC, although esophageal varices were^[28]. The latter was also observed by Lok *et al.*^[27]. Bolondi *et al.*^[29] assessed the cost-effectiveness of HCC screening by comparing 313 patients with cirrhosis and 104 patients with cirrhosis and HCC, and identified the functional classes Child-Pugh B and C as independent risk factors for HCC. Our results are different, possibly due to the small number of patients and also because most of them had preserved liver function (Child-Pugh A). However they do point to the need for identifying multiple risk factors, beyond the clinical stage of cirrhosis to allow earlier identification of risk. This is of great importance in improving the management and prognosis of patients with HCC.

Sustained virological response (SVR) occurred in 24% of the control group, while no patients in the HCC group exhibited SVR. Several studies have demonstrated the beneficial impact of HCV clearance with interferon in reducing HCC occurrence^[30]. In a multiple logistic regression analysis, AFP, ALT and platelet count were related to higher risk of HCC. In our previous cohort study of patients with cirrhosis, we found the following risk factors for HCC; AFP > 20 ng/mL, albumin < 3.4 g/dL and patients of East Asian ethnicity as the best of seven possible models applied to predict HCC risk^[16]. In the present study AFP > 20 ng/mL was confirmed as a predictive risk factor for the presence of HCC. The diagnostic importance of AFP has been the subject of much scientific debate in recent years. In some studies, a high base value of AFP has been considered a risk factor for HCC, with a cut-off level of 20 ng/mL for determining groups of high and low risk^[29]. AFP levels above 400 ng/mL in the presence of a hepatic nodule in imaging finding, is a conclusive HCC diagnosis^[28]. However, small HCC tumors (< 2 cm) involve low-level secretion of AFP and thus, in most cases the patients cannot be diagnosed using this test alone^[31]. In a prospective study, Tong *et al.*^[32] analyzed 31 patients with cirrhosis and hepatitis B virus or HCV who had developed HCC; they found AFP values above 400 ng/mL in only 4(13%). It is important to note that the AFP levels may be higher in individuals

with chronic viral hepatitis (B or C), but without HCC compared with similar patients with other etiologies of cirrhosis. This is caused by the inflammatory activity and hepatocyte regeneration in the most severe cases of viral hepatitis. Gupta *et al.*^[31] conducted a systematic review evaluating AFP as an instrument for the detection of HCC in patients with hepatitis C; they concluded that AFP has limited utility in this setting. Most authors have found that an isolated measurement of serum AFP levels had limited success for early HCC screening^[14,33], but even small changes in AFP levels may be a predictor for HCC^[34,35]. In fact, dynamic AFP measurement could identify patients at higher risk of HCC occurrence, as recently shown by Bird *et al.*^[36].

Early HCC detection remains challenging, but novel serum biomarkers are under evaluation, such as microRNAs (miRNAs)^[37,38], creatine/betaine ratio^[39], the combination of chaperonin containing TCP1 complex (CCT) and IQ-motif-containing GTPase-activating protein-3 (IQGAP3)^[40] and circulating c-Myc and p53 proteins^[41].

The lower blood platelet count in HCC patients can be explained by a longer evolution of chronic liver disease with subsequent advanced portal hypertension and hypersplenism. Velázquez *et al.*^[26] showed that platelet count $< 75,000/\text{mm}^3$ was an independent positive predictive value for HCC development. In this analysis, the cut-off level for platelet count was $100,000/\text{mm}^3$ according to previously defined levels^[42,43]. Lok *et al.*^[27] also demonstrated the association of HCC risk with low platelet count through the HALT-C study cohort. In a recent prospective study of the ANRS CO12 CirVir cohort including 1323 patients with HCV cirrhosis, Ganne-Carrié *et al.*^[44] found five variables independently associated with HCC development at 1, 3, and 5 years: age > 50 years, past excessive alcohol intake, GGT above the upper limit of normal, absence of SVR during follow-up and platelets $< 100,000/\text{mm}^3$. The latter was also evidenced in our work and in the retrospective study by Noh *et al.*^[45] as a predictor of HCC.

This study found that serum levels of ALT, AFP and platelet count could be used to determine the risk of small HCC with a sensitivity of 81% and specificity of 60%. The major strength of this formula is the tests are easy to apply, and the score is simple to calculate. Therefore, this model is an auxiliary tool for identification of patients with HCV at elevated risk of HCC by applying a formula with three serum exams used in routine outpatient clinical practice throughout the world. An even better application of the aforementioned model would be to rule out the presence of small HCC in the initial evaluation of the patient, since the negative predictive value was 99.1% for those stratified as low risk (a score of 26). For example, in a patient with HCV and cirrhosis, the presence of two abnormal variables, imply a higher risk of HCC with a score of 54. In another hypothetical scenario with a patient score of 26, due to no abnormal variables, the patient could be excluded from the high risk group. For maximization of the specificity of the model score, the cut-off of 100 reflects, for instance, the three abnormal variables. We tested the score performance based on a HCC prevalence of 3% (Brazil) and in another scenario with an HCC prevalence of 10% (Japan), showing that the higher the HCC prevalence, better the score performs in identifying individuals with HCC. Recently, El-Serag *et al.*^[34] proposed models to predict HCC risk with the same variables we found (AFP > 20 ng/mL, platelets $< 100,000/\text{mm}^3$ and higher ALT) from the analysis of the change in AFP values according to HCC development. Flemming *et al.*^[46] evaluated a risk model using six baseline clinical variables, including age, diabetes, gender, ethnicity, etiology of cirrhosis, and severity of liver dysfunction independently associated with HCC occurrence. The authors showed C-indices of 0.704 and 0.691 in the derivation and internal validation cohorts, respectively^[46]. By comparison, the score proposed in this paper achieved a C-index of 0.79 (0.7-0.89). Attallah *et al.*^[47] reported the simplified HCC-ART score for HCC detection in chronic hepatitis C patients from Egypt based on age, AFP, AST/ALT ratio, albumin and alkaline phosphatase. The AUROC curve for discriminating patients with HCC ($n = 227$) from those with liver cirrhosis ($n = 341$) was 0.95. Like our work, they used easily obtainable laboratory tests.

Our study is somewhat limited by the fact that the model score was developed only on a Brazilian HCV

population between ages of 38 and 77 years, and still requires external validation with other etiologies, but a bootstrap internal validation was applied and we accessed the optimal diagnostic measures such that our model score is still useful, practical, readily available and easy to apply in primary or tertiary health centers in developing countries.

In conclusion, a score model was created from the results of the case control study based on serum levels of ALT, AFP and platelet count. This score facilitates the identification of patients with small diameter HCC (≤ 3 cm), and mainly those at lowest risk of its presence in the absence of ALT, AFP and platelet count alterations in the thresholds defined in this study. The score is not intended to predict HCC development. Instead, its strength is to rule out small HCC in HCV cirrhotic patients, considering that the negative predictive value of those classified as low risk of HCC presence was 99.1%. This information may assist screening strategies in the population of patients with HCV-related cirrhosis. Further studies in other populations, including non-HCV related cirrhosis are needed to address its role in HCC detection.

DECLARATIONS

Authors' contributions

Designed and performed the research: Paranaguá-Vezozzo DC, Matielo CEL

Analyzed data and wrote the article: Paranaguá-Vezozzo DC, de Campos Mazo DF

Revised the article critically: Nacif LS, Pessoa MG, Pereira GLR, de Lima RGR, Zitelli PMY, Ono SK, Carrilho FJ

Approved the final version of the manuscript: all authors

Data source and availability

The relevant raw data from this study can be available upon request for non-commercial purpose to the corresponding author.

Financial support and sponsorship

This study was in part supported by Alves de Queiroz Family Fund for Research. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest in this work.

Patient consent

Informed consent was obtained.

Ethics approval

This study was approved by the Institutional Review Board fulfilling all of the requirements for retrospective studies in humans, according to the guidelines of the 1975 Helsinki Declaration.

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Case Report

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Beta2-glycoprotein I cooperate with hepatitis B surface antigen promotes hepatocellular carcinogenesis via the nuclear factor kappa B signal pathway were enhanced by the lipopolysaccharide

Xue-Li Ding^{1*}, Xue Jing^{1*}, Nai-Jun Han², Zi-Bin Tian¹, Pu-Jun Gao³, Lin Yang¹, Ya-Nan Yu¹

¹Department of Gastroenterology, the Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong, China.

²Department of Research and development, Yebio Bioengineering Co., Ltd. of Qingdao, Qingdao 266114, Shandong, China.

³Department of Hepatology, the First Hospital of Jilin University, Changchun 310021, Jilin, China.

*Authors contributed equally

Correspondence to: Dr. Xue Jing, Department of Gastroenterology, the Affiliated Hospital of Qingdao University, No.16 Jiangsu Road, Qingdao 266003, Shandong, China. E-mail: qdu-jingxue@foxmail.com

How to cite this article: Ding XL, Jing X, Han NJ, Tian ZB, Gao PH, Yang L, Yu YN. Beta2-glycoprotein I cooperate with hepatitis B surface antigen promotes hepatocellular carcinogenesis via the nuclear factor kappa B signal pathway were enhanced by the lipopolysaccharide. *Hepatoma Res* 2018;4:12. <http://dx.doi.org/10.20517/2394-5079.2018.07>

Received: 11 Feb 2018 **First Decision:** 8 Mar 2018 **Revised:** 30 Mar 2018 **Accepted:** 2 Apr 2018 **Published:** 28 Apr 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Guang-Zhe Zhu **Production Editor:** Huan-Liang Wu

Abstract

Aim: We aimed to elucidate whether beta2-glycoprotein I (β 2GPI) cooperation with hepatitis B surface antigen (HBsAg) promoted hepatocellular carcinogenesis enhanced by the lipopolysaccharide (LPS) via activation of nuclear factor kappa B (NF- κ B) and expression of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and alpha fetal protein (AFP) in liver cancer cells.

Methods: Liver cancer cells (SMMC-7721) were transiently transfected with β 2GPI and/or HBsAg and were subjected to LPS treatment. TNF- α , IL-1 β , and AFP expression were measured in all groups by ELISA. NF- κ B activation was assessed by non-radioactive electrophoretic mobility shift assay (EMSA) and was quantified in all groups.

Results: Cells transfected with β 2GPI and/or HBsAg induced activation of NF- κ B, with the highest activation seen in the doubly β 2GPI- and HBsAg-transfected cells treated with LPS. Non-transfected cells treated with LPS exhibited lower activation compared to either β 2GPI- or HBsAg-transfected cells with LPS treatment. In addition, cells transfected with β 2GPI and/or HBsAg induced significantly increased expression of TNF- α , IL-1 β and AFP, with the highest levels again seen in the doubly β 2GPI- and HBsAg-transfected cells treated with LPS.

Conclusion: These observations suggest that the activity of NF- κ B induced by β 2GPI and HBsAg was enhanced by LPS. Expression of TNF- α , IL-1 β and AFP increased in β 2GPI and HBsAg cotransfected liver cancer cells.



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Keywords: Beta2-glycoprotein I, hepatitis B surface antigen, lipopolysaccharide, liver cancer SMMC-7721, nuclear factor kappa B

INTRODUCTION

Beta2-glycoprotein I (β 2GPI) also known as apolipoprotein H (apoH), is an abundant glycoprotein in the plasma^[1]. To date, most studies of β 2GPI have focused on its role in anti-phospholipid antibody-thrombosis syndrome^[2,3], lipid metabolism, coagulation, and/or regulation of the fibrinolysis system^[4]. Mehdi *et al.*^[5] found hepatitis B surface antigen (HBsAg) bound β 2GPI, an interaction that has been of interest to our research group. We previously found that there was a substantially increased level of β 2GPI in hepatitis B-related hepatocellular carcinoma (HCC) tissue^[6]. The combination of β 2GPI and HBsAg substantially activated nuclear factor kappa B (NF- κ B)^[6], suggesting that β 2GPI played a role in the pathogenesis of hepatitis B-related HCC.

A recent study^[7] showed that lipopolysaccharide (LPS) specifically interacted with β 2GPI, activating NF- κ B via toll-like receptor 4 (TLR4) signaling pathway in macrophages. NF- κ B is a pleiotropic transcription factor involved in inflammation-associated tumor promotion and progression in HCC^[8]. Most hepatitis B-related liver cancer patients experience dysbacteriosis, resulting in increased levels of and sensitivity to LPS. In the present study, we further examined whether LPS enhanced the effect of β 2GPI and HBsAg on activation of NF- κ B, as well as the expression of cytokine factors in the liver cancer cells.

METHODS

Experimental groups

The human hepatoma cell line SMMC-7721 maintained in our laboratory were gifts from the central laboratory of the First Affiliated Hospital of Jilin University. The cells were incubated with Iscove's modified Dulbecco's medium (IMDM) culture media purchased from Gibco, containing 10% fetal bovine serum (FBS), and maintained at 37 °C in a 5% CO₂ incubator. All cells were grown to adherence and were passaged every 2-3 days. Cells in the logarithmic growth phase were selected for experimental use. SMMC-7721 cells were divided into six experimental groups. Group A was the control group, neither transfected nor treated; group B was co-transfected with β 2GPI- and HBsAg plasmids without LPS treatment; group C was treated with 500 μ L (100 ng/mL) LPS and incubated for 6 h^[9]; group D was transiently cotransfected with β 2GPI- and HBsAg plasmids after treatment with 500 μ L (100 ng/mL) LPS and incubated for 6 h; group E was transiently β 2GPI-transfected after treatment with 500 μ L (100 ng/mL) LPS and incubated for 6 h; group F was transiently HBsAg-transfected after treatment with 500 μ L (100 ng/mL) LPS and incubated for 6 h.

Cell transfection

Groups B, D, E, and F were respectively transfected. The vector pcDNA3.1(-) was obtained from Invitrogen. The pcDNA3.1(-)-beta2-GPI and pcDNA3.1(-)-HBsAg eukaryotic expression plasmids were constructed previously in our laboratory. The recombinant plasmids, pcDNA3.1(-)- β 2GPI, or pcDNA3.1(-)HBsAg at 1 μ g/well, and both at 3 μ g/well (1:3) were dissolved in 50 μ L IMMD basal media that was mixed to become Solution A. 2 μ L FuGENE HD transfection reagent was dissolved in 50 μ L IMMD basal media, mixed gently, incubated at room temperature for 5 min, labeled as Solution B. Solution A and Solution B were mixed gently to become Solution C, incubated at room temperature for 20 min. The cells were washed 3 times in serum-free IMMD culture media, and Solution C was slowly added to the cells that were incubated at 37 °C in a 5% CO₂ incubator. Transfection media was removed after 6-8 h and was replaced with 500 μ L 10% FBS IMMD media. Cell supernatants were collected at 24 h after transfection. A previous study^[6] from our lab found β 2GPI protein expression was the highest 24 h after transfection.

Enzyme-linked immunosorbent assay analysis

Enzyme-linked immunosorbent assay (ELISA) detection of targets of interest was performed according to the manufacturers' instructions. β 2GPI was measured in groups A, B, D, and E; HBsAg in groups B, D and F; and tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and alpha fetal protein (AFP) in all groups. Once β 2GPI reached the highest expression level, as determined from previous studies^[6], cell supernatants from each group were collected for ELISA analysis. Triplicates of standards, samples and blank groups were prepared. The optical density (OD) value of each well was measured at 450 nm. Data were presented as means \pm SD.

Non-radioactive NF- κ B EMSA and NF- κ B relative quantification

Assays were performed only with nuclear extracts according to the manufacturer's instructions. Nuclear extracts (5 μ g) were used for each reaction with 400 fmol bio-labeled (hot) oligonucleotide NF- κ B probe (5'-AGT TGA GGG GAC TTT CCC AGGC-3') and unlabeled (cold)-NF- κ B probe (5'-AGT TGA GGG GAC TTT CCC AGGC-3'). Poly(dI-dC): poly(dI-dC) was used as a nonspecific competitor. A 25-fold molar excess of unlabeled homologous oligonucleotide was used as a specific competitor. Non-homologous oligonucleotide sequences were also used to validate the specificity of the binding of each transcription factor in the competition assays. Binding reaction resolved by 6.5% acrylamide/bis (30:1 ratio) electrophoresis in 0.25 \times TBE on ice. The gel was transferred to nitrocellulose membranes in 0.5 \times TBE. The membrane was then UV crosslinked for 10 min, blocked with 1 \times blocking buffer for 30 min, and then incubated with streptavidin-HRP in blocking buffer (1:750) at room temperature for 30 min. The membrane was washed four times with 1 \times washing solution and was equilibrated with 1 \times equilibration solution for 5 min with shaking. Finally, the membranes were incubated with chemiluminescence substrate buffer, and the bands were visualized using Viagene CoolImager (Viagene Biotech Co., China). NF- κ B relative quantification was based on relative activity of the combination of NF- κ B and DNA. The last result was represented by $\Delta\Phi$ (gray value). The gray values of the image were measured after film exposure by the imaging system CoolImger. Data were presented as means \pm SD.

Statistical analysis

SPSS 22.0 software was used for data processing and statistical analysis. Cell assay data were presented as means \pm SDs and the variance was analyzed. Comparison between groups was measured using Fisher's least significant difference (LSD) test. Differences were significant at $P < 0.05$.

RESULTS

Expression of β 2GPI and HBsAg in transfected cells

We used ELISA to measure expression of β 2GPI and HBsAg 24 h after transfection of recombinant plasmids in cell supernatants. β 2GPI protein expression was found in group B, D, and E, significantly different from non-transfected, non-treated group A ($P < 0.001$). There were no differences in expression levels of β 2GPI in groups B, D, and E ($P > 0.05$) suggesting similar transfection efficiency. HBsAg protein expression was found in groups B, D, and F. Expression was determined using a cutoff value (COV) that equal to the average absorbance value of the negative control (0.532). The absorbance of specimen \geq COV indicated positive expression of HBsAg.

Activation of NF- κ B in β 2GPI- and/rHBsAg-transfected cells following LPS stimulation

A representative image of non-radioactive NF- κ B EMSA in the six groups is shown in [Figure 1](#), and NF- κ B relative quantification was represented by gray value is shown in [Figure 2](#). Groups B, C, D, E, and F induced differential levels of activation of NF- κ B, with the highest relative activity of NF- κ B observed in group D (1404.5 ± 11.28); this was significantly different compared with the other five groups ($P < 0.05$). The relative activity of NF- κ B in group B was 914.57 ± 12.51 , significantly higher than levels in groups A, C, E, and F ($P < 0.05$). The levels in group E (867.76 ± 6.27) and F (882.52 ± 7.92) were much higher than those of group

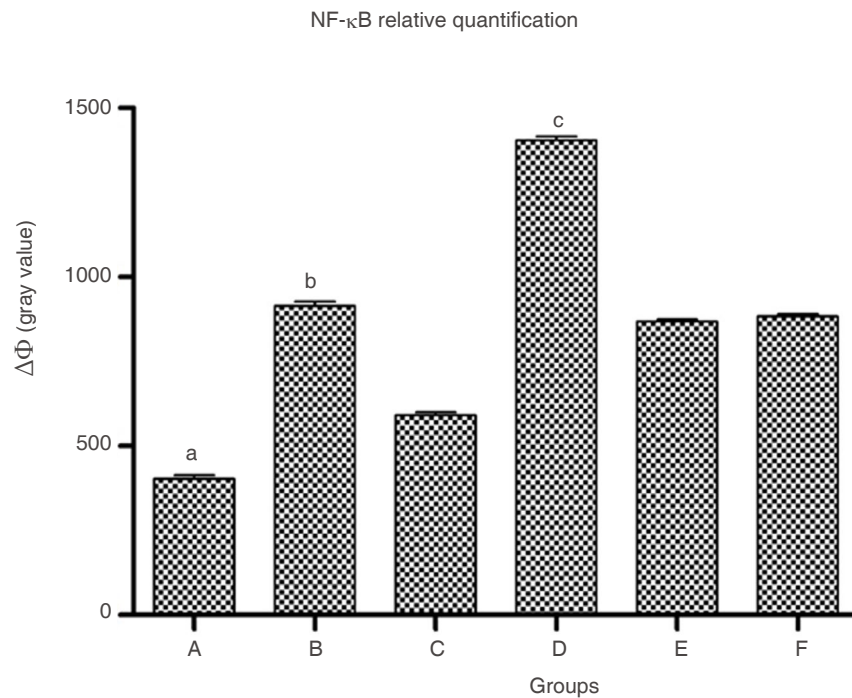


Figure 1. Detection of non-radioactive NF- κ B by EMSA in six groups. Group A: non-transfected, non-treated cells; group B: transient β 2GPI- and HBsAg-transfection without LPS treatment; group C: non-transfected cells treated with 100 ng/mL LPS; group D: transient β 2GPI- and HBsAg-transfection and treated with 100 ng/mL LPS; group E: transient β 2GPI-transfection and treated with 100 ng/mL LPS; group F: transient HBsAg-transfection and treated with 100 ng/mL LPS. β 2GPI: beta2-glycoprotein I; HBsAg: hepatitis B surface antigen; LPS: lipopolysaccharide

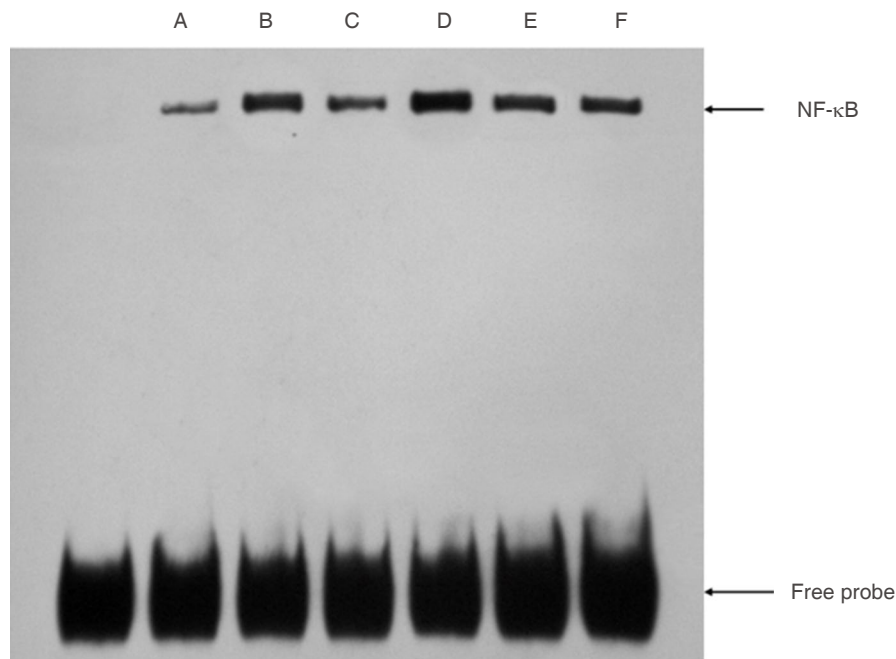


Figure 2. NF- κ B relative quantification in six groups. Group A: non-transfected, non-treated cells; group B: transient β 2GPI- and HBsAg-transfection without LPS treatment; group C: non-transfected cells treated with 100 ng/mL LPS; group D: transient β 2GPI- and HBsAg-transfection and treated with 100 ng/mL LPS; group E: transient β 2GPI-transfection and treated with 100 ng/mL LPS; group F: transient HBsAg-transfection and treated with 100 ng/mL LPS. Data presented as means \pm SD; a: groups B, C, D, E, and F compared with group A, $P < 0.05$; b: group B compared with groups A, C, E, and F, $P < 0.05$; c: group D compared with other five groups, $P < 0.05$. β 2GPI: beta2-glycoprotein I; HBsAg: hepatitis B surface antigen; LPS: lipopolysaccharide

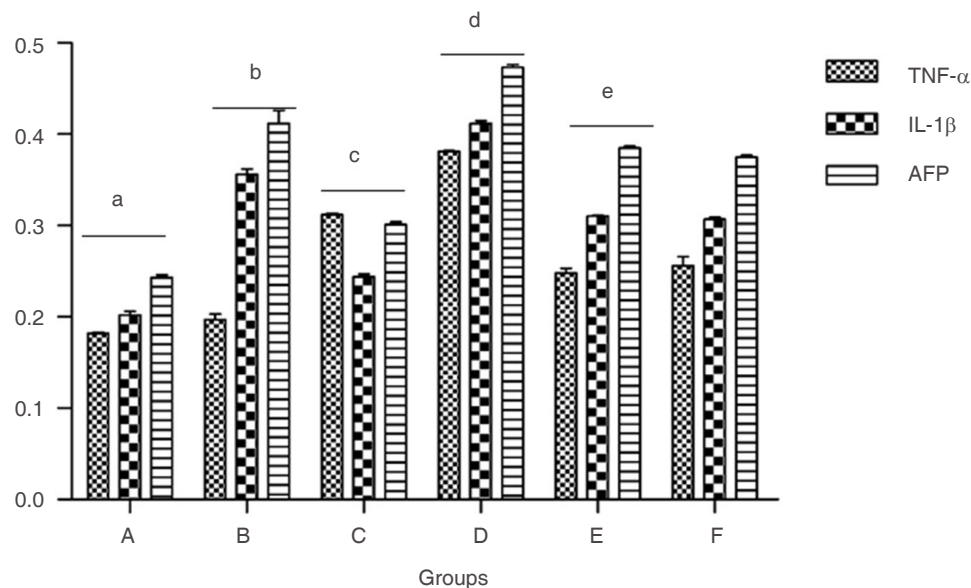


Figure 3. Expression of TNF- α , IL-1 β and AFP by ELISA in six groups. Group A: non-transfected, non-treated cells; group B: transient β 2GPI- and HBsAg-transfection without LPS treatment; group C: non-transfected cells treated with 100 ng/mL LPS; group D: transient β 2GPI- and HBsAg-transfection and treated with 100 ng/mL LPS; group E: transient β 2GPI-transfection and treated with 100 ng/mL LPS; group F: transient HBsAg-transfection and treated with 100 ng/mL LPS. Data presented as means \pm SD. a: groups B, C, D, E, and F compared with group A, $P < 0.05$; b: group B compared with groups A, C, E, and F, $P < 0.05$; c: groups E and F compared with group C, $P < 0.05$; d: group D compared with groups A, B, C, E, and F, $P < 0.001$; e: group E compared with group F, $P > 0.05$; f: group C compared with groups B, E, and F, $P < 0.05$. β 2GPI: beta2-glycoprotein I; HBsAg: hepatitis B surface antigen; LPS: lipopolysaccharide

C (590.4 ± 9.49) ($P < 0.05$). The level of NF- κ B activation in group E and F were similar ($P > 0.05$). Taken together, these data suggest that LPS alone induced activation of NF- κ B, which enhanced by either β 2GPI- or HBsAg-transfection. However, the highest effect was seen in doubly-transfected cells, suggesting synergism between LPS, β 2GPI and HBsAg with respect to activation of NF- κ B in HCC.

LPS induced increased expression of TNF- α , IL-1 β , and AFP in β 2GPI- and/or HBsAg-transfected cells

Cell supernatants from the six groups were collected and levels of TNF- α , IL-1 β and AFP were assayed 24 h after transfection of respective recombinant plasmids. As depicted in Figure 3, groups B, C, D, E, and F induced expression of TNF- α , IL-1 β and AFP more than did group A ($P < 0.05$). The highest expression levels of all three cytokines was seen in group D (doubly transfected with β 2GPI and HBsAg and treated with LPS) ($P < 0.001$). The expression levels of IL-1 β and AFP in group B was higher ($P < 0.05$) were higher than those of groups A, C, E, and F, while their expression in groups E and F were higher than those of group C ($P < 0.05$). The expression of TNF- α in group C was higher than that of groups B, E, and F ($P < 0.05$). TNF- α and IL-1 β levels were similar in groups E and F ($P > 0.05$), while AFP in these groups were significantly higher than in group A ($P < 0.05$).

DISCUSSION

HCC, one of the most common tumors, is currently the fifth most common malignant tumor worldwide, with morbidity increasing every year. Hepatitis B virus (HBV) and hepatitis C virus (HCV) are major causes of HCC^[10]. Therapeutic options include etiological treatment, resection, percutaneous ablation, trans-arterial chemoembolization (TACE), and targeted therapy. The overall efficacy of these therapies is poor, and five-year survival rates for early treatment of HCC are not favorable^[11]. Therefore, understanding the pathogenesis of HCC (abnormal neovascularization, genomics, proteomics and signal transduction pathways) is necessary to understand how HCC occurs and to develop new therapeutic approaches.

β 2GPI is synthesized by liver cells and plays roles in anticoagulation, cell clearance, and lipid metabolism under normal physiological conditions^[4]. β 2GPI is also involved in the pathogenesis of chronic viral hepatitis, alcoholic liver disease, autoimmune liver disease, liver cirrhosis and liver cancer^[12]. A previous study showed that a fraction with maximal apoH (β 2GPI)-binding predominantly contained full Dane particles in HBV patients^[13]. Gao *et al.*^[14] found there was a specific binding event between HBV and β 2GPI. Gao *et al.*^[15,16] provided the first evidence that a protein existed on SMMC-7721 cell membrane that could specifically bind β 2GPI. The binding protein was later identified as annexin II. A previous study from our lab^[6], demonstrated strong β 2GPI expression in hepatitis B-related HCC tissue. In addition, the combination of β 2GPI and HBsAg was shown to significantly activate NF- κ B and expression of AFP, suggesting that β 2GPI may be involved in the pathogenesis of hepatitis B-related HCC. However, it is unknown whether β 2GPI directly interacts with HBsAg or if other proteins are involved in NF- κ B activation.

β 2GPI is physically closed in a circular conformation, with low activity^[17]. β 2GPI opens and adopts a J-like conformation and becomes active when combined with antibodies or anionic phospholipids. In a study^[17], it was found that LPS opened β 2GPI, exposed its binding sites in domain V, and interacted with β 2GPI to participate in physiology and pathology. The β 2GPI and LPS complex relied on the TLR4 signaling pathway to activate NF- κ B in macrophages. A previous study from our lab^[9] found that LPS enhanced signal transduction in β 2GPI in liver cancer cells leading to activation of NF- κ B, triggering downstream signal transduction and increasing the expression of downstream factors. This activation was related with LPS concentration. This suggests that LPS enhancement of β 2GPI signal transduction may participate in the development of liver cancer.

LPS, a component of the cell wall of gram-negative bacteria, is an important mediator of the host inflammatory response to infection. A study of 169 patients with chronic hepatic disease found elevated levels of LPS in 27%, 85%, and 41% of patients with chronic hepatitis, chronic hepatitis with acute exacerbation and cirrhosis, respectively^[18]. In patients with chronic liver diseases, elevated levels of LPS in the portal and/or systemic circulation are common because of increases in intestinal permeability and bacterial translocation. LPS from gut microbiota contributed to HCC promotion by activating TLR4 signaling. Classically, TLR4 recognizes microbial lipids in homodimer configuration, thus activating various intracellular signaling pathways, such as the NF- κ B and MAPK pathways. TLR4 has been identified in HCC and may play a role in progression of HCC. LPS-induced activation of TLR4 signaling promoted HCC cell survival and proliferation associated with regulation of the activation of the NF- κ B and MAPK pathways^[19-22].

In the present study, we demonstrated substantial activity of NF- κ B in cells transfected with both β 2GPI and HBsAg and treated with LPS. Our data suggested that the combined action of β 2GPI and HBsAg were enhanced by LPS in the progression of carcinogenesis. Constitutive expression of NF- κ B is emerging as a hallmark of cancer. In fact, constitutive NF- κ B activation is generally associated with cancer proliferation, survival, chemoresistance, and progression of HCC^[23].

NF- κ B is another pro-inflammatory transcription factor that triggers downstream signal transduction and increases expression of downstream factors. In the present study, inflammatory cytokines (TNF- α , IL-1 β , and AFP) were substantially elevated in cells transfected with both β 2GPI and HBsAg and treated with LPS, more so than by single transfections with either factor. The action of various inflammatory mediators is known to occur in carcinogenesis. TNF- α has been postulated to have a crucial role in the pathogenesis of various cancers. It is one of the most important pro-inflammatory cytokines involved in the growth, differentiation, cellular function and survival of many cells. It is produced by several types of cells, including macrophages, neutrophils, fibroblasts, keratinocytes, NK cells, T and B cells, and tumor cells^[24]. IL-1 β is also known to mediate several immune responses in HCV/HBV infection. There is a network of TNF- α and IL-1 β secretion and interactive bio-functions in immune responses^[24].

We found that LPS enhanced the effect of β 2GPI- and HBsAg in development of liver cancer by increasing the activity NF- κ B and elevating levels of TNF- α , IL-1 β , and AFP. We predict that LPS may be an initiating agent in the pathogenesis of HCC, combining with β 2GPI to activate and expose β 2GPI binding sites to HBsAg, in turn interacting with HBsAg to further modulate NF- κ B. Further studies are needed to uncover the specific mechanisms of interaction of β 2GPI, HBsAg and LPS, and the role of β 2GPI in liver cancer and other hepatic diseases.

DECLARATIONS

Authors' contributions

Designed the study protocol, performed the studies and wrote the manuscript: Jing X, Ding XL
Gathered the data and performed the statistical analyses: Han NJ, Yang L, Yu YN Improved the final version of the manuscript: Tian ZB, Gao PJ

Data source and availability

The data presented is original and obtained in our laboratory. It is available with the authors and can be made available if required.

Financial support and sponsorship

This work was supported by the National Natural Science Youth Foundation of China (No. 81101853) and Natural Science Youth Foundation of Shandong Province (No. ZR2016HQ35).

Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Original Article

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Neoadjuvant hepatic arterial infusion chemotherapy for resectable hepatocellular carcinomas

Rina Tsutsui¹, Hiroaki Nagamatsu², Osamu Itano³, Akihiro Deguchi⁴, Tsubasa Tsutsumi⁵, Mamoru Hiraki⁶, Naohisa Mizukami⁷, Jun Akiba⁸

¹Department of Digestive and Transplantation Surgery, Tokyo Medical University Hachioji Medical Center, Tokyo 193-0998, Japan.

²Department of Gastroenterology, Juntendo University Hospital, Tokyo 113-8431, Japan.

³Department of Hepato-Biliary-Pancreatic and Gastroenterological Surgery, International University of Health and Welfare School of Medicine, Chiba 286-8686, Japan.

⁴Department of Medicine, Kagawa Rosai Hospital, Kagawa 763-0013, Japan.

⁵Department of Medicine, Kurume University School of Medicine, Fukuoka 830-0011, Japan.

⁶Department of Surgery, Yame General Hospital, Fukuoka 834-0034, Japan

⁷Department of Radiology, Yame General Hospital, Fukuoka 834-0034, Japan

⁸Department of Pathology, Kurume University School of Medicine, Fukuoka 830-0011, Japan.

Correspondence to: Dr. Hiroaki Nagamatsu, Department of Gastroenterology, Juntendo University Hospital, 3-1-3 Hongo, Bunkyo-ku, Tokyo 113-8431, Japan. E-mail: h-nagamatsu@juntendo.ac.jp

How to cite this article: Tsutsui R, Nagamatsu H, Itano O, Deguchi A, Tsutsumi T, Hiraki M, Mizukami N, Akiba J. Neoadjuvant hepatic arterial infusion chemotherapy for resectable hepatocellular carcinomas. *Hepatoma Res* 2018;4:13. <http://dx.doi.org/10.20517/2394-5079.2018.20>

Received: 15 Mar 2018 **First Decision:** 10 Apr 2018 **Revised:** 3 May 2018 **Accepted:** 7 May 2018 **Published:** 11 May 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Guang-Zhe Zhu **Production Editor:** Huan-Liang Wu

Abstract

Aim: To evaluate the effect of neoadjuvant hepatic arterial infusion chemotherapy (HAIC) on the survival of patients with resectable hepatocellular carcinoma (HCC).

Methods: Between January 2003 and January 2014, 80 patients underwent hepatic resection for HCC. Of these patients, we evaluated 49 patients who met the following inclusion criteria: (1) preserved liver function (Child-Pugh A); (2) resectable HCC (≤ 3 nodules, regardless of the size); and (3) HCC with high-grade malignant potential. Among them, 13 patients underwent neoadjuvant HAIC and curative hepatectomy (treatment group). The remaining 36 patients underwent curative hepatic resection without neoadjuvant therapy (control group). Survival after hepatic resection was compared retrospectively between the groups.

Results: During follow-up, 2 (15.4%) patients in the treatment group and 25 (69.4%) patients in the control group developed recurrence. The 1-, 3-, and 5-year disease-free rates (100%, 78.6%, and 78.6%, respectively vs. 65.8%, 33.7%, and 26.6%, respectively; $P = 0.003$) and overall survival rates (100%, 100%, and 100%, respectively vs. 91.7%, 77.8%, and 55.3%, respectively; $P = 0.037$) were significantly better in the treatment group than in the control group.



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Conclusion: Neoadjuvant HAIC decreased the risk of recurrence and improved survival in patients with HCC with high malignant potential.

Keywords: Hepatocellular carcinoma, transcatheter arterial chemoembolization, hepatic arterial infusion chemotherapy

INTRODUCTION

Surgery is the standard treatment for hepatocellular carcinoma (HCC), which offers a chance of cure with preservation of liver function^[1] and achieves the best outcome (5-year survival rate of 33%-60%)^[2]. However, after curative liver resection for HCC, the incidence of recurrence in the remnant liver is as high as 60% within 3 years^[3-5]. Among all cases of recurrence, approximately 90% are intrahepatic recurrences, which contribute to the high mortality rate in patients with HCC^[6-9]. The risk factors for early-phase recurrence of HCC depend on the malignant potential of the tumor, including the presence of microscopic vascular invasion (MVI), serum alpha-fetoprotein (AFP) levels, tumor number, and tumor size^[3,10,11]. Among these, the presence of MVI is an important risk factor affecting survival throughout the entire postoperative period^[12], and the gross classification of HCC predicts the presence of MVI^[13].

Some studies demonstrated that preoperative transarterial chemoembolization (TACE) improved prognosis in select patients, such as those with preserved liver function and advanced-stage HCC^[14-17]. However, according to the 2012 European Association for the Study of the Liver (EASL) and European Organization for Research and Treatment of Cancer clinical practice guidelines, neoadjuvant chemoembolization has not proven to improve the outcomes of patients who underwent resection^[1]. Additionally, neoadjuvant TACE is associated with the disadvantages of delaying surgery and increasing complications during surgery because of inflammatory pediculitis, perihepatic adhesions, or arterial thrombosis; moreover, if the tumor fails to respond to therapy, it continues to grow and becomes incurable^[18,19]. Moreover, TACE also has the potential to cause adverse effects on liver function. Hepatic arterial infusion chemotherapy (HAIC) may sometimes be chosen as a therapeutic option for advanced HCC because of poor liver function. It allows the direct delivery of high doses of chemotherapeutic agents to the tumor site and reduces the systematic concentration of chemotherapeutic agents to a low level, which may result in a lower incidence of adverse drug reactions and early appearance of the chemotherapeutic effects in the early stage of treatment.

In this retrospective study, we evaluated the safety, feasibility, and surgical complications of neoadjuvant HAIC, and investigated the effect of it on survival without recurrence after resection of the lesion.

METHODS

Patients

Between January 2003 and January 2014, 80 patients underwent hepatic resection for HCC at our hospital. Of these patients, we investigated 49 patients who met the following inclusion criteria: (1) preserved liver function (Child-Pugh A); (2) resectable HCC (≤ 3 nodules, regardless of the size); and (3) HCC with high-grade malignant potential. High-grade malignant potential refers to HCC with MVI. The patients were diagnosed on the basis of fan-shaped portal perfusion defects, which appeared in the periphery of the tumor on computed tomography (CT) scans during arterial portography and showed tumorous arteriportal shunts caused by microscopic portal vascular invasion. In terms of gross appearance, the simple nodular type with extranodular growth or confluent multinodular type predicted the presence of MVI^[13,20].

Of the 49 patients, 13 patients who were preoperatively diagnosed as having HCC with high-grade malignant potential, between June 2009 to January 2014, were treated with neoadjuvant HAIC (treatment group).

Another 36 patients who met the inclusion criteria, between January 2003 and May 2009, had a curative hepatic resection (control group). This was a retrospective study of HCC patients at Yame General Hospital. The institutional review board approved this study, and written informed consent was obtained from the treatment group. Regarding the control group, the Ethics Committee waived the requirement for ethical approval and informed consent due to the retrospective nature of the study.

Preoperative evaluation

Baseline imaging examinations [CT angiography, dynamic CT, or/and dynamic contrast-enhanced magnetic resonance imaging (MRI)] were performed before surgery. HCC was confirmed when at least 2 radiographic images revealed the hallmarks of HCC or 1 radiographic image revealed the hallmarks of HCC together with AFP levels > 400 ng/mL^[1]. HCC staging was performed according to the Barcelona clinic liver cancer (BCLC) staging classification^[21,22] and the 6th edition of the American Joint Committee on Cancer staging system of tumor nodes metastasis. Laboratory blood tests, including tests for hepatitis B surface antigen, hepatitis C virus antibodies, serum AFP, serum des-gamma-carboxyprothrombin (DCP), serum albumin, serum total bilirubin, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), prothrombin time, C-reactive protein, and platelet counts, were performed.

Neoadjuvant hepatic arterial infusion chemotherapy protocol

In the treatment group, a temporary indwelling catheter system^[23] was implanted via the left brachial artery under fluoroscopic guidance and was used for HAIC. A polyurethane-covered catheter, called anthron P-U catheter (APUC), 5 Fr (100 cm) (Toray Medical Co., Ltd., Tokyo, Japan) with a tapered tip (5- and 3.3-French outer diameters of the shaft and tip, respectively, and 0.035-/0.021-in inner diameters of the shaft and tip, respectively) was used as the indwelling catheter. This catheter was 100-cm long and tapered to a 3.3-French microcatheter 60 cm from the tip. The tip of the catheter was inserted into the right or left hepatic artery, corresponding to the side on which the main tumor was located, via the celiac artery. In the case of multiple tumors, one or two side holes were manually created with a surgical knife to supply the rest of the tumor with chemotherapeutic agents.

The treatment regimen included low-dose 5-fluorouracil (5-FU) and cisplatin (low-dose FP), specifically, the regimen featured daily administration of cisplatin (10 mg for 30 min) and a subsequent infusion of 5-FU (250 mg for 3 h) on days 1-10. We named this treatment regimen as 2 weeks of low-dose FP. After the administration of chemotherapeutic agents, the catheter was removed under fluoroscopic guidance. No prophylactic antibiotics were administered during the catheter placement.

Laboratory variables were assayed once in several days, and the tumor marker was measured before and after the treatment regimen. HAIC was discontinued or reduced in case of adverse events higher than grade 3/4 of the common terminology criteria for adverse events (CTCAE).

Surgical procedure

Curative liver resection was performed after a mean delay of 24 ± 12 days after catheter removal. A single surgeon performed all surgeries. Anatomic resection was defined as hemihepatectomy, extended hemihepatectomy, sectionectomy, or segmentectomy, and all other non-anatomic resections were classified as partial resections.

To determine the operative outcome, data regarding the operative time, intraoperative blood loss, red blood cell transfusion, complications, type of resection, hospital mortality, and hospital stay were collected for both groups.

Pathologic assessment

Two senior pathologists reviewed each specimen for histologic confirmation of the diagnosis. Clinicopathologic data such as tumor size recorded as the maximum diameter, vascular invasion, intrahepatic metastasis, gross

classification, histologic grade, and the degree of liver cirrhosis were collected. The therapeutic effect was classified into 4 categories based on the Japanese breast cancer society criteria^[24].

Follow-up

Laboratory variables such as serum AFP, serum DCP, serum albumin, serum total bilirubin, serum AST, serum ALT, prothrombin time, and C-reactive protein levels and platelet counts were measured for both groups on postoperative days 1, 3, 7, and 30.

After discharge from our hospital, all patients were followed up in the outpatient clinic. Ultrasonography, 4-phase CT, or dynamic contrast-enhanced MRI was performed every 2 to 3 months, and serologic tests such as AFP and DCP measurements were performed at that time. In cases of recurrence, the patients were treated accordingly.

Survival was defined as the time from surgery to death, and disease-free survival (DFS) was defined as the time from surgery to either recurrence or death. Patients who were alive and free of recurrence at the end of follow-up were censored for DFS^[22].

Statistical analysis

Continuous data were presented as the mean \pm standard deviation or median and range and were compared using the *t*-test or Mann-Whitney's *U* test, respectively. Categorical data were compared using Pearson's χ^2 test or Fisher's exact test, as appropriate.

The Kaplan-Meier method was used to calculate the survival curves, and the log-rank test was used to assess the prognostic predictors of DFS. Variables with $P < 0.10$ in univariate analysis were included in the multivariate analysis.

Differences were considered significant when the 2-sided *P*-value was < 0.05 . Descriptive statistical analyses were performed using the IBM statistical package for the social sciences, version 20.0 (SPSS, IBM Co., Armonk, NY, USA).

RESULTS

The baseline characteristics of the patients are shown in Table 1. No significant differences were observed between the 2 groups.

Outcomes and complications associated with neoadjuvant HAIC

In the treatment group, all catheterization procedures were performed without critical complications. The median procedure time for implantation of the system was 80 min (range 43-180 min). The system was successfully implanted and used for treatment in all patients. The median catheter dwell time was 10 days (range 9-13 days). The median time to surgery after catheter removal was 21 days (range 12-34 days). Major complications associated with a temporary indwelling catheter system, such as hematoma, bleeding, hepatic arterial occlusion, dislocation of the catheter, and thrombosis, did not occur. Infection was suspected in 1 patient (7.7%), and fever and flares in the left brachial artery appeared 8 days after the procedure in this patient. The patient's symptoms improved soon after catheter removal, which was 9 days after the chemotherapy [Table 2]. One patient (7.7%) experienced CTCAE grade 2 gastritis. The most common side effects were nausea and loss of appetite; however, these symptoms were mostly CTCAE grade 1/2, and they resolved after chemotherapy was completed.

The mean plasma AFP and DCP levels tended to decrease following neoadjuvant HAIC (415.3 ± 1086 ng/mL and 451.4 ± 892.4 mg/mL, respectively, prior to HAIC vs. 158.8 ± 404.7 ng/mL and 118.0 ± 237.9 mg/mL, respectively, after HAIC; $P = 0.468$ and $P = 0.243$, respectively), but the differences were not significant. No

Table 1. Patient characteristics, baseline liver function, and tumor characteristics: treatment group versus control group

Variables	Treatment group (n = 13)	Control group (n = 36)	P
Age (years)*	69 (50-81)	74 (50-78)	0.128
Gender (male/female)	10/3	29/7	1.000
Etiology			
Hepatitis B carrier	1	6	0.298
Hepatitis C carrier	11	22	
Others	1	8	
Cirrhotic liver	10	16	0.054
Child-Pugh score at time of hepatectomy*	5.0 (5.0-6.0)	5.0 (5.0-6.0)	0.481
AFP level (ng/mL)*	6.6 (2.0-3921.0)	14.3 (2.0-2720.0)	0.504
DCP level (ng/mL)*	130 (13-3252)	74 (1.0-5940)	0.548
Tumor diameter (mm)*	27.0 (14.0-50.0)	25.0 (10.0-58.0)	0.666
Tumor number*	1 (1-2)	1 (1-3)	0.708
Presence of portal vein tumor thrombosis (Vp2-4)	0	0	1.000
Presence of satellite nodules	6	12	0.411
TNM pathological staging (stage I/II/IIIA/IIIB/IIIC/IV)	3/10/0/0/0/0	15/19/2/0/0/0	0.278

*Median with range. AFP: alpha-fetoprotein; DCP: des-gamma-carboxyprothrombin; TNM: tumor nodes metastasis [6th edition of the American Joint Committee on Cancer (AJCC) staging]

Table 2. Outcomes of temporary indwelling catheter system implantation: treatment group

Variables	n
Puncture region (left brachial artery/right femoral artery/others)	13/0/0
Procedure time (min)*	80 (43-180)
Number of catheter days (day)*	10 (9-13)
Time to operation from procedure (day)*	21 (12-34)
Complications	1 (7.7 %)
Procedure-related complications	
hematoma formation	0
Complications during chemotherapy	
Hepatic arterial occlusion	0
Gastroduodenal ulcer	0
Cerebral infarction	0
Infection	1
Catheter dysfunction	
Catheter dislodgement	0
Occlusion of catheter	0

*Median with range

liver function impairment and liver failure occurred after HAIC, and all patients underwent hepatectomy as expected [Table 3].

Operative and perioperative outcome

The operative outcomes and perioperative changes in liver function are presented in Tables 4 and 5. All patients with liver function impairment recovered. No adverse effect on liver function attributable to HAIC occurred after surgery. There was no difference in the operative outcomes of the 2 groups, and no hospital mortality was observed.

Pathologic assessment

The histopathologic findings of the resected livers are shown in Table 6. The histologic grade for patients in the treatment group after treatment was determined to be grade 0 for 3 patients (23%), grade 1a for 3 patients (23%), grade 1b for 3 patients (23%), grade 2a for 2 patients (15%), grade 2b for 1 patient (8%), and grade 3 for 1 patient (8%).

Table 3. Preoperative liver function and tumor marker levels in the treatment group

Variables	Before HAIC	Before operation	P
Total bilirubin (mg/dL)	0.6 (0.3-1.1)	0.6 (0.3-0.8)	0.511
Serum albumin (g/dL)	4.3 (3.7-4.9)	4.1 (3.4-5.1)	0.448
Serum AST (U/L)	32 (18-99)	31 (20-58)	0.762
Serum ALT (U/L)	34 (9.0-120)	32 (12-61)	0.801
Prothrombin time (%)	88 (72-105)	92 (78-120)	0.336
Platelet ($\times 10^4/\mu\text{L}$)	15.8 (11.3-27.0)	13.1 (10.2-22.1)	0.204
C-reactive protein (mg/dL)	0.05 (0.01-0.18)	0.06 (0.04-0.60)	0.418
AFP level (ng/mL)	6.6 (2.0-3921)	9.7 (2.4-1365)	0.776
DCP level (ng/mL)	130 (13-3252)	54 (12-832)	0.106

All data shown as median with range. HAIC: hepatic arterial infusion chemotherapy; AST: aspartate aminotransferase; ALT: alanine aminotransferase; AFP: alpha-fetoprotein; DCP: des-gamma-carboxyprothrombin

Table 4. Intraoperative and postoperative outcomes of hepatectomy: treatment group versus control group

Variables	Treatment group (n = 13)	Control group (n = 36)	P
Operative duration (min)*	355 (125-465)	316 (127-590)	0.389
Intraoperative blood loss (mL)*	860 (41-2582)	528 (150-3320)	0.118
Red blood cells transfusion	4	3	0.070
Anatomical hepatectomy	11	31	0.608
Complications			0.663
Postoperative hemorrhage	0	1	
Bile leak	0	0	
Subphrenic collection	0	0	
Wound infection	0	1	
Transient liver impairment	0	0	
Ascites	0	4	
Ileus	1	1	
Hospital mortality	0	0	
Hospital stay*	12 (9-25)	12 (8-20)	0.297

*Median with range

Survival

During the follow-up period, 2 (15.4%) patients in the treatment group and 25 (69.4%) patients in the control group experienced recurrence. The pattern of initial recurrence in the treatment group revealed that 1 patient each had intrahepatic recurrence and simultaneous intrahepatic and extrahepatic recurrence (multiple bone metastases).

The 1-, 3-, and 5-year DFS rates were 100%, 78.6%, and 78.6%, respectively, for the treatment group and 65.8%, 33.7%, and 26.6%, respectively, for the control group. The DFS rates were significantly better in the treatment group than in the control group ($P = 0.003$) [Figure 1]. The 1-, 3-, and 5-year overall survival (OS) rates were 100%, 100%, and 100%, respectively, for the treatment group and 91.7%, 77.8%, and 55.3%, respectively, for the control group, respectively. The OS rates were significantly better in the treatment group than in the control group ($P = 0.037$) [Figure 2].

The results of univariate analyses of the predictors of DFS are shown in Table 7. Using factors identified as significantly associated with DFS, multivariate analyses revealed that neoadjuvant HAIC [$P = 0.039$, hazard ratio (HR) = 0.215; 95% confidential interval (CI) = 0.050-0.928], age ($P = 0.017$, HR = 0.374; 95% CI = 0.166-0.842), and tumor number ($P < 0.001$, HR = 7.731; 95% CI = 2.474-14.161) were independent predictors of DFS [Table 7].

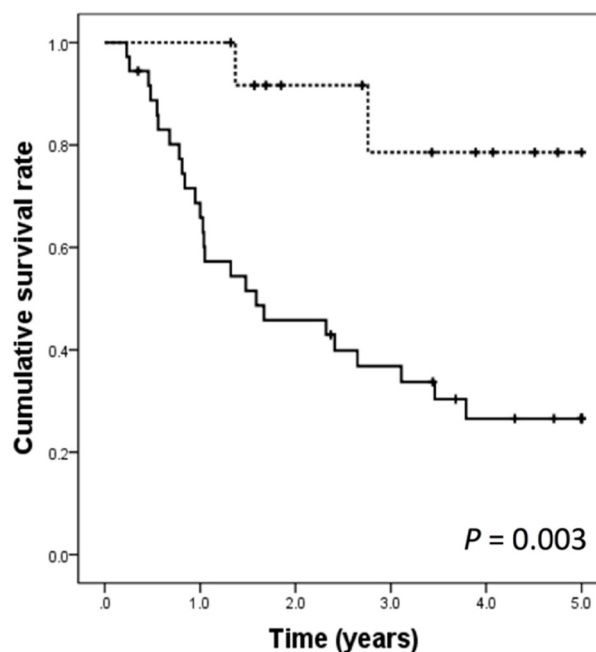
DISCUSSION

The present study evaluated the effect of neoadjuvant HAIC for patients who had HCC with high malignant

Table 5. Postoperative liver function: treatment group versus control group

	1 POD			3 POD			7 POD			1 POM		
	Treatment group (n = 13)	Control group (n = 36)	P	Treatment group (n = 13)	Control group (n = 36)	P	Treatment group (n = 13)	Control group (n = 36)	P	Treatment group (n = 13)	Control group (n = 36)	P
Total bilirubin (mg/dL)	1.77 ± 0.96	1.69 ± 0.89	0.801	1.40 ± 0.70	1.44 ± 0.78	0.867	0.90 ± 0.26	0.87 ± 0.34	0.770	0.65 ± 0.28	0.68 ± 0.30	0.594
Serum albumin (g/dL)	3.65 ± 0.29	3.50 ± 0.37	0.206	3.61 ± 0.29	3.35 ± 0.45	0.069	3.40 ± 0.25	3.15 ± 0.40	0.057	3.86 ± 0.46	3.69 ± 0.39	0.227
Serum AST (U/L)	220 ± 161	254 ± 168	0.534	71.2 ± 55.7	84.1 ± 35.0	0.339	41.5 ± 29.0	36.1 ± 14.3	0.526	28.6 ± 8.27	46.0 ± 22.4	0.008
Serum ALT (U/L)	147 ± 117	193 ± 143	0.303	94.4 ± 66.2	118.7 ± 73.6	0.300	55.8 ± 48.1	55.0 ± 30.7	0.940	22.0 ± 8.50	40.1 ± 21.5	0.004
Prothrombin time (%)	69.5 ± 20.4	65.5 ± 10.9	0.505	81.4 ± 9.35	77.6 ± 13.4	0.357	80.5 ± 9.9	94.7 ± 118.7	0.671	84.7 ± 9.57	76.3 ± 14.5	0.079
Platelet ($\times 10^4/\mu\text{L}$)	11.6 ± 3.50	11.8 ± 3.17	0.843	5.76 ± 1.60	12.1 ± 3.38	0.223	18.7 ± 9.85	15.7 ± 4.78	0.312	18.5 ± 5.27	15.3 ± 4.80	0.056
C-reactive protein (mg/dL)	-	-	-	-	-	-	2.23 ± 1.68	2.48 ± 2.05	0.709	0.75 ± 1.82	0.41 ± 0.63	0.329

AST: aspartate aminotransferase; ALT: alanine aminotransferase; AFP: alpha-fetoprotein; POD: post-operative day; POM: post-operative month

**Figure 1.** Disease-free survival curves after hepatic resection in the treatment group (dashed line) and the control groups (solid line)

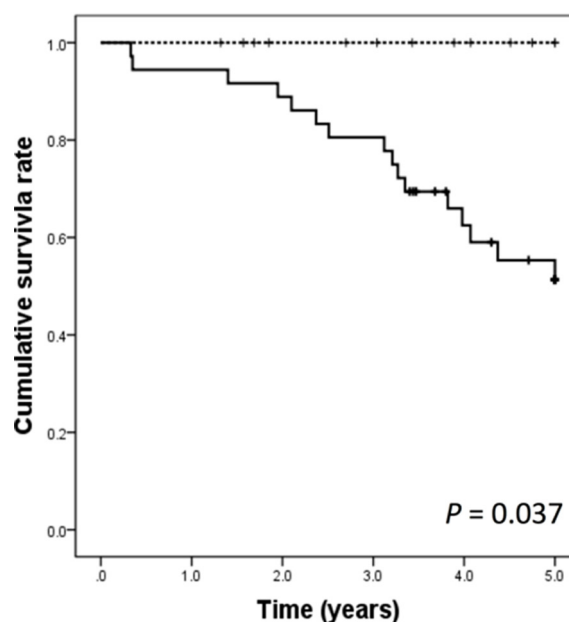
potential. In the treatment group, the tumor marker levels decreased after chemotherapy, and the 5-year DFS and OS rates after surgery were improved significantly.

In this study, we administered short-term HAIC using a temporary indwelling catheter system. Almost all previous reports about neoadjuvant chemotherapy for HCC revealed that lesions were scheduled for TACE and that related complications such as liver function impairment or surgical delay sometimes made resectable tumors unresectable. HAIC is considered to cause fewer liver function complications than TACE^[25-28]. In fact, this study illustrated that liver function was not adversely affected by neoadjuvant HAIC.

Table 6. Histopathology of resected livers: treatment group versus control group

Variables	Treatment group (n = 13)	Control group (n = 36)	P
Tumor size (mm)*	27 (14-50)	25 (10-58)	0.666
Number of tumor (n)*	1.0 (1-2)	1.0 (1-3)	0.560
Microscopic vascular invasion	3	18	0.131
Intrahepatic metastasises	5	10	0.476
Gross classification			
SN/SNEG/CMN	4/4/4	7/21/8	0.535
Histologic grade			0.202
Well differentiated	1	1	
Moderately differentiated	8	32	
Poorly differentiated	3	3	
Liver cirrhosis**			0.227
F0	2	3	
F1-F2	6	8	
F3-F4	5	25	
JBCS			
Grade 0	3	-	
Grade 1 (1a/1b)	3/3	-	
Grade 2 (2a/2b)	2/1	-	
Grade 3	1	-	

*Median with range; **new Inuyama classification. SN: simple nodular type; SNEG: simple nodular type with extranodular growth; CMN: confluent multinodular type; JBCS: Japanese Breast Cancer Society

**Figure 2.** Overall survival curves after hepatic resection in the treatment group (dashed line) and the control groups (solid line)

The regimen selected for this study was 2 weeks of low-dose FP. Ishikawa *et al.*^[29,30] first reported that HAIC with cisplatin before radical local treatment (radiofrequency ablation/percutaneous ethanol injection therapy) for early-stage HCC prevented intrahepatic metastasis and prolonged the survival time. According to some clinical studies, the efficacy of low-dose FP is better than that of cisplatin alone^[28]. Ueshima *et al.*^[31] reported that HAIC using low-dose FP (continuous arterial infusion of 5-FU and cisplatin for the first 2 weeks followed by a single dose of cisplatin and 5-FU once a week) is an effective treatment for locally advanced HCC. In our experience, almost all HAIC responders exhibited a decrease in tumor marker ratios in the early stage of treatment; thus, we believe 2 weeks of low-dose FP was sufficient to observe the effect of chemotherapy. HAIC-related liver toxicity is caused by complications associated with catheter placement, such as catheter dislocation, hepatic artery occlusion and stenosis, and infection. The 2-week regimen

Table 7. Univariate and multivariate analysis of the prognostic predictors of disease-free survival

Variables	Condition	95% CI	P	HR	95% CI	P
Age (years)	> 70	2.971-4.335	0.002	0.374	0.166-0.842	0.017
	≤ 70	1.296-2.684				
Gender	Male	2.064-3.300	0.192			
	Female	2.814-4.712				
Etiology	Hepatitis B carrier	0.912-3.782	0.444			
	Hepatitis C carrier	2.485-3.769				
	Others	1.153-3.829				
AFP level (ng/mL)	> 200	1.866-4.274	0.699			
	≤ 200	2.242-3.454				
DCP level (ng/mL)	> 400	1.546-4.097	0.684			
	≤ 400	2.276-3.433				
Tumor diameter (cm)	≥ 3	1.853-3.750	0.766			
	< 3	2.342-3.644				
Tumor number	> 3	2.609-3.716	< 0.001	7.731	2.474 - 14.161	< 0.001
	≤ 3	0.343-0.889				
Microvascular invasion	(+)	3.383-3.684	0.631			
	(-)	1.696-3.494				
Intrahepatic metastasis	(+)	1.145-3.178	0.094			
	(-)	2.571-3.805				
Differentiation grade	Poor	1.345-4.292	0.832			
	Others	2.303-3.481				
Neoadjuvant HAIC	(+)	3.662-5.147	0.003	0.215	0.050-0.928	0.039
	(-)	1.783-2.988				
Liver cirrhosis	(+)	1.854-3.511	0.482			
	(-)	2.352-3.775				
TNM pathological staging	I	2.529-6.953	0.058			
	II	4.209-7.588				
	IIIA	0.545-0.545				

HAIC: hepatic arterial infusion chemotherapy; AFP: alpha-fetoprotein; DCP: des-gamma-carboxyprothrombin; TNM: tumor nodes metastasis [6th edition of the American Joint Committee on Cancer (AJCC) staging]

enabled us to use a temporary indwelling catheter system, and after the administration of chemotherapy, the catheter system was removed easily under fluoroscopic guidance. In this study, the complication rate related to the temporary indwelling catheter system was also low.

Our data demonstrated the definitive improvements of DFS and OS after HAIC. There are two predicted reasons for this effect: (1) prevention of tumor cell dissemination during surgery, and (2) effectiveness in eradicating undetectable intrahepatic metastases. Concerning adjuvant HAIC, 2 non-randomized control trials reported that adjuvant HAIC after hepatic resection for HCC with macroscopic vascular invasion might reduce the risk of recurrence^[32,33]. However, among patients with Vp2 or invasion of the main trunk of the hepatic vein (Vv2), the 3-year DFS and OS rates were not significantly different between the 2 groups^[33]. Dislodging of tumor cells during surgery is considered one of the main causes of postoperative intrahepatic metastasis^[34,35]; thus, neoadjuvant HAIC is theoretically effective for preventing tumor cells from dislodging and disseminating into the portal venous stream.

In the present study, complete necrosis (grade 3) was observed in 1 patient, and a shift from a viable tumor lesion to necrosis (grade 1a, 1b, 2a or 2b) was noted in 9 patients. Even when a pathomorphologic therapeutic effect did not appear in the main tumor, the effect of the chemotherapeutic agent might contribute to the suppression of cellular motility and invasiveness, facilitating the eradication of undetected intrahepatic metastases.

Multivariate analysis revealed that neoadjuvant HAIC was one of the independent favorable prognostic factors for DFS. However, there are several limitations to this study. First, our study was retrospective in nature and some biases may be present, including selection biases leading to the overestimation of the apparent importance of preoperative HAIC. Second, the sample size was still small ($n = 13$). Although

we think that effective adjuvant therapy in addition to preoperative HAIC is crucial for further improved prognosis, we could not show the sufficient efficacy of adjuvant chemotherapy. Further prospective multicenter trials are required to establish the effectiveness of neoadjuvant HAIC for the treatment of HCC.

In conclusion, neoadjuvant HAIC for patients with HCC with a high-grade malignant phenotype decreases the risk of recurrence and improves survival without serious complications. However, a prospective randomized study is required to confirm our findings.

DECLARATIONS

Author's contributions

Conceived of the presented idea and developed theory: Tsutsui R, Nagamatsu H, Itano O

Contributed to the interpretation of the results: Deguchi A, Tsutsumi T, Hiraki M, Mizukami N, Akiba J

Provided critical feedback and helped shape the research, analysis and manuscript: Tsutsui R, Nagamatsu H, Itano O, Deguchi A, Tsutsumi T, Hiraki M, Mizukami N, Akiba J

Data source and availability

The data presented is original and obtained in our laboratory. It is available with the authors and can be made available if required.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Written informed consent from all the patients of the treatment group was obtained as part of the involvement in this study.

Ethics approval

The study was reviewed and approved by the Yame General Hospital Institutional Review Board.

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Review

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New strategy to distinguish clonal origin of RHCC/MHCC between intrahepatic metastasis and multicentric occurrence

Han Wang, Wen-Ming Cong

Department of Pathology, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China.

Correspondence to: Dr. Wen-Ming Cong, Department of Pathology, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Changhai Road 225, Shanghai 200438, China. E-mail: wmcong@smmu.edu.cn

How to cite this article: Wang H, Cong WM. New strategy to distinguish clonal origin of RHCC/MHCC between intrahepatic metastasis and multicentric occurrence. *Hepatoma Res* 2018;4:14. <http://dx.doi.org/10.20517/2394-5079.2018.16>

Received: 5 Mar 2018 **First Decision:** 13 Apr 2018 **Revised:** 19 Apr 2018 **Accepted:** 20 Apr 2018 **Published:** 28 May 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Guang-Zhe Zhu **Production Editor:** Cai-Hong Wang

Abstract

Hepatocellular carcinoma (HCC) is a kind of malignancy with high potential of metastasis and multicentric occurrence. The treatment of recurrent hepatocellular carcinoma (RHCC) and multinodular hepatocellular carcinoma (MHCC) is always a nodus because of the diverse clonal origin of RHCC/MHCC. Theoretically, the RHCC/MHCC can originate from intrahepatic metastasis (IM type) or multicentric occurrence (MO type). Our previous study proposed that there are at least 6 subtypes of clonal origin patterns in RHCC. RHCC and MHCC with different clonal origins have variant biological behaviors, clinical prognosis as well as treatment strategy. Generally speaking, patients with IM type HCC have a poorer prognosis compared with those with MO type HCC. Therefore, it is essential to emphasize the distribution of the clonal origin in HCC in order to determine the choice of clinical treatment. Undoubtedly, the detection of clonal origin pattern will become a promising breakthrough in the molecular pathological diagnosis of HCC. We should attach more attention to the establishment of a standardized molecular pathological clonal origin detection method and a new stratification of clinical treatment choice for RHCC/MHCC in future.

Keywords: Hepatocellular carcinoma, clonal origin, molecular pathology, recurrent hepatocellular carcinoma, multinodular hepatocellular carcinoma, intrahepatic metastasis, multicentric occurrence

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancer related fatal diseases in the world, especially in China. The recent cancer statistics of China showed that its incidence was in the fourth place,



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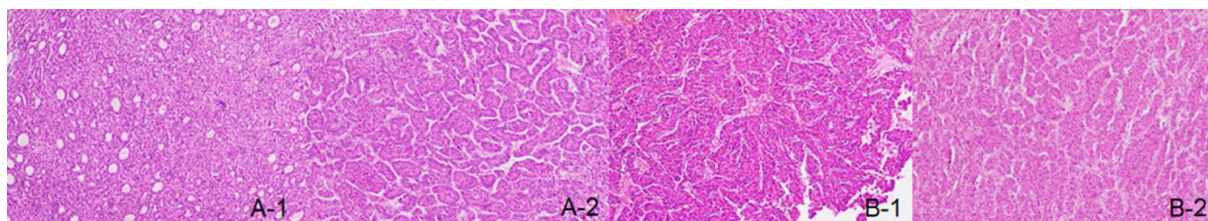


Figure 1. Hepatocellular carcinoma with different histological appearance and similar histological appearance. A-1: pseudoglandular pattern; A-2: thick trabecular pattern; B-1: thick trabecular pattern; B-2: thick trabecular pattern

and the mortality rate ranked the third^[1]. With the development of time, the hepatic surgery has made great progress, and liver resection has become a routine method for the treatment of HCC^[2]. However, the hepatic surgery is still facing two major obstacles. One is the treatment of recurrent hepatocellular carcinoma (RHCC). It was reported that the 5-year recurrence rate after hepatic resection of HCC is about 70% to 80%, or even higher^[3-7]. Meanwhile, there is no consensus on the clinical treatment options for RHCC. Secondly, it is the treatment of multinodular hepatocellular carcinoma (MHCC). It has approved that the patient's prognosis is poorer accompanied by the increased tumor nodules, especially > 3 foci^[8]. One of the material causes for two major obstacles stems from the unprecise judgment of the clonal origin of RHCC and MHCC. It has affirmed the secondary tumor (synchronous or metachronous) was the core to directly reflect the biological behavior and determine patient's prognosis^[9-12]. For our practice, we found that two tumor nodules in one patient may have similar or different histological appearance, which may suggest the clonal origin of the tumors [Figure 1]. However, this judging method largely depends on the experience of the pathologist, which is not objective and accurate. Obviously, the clonal origin detection is unquestionably the check point to explore the biological behavior of HCC.

HCC is a malignant tumor with high potential of recurrence and metastasis^[13]. However, the clonal origin of RHCC/MHCC cannot be determined by simple clinical indicators and histopathology^[14]. Consequently, the molecular pathological clonal origin detection is a new method to objectively determine the early, intermediate, and advanced stage of HCC in biological behavior and construct the basement of HCC molecular classification^[15]. In other word, the clonal origin model directly affects the choice of clinical treatment.

Therefore, this review article briefly summarizes some relevant progresses of molecular pathological clonal origin of RHCC and MHCC. We searched all available publications regarding “clonal origin”, “recurrent hepatocellular carcinoma”, “multinodular hepatocellular carcinoma”, “intrahepatic metastasis”, and “multicentric occurrence” in the PubMed and focused the data mainly based on the high quality full-text format.

THE CLONAL ORIGIN OF HCC

The exploration of the clonal origin of the malignancy started in the blood system tumor^[16,17]. Currently, it has approved that multiform clonal origins exist in malignant tumor. Identifying the clonal origin is of great significance for exploring tumor occurrence and evaluating tumor evolution^[18-22]. For solitary tumor, there are two types of clonal origin, monoclonal origin and polyclonal origin^[23]. Whether the secondary tumor is synchronous or metachronous, it may originate from intratumor metastasis of primary tumor (IM type); peradventure, it may be unrelated to the primary tumor, but from the normal cells which have adequate malignant mutation accumulation (MO type)^[24]. Similarly, IM type HCC originates from the primary HCC with low degree of differentiation, incomplete envelope, widespread microvascular invasion (MVI) or even portal vein invasion. Among all of risk factors, MVI is considered to be the core factor in the occurrence of IM type HCC. According to our research on 686 HCC patients, the incidence of MVI was about 42%^[25].

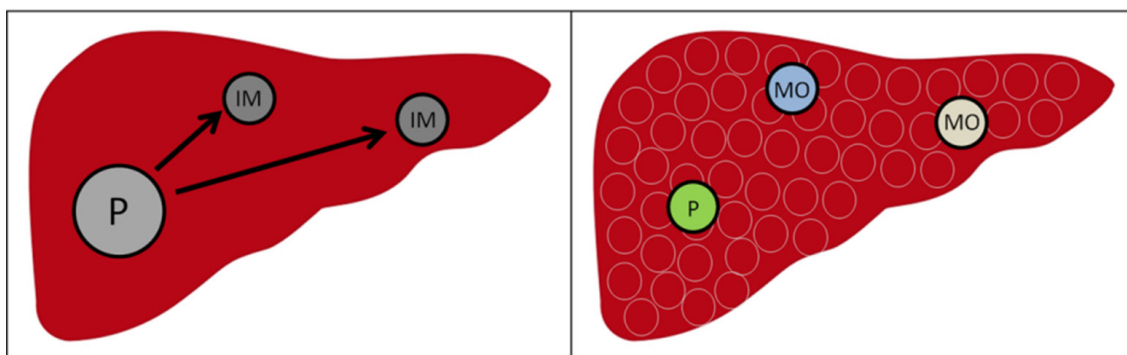


Figure 2. Mechanism of clonal origin with IM type and MO type in recurrent hepatocellular carcinoma/multinodular hepatocellular carcinoma. P: primary hepatocellular carcinoma; IM: intrahepatic metastasis; MO: multicentric occurrence

Remarkably, the incidence of MVI in single nodule HCC and MHCC are 40.4% and 55.6%, respectively. Higher incidence of MVI in MHCC indicates the possibility of IM type clonal origin in MHCC; MO type HCC is derived from the continuous blow of inflammation and fibrosis. Among the pathogenesis of inflammation, hepatitis viral is the most important reason and the most common cause of HCC. According to our statistics of 30 years' HCC patients in the Eastern Hepatobiliary Surgery Hospital, the infection rate of hepatitis B virus (HBV) and hepatitis C virus (HCV) was 85.86% and 9.76%, respectively^[26]. Therefore, effective inhibition of hepatitis virus replication is a key factor in the prevention of the occurrence of MO type HCC [Figure 2].

With the theory about the origination of malignant tumor constant improvement, such as tumor heterogeneity, cancer stem cells, circulating tumor cells, increased evidence suggests that there may be more complex clonal origin patterns in malignant tumor^[27-29]. For example, heterogeneous clonal origin in single nodule HCC and IM-MO mixed clonal origin in RHCC and MHCC^[30-32]. HCC with different clonal origin may engender variant clinical prognosis and therefore, different therapy method^[33,34]. Consequently, it is a crucial cooperation for hepatic surgery and molecular pathology to formulate rational treatment strategy for RHCC and MHCC with different clonal origin.

THE CLONAL ORIGIN OF RHCC

The postoperative recurrence of HCC is likely to be an important indication of enhanced invasiveness of HCC and poor prognosis^[35]. As a result, the current treatment strategy for primary HCC may not be suitable for RHCC. In view of this, scholars established many assessment systems for the prognosis of RHCC^[36-41]. However, many studies focused on exploring the rational treatment of RHCC did not screen out the suitable groups for traditional treatments, such as hepatic resection, liver transplantation, transhepatic arterial chemotherapy and embolization (TACE), and radiofrequency ablation (RFA)^[42-45]. It may attribute to the ignorance of great impact of clonal origin on the prognosis of patients.

Therefore, studies based on pathomorphology to predict the clonal origin of RHCC suggested that the incidence of IM type and MO type HCC is about 60% and 40%, respectively; IM type RHCC has poorer prognosis than MO type RHCC. Meanwhile, MO type RHCC and IM type RHCC are suitable for hepatic resection and TACE, respectively^[46-48]. Based on above studies, to some extent, it is meaningful to judge the clonal origin of RHCC by histopathology. However, the experience of pathologist may affect the judgment of the clonal origin pattern. Therefore, histopathology cannot objectively and quantitatively reflect the real biological behavior of RHCC. To sum up, it is necessary for us to establish therapeutic strategy for RHCC with different clonal origin according to molecular pathological examination, so as to enable patients to get the best prognosis.

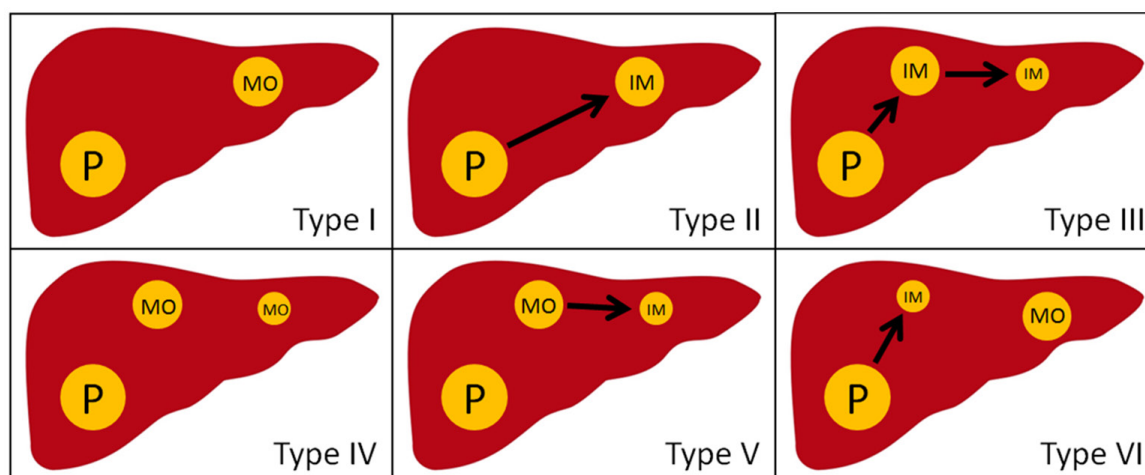


Figure 3. Six subtypes of clonal origin in recurrent hepatocellular carcinoma/multinodular hepatocellular carcinoma. P: primary hepatocellular carcinoma; IM: intrahepatic metastasis; MO: multicentric occurrence

Molecular pathology applies a variety of methods to determine the clonal origin of RHCC. The HBV infection is present in most patients with HCC. Chen *et al.*^[49] used southern-blot to detect the hepatitis B virus DNA (HBV-DNA) integration site in 5 cases of RHCC. Compared with 2 cases of IM type, 3 cases were MO type. Yamamoto *et al.*^[50] checked the HBV-DNA integration site and its flanking genomic DNA, and found that 6 of 8 cases of RHCC were MO type and 2 were IM type. Interestingly, Liang *et al.*^[51] used the same method, and found that, for multiple nodular RHCC, there are some nodules with the same clonal origin of primary HCC while other nodules is different, which is IM-MO mixed type RHCC. These studies provide a basis for the study of the clonal origin pattern of RHCC. However, HBV-DNA integration site detection is only suitable for HBV-related HCC. Referring to the distributed gene expression between primary HCC and RHCC, the scholars explored the clonal origin of RHCC by DNA ploidy analysis and p53 gene mutation site analysis^[52-54]. However, the case of RHCC in these studies is little (< 20 patients). Moreover, the above studies only explained two clonal origin patterns of RHCC, but not integrated with prognosis of patients. Therefore, we adopted microdissection-based PCR single-strand conformation polymorphism assay to check fifteen high-frequency of loss of heterozygosity (LOH) of DNA microsatellites on 100 tumor nodules in 60 matched pairs of RHCC from 40 patients who underwent liver re-resection. The definitions of the MO type and the IM type of RHCC were as follows: a $\geq 30\%$ difference (number of different LOH loci/number of informative loci $\times 100$) between primary HCC and any recurrent nodule was defined as MO type, on the contrary, IM type. Among all the patients, the percentage of IM type RHCC and MO type RHCC was 76.7% and 23.3%, respectively. MO type RHCC had a better prognosis than IM type RHCC (OS 130.8 ± 8.5 months vs. 80.8 ± 8.5 months; RFS 33.8 ± 4.5 months vs. 14.2 ± 2.5 months)^[33]. Then, we classified 2 clonal patterns into 6 subclonal types: type I, single-nodular MO-RHCC; type II, single-nodular IM-RHCC; type III, single-nodular IM-RHCC spreading intrahepatic metastasis; type IV, multinodular MO-RHCC; type V, single-nodular MO-RHCC spreading intrahepatic metastasis; and type VI, single-nodular MO-RHCC combined with IM-RHCC [Figure 3]. Among them, type I, IV, and VI is MO type; Type II, III, and V is IM type. We recommended liver re-resection for MO type RHCC, and interventional therapy for IM type RHCC. This classification provided a theoretical basis for the selection of clinical treatment.

With the development of the next-generation sequencing technology, we can explore the clonal origin of RHCC from the level of the whole genome expression spectrum. Shi *et al.*^[55] sequenced the whole exome with 1 case of RHCC patient of MHCC after resection. The gene expression profile of two RHCC nodules was highly similar with one primary nodule (86.7% and 86.6% respectively), rather than other primary nodule which pointed out the clonal origin of RHCC.

THE CLONAL ORIGIN OF MHCC

MHCC is a common clinical form of HCC. At present, scholars in various countries, including some international standards, have not yet reached a consensus on the clinical diagnosis and staging of MHCC. For example, there is controversy about ≥ 2 nodules or ≥ 3 nodules as the standard of MHCC^[56]. The Barcelona clinic liver cancer (BCLC) staging classification defined ≤ 3 nodules, ≤ 3 cm as stage A, called the early stage; ≥ 4 tumors of any size, or > 3 cm, 2-3 tumors are classified as stage B, called the intermediate stage, and defined as MHCC^[57]. Therefore, MHCC is not considered as early form of HCC in BCLC staging classification. Accordingly, the guidelines of HCC in Europe and America also recommend TACE/sorafenib as a first-line treatment for MHCC^[58,59]. However, if such kind of HCC occurred based on clonal origin of MO type, then they should not be considered pathobiologically as in the intermediate progression stage, and their treatment strategy will also be different accordingly. As the exploration of different treatment with BCLC intermediate stage of HCC, hepatic resection for some patients can obtain better prognosis than conservative treatment^[60,61].

With the increase of nodule and the scattered nodule, the prognosis of the patients is worse^[62-64]. Therefore, the current clinical study is paying more attention to the screening of radical treatment for MHCC^[65,66]. Huang *et al.*^[67] studied 102 MHCC patients with less than 3 nodules, and found that the presence of MVI is an independent risk factor for the patients of early recurrence (< 1 year) (HR, 4.02, 95% CI, 1.42-11.39, $P = 0.009$). Nojiri *et al.*^[68] retrospectively analyzed 107 patients of MHCC who underwent R0 resection and found that, for the patients with > 4 nodules, vascular invasion was an independent risk factor for long-term survival (1-year overall survival 71.1% vs. 82.4%, 3-year overall survival 36.9% vs. 61%, 5-year overall survival 0% vs. 25.4%, $P = 0.0035$). In view of vascular invasion, it is an important indication for the occurrence of MHCC as IM type. To sum up, no matter the number of nodules, vascular invasion are always the important prognostic factors for MHCC. Referring to the correlation between vascular invasion and IM type clonal origin, effective screening of MO type MHCC patients for actively radical treatment has become an important point of MHCC clonal origin research.

Similar to the research of RHCC clonal origin, the study of MHCC clonal origin also begins with the HBV-DNA integration site analysis. Govindarajan *et al.*^[69] and Aoki *et al.*^[70] analyzed the HBV-DNA integration sites in 2 cases of MHCC, respectively, and preliminarily established the concept of IM type and MO type in MHCC. After that, some scholars used different methods, such as analysis of methylation pattern of X-chromosome-linked human androgen receptor gene, mitochondrial D-loop mutations analysis, DNA fingerprinting analysis, analysis of difference of tumor suppressor gene promoter region methylation, to confirm the existence of IM type and MO type MHCC^[71-74]. Subsequently, scholars began to pay attention to the proportion of IM type and MO type in MHCC. Hsu *et al.*^[75] analyzed the HBV-DNA integration site of 25 cases of MHCC, including the main tumor, satellites and metastatic loci, and found that the IM type and MO type accounted for 60.7% and 39.3%, respectively. Tsuda *et al.*^[76] detected the alleles LOH of chromosome 16 in 19 MHCC patients, and found that the IM type and MO type accounted for 52.4% and 47.6%, respectively. Hui *et al.*^[77] performed DNA ploidy analysis of 62 tumor nodules in 26 MHCC patients, and found that IM type and MO type accounted for 53.8% and 46.2%, respectively. Based on our detection of the clonal origin of 439 cases of MHCC in Eastern Hepatobiliary Surgery Hospital, IM type and MO type MHCC account for 51.9% and 48.1%, respectively (unpublished data). Referring to the clonal origin of RHCC, we believe that MHCC is likely to have the same clonal origin patterns with RHCC [Figure 3]. Therefore, the choices of clinical treatment patterns for patients with MHCC should be based on the clonal origin patterns of MHCC in order to get better prognosis for these patients.

With the development of the next-generation sequencing technology, the understanding of clonal origin of MHCC can be penetrated into the level of specific gene and whole gene expression profiles. Xue *et al.*^[31] performed exome and low-depth, whole-genome sequencing for 43 nodules of primary tumors, satellite foci,

Table 1. Techniques of clonal origin detection

Technique	Method	Material	Genomic loci	Reference
HBV-DNA integration pattern	Southern blot analysis	Freshly frozen tissue	HBV DNA	[49,51,69,70,75]
HBV-DNA and flanking human DNA junctions	PCR	Paraffin-embedded tissue	HBV DNA	[50]
DNA fingerprint analysis	AP-PCR	Paraffin-embedded tissue	Nuclear DNA	[72]
DNA ploidy analysis	Feulgen-DNA analysis; flow cytometric method	Paraffin-embedded tissue	Nuclear DNA	[52,53,77]
X-chromosome inactivation pattern	PCR	Freshly frozen tissue	The HUMARA locus of exon 1 of the X-chromosomelinked human androgen receptor gene	[74]
Chromosomal alterations	Comparative genomic hybridization	Freshly frozen tissue	Nuclear chromosome	[80]
Chromosomal LOH	RFLP analysis	Freshly frozen tissue	HBA1, D16S32, D16S34, D16S35, CETP, MT2, D16S4, HP, TAT, CTRB, APRT	[76]
Mitochondrial D-loop mutations	PCR	Freshly frozen tissue	Mitochondrial DNA D-loop region	[73]
Allelotype and LOH of p53 gene	BanII RFLP analysis	Freshly frozen tissue	Sequencing of exons 5, 7, and 8 of the TP53 gene	[54]
Microsatellite LOH	PCR	Paraffin-embedded tissue	D1S243, D1S507, D4S402, D4D406, D4S415, D8S264, D8S277, D8S520, D13S268, <i>et al.</i>	[33,34,82,83]
Tumor genomic heterogeneity analysis	Next-generation sequencing technology	Freshly frozen tissue	Whole-genome sequencing	[31,55,78,79]

LOH: loss of heterozygosity; HBV: hepatitis B virus; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism

metastatic foci and multiple foci in 10 patients with MHCC. They found that the proportion of ubiquitous mutations in different tumor nodules in the same patient varied with 8%-97%. Furuta *et al.*^[78] performed whole genome sequencing and RNA sequencing for 49 nodules from 23 MHCC patients, which provides more detailed genetic information for clonal origin of MHCC. Lin *et al.*^[79] applied the whole exome sequencing to analyse 69 lesions from 11 MHCC patients, and found that 29% of driver mutations is heterogeneous. The heterogeneity of methylation level may be a key for the occurrence and progress of MHCC.

TECHNIQUES OF CLONAL ORIGIN DETECTION

The criteria for judging the clonal origin of IM type and MO type HCC have not been widely accepted. Some studies based on whether the recurrent time < 1 year or histopathology to define IM type and MO type RHCC^[6,47]. However, these classification methods can not accurately and objectively reflect the clonal origin of HCC. Therefore, molecular pathology uses a variety of methods to confirm it: HBV-DNA integration site analysis, DNA ploidy analysis, DNA fingerprint analysis, X-chromosome inactivation pattern detection, chromosomal LOH analysis, p53 gene mutation analysis, mitochondrial D-loop mutations analysis, microsatellite LOH analysis, next-generation sequencing technology, and so on [Table 1]. Some scholars has compared various kinds of methods^[80,81]. According to our experience, we recommended the microsatellite LOH detection^[33,82,83]. It is not only suitable for paraffin embedded tissues, resolves the restriction of gender and HBV infection, but also it can select a set of microsatellite profile to improve the diagnostic accuracy. In addition, microsatellite DNA is a suitable marker to reflect the overall stability of genome. To sum up, microsatellite LOH detection is the relatively ideal method to reduce the bias of HCC heterogeneity to the clonal origin in various methods.

CONCLUSION

With the development of time, the molecular biological behavior and characteristics of HCC has become an important guide for hepatic surgery. Among them, RHCC and MHCC will be an important breakthrough

in improving the long-term effect of HCC. Molecular cloning detection is an important theoretical and technical support to break this bottleneck. Therefore, strengthening the study of clonal origin of HCC and establishing a scientific and precise molecular cloning detection technology will be an important task in the field of HCC pathology. The innovation of molecular cloning technology provides guidance for the individualized treatment strategy of RHCC and MHCC. The overall view is that the IM type HCC has a more malignant biological behavior, and poorer clinical prognosis than the MO type HCC, no matter RHCC or MHCC.

The molecular pathological technical standards for evaluating the clonal origin of HCC have not yet been unified. Microsatellite LOH detection is currently the most widely used method in clinical practice. We should explore the method to unite high sensitivity and specificity, low cost, convenient and quick to serve the clinical practice better in future.

DECLARATIONS

Authors' contributions

Reviewed the literature and wrote the manuscript: Wang H, Cong WM

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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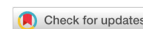
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Original Article

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Hepatocellular carcinoma in patients without cirrhosis: relevance and clinical characteristics

Kellyane Santana Dias Carvalho¹, Luciano E. Fonseca², Helma P. Cotrim¹

¹Medicine School, Universidade Federal da Bahia, Salvador 40110-060, Brazil.

²University Hospital Prof. Edgar Santos, Universidade Federal da Bahia, Salvador 40110-060, Brazil.

Correspondence to: Dr. Helma P. Cotrim, Programa de Pós Graduação em Medicina e Saúde – PPgMS, Complexo Universitário Professor Edgard Santos, Rua Augusto Viana, 50 Andar, Canela, Salvador 40110-060, Brazil. E-mail: helmacotrim@gmail.com

How to cite this article: Carvalho KSD, Fonseca LE, Cotrim HP. Hepatocellular carcinoma in patients without cirrhosis: relevance and clinical characteristics. *Hepatoma Res* 2018;4:15. <http://dx.doi.org/10.20517/2394-5079.2018.13>

Received: 24 Feb 2018 **First Decision:** 21 Mar 2018 **Revised:** 8 May 2018 **Accepted:** 8 May 2018 **Published:** 29 May 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Guang-Zhe Zhu **Production Editor:** Huan-Liang Wu

Abstract

Aim: The present study evaluated the frequency of hepatocellular carcinoma (HCC) in patients without cirrhosis.

Methods: HCC patients were recruited from two reference centers for liver disease in Northeast Brazil from 2010 to 2016. The diagnosis of HCC and cirrhosis was based on international criteria.

Results: A total of 169 patients were included, and 16% (27) of the patients did not have hepatocellular carcinoma in non-cirrhosis (HCC-NC). The mean age of HCC-NC was 64.4 ± 11.3 years, and 74.1% of the patients were male. The main risk factors were hepatitis C virus (HCV) in 29.6% (8), nonalcoholic steatohepatitis (NASH) in 14.8% (4) and hepatitis B virus (HBV) in 11.1% (3). Histological HCC diagnosis was performed in 81.5% (22) of the patients, and in 18.5% (5) of these patients, the diagnosis was performed by ultrasonography, computed tomography or nuclear magnetic resonance imaging methods. Single nodules were found in 56% of HCC-NC (14) when assessed by imaging methods.

Conclusion: The frequency of HCC-NC was elevated and more common in males. HCV, NASH and HBV were the most frequent risk factors. These data contribute to discussion on future protocols and criteria for the early diagnosis and treatment of HCC in patients with chronic liver disease without cirrhosis.

Keywords: Hepatocellular carcinoma, primary liver tumor, liver cirrhosis

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary malignant tumor found in the liver. HCC is



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also the second cause of deaths related to cancer, accounting for 700,000 deaths every year worldwide^[1].

In Brazil, HCC is the 8th most frequent malignant neoplasm and represents approximately 10,000 cases per year^[2].

A Brazilian national survey conducted in 2009 showed that hepatic cirrhosis was present in 98% of HCC patients, and this tumor was more frequent in cirrhosis patients with hepatitis C virus (HCV), hepatitis B virus (HBV) chronic hepatitis and alcoholic liver disease^[3].

However, HCC can also be associated with other liver diseases, such as non-alcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), and hemochromatosis as well as toxins^[4].

In patients without cirrhosis, the prevalence of HCC varies between 7% to 54% of the cases and can have a major influence on the geographical area^[5]. In Western countries, the prevalence of hepatocellular carcinoma in non-cirrhosis (HCC-NC) patients was estimated in 15% to 20% of cases^[6-8], and the most common risk factors were HBV and HCV. However, a majority of the information was obtained from Asia and Africa, where the prevalence of hepatitis B and C viral infections is also elevated^[9-11].

NASH is considered a relevant risk factor of liver disease worldwide^[12]. Associated metabolic syndrome manifestations may also contribute to the development of HCC in patients without cirrhosis^[13].

The present study evaluated the frequency, associated factors and clinical characteristics of HCC in Brazilian patients without cirrhosis.

METHODS

Design and population study

The present cross-sectional study included patients with HCC diagnosis from two reference centers for liver disease in Northeast Brazil from 2010 to 2016.

Inclusion criteria were as follows: patients diagnosed with hepatocellular carcinoma of different etiologies (NAFLD, HBV, HCV, alcohol, hemochromatosis, and etiology related to toxic agents)

Exclusion criteria were as follows: patients diagnosed with hepatocellular carcinoma and cirrhosis.

Diagnostic criteria

The diagnostic criteria for HCC were according to European Association for the Study of the Liver (EASL) recommendations^[14].

The criteria for the diagnosis of cirrhosis was histological and/or by the evaluation of non-invasive markers, such as FIB-4 {FIB-4 = age (years) × aspartate aminotransferase (AST) (U/L)/[Platelets (PLT) (10⁹/L) × alanine transaminase (ALT)^{1/2} (U/L)]}.

Clinical assessment

All the data were obtained from a questionnaire containing the following variables: gender, age, and risk factors for liver diseases (HBV, HCV, NASH, alcohol, and metabolic- and toxic-related factors). The data from physical examinations and completed additional tests [liver, lipid, and glycemic profiles, serum insulin, hepatitis B surface antigen (HBsAg), anti-HCV, ferritin, and transferrin saturation index] were considered. All the patients were also evaluated by at least two imaging methods, such as total abdominal ultrasonography (US), computed tomography (CT) or magnetic resonance imaging (MRI).

Table 1. Clinical characteristics of hepatocellular carcinoma patients with and without cirrhosis

Variables	Without cirrhosis	With cirrhosis
Gender		
Male, <i>n</i> (%)	20 (74.1)	110 (77.5)
Female, <i>n</i> (%)	7 (25.9)	32 (22.5)
Age, median \pm SD (years)	64.4 \pm 11.3	58.8 (\pm 10.9)
Size, median \pm SD (cm)	5.3 \pm 2.9	5.49 (\pm 4.0)
Etiology, <i>n</i> (%)	20 (74)	125 (88)
HCV	8 (29.6)	59 (48.5)
NASH	4 (14.8)	4 (2.8)
HBV	3 (11.1)	14 (10)
Cryptogenic	3 (11.1)	22 (15.5)
ALD	2 (7.4)	24 (17)
Hemochromatosis	-	1 (0.7)
Risk factor unknown, <i>n</i> (%)	7 (26)	17 (12)

SD: standard deviation; HCV: hepatitis C virus; NASH: non-alcoholic steatohepatitis; HBV: hepatitis B virus; ALD: alcoholic liver disease

Histological assessment

Histological evaluation was performed on liver biopsies or surgical samples. The diagnostic criteria for HCC were based on the recommendations of the International Consensus Panel^[15].

Statistical analysis

The statistical analyses were descriptive and performed with the Statistical Package for the Social Sciences (SPSS) software (version 22.0, IBM Corp., USA). The data were analyzed, and the results are expressed as the mean values, standard deviations, and medians according to the distribution of the variables.

The present study was conducted according to the guidelines established in the 1964 declaration of Helsinki. The project was approved by the Research Ethics Committee at Bahia Medicine School, Federal University of Bahia, Brazil. All the participants signed letters of informed consent.

RESULTS

A total of 169 patients with HCC were evaluated, and 16% (27) of the cases were HCC-NC. Table 1 shows the main clinical characteristics and risk factors of the patients without and with cirrhosis.

Histological analysis was performed in 81.5% of the cases (*n* = 22). A diagnosis was made by imaging methods (CT or MRI) in 18.5% of the cases [Table 2]^[16].

DISCUSSION

The prevalence of HCC-NC in this Brazilian study was elevated (16%), and the results were similar to those found in other studies conducted in Western countries^[6-8]. The patients were most frequently of advanced ages (mean of 64.4 years) and predominately male. These data are consistent with the findings of previous studies, although in other studies, the diagnosis of HCC-NC was more frequent in younger individuals and in women^[5]. This difference may be due to the geographical variations in the prevalence of HCC and its risk factors.

Chronic HBV and HCV infections are the most frequent risk factors for HCC in patients with and without HCC-NC. An estimated 0.1% of individuals with HBV without cirrhosis develop HCC^[9], likely due to the carcinogenic effect of the virus^[10]. HCV is described in most studies as being of low potential for developing HCC in the absence of cirrhosis. However, more recent studies have shown the existence of HCC-NC in patients with chronic hepatitis HCV, suggesting that other mechanisms independent of cirrhosis would affect hepatocarcinogenesis^[5,11].

Table 2. HCC in patients without cirrhosis from imaging methods (CT and/or MR)

Tumor numbers	Value, n (%)
1	17 (68)
2	3 (11.1)
3 or more	5 (20)
Size, median \pm SD (cm)	5.1 \pm 2.7
BCLC, n	
0	0
A	11 (40.7)
B	8 (29.6)
C	5 (18.5)
D	0

BCLC: Barcelona Clinic Liver Cancer^[16]; CT: computed tomography; MR: magnetic resonance; SD: standard deviation

However, this scenario could change over the next few years or decades, since effective treatments for the elimination of this virus are currently being used. However, there is a growing increase in NAFLD with the prospect of becoming the leading cause of liver disease worldwide associated with risk factors, such as dyslipidemia, central obesity, diabetes and metabolic syndrome.

In the present study, HCV was also the main risk factor for HCC-NC cases, even in areas of Northeast Brazil, where the prevalence of HCV is low^[17]. Perhaps, the prevalence has been influenced by the origin of the patients. The patients in the present study were recruited from reference centers for liver disease.

Chronic HBV infection was also a relevant risk factor for HCC-NC in this patient sample, even after national vaccination programs for this virus. These data are extremely concerning. HBV has a direct oncogenic effect^[18], and patients without cirrhosis are frequently not included in protocols for the early diagnosis of this neoplasm.

NASH, as the second most frequent risk factor after HCV, in the present series of HCC-NC patients, was observed in 14.8% of the cases. Although the prevalence of HCC without cirrhosis in patients with NASH is considered low, in some studies^[19-21], NASH also has been recognized as a relevant cause of this liver tumor in patients without cirrhosis. In addition, obesity and diabetes, the major risk factors associated with NAFLD (steatosis and NASH), are also independent risk factors for HCC^[13,22]. In the present study, 33% of the HCC-NC patients had diabetes.

In Brazil, a recent national survey that included 110 cases of HCC associated with NAFLD showed that 31% of the cases, diagnosed through liver biopsy, did not present cirrhosis^[23].

In the present study, a single nodule was observed in 68% of the HCC cases. Treatment with curative intent (resection) occurred in 59.3% of the cases. Histopathological evaluation was performed in 81.5% of the cases, and 51.9% of the HCC cases were classified as moderately differentiated tumors. This finding is interesting since the HCC diagnosis was conducted in patients without cirrhosis, who were not included in protocols for early diagnosis and treatment.

Previous studies have also shown that the majority of HCC-NC cases are diagnosed as a single and larger tumor^[24,25], it could be explained because patients with chronic liver disease without cirrhosis are not part of the surveillance protocol, and the diagnosis was performed in patients with more advanced stages.

Although the study presents relevant data, it has some limitations. A lack of knowledge of the prevalence of HCC in the reference population is important because the frequency of HCC-NC may be underestimated.

The majority of the clinical information was obtained from patient records, and some of the patients presented incomplete data.

In conclusion, the frequency of HCC-NC in these Brazilian patients was elevated and more commonly observed in men. HCV, NASH, and HBV were the most frequent risk factors associated with HCC-NC. These data contribute to discussions on future protocols and criteria for the early diagnosis and treatment of HCC patients with chronic liver disease without cirrhosis.

DECLARATIONS

Authors' contributions

Concept and design: Cotrim HP

Data acquisition: Carvalho KSD, Fonseca LE

Data analysis: Carvalho KSD, Cotrim HP

Manuscript preparation: Carvalho KSD, Cotrim HP

Critical revision and finalizing of the manuscript: Cotrim HP

Data source and availability

The data were strictly obtained from medical records according to the privacy policy and ethics code of our institute.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Consents from all of the patients were established prior to submission and all records were confidential.

Ethics approval

The present study was approved by the Ethics Committee and Research at Bahia School of Medicine, Universidade Federal da Bahia, Brazil.

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Review

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T cell immunotherapy in hepatitis B virus related hepatocellular carcinoma

Morteza Hafezi, Antonio Bertolotti, Anthony T. Tan

Emerging Infectious Diseases Programme, Duke-NUS Medical School, Singapore 169857, Singapore.

Correspondence to: Dr. Anthony T. Tan, Emerging Infectious Diseases Programme, Duke-NUS Medical School, 8 College Road, Singapore 169857, Singapore. E-mail: anthony.tan@duke-nus.edu.sg

How to cite this article: Hafezi M, Bertolotti A, Tan AT. T cell immunotherapy in hepatitis B virus related hepatocellular carcinoma. *Hepatoma Res* 2018;4:16. <http://dx.doi.org/10.20517/2394-5079.2018.55>

Received: 11 May 2018 **First Decision:** 22 May 2018 **Revised:** 22 May 2018 **Accepted:** 22 May 2018 **Published:** 30 May 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Chronic hepatitis B virus (HBV) infection is a major risk factor for hepatocellular carcinoma (HCC). While multiple treatment modalities are available, liver transplantation remains the sole curative treatment for advanced stages of HCC, and hence new treatment approaches are required to fulfill this unmet need of curative HCC therapy. Our first-in-man proof-of-concept adoptive T-cell immunotherapy against HBV related hepatocellular carcinoma metastases has shown promising results. Here, we review the development of T-cell immunotherapy targeting HBV antigens for the treatment of HBV-HCC and discuss the practical considerations for the safe and effective use in clinics.

Keywords: Chronic hepatitis B virus, hepatocellular carcinoma, T-cell immunotherapy

INTRODUCTION

Hepatocellular carcinoma (HCC) is the primary liver malignancy in adults, and it occurs predominantly in patients with chronic liver inflammation and cirrhosis. It accounts for approximately 800,000 deaths annually worldwide and in the majority of these cases, hepatocellular carcinoma (HCC) occurrence is linked to chronic hepatitis B virus (HBV) infection^[1]. HBV is a non-cytopathic DNA virus from the Hepadnaviridae family that specifically infects hepatocytes. Patients with chronic HBV infection can remain largely asymptomatic, but viral persistence increases the risk of developing liver complications like fibrosis, cirrhosis and hepatocellular carcinoma^[2,3]. Despite prophylactic vaccination against HBV, approximately 300 million people globally have been infected with this virus^[2] and among chronically infected individuals, approximately 25% will develop HCC neoplasm^[4].



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Due to the lack of effective diagnostic and screening strategies, most of the HCC patients are diagnosed late. Depending on the stage of the cancer, multiple different treatment modalities like transplantation, partial hepatectomy, chemo-embolization, systemic chemotherapy could be given^[5,6]. Among these approaches, the only potentially available curative therapy is resection and liver transplantation which can only be applied in the early stages of HCC before metastasis are detected^[5]. However, for the majority of patients who are diagnosed at later stages, or with metastases, current available therapy is ineffective and even first line drugs like sorafenib can only increase the survival for up to 3 months in these patients^[7]. In addition to the lack of an effective therapy for the majority of HCC patients, the increasing supply of donor livers with the advent of living donor transplantation has resulted in a change of the liver transplantation criteria. New Criteria were developed to include patients with more advanced disease. Though this has opened up the option of liver transplantation for more HCC patients, it also has a negative impact on the post-transplantation HCC recurrence rates. In most cases, therapeutic options for patients who have tumour recurrence post liver transplantation are even more limited^[8-11]. Therefore, there is a clear unmet need which supports the development of new effective therapeutic approaches. In this review, we focus on the use of adoptive T-cell immunotherapy targeting HBV antigens for the treatment of HBV-HCC and discuss the practical considerations for their use in clinics.

T-CELL IMMUNOTHERAPY FOR HCC

Immunotherapy has shown promising outcomes in different hematologic malignancies, demonstrating its high potential for curative HCC therapy^[12,13]. Major progress have been made in the development of immunotherapy approaches that attempts to rejuvenate and/or induce anti-tumour T cell responses in the HCC microenvironment, like immune checkpoint inhibitors (ICIs)^[14]. However, this approach requires a pre-existing inflammatory tumour microenvironment with significant immune cell infiltration, the expression of immune checkpoints on tumour cells, and/or an existing anti-tumour immune response, in order to exert an anti-tumour effect^[13,15-18]. With the intra- and inter- HCC patient tumour heterogeneity, it would be difficult to expect the mechanism of action for the therapy to be intact for all tumour nodules, especially in metastatic nodules that develop in different anatomical environments^[19]. Some tumours will be inherently devoid of infiltrating T-cells and hence will not respond to such treatments^[20].

Furthermore, this approach is non-specific. It aims to augment the general anti-tumour immune response. This comes with its own drawbacks as the enhanced immune response is a double edged sword. On one hand, it provides the desired anti-tumour effect, on the other, it could result in uncontrolled autoimmune effects^[21]. This is particularly important in patients with HCC recurrence post liver transplantation. In these patients, immunosuppressive agents are given to control graft rejection, but the very same enhanced anti-tumour response due to checkpoint inhibitors could also lead to uncontrolled inflammation and even graft rejection^[22,23], which is why the use of checkpoint inhibitors is at the moment not indicated for liver transplanted patients.

In such scenarios, the adoptive transfer of personalized autologous engineered T cells maybe a suitable strategy. Currently, multiple clinical trials using autologous engineered T cells against HCC are ongoing^[24]. Unlike others, this strategy does not rely on the immune pre-requisites above, instead new anti-tumour T-cells are engineered *in vitro* and reinfused back into the patient to combat the tumour [Figure 1]. In addition, the extensive body of work involved in the development of CD19-specific T-cell immunotherapy for B-cell leukemia has clearly demonstrated the potent cytotoxic function of autologous engineered T-cell^[25,26]. At present, adoptive T-cell therapy comprises of introducing either chimeric antigen receptors (CAR) or T-cell receptors (TCR) to re-direct the specificity of T cells towards the tumours, each with its own advantages or limitations.

CARs are membrane-bound proteins composed of an ectodomain, typically derived from a single-chain

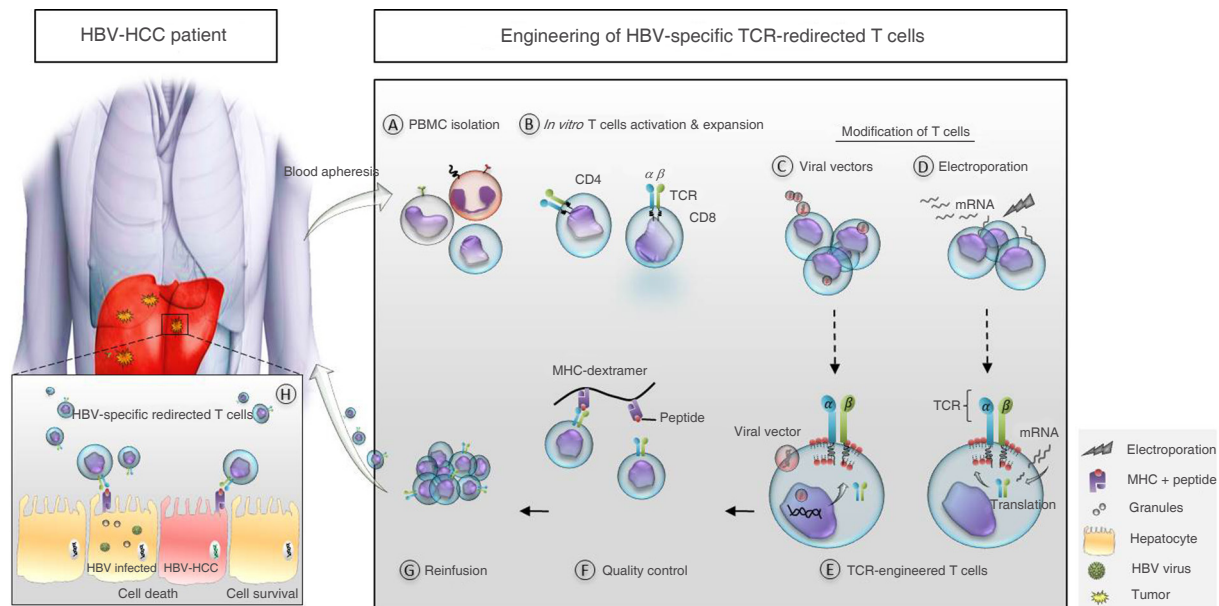


Figure 1. Schematic illustrating the production of personalized HBV-specific TCR redirected T cells. A: PBMC isolation from HCC patients; B: activation and expansion of $\alpha\beta$ TCR T cells for modification; C: transduce activated T cells with viral vectors encoding HBV-specific TCRs; D: electroporate activated T cells with in vitro transcribed mRNA; E: TCR-T cells engineered through viral transduction has the gene encoding the TCR integrated into the genome while electroporation only results in the translation of the introduced mRNA; F: analysis of the expression kinetics and function of HBV-specific TCR-T cells by tetramer staining and immune assays; G: adoptive transfer of autologous HBV-specific TCR-T cells back into the HBV-HCC patient; H: cytolysis of HBV expressing hepatocyte or HCC cells. HBV: hepatitis B virus; TCR: T-cell receptor; PBMC: peripheral blood mononuclear cell; HCC: hepatocellular carcinoma

variable fragment (ScFv), hinge and transmembrane domain. The ectodomain enables CARs to recognize cancer antigens in a HLA-independent manner. This particular feature enables CAR redirected T cells to be used in more patients without being restricted by their HLA haplotype, and also to target tumor cells that down-regulate their HLA expression^[27,28]. However, CAR recognition is limited only to conformationally intact antigens, both cell membrane bound or soluble forms. This represents only a small fraction of the total cellular proteins which limits the pool of antigens that can be targeted by CARs. In contrast, the TCR consist of alpha-beta chain heterodimeric glycoprotein which recognizes almost any degraded intracellular protein via the HLA system. This means that a greater degree of personalization is required when applied in patients, but at the same time, a larger number of tumour-specific T cell epitopes could potentially be targeted^[29].

These advantages of T-cell immunotherapy makes it a highly promising approach as a curative HBV-HCC treatment. However, choosing the appropriate tumour-specific antigen to redirect the T-cells towards remains a critical decision that dictates both the efficacy and safety of the approach.

TARGETING HBV ANTIGENS AS A TUMOUR ANTIGEN

Several clinical trials have shown that both CAR and TCR redirected T-cell therapy can cause substantial solid tumour regression^[30]. In all these cases, tumour discrimination is determined by the recognition of classical tumour associated antigens (TAA; alpha-fetoprotein, NY-ESO, MAGE, EGFR), essentially self-antigens that are aberrantly expressed in tumour cells, by high affinity CARs or TCRs. Such aberrant expression includes the overexpression of certain cell surface proteins at high levels, or the expression of fetal antigens that are typically not found in normal cells at a steady state^[31]. In both cases, due to the self-nature of the TAA, one cannot reliably predict and hence exclude the expression of the supposed tumour-specific antigens on other healthy cells. Adult cells undergoing active division could re-express fetal antigens which otherwise remains non-expressed when at a steady state. This is made even more challenging when high affinity CARs or TCRs, which recognizes pico-molar quantities of TAA, are used. For instance, clinical trials from NCI and Adaptimmune has shown the unexpected binding of high affinity MAGE-A3 TCR to similar epitopes like MAGE-A12 and titin in the brain and heart respectively, resulting in severe off-tumour

responses and a fatal outcome^[29]. Targeting overexpressed self-antigens in tumours might not be ideal due to potential off-tumour responses against normal cells^[29,32].

Recently, the discovery of altered self-antigens in highly mutated tumours have sparked an interest in their use as a TAA for T-cell immunotherapy^[33]. The continuous evolution and accumulation of mutations in tumours can result in the modification of self-antigens to an extent where they are no longer perceived as self-antigens, and hence become immunogenic. These neoantigens have been reported to be capable of inducing a robust T-cell response which mediates an anti-tumour effect. Since neoantigens are sufficiently different from self-antigens, this makes them a better TAA with far lower risks of on-target off-tumour responses observed when targeting classical TAAs. However, the generation of neoantigens involves the accumulation of random mutations which differs between tumour nodules and patients, making their discovery and characterization difficult and cumbersome^[34].

A possible alternative approach is to target HBV antigens as a TAA. In the natural history of chronic HBV infection, the virus integrates itself into the human genome, hence the HCC cells that eventually develop from chronically infected hepatocytes will carry these integrations and can be targeted by HBV-specific TCR T-cells^[32,35]. This integration results in either the expression of whole HBV antigens when the complete open reading frame is integrated, or the production of chimeric HBV-host proteins when only short fragments of HBV are integrated^[36]. In any case, the integration process inadvertently marks the HBV-HCC cells with a foreign antigen through a mechanism that is highly hepatotropic as dictated by the infectivity of HBV^[32]. This liver-specific marking would mean that the on-target off-tumour adverse events is largely predictable and would primarily be limited to the liver compartment, with little or no involvement of other organs^[29]. However, since HBV-specific TCR-T cells are unable to discriminate between HBV-infected hepatocytes and HBV-HCC cells, the risk of on-target off-tumour lysis of infected hepatocytes is also of concern. At present, this issue can be circumvented by treating only HBV-HCC patients with tumour recurrence post liver transplantation and by selecting HBV-specific TCRs restricted by HLA molecules present on the patient cells and not on the donor liver. Extending this approach to HBV-HCC patients without liver transplantation will have to be properly evaluated after more clinical data and experience have been accumulated. In addition to the desirable safety considerations above, targeting HBV antigens would also provide a commonality across multiple tumour nodules and patients, unlike the diverse and somewhat random nature of neoantigens, making it less complicated to use in clinics.

It is also important to note that the targeting of HBV antigens for HBV-HCC treatment comes with its own restrictions due to the natural virological characteristics of chronic HBV infection. In chronic HBV patients, microgram quantities of HBV envelope antigens are circulating in the serum. These soluble HBV antigens can interfere with the function of HBV-envelope specific CAR-T cells by either blocking and sequestering of the cell surface CARs, or by the inappropriate activation of the CAR-T cells^[32,37,38]. However, the obligate requirement for HLA presentation of T cell epitopes to TCRs would render the TCR-T cells insensitive to soluble HBV antigens in the serum^[39]. To capitalise on the better safety considerations associated with targeting HBV antigens, one would have to employ the use of HBV-specific TCRs and not CARs recognizing HBV antigens for HBV-HCC T-cell immunotherapy.

FIRST-IN-MAN PROOF-OF-CONCEPT TCR-T CELL IMMUNOTHERAPY OF HBV-HCC

The feasibility of using HBV-specific TCR-T cells for the treatment of HBV-HCC was first demonstrated in a compassionate therapy of a chronic HBV patient who has widespread extrahepatic HCC metastasis post-liver transplantation^[40]. The combination of several clinical features of the patient makes him an ideal candidate for the first-in-man proof-of-concept therapy where an emphasis on safety is essential. First, the patient had undergone liver transplantation. This means that the main bulk of HBV infected

hepatocytes have been removed, reducing the risk of overt destruction of functional hepatocytes. This risk is further lowered by confirming the absence of HBcAg, HBsAg and HBV DNA from a biopsy sample of his transplanted liver. Second, immunohistochemistry analysis of his metastatic tumour nodules shows the expression of HBsAg. This not only suggest that the tumour cells can be recognized by HBV-specific TCR-T cells, it also means that the serum levels of HBsAg could be used as a surrogate to monitor the efficacy of therapy as HBsAg is only produced by the tumour cells. With a single infusion of small numbers (10^4 HBV-specific TCR-T cells/kg) of retroviral transduced TCR-T cells, the cells expanded efficiently *in vivo* (~2% of CD8 T cells), and a reduction of over 90% of the serum HBsAg levels was achieved within 30 days without exacerbation of liver inflammation or any detectable on/off-target toxicities. This was not observed over the duration of one year when multiple radiotherapy and surgical resections of the tumours were performed. Unfortunately, the patient was treated at a very late stage and he succumbed to his disease after 8 weeks of monitoring. Nonetheless, the promising results obtained from this proof-of-concept therapy warranted further development of this treatment approach.

PRACTICAL CONSIDERATIONS FOR SAFE AND EFFECTIVE T CELL IMMUNOTHERAPY OF HBV-HCC

Despite the encouraging data obtained from the proof-of-concept therapy described above, additional considerations will have to be addressed in order to develop a safe and effective TCR-T cell immunotherapy for HBV-HCC. The first consideration is the issue of safety associated with the use of viral vectors for the delivery of the TCR gene construct. The oncogenic effect mediated by the insertion of the TCR gene into the host genome is a potential concern. More importantly, viral transduction generates T cells that stably expresses the HBV-specific TCR, allowing them to expand *in vivo*. This *in vivo* persistence may be beneficial for tumour eradication, but it would also pose a safety concern as the quantity and function of the modified T cells could not be easily controlled if a treatment related adverse event were to occur^[32]. An alternative is to introduce the TCR gene via the electroporation of *in vitro* transcribed functional mRNA [Figure 1]^[41]. This approach will not result in insertional mutagenesis and the expression of the introduced TCR is transient, while maintaining the anti-tumour effects. Not only will you have better control of the TCR-T cell function, the transient expression also allows clinical trials to be designed with an intra-patient dose-escalation protocol and thereby improving the safety. At the moment, HBV-specific TCR-T cells modified through mRNA electroporation have been extensively characterized *in vitro* and in *in vivo* pre-clinical models and is currently utilized in clinical trials for the treatment of HBV-HCC in liver transplanted patients^[42]. In addition, the transient function of mRNA electroporated T cells is ideal for the treatment of the majority of HBV-HCC patients who have not undergone liver transplantation, where the risk of on-target off-tumour lysis of functional but HBV infected hepatocytes is high.

Patient selection is also a critical issue that needs to be addressed. Barring inclusion and exclusion criteria associated with clinical parameters, at the moment, patient eligibility is dictated solely by the HLA haplotype of the patient^[29]. In which case, the patient is suitable for therapy if he/she expresses the appropriate HLA molecule capable of presenting the T-cell epitope recognized by the TCR-T cells. This simplistic criteria only takes into consideration the HLA component of the complex recognized by the TCRs^[29]. For the therapy to be effective, one has to be able to account for the presence or absence of the T cell epitope on the HCC cells. Ideally, this can be achieved using TCR-like antibodies specific for every HLA/HBV-epitope complex^[43,44] but the diversity of complexes makes this approach unfeasible^[45]. Peptide elution and mass spectrometry strategies^[46] might seem possible, but such techniques is highly specialized and complex, and at the moment restricted primarily to academic research and not clinical application. As a compromise, the detection of HBV proteins, DNA or mRNA would suffice with the assumption that antigen processing and epitope presentation occurs as expected. This is simpler in the situation where the complete open reading frame of a HBV antigen is integrated in the HCC cells. Detection of HBV antigens by serological means through immunohistochemistry analysis of tumour tissues would be sufficient. However, a recent study demonstrated

the existence of HBV-host chimeric proteins in HCC^[47-49], where only short fragments of HBV DNA are integrated into the host genome. In such situations, serological assays will fail to detect the presence of HBV antigens and the patient would be deemed unsuitable for TCR-T immunotherapy. However, these chimeric proteins could potentially be processed and presented on the HCC cell surface, rendering the tumours recognizable by TCR-T cells. These integrations could only be detected through genetic means. As such, to have better patient selection, it is essential to develop new genetic based assays for the rapid detection of short HBV integrations and determine whether the appropriate HBV T-cell epitopes could be potentially produced by HBV-HCC cells.

Lastly, it is also necessary to understand how the basal biochemical parameters of HBV-HCC patients could influence the function of TCR-T cells. It is common to have HBV-HCC patients with, elevated serum alpha-fetoprotein levels, to be treated with multi-kinase inhibitors or with immunosuppressive agents if they have been liver transplanted. The effects of such variables have remained largely unexplored in the context of TCR-T cell immunotherapy, but it could have important impacts on the treatment efficacy.

CONCLUSION

In this short review, we have discussed the need for new treatment strategies against HBV-HCC, the scientific rationale that guides the development of HBV-specific TCR-T cell immunotherapy and some practical considerations surrounding its use in patients. It is in our opinion that many unknowns still remain. At the moment, dosing and infusion frequencies are still determined arbitrarily, or extrapolated from T-cell immunotherapies for other cancers, while the accessibility of tumours at different anatomical locations, and even the function of TCR-T cells in different tumour microenvironments remains a subject of continuous investigation. We are however confident that the promising potential of T-cell immunotherapy will stimulate further research and development making its use in the treatment of HBV-HCC a reality.

DECLARATIONS

Authors' contributions

Hafezi M, Bertoletti A and Tan AT wrote the manuscript.

Financial support and sponsorship

This work was supported by a Singapore Translational Research (STaR) Investigator Award (NMRC/STaR/013/2012) to Bertoletti A.

Conflicts of interest

Bertoletti A participates in Advisory Boards on hepatitis B virus immune therapy for Gilead, Janssen, Medimmune and is a co-founder of Lion TCR Private Limited, a biotech company developing T cell receptors for treatment of virus-related cancers and chronic viral diseases. Tan AT is a consultant of Lion TCR Private Limited. Hafezi M discloses no conflicts.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Review

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Pre-S2 and HBV associated hepatocellular carcinoma

Ying Zheng, Yan-Yan Qian, Hong Fan

The Key Laboratory of Developmental Genes and Human Diseases, Department of Medical Genetics and Developmental Biology, Medical School, Southeast University, Nanjing 210009, China.

Correspondence to: Prof. Hong Fan, The Key Laboratory of Developmental Genes and Human Diseases, Department of Medical Genetics and Developmental Biology, Medical School, Southeast University, Dingjiaqiao #87, Nanjing 210009, China.
E-mail: fanh@seu.edu.cn

How to cite this article: Zheng Y, Qian YY, Fan H. Pre-S2 and HBV associated hepatocellular carcinoma. *Hepatoma Res* 2018;4:17. <http://dx.doi.org/10.20517/2394-5079.2018.08>

Received: 24 Feb 2018 **First Decision:** 28 Mar 2018 **Revised:** 14 May 2018 **Accepted:** 14 May 2018 **Published:** 31 May 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Guang-Zhe Zhu **Production Editor:** Cai-Hong Wang

Abstract

Hepatitis B virus (HBV) infection is a primary cause of hepatocellular carcinoma (HCC). Under selection pressures of host immunity and/or immunoprophylaxis and antiviral therapies, HBV evolves by accumulating mutations in its genome. Several studies highlighted the considerable importance of HBV surface (HBs) protein mutants (pre-S/S variants) in tumorigenesis. Among those mutants, pre-S2 mutants have been recognized as "precursor lesions of HCC" and as risk factors for post-operative recurrence of HCC. Pre-S2 mutants play important roles in tumor progression and induce various mechanisms of tumorigenesis. These roles include that the cytoplasmic orientation of the pre-S2 domain is essential for the transcriptional activator C-terminally truncated middle surface protein (MHBst) which participates in the development of hepatocellular carcinoma. Pre-S2 mutants may also play important roles in HBV tumorigenesis by inducing both endoplasmic reticulum stress-dependent and endoplasmic reticulum (ER) stress-independent pathways. Because HCC has poor prognosis and its incidence is increasing, methods for the prevention and treatment of HCC should be comprehensive. Emerging treatments based on ER stress may provide a new strategy.

Keywords: Pre-S2, hepatocellular carcinoma, hepatitis B virus, endoplasmic reticulum stress

INTRODUCTION

More than 240 million individuals worldwide are infected with chronic hepatitis B virus (HBV)^[1]. Chronic HBV infection progresses to cirrhosis in up to 40% of untreated patients, and there is an associated risk of decompensated cirrhosis and hepatocellular carcinoma^[2-6]. Several hypotheses have been proposed to



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explain the mechanisms of HBV related to tumorigenesis, including inflammation, liver regeneration associated with cytotoxic immune injuries and transcriptional activators of mutant HBV gene products^[7-10]. The HBV genome consists of a circular, partly double-stranded DNA with four overlapping open reading frames: (1) the pre-S/S open reading frame (ORF) encodes three viral surface proteins [including hepatitis B surface antigen (HBsAg)/HBV surface (HBs)], (2) the pre-C/C ORF encodes the hepatitis B e antigen (HBeAg) and the hepatitis B core antigen (HBcAg), (3) the P ORF encodes the terminal protein (TP) and the viral polymerase that possess DNA polymerase and reverse transcriptase and RNaseH activities, and (4) the X gene encoding for a transcriptional transactivator, hepatitis B virus X protein (HBx), which is essential for virus replication^[11,12].

Among the four functional proteins encoded by HBV (X, surface, core, and polymerase), HBx and HBs (mutant) proteins are designated “viral oncoproteins”^[13]. The pre-S/S mutants of HBV are considered “precursor lesions” of hepatocellular carcinoma (HCC)^[14] and as risk factors for the post-operative recurrence of HCC^[15,16]. Various pre-S/S mutants contribute to HCC tumorigenesis via various mechanisms, including transactivation of transcription factors, the endoplasmic reticulum (ER) stress-dependent pathway, the ER stress-independent pathway, and others. Among these mutants, pre-S2 mutants showed significant correlations with HCC and have been widely considered novel biomarkers of HBV-associated HCC^[13,17]. The malignant transformation potential of pre-S2 mutation has been confirmed in an immortalized human hepatocyte line HH411^[18]. In transgenic mice, pre-S2 mutants induced dysplasia of hepatocytes and development of HCC^[19], suggesting that pre-S2 plays a key role in HCC tumor progression.

In this mini-review, we discussed the relationship between pre-S2 mutations and HCC, as well as the underlying molecular mechanisms and treatments based on HBV tumorigenesis induced by pre-S2.

STRUCTURE AND ROLE OF PRE-S IN HBV

HBV is a small, enveloped 3.2-kb DNA virus with four open reading frames. The HBV envelope is composed of three forms of HBsAg, including the large (encoded by the pre-S1/S2/S gene), middle (pre-S2/S gene) and small (S gene) envelope proteins^[20,21]. In addition, truncated and mutated pre-S2/S [the large HBV surface protein (LHBs) and truncated middle surface protein (MHBs)] or HBx proteins are produced by integrated viral sequences^[22-24]. The pre-S region has been reported to mediate hepatocyte attachment of the virus, containing B cell and T cell epitopes^[25,26], a binding site for neutralizing anti-pre-S2 antibody^[27,28], and an S promoter for controlling the production of middle and small HBs proteins. Under endogenous (host immunity) and/or exogenous (immunoprophylaxis and antiviral therapies) selection pressures, HBV evolves by accumulating mutations in its genome, resulting in HBV variants with altered epitopes providing higher pathogenicity^[29-31]. In this context, a growing number of studies were performed to evaluate various HBV genotypes; these pointed out the considerable importance of HBV envelope protein mutants (preS/S variants)^[32,33]. Naturally occurring pre-S mutations are frequently detected in serum obtained from patients with chronic HBV infection^[34]. Furthermore, pre-S mutations were more common in chronic HBV infection and were related to disease progression and HCC. Currently, the most frequently reported variations are the pre-S deletion mutation and the pre-S2 start codon mutation^[19,31,35-37]. In particular, the pre-S2 mutation often coincides with changes in human immune cell epitopes^[38] and is more significantly correlated with HCC than pre-S1 mutation^[39].

THE ASSOCIATION BETWEEN PRE-S MUTATIONS AND HCC

The notion of pre-S/S mutations as causes of HBV immune escape was supported by the identification of individuals who developed HBV infection in spite of having vaccine-induced circulating anti-HBs antibodies^[31,32,40]. Apart from the ability to avoid neutralization by vaccine-induced anti-HBs, these pre-S/S mutations may also have accounted for cases of occult HBV infection^[31,41]. Furthermore, pre-S/S mutations

have been found in association with various forms of acute and chronic liver disease, including fulminant hepatitis (FH), fibrosing cholestatic hepatitis (FCH) and cirrhosis^[42-44]. Both pre-S1 and pre-S2 mutants led to defective secretion of mutant large surface antigens which then accumulated in ER, leading to ground glass hepatocytes (GGH) formation in chronic HBV infection^[45,46]. Under electron microscopy, GGHs were characterized by an abundance of ER, and overloaded ER made the cytoplasm of GGH become “foggy” or “glassy”. GGH was recognized as a risk factor for HCC, in particular, type II GGHs that harbor pre-S2 mutations accumulated on the ER of hepatocytes were considered biomarkers of HCC and were helpful in predicting recurrence and survival in HBV-infected HCC patients^[47]. Previous studies reported several tumorigenic mutants, including sL95*, sW182*, and sL216*, that did not promote ER stress but rather activated cell proliferation and transformational abilities; the sW182* mutant was demonstrated to have potent tumorigenic activity^[48]; MHBst167 mutants have been shown to interact with proteins associated with tumor progression/progression *in vitro*^[49]. A recent study reported that a pre-S2 start codon mutation of HBV subgenotype B3 affected nuclear factor κB (NF-κB) expression and activation in Huh7 cell lines^[50].

The frequency of pre-S mutations increased successively in the various stages of chronic hepatitis B (CHB) infection. A meta-analysis showed that the frequency of pre-S mutants was approximately 10%, 20%, 35%, and 50% in asymptomatic HBsAg carriers, CHB patients, patients with liver cirrhosis and HCC patients, respectively^[39]. The prevalence of pre-S mutants varied among countries with endemic HBV genotypes with a higher prevalence of genotypes B and C^[51]. Pre-S deletion mutants detected in serum were also reported to increase the risk of post-operative recurrence of HCC^[15]. To efficiently detect pre-S deletion mutants in serum, Su *et al.*^[7] successfully developed an oligonucleotide pre-S gene chip to detect pre-S deletion mutations in sera as a predictive hallmark of HCC. Combined detection of pre-S mutations and other markers of HBV replication such as HBeAg and viral loads may offer a reliable method for predicting HCC risks in chronic HBV carriers. Among those mutants, the pre-S2 mutation in particular was found to be significantly associated with the risk of HCC development^[20,31,52-55]. Pre-S2 deletion mutations in sera can be detected in nearly half of children with HCC^[56], and in tissue samples, pre-S2 deletion mutations can be detected in about 80% of pediatric HCC^[57].

VARIOUS MECHANISMS OF PRE-S2 CONTRIBUTING TO HCC

Pre-S2 transcriptional activator proteins

During the infectious process, HBV DNA integrates into hepatocellular chromosomes and encodes two transcriptional activators: the HBV X protein and the family of the pre-S2 activator proteins of HBV, including the LHBs and C-terminally MHBst^[23]. The pre-S/S genomic region, when deleted in the C-terminus portion (including the viral transmembrane hydrophobic region III of the S domain) produces C-terminally truncated middle surface protein^[31]. HBs transactivators (LHBs and MHBst) function based by cytoplasmic orientation of the pre-S2 domain^[58]. Unlike full-length MHBs, truncated MHBst is retained in the endoplasmic reticulum and is not secreted. Therefore, the pre-S2 region of MHBst can interact with the cytoplasmic protein in the cytoplasmic region, resulting in transcriptional activation^[59,60].

The discovery of transactivating functions exerted by LHBs and MHBst supports the notion that transactivation of cellular gene expression could be relevant to hepatocarcinogenesis. Pre-S2 activators LHBs and MHBst exerted tumor promoter-like functions by activating c-Raf-1/Erk2 signaling in transgenic mice, leading to enhanced proliferative activity of hepatocytes^[58]. Liang *et al.*^[61] found that overexpressing MHBst in hepatoma cells enhanced TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. In addition, a study showed that pre-S2, functioning as a transcriptional activator, promoted the development of hepatocellular carcinoma by activating oncogenes, including c-myc, human telomerase reverse transcriptase (hTERT) and forkhead box P3(Foxp3)^[18,22,23,62]. Another recent study provided evidence that HBV protein pre-S2 was responsible for reactivation of two oncogenes, alpha-fetoprotein (AFP) and glypican 3 (GPC3), in HCC^[63]. Other studies reported that pre-S2 increased protein levels of transcriptional co-activators with

PDZ-binding motifs (TAZ), thereby playing oncogenic roles in HCC cells by repressing miRNA-338-3p expression, implicating hepatocarcinogenesis^[64-66].

Pre-S2 mutants

Both pre-S1 and pre-S2 mutants led to defective secretion of mutant large surface antigens that then accumulated in the ER, leading to GGH formation in chronic HBV infections. As mentioned above, type II GGHs that harbored pre-S2 mutations accumulating on the ER of hepatocytes were considered biomarkers of HCC^[47]. HBV proteins utilize the ER protein folding machinery and cellular secretory pathway^[67]. Therefore, the underlying mechanisms of pre-S mutations contributing to HCC may be involved in ER stress^[7]. ER stress, also called the UPR in mammalian cells, is a cellular defense mechanism that responds to unfolded viral proteins or perturbed ER functions^[19]. Expression of viral gene products is detected by three UPR sensors, including two ER transmembrane kinases (IRE1 and PERK), and one ER transmembrane transcription factor (ATF-6). The three UPR sensors are associated with ER chaperone GRP78/BiP at rest, and are dissociated from GRP78 upon ER stress^[68]. Induction of GRP78 prevented cells from apoptosis, and ER stress-regulated translation increased tolerance to extreme hypoxia and then promoted tumor growth^[69,70]. The activation of ER-stress downstream molecules such as ATF-6, GRP78 and XBP-1 is believed to be involved in hepatocarcinogenesis^[71].

Both types of pre-S mutants cause overproduction and accumulation of mutated envelope proteins in the ER, and the accumulation of mutant or unfolded proteins cause stress in the ER that is sensed by the glucose-regulated protein 78 (GRP78). Unfolded proteins sequester GRP78 and dissociate from three ER transmembrane transducers leading to their activation; this leads to significant ER stress that may lead to oxidative stress and DNA damage^[72], resulting in genomic instability^[73] and ultimately development of HCC^[74,75]. A detailed study aimed at delineating the molecular mechanisms of pre-S mutant-induced genomic instability suggested that pre-S2 mutant large surface protein inhibited DNA double-strand break repair and led to genome instability in hepatocarcinogenesis; this represented a promising high-risk HCC biomarker in chronic HBV carriers^[76]. The ER stress initiated by the pre-S mutants activated two pathways that protect hepatocytes from apoptosis, one involving nuclear factor (NF)- κ B to upregulate cyclooxygenase-2 (COX-2)^[45,77] and the other involving vascular endothelial growth factor to activate AKT/mammalian target of rapamycin (mTOR) signaling^[74]. The mammalian target of mTOR is a highly conserved serine/threonine kinase that controls cell growth and proliferation^[78]. Pre-S2 mutations promoted tumorigenesis by sustaining high activation rates of aerobic glycolysis through the mTOR signal cascade^[79]. In addition, the pre-S2 mutation LHBs induced an ER stress-independent c-Jun activation domain binding protein 1 (JAB1)/p27/retinoblastoma (Rb)/adenovirus E2 promoter binding factor/cyclin A signal to initiate cell cycle progression^[75]. These studies suggested that the combined effects of genomic instability and cell proliferation potentially resulted in carcinogenesis^[7].

TREATMENT STRATEGIES BASED ON ER STRESS

One of the strategies used to prevent HBV-associated liver diseases and HCC is vaccination^[80]. The effectiveness in preventing blood-borne transmission from an infected mother to her newborn was about 90%^[81], however therapeutic vaccines for the treatment of established HBV infection are not available^[82,83]. Two antiviral therapies have been approved: pegylated alpha interferon and nucleoside/nucleotide analogues (NA)^[84]. NA therapy has antiviral effects that reduce HCC development and post-operative recurrence of HCC^[85]. NA treatment affects the reverse transcription of pregenomic RNA but does not affect cDNA and subgenomic RNA that have translational activity associated with HBsAg levels. Thus, current NA therapy can hardly clear HBsAg^[13]. Subsequent studies also showed that pre-S2 mutations induced resistance to NAs and predicted HCC development^[86]. Related studies showed that interferon treatment, more than NA treatment, inhibited HBsAg and pre-S mutant protein^[53,87,88]. However, these antiviral therapy often failed to eradicate the virus completely, and their efficacy in preventing liver cirrhosis and HCC was limited^[89,90].

Thus, it is necessary to clarify the details of the host-virus relationship during HBV infection to facilitate the development of efficient therapeutic strategies for HBV infection.

To prevent HCC, targeting HBV-induced ER stress may provide novel strategies in high-risk CHB. Antioxidants may be such ideal agents, because they reduce ER stress, thereby improving protein folding^[91]. Natural products, including silymarin and resveratrol, have been used in HCC. The two drugs target ER stress-associated signal pathways^[7]. The pre-S2 mutant initiated an mTOR-dependent glycolytic pathway to activate the solute carrier family 2 member 1 (SLC2A1), contributing to aberrant glucose uptake and lactate production in advanced stages of pre-S2 mutant transgenic tumorigenesis; the mTOR signaling cascade in pre-S2 mutant-mediated hepatocarcinogenesis was inhibited by the combined treatment of resveratrol and silymarin^[79]. However, these findings require further validation. Glycyrrhizin acid (GA) has also been reported to suppress ER stress in acute liver injury via several functions, including effective hepato-protection and the reduction of elevated transaminases^[92]. Long-term treatment with glycyrrhizin prevented HCC development in chronic hepatitis C infection^[93]. Together, these strategies for prevention and treatment of HBV-related HCC should be further investigated.

DECLARATIONS

Authors' contributions

Drafted the manuscript: Zheng Y

Revised the manuscript: Qian YY

Revised and approved the final version: Fan H

Financial support and sponsorship

This work was funded by the grants from National Natural Science Foundation of China (81672414, 81472548, and 81702906).

Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Review

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Ablative techniques in hepatocellular carcinoma treatment

Ehsun Naeem, Shahab Abid

Section of Gastroenterology, Department of Medicine, Aga Khan University, Karachi 74800, Pakistan.

Correspondence to: Prof. Shahab Abid, Section of Gastroenterology, Department of Medicine, Aga Khan University, Stadium Road P.O Box 3500, Karachi 74800, Pakistan. E-mail: shahab.abid@aku.edu

How to cite this article: Naeem E, Abid S. Ablative techniques in hepatocellular carcinoma treatment. *Hepatoma Res* 2018;4:18. <http://dx.doi.org/10.20517/2394-5079.2018.22>

Received: 17 Mar 2018 **First Decision:** 22 Mar 2018 **Revised:** 8 May 2018 **Accepted:** 17 May 2018 **Published:** 13 Jun 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Hepatocellular carcinoma has been known to arise commonly in the setting of chronic liver disease. Due to its association with cirrhosis, patients with hepatocellular carcinoma often present with markedly diminished hepatic functional reserve, making them poor surgical candidates. For such patients, image-guided percutaneous ablative modalities have provided a viable alternate curative therapy. Although treatment allocation is a decision based on a number of factors, patients eligible for percutaneous ablation generally include those with early stage disease, hepatocellular carcinoma with disease limited to the liver and no extra-hepatic metastases. While percutaneous ethanol injection is the seminal technique, newer developments have led to it being replaced by percutaneous radiofrequency ablation as the most commonly employed procedure, due to a better efficacy as well as safety profile. Other ablative modalities including microwave ablation, laser ablation and cryotherapy are not as widely available. Furthermore, data comparing their effectiveness with well-established procedures like radiofrequency ablation is limited.

Keywords: Barcelona Clinic liver Cancer staging, chronic liver disease, hepatocellular carcinoma, Milan Criteria, percutaneous ethanol injection, radiofrequency ablation, surgical resection

INTRODUCTION

The past two decades have seen percutaneous ablation emerge as an exciting new therapeutic approach for the treatment of hepatic malignancies worldwide. While surgery is still regarded as the mainstay of therapy for hepatocellular carcinoma (HCC), high tumor burden and reduced hepatic functional reserve, as often encountered in such patients, precludes surgical resection in a significant proportion of patients^[1]. For such



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patients, image guided local ablation has provided a viable curative option that has significantly prolonged survival and improved cure rates^[2].

As the fourth leading cause of cancer related deaths in the world^[3], hepatocellular carcinoma remains one of the most feared complications of liver cirrhosis to date. The tremendously high case-fatality rate of this malignancy is often attributed to the relatively advanced stage of disease at the time of diagnosis in most cases^[3,4]. Although adoption of intensive surveillance programs for patients with underlying chronic liver disease have allowed for earlier detection of HCC^[5], prognosis remains poor for most patients, as evidenced by the short median survival following diagnosis, ranging from 6-20 months^[6]. Nevertheless, most guidelines recommend screening at-risk individuals, such as those with chronic liver disease, with a non-invasive and cost-effective radiological investigation like ultrasound every 6 months.

HCC arises most often in the setting of cirrhosis, with an incidence of HCC development being as high as 1%-8% per year in chronic liver disease patients. Furthermore, the disease prevalence has been found to reflect the geographical distribution of the risk factors for cirrhosis^[7]. Areas with a high prevalence rate include Eastern Asia and Sub-Saharan Africa due to the presence of chronic HBV infection, which is considered to be the dominant risk factor for chronic liver disease^[8,9].

Optimal therapeutic approach is individualized to each patient, and should ideally be determined by a multi-disciplinary team comprising of hepatologists, surgeons, oncologists, radiologists, interventional radiologists and pathologists due to the complexity of the disease. Factors that need to be considered when determining treatment approach include liver function, size and number of nodules, tumour extension, age and co-morbid conditions of the patient. Nature of the underlying chronic liver disease may also play a part in this decision, particularly in cases where the oncogenic agent is expected to persist following treatment, reducing the viability of invasive procedures like surgical resection.

Guidelines such as The European Association for the study of Liver and The American Association for the Study of Liver Diseases recommend algorithms based on the Barcelona Clinic Liver Cancer staging system for the purpose of staging and treatment allocation. Although it has a number of limitations, the BCLC staging system has been validated in different settings and is commonly employed in many countries^[10]. The algorithm stratifies patients into five categories, based on the disease stage. In general, potentially curative treatments such as tumor resection, liver transplantation and percutaneous ablation are reserved for patients with early stage disease, classified as BCLC stage 0 and BCLC stage A, while patients in BCLC stage B, C and D presenting with advanced disease are offered palliative treatment options like chemoembolization and Sorafenib or supportive care [Figure 1].

Since their introduction, The Milan Criteria have become the standard guidelines for hepatic transplantation^[11]. These criteria restrict liver transplant to patients with either a single tumor less than 5 cm in diameter or less than three foci of tumor each with a diameter of no more than 3 cm, absence of angio-invasion and extra hepatic involvement. Using these criteria, excellent 5-year survival rates of 70% or greater and a 15% recurrence rate have been demonstrated by multiple studies, indicating their importance in predicting prognosis in HCC patients undergoing liver transplant. The Milan Criteria has also been found to produce excellent results when used for treatment allocation of patients with early stage disease, who may be candidates for other curative procedures like surgical resection or loco-regional ablative treatments

PERCUTANEOUS LOCAL ABLATION

Since their advent in the 1990s, percutaneous local ablative techniques have been continuously evolving owing to rigorous research and clinical testing in this area^[12]. While percutaneous ethanol injection was regarded as the primary ablative therapy up until the turn of the century, recent years have seen it largely

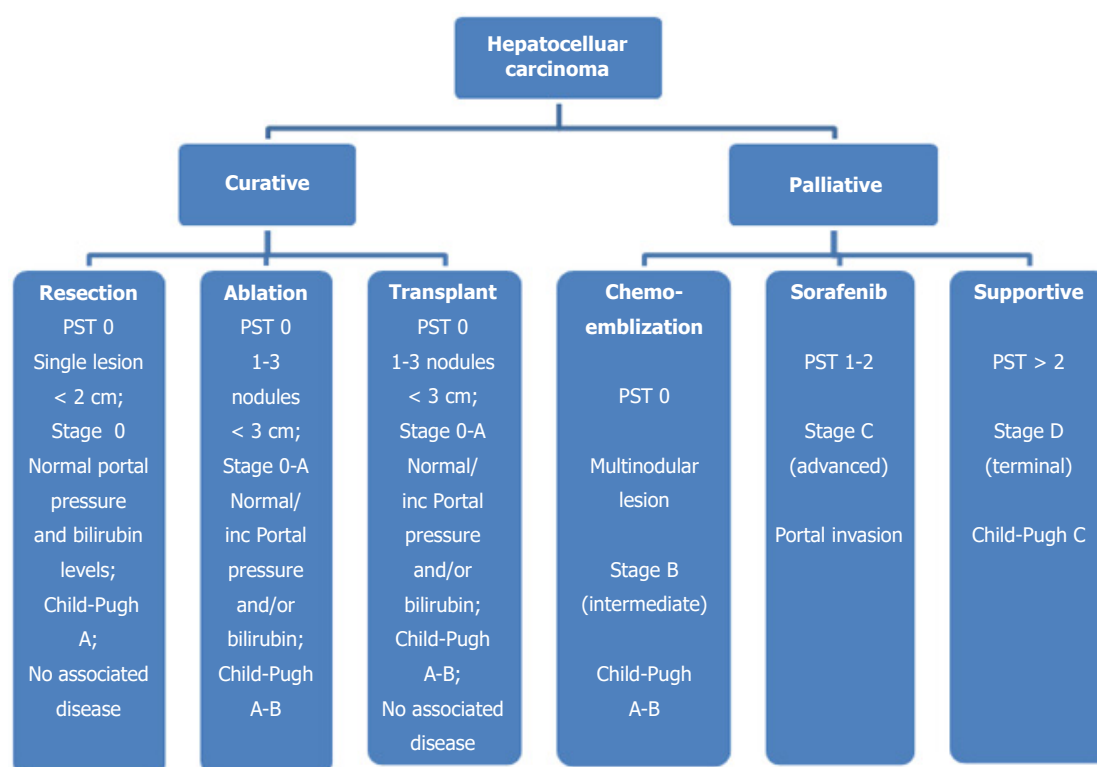


Figure 1. Treatment algorithm based on disease and patient characteristics, adapted from the BCLC staging system

being replaced by newer modalities like Radiofrequency ablation. Although encouraging results have been reported for both in terms of treatment response and long-term survival, differences exist in terms of applicability and adverse effects of each, and the decision to use one over the other is often individualized to each patient and requires careful patient evaluation and triage.

PERCUTANEOUS ETHANOL INJECTION

Percutaneous ethanol injection (PEI), performed under local anaesthesia with ultrasound guidance, involves injecting ethanol intra-lesionally using non-cutting needles over multiple sessions in the outpatient setting. By promoting cellular dehydration and occlusion of smaller tumor vessels, ethanol induces coagulative necrosis and a fibrous reaction leading to complete necrosis of most lesions. PEI is a well-established therapy, particularly for the treatment of nodular HCC, owing to the ability of ethanol to diffuse through the soft malignant tissue and the firm consistency of the surrounding cirrhotic liver parenchyma^[13].

In general, tumour response following PEI is determined by the size of the lesion as well the degree of hepatic dysfunction. Tumours smaller than 2 cm show the best response in terms of disease eradication with 90%-100% of lesions showing complete response, while larger lesions have shown a high rate of local recurrence when treated with PEI^[14-16]. This is postulated to be due to septae within larger lesions, presenting barriers to the diffusion of ethanol, leading to an incomplete response. With recent technological advances however, including the introduction of a multipronged needle with retractable prongs, even larger tumors up to 4 cm in size have demonstrated complete response rates as high as 80%-90%^[17].

With 5-year survival rates as high as 47%-53% in patients with early stage disease, PEI has shown encouraging results^[18,19]. It is however, associated with a high local recurrence rate of 43%, particularly for lesions larger than 3 cm in diameter, which undermines its curative capacity when compared with newer ablative modalities like radiofrequency ablation (RFA)^[20].

PEI may be considered as an alternate curative approach for patients with limited hepatic malignancies who are poor surgical candidates. High local recurrence rates preclude PEI in patients with tumours larger than 5 cm, or with a volume in excess of 30% of the total liver volume. Other contraindications include extra-hepatic disease, involvement of the dome of the liver, portal vein thrombosis and Child-Pugh class C cirrhosis.

Although rare, serious adverse effects associated with PEI include intra-peritoneal haemorrhage, liver failure, bile duct necrosis or biliary fistula, portal vein thrombosis, hepatic infarction, hypotension and renal failure^[21]. The incidence of such major complications has been found to be as low as 2.2% according to some studies. Other minor side effects experienced more commonly include localized pain and peritoneal irritation secondary to ethanol leakage^[22].

THERMAL ABLATION

Thermal ablative therapies for HCC include hyper-thermic treatments like radiofrequency ablation, microwave ablation, and laser ablation as well as cryotherapy. Hyper thermic modalities typically achieve destruction of the tumor by exposing the tissues to cytotoxic temperatures. While hyper-thermic techniques are mostly administered using a percutaneous approach, open or laparoscopic approach is often employed for cryotherapy^[23]. Compared to RFA, Laser and microwave ablation have not been as well studied and are not widely available.

RFA

By generating an alternating electric field within the tissues using a needle electrode, radiofrequency ablation relies on ionic vibrations to generate large amounts of frictional heat, inducing temperatures in excess of 60 C, leading to irreversible cellular damage^[24]. By producing a safety ring within the peri-tumoral tissue, RFA is better able to achieve complete eradication of the primary lesion, as well as micro-satellites located within its proximity. Due to the larger ablation area of up to 3 cm with each application, RFA is also able to achieve complete eradication of the disease, requiring fewer number of electrode insertions, when compared with PEI.

Using RFA, treatment response has been found to correlate best with the size of the lesion; a complete response rate between 80%-90% in tumors up to 3 cm in diameter^[24-27], and 50%-70% in lesions between 3 and 5 cm in diameter^[25,28-31]. Five-year survival rates following RFA were reported as 48%-71% by some studies^[32-34]. As with local tumor control, survival following RFA was also found to correlate best with the size of the lesions. For instance, three-year survival rates for lesions > 5 cm, 2.1 to 5 cm, and ≤ 2 cm have been reported as 59%, 74%, and 91%, respectively by a study comprising 302 patients^[35].

When compared with PEI, significant differences have been observed particularly in terms of local control of the disease, as evidenced by local recurrence rates of only 2%-18% following RFA, as compared to 11%-45% in case of PEI^[36-40].

Like PEI, radiofrequency ablation is indicated in patients with early stage liver-only disease, who are candidates for curative therapy but do not meet the resectability criteria. It has also proven efficacious in the treatment of recurrent HCC lesions following partial hepatectomy^[35,41]. It should however, be avoided in case of lesions located in the dome or the inferior edge of the liver due to the risk of diaphragmatic injury^[42]. It is also advisable to avoid RFA in case of sub-capsular tumors located within 1 cm of the hepatic capsule, due to the risk of needle-track seeding, which was observed in 4 out of 32 patients in a series^[43] [Table 1].

Table 1. Contraindications to radiofrequency ablation. Adapted from reference^[47]

Absolute	Relative
Decompensated liver disease (Child-Pugh C)	Lesions larger than 5 cm in diameter
Proximity to major hepatic ducts	> 3 lesions
Extrahepatic disease	Severe coagulopathy
Altered mentation	Sub-capsular tumors
Active infection	Tumors within the dome of liver

With RFA, severe complications have been thought to occur at a rate of 2.2%-11%, with procedural mortality rates of 0.1%-0.8%. These include fatal, such as liver failure, colon perforation, and Portal Vein Thrombosis as well as non-fatal complications like liver abscesses, pleural effusion, skin burns, hypoxemia, pneumothorax, sub-capsular hematoma and hemo-peritoneum^[30,44-46]. RFA, when employed for the ablation for sub capsular tumors located within 1 cm of the hepatic capsule, can also potentially lead to needle-track seeding, as has been observed in several studies.

LASER ABLATION

Percutaneous laser ablation employs laser fibers inserted directly into the tissues to deliver light energy capable of inducing coagulative necrosis within the malignant tissue. While the volume of necrosis that can be achieved with a single bare laser fiber is 2 cm, a greater area of ablation can be achieved with the use of multiple fibers^[18].

The safety and efficacy of this technique are not as well-documented, and the availability of data comparing its effectiveness with other ablative modalities is limited. A complete response rate of 78% was observed following laser ablation in a study of 432 patients, while the local recurrence rate was found to be 20%, with 3- and 5-year survival rates as high as 61% and 34% respectively^[48-50]. The safety of laser ablation has also been found to be comparable with other percutaneous modalities like RFA with major and minor complication rates of 1.5% and 6.2% respectively, and a mortality rate of 0.8% as reported by an Italian study^[51].

While these results may be encouraging, they do not provide evidence of greater efficacy or a better safety profile over alternate technology that is available at a much cheaper cost and hence, much more readily than percutaneous laser ablation^[52]. These factors have restricted the use of laser ablation mostly to European countries.

MICROWAVE ABLATION

Most commonly used in China and Japan^[53], microwave ablation generates microwaves using implanted electrodes to induce molecular rotation, generating heat which is even being distributed evenly. By doing so, it creates an ablation area in the shape of the needle.

Like laser ablation, data for microwave ablation is also limited, but studies have indicated complete response rates between 89% and 95%, while three and five-year survival rates have been reported as 73% and 57% respectively^[25,54-58]. As seen in case of RFA, survival following microwave ablation was also affected by tumor size, number of nodules and Child-Pugh class.

When compared with RFA, although no significant differences in efficacy were observed, local recurrence and complication rates were found to be lower in case of RFA. Nevertheless, an important advantage favouring microwave ablation (MWA) over RFA is that its effectiveness is not limited by the proximity of the tumor to large vessels. Unlike RFA, MWA can also be used to perform multiple ablations simultaneously, in case of tumors with multiple foci, however this technique is not as widely available.

Table 2. Comparison of overall 3- and 5-year survival rates following resection and radiofrequency ablation. Adapted from reference^[60]

	Resection	Radiofrequency ablation
Number of patients	115	115
3-year survival rate	92.2%	69.6%
5-year survival rate	75.7%	54.8%

RFA VS. SURGICAL RESECTION

Underlying chronic liver disease presents a significant challenge in the treatment of hepatocellular carcinoma. Hepatic failure often complicates surgical resection in cases where hepatic functional reserve is significantly depleted. The decision to avoid surgery and opt for alternate loco-regional ablative procedures in such cases thus seems rather prudent.

Where hepatic function is relatively preserved and lesions are amenable to resection, surgery is still regarded as the mainstay of therapy, although a case can be made to opt for Radiofrequency ablation here in light of the unavoidable risks of the procedure and the hospitalization. Even when performed by highly experienced surgeons, operative mortality rates ranging from 1.6%-10% have been observed in various studies^[59]. Whereas percutaneous radiofrequency ablation is much less invasive, is associated with a lower rate of complications and mortality, and usually involves short hospital stays if needed at all. Unlike resection, it can also be used in cases where HCC arises in the setting of cirrhosis secondary to oncogenic stimuli expected to persist following treatment, such as metabolic conditions like hemochromatosis. Furthermore, RFA as well as PEI may be used as bridging therapies for patients with HCC scheduled to undergo liver transplant.

Unfortunately, studies directed at comparing the efficacy of RFA relative to surgical resection have failed to provide sufficient evidence to support its use in cases where patients may be candidates for both. In fact, some studies have even reported better outcomes, in terms of 3- and 5-year survival rates following surgical resection, as compared to RFA. The results of one such study performed on a cohort of 225 participants fulfilling the Milan criteria have been presented in Table 2 and show significantly higher survival rates for patients following resection^[60].

With careful patient selection and good operative technique, surgical resection has been shown to achieve 5-year and long-term survival rates of 78% and 40% respectively. Such optimal criteria for patient selection include patients with solitary lesions less than 5 cm in diameter, absence of angio-invasion or hepatic metastases, and adequate surgical margins of at least 1 cm. Current guidelines such as AASLD also recommend hepatic resection over RFA for patients with resectable T1 or T2 HCC and Child-Pugh A cirrhosis.

RFA VS. PEI

While PEI has shown to be almost as equally effective as RFA for small tumours, and costs much less since it requires a minimal amount of equipment, its use has largely been restricted to situations where RFA might not be available or for lesions located near the gall bladder, hepatic hilum or major vessels, precluding thermal ablation^[25]. Factors responsible for this may include peri-procedural pain and the need for multiple settings, both of which contribute to non-compliance, as well as higher local recurrence rates in comparison with RFA as observed by various randomized trials and meta-analyses^[61].

While both RFA and PEI have proven their feasibility and applicability in cases where surgical resection is not a viable option, some studies do provide evidence of greater efficacy with the use of RFA, as evidenced by greater 3-year survival rates as well as the lower rate of local recurrence following its use, as summarized in Table 3^[62].

Table 3. Differences in outcome following RFA and PEI, as reported by an Italian study comprising of 271 patients with a single lesions up to 3 cm in size. Adapted from reference^[62]

	RFA	PEI
Number of patients	128	143
3-year survival	83	78
5-year survival	70	68
Average number of sessions	5	8
Rate of major complications	0.9%	1.9%
Recurrence rate at 3 years	7.8	9.4

PEI: percutaneous ethanol injection; RFA: radiofrequency ablation

CONCLUSION

While surgical resection is still considered the standard of care for patients with early stage Hepatocellular carcinoma, percutaneous ablation has emerged as a viable alternative for the management of patients who are poor surgical candidates. Among percutaneous therapies, radiofrequency ablation has now replaced percutaneous ethanol injection as the treatment of choice for patients with BCLC 0-A tumors, not amenable to surgical resection, while ethanol injection is still recommended in cases where RFA is not technically feasible due to the inaccessible location of the lesions.

Radiofrequency ablation has shown the best results when used for smaller tumors, particularly those smaller than 3 cm. While the percutaneous approach is employed most frequently, RFA can be administered via the laparoscopic or open approach as well, preferred in case of lesions located near the inferior edge of the liver, in close proximity to adjacent organs. Other emerging loco-regional procedures like microwave ablation, percutaneous laser ablation and cryotherapy are not as widely available and have not been studied as well.

DECLARATIONS

Authors' contributions

Developed the first draft: Naeem E

Modified and layout the concepts of tables and figure: Abid S

Reviewed the subsequent versions and final draft: Naeem E, Abid S

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Mitoepigenetics and hepatocellular carcinoma

María Guadalupe Lozano-Rosas, Enrique Chávez, Alejandro Rusbel Aparicio-Cadena, Gabriela Velasco-Loyden, Victoria Chagoya de Sánchez

Department of Cellular Biology and Development, Institute of Cellular Physiology, National Autonomous University of Mexico, Mexico City 04510, Mexico.

Correspondence to: Dr. Victoria Chagoya de Sánchez, Department of Cellular Biology and Development, Institute of Cellular Physiology, National Autonomous University of Mexico, Mexico City 04510, Mexico. E-mail: vchagoya@correo.ifc.unam.mx

How to cite this article: Lozano-Rosas MG, Chávez E, Aparicio-Cadena AR, Velasco-Loyden G, Chagoya de Sánchez V. Mitoepigenetics and hepatocellular carcinoma. *Hepatoma Res* 2018;4:19. <http://dx.doi.org/10.20517/2394-5079.2018.48>

Received: 30 Apr 2018 **First Decision:** 21 May 2018 **Revised:** 7 Jun 2018 **Accepted:** 10 Jun 2018 **Published:** 19 Jun 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Mitochondria are the center of energy production in eukaryotic cells and are crucial for several cellular processes. Dysfunctional mitochondria have been associated with cancer progression. Mitochondria contain their own circular DNA (mtDNA), which codes for 13 proteins, 2rRNA, 22tRNA and non-coding RNAs. Recent evidence showed the presence of 5-methylcytosine and 5-hydroxymethylcytosine in mtDNA suggesting that the level of gene expression could be modulated like a nuclear DNA by direct epigenetic modifications. Mitoepigenetics is a bidirectional phenomenon in the epigenetic regulation of mitochondrial genes encoded in both the nucleus and the mitochondrion. This process is affected by SAM-mediated methylation and hydroxymethylation of mtDNA and by nuclear chromatin modulators from mitochondria, such as Acetyl-CoA and NAD⁺. There is some information about physiological and pathological methylated profiles, but information is scarce for hepatocellular carcinoma (HCC). The aim of this review is to summarize the mitoepigenetic knowledge in HCC already reported so far, through a keywords search in Medline. In addition, the deregulation of energy intermediaries needed for the mitoepigenetic regulation is described. As this is a new area of study, a rigorous analysis and careful interpretation and integration of results are needed.

Keywords: Hepatocellular carcinoma, mitochondrial genome, mitochondrial epigenome, microRNAs

INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly malignant cancer, with high recurrence rate and a poor prognosis. HCC is a complex pathology associated with chronic liver disease, 80% to 90% are originated from cirrhosis of diverse etiology, most frequently infections with hepatitis B virus, hepatitis C virus, the mycotoxin, aflatoxin B1, and the metabolic syndrome^[1,2].



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Mitoepigenetics events include the interplay between mitochondrial-derived substrates and the nuclear epigenetic landscape. This includes all epigenetic events that affect the expression of the mitochondrial genome and the nuclear-encoded mitochondrial genes^[3,4]. The importance of mitoepigenetics lies not only in the functions described for this organelle, but also in the generation of intermediaries that serve to regulate the function of other cellular components, as will be mentioned later.

Mitochondrial dysfunction is involved in several diseases including cancer^[5]. The ability of mitochondria to regulate the energetic redox state and the metabolism of cells could result in the production of epigenetic intermediates that participate in normalization of the mitochondrial function. Studies have revealed several metabolic alterations in liver diseases including modification in energy supply^[6,7]. A sequential model of cirrhosis-HCC induced by diethylnitrosamine (DEN) revealed that cancer progression is associated with mitochondrial dysfunction^[8].

Multiple insults to the mitochondrial genome have been associated with different pathophysiologies, and have been described as one of the most common and consistent phenotypes of cancers^[9-13].

Mitochondria are vital for the cell because they are responsible for its metabolic activity, as well as for producing the bulk of the energy requirements in the form of ATP, maintaining calcium homeostasis, and inducing apoptosis^[14,15]. In mitochondria, ATP is generated through the process of oxidative phosphorylation (OXPHOS), which occurs via the electron transport chain (ETC). Mitochondria contain their own genome (mitochondrial DNA, mtDNA). Each organelle contains about 1-10 copies of mtDNA^[16]. MtDNA is distinctly different from the nuclear DNA (nDNA), the mtDNA is a circular, double-stranded DNA molecule of approximately 16.6 kb in size and it is inherited only through the mother. MtDNA is found associated and packed with proteins in a nucleoid, where an encoded nuclear protein known as mitochondrial transcription factor A (TFAM) is the major protein component^[17]. The mtDNA comprises a heavy (H) strand and a light (L) strand, which encode 13 of the polypeptides that constitute the complexes I, III, IV, and V of the ETC. MtDNA also encodes some of its own transcriptional and translational machinery, which includes 22 tRNAs and 2 rRNAs^[12,13,18]. The rest of the mitochondrial proteins (~1500), involved in the mitochondrial function, replication, transcription and translation of mtDNA, are encoded by nuclear genes and are targeted to the mitochondrion by a specific transport system^[19].

METHODS

A bibliographic search was performed of the Medline database (US National Library of Medicine, <http://www.ncbi.nlm.nih.gov>). The keywords used or combinations of them were: cancer, hepatocellular carcinoma, epigenetics, mitoepigenetics, mitochondria, methylation, hydroxymethylation and miRNA. All the articles that included the terms and/or combinations referring to the metabolic regulation, as well as to mitoepigenetics in HCC were selected.

EPIGENOME SUBSTRATES GENERATED BY MITOCHONDRIA

Cellular growth and replication depends on the energetic state through epigenetic modifications in the DNA chromatin structure^[20]. This is achieved by coupling modulation of nDNA chromatin structure and function by modification via high energy intermediates: phosphorylation by ATP, acetylation by acetyl-coenzyme A (Ac-CoA), deacetylation by nicotinamide adenine dinucleotide (NAD⁺), and methylation by S-adenosyl-methionine (SAM)^[20]. As afore mentioned, the mitochondrion is responsible for ATP production as part of the energetic metabolism. However, the role of ATP is not just to be the main energy provider, but it also regulates multiple cellular functions through phosphorylation and dephosphorylation reactions (when there are low levels of ATP), as part of what is known as post-translational modifications.

Glucose is the main source of Ac-CoA and is the link between glycolysis and the tricarboxylic acid cycle (TCA). In addition to its energetic role, the Ac-CoA is the substrate for acetylation reactions that are important to modulate gene expression and the function of some proteins^[21]. Acetylation of mitochondrial substrates is controlled by a NAD⁺-dependent deacetylase sirtuin-3 (Sirt-3). Therefore, the mitochondrial redox state must be controlled to maintain the NAD⁺ availability for the Sirt-3 activity.

On the other hand, SAM is the physiological methyl donor group synthesized from L-methionine and ATP in a reaction catalyzed by the methionine adenosyltransferase (MAT) enzyme^[22]. SAM is synthesized in the cytosol and imported to the mitochondrial matrix via the mitochondrial SAM carrier, likely via exchange for its metabolized variant S-adenosyl-homocysteine (SAH)^[23]. SAM synthesis is regulated in part by the mitochondrial one-carbon (folate) metabolism^[24]. An enzyme that participates in mitochondrial folate metabolism, the mitochondrial bifunctional enzyme (MBE), regulates the change between SAM and the nucleotide synthesis. In proliferative cells, such as embryonic or cancer cells, MBE is expressed, and the one-carbon units are shuttled predominantly towards nucleotide synthesis. Under these conditions, less one-carbon units are available for SAM synthesis and DNA methylation. Conversely, in differentiated cells, MBE is turned off, less mitochondria toward nucleotides synthesis are produced, and one-carbon units are directed through increased SAM synthesis and increased DNA methylation^[25]. SAM is important for DNA epigenetic, methylation of phospholipids^[26], and proteins; thus, modulating relevant cellular functions. For example, the relationship between methylation and mitochondrial dysfunction being so close that deficiency of SAM may lead to mitochondrial damage and, finally, to insulin resistance^[27].

PRINCIPAL MITOEPIGENETICS PROCESSES

mtDNA methylation and hydroxymethylation

DNA methylation is an epigenetic modification of the DNA that is frequently disrupted in nearly all types of cancer. Hypomethylation of the repetitive elements associated with increased genomic instability is frequently observed in cancer cells^[28]. The hypermethylation of specific CpG islands in promoter regions of several tumor-suppressor genes is commonly observed to be associated with transcriptional silencing of the gene^[29,30]. Epigenetic regulation of the mitochondrial genome was an enigma, until recent studies^[31]. For example, there is no evidence of post-translational modifications of TFAM as it happens in nuclear DNA histones, and the most important epigenetic regulation of mtDNA is DNA methylation and hydroxymethylation. DNA methylation is regulated by four DNA methyltransferases (DNMT1, 3A, 3B, and 3L) and three demethylases, that is, ten-eleven translocases (TET1-3)^[32].

The mtDNA methylation is accomplished by the mitochondrial DNA methyltransferase (mtDNMT1), a nuclear encoded DNMT1 that contains a mitochondrial targeting sequence^[33]. Methylation of nDNA occurs principally in cytosines (5mC) of CpG dinucleotides, but recently it has been shown that mtDNA methylation is found predominantly in non-CpG sites and that it is DNMT independent^[4]. In general, mtDNA is undermethylated, with only 1% to 5% of methylated cytosines. Several factors increase mtDNMT1 transcription and translocation to the mitochondria, like p53, oxidative stress-responding transcription factors, nuclear respiratory factor 1 (NRF1), peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1 α), and p16 cell cycle inhibitor^[33]. The methyltransferase DNMT3a may also be involved in the methylation of mtDNA since it has been found in mitochondrial fractions from mouse cell lines and from the human central nervous system^[32]. In 2011, Shock *et al.*^[33] reported the presence of 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) in mammalian mtDNA, further demonstrating the translocation of methyltransferase 1 (DNMT1) to mitochondria. Alterations in mtDNMT expression affect transcripts of the heavy and light strands of mtDNA. The modulation of mtDNA methylation has been studied in response to oxidative stress, where there seems to be a decrease in this modulation^[34], this response can be a compensatory response to mtDNA damage by increasing the expression of residual mtDNA genes. The

DNMT enzymes use SAM as the methyl donor. However, the importance of mtDNA in methylation not only lies in the mitochondrial function but it can modify the overall epigenetic state of the cell. In fact, it has been observed that the decrease of mtDNA leads to altered levels of methylation in the genomic DNA, which is normalized once the mtDNA content is reestablished^[33]. In 2013, Bellizzi *et al.*^[35] studied the methylation patterns in mitochondrial cytosines in humans and mice and the effect of suppressing DNMT1, DNMT3a, and 3b. In general, a preponderant methylation of CpG dinucleotides and its inhibition was found in knockout mice without affecting methylation in non-CpG sites^[35].

The DNA methylation pattern of the human mitochondrial genome remains relatively constant; however, there are some loci that are differentially methylated in different tissues and over time^[36]. For example, subunit 6 of NADH dehydrogenase, a crucial subunit for the assembly of complex I, is suppressed due to hypermethylation by an increase in the expression of DNMT1^[33]. It is also known that the 12S rRNA gene is methylated by the rRNA methyltransferase-related transcription factor 1 (mt-TFB1)^[37]. This epigenetic regulation is important for ribosomal biogenesis and mitochondrial translation and has been related to aging; this modification alters the efficiency of the ETC by hampering the assembly of complex I^[38]. In 2016, Liu *et al.*^[39] determined methylation of the human mitochondrial genome in blood and saliva samples; by bisulfite pyrosequencing, 9 regions in human mtDNA were detected including a D-loop, 12S rRNA, 16S rRNA, ND1, COXI, ND3, ND4, ND5, CYTB.

Hydroxymethylation is another important epigenetic modification described for mtDNA, where 5mC is oxidized into 5-hmC by the TET family of methylcytosine dioxygenases. The presence of TET1 and TET2 has been described in neuronal mitochondrial fractions^[32,40]. TET proteins are members of the family of 2-oxoglutarate-dependent dioxygenases (2-OGDO) that can oxidize 5mC to generate 5hmC, 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), mediating DNA demethylation by oxidation in cooperation with the BER repair pathway^[40]. The reaction is dependent on the presence of oxygen, 2-oxoglutarate, and Fe²⁺. 2-Oxoglutarate is a key metabolite in the Krebs cycle that occurs in the matrix of mitochondria; on the other hand, succinate and fumarate, also TCA intermediates, are potent inhibitors of 2-OGDO enzymes, in this manner TCA cycle controls the DNA and histone methylation and, thus, controls gene expression^[40].

The kind of hydroxymethylation of cytosines in mitochondria has also been reported^[33]. The profile of modifications in the D-loop region (a non-coding region that acts as a promoter for the H and L strands of the mtDNA and contains transcription and replication elements), and the similarity of this profile in cells of similar tissues and how the profile is different in cells of different tissues have been described recently^[41].

Given the relevance of mitochondrial DNA and its regulation at different levels, recent studies have also proposed mitochondrial DNA methylation as a potential biomarker^[42].

The central role of microRNAs in modulating mitochondria

Other regulators that have recently been studied in mitochondria are microRNAs (miRNAs)^[43]. miRNAs are small non-coding RNAs, implicated in gene post-transcriptional regulation and the conformation of genetic expression patterns with physiological relevance. They derive from longer RNAs, primary miRNAs (pri-miRNAs), and are sequentially cleaved by ribonuclease III (RNase III) enzymes or processed for pre-miRNA splicing and RNA degradation pathways^[44].

It has been found that both pre-miRNAs and mature miRNAs can be found in mitochondria, suggesting that this organelle can synthesize them and keep them active in their transcriptional machinery or export them to the cytosol. Likewise, the possibility arises that the miRNAs processed in mitochondria regulate the expression of genes related to the function of the same organelle^[45]. On the other hand, to mention some examples, miR-181c-5p regulates mitochondrial energy metabolism through mt-COX1 mRNA; although

its origin is nuclear it can be exported to the mitochondria and execute its effect^[46,47]. In addition, miR-499 regulates mitochondrial dynamics through mitochondrial fission protein and apoptosis^[48].

Epigenetic regulation of nuclear-encoded mitochondrial genes

Mitoepigenetics includes the regulation of nuclear-encoded mitochondrial genes as previously mentioned in its definition; importantly, two of the transcription factors that carried out this modulation are: PGC-1 α and NRF-1. However, as we describe in this section, little is known about its epigenetic regulation in HCC, then, it represents an area of study to explore.

PGC-1 α

PGC-1 α is critical for the expression of genes involved in fatty acid oxidation, as well as in mitochondrial gene expression through the coactivation of major transcription factors, controlling the complex program of mitochondrial biogenesis^[49]. It has been suggested that biogenesis induced by PGC-1 α is tumor promoting^[50]. Sirtuin-1 (Sirt-1), a NAD⁺-dependent deacetylase, targets several transcription factors, like PGC-1 α , both proteins have been found overexpressed in HCC and are related to defective mitochondrial accumulation^[51]. Non-CpG methylation of the PGC-1 α promoter controls mitochondrial density and has been detected in pathological conditions such as obesity^[52]. It may be interesting to obtain an epigenetic PGC-1 α pattern in HCC to know if its modulation is carcinogenesis stage-dependent and to establish the accurate way to be pharmacologically controlled.

NRF-1

NRF-1 binds to the cytochrome C promoter and positively regulates nuclear-encoded mitochondrial genes^[53]; low-levels of NRF-1 cause mitochondrial dysfunction. The NRF-1 gene sequence has several CpG islands which are susceptible to methylation and demethylation processes; it has been shown that hypermethylation of the promoter region of NRF-1 causes a decrease in its expression^[54]. Further studies of the epigenetic regulation of NRF-1 in HCC are needed.

MITOPIGENETICS IN HCC

One of the most and consistent phenotypes of cancer are defective mitochondria. There is evidence that the loss of mitochondrial function and epigenetic alterations in this organelle are related to the process of carcinogenesis because of their vital role in energy production and contribution to the metabolism of epigenome effectors^[1,55,56]. Mitochondrial dysfunctions also lead to resistance to apoptosis. Since Warburg's hypothesis, a number of mitochondrial abnormalities in cancers, both at the genetic and metabolic levels, have been reported^[8,9,11].

Moreover, progressive mitochondrial dysfunction has been linked to an enormous variety of diseases, such as mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes syndrome (MELAS), Leber's hereditary optic neuropathy (LHON), deafness, diabetes, Alzheimer, and Parkinson disease^[57,58].

Alterations observed in the epigenetic substrates in HCC

As previously reported, metabolic alterations have been found in liver diseases^[6,7] and the HCC is not the exception; the metabolic reprogramming that happens in cancer cells implies a decrease in ATP^[8,59]. However, some studies have not found significant changes in acetyl-CoA levels that normally depend on energy metabolism, but which, in HCC, may have a nuclear origin and play a fundamental role in the progression of the cell cycle and DNA replication^[8,60].

We have mentioned that the mitochondrial redox state is fundamental for the proper functioning of mitochondria; with respect to HCC, it is known that the NAD⁺/NADH ratio decreases significantly^[8], which could modify the activity of enzymes that depend on the cellular redox state. On the other hand, currently a

change in the levels of an enzyme called MAT that catalyzes the formation of SAM has been implicated also. MAT is encoded by two genes, *Mat1a* and *Mat2a*. In HCC, the liver decreases the amount of MAT1A and increases MAT2A through epigenetic mechanisms alone; this switch is responsible for the decreasing level of SAM, favoring the development of this pathology^[61,62].

Due to the above, it is necessary not only to consider the epigenetic modifications but also the generation of intermediaries by mitochondria that allow for the appropriate epigenetics control of both the mtDNA and the nDNA.

Altered mitoeptigenetics in HCC

As afore mentioned, NRF1 and PGC1- α act on nuclear genes encoding respiratory subunits from ETC and are involved in the transcription and replication machinery. An up-regulation of this protein in HCC has been demonstrated^[8,63]. An increased PGC1 α level has been suggested to be an important inducer for the accumulation of dysfunctional mitochondria^[8]. Although the role of *pgc1- α* and *nrf1* genes methylation has not been studied in HCC, this is an interesting area to be investigated. A meta-analysis of DNA methylation in HCC revealed a correlation between several aberrant methylated genes and the risk of HCC, among them p53 was hypermethylated in HCC tumor tissue compared to the adjacent tissue. It is important to consider that, in turn, this gene is implicated in the transcription and translocation of mtDNMT1 to mitochondria, and, in this way, besides of its role as tumor suppressor it could be modulating the methylation status of mitochondrial genes^[64].

Under conditions of oxidative stress, which may be a factor for the development of HCC, the transcriptional and mitochondrial DNA replication machinery is altered. Consequently, the ETC loses its functionality and favors the accumulation of reactive oxygen species (ROS). In addition, the mtDNA may suffer injuries because of the accumulation ROS^[1,65]. On the other hand, the mitochondrial damage observed in cancer cells can have consequences on the expression of nuclear genes. There are studies that indicate that the removal of mtDNA responds to changes in the nuclear genome^[56,66].

A genome-wide mapping of DNA methylation and hydroxymethylation in a study on HBV-related HCC revealed that the metabolic pathways that include glycolysis, gluconeogenesis, oxidative phosphorylation, and TCA contained the largest number of (hydroxy) methylation-altered genes, indicating the crucial roles of metabolic processes that implicate mitochondria in the progression of HCC, which, in turn, are regulated by epigenetic mechanisms. The authors propose that some of the identified (hydroxy) methylation-altered genes may serve as biomarkers for the diagnosis and prognosis of HCC^[67]. Among the 5-mC and 5-hmC altered genes related to OXPHOS were the following: NADH dehydrogenase [ubiquinone] 1 subunit C2 (NDUFC2), NADH dehydrogenase [ubiquinone] flavoprotein 1 (NDUFB1), NADH: ubiquinone oxidoreductase subunit S6 (NDUFS6) from complex 1 and succinate dehydrogenase complex flavoprotein subunit A (SDHA) from complex II. Among the TCA genes: succinyl-CoA ligase [GDP-forming] subunit beta (SUCLG2) and pyruvate carboxylase.

Also, in HBV-induced hepatic carcinogenesis, protein X (HBx), encoded by the virus, has been proposed as an epigenetic regulator for tumor suppressor genes, by hypermethylation. It has been suggested that, in hepatomas, NQO1 (NAD(P)H: quinone oxidoreductase 1), which is a cytosolic protein that catalyzes two-electron reduction, can be deregulated by induction of HBx, generating mitochondrial damage and increasing oxidant stress in cells through hypermethylation of the NQO1 promoter^[68].

Specifically in mitochondria, some epigenetic modifications have been described in HCC, such as hypermethylation of the *Mrps12* (mitochondrial ribosomal protein S12), *Mgrap* (mitochondria-localized glutamic acid-rich protein), and *Tmem70* (transmembrane protein 70) genes^[69]. The TMEM70 protein,

encoded by the *Tmem70* gene, is a protein of the mitochondrial inner membrane that participates in mitochondrial biogenesis and whose mutations can be associated with the deficiency in the synthesis of ATP^[70,71]. MRPS12 is a mitochondrial conserved protein^[72] and MGRAP is an important protein for the maintenance of mitochondrial morphology and quantity, as well as for the process of steroidogenesis^[73].

Enzymes that catalyze acetylation, methylation, or their loss also regulate epigenetic changes^[74,75]. For example, the enzyme LSD1 (lysine-specific demethylase 1) uses the mitochondrial cofactor FAD to carry out the demethylation of modified histones such as H3K4me1 and H3K4me2^[76].

LSD1 has been proposed as a regulator of cell proliferation in several cancer types, as well as its metabolic reprogramming^[77,78]. In HCC, it has been determined that LSD1 regulates energy production and suppresses mitochondrial respiration^[78]. These studies also determined that the demethylase activity by LSD1 represses mitochondrial metabolism genes and induces the expression of glycolytic genes^[78].

In HCC, differential expression of different miRNAs has been observed, as well as epigenetic modifications^[79].

miR-122 regulation of PGC-1 α (peroxisome proliferator activated receptor gamma, coactivator 1 alpha) and SDH (succinate dehydrogenase) subunits A and B is necessary for mitochondrial metabolism. In HCC, this miRNA is scarcely expressed. Studies propose this microRNA as a tumor suppressor, since in primary HCC tumors, in both human and rodents, it is seen at low levels, compared to its healthy controls. Also, in HBV-infected patients, this miRNA is reduced in hepatic tissue^[45,80,81]. miR-122 is implicated in the control of lipid metabolism and circadian regulation in the liver. This microRNA has been observed in steatohepatitis and liver fibrosis, in addition to HCC^[80,82]. Experimentally, the genetic deletion of miR-122 in mice has important effects on lipid metabolism, as well as on the progression of liver disease, from microsteatosis to HCC^[80,82].

miR-33a/b regulates lipid metabolism through the ABCA1 cholesterol transporter. Its overexpression inhibits the oxidation of fatty acids in the liver cancer cell line HuH7, favoring the accumulation of triglycerides in larger lipid droplets. MiR-33 binding sites have been identified in the 3'UTR of genes for mitochondrial proteins such as carnitine O-octaniltransferase (CROT) and carnitine palmitoyltransferase 1A (CPT1a). This miRNA inhibits also the insulin receptor substrate 2 (ISR2) and regulates insulin signaling^[83,84]. There is very little information about the role of this miRNA in HCC, so we suggest that this could be studied more extensively to determine its importance in this pathology.

On the other hand, the role of mitochondrial genetic alterations has been investigated in HCC, and tumors contained significantly reduced mtDNA and TFAM overexpression, although neither condition correlated with the degree of cell differentiation; TFAM expression correlated with tumor size^[85].

For all the above, the field of mitoeugenetics has attracted research with the aim of having more effective therapeutic targets in the treatment of HCC.

Mitoeugenetics in tumor-initiating stem-like cells in HCC

The liver cancer, as other epithelial cancers, has tumor-initiating stem-like cells (TICs) that are implicated in tumorigenesis and drug resistance^[86]. TICs share some characteristics with embryonic stem cells (ESC) including expression of the pluripotency transcription factors, NANOG, OCT4, MYC, and SOX2. The expression of pluripotency transcription factors contributes to cancer progression by reprogramming mitochondrial metabolism^[87,88].

NANOG represses OXPHOS genes, prevents mitochondrial ROS production, and activates fatty acid oxidation (FAO), contributing to TIC self-renewal and drug resistance^[87]. Low levels of ROS are necessary to preserve

stemness and self-renewal characteristics in TICs and protect them from drug-induced cell death. Elevated levels of ROS inhibit stemness genes by activation of the p38 MAPK pathway leading to polycomb suppressor protein complex I (BMI) protein degradation and FOXO3 activation. NANOG also acts synergistically with p53 inactivation and b-catenin activation to reprogram cellular metabolic pathways, since p53 promotes glycolysis and OXPHOS^[88]. Other pluripotency transcription factors that are being studied that contribute to TICs metabolism reprogramming are MYC and OCT4. MYC regulates the glutaminolysis and glycolysis pathways and OCT4 also regulates OXPHOS^[29,88].

MtDNA copy number has a very important role in tumorigenesis. Depending on the cancer types, the mtDNA copy number varies, an increase in prostate and endometrial cancer has been reported, whereas a decrease has been shown in HCC and gastric cancer^[89]. Cancer stem like cells have a low mtDNA copy number that promotes their high proliferation rate and shifts their energy production by glycolysis. This low mtDNA copy number downregulates the expression of the catalytic subunit of the mitochondrial-specific polymerase POLGA by hypermethylation at exon 2^[89]. The reduced expression of POLGA is necessary to maintain pluripotency of cancer stem like cells. Yamada *et al.*^[90] reported a reduced copy number of mtDNA in patients with HCC, which correlated with malignant potential.

The removal of mtDNA from cells in culture induces alterations in nDNA methylation. For example, the content of 5mC in the genomic DNA of HCC (tumor tissue) is negatively correlated with the content of mtDNA^[91]. These changes are reversible upon re-establishment of mtDNA^[56]. One possible mechanism is that the expression of the enzyme DNMT1, crucial in DNA methylation, is dependent on the copy number of mtDNA^[92].

miR122 is the most important miRNA in adult healthy liver and is associated with liver stem cells differentiation towards hepatocytes. In HCC, miR122 expression is lost. When miR122 expression is reestablished in a stem-like cell line derived from human HCC (BCLC9 cells), it decreases cell proliferation rate and reduces tumor size *in vivo*. This effect is achieved by down-regulating MYC, KLF4, FOXM1, AKT2, and AKT3 and up-regulating FOXO1 and FOXO3A gene expression^[93].

POTENTIAL VALUE OF MITOPIGENETICS AS BIOMARKERS FOR CANCER DIAGNOSIS AND THERAPY

The main problem of HCC is the absence of early detection and effective therapies. The Asian Pacific Association for the Study of the Liver has recommended the use of alpha-fetoprotein (AFP) as a diagnostic biomarker for early detection of HCC complemented with orthodox imaging-based tools^[94]. There are other candidates with clinical value for early HCC diagnosis; in this regard, glycoforms of AFP, des-γ-carboxyprothrombin, glypican-3, cytokeratin 19, annexin A2, and circulating miRNAs have been proposed, among others, to be used alone or in combination^[95].

Cancer metabolic reprogramming regulated by mitochondrial enzymes is now one of the hallmarks of cancer. Tumor cells can acquire functional mtDNA from healthy cells to restore respiratory function and metabolic activity, which enabled them to proliferate^[96].

The mtDNA acts as a critical message to travel and communicate between tumor cells and neighbor non-tumor cells. The outcome of mtDNA horizontal transfer could induce chemoresistance in the treatment^[97].

In addition to the above mentioned, the mitochondrial cellular content and mutations have also been suggested as novel molecular markers^[98]. Moreover, reduced expression of OXPHOS complexes has been

associated with various form of cancer, including HCC. Carcinogenesis is a complex process that can be accompanied by epigenetic modifications. According to what has been described in this review, the epigenetic regulation of carcinogenesis can be used not only as a biomarker of cancer, but also to determine the stage of the carcinogenic process, because epigenetic patterns may be associated. Although there is very little information about mitoepigenetics in HCC, there are data that may be promising as a biomarker and even as a therapeutic target. The mtDNA heterogeneity epigenetics should be investigated by measuring single-cell DNA sequencing, comprehensive characterizations of mtDNA, and bidirectional effects between mtDNA and 3D genome, instability, and gene editing. It would be more helpful to combine the single-cell biology of CRIPRS to mtDNA function, given that copy number changes can also be regarded as biomarkers in cancer diagnosis and treatment^[99-102].

Ye *et al.*^[67] reported the methylation and hydroxymethylation profile of DNA in HCC related to HBV; they found some hypermethylated genes associated with metabolic pathways. Of these genes, the *pc* gene that codes for the enzyme pyruvate decarboxylase (PC) should be considered. PC is a nuclear-encoded mitochondrial enzyme involved in gluconeogenesis, it catalyzes the conversion of pyruvate to oxaloacetate in an adenosine triphosphate (ATP)-dependent form. Being a liver-specific enzyme, the hypermethylation of this gene could provide specificity as a biomarker of HCC.

Further studies are needed to find correlations between mtDNA methylation patterns and HCC in such a way as to get diagnostic tools through non-invasive techniques. Let us recall the work of Liu *et al.*^[39], in which the detection of mtDNA methylation was below 2% in blood and saliva. However, there are other mitoepigenetic parameters in which significant correlations have been found with HCC and that point them out as potential biomarkers, such is the case of miR-122 that has been considered a molecule with great potential for diagnosis, prognosis of liver disease, and therapy. Studies demonstrated that miR-122 is reduced in rodent and human primary HCC^[103]. Being it a miRNA that regulates hepatic homeostasis and having been found under-regulated in diseases. such as HCC, it becomes a possible biomarker and possible therapeutic target^[104].

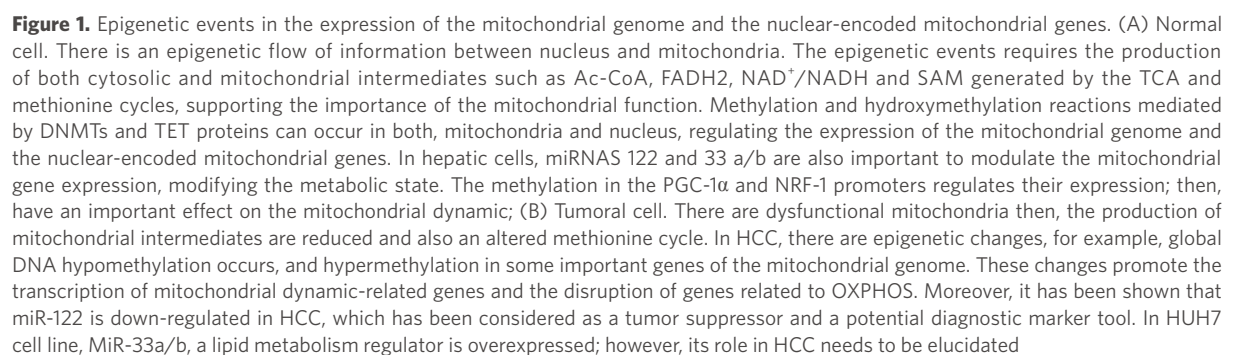
FINAL REMARKS

This review supports the suggestion that disrupted mitoepigenetics may contribute to tumorigenesis. The next generation experiments may elucidate the functional implications of mtDNA methylation and hydroxymethylation and could help clarify the role of these epigenetic markers.

The role of mtDNA is pluripotent, because it can affect processes like cellular differentiation, energy production, oxidative stress, metabolism, inflammation, and carcinogenesis. However, most of these emerging evidences could be modulated, at least in part, through changes in mitochondria, they could offer also a new opportunity to understand the causality of cancer [Figure 1].

The studies of the role of mitoepigenetics modifications and the metabolic processes in the pathogenesis of HCC could be a relevant advancement in the diagnosis and future therapy for this and other types of cancer.

Given the impact of mitochondrial biology and genome, the mitoepigenetics field offers a new opportunity to understand mitochondrial diseases and others that are not known as mitochondrial diseases. In addition, the mitochondrial epigenome also provides new clues for possible therapeutic targets and favors the appearance of new pharmacological options.



DECLARATIONS

Authors' contributions

All authors contributed actively in the search of useful information and participated in the writing of this review.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by Consejo Nacional de Ciencia y Tecnología (240315) and Dirección General de Asuntos del Personal Académico-Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (DGAPA-PAPIIT) (IN208915).

Conflicts of interest

All authors declare that they have no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Intrahepatic cholangiocarcinoma: review and update

Vincenzo Cardinale^{1*}, Maria Consiglia Bragazzi^{1*}, Guido Carpino², Sabina Di Matteo³, Diletta Overi⁴, Lorenzo Nevi³, Eugenio Gaudio⁴, Domenico Alvaro³

¹Department of Medico-Surgical Sciences and Biotechnologies, Polo Pontino, Sapienza University of Rome, Rome 00198, Italy.

²Department of Movement, Human and Health Sciences, Division of Health Sciences, University of Rome "Foro Italico", Rome 00100, Italy.

³Department of Medicine and Medical Specialties, Sapienza University of Rome, Rome 00198, Italy.

⁴Division of Human Anatomy, Department of Anatomical, Histological, Forensic Medicine and Orthopedics Sciences, Sapienza University of Rome, Rome 00198, Italy.

*Authors contributed equally.

Correspondence to: Prof. Domenico Alvaro, Department of Medicine and Medical Specialties, Sapienza University of Rome, Rome 00198, Italy. E-mail: domencio.alvaro@uniroma1.it

How to cite this article: Cardinale V, Bragazzi MC, Carpino G, Di Matteo S, Overi D, Nevi L, Gaudio E, Alvaro D. Intrahepatic cholangiocarcinoma: review and update. *Hepatoma Res* 2018;4:20. <http://dx.doi.org/10.20517/2394-5079.2018.46>

Received: 30 Apr 2018 **First Decision:** 21 May 2018 **Revised:** 1 Jun 2018 **Accepted:** 1 Jun 2018 **Published:** 19 Jun 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Cholangiocarcinoma (CCA) is a heterogeneous group of malignancies that could develop at any level from the biliary tree. CCA is currently classified into intrahepatic (iCCA), perihilar and distal on the basis of its anatomical location. Of note, these three CCA subtypes have common features but also important inter-tumor and intra-tumor differences that can affect the pathogenesis and outcome. A unique feature of iCCA is that it recognizes as origin tissues, the hepatic parenchyma or large intrahepatic and extrahepatic bile ducts, which are furnished by two distinct stem cell niches, the canals of Hering and the peribiliary glands, respectively. The complexity of iCCA pathogenesis highlights the need of a multidisciplinary, translational and systemic approach to this malignancy. This review will focus on the advances of iCCA epidemiology, histo-morphology, risk factors, molecular pathogenesis, revealing the existence of multiple subsets of iCCA.

Keywords: Cholangiocarcinoma, classifications, inflammation, cells of origin, stem cells, molecular profiling

INTRODUCTION

Cholangiocarcinoma (CCA) is a heterogeneous group of malignancies emerging at any level from the biliary tree^[1-3] [Figure 1]. CCA is classified into intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) based on its anatomical location^[1-3]. Of note, these three CCA subtypes have common features but also important inter-tumor and intra-tumor differences that can affect the pathogenesis and outcome^[4-9]. The complexity



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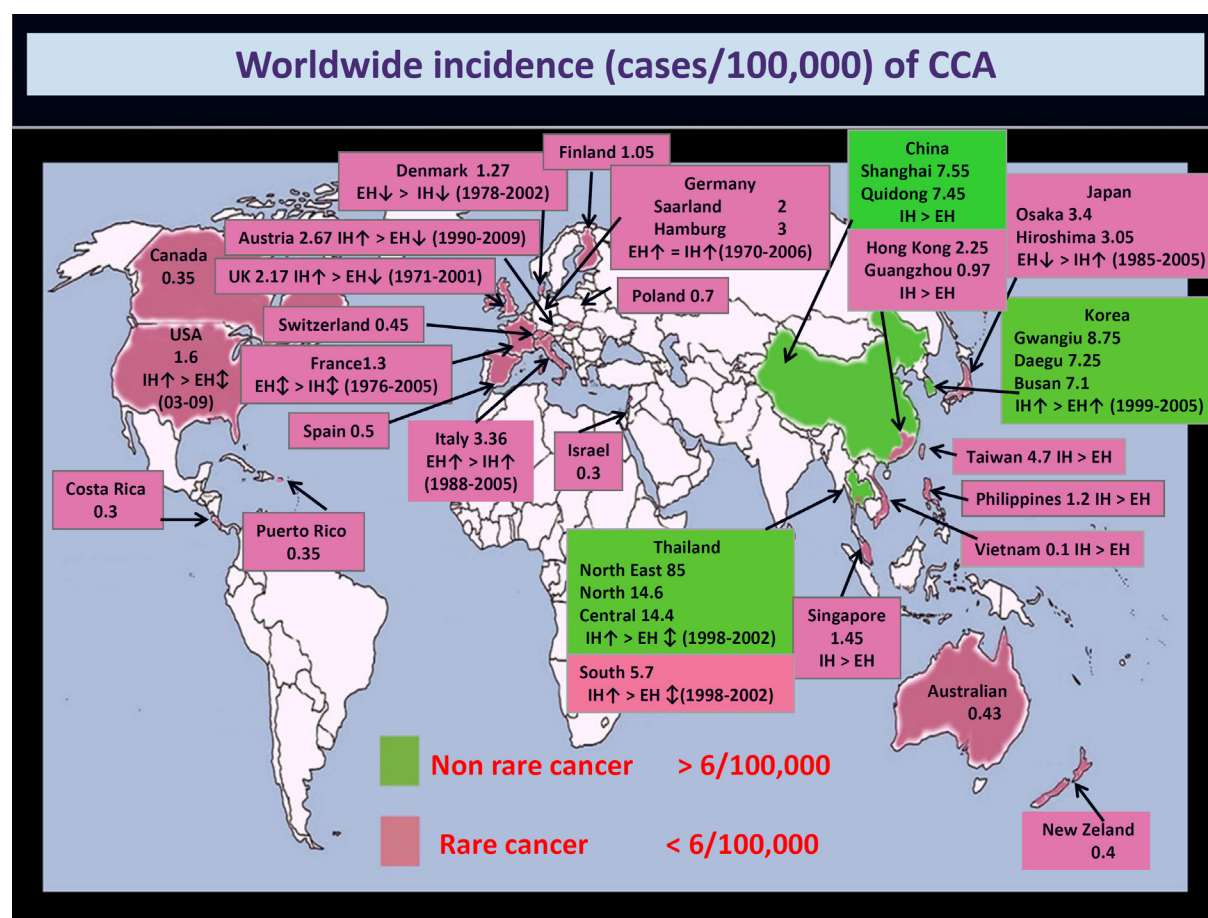


Figure 1. Worldwide incidence (cases/100,000) of cholangiocarcinoma (CCA). Data refer to the period 1971-2009. Green colour identifies areas with lower incidence (< 6/100,000 cases, rare cancer), while pink colour indicates countries where CCA is not a rare cancer (> 6/100,000 cases). Diagnoses have been classified according to the International Classification of Diseases (ICD-O-1, ICD-O-2, ICD-O-3, ICD-10, ICD-V9, ICD-V10, ICD-O). Where available, the more incident form [intrahepatic (IH) vs. extrahepatic (EH) CCA] and the temporal trend of incidence (↑increasing trend; ↓stable trend; ↓decreasing trend) have been reported. This figure was modified from Banales *et al.*^[3] with permission

of the pathogenesis and the pronounced heterogeneity affected in particularly iCCAs had impeded clinical goals in iCCA^[10]. This review will focus on the advances of iCCA epidemiology, classifications and histomorphology, risk factors, molecular pathogenesis and clinical presentation revealing the existence of multiple subtypes of iCCA.

THE BURDEN OF iCCA

The epidemiologic trend of CCA shows a constant and dramatic increase in incidence and mortality worldwide^[1-3], clearly depicting CCA relevance among others types of cancer. A progressive increase in intrahepatic CCA incidence was reported, while the incidences of both perihilar CCA and distal CCA seem to be stable^[1-3]. The incidence of CCA in European countries ranges from 1 to more than 4 cases/100,000^[1-3] [Figure 1]. However, the difficulties with classification coding for CCA, and with the various terminology that is used, determined an underestimation of CCA burden. In a recent report, the four ICD-10 (International Classification of Diseases) sub codes were agreed on for CCA and used^[11]. This report showed that in England alone (not the whole of the UK), in 2013, 1965 new CCAs were diagnosed with an incidence rate of 3.65 per 100,000 population, while, 2161 deaths and a mortality rate of 4.01 per 100,000 population were registered. The number of deaths per 100,000 population for the CCA in the period from 2010 to 2013 in England tragically surpassed the ones for the hepatocellular carcinoma (HCC), with 7743 vs. 6899 deaths in 2013 for

CCA and HCC respectively^[11]. The trend in iCCA incidence is paralleled also by the fact that mortality for primary liver cancer has become more uniform across Europe over recent years with an evident decline of HCC mortality, but, in contrast, intrahepatic CCA mortality has substantially increased for the most part of Europe^[12,13]. Over recent years intrahepatic CCA accounted for over a fourth of all liver cancer deaths in men and 50% in women^[12]. Liver cancer mortality rates are expected to rise by 58% in the UK between 2014 and 2035, i.e., to 16 deaths per 100,000 people by 2035^[14]. Considering epidemiology trend in primary liver cancer, half of deaths for primary liver cancer will be determined by intrahepatic CCA^[12-14]. Furthermore, when the mortality rates for all malignancies are considered, the untargeted problem of CCA emerged clearly. Indeed, while a reduction of the mortality rate from 19 malignancies (comprising breast, lung, colon, *etc.*) was shown from 1990 to 2009 (US data), the mortality rate for malignancies of liver and bile ducts increased by more than 40% and 60% in females and males, respectively^[15]. Finally, it is noteworthy to mention that CCA is the most frequent cause of metastasis of unknown origin, and thus further highlights how we still do not know the real burden of CCA^[16].

NEW INSIGHTS INTO iCCA CLASSIFICATIONS

A huge number of different classifications have been proposed for CCA^[1-10,17]. The most updated one, but still discussed, identify on the basis of the anatomical localization the iCCA, the pCCA and the dCCA^[1-3]. However, being a topographic classification it suffers several pitfalls, and, of course, it does not reflect different biological features. Firstly, it should be noted that, the diagnosis of CCA frequently occurs at an advanced stage, where, the differentiation between the intra-hepatic or extra-hepatic location results is very difficult, and sometimes impossible^[1-3]. Since, small bile ducts and ductules are also present in the perihilar liver parenchyma, then, pCCA as iCCA, may originate either from these smaller ducts and this cannot be discriminated based on gross morphology. Similarly, the iCCA may originate from larger or smaller portion of intrahepatic biliary tree. Third, recent studies demonstrated how, from a pathological and molecular point of view, differences between pCCA and the iCCA originated from larger bile ducts ceased to exist and, therefore, the distinction between these two forms of CCA is losing relevance^[4,9]. Taking into consideration the macroscopic pattern of growth, iCCA has been classified in mass-forming (MF), periductal infiltrating (PI), and intraductal growing (IG)^[2,3]. As far as pCCA and dCCA are concerned, either a PI or IG pattern has been recognized. For pCCA a nodular + PI growth pattern predominates (> 80%)^[2,5,17,18].

On the histological level, while, the vast majority of pCCA and dCCA are mucinous adenocarcinomas, iCCAs are highly heterogeneous tumors and several classifications have been proposed^[4,5,9,19]. The small bile duct type (mixed) iCCAs display an almost exclusively MF growth pattern^[4,5,9,19], and are frequently associated with chronic liver diseases (viral hepatitis or cirrhosis)^[4,5,9,19,20]. Notably, this subtype shares clinic-pathological similarities with cytokeratin (CK) 19-positive hepatocarcinoma (HCC)^[4,21]. On the other hand, large bile duct type (mucinous) iCCAs may grossly appear as MF, PI or IG types; they are more frequently associated with PSC and can be preceded by pre-neoplastic lesions such as biliary intraepithelial neoplasm (BiIN) or intraductal papillary neoplasm (IPNB)^[4,5,9,19]. Interestingly, the large bile duct type (mucinous) iCCAs share phenotypic traits with pCCA and pancreatic cancers^[4].

In our opinion, this histological subtyping should be taken into serious consideration because it underlines different risk factors, molecular profile, and clinical management^[3,4,9,14,22-28].

MULTIPLE RISK FACTORS REVEAL iCCA SUBTYPE-SPECIFIC PATHOGENESIS

Although CCA is a rare cancer (incidence < 6/100,000) in most countries, its incidence may reach an extremely high in some populations of Chile, Bolivia, South Korea and North Thailand^[29] [Figure 1]. The different prevalence of risk factor in geographic areas may explain the variation in incidence rates of CCA. For example, in Thailand regions, the very high incidence of CCA is closely related to the incidence of liver flukes^[30-32].

In order to review literature on risk factors associated with iCCA we have searched for case series of iCCA or case series with appropriate topographic classification of histologically verified iCCA. The risk factors of iCCA (diagnosed according the current recognized criteria, i.e. European RARECARE^[33]) could be classified on the basis of the tissue or the cell which is primarily targeted by diseases or conditions and therefore likely involved in the carcinogenic process as cell or tissue of origin. For instance, biliary diseases as cholangitis/PSC, secondary biliary cirrhosis, choledocholithiasis, hepatolithiasis, cholecystitis, and liver flukes are pathologic conditions primarily affecting large intra-hepatic bile ducts [Table 1]^[34-46], and are risk factors for both iCCA and p/dCCA. Parenchymal liver diseases include chronic viral and non-viral liver diseases, recognize the interlobular bile ducts, bile ductules and the canals of Hering as the primary targets. Accordingly, these conditions are specific risk factors for iCCA [Table 1].

Other risk factors, like several toxic and environmental factors; amongst them nitrosamine-contaminated food, asbestos, dioxins, vinylchlorides, and thorotrast as was always the case in the past^[47], which hit multiple cellular targets, are considered risk factors associated to all CCA subtypes.

PSC, a disease affecting both intra-hepatic and extra-hepatic bile ducts, represents the strongest independent risk factor both for iCCA and for pCCA [Table 1]. Most of the studies evaluated the cumulative risk of CCA in PSC patients, but not the discrete risk of iCCA and/or pCCA to PSC^[48-51]. The cumulative incidence of CCA in PSC patients ranges from 5% to 10%^[52-55]. Clinical and pathological observations suggested that PSC is specifically associated with the development of bile duct (mucinous) type CCA^[4,56]. Data on the role of inflammatory bowel diseases (IBD), associated with or preceding PSC, in affecting the risk of CCA are controversial. The coexistence and duration of IBD significantly increased the risk of CCA in PSC patients^[51]. In IBD patients the RR estimated was 2.61 for iCCA vs. 1.47 for pCCA^[57]. Crohn's disease (CD) seemed to have a lower risk of CCA than ulcerative colitis (UC)^[57,58]. In contrast, in a study carried out in the USA, neither IBD nor its duration confers additional risk of CCA in PSC patients^[59].

In a study, Welzel *et al.*^[36] described that duodenal ulcer disease was significantly more common among pCCA and iCCA cases than controls. Many studies have demonstrated associations between CCA and *H. pylori* but the correlation remains controversial and a direct cause-and-effect relationship has not been established^[60-66]. In particular, in East-Asia, where iCCA represents a large proportion of primitive liver cancers, a strong association exists between liver fluke infestation (*Ophisthorchis viverrini* and *Clonorchis sinensis*) and the development of CCA [Table 1]^[67,68]. Several epidemiological studies estimated the relationship between type II diabetes and CCA [Table 1]^[36,69-71]. Notably, a possible explanation of this association is attributable to a recent demonstration that in a diabetes model and in human subjects affected by type II diabetes, PBGs underwent proliferation and expansion in relation to hyperglycemia^[72]. It's worthy to note that metformin reduced the risk of iCCA in diabetic patients by a significant margin up to 60%^[73,74]. A recent meta-analysis confirmed that, in addition to type II diabetes, even obesity, alcohol use and smoking, have an association with iCCA^[75].

It is becoming increasingly evident that metabolic conditions predispose to the development of primary liver cancers^[3,44,76]. Nonalcoholic fatty liver disease/non alcoholic steato-hepatitis (NAFLD/NASH) resulted in independent predictors of iCCA (not of pCCA development), even if with a less strong association compared with other risk factors (viral hepatitis, cirrhosis) [Table 1]^[76]. Hemochromatosis resulted in an independent predictor of iCCA development, and it failed to predict pCCA [Table 1].

It has long been known that the presence of cirrhosis increases the risk of iCCA^[36,37,40,44,75]. HBV- and HCV-related liver diseases have been identified as definitive risk factors for CCA, with a stronger association for iCCA than pCCA^[77,78]. A meta-analysis by Palmer and Patel^[75] concerning 8 case control studies indicated that HCV was associated with an overall OR of 4.84 for iCCA. Where the prevalence of the HBV infection is higher, the association with iCCA and HBV is more significant (e.g. Asian countries)^[79,80]. The range of the

Table 1. Summary of risk factors significantly associated to iCCA* as assessed by case control studies (odd ratios by multivariate analyses)

Risk factors for iCCA	Odds ratios for increased risk
Bile duct diseases and conditions	
Cholecystitis ^[36]	8.5
Cholelithiasis ^[35,40]	10.23-13.5
Hepatolithiasis ^[37,39,40,43,77§]	50.0-4.8; 6.7§
Choledochal cysts ^[36,37,44,59]	10.7-43.03; 36.9
Choledocholithiasis ^[35,43]	4.17-33.35
Cholangitis/primary sclerosing cholangitis ^[36,44]	64.2-75.23
Biliary cirrhosis/PBC ^[36,44]	17.08-19.8
Cholecystectomy ^[36,39]	3.6-5.4
Digestive diseases	
Inflammatory bowel diseases ^[36,58]	1.72-3.95
Crohn's disease ^[36,44]	1.68-2.4
Ulcerative colitis ^[36,44]	3.3-4.5
Duodenal ulcer ^[36]	3.4
Chronic pancreatitis ^[36]	5.9
Liver flukes	
Clonorchis sinensis infection ^[38,42]	8.6-13.6
Endocrine disorders	
Thyrotoxicosis ^[36]	1.5
Diabetes mellitus type II ^[37-39,43,75,86]	1.8-3.2
Metabolic conditions and general risks	
Obesity ^[36,44]	1.7-1.71
Alcohol intake > 80 g/day ^[37,39,75]	1.52-5.21
Smoking ^[36,44]	1.3-2.1
Metabolic syndrome ^[44#]	1.32-1.83
Dyslipoproteinemia ^[44]	1.65
Hypertension ^[44]	1.63
Chronic liver diseases	
Alcoholic liver disease ^[36,44]	3.1-5.69
Non specific cirrhosis ^[36,37,43,44,75]	18.24-28.79
Hemochromatosis ^[36]	2.6
Hepatic schistosomias ^[43]	11
Non alcoholic liver disease ^[36]	3
Unspecified viral hepatitis ^[44]	7.66
HCV infection ^[36-40,44,75,77§]	2.41-9.71; 9.7§
HCV infection plus cirrhosis ^[40]	8.53
HBsAg positive ^[35,37-40,44,75,81°]	2.3-9.7; °2.35-4.3
HBsAg positive plus cirrhosis ^[35,40,41]	13-18
HBsAg negative/HBcAb positive ^[45,81°]	1.09-1.81°
Occupational exposure	
Occupational exposure to asbestos ^[46]	4.81

*Histological verified cases; SiCCA cases comprise 2 cases of cHCC-CCA; #according the 2001 U.S. NCEP-ATP III definition; °Risk of CCA only in Asia. The table was prepared summarizing findings by case control studies investigating risk factors associated to iCCA as assessed by multivariate analyses. The case-control studies were selected from the papers individuated by the following terms, that were searched on PubMed: ("cholangiocarcinoma"[MeSH Terms] OR "cholangiocarcinoma"[All Fields]) AND ("risk factors"[MeSH Terms] OR ("risk"[All Fields] AND "factors"[All Fields]) OR "risk factors"[All Fields] OR ("risk"[All Fields] AND "factor"[All Fields]) OR "risk factor"[All Fields])) NOT ("review"[Publication Type] OR "review literature as topic"[MeSH Terms] OR "review"[All Fields]) AND English[lang]. The criteria selections of the works comprise moreover the case definition of CCA: histological verified cases series of iCCA with appropriate topographic classification (Klatskin tumours classified as pCCA and excluded from the iCCAs)

OR in the HbsAg positive subjects goes from 2.3 to 9.7 [Table 1]^[81]. The presence of cirrhosis increases the risk of CCA [Table 1] even more by 2.5 fold (95% CI: 1.2-5.1; $P = 0.02$) in HBV, and 3.2 fold (95% CI: 1.231-8.148, $P = 0.017$) in HCV patients^[41].

The burden of HCV in the last decades has been associated with the specific increase of the iCCA as well as the HCC^[81]. Accordingly, clinical and pathological observations suggested that liver cirrhosis is specifically

associated with the development of small bile duct (mixed) type iCCA^[4]. Ductular reaction is a marker strongly associated with the evolution of chronic liver disease in cirrhosis. The origin of the small bile duct type iCCA may be associated with the chronic proliferative activation of hepatic stem cells and mature hepatocytes senescence in chronic liver diseases^[12,82]. Since cirrhosis, chronic hepatitis B and C, alcohol use, diabetes, and obesity are major risk factors for iCCA and HCC^[75], a common pathogenesis of primary intrahepatic epithelial cancers has been suggested. The parallel worldwide reduction of mortality of HCC^[12], which is highly correlated to viral infection and cirrhosis, and on the pandemic of metabolic disorders, suggests that metabolic risk factors are responsible for the rising clinical impact of iCCA. Interestingly we provided the pathologic basis of this epidemiology phenomenon since we demonstrated DM-induced proliferation of PBG cells^[72].

MOLECULAR PROFILING AND THE IDENTIFICATION OF MULTIPLE iCCA SUBSETS

Although there exist enormous geographic and racial differences^[3,83], generally, the prominent genetic alterations described in CCAs affect TP53 (DNA repair)^[84-86], tyrosine kinase (KRAS, BRAF, SMAD4 and FGFR2)^[8,84-88], protein tyrosine phosphatase (PTPN3)^[89], deregulated WNT/CTNNB1^[90] and Notch pathways, epigenetic (IDH1 and IDH2)^[28,84,88,91,92], and chromatin-remodeling factors (MLLs, ARID1A, PBRM1 and BAP1)^[84-86,88,91].

Chronic bile duct inflammation characterizes CCA risk factors^[93-95]. Accordingly, it was demonstrated that the enzyme cyclooxygenase-2 (COX-2) is induced in CCA by both bile acids and oxysterols, the oxidation products of cholesterol that are increased in the bile during biliary inflammation^[96,97]. Inflammatory cytokines may also upregulate the expression of inducible nitric oxide synthase (iNOS) in CCA. Notably, nitric oxide (NO) promotes DNA damage directly by inhibiting DNA repair mechanisms, thus promoting carcinogenesis^[98,99]. Moreover, iNOS activation stimulates further the expression of COX-2^[100]. Notably, the tumoral stroma seems to have a peculiar role in the amplification of the inflammation. While the tumor epithelium was defined by deregulation of the HER2 network and frequent over-expression of EGFR, the hepatocyte growth factor receptor (HGF/MET), pRPS6, and Ki67, the stroma was enriched in inflammatory cytokines^[101].

In the chronic inflammation milieu of CCA emerging in hepatitis infection^[88], recurrent genetic variants in the promoter of the human telomerase reverse transcriptase (TERT) were described^[88]. This could be correlated with the pivotal role of this “longevity” enzyme in controlling stem cells. These cells are extremely challenged in these conditions because the senescence of the mature hepatocytes determines the secondary stem proliferative activation (e.g. ductular reaction)^[12].

A dissection of the molecular heterogeneity of iCCA, conducted by the evaluation of gene expression profile (transcriptome), clinic-pathological traits, and patient outcomes in iCCA cases, has allowed the identification of 2 main biological classes of iCCA. The first inflammation class (38% of IH-CCA), characterized by activation of inflammatory signaling pathways, overexpression of cytokines, and STAT3 activation and; the second proliferation class (62% of IH-CCA), characterized by activation of oncogenic signaling pathways (i.e. RAS, MAP-kinase and HGF/MET), DNA amplifications at 11q13.2, deletions at 14q22.1, mutations in KRAS and BRAF, and gene expression signatures previously associated with poor outcomes for patients with HCC^[7].

Molecular studies of human iCCA associated with liver flukes demonstrated over-expression of genes involved in xenobiotic metabolism (UGT2B11, UGT1A10, CHST4, SULT1C1). Whereas non-OV-associated iCCA showed enhanced expression of genes related to growth factor signaling (TGFB1, PGF, IGFBP1, IGFBP3)^[32,102]. Possible mechanism associated with liver flukes carcinogenesis may emerge from the discovery of the draft genome of *Clonorchis sinensis* and transcriptomes of *Clonorchis Sinensis* and OV^[103,104]. For instance, the evaluation of the putative signature of liver flukes associated CCA could help in screening and surveillance, with the perspective of an early diagnosis of infestation in subjects^[102]. A putative role of liver fluke infestation in modulating

epigenetic has been suggested by the demonstration of promoter hypermethylation in a handful of target genes in a large cohort of iCCA ($n = 102$) associated with liver fluke infection^[105].

CCA genetic susceptibility has been investigated in geographic areas where liver flukes are endemic. In these studies, specific haplotypes of COX2-coding gene (PTGS2) or IL8RB have been recently associated with a significant risk of CCA development^[106].

As far as CCA emerging in PSC, different molecular signatures of the high oncogenic risk were described in PSC patients. KRAS mutations were found in 30% of bile fluid of PSC patients without evidence of CCA^[107]. Since KRAS mutations are frequently observed in CCA, and since the mutational profiling can be performed in cell-free DNA of bile supernatant, this early mutagenic event into the bile duct carcinogenesis could be evaluated for screening purposes in PSC patients^[108]. The inflammatory microenvironment has also been associated with an aberrant DNA methylation profile in CCA emergence in PSC patients, which provides survival signals for the tumor^[109]. Even, an inherited increase in the risk of CCA development in PSC patients was demonstrated by studies concerning the natural killer cell receptor G2D receptor, where specific genetic variants have been described in PSC patients^[110].

Heterogeneity of molecular profile of CCA provides a demonstration of how somatic mutagenesis and epigenome features are highly cell/lineage type-specific, and are largely driven by the pre-neoplastic tissue pathologic milieu (see inflammation). Indeed, at a molecular level, distinct patterns of genetic mutations, methylation, and expression profiling may differentiate iCCA from pCCA. iCCAs were significantly more frequently bcl-2+ and p16+, whereas pCCAs were more often p53+^[111]. Miller *et al.*^[112] revealed 545 genes with altered expression in p/dCCA and 2354 in iCCA. Mutations in IDH1 and IDH2 were found only in iCCA ($n = 9$), but in none of the examined p/dCCA ($n = 22$) and gallbladder cancer ($n = 75$)^[113]. Recent papers confirmed liver fluke negative iCCAs are enriched for IDH mutants^[14,28]. A cross-platform comparison of iCCA with pancreatic cancer and HCC further emphasizes the presence of distinct tumor subsets, suggesting similarities of the IDH mutants CCAs with the HCCs rather than pancreatic cancers^[28]. Conversely, mutations in KRAS by tumor site demonstrated predominance in pCCAs (53.3% of hilar vs. 6.7% of peripheral type)^[7]. As far as epigenetic abnormalities are concerned, methylation of RASSF1A was more common in pCCA than in iCCA, while the opposite was demonstrated for methylation of GSTP gene^[114]. Other reported alterations uniquely associated with iCCA, comprised fibroblast growth factor receptor (FGFR) pathways and ephrin type-A receptor 2 mutations^[115].

Finally, the histopathological distinction of cholangiolocellular differentiation of iCCA has been correlated with molecular features^[115]. iCCA with cholangiolocellular differentiation resembling an inflammation-related subtype revealed less aggressive histopathological features compared to iCCA without cholangiolocellular differentiation resembling a proliferation subtype. Accordingly, the former showed more favorable clinical outcomes, including overall survival, than iCCA without cholangiolocellular differentiation^[116]. The emerging therapeutic approaches based on the molecular targets in CCA have been recently reviewed by Rizvi and Gores^[117].

VARIABLE CLINICAL PRESENTATIONS AND DIAGNOSTIC FEATURES

Clinical presentation of CCA is largely influenced by anatomic location and pattern of growth, which ultimately belong from the cells of the origin. Accordingly, emerging concepts into CCA origins demonstrated that it comprises at least two separate entities which a distinct histology, progression and risk factors. These sub-types have been recently classified in large bile duct (mucinous) type CCAs and the small bile ducts or mixed-CCAs. According to different observations, pCCAs are more likely associated with pre-neoplastic lesions emerging in surface epithelium^[2,3] and PBGs^[118]. On the other hand, iCCAs show inter-tumor heterogeneity leading to the classification into two main different histological subtypes^[4,119], with likely different cells of origin^[4]: the CCAs of the small bile ducts or mixed-CCAs and the large bile duct

(mucinous) type iCCAs^[22,119]. The last iCCA subtype displays IHC, gene expression and clinic-pathological profile that can be superimposed on pCCA^[4,120-122]. Small bile ducts or mixed-CCAs usually showed a peripheral localization and a mass forming growing pattern. Differently, the large bile duct (mucinous) type usually showed a peri-ductal infiltrating and/or mass forming growth pattern^[4]. Importantly, these separate entities displayed different prognosis (being worst the one of the mucin-producing iCCAs) and different associated diseases^[4,10,82,123]. Indeed, parenchymal liver diseases, including chronic viral and non-viral liver diseases and liver cirrhosis, characterize the clinical-pathologic background for mixed-iCCAs^[4,10,82,123]. In contrast, chronic biliary diseases or pathologies and conditions affecting the intrahepatic medium-large and extrahepatic bile ducts characterize the clinical-pathologic background for mucin-producing iCCAs and pCCAs^[4,10,82,123].

As far as the mixed type-mass-forming iCCA is concerned, the clinical presentation is similar to other intrahepatic liver malignancies, but different from that of pCCA^[4,10,82,123]. iCCAs are usually asymptomatic in early stages (20%-25% of cases are incidental finding). Malaise, cachexia, abdominal pain, night sweats, fatigue and/or jaundice, associated or not with systemic manifestations, represent the clinical onset of symptomatic iCCA^[4,10,82,123]. In contrast, a typically painless jaundice is the most frequent clinical onset in pCCA^[4,10,82,123]. Regarding patients with PSC, CCA may present as the development of a rapid deterioration of clinical conditions or a dominant stricture during follow-up^[3]. In general, the MF type represents the most frequent macroscopic presentation of iCCA (> 90%) appearing, at imaging, as a nodule^[3,123]. In the context of cirrhotic liver, the first diagnostic challenge is the differential diagnosis of iCCA *vs.* HCC. In the cirrhotic liver it was demonstrated that by contrast, enhanced MRI iCCAs showed constantly a lack of HCC hallmarks; however, by CT, this occurs only in large nodules (> 3 cm)^[124-126]. Although, the HCC diagnosis belong from the demonstration of the typical contrast agent uptake, the identification of HCC with stem cell features (CK19+HCC), combined HCC-CCA, cholangiolocellular carcinoma and bile duct mixed type iCCA, by imaging procedures, still remains an unsolved challenge^[3,4,10,123,127,128]. Biopsy is, therefore, necessary after excluding HCC in cirrhosis, or in the context of a nodule in non-cirrhotic liver^[3,129]. From a histological point of view, differential diagnosis of iCCA *vs.* HCC or metastasis represents an unsolved problem^[2,3,129,130], also due to the lack of validation of specific markers.

Radiologically, iCCA may appear as a dominant stricture in the context of PSC or in patients without a documented specific hepato-biliary disease. This is a typical presentation of the pCCA. When a dominant stricture of the intrahepatic biliary tree is suspected, the MRI + MRCP represents the imaging procedure with the highest diagnostic accuracy for localizing and sizing the stricture^[3]; the challenge being the definitive demonstration of malignancy^[3]. In this respect, ERCP enables a number of procedures in order to obtain a microscopic confirmation, comprising, cytology, brushing, FISH-polisomy, biopsy, or further innovative techniques^[3]. However, all these techniques show an unsatisfactory sensitivity^[54,130-133], and even, the FISH-polisomy in detecting CCA in PSC patients demonstrated a low sensitivity in a meta-analysis^[133].

In substance, diagnosis of CCA still requires a combination of clinical, radiologic and non-specific histologic/biochemical markers (see review by Banales *et al.*^[3]).

As already mentioned, no specific serum, urine, biliary or histological biomarkers are currently available for the diagnosis of CCA and a proposal by our group which has been recently refreshed by new confirmation, identifies biliary IGF1 as specific markers of CCA. However, the very promising role of biliary IGF1 has been confirmed only in CCA without PSC. Recently, Arbelaiz *et al.*^[134] evaluated the serum concentration of extracellular vesicles (EVs) and performed a careful analysis of the protein content in patients with CCA, PSC, and HCC. Proteomic signatures found in serum EV of CCA, PSC, and HCC patients show potential usefulness as diagnostic tools. As noted previously, the EV cargo in the two distinct EV populations (*i.e.*, basolateral and apical) is evidently different as a large difference exists between the protein content of EVs released by normal cholangiocytes and cholangiocytes involved in chronic inflammation (*i.e.*, PSC) or

neoplastic transformation (i.e., CCA)^[135]. Further validation studies will be necessary to bring this important scientific advance into the clinical approach of CCA differential diagnosis.

NEW ADVANCES INTO CCA THERAPY

Surgery with complete resection, including liver transplantation in highly selected cases, is the only curative therapy for CCA. In patients with unresectable tumours, several types of loco regional therapy or chemotherapy (such as trans arterial chemoembolization, trans arterial radio embolization or radiofrequency ablation) can be considered. In substance, CCAs must be managed by dedicated centres with multidisciplinary expertise in which personalized diagnostic work-up and management can be performed, as clearly stated by a European Consensus (see review by Banales *et al.*^[3]).

Recently two important advances have been reached in therapy of iCCA. On one hand, the first clinical trial of adjuvant therapy has been concluded^[136]. In this clinical trial, 447 surgically resected patients were randomly assigned to capecitabine for 6 months or observation (> 80% of the patients were followed for at least 3 years). Interestingly, results showed a survival of 51 vs. 36 months in capecitabine arm vs. observation, and median time to cancer recurrence of 25 vs. 18 months, respectively. In 430 patients who received treatment per study protocol, capecitabine is associated with a 25% lower chance of death than observation^[136]. On the other, the first report of a molecular target therapy in chemotherapy-refractory CCA appeared. BGJ398 was a first-in-class FGFR kinase inhibitor with manageable toxicities showing meaningful clinical activity against chemotherapy-refractory CCA containing FGFR2 fusions. This promising antitumor activity supports continued development of BGJ398 in this highly selected patient population^[137]. Emerging therapeutic approaches based on the molecular targets are still in early phase of clinical study and have been recently reviewed by Rizvi and Gores^[117].

PERSPECTIVES

A unique feature of CCA is that it recognizes as origin tissues, the hepatic parenchyma or large bile ducts, which are furnished by two distinct stem cell niches, the canals of Hering and the peribiliary glands (PBGs), respectively^[138].

Stem cells have been identified as cells of origin of different cancer types, comprising primary liver cancers, both in experimental studies and in humans^[139-147]. Based on the grade of maturation of the cells of origin within the two lineages of the liver (hHpSC-derived and hBTSC-derived lineages), we have proposed that CCAs could be classified as:

- Primary liver parenchymal CCA: cholangiolo-carcinoma, small bile duct type (mixed) CCA. These tumors emerge within the liver parenchyma from canals of Hering, bile ductules and interlobular bile ducts and indeed originate from hHpSCs, immature NCAM+ cholangiocytes, or mature (NCAM-) interlobular cholangiocytes. A rigorous study, based on an integrative genomic analysis of HCC-CCAs, demonstrated that cholangiolo-carcinoma represents a distinct biliary-derived entity compared with the mixed/combined HCC-CCA, which, on the other side, comprised the stem-cell type, with an aggressive nature and poor outcome, and the classical type, with common cell lineage for both the HCC and the iCCA component^[148].
- Primary biliary CCA: dCCA, pCCA, and large bile duct (mucinous) type iCCA. These tumors emerge from extra-hepatic biliary tree and larger intra-hepatic bile ducts and originate from PBGs or surface epithelium of corresponding bile ducts.

Thus, facing the origin of iCCA, a physiopathology concept should be considered, instead of the cell of origin, the lineage of origin^[10,12,13,138]. An iCCA classification based on the cell-lineages-of origin is more coherent with current knowledge on the epidemiology and risk factors and may have important clinical implications for the definition of specific therapeutic targets. Moreover, it highlights a lineage dependency of the chronic

liver diseases and related molecular carcinogenesis^[12]. Being somatic mutagenesis and epigenome features highly cell/lineage type-specific^[149], and largely driven by the pre-neoplastic tissue pathologic milieu (see inflammation), finally, the multiple lineages of origin plus the related diseases may explain the intertumoral heterogeneity observed at any level in iCCA, comprising molecular profiling, with clear implication into preventive strategies in patient with clinical or subclinical underlining hepatic or biliary diseases, therapy and in near future approaches of personalized medicine in iCCA patients.

DECLARATIONS

Authors' contributions

Concept, design, definition of intellectual content, literature search, data acquisition and analysis, statistical analysis, manuscript preparation, editing and review: Cardinale V, Bragazzi MC, Carpino G

Data acquisition and analysis: Di Matteo S, Overi D, Nevi L

Manuscript editing and review: Gaudio E

Definition of intellectual content, literature search, manuscript preparation, editing and review: Alvaro D

Availability of data and materials

Not applicable.

Financial support and sponsorship

The study was supported by Consorzio Interuniversitario Trapianti d'Organo, Rome, Italy and by a sponsored research agreement from Vesta Therapeutics (Bethesda, MD).

Conflicts of interest

None.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Pathway analysis provides insight into the genetic susceptibility to hepatocellular carcinoma and insight into immuno-therapy treatment response

Yih-Kuang Lu¹, Jacob Morris Brill¹, Ardesher Aghili¹, Kenneth Howard Buetow^{1,2,3}

¹Computational Sciences and Informatics, Complex Adaptive Systems, Arizona State University, Tempe, AZ 85287, USA.

²Center for Evolution and Medicine, Arizona State University, Tempe, AZ 85287, USA.

³School of Life Sciences, Arizona State University, Tempe, AZ 85287, USA.

Correspondence to: Dr. Kenneth Howard Buetow, Computational Sciences and Informatics, Complex Adaptive Systems, Arizona State University, Tempe, AZ 85287, USA. E-mail: kenneth.buetow@asu.edu

How to cite this article: Lu YK, Brill JM, Aghili A, Buetow KH. Pathway analysis provides insight into the genetic susceptibility to hepatocellular carcinoma and insight into immuno-therapy treatment response. *Hepatoma Res* 2018;4:21.
<http://dx.doi.org/10.20517/2394-5079.2018.44>

Received: 26 Apr 2018 **First Decision:** 28 May 2018 **Revised:** 11 Jun 2018 **Accepted:** 12 Jun 2018 **Published:** 22 Jun 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Clear evidence exists for genetic susceptibility to hepatocellular carcinoma (HCC). Genome-wide association studies have identified multiple candidate susceptibility loci. These loci suggest that genetic variation in the immune system may underpin HCC susceptibility. Genes for the antigen processing and presentation pathway have been observed to be significantly enriched across studies and the pathway is identified directly through genome-wide studies of variation using pathway methods. Detailed analysis of the pathway indicates both variation in the antigen presenting loci and in the antigen processing are different in cases in controls. Pathway analysis at the transcriptional level also shows difference between normal liver and liver in individuals with HCC. Assessing differences in the pathway may prove important in improving immune therapy for HCC and in identifying responders for immune checkpoint therapy.

Keywords: Hepatocellular carcinoma, genetic susceptibility, genome-wide association study, pathway analysis, antigen presentation and processing, immune checkpoint therapy

Hepatocellular carcinoma (HCC), the most common form of primary liver cancer, is ranked 5th in global incidence and 2nd in mortality^[1]. With the exception of East Asia, the incidence of HCC is increasing in almost all regions of the world and has doubled in the USA since the early 1980s^[2]. This increase is attributable to increases in obesity and type II diabetes^[3,4]. Liver cancer's 5-year survival is the second worst among all cancers (18.1%)^[5].



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In this manuscript, the role of genetic susceptibility to HCC is examined. Novel tools that evaluate genetic data using collections of genes and their interactions within biologic networks are used to identify key biologic processes driving susceptibility. The relationship of germline and somatic variation is explored. The importance of these findings is assessed in the context of current therapeutic interventions for HCC.

SOMATIC GENETIC ETIOLOGY OF HCC

Like other solid tumors, at a somatic level, HCC appears to arise via alterations in numerous genes that modify multiple biologic processes. An early whole-genome sequencing effort identified an average of 9718 nucleotide alternations, 271 insertion/deletions, and 41 structural variations per tumor, with substantial variability from tumor to tumor^[6]. Within coding sequences, it has been reported that there are an average of 21 synonymous and 64 non-synonymous mutations per tumor^[7]. Tumors of larger size are observed to have greater numbers of point mutations, which are speculated to contribute to heterogeneity within the tumors. The Cancer Genome Atlas (TCGA) Research network's evaluation of HCC^[8] finds alterations over-represented in the RAS pathway, WNT pathway, cell cycle regulation pathways and chromatin modification pathways with high mutation rates in TP53 (31%), CTNNB1 (27%), AXIN1 (8%), ARID1A (7%), ARID2 (5%), RB1 (4%), PIK3CA (4%), CDKN2A (2%), KRAS (1%), NRAS (1%), high deletion frequencies of RB1 (19%), CDKN2A (13%), PTEN (7%) and amplification of CCND1 (6%). The most commonly mutated locus was TERT with promoter mutations found in 44% of tumors^[8]. The TCGA data unexpectedly also showed high mutation rates in ALB (13%) and APOB (10%).

GENETIC SUSCEPTIBILITY TO HCC

In contrast to other common tumors, genetic susceptibility to HCC remains poorly characterized. Studies have identified evidence for familiarity of HCC, over and above familial exposures such as HBV infection^[9-14]. For example, after accounting for HBV infection, individuals with a family history of HCC have a rate ratio of 2.4^[10]. To date, these studies have examined only hepatitis virus associated HCC and have yet to explore the role of obesity and diabetes related susceptibility.

A limited number of studies have been conducted to identify the loci underpinning this familiarity. Original studies focused on candidate genes whose observed single nucleotide polymorphisms (SNPs) could plausibly modify known environmental risk factors for HCC including aflatoxin, alcohol, or tobacco. A meta-analysis of these studies found associations with 5 genes *HFE*, *IL-1B*, *MnSOD*, *MDM*, and *2UGT1A7*^[15].

HCC has had a small number of genome wide association studies (GWAS) conducted with modest success in identifying risk loci. The NHGRI-EBI Catalog lists a total of 11 studies that have identified 22 loci^[16]. These studies examine East Asian populations and have included HCC associated with hepatitis B virus (HBV), hepatitis C virus (HCV), and non-alcoholic steatohepatitis (NASH) etiologies. The studies have identified SNPs in the genomic proximity (intronic, upstream and/or downstream) of twenty protein coding loci.

Clues to the biologic basis of HCC susceptibility across GWAS studies can be identified by looking for non-random enrichment. Using the resources of the Gene Ontology consortium (GO) (<http://geneontology.org>), the twenty protein coding loci were examined for biologic process enrichment in *Homo sapiens*. This enrichment analysis uses the tools of Panther (<http://pantherdb.org/webservices/go/overrep.jsp>). Four high level GO processes were observed to be significantly enriched "T cell receptor signaling pathway" ($P = 0.0366$), "interferon-gamma-mediated signaling pathway" ($P = 0.0026$), "T cell costimulation" ($P = 0.0020$), and "antigen processing and presentation of exogenous peptide antigen via MHC class II" ($P = 0.0001$).

We have previously looked for inherited susceptibility using genome-wide genotyping and a novel analytic approach that uses biologic networks - Pathways of Distinction Analysis (PoDA)^[17]. In PoDA, the network is

Table 1. Updated significant networks identified through pathway of distinction analysis

PoDA pathway name	Source	DS	OR	No. of genes	No. of SNPs
Axon guidance	KEGG	1.888	3.1699	245	13,044
GPCR downstream signaling	REACTOME	1.706	2.4122	695	16,949
Focal adhesion	KEGG	0.802	2.3329	197	7999
Pathways in cancer	KEGG	0.570	2.2487	284	10,406
MAPK signaling pathway	KEGG	0.620	2.1152	245	7368
PI3K-Akt signaling pathway	KEGG	-0.339	2.0837	314	10,409
Calcium signaling pathway	KEGG	-1.030	1.8479	163	8684
Regulation of actin cytoskeleton	KEGG	-1.004	1.8207	195	5681
Glycerolipid metabolism	KEGG	2.003	1.7607	55	1590
Mechanism of gene regulation by peroxisome proliferators via ppara	BIOCARTA	2.371	1.7272	49	1076
Interleukin-3, 5 and GM-CSF signaling	REACTOME	2.969	1.7235	41	1188
Glycerophospholipid biosynthesis	REACTOME	2.201	1.7208	70	1714
T cell receptor signaling pathway	BIOCARTA	2.493	1.6792	55	1500
Dopaminergic synapse	KEGG	-1.348	1.6651	116	5396
Stabilization and expansion of the E-cadherin adherens junction	NCI/NATURE	2.142	1.6630	40	1449
Eicosanoid metabolism	BIOCARTA	3.026	1.6620	16	800
Netrin-mediated signaling events	NCI/NATURE	1.965	1.6620	28	2400
Pre-NOTCH expression and processing	REACTOME	3.240	1.6343	45	1451
Purine metabolism	KEGG	-1.190	1.6284	150	4726
Toxoplasmosis	KEGG	2.470	1.5901	110	2088
Angiopoietin receptor Tie2-mediated signaling	NCI/NATURE	2.163	1.5806	47	1331
Circadian entrainment	KEGG	-1.498	1.5738	88	5919
Systemic lupus erythematosus	KEGG	3.873	1.5688	82	1185
Bioactive peptide induced signaling pathway	BIOCARTA	2.276	1.5677	42	1260
Role of mef2d in t-cell apoptosis	BIOCARTA	2.138	1.5522	30	946
Herpes simplex infection	KEGG	2.816	1.5388	170	1994
Glycosphingolipid biosynthesis - lacto and neolacto series	KEGG	2.756	1.5285	23	535
Multi-step regulation of transcription by pitx2	BIOCARTA	2.935	1.5253	22	526
Retrograde endocannabinoid signaling	KEGG	-1.990	1.5208	94	4960
TCR signaling	REACTOME	3.001	1.4913	51	1226
TPO signaling pathway	BIOCARTA	2.556	1.4896	23	635
Growth hormone signaling pathway	BIOCARTA	2.144	1.4813	28	768
Rheumatoid arthritis	KEGG	2.895	1.4801	84	978
Huntington's disease	KEGG	2.156	1.4658	152	1647
Inactivation of gsk3 by akt causes accumulation of b-catenin in alveolar macrophages	BIOCARTA	2.467	1.4628	32	709
Chaperones modulate interferon signaling pathway	BIOCARTA	2.486	1.4615	18	313
Phospholipase c signaling pathway	BIOCARTA	2.886	1.4577	10	849
GnRH signaling pathway	KEGG	-1.259	1.4488	84	3622
Oocyte meiosis	KEGG	-1.285	1.4371	102	2727
Biosynthesis of unsaturated fatty acids	KEGG	1.927	1.4342	19	495
GMCSF-mediated signaling events	NCI/NATURE	1.843	1.4339	30	841
p75 NTR receptor-mediated signalling	REACTOME	-1.195	1.4335	76	2466
E-cadherin signaling in keratinocytes	NCI/NATURE	2.123	1.4326	21	477
Signaling events mediated by HDAC Class III	NCI/NATURE	1.927	1.4323	26	565
Keratan sulfate/keratin metabolism	REACTOME	1.962	1.4251	28	447
Morphine addiction	KEGG	-3.158	1.4243	86	4524
IL3-mediated signaling events	NCI/NATURE	2.199	1.4233	22	399
Intestinal immune network for IgA production	KEGG	3.740	1.4224	45	506
lectin induced complement pathway	BIOCARTA	2.541	1.4167	11	359
Leishmaniasis	KEGG	2.734	1.4130	68	927
Alternative complement pathway	BIOCARTA	2.196	1.4075	11	236
Autoimmune thyroid disease	KEGG	2.835	1.4056	39	513
Graft-versus-host disease	KEGG	3.079	1.4025	33	240
Activation of pkc through g-protein coupled receptors	BIOCARTA	1.733	1.3968	11	892
Allograft rejection	KEGG	3.327	1.3952	30	253
Costimulation by the CD28 family	REACTOME	2.648	1.3931	62	1270
Eicosanoid ligand-binding receptors	REACTOME	2.798	1.3899	11	174

Staphylococcus aureus infection	KEGG	3.410	1.3766	52	504
Serotonergic synapse	KEGG	-1.854	1.3733	73	3128
N-glycan antennae elongation in the medial/trans-Golgi	REACTOME	2.122	1.3729	14	396
Integrins in angiogenesis	NCI/NATURE	-1.929	1.3622	74	2110
Tandem pore domain potassium channels	REACTOME	2.299	1.3589	4	206
Fatty acid elongation in mitochondria	REACTOME	2.226	1.3579	12	170
IL5-mediated signaling events	NCI/NATURE	2.080	1.3568	12	304
Antigen processing and presentation	KEGG	3.506	1.3397	65	400
Asthma	KEGG	3.713	1.3246	31	200
Neurotransmitter release cycle	REACTOME	1.846	1.3233	9	326
Classical complement pathway	BIOCARTA	2.682	1.3164	12	239
Antigen processing and presentation	BIOCARTA	2.938	1.2857	9	52
Interferon gamma signaling	REACTOME	3.080	1.0558	61	2598
Antigen processing-cross presentation	REACTOME	2.187	1.0371	59	1962

the unit of analysis and accounts for interactions among features within the network. In this analysis “antigen processing and presentation” was identified as having significant differences in variability in a population of Korean HBV associate HCC cases and controls. Consistent with the results of the enrichment analysis, re-analysis of this dataset with an extended set of 1200 pathways again identified “antigen processing and presentation”, but also “interferon gamma signaling”, “TCR signaling”, and “T cell receptor signaling pathway” [Table 1] suggesting that immune response may be a key driver of HCC susceptibility.

THE ROLE OF ANTIGEN PROCESSING AND PRESENTATION IN HCC

To assess what might be the key factors within “antigen processing and presentation”, we performed analysis utilizing a modified version of PoDA using the Korean HCC dataset. In this analysis, all 400 of the SNPs genotyped in the data set for the 65 genes in the pathway were contrasted in the cases and controls. After assessing significance of the odds ratio for the entire set of SNPs, each individual SNP was removed one at a time from the dataset and the significance was re-assessed. The SNP which least affected the significance of the odds ratio was then removed and the process was repeated. SNPs were progressively removed in this “stepdown” procedure until the significance of the odds ratio was no longer improved. Interestingly, it was observed that initial removal of SNPs substantially improved significance of the difference between cases and controls. When stepdown was completed, a total of 49 SNPs in 26 genes were observed [Table 2].

While the genes identified included key genes seen in the GWAS catalog, specifically members of HLA class II, other genes associated with antigen processing were also observed [Figure 1]. The design of Genome-wide association studies does not permit the specific etiologic effects of the variation. By design, the variation used in the studies is not chosen for function, but instead the ability to test differences between populations. The high linkage disequilibrium observed between variations in humans further complicates the capacity to interpret the molecular mechanisms of action.

Nevertheless, this study identifies variation of genes of potential significance in etiology. Of particular interest are the proteasome (HSPA2, HSPA4, HSPA5 HSP90AB1), endoplasmic reticulum TAP1, TAP2, CANX), and exosome (LGMN) genes associated with the processing of antigens so that they may be presented by HLA loci. The pathway also identifies genes on the surface of immune cells - NK cells (KIR2DL3, KIR2DL4, and KIR2DL5) and CD4 T cells (CD4) that may compromise immune surveillance and regulation.

It is possible to examine the intra-pathway associations of the variants. Using the analytic tool PLINK^[18], one can estimate the association (r^2) between loci in cases and controls [Table 3]. As expected by the PoDA analysis, variants within the pathways are associated with one another. Both variants within loci and between loci are observed to be associated. Interestingly, the magnitude of associations differs between cases and

Table 2. Significant genes and SNPs within the KEGG antigen processing and presentation pathway

Gene symbol	Gene name	SNP (rs id)
CANX	Calnexin	rs7734102
CD4	CD4 molecule	rs1075835
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	rs2748249
CIITA	Class II, major histocompatibility complex, transactivator	rs6498122
CIITA	Class II, major histocompatibility complex, transactivator	rs7203275
CIITA	Class II, major histocompatibility complex, transactivator	rs11074934
CIITA	Class II, major histocompatibility complex, transactivator	rs6498119
CTSS	Cathepsin S	rs11204722
HLA-A	Major histocompatibility complex, class I, A	rs12202296
HLA-DMA	Major histocompatibility complex, class II, DM alpha	rs11539216
HLA-DMA	Major histocompatibility complex, class II, DM alpha	rs17617515
HLA-DMB	Major histocompatibility complex, class II, DM beta	rs3132132
HLA-DMB	Major histocompatibility complex, class II, DM beta	rs714289
HLA-DOA	Major histocompatibility complex, class II, DO alpha	rs3129304
HLA-DOA	Major histocompatibility complex, class II, DO alpha	rs3129303
HLA-DOA	Major histocompatibility complex, class II, DO alpha	rs3130602
HLA-DOA	Major histocompatibility complex, class II, DO alpha	rs3129302
HLA-DPB1	Major histocompatibility complex, class II, DP beta 1	rs9277378
HLA-DQA2	Major histocompatibility complex, class II, DQ alpha 2	rs9275356
HLA-DQA2	Major histocompatibility complex, class II, DQ alpha 2	rs9276427
HLA-DQA2	Major histocompatibility complex, class II, DQ alpha 2	rs9469266
HLA-DRA	Major histocompatibility complex, class II, DR alpha	rs7194
HLA-G	Major histocompatibility complex, class I, G	rs2517898
HSP90AB1	Heat shock protein 90kDa alpha (cytosolic), class B member 1	rs504697
HSPA2	Heat shock 70kDa protein 2	rs4313734
HSPA4	Heat shock 70kDa protein 4	rs7702889
HSPA5	Heat shock 70kDa protein 5	rs12009
HSPA8	Heat shock 70kDa protein 8	rs4936770
KIR2DL3	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	rs9797797
KIR2DL3	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	rs13344915
KIR2DL4	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4	rs10500318
KIR2DL4	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4	rs3865509
KIR2DS4	Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 4	rs11673276
KLRD1	Killer cell lectin-like receptor subfamily D, member 1	rs17206564
LGMN	Legumain	rs8177528
LGMN	Legumain	rs2250672
LGMN	Legumain	rs716097
LGMN	Legumain	rs12885208
LGMN	Legumain	rs9791
LOC100509457	HLA class II histocompatibility antigen, DQ alpha 1 chain-like	rs2647015
LOC100509457	HLA class II histocompatibility antigen, DQ alpha 1 chain-like	rs2859090
LOC100509457	HLA class II histocompatibility antigen, DQ alpha 1 chain-like	rs9272219
RFXAP	Regulatory factor X-associated protein	rs6563500
TAP1	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	rs4148882
TAP2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	rs3819720
TAP2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	rs2228396
TAP2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	rs241428
TAP2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	rs9784758
TAP2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	rs241431

controls. This confirms that the pathway utilizes information (interactions between loci) that would not be observed in simple single locus GWAS assessments.

“ANTIGEN PROCESSING AND PRESENTATION” TRANSCRIPTIONAL ACTIVITY

It is possible to assess whether the germline variation in “antigen processing and presentation” translates into functionally significant difference in normal liver when contrasted to tumor adjacent liver and HCC.

Table 3. Association of case and control SNP variation with r^2 greater than 0.1 within the KEGG antigen processing and presentation pathway

SNP_A	SNP_B	Case r^2	Control r^2
SNP_A-4289896 - KIR2DL3	SNP_A-8561730 - KIR2DL3	0.88	0.95
SNP_A-8566010 - HLA-DQA1L	SNP_A-2200530 - TAP2	0.38	0.20
SNP_A-8515749 - HLA-G	SNP_A-8649593 - HLA-A	0.16	0.37
SNP_A-2214036 - HLA-DQA1L	SNP_A-4206711 - HLADQA1	0.16	0.14
SNP_A-8524421 - KIR2DL4	SNP_A-8613821 - KIR2DS4	0.14	< 0.1
SNP_A-1985650 - HLA-DOA	SNP_A-8430032 - KIR2DL3	0.12	< 0.1
SNP_A-2214036 - HLA-DQA1L	SNP_A-2200530 - TAP2	0.11	< 0.1
SNP_A-8451478 - TAP2	SNP_A-8415280 - TAP2	0.10	< 0.1
SNP_A-2305613 - CSTB	SNP_A-1944939 - CSTB	< 0.1	1.00
SNP_A-8566010 - HLA-DQA1L	SNP_A-1985650 - HLA-DOA	< 0.1	0.28
SNP_A-4223083 - HLA-DQA1L	SNP_A-8415280 - CIITA	< 0.1	0.18
SNP_A-4206711 - HLA-DQA1	SNP_A-8451478 - TAP2	< 0.1	0.16
SNP_A-4277940 - HLA-DQA1L	SNP_A-1985650 - HLA-DOA	< 0.1	0.14

This can be done by looking at the transcriptome of these tissues using publicly accessible data from the Gene Tissue Expression project (GTEx)^[19-21] and the TCGA^[8]. Data from both sources were processed with a common analytic pipeline that included realignment of sequencing reads to Hg38^[22,23], uniform count scoring^[24] and adjustment for over-dispersion^[25,26].

The scored transcript data was then evaluated using the novel pathway analysis tool PathOlogist^[27-29]. PathOlogist utilizes the logical information contained within networks to compute network scores. By utilizing the structure of a network, in this approach the conditional state of genes determines expectations for the state of other members of the network. Two different scores are provided. The first assesses whether the activity state of the network differs. In the second, an assessment of the logical state of the network is measured as consistency. Consistency determines whether the transcription patterns follow the expected logic of the network.

Examination of the transcriptional state of “antigen processing and presentation” provides additional insight into the susceptibility findings. First, “antigen processing and presentation” activity is observed to be significantly higher in normal liver (GTEx) compared to TCGA tumor-adjacent (adjusted $P < 0.0001$) and tumor (adjusted $P < 0.0001$) while no difference is observed between tumor adjacent and tumor (adjusted $P = 0.87$). This suggests that individuals with HCC have a different “antigen processing and presentation” profile in both their non-tumor and tumor than normal liver.

No significant difference is observed between the consistency scores of normal liver (GTEx) and TCGA tumor-adjacent (adjusted $P = 0.64$) and tumor adjacent and tumor (adjusted $P = 0.89b$) for “antigen processing and presentation”. However, significant difference is observed between normal liver and tumor (adjusted $P < 0.0001$). This suggests that “antigen processing and presentation” may be a target of mutagenesis in HCC.

IMMUNE CHECKPOINT THERAPY AND “ANTIGEN PROCESSING AND PRESENTATION”

“Antigen processing and presentation” may be an important mediator of treatment response for HCC. Immune checkpoint therapy is dramatically altering the cancer therapeutic landscape^[30]. Checkpoint therapy targets inhibitory signals to the immune system such as CTLA-4 and PD-1/PD-L1. These treatments show promising, durable response results in previously treatment resistant cancers such as melanoma^[31] and non-small cell lung cancer^[32]. The US FDA has approved checkpoint therapy for second line treatment of HCC. Numerous studies are in progress to assess the efficacy as 1st line treatment (clinicaltrials.gov).

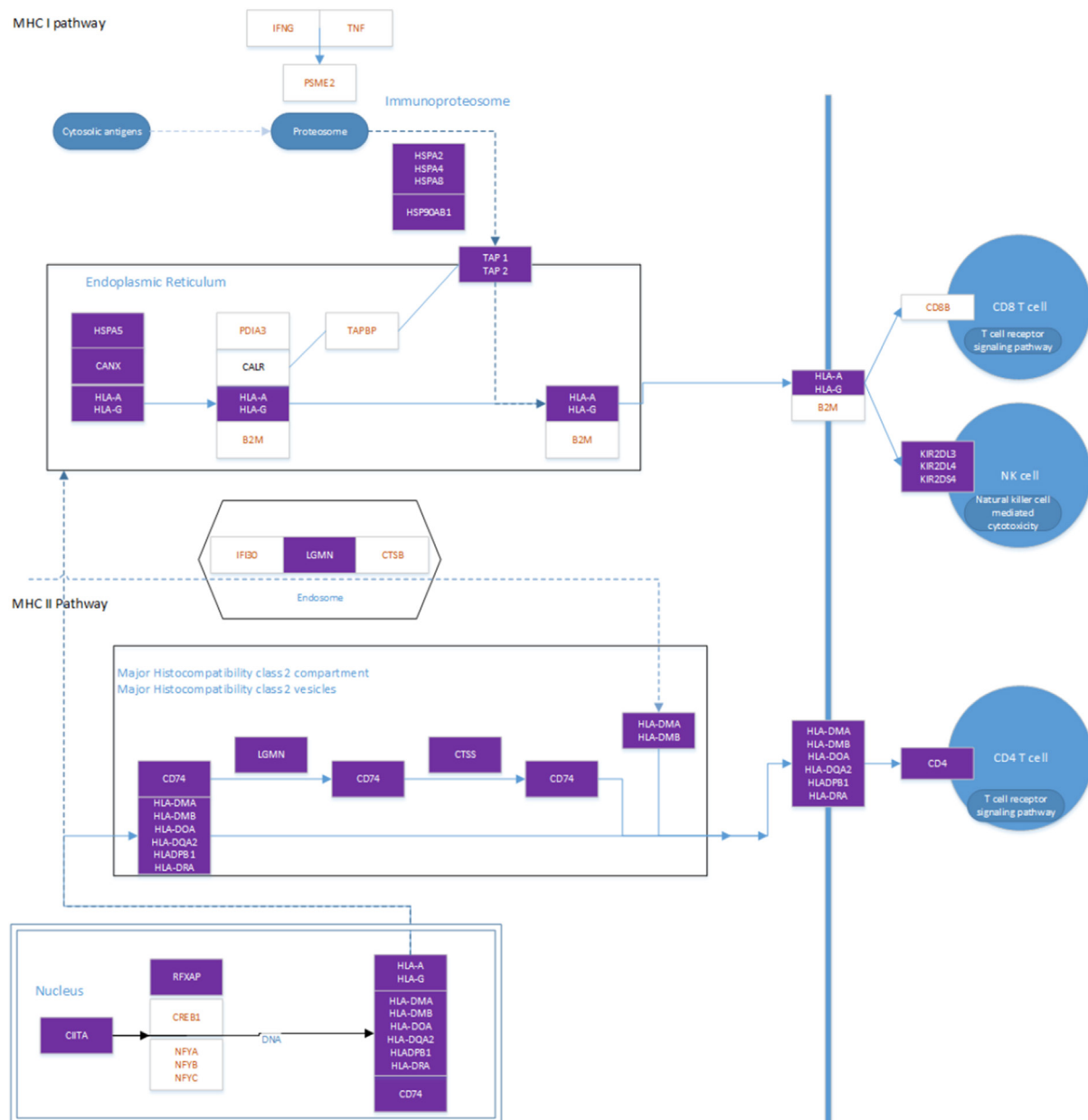


Figure 1. Gene-based SNPs associated with HCC in the antigen processing and presentation pathway. The genes and their relationships obtained from KEGG's antigen processing and presentation pathway. Purple boxes with white letters indicate genes SNP variations associated with HCC from the PoDA stepdown analysis. Removal of these loci reduced the overall threshold of significance below that observed for the entire pathway. Genes in open boxes (with orange letters) indicate genes which could be removed without altering significance of the pathway's association. HCC: hepatocellular carcinoma

Unfortunately only a minority of individuals respond to the treatments^[33]. It is unknown what mediates response. Indicators of response include DNA mismatch repair capabilities^[34] and tumor mutational burden^[35]. But these have poor predictive capabilities.

For checkpoint therapy to work, an intact immune response is required. As implied from the indicators of response, the immune system must have the capacity to recognize tumor antigens as foreign. This recognition is mediated through antigen processing and presentation. Inherited variability may indicate individuals in which this capacity is compromised. Moreover, variation in these processes may indicate individual response to immune directed therapeutic interventions.

In conclusion, the results of the germline variation studies suggest that immune mediating processes are polymorphic in the population and systematically different in HCC. Individuals with HCC have significantly lower activity for these processes and HCC shows alterations in the “logic” of the processing and presentation pathways. As such, it may be possible to predict response to checkpoint therapy through the evaluation of the inherited genetic state of “antigen processing and presentation”. Understanding these differences may provide opportunities designing new immune checkpoint modulators and provide a rational basis for combinatorial therapy.

DECLARATIONS

Acknowledgments

The Korean HCC case-control study was collected by Dr. Myung Lyu (NCI/NIH/DHSS) and Dr. Young-Hwa Chung (Asan Medical Center, Seoul, South Korea).

Authors' contributions

Data analysis: Lu YK, Brill JM, Aghili A

Design of the work, data analysis, manuscript drafting and revising, and final approval of the version to be published: Buetow KH

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Buetow KH is an advisor for the Bristol Myers Squibb IO-ICON project.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Time for hepatocellular carcinoma immunotherapy: insights for successful clinical applications in this challenging tumor

Gabriele Missale¹, Elisabetta Cariani²

¹Laboratory of Viral Immunopathology, Unit of Infectious Diseases and Hepatology, Azienda Ospedaliero-Universitaria di Parma, Parma 43126, Italy.

²Toxicology and Advanced Diagnostics, Ospedale Civile S. Agostino-Estense, Modena 41126, Italy.

Correspondence to: Dr. Gabriele Missale, Laboratory of Viral Immunopathology, Unit of Infectious Diseases and Hepatology, Azienda Ospedaliero-Universitaria di Parma, Parma 43126, Italy. E-mail: missalegabriele@gmail.com

How to cite this article: Missale G, Cariani E. Time for hepatocellular carcinoma immunotherapy: insights for successful clinical applications in this challenging tumor. *Hepatoma Res* 2018;4:22. <http://dx.doi.org/10.20517/2394-5079.2018.72>

Received: 1 Jun 2018 **First Decision:** 12 Jun 2018 **Revised:** 15 Jun 2018 **Accepted:** 19 Jun 2018 **Published:** 25 Jun 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

The multiplicity and phenotype of intratumoral immune infiltrate have been shown to influence the clinical outcome of hepatocellular carcinoma (HCC), thus providing a strong rationale to therapeutic interventions aimed at restoring the dysfunctional immune response against the tumor. Improving the knowledge of the complex interactions between transformed hepatocytes, nonparenchymal resident cells, and infiltrating immune cells (characterizing the HCC microenvironment) will be instrumental to increase the success rate of existing immunotherapeutic strategies and to identify new potential targets for intervention or biomarkers to optimize the selection of candidate patients.

Keywords: Hepatocellular carcinoma, immune checkpoint inhibitors, T lymphocytes, cytotoxic T lymphocytes, natural killer cells, macrophages, cytokines

INTRODUCTION

The liver immune landscape fosters tolerance towards foreign antigens driven by portal blood. Liver sinusoidal endothelial cells (LSECs) that separate liver parenchyma from sinusoidal blood, liver resident macrophages (Kupffer cells), hepatic stellate cells (HSCs), and dendritic cells (DCs) exert antigen presenting cell (APC) function and participate in the tolerogenic liver environment^[1]. Innate immune cells such as natural killer (NK), NKT and γ/δ T cells are found at higher frequency in the liver, as Foxp3⁺ regulatory T cells (Tregs). The liver environment is also characterized by increased expression of immunosuppressive cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)- β ^[2].



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Hepatocellular carcinoma (HCC) initiation and progression are multi-step processes profoundly influenced by the interplay between hepatocytes and immune cells. Immunotolerance is disrupted in chronic liver disease where persistent infections with hepatitis B virus (HBV) or hepatitis C virus (HCV), accumulation of fat, exogenous toxic substances (alcohol) or iron overload (haemochromatosis) enhance inflammatory signals triggering a cycle of cell death/regeneration and compensatory fibrosis, leading to liver cirrhosis, that represents a pre-neoplastic state. Chronic inflammation induces the accumulation of reactive oxygen species, generating epigenetic changes and chromosomal instability that contribute to tumor initiation, with expression of neo-antigens and/or deregulation of the expression of oncofetal and cancer testis antigens such as alpha-fetoprotein (AFP), glypican-3 (GPC-3), melanoma-antigen gene (MAGE) family, NY-ESO.1^[3].

IMMUNE RESPONSE AGAINST HCC

Cytotoxic T cells (CTLs) recognizing tumor-associated antigen (TAAs) have been detected in HCC patients and their abundance is associated with patient survival^[3-5]. CD8+ and CD4+ T cells were shown to accumulate in early HCC with a progressive decrease in late stages, that represents a negative predictor for disease outcome^[3,6]. TAA-specific CD8+ T cells from peripheral blood produce interferon (IFN)- γ upon stimulation, but tumour-infiltrating lymphocytes fail to do so, indicating the progressive exhaustion of intratumour CD8+ T cells^[3]. Exhausted T cells are characterized by impaired effector function and sustained expression of co-inhibitory receptors, and cannot mature into memory T cells.

NK cells account for 25%-50% of the total number of liver lymphocytes and are strongly implicated in the anti-tumor response. Impaired effector function of NK cells was reported in HCC and related to disease outcome^[7,8]. Several mechanisms have been implicated in NK cell dysfunction: the genetic make-up of KIR NK cell receptors^[9-11], a higher percentage of NK-cells co-expressing inhibitory NKG2A and activating NKp30-NKp46 receptors^[12,13], myeloid-derived suppressor cells (MDSCs)-mediated suppression^[14-16], increased prevalence of a dysfunctional CD11b^{neg}CD27^{neg} NK-cell subset^[17].

Among factors contributing to the immune suppressive microenvironment of HCC are cell-mediated mechanisms, the secretion of cytokines and chemokines by tumor, stromal, and infiltrating cells, and the immunoediting of TAAs^[18,19]. In this context, adaptive immune response exerts a dual role with seemingly opposite functions, being part of the inflammatory environment that likely plays a major role in tumor promotion, but hampering tumor dissemination through cytotoxic function against transformed cells^[20].

MECHANISMS OF IMMUNE IMPAIRMENT IN HCC

The development and progression of HCC evolve through a dynamic interaction between tumor cells, non-parenchymal resident cells such as Kupffer cells (KCs), HSCs, LSECs, infiltrating immune cells and immune mediators. All these elements participate in the tumor microenvironment that exerts a profound influence on the evolution of disease. The many factors that co-operate to the immune landscape of HCC represent potential targets for therapeutic intervention.

Immunosuppressive molecules

Immune checkpoints are coinhibitory molecules that control the duration and the strength of immune response to prevent over-activation of T cells. This class of molecules includes CTLA-4, PD-1, TIM-3, lymphocyte activation gene 3 protein (LAG-3) and B and T lymphocyte attenuator (BTLA). Immune checkpoints are exploited by tumors as mechanisms of immune evasion and may therefore become major targets of immune therapeutic strategies.

CTLA-4 is expressed by activated T cells and by Treg cells. It competes with the activating molecule CD28 for binding CD80 and CD86^[21] and activates Tregs^[22,23].

PD-1 is expressed by activated T and B lymphocytes, NK cells, Treg cells, MDSCs, monocytes and DCs^[24,25]. The expression of PD-1 is induced by several cytokines including IFN- γ ^[26,27]. Under hypoxic conditions, production of hypoxia-inducible factor (HIF)-1 induces the expression of PD-L1, the PD-1 ligand, in MDSCs and tumor cells^[22,27,28]. The interaction between PD-1 and PD-L1 inhibits T cell effector function and leads to T cell exhaustion^[29]. The immune infiltrate of HCC is enriched in PD1+ CD8+ cells and their abundance is associated with disease prognosis^[30]. The expression of PD-L1 in HCC has also been reported as a prognostic factor of shorter disease-free and overall survival^[31-34].

TIM-3 is a transmembrane protein expressed on various immune cells and interacting with multiple ligands among which galectin-9, a soluble protein expressed by several tissues including liver^[35] that negatively regulates Th1 cell function^[36]. In addition, TIM-3+ Treg cells exhibit enhanced suppressor activity^[37]. A role of the TIM-3/galectin-9 pathway in the determination of HCC-infiltrating T cell dysfunction has been reported^[38].

LAG-3 binds MHC class II molecules with high affinity^[25,39] thus reducing co-stimulatory function of DCs. LAG-3 is upregulated upon activation of T cells^[40] and is a marker of exhausted T cells^[41]. Its activation and, as a consequence, its blockade are synergistic with PD-1^[42,43]. BTLA is upregulated on activated lymphocytes and on tumour-specific CD8+ T cells in patients with cancer^[44]. High expression of the BTLA ligand HVEM (herpesvirus entry mediator) has been reported in patients with HCC and is associated with reduced lymphocyte infiltration and poorer prognosis^[45].

Cytokines are membrane-bound or secreted proteins involved in the regulation of immune cell function, inflammation and angiogenesis. Their pleiotropic roles include pro- and anti-inflammatory functions. CD4+ T helper cells produce either Th1 cytokines [e.g., interleukin (IL)-1, IL-2, IL-12, IL-15, tumor necrosis factor (TNF)- α and IFN- γ] usually defined pro-inflammatory, or Th2 cytokines (e.g., IL-4, IL-8, IL-10 and IL-5) mainly exerting anti-inflammatory functions^[46].

Increased levels of IL-10 and TGF- β and reduced levels of IFN- γ have been detected in plasma from HCC patients^[47]. In liver tissue IL-10 is produced by DCs, KCs, HSCs, LSECs, MDSCs and T cells, inducing tolerance^[48,49]. Tolerogenic effect of IL-10 is linked to inhibition of CD4+ T cell activation^[50] and, as a consequence, of cytotoxic CD8+ T-cell function^[51]. In addition IL-10 further interferes with T cell activation by downregulating the expression of MHC-II and CD80/CD86 on APCs^[52] and of NF- κ B^[53], a transcription factor strongly implicated in inflammatory responses. Despite its immunosuppressive activity in the context of inflammation, several studies report an immune-stimulatory role of IL-10 on CD8+ T-cell and NK-cell cytotoxic activity in experimental tumor models^[54-56].

TGF- β , produced by parenchymal and non-parenchymal liver cells, is implicated in the maintenance of liver immune homeostasis^[57] and may exert a suppressive function towards anti-tumor immune reaction. TGF- β inhibits the expression of the transcription factors T-bet and GATA3, essential for the conversion of naive CD4+ T cells into Th1 and Th2 CD4+ T cells, respectively^[58,59]. Conversely, TGF- β induces the differentiation of naive CD4+ T-cells into Tregs, inhibits the differentiation of naive CD8+ T cells to effector cells^[60,61] and decreases perforin and IFN- γ expression, further impairing cytotoxic CD8+ T-cell activity^[62].

Cell-mediated immune suppression

Immune evasion of tumor cells may be linked to altered antigen processing and presentation, deriving from HLA class I downregulation or from 2 microglobulin mutation/deletion^[63]. HLA class I expression is essential for antigen presentation to CTLs and for tumor cell recognition by NK cells^[64]. Tumor cell elimination by NK cells may be also impaired by decreased expression of the NKG2D ligand ULBP1 that correlates with early recurrence of HCC^[65]. Another mechanism of HCC immune evasion from NK cell killing has been

ascribed to the impaired interaction between NKG2D and its stress-induced ligands MIC-A and -B, that are upregulated on tumor cells. In advanced HCC, tumor cells escape from NK-mediated immunosurveillance through shedding of MIC-A that induces downregulation of NKG2D thus affecting NK cell effector function^[66].

Together with shared oncofetal and cancer-testis antigens, driver and passenger mutations occurring in the tumor cell genome can generate tumor-specific neoantigens that can contribute to tumor immunogenicity and represent potential immunotherapeutic targets^[67]. Like viral antigens, TAAs undergo immune selective pressure that triggers the selection of resistant variants with survival advantage due to lower immunogenicity or immunosuppressive activity. The genetic instability of transformed cells favors this phenomenon of antigenic immunoediting^[68]. Immune escape may also result from the secretion by HCC cells of immunosuppressive molecules as TGF- β , IL-10, indoleamine 2,3-dioxygenase (IDO), arginase, or from decreased co-stimulatory/increased inhibitory checkpoint signaling^[69].

MDSCs represent a heterogeneous population of immature myeloid cells^[70] that share suppressive functions^[71,72] through different mechanisms: depletion of arginine^[73] and cysteine^[74] that are essential for T cell function, and release of reactive oxygen and nitrogen species that disrupt TCR signaling^[75]. In addition, MDSCs promote tumor progression through neo-angiogenesis due to vascular endothelial growth factor (VEGF) production, and through enhanced tumor cell survival and dissemination^[76].

In HCC MDSCs have been shown to inhibit NK cell function via NKp30 receptor^[14] or through membrane-bound TGF- β ^[15] and to induce Tregs by IL-10 and TGF- β production^[77]. A specific CD14pos HLA-DRneg/low MDSC subset increased in tumor tissue and peripheral blood of patients with HCC was implicated the induction of Tregs^[77]. The multiplicity of this MDSC subset was reported as a negative prognostic factor for HCC recurrence after resection^[78], radiation therapy^[79], hepatic arterial infusion chemotherapy^[80], as well as for tumor progression^[81].

MDSCs are recruited by cytokines and chemokines secreted by tumor cells^[72,82]. Senescent hepatocytes were shown to recruit immature MDSCs able to differentiate into macrophages through C-C motif chemokine ligand 2 (CCL2)-CCR2 signaling, thus preventing HCC initiation. However, in the presence of HCC, immature MDSCs do not differentiate thus contributing to the immunotolerant environment through NK-cell inhibition^[83].

Kupffer cells (KCs), the liver resident macrophages, represent about 80% of the macrophages in the body^[84] and contribute to the maintenance of liver immune tolerance through their anti-inflammatory function^[85] exerted by upregulation of PDL-1 expression, downregulation of costimulatory molecules^[86], secretion of IDO^[87] and IL-10^[88]. In human HCC, Kupffer cells in the peritumoral margin express higher levels of PDL-1 compared to non-tumorous liver, thus inhibiting CD8+ T cell effector function. Blockade of PD-1/PDL-1 interaction *in vitro* was able to restore T cell killing *in vitro*^[89].

The HCC immune microenvironment induces the polarization of macrophages towards the M2 phenotype typical of the tumour-associated macrophages (TAMs). M2 macrophages are characterized by producing high levels of IL-10 that induce Treg expansion and impairs NK cell activation^[90]. In addition, TAM promote tumor angiogenesis and dissemination^[91,92]. A distinct subset of monocytes expressing TIE2 with enhanced pro-angiogenic properties has been described in peripheral blood and in tumor infiltrate^[93-95]. In human HCV-related HCC this monocyte subpopulation was related to neo-angiogenesis and to prognosis^[96].

Tregs are CD4+ T cells expressing CD25, CTLA-4, CD62L and FoxP3. Tregs exert inhibitory functions through multiple mechanisms, among which IL-2 depletion by CD25 (IL-2 receptor), competition with

CD28 by CTLA-4, CTLA-4-mediated downregulation of CD80 and CD86^[97], expression of TGF- β and IL-10^[98]. The recruitment of Tregs in HCC occurs via the CCR6-CCL20 axis^[99] and CCL22 induction by tumor cell-secreted IL-1 α ^[100]. In addition, FoxP3 upregulation and conversion of CD4+ T cells into Tregs may be fostered by poor stimulation of naive CD4+ T cells combined with TGF- β signalling by tumor cells^[101].

In patients with HCC, FoxP3+ Tregs are increased both in peripheral blood^[102,103] and in tumor tissue^[31,103], and the abundance of tumour-infiltrating Tregs is associated with intra-tumoral macrophages^[104]. Several studies support a negative correlation between Treg infiltrate and effector function of intra-tumoral CD8+ T cells^[47,103] and a direct role of Treg infiltration over disease progression and overall survival^[99,103-105].

HSCs play a role in HCC progression through release of hepatocyte growth factor^[106] and induction of both MDSC^[107,108] and Treg accumulation^[109]. In addition, HSCs can also directly induce T cell apoptosis through PD-L1 expression^[110]. Activated HSCs interact with monocytes inducing an immunosuppressive environment and contributing to poor prognosis in HCC^[111].

NKT cells are a heterogeneous group of T lymphocytes sharing properties of both T cells and NK cells. NKT cells recognize glycolipid antigens via an invariant TCR α chain. The CD4+ iNKT-cells have been found to be enriched in intrahepatic malignant tumors^[112]. Intra-tumoral CD4+ iNKT-cells produce Th2 cytokines that can inhibit expansion of tumor antigen-specific CD8+T-cells^[112]. Consistent with this view, we observed that enrichment of iNKT cells in HCC infiltrate was predictive of shorter TTR^[31].

Several other infiltrating or stromal cell types co-operate to the generation of immunosuppressive tumor microenvironment^[113]. A population of PD-1-positive B cells has been identified in HCC. This cell subset was shown to suppress anti-tumor T cell response through PD-1-PDL-1 interaction and to promote disease progression^[114].

LSECs express PDL-1 and contribute to the immunosuppressive environment by TGF- β -dependent induction of Tregs^[115]. A subset of CD14+ DCs with suppressor function has been detected in patients with HCC. These DCs expressing high levels of CTLA-4 and PD-1 inhibit T-cell response through production of IL-10 and indoleamine-2,3-dioxygenase (IDO)^[116]. Th17 cells are a IL-17-producing CD4+ T cell subset that plays an important role in the maintenance of mucosal barriers. Increased frequency of CCR4+CCR6+, but not CCR4-CCR6+ Th17+ cells was reported in peripheral blood from patients with HCC. The CCR4+CCR6+Th17+ cell subset was shown to impair CD8+ T cell effector functions^[117]. Neutrophils release cytokines that contribute to the tumor microenvironment either promoting or inhibiting tumor progression^[118]. In HCC, neutrophils have been shown to recruit macrophages and Tregs fostering tumor progression and resistance to sorafenib^[119]. Tumor-associated fibroblasts (TAFs) are essential components of the HCC microenvironment and support tumor progression through the secretion of various cytokines and growth factors. HCC-associated TAFs inhibit NK-cell function by secreting prostaglandin E2 and IDO^[120]. In addition, TAFs have been shown to release IL-6 and SDF-1 α (CXCL-12), which induce MDSC generation and activation thus impairing anti-tumor immune response^[121].

IMMUNOTHERAPEUTIC STRATEGIES

The scenario of the immune mechanisms operative in HCC is quite complex with liver intrinsic immunosuppressive environment associated with several mechanisms common to solid tumors. Physiologically, with the exception on anecdotal cases the tumor will escape, avoid, adapt to or overcome the immune mediated mechanism aimed at rejection of transformed cells. From the therapeutic perspective the problem has been approached by several strategies focusing on potentiation of different effector immune cells, tumor antigens made immunogenic, block of negative costimulatory pathways or immunosuppressive cells or soluble mediators.

Adoptive cell transfer

Adoptive transfer of autologous cytokine activated killer cells (CIK) has been one of the first immunotherapeutic approaches^[122]. Anti-CD3 antibodies in the presence of IL-2, IL-1 and IFN- γ expand and activate *ex vivo* NKT-cells that are reinfused in the patient. This approach has been performed as adjuvant treatment in patients undergoing liver resection for HCC or percutaneous ablative treatments like percutaneous ethanol injection (PEI) or transarterial chemo-embolization (TACE). A systematic review of phase II and III studies conducted in patients undergoing CIK infusion either alone or associated with resection, PEI or TACE, showed a significant effect on overall and progression free survival^[123]. More recently a randomized controlled trial of adjuvant CIK was conducted in patients undergoing curative liver resection showing a significant effect on TTR but no effect on DFS and OS^[124]. Another randomized phase 3 study from Korea could demonstrate that patients receiving CIK post-resection, PEI or radiofrequency thermal ablation (RFA) had a significantly increased recurrence-free and overall survival^[125]. Several other studies with similar methodological approach are ongoing in patients with HCC and other solid tumors.

Cancer vaccines

More than 15 years ago, discovery of TAAs raised enthusiasm on their possible use for vaccination strategies. Several different approaches have been employed from tumor lysates to individual epitopes associated with different adjuvants by parenteral route (subcutaneous, intradermal or intravenous), or intratumoral injection. Alternatively DCs pulsed with synthetic peptides or transfected with RNA vectors have been used to expand tumor-specific T-cell response. Target antigens for HCC have been cancer testis TAAs like MAGE, synovial sarcoma X breakpoint 2 (SSX-2) and NY-ESO-1, beside GPC-3, human telomerase reverse transcriptase (TERT), carcinoembryonic antigen (CEA) and AFP. Clinical trials have been conducted in patients with advanced or non-resectable HCC or as an adjuvant treatment in patients undergoing resection or RFA or TACE. Efficacy in these studies has been limited. Phase II studies with antigen-pulsed DCs^[126], intradermal GPC-3 peptide^[127], or intravenous tumor (HepG2 cell line) lysate-pulsed DCs^[128] have been conducted showing partial response associated with antigen-specific T-cells responses in PBMCs in some patients. In particular, antigen-pulsed DCs vaccination^[126] showed no tumor recurrence up to 24 weeks in 9 out of 12 treated patients. Phase I GPC-3 studies achieved their aims: safety, immunogenicity and dose finding, however phase II studies with GPC-3 aimed at relapse prevention after curative treatments (surgery or RFA) failed to achieve clinically relevant results^[129,130]. Infusion of autologous DCs pulsed with lysate of HepG2 cell line was performed in a phase II trial in patients with advanced HCC. Clinical response (either stable disease or partial response) was shown in 28% of patients performing at least three infusions. Treatment was safe and antigen-specific immune response could be demonstrated in some patients^[128].

A particular vaccination strategy has been conducted with an oncolytic, genetically modified vaccinia virus (JX-594) that has been injected in the tumor lesions of advanced HCC patients. The rationale of this approach is the release of tumor antigens from oncolytic tumor cells destruction associated with local expression of granulocyte-macrophage colony stimulating factor (GM-CSF), an inserted gene of the genetically modified vaccinia. JX-594 has been tested in a dose finding study showing improved overall survival in patients receiving a higher infectious dose compared to lower dose^[131]. A phase II trial failed to achieve survival advantage. In this trial however HCC patients were very advanced having progressed to sorafenib treatment. A phase III randomized clinical trial (NCT02562755) is now ongoing, comparing patients on sorafenib to patients undergoing three vaccination rounds followed by sorafenib treatment.

Table 1 represents a summary of completed clinical trials based on adoptive cell transfer and vaccines.

Cell therapy

More efficient adoptive cell transfer immunotherapeutic approaches, are represented by the CAR T-cell therapy which until now has been primarily used in hematologic malignancies. T cells are genetically engineered to express chimeric antigen receptors (CARs). Autologous engineered T cells are expanded *ex vivo* into the

Table 1. Adoptive cell transfer and vaccines for HCC immunotherapy

Therapeutic approach	Target	Phase	Study population	No. of patients	Results	Ref.
CIK	NA	II	Post-resection	76 + 74 controls	Improved RFS	[122]
CIK	NA	III	Post-resection	100 + 100 controls	Improved TTR	[124]
CIK	NA	III	Post-resection or RFA or PEI	114 + 112 controls	Improved RFS and OS	[125]
Peptide vaccine	GPC-3	I	Advanced HCC	11	Improved CTL response	[129]
Peptide vaccine	GPC-3	II	Post-resection or RFA	41	Improved RFS for patients with GPC-3 positive tumors	[130]
DC pulsed HepG2 protein lysate	Tumor antigens	II	Advanced HCC	35	PR 4%, SD 24%	[128]
DC pulsed AFP, MAGE-1 and GPC-3	Tumor antigens	I/II	Post-resection or RFA or PEI or TACE	12	Improved TTP vs. historical results	[126]
Oncolytic virus JX-594	Tumor antigens	II	Advanced HCC	30	Dose related improved OS	[131]

HCC: hepatocellular carcinoma; RFS: recurrence free survival; TTR: time to recurrence; TTP: time to progression; OS: overall survival; RFA: radio-frequency ablation; PEI: percutaneous ethanol injection; TACE: transarterial chemo embolization; CTL: cytotoxic T lymphocytes; NA: not applicable

hundreds of millions and finally are infused in the patient. Third generation CARs are constituted of an immunoglobulin variable heavy chain (VH), a variable light chain (VL) connected to a transmembrane domain by a spacer and the transmembrane domain to 2 costimulatory molecules (e.g., CD27, CD28, 4-1BB, OX40) and CD3. This receptor when engaged can activate the effector cytotoxic T-cell, specifically redirected to the tumor antigen recognized by the VH and VL chains. As far as HCC, CAR-T have been designed with different specificities and phase I and phase I/II clinical trials are recruiting for patients with HCC or HCC and other solid tumors, targeting GPC-3, CEA and Mucin 1, cell surface associated (MUC-1)^[132].

A different approach that engages T and NK-cells *in vivo* to direct them against tumor cells is represented by bispecific antibodies (BsAb). BsAb against HCC and other solid tumors have been generated with different specificities. One arm of antibody binds a tumor antigen [GPC-3, epithelial cell adhesion molecule (EpCAM), osteopontin, VEGF] and the second can activate cytotoxic T or NK cells binding CD3 or CD16. A phase 1 dose escalation trial with BsAb specific for GPC-3 and CD3 is ongoing (NCT02748837).

Another approach to generate tumor-specific immune cells is cloning and TCR transfection of T and NK cells that are *in vitro* expanded and reinfused in the patients. These redirected effector cells, differently from CAR-T or BsAb, recognize tumor epitopes in the context of specific HLA-class I molecules, but have advantage to recognize endogenously processed antigens, that is the case of many known epitopes from tumor associated antigens or neo-antigens from somatic mutations of the tumor-cell. In fact, cell therapies based on CARs and antibodies can only recognize conformational antigens expressed on the surface of transformed cancer cells. Redirect T-cells have been clinically tested in a patient that developed extra-hepatic metastasis after liver transplantation for HCC in HBV-related liver disease^[133]. The tumor, but not the transplanted liver, expressed HBV antigens and autologous T-cells transfected with a TCR specific for HBsAg could expand *in vivo* and determine reduction of HBsAg serum levels.

Immune checkpoint inhibitors

The first clinical study on immune checkpoint inhibitors (ICIs) in HCC has been a phase II clinical trial targeting CTLA-4 in patients with advanced tumors in HCV chronic liver disease^[134]. The study showed partial response in 17.6% of patients and a good safety profile. Transaminase flares were observed in some of the patients after the first anti-CTLA-4 administrations that however did not require any immunosuppressive intervention. Interestingly in this study an enhanced HCV-specific T-cell response associated with significant drop of HCV viremia was observed. Several other studies have started. The main target has been PD-1 and its ligand PD-L1 and recently FDA has granted accelerated approval for anti-PD-1 in patients that had been previously treated with sorafenib, based on the phase I/II Checkmate-040 study (that showed an overall

Table 2. Immuno check points inhibitors for HCC immunotherapy

Target	Number of patients	Trial	First line/Second line	Status	Results	Ref or study number
CTLA-4	20	Phase II	Adjuvant TACE and ablation	Completed	PR 17.6 %	134
CTLA-4	32	Pilot	Second	Completed	PR 15.6 %	137
PD-1	576	Phase I/II	First and Second	Completed	PR 20 % (expansion) 15% (dose escalating)	135
PD-1	723	Phase III	First vs. sorafenib	Not recruiting active	NA	NCT02576509
PD-1	660	Phase III	First vs. sorafenib	Recruiting	NA	NCT03412773
PD-1	104	Phase II	Second	Not recruiting active	PR 15.4 %, CR 1%	NCT02702414
PD-1	408	Phase III	Second	Not recruiting active	NA	NCT02702401
PD-1	530	Phase III	Adjuvant SR and ablation	Recruiting active	NA	NCT03383458
PD-L1	114	Phase I	Second	Recruiting active	NA	NCT02519348
PD-L1 ± CTLA-4	440	Phase II	Second	Recruiting active	NA	NCT02519348
PD-L1 ± CTLA-4	1200	Phase III	First	Recruiting active	NA	NCT03298451

HCC: hepatocellular carcinoma; SR: surgical resection; TACE: transarterial chemoembolization; NA: not available; PR: partial response; CR: complete response

response rate of 18.2% and acceptable safety profile)^[135]. There was concern on possible immune mediated liver toxicity in patients with liver cirrhosis and chronic HBV or HCV infection. However, until now safety profile of ICIs has not shown to be different from what observed for melanoma and non-small cell lung cancer (NSCLC) and even if substantial transaminase flares have been described, patients coming off therapy for adverse events are in line with what observed for other cancers treated with anti-PD-1 or anti-PD-L1^[136].

First line studies comparing ICIs to sorafenib treatment are ongoing in patients with advanced HCC: two studies from different companies, CheckMate-459 and NCT03412773 with anti-PD-1 and the HIMALAYA study testing the combined activity of an anti-PD-L1 and anti-CTLA-4. The adjuvant role of ICIs is also tested with anti-PD-1 versus placebo in patients with early stage HCC undergoing surgery or ablation evaluating relapse free survival as primary endpoint (NCT03383458). A study combining RFA, cryablation or TACE and-CTLA-4 in advanced HCC has been recently published^[137]. Subtotal ablative treatments were given after the second anti-CTLA4 infusion. The study demonstrated feasibility and no dose-limiting toxicity of this therapeutic approach. Moreover 5/19 evaluable patients presented partial response. Interestingly pre and post-treatment biopsy showed an enrichment of CD3 and CD8 positive T-cells infiltrating the tumor after treatment that positively correlated with clinical response. Table 2 represents a more comprehensive list of completed and ongoing clinical trials with ICIs.

Until now it is not possible to understand which immune checkpoint is the most promising for HCC patients. Experience from other solid malignancies suggests that combining different ICIs may improve clinical response, given the increased risk of severe toxicities. Vaccination protocols combined with ICIs are tested in clinical trials, representing an alternative treatment strategy expanding tumor-specific T-cell populations *in vivo*^[138].

Another immunotherapeutic approach that cannot be strictly considered an ICI is represented by an anti-TGFβRI (Galunisertib) that is expected to block the immunosuppressive and pro-tumorigenic effect of TGF-β. It has been tested in association with sorafenib in a phase II clinical trial (NCT01246986) showing a median overall survival of 17.9 months that represents an improved survival compared to sorafenib historical results.

PREDICTIVE BIOMARKERS

Although promising, the results of immunotherapy for HCC are far from optimal. Recent trials suggest that combined regimens with different ICIs would lead to higher rates of clinical response, but with increased

risk of immune-related adverse events. The development of biomarkers with acceptable predictive value will be instrumental to maximize the benefit of immunotherapy.

As previously described, the anti-cancer immune response is the results of multiple factors deriving from the antigenic characteristics of the tumor, the multiplicity and the phenotype of TAA-specific immune cells, the latter mainly dictated by the tumor microenvironment. From this perspective the use of a single analyte biomarker might not be sufficient to recapitulate the complex interplay between tumor biology and immune response. Immunostaining with anti-PD-L1 antibodies has been the first approach evaluated to predict the response to anti-PD-1 treatments. However, this marker was shown to be unreliable especially for its poor negative predictive value^[139,140]. In addition, the intrinsic variability of immunohistochemistry together with the heterogeneity and dynamic nature of PD-L1 expression in tumor and immune cells raise concern about its adequacy to clinical standards^[141].

Multianalyte profiles may represent promising tools for the accurate prediction of immunotherapy outcome. The response to PD-1 blockade has been related to the presence of immunogenic neoantigens arising from the active expression of viral genes or from increased tumor mutational burden^[140,142]. According to this view, pembrolizumab (anti-PD-1) was approved by FDA in 2017 for the treatment of unresectable or metastatic solid tumors with mutations in genes for DNA mismatch repair (dMMR) or microsatellite instability (MSI), independently from the tissue of origin. However, dMMR or MSI are infrequent in HCC^[143].

The multiplicity, composition, activity and location of tumor-infiltrating immune cells have been shown to represent prognostic markers in HCC^[4-6,104]. A subset of HCCs characterized by an inflammatory gene signature has been detected in several studies^[144-148]. A recent study identified in about 25% of patients an immune-specific molecular class of HCC including two distinct subtypes, characterized by a prevalent adaptive T-cell response and an exhausted immune response, respectively^[146]. Interestingly, immune gene profiles suggesting active anti-tumoral response have been associated with longer time to recurrence^[149,150].

Gene signatures may provide a global picture of the complex tumor immune landscape. This approach represents a tool for the discrimination of tumors with pre-existing immune infiltrate, more likely to respond to interventions aimed at overcoming inhibitory factors. In a recent paper a so-called “T cell-inflamed gene expression profile”, containing IFN- γ -responsive genes related to antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance, was shown to be necessary, but not always sufficient, for clinical response to pembrolizumab in 10 tumor types^[151].

The lack or low abundance of cellular infiltrate may indicate a defect in innate immunity or in immune cell trafficking and suggest alternative therapeutic approaches. Consistent with observations made in other tumors^[152], a molecular profile of “immune exclusion” is associated with activated Wnt/ β -catenin pathway signaling in HCC^[149]. This suggests the potential of Wnt/ β -catenin activation as a biomarker predictive of resistance to checkpoint inhibitors. As a future perspective, gene signatures integrating information about tumor cell mutational burden, presence and nature of the immune infiltrate, possibly at different investigational levels (genetic, genomic, epigenetic) would provide information for a comprehensive therapeutic stratification of HCC patients.

DECLARATIONS

Authors' contributions

The authors contributed equally to the conception, design, writing, and revision of this review article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Molecular targeting of antiviral drugs used against hepatitis C virus infection

Mohammad Irshad¹, Priyanka Gupta¹, Khushboo Irshad²

¹Clinical Biochemistry Division, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi 110029, India.

²Department of Biochemistry, All India Institute of Medical Sciences, New Delhi 110029, India.

Correspondence to: Prof. Mohammad Irshad, Clinical Biochemistry Division, Department of Laboratory Medicine, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India. E-mail: drirshad54@yahoo.com

How to cite this article: Irshad M, Gupta P, Irshad K. Molecular targeting of antiviral drugs used against hepatitis C virus infection. *Hepatoma Res* 2018;4:23. <http://dx.doi.org/10.20517/2394-5079.2018.25>

Received: 21 Mar 2018 **First Decision:** 25 May 2018 **Revised:** 11 Jun 2018 **Accepted:** 11 Jun 2018 **Published:** 26 Jun 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Present study reports an update on the molecular interaction of antiviral drugs with viral and host cell components during hepatitis C virus (HCV) infection. In addition to the traditional therapeutic drug regimen, termed as standard of care, some recent drugs have been added in the existing regimen used for HCV infection. These drugs were categorized as direct-acting antivirals (DAAs) agents and “other agents”, with their efficacious impact in the control of HCV infection. They target both viral proteases and host cell receptor proteins/enzymes involved in HCV entry into the cell, replication, and assembly to check their propagation both *in situ* as well as in cell to cell transmission. Recent studies have reported a significant rise in sustained virological response after the use of these drugs both alone and in combination with pegylated interferon- α (PegIFN- α) plus ribavirin. Recently, DAAs have been reported to be highly effective in eradication of HCV infection, especially liver cirrhosis, reducing but not avoiding the occurrence of liver cancer. Some studies have demonstrated that the presence of resistant HCV variants, arising during viral replication, may be controlled by the new drug regimen. It is important to note here that all these drugs are influenced by viral as well as host factors including basic viral load, HCV genotypes, IFN action, interleukin 28B polymorphism and some liver and metabolic diseases, *etc.* This is an area with on-going investigations to explore more antiviral agents that may address new challenges in HCV therapy.

Keywords: Hepatitis C virus, interferon, pegylated interferon, direct-acting antivirals, sustained virological response, drug-resistance

INTRODUCTION

Hepatitis C virus (HCV) infection is a known cause of serious liver diseases recorded worldwide. Majority of infections are asymptomatic and in about 80% of cases, the virus persists without the patient's



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awareness. HCV infection causes both acute as well as chronic liver diseases including cirrhosis of liver and hepatocellular carcinoma. Globally, HCV infection affects nearly 180 million people^[1] which account for 3% population of the world. Approximately 3 million new cases are added to this population every year^[2,3]. A high proportion of HCV infected patients develop chronic liver diseases and nearly 20% of them progress to cirrhosis and about 10% to liver cancer^[4,5] in later stage. The presence of HCV infection, though varies from region to region, has been noted throughout the world. Hepatitis B virus (HBV)-based prevention and control measures for viral hepatitis have achieved remarkable results, and hepatitis C has relatively little awareness. Efforts have been made to develop effective prophylactic and therapeutic measures for treatment of chronic HCV infection. There is a common belief now that HCV infection needs more attention even than human immunodeficiency virus (HIV) infection in terms of its early detection and timely remedies since both of them do not have any vaccine for prevention. Moreover, the disease burden caused by HCV is also more serious even than HIV. A high genomic variability in HCV has led to development of at least seven genotypes and many isotypes.

HCV is an RNA virus with about 9.6 kb genome. This is a single stranded, enveloped virus with positive polarity and has been categorized under flaviviridae family. Its genome has a single ORF encoding for polypeptides of 3011 amino acids. The 5'UTR region has an internal ribosomal entry site (IRES) which is involved in HCV replication. Using host and viral proteases, HCV polyprotein is cleaved into three structural proteins (Core, E1 and E2) and seven non-structural proteins (P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B)^[6]. HCV-core forms viral nucleotide that has significant role in viral pathogenesis^[7] and E1 and E2 proteins are involved in viral entry into the cell^[8]. The P7, a 63-amino acid protein, helps in translocation of NS2 into endoplasmic reticulum and also in viral assembly and release of HCV virions^[9,10]. The NS2 peptide is a transmembrane protein which plays role in viral replication. The NS3, on the contrary, is a protease and acts as ATPase/helicase^[11,12]. Usually, HCV protease disrupts interferon (IFN) and toll-like receptor-3 (TLR-3) signaling pathways. The NS4A acts as a cofactor for NS3 protease, the NS4B is needed to recruit other viral proteins^[13,14] and NS5A, a phosphoprotein, plays role in viral replication^[15,16]. The last non-structural protein i.e. NS5B is an HCV RNA dependent RNA polymerase (RdRp) which also participates in RNA replication^[17].

The studies available in last few decades have elucidated the virus specific events in infected cells. In order to use these events as targets for chemotherapy, some antiviral agents were developed and used to treat HCV infection on a line similar to the one used for other viral infections. This targeting is aimed to suppress virus reproduction without an adverse effect on the host-cell. There are a number of virus specific processes within virus replicative cycle in an infected cell that may be targeted for chemotherapeutic intervention. The major target steps include virus entry into the cell, reverse transcription, viral DNA/RNA polymerization and the reactions involved in viral DNA/RNA synthesis *etc.* At present, a variety of agents including nucleosides and non-nucleosides entities have been developed which interact with virus targets and inhibit virus replication. In case of treating HCV infection, today a variety of agents are available for use. In addition to the virus-specific events, there are several host enzymes and processes that are closely associated with viral DNA, RNA or protein synthesis. These processes may also be the targets for antiviral agents.

The recommended treatment for HCV infection includes a combination therapy with PegIFN and ribavirin^[18]. However, recently several new regimens have been evolved for treatment of HCV infection. The drugs including direct-acting antiviral agents (DAAs) like boceprevir or telaprevir as protease inhibitors have provided a new promise to aim the HCV treatment. This therapy improves sustained virological response (SVR) in patients infected with HCV genotype-1 by more than 70%. Moreover, it has an additional significance of little chances of development of drugs resistant variants^[19-21]. Several other DAAs are in clinical trials today and have been evaluated for combination therapy^[22]. The emerging new antivirals need a new trial for serious liver diseases, particularly, in those cases with poor response to current regimens^[23,24].

Present study gives an update on the availability and action of therapeutic agents targeting various steps of HCV viral life cycle and infected host cell processes that may be disrupted to check viral reproduction and underlying pathological reaction cascade. It also describes the comparative efficacies of different agents and the future of HCV- treatment under the use of these agents.

TYPES OF DRUGS

In a common practice, the combination of pegylated interferon- α (PegIFN- α) and ribavirin, is used for the treatment of HCV infection^[18]. The addition of DAAs, like boceprevir, ortelaprevir, in the drug regimen, has brought a new change in the status of HCV treatment. This regimen improves the SVR to a significant level even in genotype-1 infected patients^[19]. IFN and ribavirin can cause patients with flu-like symptoms, cognitive dysfunction, thyroid dysfunction and other adverse reactions, leading to premature termination of treatment in some patients. However DAAs were found to develop drug resistant variants^[20,21]. Subsequent studies introduced the next generation DAAs like simprevir and sofosbuvir, that were approved by FDA for treatment of HCV infection^[21,25]. An interferon free drug regimen comprising ombitasvir, paritaprevir, ritonavir and dasabuvir has been approved for HCV genotype-1 infected patients. Now it is believed that the new drug combination may consist of interferon free regimen with high viral killing efficiency, short therapy time and less adverse effect. The development of drugs and their different combinations for an effective therapy against HCV is under investigation for last several years. Some new drugs developed and used in recent past are described in Table 1. These drugs are used both alone as well as in combination to other drugs. Based on their nature, action and host response, these drugs have been classified under different categories:

Interferon

PegIFN- α , a commonly used drug increases the SVR rate by causing a delay in renal clearance. Human albumin-IFN- α (Albinterferon) is a fusion protein. This protein is used for the treatment of HCV infection. Different reports have shown that the SVR rate arising from the use of Albinterferon and Ribavirin was nearly the same as noted with use of the SOC treatments^[26,27]. Similarly, IFN- λ which is a class-III interferon, is also used for the treatment of HCV infection. The receptors of IFN- λ are mainly present in the liver and therefore very minimal extrahepatic adverse effects were recorded with the use of IFN- λ in comparison to IFN- α ^[28].

Direct acting antiviral agents

This is the class of drugs acting against viral and host proteins involved in HCV life cycle. The major inhibitors of NS3 viral protein are telaprevir and boceprevir. Telaprevir was approved and recommended for use with PegIFN- α and ribavirin in genotype-1 patients. This was classified as triple therapy. Since telaprevir treatment is reported to be effective against the resistant mutants in the short term duration, it was decided to use it for long-term and subsequently approved for the treatment^[29]. It is important to note here that the long term use of these drugs often leads to drug resistance including T54A/S, R155K/T, V36A/M, V55A, and A156/S/T/V, *etc.* Simeprevir is another NS3 protease inhibitor classified as second generation drug. This drug is a reversible inhibitor of NS3/4A protease^[30]. Danoprevir and faldaprevir are also second-generation HCV NS3/4A protease inhibitors and used in patients infected HCV genotype-1. In addition to these drugs, there are various other NS3 protease inhibitors like Vaniprevir (MK-7009), Narlaprevir (SCH 900518), Asunaprevir (BMS 650032), VX 985, and MK-5172 which are used for treatment of HCV infection. There is every possibility that these drugs may be approved for therapeutic use against HCV infection^[29].

Daclatasvir (BMS) 790052 was found to inhibit NS5A, a protein involved in HCV replication and therefore used as a drug for control of HCV infection. This particular drug has a broad genotype antiviral activity. In addition, other NS5A inhibitors include Ledipasvir (GS-5885), ABT 267, IDX791, and ACH-2928 *etc.* NS5B is a RNA-dependent RNA polymerase (RdRp) involved in HCV replication. This NS5B enzyme activity is inhibited by two categories of inhibitors that are nucleoside/nucleotide derivative inhibitors (NIs) and non-

Table 1. Mechanism of drug action to control HCV infection

Site of action (target)	Drugs	Mechanism of action
Viral entry		
Attachment	Lectin cyanovirin-N, BA-LNC, Ficolin, Heparin and heparin-derived compounds, Heparanase, EGCG and its derivatives, Lactoferrin, A p7 ion channel-derived peptide H2-3	Inhibits attachment factors reducing concentration of virions on cell surface
Post-binding interactions with entry factors		
CD81	Imidazole-based compounds, Anti-CD81 mAbs, Soluble CD81 LEL	Inhibits viral binding with entry factors
SRB1	Serum amyloid A, Anti-SRB1 pAb and mAb, ITX5061	
CLDN1	Anti-CLDN1 peptides, Anti-CLDN1 pAb and mAb	
EGFR	Erlotinib	
EphA2	Dasatinib	
TfR1	Anti-TfR1 mAbs, Ferristatin	
NPC1L1	Anti-NPC1L1 mAbs, Ezetimibe	
Clathrin-mediated endocytosis	Chlorpromazine, Arbidol	
Fusion and uncoating		
Endosome acidification	Concanamycin A, Bafilomycin A Chloroquine, Ammonium chloride	Reduces acidification of endosome required for membrane fusion between virus and host cell
Lipid composition of virus or host cell	Arbidol, Phenothiazines, RAFIs (aUY11), LJ001, Silymarin	Reduced fusion efficiency of HCV particles
Unclear mechanism	Ferroquine, PS-ONs	Exact mechanism not elucidated
Natural compounds and small molecules	Flavonoids, Terpenoids, Tannic acid, Gallic acid, PF-429242	
Viral replication		
	Interferon	IFN-alpha declines HCV RNA level
	PegIFN- α , Human serum albumin IFN- α , PegIFN- λ -1a	
	Ribavirin (Nucleoside analogue)	Mechanism unclear
	DAA's	
Viral protein	Telaprevir, Boceprevir, Faldaprevir, Simeprevir, Asunaprevir, Paritaprevir, Danoprevir, Grazoprevir, Vaniprevir, TMC435	Inhibits NS3/4A proteases involved in viral replication
NS3/4A		
NS5A	Daclatasvir, Ledipasvir, Ombitasvir, Elbasvir, Velpatasvir	Inhibits binding of NS5A to viral RNA required for RNA replication and viral assembly of HCV
NS5B	Sofosbuvir, Dasabuvir, Mericitabine BI207127, Lomibuvir/VX-222, Setrobuvir	Inhibits NS5B, RNA-dependent RNA polymerase inhibitor
NS3	3-bromo-4-hydroxyl derivative 4,5,6,7 – tetrobromo benzotriazole (TBBT), 30-methylpiperidine-10-YI QU663	NS3 helicase inhibitor Protein kinase-2 inhibitor Helicase inhibits NS3 helicase inhibitor
NS4B	Clemizole	Inhibits HCV RNA replication by blocking binding of viral RNA to NS4B
Host factors		
Cyclophilins	Cyclosporin A	Inhibit HCV replication
miRNA	Miravirsen	Reduces HCV replication
Viral assembly		
Alpha-glucosidase	UT-231B (Immino sugar) and Celgosivir (MX-3253-a castano- spermine prodrug)	Inhibits alpha glucosidase involved in HCV assembly
DGAT-1 (Cellular factor) (Diacylglycerol O- acyltransferase-1)	DGAT-1 inhibitor	Inhibits DGAT-1 needed for core protein localization around LDs
DGAT-2 (Cellular factor) (Diacylglycerol O- acyltransferase-2)	DGAT-2 inhibitor	DGAT-2 involved in LD biogenesis
VLDL biogenesis	Grapefruit flavonoid naringenin	Inhibitor of VLDL secretion disturbing viral assembly

nucleotide inhibitors (NNIs). It has been found that NIs have a similar effect for different HCV genotypes and also show low incidence of resistant genes. Sofosbuvir, a NIs, has been used in cases of HCV infection caused by non-genotype-1 HCV^[31,32]. However, DAAs are well tolerated and adverse reactions are significantly lower

than IFN, but there are still a few cases of adverse reactions and reactivation of HBV during DAAs anti-HCV treatment^[31].

Cyclosporine and miravirsen

Cyclophilins including cyclophilins A, B, and C are involved in HCV replication. An immunosuppressive compound cyclosporine A is involved in the inhibition of HCV RNA replication by interfering with cyclophilins A functions. Alisporivir (Debio-025) which is a derivative of cyclosporine A acts as antiviral agent against many HCV genotypes. The antiviral effect of cyclophilin inhibitors is increased when used in combination with PegIFN- α . Thus, in addition to many other benefits, these agents may be used as effective antiviral agents^[33,34]. Miravirsen is another drug that targets miRNA-122. It inhibits several HCV genotypes *in vitro*. Its effect lasts long simultaneous with non-appearance of resistant mutations.

Other antiviral agents

In addition to antiviral agents described above, vitamin B12 was also reported to act as an inhibitor of HCV replication. The use of vitamin B12 with SOC drugs raised the SVR rate to the level higher than the rate noted in patients treated with SOC alone^[35]. Recently, it has been observed that vitamin D also acts against HCV *in vitro*. The SVR rate of patients infected with HCV genotype-1 or 2/3 is improved once vitamin D is added to PegIFN- α and ribavirin therapy^[36,37]. A comparison of study using PegIFN- α and RBV with supplement of L-carnitine group vs. the PegIFN- α plus RBV group has shown an increase in SVR rate^[38]. This substantiates that L-carnitine may be useful for the treatment of HCV infection.

MECHANISM OF DRUG-ACTION

Targets of drugs

The basic aim of designing the drugs against HCV infection is to develop agents that can check the entry of virus into cells, blocks its replication and disrupts the viral assembly inside the cell. As such, drugs do not kill the virus or its components but prevent their formation and reproduction. In case of HCV infection, attempts were made to develop drugs that can check viral entry and replication process. Since the discovery of HCV, a number of experimental studies were conducted which reported detailed analysis of HCV life cycle and its interaction with human host. These studies revealed several targets for therapeutic intervention in HCV infection. Recent improvements in the SOC therapy have raised the hope that HCV infection can be managed with adequate medical intervention. However, the current treatment is not effective for all seven genotypes. The basic aim for HCV therapy is to achieve high SVR using traditional drugs in combination with direct acting antivirals (DAAs), without any chance of escape mutations.

HCV entry as target

The drugs inhibiting HCV entry into cells target receptors and enzymes helping in viral entry process. These entry inhibitors have prophylactic properties and show synergistic effect when combined with other agents^[39]. Circulating virions bind with glycosaminoglycans (GAGs) and LDLA^[40]. The lectin cyanovirin-N (CV-N) impairs viral binding by its interaction with E1/E2 HCV proteins to check entry^[41]. Similarly, L-ficolin proteins can neutralize HCV particles through their binding to E1/E2 proteins^[42]. Epigallocatechins gallate (EGCG), a natural polyphenol compound and abundant in green tea extract regulate lipid metabolism impairs HCV binding to host cell by interfering with HCV E1/E2 function and also block cell-to-cell transmission *in vitro*^[43-45]. This is the reason that green tea is considered as an effector against HCV infection. Lactoferrin, present in milk, also blocks HCV attachment^[46]. Like E1/E2, the P7 protein also inhibits HCV entry by directly effecting virus binding to cell surface and interfering with host-virus interaction^[47].

After attachment of virus with cell surface, its entry requires different host factors like CD81, SRB1, CLDN1 and OCCDN1, jfRI, EGFR, EphA2 and NPC1-L1, *etc.* CD81 interacts with HCV E2 helping HCV infection. Specific NTCD81 monoclonal antibodies like JS-81 or KO4 counteract HCV E2-CD81 interactions and

interfere with HCV entry during post binding process^[48-53]. SRB1 proteins, related to lipid metabolism, also affect HCV entry to host cells^[54]. Serum amyloid A, an acute phase protein and produced by liver, inhibits HCV entry^[55-57]. Similarly, ITX5061, a small molecule, also blocks uptake of HCV and functions synergistically with DAAs, thus giving a promise for future use. CLDNs and OCLNs form complex with CD81 and contribute to efficient HCV internalizations. Since CLDN1 is highly expressed in hepatocytes, it may be a potential target for antiviral agents. Antibodies vs. CLDN1 show inhibitory effect on HCV infection^[58-60]. OCLN is also a main entry factor for HCV. Recently, it has been found that mi R-122 can decrease HCV entry by inhibiting OCLN. The EGFR and EphA2, the receptor tyrosine kinases (RTKs), act as cofactors for HCV entry^[61]. These are expressed in liver and inhibited by anticancer drugs like Erlotinib and Desatinib. These drugs impair HCV cell-entry. RTKs interfere with CD81-CLDN1 complex association and block cell to cell transmission of HCV^[61]. However, their efficiency needs further authentication. After interaction with various receptors, HCV particles are internalized through clatherin-mediated endocytosis^[62]. CD81-CLDN1 complex facilitates virus entry and fusion simultaneously^[58]. The compound chlorpromazine interferes with clatherin, thus impairing HCV endocytosis^[63]. Arbidol, used as an anti-influenza drug, impairs clatherin mediated endocytosis of HCV^[64]. The fusion of virus membrane to host cell is followed by viral replication inside the cell. The indole derivative arbidol also inhibits HCV membrane fusion^[65]. Silymarin is a mixture of several flavonolignans and flavonoid taxifolines and inhibits fusion as done by arbidol^[66]. Other fusion inhibitors include feroquine and aclorocquin, *etc.*

HCV replication as target

The HCV replication cycle presents another important target for antiviral therapy. The successful use of protease inhibitors for the treatment of HIV infections prompted researchers to focus on the HCV associated enzymes including NS3-4A protease and NS5B polymerase, *etc.*^[67,68]. The HCV RdRp also became an attractive drug target. Finally, inhibitors targeting NS5A have also been developed. Simultaneous with viral proteins, several host cellular components were also used as targets while developing drugs against them.

NS3 is a component of HCV encoded polyprotein which together with NS4A, constitutes the protease NS3-4A. Its carboxy-terminal region shows RNA helicase and NTPase activity^[69]. Both these proteases are essential for HCV replication and have been pursued as drug targets. Since NS3-4A binds with its substrate by weak interactions, this restricts the development of drugs targeting NS3-4A. However, later studies could be successful in developing certain DAAs targeting NS3-4A^[70]. These drugs were put under three different categories on the ground of their properties and action^[71]. The DAAs in category I include linear peptidomimetics that bind proteases enzymes through covalent bonds. For example, telaprevir and boceprevir, the drugs of class I bind to the active-site Ser (Serine) forming a covalent enzyme - inhibitor adduct. This not only shows antiviral activity but also uses strong forces to bind the target site. DAAs under category II and III are NS3-4A specific drugs. These are linear peptidomimetics or macrocyclic inhibitors and do not bind with their target by covalent bonds. It has been reported that these drugs do not target all HCV genotypes. These NS3-4A inhibitors are two macrocycles MK-5172^[72] and ACH-2684^[73].

The NS5A replicase is the most enigmatic HCV protein. On the basis of molecular masses, their predominant forms are p56 and p58, respectively^[74]. The phosphorylation in NS5A replicase is reported to be mediated by different kinases^[75,76]. It has several sites identified as targets in the central and c-terminal part of NS5A and LCS1 region. The RdRp-NS5B is another enzyme regulating viral RNA synthesis. Several studies have demonstrated the candidate NS5B inhibitors which are nucleoside and nucleotide inhibitors (NIs) in nature and bind at active site of the enzyme. The non-nucleoside inhibitors (NNIs) bind at allosteric sites to bring conformational changes and inhibit polymerase activity^[67,68,71]. These NIs have been reported to be effective against several HCV genotypes.

HCV replication is a complex process involving many other viral proteins simultaneous with NS3-4A, NS5A and NS5B. These proteins have been pursued as drug targets. Moreover, there are some non-enzymatic

proteins which also make a suitable intervention point. Although the exact function of NS4B is not very clear, it has been found as a good drug target^[77]. NS4B also plays an important role in HCV RNA replication by forming membranous replication complexes. It has been observed that the C-terminal portion of NS4B is needed for functional HCV replication complexes^[78]. Clemizole has been found as a potent inhibitor of HCV RNA replication. This agent blocks the binding of viral RNA to NS4B^[79].

Apart from viral proteins, some host cell factors also emerged as promising targets for antiviral therapy. Among host factors contributing to the viral replication cycle, we describe here two main factors that have been studied in detail, which are cyclophilins and miR-122. Cyclophilins A (CYPA) is the primary host factor and targeted by immunosuppressive drug cyclosporin A (CsA)^[80,81] which inhibits HCV replication in cell culture^[82]. The CYPA-CsA complex also inhibits calcineurin, involved in activation of T cells. Some CYPA antagonists have been developed. These compounds are Alisporivir, NIM811 and SCY635. miRNA-122 is another important host factor that was targeted for the treatment of chronic HCV infection. miRNA-122 stimulates HCV replication by stabilizing HCV RNA^[83,84], translates of the viral genome^[85] and enhances RNA replication^[83]. Naturally, targeting miRNA-122 by antagonist disrupts HCV replication *in vitro* and *in vivo*^[86,87] and therefore becomes an effective target of therapy. miRNA-122 also shows the important role in hepatocyte lipid homeostasis and it may be taken into account when considering the therapeutic use of miRNA-122 antagonists.

HCV assembly as target

The experimental studies indicated that antiviral molecules act at different steps of HCV lifecycle. Also many cellular factors act as candidate targets. The inhibition of α -glucosidases disrupts HCV assembly^[88,89]. The α -glucosidase inhibitors including UT-231B and Celgosivir (MX-3253-a castano-spermine prodrug), were used as assembly antagonists^[90,91]. Identification of diacylglycerol O-acyltransferase-1 (DGAT1), the factor needed for core protein localization around LDs, indicates that DGAT1 may be a target for therapeutic intervention^[92]. Although diacylglycerol O-acyltransferase-2 (DGAT2) is also involved in LD biogenesis^[93], HCV targets only DGAT1. Furthermore, DGAT2-generated LDs form normally in DGAT1 inhibitor treated cells. This shows a limited effect of DGAT1 inhibitors on the cellular functions^[92].

EFFECT OF VIRAL AND HOST COMPONENTS ON DRUG ACTION

Baseline viral load

When baseline viral load is less than 400,000-800,000 IU/mL, the course of treatment may be reduced to 24 weeks in genotype-1/4 patients and to 12-16 weeks in genotype-2/3 patients. Many studies have shown that low viral load (HCV-RNA, 600,000-800,000 IU/mL is a good predictor of SVR^[94-96]. An increase in viral load decreases SVR rate.

Viral genotypes

HCV has a total of seven genotypes with more than 50 subtypes and several quasispecies. Genotypes play very important roles in deciding the host response to anti-viral treatment. Patients infected with genotype-1, -4, -5, -6 respond worse than those with genotype-2/3 infection. Although, it is not fully established, it is believed that DAAs have better effect on non-responder genotypes like genotype-1. Using sofosbuvir drug it has been altered that when it is combined with the SOC regimen, there is a good impact on SVR, both in genotype-1 and genotype-2/3 patients^[97,98].

Interferon action

Interferons are involved in host natural immune response against various pathogens including HCV^[99]. Interferon binds with receptors on the target cells and activates signaling pathways like JAK-STAT pathway. This upregulates IFN-stimulated genes (ISGs) with expression of several types of antiviral effector protein^[100-102]. This has been a basis of using IFN- α as an antiviral agent in chronic HCV infection^[103]. However,

some studies have demonstrated that IFN- α based treatment of HCV infection is influenced by several factors including viral as well as host factors. Viral load and HCV genotypes were found to be important factors influencing IFN-therapy. HCV genotype-1 responded poorly to IFN therapy achieving SVR to near about 50% in comparison to HCV genotype-2 and -3 where SVR reached up to 85%^[104]. It has been found that many HCV proteins interfere in the antiviral action of IFN- α ^[105]. Subsequently, it was noted that various HCV proteins including Core, E2, NS3/4A, NS5A/5B, antagonize antiviral effect of IFN- α . It may be illustrated more specifically in reference to individual HCV viral proteins. For example, HCV core induces expression of Suppressor of cytokine signaling-3 and -1 (SOCS-3 and SOCS-1), which antagonize IFN- α action by blocking JAK/STAT-pathway and ISGs expression^[106,107]. HCV core also inhibits IFN induced phosphorylation and nuclear translocation of STAT-1. Binding of HCV core to STAT-1 decreases its phosphorylation and ISGs transcription^[108,109]. Another important structural protein HCV E2 was also found inactivating IFN- α through inhibition of PKR^[110]. This effect of E2 was detected prominently in patients infected with HCV-1 isolate. HCV genotype-2 and -3 could not show the same effect^[110]. Of the nonstructural proteins, HCV NS3/4A was found to disrupt the IFN induction pathway. HCV NS3/4A protease cleaves various proteins including antiviral signaling proteins (MAVs)^[111,112], TIR domain containing adaptor inducing IFN- α (TRIF)^[113] and adapter protein of RIG-1 TLR-3 signaling pathways *etc.* This cleavage disrupts not only innate immune response but also IFN-induction pathway, ultimately resulting in down regulation of the transcription of IFN-alpha inducible genes^[114,115]. In addition, HCV NS4B and NS5A were also found to inhibit protective action of IFN- α . NS4B reduces IFN- α induced phosphorylation of STAT-1 and expression of IFN receptors. On the other hand NS5A binds and inactivates PKR^[116-118]. Several studies have shown inhibitory effect of NS5A on IFN induced JAK-STAT signaling pathway^[119-121]. NS5A usually blocks IFN-1 induced STAT-1 phosphorylation and its nuclear translocation resulting in downregulation of ISGs induced expression.

IL28B polymorphism

Single nucleotide polymorphism (SNP) in IL28B gene present on chromosome 9 has an impact on HCV treatment response. The SVR rate of SOC in HCV patients carrying CC genotypes was 2-3 times higher as compared to the one with its clearance. There is high frequency of CC genotypes^[122] in comparison to European and African. IL28B polymorphism is the best predictor of treatment response, better even than viral load, liver fibrosis, glucose level *etc.* EASL guidelines showed that IL28B polymorphism can be used to give a predictive value. Thus IL28B gene has a better predictive value in comparison to SOC and DAAs.

Hepatic steatosis

Patients with hepatic steatosis usually do not respond well to HCV infection treatment. The presence of steatosis does not allow the EVR or SVR to attain in genotype-1 infected patients when treated with SOC. Similarly, steatosis affects negatively in patients infected with other genotypes. It causes relapse after discontinuation of treatment in patients with genotype-3. This all indicates that pathogenesis of steatosis differs in different genotypes and influences the treatment. In addition to all above factors influencing the treatment response, other conditions like age, insulin resistance, and metabolic syndrome *etc.* also have negative impacts on treatment.

Virological response to therapy

The therapy of HCV infection is basically aimed to eradicate the virus and prevent the ensuing disease complications. The success of therapy is monitored by SVR rate which is defined as the absence of the HCV RNA in serum post 24 weeks of stoppage of treatment^[123]. The value of SVR indicated not only eradication of virion from circulation but also correlates with symptoms^[124-127]. The combination of PegIFN and ribavirin has been the SOC for all patients infected with HCV irrespective of viral genotypes^[123]. This regimen produces SVR to 70%-80% in patients with HCV genotype-2 or -3 infection. However, SVR reached only 45%-70% in patients infected with other genotypes^[123]. In recent trials of boceprevir and telaprevir in patients with cirrhosis it was noted that SVR was low in comparison to that in non-cirrhotic patients.

Drug resistance

HCV is a highly variable virus with a large viral population and numerous quasispecies turnover in an infected individual. Its life cycle remains confined to the cytoplasm in cell with little possibility of its genome integration with host genome. Treatment of chronic HCV infection is based on the combination of PegIFN- α and ribavirin. The use of DAAs against HCV demonstrates that these agents may give rise to drug resistant viral species. These viral variants have different amino acid composition on target sites and so, are less susceptible to drug action^[128]. In fact, the variants preexist before treatment, possibly arising from error prone activities of HCV-RNA dependent RNA polymerase (RdRp)^[129] and rarely detected by current techniques. Drug exposure inhibits replication of the dominant drug-sensitive viral population to the level of appearance of resistant variants. *In vivo*, viral resistance is influenced by three major factors including the genetic barrier to resistance, *in vivo* fitness of the viral variant population and drug exposure. Different studies have indicated that the variants show resistance to NS3/4A protease inhibitors, nucleoside/nucleotide analogues, non-nucleoside RNA-dependent RNA polymerase inhibitors, NS5A as well as cyclophilin inhibitors^[130]. In view of these alterations, the drug resistant variants may cause a serious challenge to infection and therefore, this problem needs a solution by more extensive investigations.

CONCLUSION

This study concludes that the use of PegIFN- α and ribavirin is still a major part of standard of care (SOC) and the control of HCV infection. The addition of new drugs including DAAs, cyclophilins and miravirsin, *etc.* has made a significant improvement in SVR even in those patients where HCV genotypes remain resistant to PegIFN- α plus ribavirin drug regimen. These drugs target and inhibit viral proteases and cell receptor proteins as well as enzymes facilitating viral entry into the cell and viral replication and assembly inside the cell. A check on viral entry as well as their cell to cell transmission or further replication by the use of these drugs achieves the aim of treatment. In spite of an increase in SVR, the effect of DAAs is altered by the viral and cellular factors. Basic viral load and viral genotypes were found to show a significant effect on therapeutic outcome. Similarly, some disease conditions or cellular genomic polymorphism like IL28B polymorphism also have an impact on drug therapy. The development of drug resistant HCV variants during viral propagation still remains a serious challenge and needs to be resolved by different combination or development of new drugs. Studies are in progress looking towards new aspects of drug therapy against HCV infection.

DECLARATIONS

Acknowledgments

We appreciate the infrastructure provided by All India Institute of Medical Sciences, New Delhi, India, for the conduct of this study.

Authors' contributions

All authors have made equal contributions in the performance of study, compilation of data and preparation of the manuscript.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

The authors declare that there are no conflicts of interest related to this study.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Epidemiology and viral risk factors for hepatocellular carcinoma in the Eastern Mediterranean countries

Suna Yapali, Nurdan Tozun

Division of Gastroenterology, Acibadem University School of Medicine, Istanbul 34752, Turkey.

Correspondence to: Dr. Suna Yapali, Division of Gastroenterology, Acibadem University School of Medicine, Istanbul 34752, Turkey. E-mail: sunayapali@gmail.com

How to cite this article: Yapali S, Tozun N. Epidemiology and viral risk factors for hepatocellular carcinoma in the Eastern Mediterranean countries. *Hepatoma Res* 2018;4:24. <http://dx.doi.org/10.20517/2394-5079.2018.57>

Received: 12 May 2018 **First Decision:** 29 May 2018 **Revised:** 13 Jun 2018 **Accepted:** 14 Jun 2018 **Published:** 27 Jun 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Given the high prevalence of viral hepatitis in the Eastern Mediterranean countries, hepatitis B and C infections are the major causes of hepatocellular carcinoma (HCC) in the region. Most cases are associated with cirrhosis related to hepatitis B or C infection. Environmental, host genetic and viral factors can affect the risk of HCC in patients with hepatitis B and C infection. Understanding the epidemiology and viral risk factors in the region provides the implementation of strategies for prevention and treatment of viral hepatitis. Herein, we reviewed the epidemiology, burden of disease and viral risk factors for HCC.

Keywords: Viral hepatitis, Eastern Mediterranean countries, hepatocellular carcinoma, epidemiology, risk factors, burden

INTRODUCTION

Hepatocellular cancer (HCC) is the fifth most common cancer in men and the seventh most common cancer in women worldwide accounting for 90% of all primary liver cancers. Furthermore, HCC is the third leading cause of cancer-related death^[1-3]. Because of the low resectability rate, high recurrence rate after resection and poor response to the conservative treatment, the prognosis of HCC is poor with a 5-year survival rate of 6.9%^[1-3]. The burden of HCC is higher in developing countries and varies markedly by age, gender, race and exposure to risk factors in different geographic regions. In the Eastern Mediterranean countries, HCC has a lower prevalence compared to the highly prevalent regions like Eastern Asia and sub-Saharan Africa. However, HCC remains to be a major concern for countries like Egypt and Saudi Arabia. This article reviews the epidemiology and viral risk factors of HCC in Eastern Mediterranean countries.



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Table 1. ASIR with 95% UI and male to female ratio by region, by sex in 2015

	ASIR 2015 (95% UI): male	ASIR 2015 (95% UI): female	ASIR ratio: male/female
HCC	8.1 (7.1-9.1)	4.7 (3.7-5.4)	1.7
HCC due to alcohol	1.4 (1.0-1.8)	0.3 (0.2-0.6)	4.7
HCC due to HBV	2.2 (1.7-2.6)	1 (0.7-1.2)	2.2
HCC due to HCV	3.5 (3.0-4.1)	2.4 (1.9-2.9)	1.5

Adapted from reference^[8]. ASIR: age-standardized incidence rates; UI: uncertainty interval; HCC: hepatocellular carcinoma; HBV: hepatitis B virus; HCV: hepatitis C virus

GLOBAL EPIDEMIOLOGY AND BURDEN OF HCC

High-incidence regions of HCC are sub-Saharan Africa and Eastern Asia with 25 and 35 cases per 100,000 population/year, respectively. In these regions, high incidence rate of HCC is associated with high hepatitis B virus (HBV) prevalence. China has the highest incidence of HCC in the world, accounting for more than 40% of all HCC cases and 55% of liver cancer deaths^[3,4]. Southern European countries have an intermediate-incidence (10-20 cases per 100,000 population/year); while North America, South America, Northern Europe, and parts of Middle East have low-incidence rates (< 5 cases per 100,000 population/year)^[3-5]. The incidence in Asian countries tends to decline in the past 2 decades whilst it increased in United States and Canada because of high rate of chronic hepatitis C virus (HCV)-related cirrhosis, nonalcoholic fatty liver disease and immigrants from HBV endemic regions^[4].

Hepatitis B and hepatitis C infections are the most important risk factors for HCC. Geographic distribution of HBV and HCV infections is the major factor, which determines the incidence of HCC. Owing to the high hepatitis B surface antigen (HBsAg) seroprevalance rates, HCC incidence is highest in East Asia and Africa. On the other hand, HCV is the etiological factor in approximately 20% of all HCC cases, particularly in the low-incidence regions such as Western Europe and North America^[3,6].

The mean age of HCC diagnosis was 55-59 years in China and 63-65 years in Europe and North America^[7]. Men were found to have 2-4 fold increased incidence of HCC than women. The results of the global burden of disease (GBD) study for 195 countries or territories from 1990 to 2015 showed that HCC was more common in men with 591,000 incident cases compared to women with 264,000 cases^[8]. Similarly, mortality rates were higher among men. The gender disparity was also notable for high rates of HBV-related and alcohol-related HCC in men^[8]. The variations in hepatitis carrier state, sex steroid hormones, immune responses and epigenetics were linked to higher HCC incidence rates among men^[7].

EPIDEMIOLOGY AND BURDEN OF DISEASE IN THE EASTERN MEDITERRANEAN COUNTRIES

According to GBD study 2015 report, in the Eastern Mediterranean countries age-standardized incidence rate (ASIR) of HCC was 8.1 per 100,000 in men, and 4.7 per 100,000 in women [Table 1]^[8]. HCC is a major health problem especially in certain countries such as Egypt and Saudi Arabia. In Egypt, HCC is the fourth most common cancer and is the second cause of cancer mortality in both sexes^[8]. In the last decades, a twofold increase of HCC was reported among chronic liver disease patients in Egypt with a significant decline of HBV and slight increase of HCV as risk factors^[9]. HCV is an important risk factor for HCC in Egypt where 71% of HCC cases were positive for anti-HCV antibodies^[10]. Likewise, in the Nile delta, hepatitis C rather than hepatitis B was linked to the development of HCC^[11]. In Saudi Arabia, and according to the National Cancer Registry, HCC is ranked the sixth most common cancer in males and thirteenth in females with a male to female ratio of 2.6:1. The overall age-standardized rate (ASR) is 3.5/100,000. ASR is 4.9/100,000 for males and 1.8/100,000 for females. The median age of diagnosis is 66 years^[12]. The results of a tertiary center in Saudi Arabia showed that most of the patients diagnosed with HCC presented at late tumor stages with advanced liver disease and had poor prognosis with an average of 33-month survival^[12]. This prompts the implementation of HCC surveillance strategies in this geographic region.

Table 2. Etiology of HCC in Eastern Mediterranean countries

Location	Alcohol	HBV	HCV	Other
Afghanistan	11%	36%	32%	21%
Bahrain	17%	39%	28%	16%
Cyprus	32%	19%	39%	14%
Djibouti	13%	33%	36%	18%
Egypt	12%	13%	63%	12%
Iran	6%	44%	24%	26%
Iraq	12%	37%	32%	19%
Israel	15%	20%	49%	17%
Jordan	15%	35%	31%	19%
Kuwait	15%	37%	31%	18%
Lebanon	17%	28%	40%	15%
Libya	15%	33%	34%	18%
Morocco	14%	31%	36%	19%
Oman	17%	39%	28%	16%
Pakistan	7%	16%	54%	23%
Qatar	18%	38%	28%	15%
Saudi Arabia	17%	41%	17%	25%
Somalia	15%	36%	30%	18%
Sudan	18%	35%	30%	16%
Syria	14%	32%	34%	19%
Tunisia	18%	20%	44%	18%
Turkey	19%	26%	44%	11%
United Arab Emirates	21%	44%	22%	13%
Yemen	8%	44%	35%	12%

Adapted from reference^[8]. HCC: hepatocellular carcinoma; HBV: hepatitis B virus; HCV: hepatitis C virus

RISK FACTORS FOR HCC

The major risk factors for HCC are the presence of cirrhosis, and HBV/HCV infection. Other factors, such as aflatoxin B exposure and nonalcoholic steatohepatitis (NASH) are important in certain regions of the world. In the high-incidence countries of Asia and Africa, chronic HBV infection and aflatoxin B exposure are the major risk factors. Exceptionally, in Japan and Egypt the most common risk factor is HCV infection. On the contrary, excessive alcohol consumption and metabolic syndrome play more important roles in the low-incidence regions. In addition, inherited metabolic disorders such as hemochromatosis, A1AT deficiency, tyrosinemia, several porphyrias also increase the risk of HCC^[13].

The distribution of viral and other risk factors of HCC in the Eastern Mediterranean countries are summarized in Table 2.

Chronic hepatitis B

Countries with HBV prevalence of greater than 2% have increased incidence and mortality rates of HCC. The majority (70%-90%) of HBV-related HCC develops in patients with cirrhosis^[14,15]. In persons chronically infected with HBV, the risk of HCC has been shown to increase up to 30-fold^[14,15]. As a result of hepatic inflammation and liver damage, genetic and epigenetic defects lead to development of HCC^[16-18]. However, in the absence of cirrhosis HCC can develop in 10%-20% of HBV-infected individuals as a result of integration of HBV into the host genome that induces chromosomal alterations and insertional mutagenesis of cancer genes^[17-19]. The genetic instability of the hepatocyte triggers the clonal growth of hepatocytes before the liver damage occurs. HBV-encoded X protein (HBx) which is a multifunctional protein that regulates the expression of genes in the involved in the signal cascades, has a pivotal role in the pathogenesis of HBV-related HCC^[17-19]. In addition to cirrhosis, other factors reported to increase HCC risk among patients with chronic HBV comprise; demographic (male sex, older age, Asian or African ancestry, family history of HCC), viral [higher levels of HBV replication, HBeAg positivity, HBV genotype, longer duration of infection, co-

infection with HCV, human immunodeficiency virus (HIV), or hepatitis D virus] and environment related factors (exposure to aflatoxin, excessive alcohol or tobacco consumption).

A population-based study of untreated chronic hepatitis B (CHB) patients from Taiwan named the risk evaluation of viral load elevation and associated liver disease/cancer-hepatitis B virus (REVAL-HBV), first reported that high baseline serum HBV DNA level was associated with the risk of cirrhosis and HCC^[20]. The risk began to increase in a dose-response relationship from < 300 (undetectable) to $\geq 1,000,000$ copies/mL. Furthermore, patients with persistently high HBV DNA levels had the highest risk of HCC. The role of viral load on HCC development was also confirmed in several cross-sectional and longitudinal cohort studies from Taiwan, Hong Kong, and China^[21-23]. HBeAg-positivity, which shows active viral replication, is also associated with the development of HCC^[24]. Although long-term suppression of viral replication can be achieved with the use of potent oral antiviral therapies, the risk of HCC is not eliminated. This was clearly demonstrated in a study of 1378 patients comparing the incidence of HCC between patients who received oral antiviral treatment and inactive carriers^[25]. The study found a higher risk of HCC development in patients treated with oral antiviral drugs than those with inactive CHB and indicated that the risk of HCC is not eliminated in patients receiving oral antiviral treatment. These patients should continue to be screened for HCC.

HBV genotype is also important in determining the risk for HCC^[26,27]. The risk is higher in patients with genotype C than patients with genotype B. High viral load and genotype C have an additive role in increasing the risk of HCC^[28]. Genotype D patients carry a higher risk for HCC than patients with genotype A^[28]. HBV genotype D was found to be the most prevalent genotype in studies reported from Turkey, Iran, Pakistan and Saudi Arabia^[29-32]. There is rare evidence to show the association genotypes with the risk of HCC in the Middle Eastern countries. Studies from Iran have also demonstrated a strong relationship of genotype D and mutations in basal core promotor (BCP) and precore regions with the disease outcomes^[33,34].

The prevalence of HBV infection is complex and a major public health problem in Eastern Mediterranean countries. In the early studies, reported HBV prevalence rates ranged from $< 2\%$ to $2\%-8\%$ in most countries, reaching up to $\geq 10\%$ in Saudi Arabia, Yemen and Sudan^[29,35-38]. In the latest report of World Health Organization (WHO), Eastern Mediterranean countries have a prevalence of chronic HBV infection ranging from low intermediate ($2\%-4\%$) in most countries to high intermediate ($5\%-7\%$) in Somalia and Sudan^[39,40]. The WHO estimates that more than four million people are infected yearly with HBV in this region^[41]. The lifetime risk of HBV infection in the pre-vaccination era ranged from 25% to $> 75\%$, with continued transmission from the perinatal period throughout early children and adult life. It was estimated that around 100,000 persons from each birth cohort in the region would die from HBV-related liver disease and HCC during their lifetime. In these high-risk regions, the primary transmission routes are perinatal, child-to-child, sexual contact and percutaneous exposures (e.g., unsafe injections and blood transfusions).

Despite the introduction of hepatitis B vaccination programs, HBV continues to be transmitted among unvaccinated older children and adults. Therefore, in 2009 WHO Eastern Mediterranean regional committee implemented a regional target, to reduce the prevalence of CHB infection to less than 1% among children below 5 years of age by 2015^[39]. The national health agencies in the region supported the program with hepatitis B vaccination of newborns. By the end of 2014, 68% of the countries achieved the target. The rate of hepatitis B birth dose vaccination coverage in the region increased to 24% in 2014 compared to 14% in 2000^[39]. In 2014, 71% of newborns received a birth dose within 24 h in the countries, which had $< 80\%$ birth dose coverage^[39].

A systematic review examining the viral etiologies of HCC in the Eastern Mediterranean countries indicated HBV as a major cause in 35% , 42.5% , 55% and 52% of HCC cases in Saudi Arabia, Yemen, Turkey and Iran,

respectively^[42]. But the lack of high quality data and data registry systems represent a major challenge to determine the epidemiology of HCC in this region. Universal HBV vaccination is the most effective strategy to reduce the incidence of HCC. A 20-year follow-up report from Taiwan - an endemic region - clearly showed that HCC incidence among subjects 6-19 years of age decreased in the vaccinated cohort (64 HCC in 37,709,304 person-years), compared to the non-vaccinated cohort (444 HCC in 76,496,406 person-years), with the adjusted relative risk (RR) of 0.31^[43].

The impact of vaccination programs on the incidence of HCC development in the Eastern Mediterranean countries needs to be clarified in future studies. However, many challenges remain. The war in this region leads to low or decreased coverage of vaccination programs. Furthermore, immigration after war is a major threat for the application of immunization programs, identification and treatment of CHB patients that will change the epidemiological trends for HBV and HBV-related HCC in the Eastern Mediterranean countries.

Chronic hepatitis C

HCV is one of the major global causes of liver-related death and morbidity. The risk of HCC is increased 15-20 fold in patients chronically infected with HCV infection. Over the last decade, HCV seroprevalence is estimated to increase by 2.8%, accounting for more than 185 million infections worldwide^[44]. A systematic review analyzing the studies published between 2000 and 2015 from 138 countries (representing the 90% of the global population) estimated global HCV prevalence at 2.5%. Central Asia and Central Africa are estimated to have the highest prevalence (> 3.5%); East, South and Southeast Asia, West and East Africa, North Africa and Middle East, Southern and Tropical Latin America, Caribbean, Australasia, and Eastern Europe moderate prevalence (1.5%-3.5%); while Southern Africa, North America, Andean and Central Latin America, Pacific Asia and Western and Central Europe have low prevalence (< 1.5%). The global viremic rate was 67%, with HCV varying from 48.7% in Central Asia to 80.2% in Tropical Latin America^[45]. HCV genotype 1 is the most frequent genotype followed by genotype 3 (17.9%), genotype 4 (16.8%), genotype 2 (11%), genotype 5 (2%) and genotype 6 (1.4%)^[45]. The genotypes reported to be associated with high risk of HCC are genotype 1b and genotype 3^[46-48].

Chronic HCV infection causes increased inflammation and cell-turnover leading to cirrhosis and development of dysplastic nodules and HCC^[39]. Unlike HBV, HCV-associated hepatocarcinogenesis is more likely to be related to the indirect effects of the virus on the host cellular processes such as increased hepatocyte proliferation and steatosis, virus-induced inflammation and oxidative stress inducing genomic mutations and genome instability, mitochondrial damage and induction of reactive oxygen species, and virus-induced host immune responses^[19]. In untreated patients, cirrhosis develops in 14%-45% of patients 20 years after transmission of HCV^[49]. In patients with HCV-related cirrhosis, annual rate of HCC is 1%-4%, therefore patients with advanced fibrosis and cirrhosis should undergo HCC surveillance. The risk factors for HCC are older age, black race, HCV genotype 1b, co-infection with HBV or HIV, diabetes, obesity, steatosis, heavy alcohol consumption and low platelet levels in patients with cirrhosis^[49-52].

The HCV prevalence in the Eastern Mediterranean region ranges from 1% to 2.5% in most countries, with higher prevalence reported in Egypt (> 10%), and in Libyan Arab Jamahiriya, Sudan and Yemen (2.5%-10%)^[53]. In the Eastern Mediterranean region of WHO, it is estimated that at least 23 million people have HCV infection^[53]. This represents almost the total of HCV patients in Europe and US. Regarding the parenteral spread by the previous use of intravenous anti-schistosomal treatment campaigns, HCV prevalence is very high in Egypt, particularly in the age group of 40-60 years^[54-56]. A high prevalence of HCV among children born after these campaigns is explained by unsafe injections^[54-56]. In Pakistan, the prevalence of HCV is variable from 2% to 14%, and HCV transmission in this region is due to unsafe injections^[57].

HCV genotype is an important epidemiological determinant for the source and the possible mode of transmission. Furthermore, genotype has a substantial role in predicting the treatment response. Six major

genotypes of HCV were described. In the Eastern Mediterranean countries, there are 2 predominant genotypes; genotype 4 in the Arab countries (except Jordan) and genotype 1 in non-Arab countries (Islamic Republic of Iran, Israel and Turkey)^[58]. Egypt is of particular importance with more than 90% of genotype 4 HCV infection^[59]. The distribution of HCV genotype in Jordan differed from the other Arab countries, predominantly genotype 1a (40%), followed by genotype 1b (33%) and genotype 4 (33.3%)^[60]. The most common genotype in Southern Israel was genotype 1b (62%) while genotype 4 (78%) was predominant in the Gaza Strip^[61]. Turkey serves as a bridge between Europe and Asia, and HCV genotype pattern is similar to Eastern and Southern European countries, having genotype 1b as the most frequent genotype (> 70%) followed by genotype 1a^[62]. HCV genotype 3a is the most common subtype in Iran followed by genotype 1a, 1b and 4^[63]. The predominant genotypes (1a and 4) are the most difficult-to-treat groups. The association between the HCV genotype and the risk of HCC is based on the epidemiological data however one can speculate that the poor response to interferon (IFN)-based regimens in genotype 1 and 4 patients may explain the disease progression and high risk of HCC development.

Chronic HCV infection leads to HCC following a multistep carcinogenesis pathway. Interferon (IFN)-based regimens provided sustained virologic response (SVR) in 40%-50% of patients^[64]. Recently developed direct-acting antivirals (DAAs), which directly target the viral protease, polymerase, or non-structural proteins, have achieved a revolutionary improvement of SVR rate over 90%^[65].

In developing countries, less than 10% of HCV-infected patients can access to DAAs. Despite the high antiviral efficacy, high cost of the medications is a major barrier to the access to treatment of the sufferers^[66,67]. In addition, more than 50% of infected individuals have unrecognized HCV infection^[68]. These patients generally present with advanced liver disease. Each year approximately 3-4 million newly infected cases are expected, the burden of HCV-related liver disease will remain to be high, even in the developed countries.

A systematic review including 13 studies on 2386 patients in Egypt estimated the annual rates of death/transplantation, decompensation and HCC in patients with compensated HCV cirrhosis to be 4.58%, 6.37% and 3.36%, respectively^[69]. In 2014, an estimated 125,000 viremic individuals/year were diagnosed with HCV infection. Of these 10% had chronic hepatitis, 30% had compensated cirrhosis, and the majority (60%) were diagnosed with decompensated cirrhosis or HCC^[70,71]. The high prevalence of HCC in HCV patients was reported to be associated with decompensated cirrhosis in Egypt^[72].

In the Eastern Mediterranean countries, treatment strategies are determined by the availability of resources, availability of medications and expected number of cases. In the countries, which have access to DAAs, treatment is prioritized for patients with advanced fibrosis and cirrhosis. In 2014, national committee for control of viral hepatitis (NCCVH) in Egypt negotiated with the industry to decrease the price of DAAs. Furthermore, local generic treatments were encouraged and decreased the cost of treatment. This program provided treatment of large number Egyptian genotype 4 HCV patients. This model needs to be reproduced in other developing countries to decrease the risk of cirrhosis and HCC in HCV-infected individuals. Elimination of HCV by 2030 is one of the major targets of WHO by implementing models to reduce the rate of new infections and provide treatment access in middle and low income countries. Many countries including Australia, Brazil, Egypt, Georgia, Germany, Iceland, Japan, the Netherlands and Qatar are on the track to eradicate hepatitis C by 2030.

Hepatitis D virus

The hepatitis D virus (HDV) is an incomplete RNA virus, which is dependent on HBsAg for transmission and replication^[73,74]. HDV leads to fulminant hepatitis and further disease progression among hepatitis B infected patients. The long-term co-infection of HBV and HDV presents a worse prognosis than CHB

infection. Up to 80% of HBV and HDV co-infected patients progress to cirrhosis^[73,74]. It has been estimated that almost 5% of HBV infected patients have HDV co-infection^[73,74].

The epidemiologic distribution of HDV infection is variable throughout the world. HDV is highly endemic in the Eastern Mediterranean countries^[75]. Two studies from Turkey show prevalence of anti-HDV in 18.8% to 23.0% of HBsAg positive HCC^[37,76]. A Jordanian study reported the prevalence of anti-HDV in a small group of HBsAg positive HCC patients was 67%, but the sample size was very small^[77]. The risk of HCC is increased in HDV infection compared to HBV monoinfection. HDV infection increases the risk for HCC threefold and for mortality two fold in patients with hepatitis B cirrhosis^[78,79]. However, the pathogenetic mechanism of HDV in HCC development has not been clarified yet. Oxidative stress as a result of severe necroinflammation, epigenetic mechanisms like DNA methylation and histone modification are the proposed mechanisms^[80].

The only available treatment for HDV is interferon with a very low efficacy^[81]. Therefore, the spread of HDV can be prevented by effective HBV vaccination programs leading to a decrease in the incidence of HCC^[82]. Health-care providers should be educated to check for HDV infection in chronic HBV carriers. In addition, patients should be informed about the risk of superinfection from carriers co-infected with HDV and educated about preventive practices.

SUMMARY

HBV and HCV infections are the most important etiologies for HCC in Eastern Mediterranean and Middle Eastern countries. Implementation of screening programs for individuals at high risk, maintaining HBV suppression in chronic hepatitis B and sustained viral response in CHC, surveillance of patients at high risk for developing HCC are recommended to prevent progression to cirrhosis and HCC development. The lack of data registry systems in the region resulted in limited understanding of the exact epidemiology of disease. Furthermore, the political and social unrest in the region and the immigrations after the wars may restrict the application of preventive programs and may lead to increased incidence of hepatitis. Public health policies should consider the future impact of the current situations.

DECLARATIONS

Authors' contributions

Literature research, drafting and revision of the manuscript: Yapali S

Idea of the review, critical revision of the manuscript: Tozun N

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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HCV clearance by direct antiviral therapy and occurrence/recurrence of hepatocellular carcinoma: still an issue?

Francesco Paolo Russo, Martina Tessari, Angela Imondi, Erica Nicola Lynch, Fabio Farinati

Department of Surgery, Oncology and Gastroenterology, Section of Gastroenterology, Padua University Hospital, Padua 35128, Italy.

Correspondence to: Prof. Fabio Farinati, Department of Surgery, Oncology and Gastroenterology, Section of Gastroenterology, Padua University Hospital, Via Giustiniani 2, Padua 35128, Italy. E-mail: fabio.farinati@unipd.it

How to cite this article: Russo FP, Tessari M, Imondi A, Lynch EN, Farinati F. HCV clearance by direct antiviral therapy and occurrence/recurrence of hepatocellular carcinoma: still an issue? *Hepatoma Res* 2018;4:25. <http://dx.doi.org/10.20517/2394-5079.2018.52>

Received: 4 May 2018 **First Decision:** 15 May 2018 **Revised:** 10 Jun 2018 **Accepted:** 16 Jun 2018 **Published:** 27 Jun 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

New regimens with direct-acting antivirals (DAAs) agents have changed both efficacy and safety of hepatitis C virus (HCV)-treatment, as almost all patients can be treated and cured at any stage of liver disease. The rates of sustained virological response to currently available combinations exceed 95% in real-life practice. However, conflicting results have been produced on the occurrence/recurrence of hepatocellular carcinoma (HCC) in patients with HCV-associated cirrhosis treated with DAAs. In this review we analyse the data available in the literature in order to elucidate the impact of DAAs on the risk of HCC occurrence in patients without previous history of tumor, and of recurrence after successful treatment of the tumor. Data on “*de novo*” HCC incidence were quite homogeneous, suggesting that the treatment with DAAs does not modify the risk of HCC developing during the first 6-12 months after HCV eradication. On the contrary, HCC recurrence rates after DAAs were extremely variable across different studies, reflecting a large heterogeneity in this clinical setting. The possibility that treatment with DAAs may favour tumour growth and spread in individual patients with active HCC foci is supported by some observations but remains unproven.

Keywords: Hepatitis C virus, direct-acting antivirals, eradication, hepatocellular carcinoma, occurrence, recurrence

INTRODUCTION

Patients with hepatitis C infection (HCV) and cirrhosis have an increased risk of developing liver decompensation (LD), hepatocellular carcinoma (HCC) and liver-related death (LRD). Cirrhosis is a major risk factor for the development of HCC in Western countries, where HCC occurs at an average annual rate of 3%-5% in cirrhotic patients^[1,2]. Data on the long-term outcome of patients with HCV infections,



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treated with Peg-interferon (Peg-IFN) and ribavirin, demonstrated a reduction in LRD and non-LRD, with patients achieving sustained virological response (SVR) showing nearly the same life expectancy as the general population^[3,4]. Patients with compensated cirrhosis, and without the clinical manifestations of portal hypertension, are those who experience the greater clinical benefits of HCV eradication, as they do not develop LD and rarely HCC^[5].

This was confirmed in a meta-analysis of 12 studies including 25,497 patients that demonstrated a relative risk reduction for HCC at all stages of liver disease [hazard ratio (HR): 0.24; 95%CI: 0.18-0.31; $P = 0.001$] and an absolute risk reduction of 4.6% (95%CI: 4.2%-5.0%) in patients who achieved SVR compared to those who did not respond^[6].

DIRECT-ACTING ANTIVIRAL THERAPY: A NEW STORY HAS STARTED

The more widely extended indication criteria and the greater affordability of direct-acting antiviral (DAA) therapy is leading to higher rates of HCV eradication and is expected to reduce the risk of HCC by preventing, at least in part, liver cirrhosis. Nevertheless, in cirrhotic patients, particularly in those with concurrent pro-carcinogenic co-factors, such as diabetes and/or advanced age, the risk of HCC remains elevated for several years after SVR. This underlines the importance of HCC surveillance even after SVR in cirrhotic patients and highlights the need for early initiation of DAA therapy, before cirrhosis is established^[7]. Cabibbo *et al.*^[8] published a meta-analysis of the HCV-untreated arms of the studies evaluating the outcomes of patients with early HCC who, after a successful treatment of the tumour, did not receive any antiviral treatment. This study provides a benchmark for indirect future comparisons aimed to determine the actual benefit of HCV eradication by antiviral treatment. They found an extremely variable 2 and 3-year HCC recurrence rate, respectively at 47% and 79.8%, in patients HCV-infected who did not receive antiviral therapy, and this heterogeneity was not completely explained by any single patient or study characteristic.

DAA AND HCC: HIGHER OCCURRENCE/RECURRENCE

In 2014, the introduction of DAAs has revolutionized the standard of care of HCV infection, allowing to reach SVR rates of over 90% in patients with genotype 1. Multiple oral interferon-free HCV regimes are now available and, although there is some evidence of response variability, related to specific patient characteristics and HCV genotypes, the SVR rates are high for all approved DAAs. Due to their high efficacy, tolerability and the relatively short treatment duration, DAAs are now the standard care for patient populations that were historically considered difficult to cure^[9], even though data on the long-term outcome of patients with advanced liver disease treated with DAAs are still lacking.

The assumption that HCV eradication would translate into a reduced incidence of newly developed tumours in HCV patients as well as into a reduced HCC recurrence rate, is to be considered in the context of the controversy about a potential association between DAA treatment and an increased HCC risk, overall [Table 1].

In fact, in 2016 a report^[10] described a totally unexpected early tumor recurrence in patients with HCV-related HCC undergoing DAA treatment.

In a cohort of patients who achieved a complete HCC radiological response before starting antiviral treatment with DAAs, Reig *et al.*^[10] described an HCC recurrence rate of 28% (16 of 58 patients), with a median follow-up of only 6 months, recurrence rate that was extremely higher than expected and previously observed. Furthermore, the pattern of relapse was described as infiltrative or multinodular in 25% cases, this also being an unexpected finding. Even though the authors concluded that their data should only be taken as a “note of caution”, and that large-scale studies were necessary to confirm their results, this report raised a debate about the risk of DAA treatment and suggested that a stricter pharmacovigilance action

should be undertaken by all prescribers and regulators involved in this issue. The above-mentioned study also led to the hypothesis that HCV eradication mediated by DAAs may induce a sudden modification in HCV-dependent inflammatory status and immune surveillance, dysregulating the anti-tumor response and boosting the growth of still undetected HCC foci. Recurrence after initial complete response may be explained by the dissemination of cells prior to treatment, and by the development of new oncogenic clones within a cirrhotic liver that has already suffered genetic damage. Immune surveillance plays a major role in regulating survival and growth of metastatic cells; DAA-based antiviral treatment, as a consequence of the inhibition of HCV replication and the abrupt resolution of chronic inflammation, may disrupt cancer immunosurveillance. This may lead to tumor progression.

A similar report on the management of patients with HCC undergoing DAA treatment was elaborated by Conti *et al.*^[11]. Their data suggested that DAA-induced resolution of HCV infection did not decrease the occurrence of HCC in the short term and that HCC curative treatments, in patients undergoing antiviral therapy, do not reduce the risk of recurrence. In their study the recurrence rate of patients with a history of previous liver cancer was around 30% (17 of 59 patients) within 24 weeks. In contrast with the study by Reig *et al.*^[10], the patients who experienced HCC recurrence were younger and had a more severe liver stiffness. Another study, published by Kozbial *et al.*^[12], reported an unexpected high incidence of HCC occurrence and recurrence in patients treated with DAAs. Even though the study included a small number of patients, the authors concluded that decreasing inflammation could have a role in modulating liver regeneration and that the change in the immunological environment could induce the progression of pre-existing pre-cancerous changes^[12,13]. The authors also noticed the reduction of miR-22 levels in patients treated with DAAs, which could play a possible role in tumor development, since miR-22 is involved in suppressing the replication of virus-infected cells and controlling carcinogenesis^[14].

As suggested by Reig *et al.*^[15], tumor dormancy derives from a dynamic equilibrium between cancer cells growth and immune cells infiltration; several conditions and DAA treatment could disturb this equilibrium by causing immunological changes, connected to the fall of the antigenic load due to HCV eradication. This phenomenon was not observed in patients who underwent IFN-based therapy, probably because of the immune-modulatory and anti-proliferative properties of IFN^[16]. Moreover, chronic HCV infection activates the most prevalent innate cells in the liver, the NK cells, as well as increases the expression of IFN-stimulated genes, suggesting that infection activates an intrahepatic immune response. In fact, DAA-mediated HCV eradication is characterized by decreased levels of CXCL10 and CXCL11 and normalization of NK-cell phenotype and function, a fact that could explain the association between HCV clearance and loss of intrahepatic immune reactivation^[17,18].

Cardoso *et al.*^[19] in a study of a cohort of 54 patients successfully treated with IFN-free antiviral therapy, reported an HCC incidence of 7.4% after a median time of 7.6 months (IQR 6.3-10.6 months), in a median follow-up of 12 months (IQR 9.4-12.5 months). The authors, in agreement with Reig *et al.*^[15], speculated that an oncogenic effect of the antiviral therapy was highly unlikely, but at the same time, due to the coincidence with viral elimination, the mechanisms responsible could be those previously hypothesized.

A recent report by Abdelaziz *et al.*^[20] distinguished *de novo* vs. recurrent HCC following DAA treatment and evaluated their behaviour. No difference was found regarding patient baseline and tumor characteristics (age, gender, hepatic function assessed by Child Pugh Score, performance status, number or size of lesions) or their response to DAAs. On the opposite, a significantly different time before detection of HCC emerged between the two groups. *De novo* lesions developed later than recurrent tumors (14 ± 16.02 vs. 6.7 ± 5.1 months, $P = 0.008$) and showed a better response to ablation ($P = 0.03$). The above-mentioned studies represent the current bulk regarding the evidence of DAAs promoting liver carcinogenesis.

DAA AND HCC: JUST FICTION?

On the other hand, other published articles did not confirm the higher occurrence or recurrence of HCC in patients treated with IFN-free, DAA-based therapy. Strong evidence against this assumption was given by three French prospective multicentre studies by the Agency for Research on AIDS and Viral Hepatitis (ANRS in the French acronym) cohorts of DAA-treated HCV-infected patients treated with curative HCC therapies^[21]. In detail, the rates of recurrence in the ARNS CO22 HEPATHER cohort (including 189 DAA+ and 78 DAA- patients) were 0.73/100 and 0.66/100 person-months respectively in the DAA+ and the DAA- group. In the ARNS CO12 CirVir cohort, the rates were 1.11/100 in 13 DAA+ and 1.73/100 person-months in 66 DAA-. Finally, the ARNS CO23 CUPILT cohort of HCC liver transplant recipients, successively treated with IFN-free antiviral therapy, showed a recurrence rate of 2% (7/314 patients). Notwithstanding the large number of patients that were analysed in these studies, no increase in the risk of HCC recurrence after antiviral therapy was detected in any of the cohorts. The recurrence rates did not differ between treated and untreated patients. A sharp criticism towards the ANRS study design was made by Kolly and Dufour^[22] stating that it artificially decreased the rate of HCC recurrence in untreated patients. The ARNS collaborative group argued, as a defence of the accuracy of the design of the study, that treatment was considered as time-dependent variable and that patients who underwent treatment were considered part of the untreated group until the therapy started^[23]. A study in an English cohort, including more than 400 treated patients, supported the French findings. Also, Cheung *et al.*^[24], after a follow-up of 12 months, revealed a reduction in HCC rates after HCV eradication in DAA-treated patients. The preliminary data of another prospective observational study of patients with liver cancer and HCV infection treated with DAAs, show no HCC recurrence after curative treatment in a median follow-up of 12 months^[25]. Furthermore, the study by Zavaglia *et al.*^[26] did not confirm the alarming findings of Reig and Conti: amongst the 31 patients they followed, they only observed 1 case of liver cancer, with a median follow-up of 8 months. The longer interval between complete HCC curative treatment and antiviral therapy (median 19 months in Zavaglia's experience vs. 11 months in Reig's study) could partly explain the contrasting results. It appears that, the longer the interval between tumor eradication and antiviral therapy initiation, the lower the risk that residual tumoral cells are still present at the beginning of DAA treatment^[26], and this is highly conceivable.

Cabibbo *et al.*^[27] in a prospective study of 143 patients with previously successfully treated HCC, then treated with DAAs, showed 6-, 12- and 18-month recurrence rates of 12%, 26% and 29.1% respectively; in this group of patients, the authors found comparable results to those observed in DAA-unexposed patients. Previous history of HCC recurrence (HR: 2.22; 95%CI: 1.02-4.83; $P = 0.043$) and tumor size (HR: 2.73; 95%CI: 1.23-6.06; $P < 0.014$) were the two independent risk factors for HCC early recurrence that could be used to stratify the risk of HCC recurrence. A large amount of data was analysed by Waziry *et al.*^[28] in a meta-analysis and meta-regression analysis based on 41 studies: no evidence of increased HCC occurrence or recurrence risk after DAA therapy vs. INF-based therapy was found. HCV eradication was confirmed to decrease HCC risk in patients who achieved SVR, whereas older age, advanced cirrhosis and worse patient baseline characteristics in DAA-treated population were independent predictors of HCC development and provide an explanation of the apparently higher risk (3.1 vs. 1.1/100 per years). Another study was conducted by Ioannou *et al.*^[29] on a large cohort of HCV infected cirrhotic patients from the Veterans Affairs national healthcare system, treated with IFN regimen alone, DAA regimen or INF+DAAs, during a 6.1 years mean follow-up. A 71% HCC occurrence risk reduction was associated with DAAs-induced SVR compared to treatment failure, but the reduction was similar, irrespective of how SVR was achieved (DAA-only AHR: 0.29; 95%CI: 0.23-0.37; DAA + INF AHR: 0.48; 95%CI: 0.32-0.73; IFN-only: 0.32; 95%CI: 0.28-0.37). Maan and Feld^[30] are also amongst the authors supporting the association between SVR achievement and HCC risk reduction due to the analysis of a retrospective study on cohorts of veterans treated with DAAs. In a study by Kobayashi *et al.*^[31], 77 patients treated with DAAs, who achieved SVR, were compared to 528 patients who underwent viral eradication with Peg-IFN/RBV during a median follow-up of 4 years. Amongst DAA-treated patients, 2.6% developed liver cancer, while the 3- and 5-year cumulative HCC development rates were 1.30%

and 3% in the IFN-free treatment group and 1% and 2.2% in the Peg-IFN/RBV group, with no statistically significant differences^[31]. A European multicentre study by Kolly *et al.*^[32] assessed the HCC recurrence rate after DAA treatment, reported as the cumulative disease-free survival during the follow-up. In 47 patients previously treated for HCC, the time between tumor treatment and the initiation of DAAs was a predictor of recurrence, but whether this effect was due to the anti-viral therapy, or due to foci of HCC which were undetectable before treatment was undefined. Petta *et al.*^[33], using the ITA.LI.CA liver cancer collaborative database, demonstrated that the eradication achieved by both IFN-based therapy and DAAs resulted in an increased time before tumour recurrence in patients with HCC curatively treated by radical ablation. Data deriving from their observation showed 16 (28%) and 22 cases (39%) of HCC, after a median follow-up of 18 months in DAA, and 34 months in IFN-based SVR, respectively.

Also, the retrospective large cohort study performed by Kanwal *et al.*^[34] on DAA-treated patients from 129 Veterans Health Administration centres confirmed the lack of evidence that DAAs promote HCC and the preventive effect of the HCV eradication on HCC occurrence, with a 76% risk reduction. On the other hand, their analysis confirmed that the HCC risk persists despite SVR in DAA-treated patients, with an annual HCC incidence after HCV eradication with DAAs of 0.90%, compared to 0.3% in IFN-treated patients (as reported by previous studies). It must be said that the treated population has changed since the advent of DAAs, thus giving patients with other independent HCC risk factors, such as advanced cirrhosis, a chance to be treated. The incidence rate was greater in cirrhotic patients, underlying the importance of HCC surveillance in this scenario, as well as the need of not delaying treatment in order to avoid liver deterioration.

A retrospective population-based cohort study using the Electronically Retrieved Cohort of HCV Infected Veterans (ERCHIVES) investigated whether DAA use was associated with higher rates of incident HCC compared to treatment with IFN-based regimes, the primary outcome being the development of incident HCC cases. A series of 17,836 persons was included, and amongst cirrhotic patients DAA treatment was not associated with higher risk of HCC compared to the IFN-treatment group (HR: 1.07; 95%CI: 0.55-2.08). The risk of incident HCC was higher, among patients with known HCC risk factors including older age (HR, per 10 years increased: 1.76; 95%CI: 1.26-2.16) and AFP > 20 (HR: 4.1; 95%CI: 2.75-6.10), but when an analysis in cirrhotics was performed, there were no differences in HCC-free survival between the DAA-treated and IFN group. According to the authors, this suggests that pre-treatment HCC risk is the factor that determines post-treatment risk. In contrast, untreated cirrhotic patients had a significantly higher incidence rate of HCC compared to both DAA and IFN treated groups (45.31 per 1000 person-years; $P = 0.03$)^[35].

Very recently large cohort studies using real-world data demonstrated that DAA-based HCV treatment is not associated with an increased risk of incident liver cancer and suggested that DAA-based HCV treatments are associated with a reduced risk of incident liver cancer, irrespective of co-medication with interferon. Male gender, older age and baseline cirrhosis were the strongest predictors independently associated with subsequent incident liver cancer^[36]. It's been demonstrated that reaching SVR allows all-cause mortality reduction, including HCC-related mortality, for all stages of hepatic disease. In advanced liver disease, this was first proven when SVR was reached with IFN-based regimens^[37]. In the above mentioned study by Cheung *et al.*^[24] on DAA treatment in patients with decompensated hepatic disease, HCC incidence in patients with SVR24 was lower than in those who did not accomplish it (17/317, 5.4% vs. 10/89, 11.2%; $P = 0.049$; HR: 0.33; 95%CI: 0.13-0.87). The results were compared to HCC incidence in untreated patients (4.2%). There was no evidence of a significant increase in HCC occurrence in treated patients^[24].

Another interesting scenario is liver transplantation, and the clinical impact of viral eradication in patients on waiting list is still poorly evaluated.

Table 1. Current literature about the possible association between antiviral therapy DAAs based and the risk of HCC development

Authors (country)	Study population	Mean FU after DAAs (months)	Occurrence (DAAs)	Occurrence (controls)	Recurrence (DAAs)	Recurrence (controls)	Pos-LT recurrence (DAAs)	Post-LT recurrence (controls)
Reig <i>et al.</i> ^[10] (Spain)	58 cirrhotic patients with previous HCC (complete radiological response)	5.7	NA	NA	16/58* (28%); 25% were multinodular /infiltrative *Median time interval between HCC complete eradication and the start of therapy was 11.2 months	NA	NA	NA
Conti <i>et al.</i> ^[11] (Italy)	344 cirrhotic patients: <ul style="list-style-type: none">• 59 with history of HCC• 285 without previous history of HCC	6	9/285 (3.2%)	NA	17/59* (28.8%) *Younger age and severe fibrosis associated with recurrence	NA	NA	NA
Kozbial <i>et al.</i> ^[12] (Austria)	16 patients who developed HCC (3 of them with previous history of HCC but successfully treated and in complete remission for > 3 years; 3 patients were F3, 5 patients relapsed)	NA	NA	NA	NA	Historical group of 94 cirrhotic pts with SVR after with IFN/RBV 10 developed a HCC within a mean follow-up of 7.8 years	NA	NA
Cardoso <i>et al.</i> ^[19] (Portugal)	54 patients (patients with "non-characterized nodules" and/or a previous diagnosis of HCC were excluded)	12	4/54* (7.4%) *No significant differences in baseline variables that could be associated with an increased HCC risk were found	NA	NA	NA	NA	NA
Yang <i>et al.</i> ^[38] (USA)	81 patients who underwent LT for HCC: <ul style="list-style-type: none">• 18 → pre-LT DAA (3 of them treated with IFN based therapy)• 63 → no pre-LT therapy		NA	NA	NA	NA	5/18#* (27.8%) *Proportion of pta beyond Milan (explant pathology) higher in DAA than controls; no difference in terms of microvascular invasion and HCC differentiation	6/63#* (9.5%) #P = NS

Carrat <i>et al.</i> ^[23] (French)	(1) 267 patients with previous history of HCC (HEPATHER cohort): <ul style="list-style-type: none"> 189 treated 78 untreated (2) 79 patients with previous history of HCC (CirVr cohort) <ul style="list-style-type: none"> 13 treated 66 untreated (3) 214 patient who underwent LT for HCC treated (CUPILT cohort)	(1) 20	NA	NA	(1) 24/189 (0.73/100 person-month) (2) 1/13 (1.1/100 person-month)	(1) 16/78 untreated (0.66/100 person-month) (2) 31/66 untreated (1.73/100 person-month)	NA	NA
Cheung <i>et al.</i> ^[24] (UK)	406 cirrhotic patients [29 (7.1%) with baseline HCC] with decompensated cirrhosis (317 achieved SVR 24)	6-15 (range)	15/288 (5.2%)	11/261 (4.2%)	2/18 (11.1%)	0/11 (0%)	NA	NA
Torres <i>et al.</i> ^[25] (USA)	Prospective observational study of 8 patients with HCC (curative treatment#) treated (1 non cirrhotic) #DAA not offered to patients receiving palliative treatment (i.e., TACE)	12 months* *From DAA start	NA	NA	No recurrence	NA	NA	NA
Zavaglia <i>et al.</i> ^[26] (Italy)	31 patients (4 patients underwent LT during FU)	8 months* *From DAA start	NA	NA	1* (3.2%) *Median time interval between HCC complete eradication and the start of therapy was 19.3 months	NA	Not reported	NA
Kobayashi <i>et al.</i> ^[31] (Japan)	SVR + patients *# (retrospective evaluation): <ul style="list-style-type: none"> 77 DAA 528 Peg-IFN/RBV *No previous history of HCC #Fib-4 score > 3.25 in 29.9% and 14.8%, respectively (< 0.001)	48	2 (2.6%) in DAA group 5-year cumulative HCC development rate 3% In high Fib-4 score group 5-year cumulative rate was 9.7%	5-year cumulative HCC development rates 2.2% (<i>P</i> = NS) In high Fib-4 group 5-year cumulative rate was 8.4% (<i>P</i> = NS)	NA	NA	NA	NA
Petta <i>et al.</i> ^[33] (Italy)	SVR + patients: <ul style="list-style-type: none"> 58 DAA 57 Peg-IFN/RBV 	18 (DAA) 34 (Peg-IFN/RBV)	16 (28%)	22 (39%)	NA	NA	NA	NA

Li <i>et al.</i> ^[35] (USA)	Retrospective population-based cohort study		50 (0.86%)	436 (5.04%)	NA	NA	NA	NA
	17,836 patients: IFN 3534 DAA 5834 Untreated 8468		22.8 per 1000 person year for cirrhotic patients ($P = 0.03$ using IFN as control)	45.3 per 1000 person year for cirrhotic patients ($P = 0.03$ using IFN as control)				
	#Fib-4 score > 3.5 in 13.1%, 19.1% and 14.6% respectively							
	*Excluded if baseline or prior HCC							
Kanwal <i>et al.</i> ^[34] (USA)	Retrospective cohort study		271 (1.18 %)	NA	NA	NA	NA	NA
	22,500 patients DAA (39% diagnosis of cirrhosis, Fib-4 score > 3.25 in 29.7 %)		(3.45% among who did not achieve SVR vs. 0.90% who achieved SVR, $P < 0.0001$)					
	*No previous history of HCC							
Zanetto <i>et al.</i> ^[41] (Italy)	46 patients who underwent LT for HCC: (1) 23 pre-LT DA (2) 23 no pre-LT therapy	(1) 10 months (2) 7 months	NA	NA	NA	NA	1/8 (12.5%)#	1/12 (8.3%)# #($P = NS$); no difference in terms of number, TTV of HCC nodules, microvascular invasion and HCC differentiation
Ioannou <i>et al.</i> ^[29] (USA)	62,354 treated from 1999 and 2015: 35,871 (58%) IFN 4535 (7.2%) DAA + IFN 21,948 (35%) DAA	1.53 years (180 days - 6.1 years)	445 (2%) 1.32 per 100 patient-year (AHR 1.12)	DAA + IFN 1.06 per 100 patient-years (AHR 1.04) IFN 0.81 per 100 patient-years (AHR 1)	NA	NA	NA	NA
	16.8% cirrhotic patients, 4.7% decompensated cirrhosis, 1.1% had undergone LT		DAA SVR associated with a 71% reduction in HCC risk (AHR 0.29)					
Cabibbo <i>et al.</i> ^[27] (Italy)	Prospective multicentre study 143 patients with previous HCC (curative treatment) treated with DAA, 76% BCLC stage A when HCC treated	8.7 (3-19)	NA	NA	29/143 (20.3%): 13 (9.1%) during DAA therapy, 16 (11.2%) after DAA therapy. 62% BCLC A (17% infiltrative pattern)	NA	NA	NA
	138 (96%) achieved SVR				6-, 12-, 18-month recurrence: 12%, 26.6%, 29.1%			

DAAs: direct-acting antivirals; HCC: hepatocellular carcinoma; LT: liver transplantation; FU: follow-up; BCLC: Barcelona Clinic Liver Cancer; SVR: sustained virological response; AHR: adjusted hazard ratio; IFN: interferon; TACE: transarterial chemoembolization; RBV: ribavirin; NA: not available; NS: not significant

A study showed a high risk of HCC recurrence in patients treated with DAAs before liver transplantation (LT) (5 of 18 patients, 28%) compared to untreated (6 of 63 patients, 9.5%). However, the difference did not reach statistical significance because of the small number of patients enrolled^[38], a series definitely too limited to provide any information.

Belli *et al.*^[39] published data from a European study, investigating the probability of delisting after DAA treatment. In this study the cumulative incidences of inactivation and delisting at 60 weeks were 33% and 19.2% respectively. In another recent study published by Pascasio *et al.*^[40], 238 patients treated with DAAs while awaiting LT were enrolled, and 24% of the patients with decompensated cirrhosis were delisted after a median follow-up period of 50 weeks, as a result of clinical improvement, which appeared to remain stable in most patients. Indeed, only 9% of the patients were delisted because of HCC progression and the rate of microvascular invasion was 11%, similar to what reported in previous studies. Although these data do not indicate an increase in HCC progression, the lack of untreated patients as a control group is a limitation. As a consequence, the use of DAA therapy in HCC patients awaiting LT cannot be strongly recommended. As far as SVR is concerned, in the above-mentioned study SVR rates were similar in patients with and without HCC (87% vs. 84%, $P = 0.560$), and amongst patients with HCC there were no significant differences regarding tumor characteristics or BCLC staging comparing those with or without SVR.

In a recent retrospective study, conducted at the Padua Liver Transplant Centre, we investigated whether patients, listed for HCC and treated with DAAs, have an increased rate of tumor progression and consequently drop out from waiting list. Two groups (including 23 patients each) were evaluated, who underwent DAA therapy while awaiting LT or not. The two groups did not show any significant difference in terms of dropout rate, during a median follow-up of 10 and 7 months. Interestingly, a significantly lower probability of being transplanted was detected in the group of treated patients in comparison with the untreated, suggesting an improvement of liver function. With regard to post-LT recurrence of HCC, similar rates were found in the two groups (12.5% in DAA-treated vs. 8.3% in untreated group), suggesting that the risk of tumor recurrence was not higher in patients treated with DAA pre-LT than in those treated post-LT. Furthermore, liver explant histopathological analysis revealed similar HCC patterns in the 2 groups^[41].

DAA AND HCC: HISTOLOGICAL PATTERN

Reig *et al.*^[10] in their pivotal study also expressed concern about the histological pattern of HCC recurrence in patients treated with DAA therapy. In agreement with Reig's study, Romano *et al.*^[42] demonstrated that about 30% of HCC presented with an infiltrative and/or multifocal pattern in a multi-centre cohort of cirrhotic patients treated with DAAs, even though their data on HCC incidence were in contrast with Reig's results. The more aggressive pattern of HCC was seen somehow more frequently (54.6%) in patients without SVR compared to those with SVR (12.1%) in which the single nodule pattern prevailed (69.7%).

Nakao *et al.*^[43] also investigated the pattern of HCC recurrence and *de novo* development, reporting six cases of *de novo* HCC out of 242 patients. All of the patients had been submitted to DAAs treatment, and all showed SVR. In all six cases HCC was pathologically diagnosed, allowing inferences about tumor characteristics and kinetics. All tumours were single nodules, moderately differentiated and rapidly growing, the authors were therefore led to hypothesize that HCC carcinogenesis after DAA therapy occurs in a non-conventional, multi-step manner.

DAA AND HCC: ONLY IMMUNOLOGICAL ISSUE?

It is recognized that the immune system plays a key role in modulating tumour development, but a report by Debes *et al.*^[44] attempted to distinguish the immuno-related changes by measuring 22 different soluble immune mediators in patients who developed HCC (both *de novo* and recurrent) after HCV treatment

with DAAs, comparing them to matched controls without HCC. Each marker was measured before and after DAA treatment, and very interestingly 12 of them, including apoptosis markers, cytokines and growth factors, resulted significantly higher before treatment in patients who developed *de novo* HCC, compared to controls. The authors suggested that a different immunologic pattern could be already present in patients who eventually develop HCC, before the immune changes due to DAAs occur. The immune background could therefore be a decisive factor in HCC development. Individuals who develop HCC may express a different pattern of immune mediators, that induces ongoing carcinogenic or pre-carcinogenic activity, prior to the appearing of HCC. In addition, TNF alpha levels remained stable or trended up during the first month of DAA treatment (with viral load being undetectable in serum) in patients who developed HCC, while decreasing in controls. TNF alpha could therefore be directly involved in HCC development even if HCV is absent, or on the other hand its production could be stimulated by the presence of occult tumor foci in the liver. Finally, this study suggested that tumorigenesis occurred with different characteristics in HCC recurrence compared to *de novo* HCC after DAAs, as IL-6 levels, were shown to be increased at the end of therapy in patients with HCC recurrence, while the levels of the cytokine showed a trend toward reduction in patients with *de novo* tumor. Again, these results should be interpreted with caution and additional studies could help to clarify their interpretation.

HCC AND DAA: POSSIBLE ROLE IN RESPONSE TO ANTIVIRAL THERAPY AND NATURAL HISTORY OF DISEASE?

Another matter of debate is whether the presence of HCC can influence the response to HCV therapy with direct-acting antivirals, and what could be the mechanism behind it. According to Prenner *et al.*^[45], the presence of active HCC (and not merely a history of tumor) when starting HCV therapy was the strongest predictor of treatment failure, with an eight-fold increased risk of failing treatment at multivariate analysis compared to patients without tumour (OR 8.49; 95%CI: 3.90-18.49; $P < 0.001$). Interestingly, none of the well-known factors correlated with a lower SVR, and not even inadequate treatment regimens could explain the difference between the two groups. A possible explanation could be that HCC may serve as a sanctuary for HCV, where virus particles can evade DAAs, as already known for HBV cccDNA; it is also possible that DAAs may be unequally distributed within fibrotic areas, generated for instance after some loco-regional treatment, radioembolization above all, due to the decreased blood flow.

Very recently, a review by Konjeti and John^[46] on DAAs and HCC presence/occurrence suggested deferring IFN-free therapy until complete radiological response to HCC curative treatment. Therefore HCV eradication with DAAs is still recommended in patients with history of treated HCC, until proven otherwise by future studies.

Similar data (and similar explanations) to Prenner *et al.*^[45] study was obtained through the analysis of a large cohort of HCC identified by Beste *et al.*^[47] in the national Veterans Affairs health care system. This study also suggested that a greater likelihood of SVR after DAA treatment was reached in patients with HCC history undergoing LT. This evidence is not fully explained by clinical reasons.

What is the most effective timing to offer HCV treatment in patients listed for LT, whether it is better to do so before or after LT, still remain open questions. Finally, another matter has been investigated: does SVR really matter in the progression of liver disease? Nahon *et al.*^[48], in a multi-centre French cohort of 1323 Child A patients, mostly treated with IFN-based therapy, reported that viral eradication and achievement of SVR was associated with a significant reduction of HCC incidence (HR: 0.29; 95%CI: 0.19-0.43; $P < 0.001$). They also noticed that SVR was associated with a reduction in both liver- and non-liver-related mortality (HR: 0.27; 95%CI: 0.18-0.42; $P < 0.001$). Petta *et al.*^[49] seem to have come to the same conclusions: in an Italian study of 535 HCV cirrhotic patients, there was a reduction in disease progression and liver related mortality with the achievement of SVR. More specifically, the data of this report demonstrated a reduced incidence of

hepatic decompensation and HCC development, with a lower likelihood of liver related death at 10 years, in association with achievement of SVR^[49]. Similar findings were reported in the Hepatitis Testers cohort from North America^[50]. In the multivariable model, SVR was associated with reduced liver cancer risk (HR: 0.20; 95%CI: 0.13-0.30) in a median follow-up of 5.6 years.

DISCUSSION

The main points of strength of the previously mentioned studies are their prospective design and the large number of patients enrolled and the long follow-up. The largest studies included patients treated with IFN-based regimes and therefore a confirmation with DAA-based treatment only is needed.

Camma *et al.*^[51] considered Reig's observations as premature, affirmed that a comparison with untreated controls is mandatory to solve the issue without generating excessive alarm on DAAs; Nault and Colombo^[16], at the same time, did not consider the data published solid enough to confirm the increased risk in treated patients, even though they could recommend HCC surveillance after viral eradication; Alberti and Piovesan^[52] underlined the great variability in occurrence and in recurrence rates, reflecting the extreme heterogeneity of the different clinical settings and patient cohorts on which studies were based; finally, Blanco and Rivero-Juarez^[53] specified that prospective studies targeted on this problem are necessary before even considering a different therapeutic approach to patients with HCV-related liver disease.

Furthermore, the development of an aggressive tumor has been reported in some of patients^[42], although the authors cannot exclude that what they observed in these patients merely reflects the natural history of their liver disease. It is important to point out that the current clinical practice does not include IFN-based treatment any more, due to the important improvement in HCV-treatment made by DAAs. As a consequence, to compare DAA treatments with pre-DAA treatments or no treatment is meaningless. Nevertheless, the lack of randomized control trials is an important clinical and methodological issue. In conclusion, the risk reduction in hepatic decompensation as well as in HCC incidence in patients achieving SVR is the only proven evidence. The reports about the increased risk of HCC occurrence/recurrence in DAA treated patients are afflicted by selection and methodologic biases, that weaken the impact of these studies. We strongly believe that is mandatory to treat HCV-infected patients with DAAs but also to maintain an active surveillance for liver cancer as the guidelines suggest; the previously presented data must be considered with caution.

DECLARATIONS

Authors' contributions

Wrote the review and approved the final version: Russo FP, Tessari M, Imondi A

Edited the English: Lynch EN

Edited the manuscript and approved the final version: Farinati F

Availability of data and materials

Not applicable.

Finacial support and sponsorship

None.

Conflicts of interest

The authors declare no conflicts of interest in association with this study.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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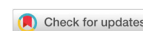
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Review

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Hypoxic microenvironment and hepatocellular carcinoma treatment

Ci-Ai Lin, Lin-Lin Chang, Hong Zhu, Qiao-Jun He, Bo Yang

Zhejiang Province Key Laboratory of Anti-Cancer Drug Research, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, Zhejiang, China.

Correspondence to: Dr. Bo Yang, Zhejiang Province Key Laboratory of Anti-Cancer Drug Research, College of Pharmaceutical Sciences, Zhejiang University, 866# Yuhangtang Rd, Hangzhou 310058, Zhejiang, China. E-mail: yang924@zju.edu.cn; Dr. Qiao-Jun He, Zhejiang Province Key Laboratory of Anti-Cancer Drug Research, College of Pharmaceutical Sciences, Zhejiang University, 866# Yuhangtang Rd, Hangzhou 310058, Zhejiang, China. E-mail: qiaojunhe@zju.edu.cn

How to cite this article: Lin CA, Chang LL, Zhu H, He QJ, Yang B. Hypoxic microenvironment and hepatocellular carcinoma treatment. *Hepatoma Res* 2018;4:26. <http://dx.doi.org/10.20517/2394-5079.2018.27>

Received: 22 Mar 2018 **First Decision:** 17 May 2018 **Revised:** 9 Jun 2018 **Accepted:** 9 Jun 2018 **Published:** 29 Jun 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Hepatocellular carcinoma (HCC) is one of the most rapidly growing and prevalent cancers in the whole world. The characterized hypoxia region inside the HCC tumors has been recently found as the key driver of HCC malignance and treatment failure, leading to a variety of hypoxia-related biological consequences including angiogenesis, metastasis, metabolism deregulation and drug resistance, which ultimately resulted in treatment failure of HCC. This review will summarize the signaling pathways involved in hypoxia-mediated malignance of HCC and discuss current advances of hypoxia-targeted therapies.

Keywords: Hepatocellular carcinoma, anti-cancer drugs, hypoxia-targeting strategie

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer globally, with a high mortality of 5-year survival rate less than 10%^[1,2]. There are various etiologies implicated in development of HCC, including infection of hepatitis B virus (HBV) or hepatitis C virus (HCV)^[3,4], chronic infection, and alcohol consumption^[5]. According to the Barcelona Clinic Liver Cancer (BCLC) staging, HCCs can be classified into five stages with each receiving different treatments^[1]. Effective therapeutic options include liver resection and liver transplantation, ablation and chemoembolization^[1]. However, for patients often diagnosed with advanced, unresectable or metastatic HCC, chemotherapeutic treatment would be the only option^[1]. Yet, as reported, significant drug resistance in these patients ultimately resulted in treatment failure^[6].



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Hypoxia is a common phenomenon in the intratumor regions of HCC patients^[6]. Abnormal microvasculature and unrestrained proliferation of HCC cells lead to oxygen deficiency^[6,7]. Hypoxia is involved in multiple biological process of HCC and promotes tumor aggressiveness, chemoresistance and immunotherapy resistance^[8,9]. Consequently, the hypoxic microenvironment has been regarded as promising target for HCC treatments. Under hypoxia, hypoxia-induced factors (HIFs) would be stabilized to trigger the transactivation of a series of hypoxia-response genes which promote the malignance of HCC. Thus, the transcription factors HIFs have been regarded as master regulators of hypoxic microenvironment^[8]. In contrast, several lines of evidence also implicated the HIF-independent hypoxia responses^[10-14]. Taken together, the mechanisms in HCC progression under hypoxia are complicated and sophisticated. This review will summarize current research advances in hypoxia-mediated molecular mechanism, how hypoxia participates in the progression of HCC and the current intervention strategies targeting intratumor hypoxia of HCC.

HYPOXIA PLAYS CRITICAL ROLES IN THE PROGRESSION AND MALIGNANCE OF HCC

Due to the rapid-growing nature of HCC, increased numbers of cells consume increased amount of oxygen, and hypoxia exists in regions of the tumor that are far away from blood vessels^[15]. Through various signaling pathways, hypoxia further triggers a series of HCC transformation, mediating its angiogenesis, metastasis, metabolism deregulation and drug resistance^[6]. Hypoxia is a major cause of hypervascularity of HCC by inducing angiogenic factors to stimulate angiogenesis and support tumor growth^[15]. In addition, a variety of genes would be transactivated under hypoxia by HIFs or the other transcriptional factor and involved in multiple steps of HCC metastasis including epithelial-mesenchymal transition (EMT), invasion of the extracellular matrix, intravasation, extravasation, and secondary growth of the metastases^[5]. Besides, hypoxia-regulated glycolysis module also contributes to HCC progression^[16]. Recent study also indicates that hypoxia promotes the differentiation and expansion of immune-suppressive stromal cells, and remodels the metabolic landscape to support immune privilege^[9]. Therefore, hypoxia can reduce the effectiveness of cancer immunotherapy. Thus, hypoxia microenvironment is highly relevant in HCC development and extensively involved in the process of HCC progression.

MOLECULAR PATHWAYS INVOLVED IN HYPOXIC HCC MALIGNANCE

The complicated and sophisticated pathways underlying hypoxia have been extensively investigated, and HIFs are identified to play pivotal roles under hypoxia, which has attracted most attention in this field for the last decades^[6,8]. Yet recently, the findings on the HIFs-independent regulation of tumor angiogenesis and chemoresistance under hypoxic conditions have challenged this notion and raised the possibility that the other important signaling pathways may also participate and promote the progression and malignance of HCC^[10-14]. Accumulating evidence shows that Yes associate-Protein (YAP)^[17], matrix metalloproteinases (MMPs), high mobility group box 1 (HMGB1) and glucose metabolism enzymes are involved in hypoxia-mediated effects in HCC^[18]. The above key molecules would be activated as sensors of intratumoral oxygen tension, and trigger the subsequent activation of hypoxia-mediated process, thus may also be regarded as potential targets for HCC therapy.

HIFs-dependent pathways

HIF system is composed of α -subunits and β -subunits. Under normoxia, HIF1 α is maintained at very low basal activities due to constitutive degradation. Prolyl hydroxylation of HIF1 α by prolyl hydroxylase domain-containing proteins (PHD1, PHD2 and PHD3) induces its ubiquitination and proteasomal degradation by an E3 ligase^[6], von Hippel-Lindau tumor suppressor protein (pVHL). Besides, asparaginyl hydroxylation of HIF1 α by factor inhibiting HIF (FIH) interferes its interaction with transcriptional coactivators, CREB-binding protein (CBP) and p300^[19,20]. Under hypoxia, lacking sufficient oxygen, hydroxylation and proteasomal degradation of HIF1 α are impaired. HIF1 α is stabilized and then translocates into nucleus,

heterodimerizes with HIF1 β and binds core hypoxia-response element [HRE, 5'-(A/G)CGTG-3']^[21]. Many HIF target genes play important roles in HCC proliferation, metabolism, angiogenesis, invasion and metastasis^[6].

Activation of Wnt/ β -catenin pathway, PI3K/AKT pathway and SNAIL1 are involved in the epithelial mesenchymal transition (EMT), increasing HCC invasion and metastasis^[22,23]. As reported, β -catenin can reinforce the transcriptional activity of HIF1 α and consequently facilitate hypoxia-induced EMT^[24]. And regulation of BCL9 expression by HIF1 α may explain the crosstalk between Wnt/ β -catenin signaling and hypoxia signaling pathways^[25]. Besides, HIF1 α activation can be regulated by PI3K/Akt pathway, and the activation of PI3K/Akt/HIF1 α pathway mediates hypoxia-induced EMT and drug resistance^[26,27]. HIF1 α also promotes EMT through increasing *SNAIL1* transcription in HCC cells under hypoxia^[28]. Angiogenic factors like VEGF, bone morphogenetic protein 4 (BMP4) and stem cell factor (SCF) can enhance HCC angiogenesis^[29]. VEGF has been well characterized as a direct target of HIF systems^[30], promoting endothelial cell proliferation and migration especially in areas of hypoxia^[31,32]. Additionally, hypoxia-induced BMP4 expression is regulated by HIF1 α ^[33] and SCF expression is HIF2 α -dependent^[34] to promote HCC angiogenesis and metastasis. Many glycolysis-related genes can be transcriptionally activated by HIF1 α , such as phosphoglycerate kinase 1 (PGK1), hexokinase-2 (HK2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphofructokinase (PFK)^[6,16]. It indicates that there is an increased glycolysis in the progression of hypoxia HCC to adapt to oxygen deficiency. HIF1 α induces growth factors, including TGF- α and IGF-2, to promote cell proliferation and survival^[35]. TGF- α /EGFR can be activated by HIF2 α and contribute to sorafenib resistance in HCC cells^[31]. Besides, HIF1 α regulates the expression of MMPs to induce extracellular matrix degradation and tumor metastasis^[36]. NKG2D is critical in directing NK cell responses against tumors. Yamada *et al.*^[37] show that hypoxia promotes downregulation of the NKG2D ligand MICA by tumor cells via a HIF1 α -dependent mechanism. Under hypoxia and in the presence of TGF- β , CD4⁺ T cells upregulate Foxp3 through direct binding of HIF1 to Foxp3 promoter region, inducing Treg formation and immune tolerance^[38]. Taken together, HIF system regulates hypoxic responses of HCC through diverse signaling pathways, and contributes to HCC progression and malignant process.

HIFs-independent pathways

HMGB1 signaling pathways

HMGB1 is a chromatin-binding nuclear damage associated molecular pattern^[39]. Its release under hypoxic condition can induce an inflammatory response to promote invasion and metastasis in HCC cells. Under hypoxic, HMGB1 activates TLR4 and RAGE signaling pathways to induce caspase-1 activation. Caspase-1 subsequently mediates the cleavage and release of a series of pro-inflammatory cytokines (IL-1 β and IL-18), which in turn promote cancer invasion and metastasis^[18,40]. Moreover, recent studies suggest that HMGB1 can also translocate from the nucleus to the cytosol under hypoxia, and then bind to mtDNA released from damaged mitochondria^[39]. Subsequent activation of TLR9 signaling pathway promotes HCC proliferation^[18,39], indicating a novel mechanism of the involvement of HMGB1 in HCC progression under hypoxia.

Hippo-YAP pathways

The Hippo pathway is a classical regulator of organ size and regeneration, and YAP is an important transcriptional co-factor locating at the downstream of Hippo pathway^[41,42]. The activation of YAP promotes survival, chemoresistance, metastasis, and the other malignant properties of HCC^[43]. It has been reported in recent studies that hypoxia induces nuclear translocation and activation of YAP in a HIF-independent way, and the subsequent activation of target genes promotes cell survival, resistance to SN38 and sorafenib in HCC^[17,43]. Meanwhile, statins (the inhibitors of hydroxymethylglutaryl-CoA reductase) can suppress YAP target genes and overcome hypoxia-induced resistance to sorafenib^[43]. Moreover, YAP could also contribute to liver tumorigenesis by inducing HIF1 α -dependent aerobic glycolysis^[44]. HMGB1 is relevant in this process by binding to GA-binding protein alpha (GABP α) to promote the expression of YAP^[44].

THERAPEUTIC STRATEGIES TARGETING-HYPOXIA FOR HCC TREATMENT

Sorafenib is the only effective first-line drug for advanced HCC^[45]. However, hypoxia-induced chemoresistance to sorafenib leads to treatment failure^[43]. Hypoxia also confers resistance to various anticancer drugs in HCC cells, including etoposide, sorafenib, SN38, cisplatin and doxorubicin^[6]. As hypoxia induces tumor malignant transformation and plays an important role in resistance to radiotherapy and chemotherapy^[14], target-hypoxia therapy is reasonable in HCC treatment. There are several approaches to target hypoxic microenvironment. One approach is to design hypoxia-activated bioreductive pro-drugs which would be activated by enzymatic reduction in hypoxic tissue; the other one is to target key molecules specifically activated in hypoxic cells, such as the most studied HIFs inhibitors. In addition, emerging new strategies such as oxygen supplement^[46] and vessel normalization^[47] were also developed to target the hypoxic cancers.

Bioreductive prodrugs

Bioreductive prodrugs generally share a common mechanism of activation. They are activated by enzymatic reduction in hypoxic tissue to form cytotoxins, resulting in hypoxia-selective cell killing^[48].

OXY111A is a synthetic allosteric effector of hemoglobin-4 and promotes normoxia in hypoxic tumors^[48]. OXY111A has been tested in several cancer animal models, showing beneficial outcomes and low side effect profiles^[49]. It is also shown to prevent HIF1 α stabilization as well as VEGF production^[6]. Tirapazamine (TPZ; SR4233) belongs to the aromatic N-oxide family and has been extensively evaluated. TPZ is reported to potentiate the antitumor efficacy of many anticancer drugs^[50-54], becoming a promising compound in combination-therapy. In addition, TPZ can also sensitize HCC cells to topoisomerase I inhibitors via cooperative modulation of HIF1 α ^[54]. As a novel hypoxia-activated prodrug, Q6^[55] arrests tumor growth *in vivo* through dual hypoxia-targeted regulatory mechanisms. Q6 exhibits potent antiproliferative efficacy and induces apoptosis in HCC under hypoxic. Besides, Q6 can induce attenuation of HIF1 α expression through autophagy-dependent degradation pathway as well. Recent study suggests that Q6 induces G2-M arrest and apoptosis via poisoning topoisomerase II^[56]. Thus Q6 shows a more potent anti-proliferative effect than TPZ.

Drugs targeting hypoxia related molecules

As a curcumin analog, diphenyl difluoroketone (EF24) is an effective and promising anticancer compound. EF24 enhance the antitumor effects of sorafenib and overcomes sorafenib resistance through VHL (Von Hippel-Lindau tumor suppressor)-dependent HIF1 α degradation and NF- κ B inactivation^[6]. Generally, EF24 exerts its effects by inhibition of proliferation and induction of apoptosis. It is reported that EF24 induces G2/M arrest and apoptosis by increasing phosphatase and tensin homologue expression (PTEN) in ovarian cancer cells^[57]. Recently, EF24 has been shown to suppress invasion and migration of HCC cells *in vitro* via inhibiting the phosphorylation of src^[58]. A series of compounds targeting hypoxia HCC are on clinical trials, such as RO7070179 and EZN-2968, both of which are antisense oligonucleotide inhibitors of HIF1 α ^[31,59]. Other compounds like Bufalin (target inhibition of PI3K-AKT-mTOR activity), ENMD-1198 (a microtubule destabilizing agent) and Metformin (an established antidiabetic drug) are involved in the suppression HIF1 α expression^[6,60].

The other treatments

Hyperbaric oxygen (HBO) treatment can enhance the amount of dissolved oxygen in the plasma and increase O₂ delivery to the tissue oxygen, so it can be used to overcome hypoxia. Both recent and previous research studies have shown that HBO can be inhibitory and reduce cancer growth in some cancer types^[46]. Granowitz *et al.*^[61] show that HBO can inhibit benign and malignant human mammary epithelial cell proliferation. In another study, Cheng *et al.*^[47] engineered VNP20009 to express histidine-proline-rich glycoprotein (HPRG) under the control of a hypoxia-induced NirB promoter. HPRG has potent antiangiogenic and tumor vessel normalization properties. Attenuated *Salmonella Typhimurium* strain VNP20009 preferentially accumulates and replicates in hypoxic tumor regions. They found that VNP20009-

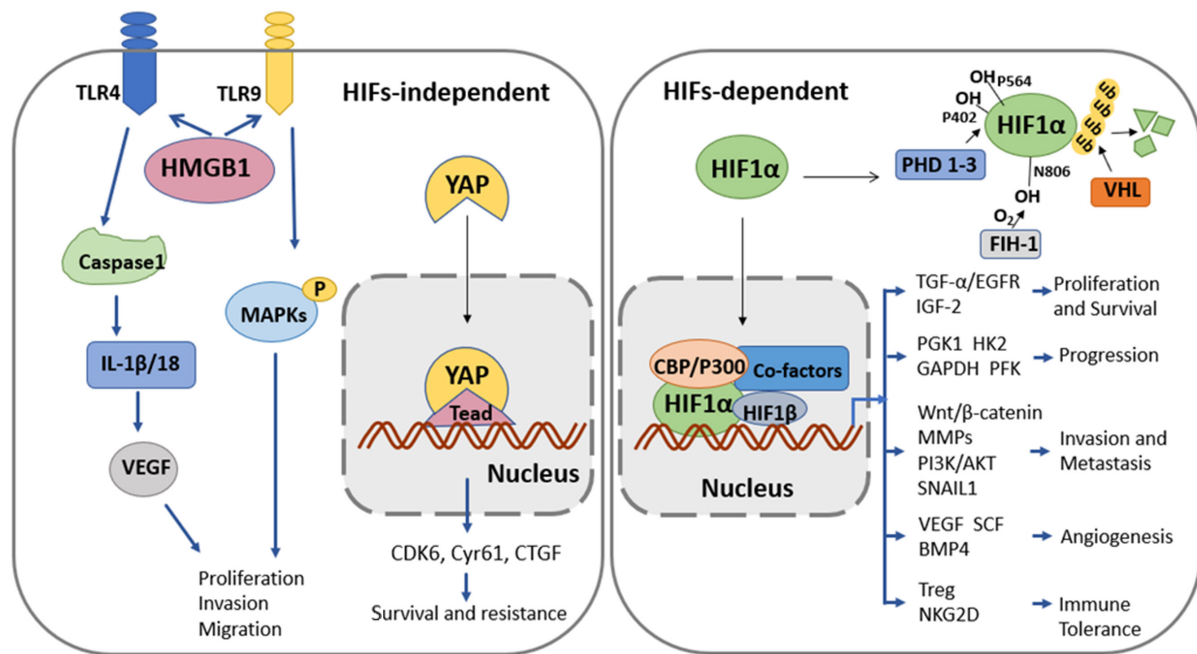


Figure 1. Molecular pathways involved in hypoxia HCC malignance. HCC: hepatocellular carcinoma; YAP: Yes associate-protein; MMPs: matrix metalloproteinases; HMGB1: high mobility group box 1; TLR: Toll-like receptor; IL: interleukin; VEGF: vascular endothelial growth factor; HIF: hypoxia-induced factor; SCF: stem cell factor

mediated targeted expression of HPRG (VNP-pNHPRG) can down-regulate the HIF1 α -VEGF/Ang-2 signal pathway by altering the hypoxic tumor microenvironment.

PERSPECTIVES

Hypoxia is highly relevant in malignant transformation of HCC and activates complicated molecular and cellular pathways through HIF-dependent or independent mechanisms [Figure 1]. Hypoxia promotes angiogenesis, invasion, metastasis, proliferation, glycolysis, drug resistance, inflammation and immune evasion. Consequently, targeting hypoxia has been regarded as promising strategies for HCC treatment. Nonetheless, there is no clear clinical evidence of efficient outcome of anti-cancer treatment due to the HIF-inhibition or the treatment of bioreductive agents. In the field of hypoxia-related studies, several concerns still remain, which need to be fully elucidated in the near future, so as to improve the clinical outcome of HCC patients, particularly those displayed intratumor hypoxia:

1. Mechanism of hypoxia response is not fully elucidated, as some recent studies have reported HIF-independent regulation of hypoxia response. It requires further investigation to further unravel the signaling pathways and crosstalk involved in hypoxic cancer;
2. There's a lack of effective treatment for hypoxic cancer. The efficacy of the bioreductive prodrugs should be improved, probably by selecting clinical cancer patients by appropriate biomarkers. In addition, more targets specifically activated under hypoxia should be exploited to seek more promising therapeutic strategies;
3. There's a problem of heterogeneity. It still remains elusive whether different quantitative levels of hypoxia in the same tumor tissue will represent similar response to hypoxia-targeted therapy. In order to guarantee the effectiveness and preciseness of treatment, we can take into consideration of researches in the relationship between the quantitative hypoxia levels and drug response.

In summary, with more profound investigations on hypoxic microenvironment, highly efficient and highly selective interventions will be developed, which will ultimately benefit those HCC patients with severe intratumoural hypoxia..

DECLARATIONS

Authors' contributions

Manuscript writing: Lin CA, Chang LL, Zhu H

Manuscript revising: He QJ, Yang B

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by the National Natural Science Foundation for Distinguished Young Scholar of China (No. 81625024).

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Expression quantitative trait loci for *PVT1* contributes to the prognosis of hepatocellular carcinoma

Ting Tian^{1,2#}, Ci Song^{1,2#}, Zhe-Ning Pu^{1,2}, Zi-Jun Ge^{1,2}, Cheng-Xiao Yu^{1,2}, Ji-Bin Liu³, Zhi-Bin Hu^{1,2}

¹Department of Epidemiology, School of Public Health, Nanjing Medical University, Nanjing 211166, China.

²Jiangsu Key Lab of Cancer Biomarkers, Prevention and Treatment, Collaborative Innovation Center For Cancer Personalized Medicine, Nanjing Medical University, Nanjing 211166, China.

³Department of Hepatobiliary Surgery, Nantong Tumor Hospital, Nantong 226361, China.

#The two authors contributed equally to this work.

Correspondence to: Dr. Zhi-Bin Hu, Department of Epidemiology and Biostatistics, Jiangsu Key Lab of Cancer Biomarkers, Prevention and Treatment, Collaborative Innovation Center For Cancer Personalized Medicine, School of Public Health, Nanjing Medical University, 101 Longmian Rd., Nanjing 211166, China. E-mail: zhibin_hu@njmu.edu.cn

How to cite this article: Tian T, Song C, Pu ZN, Ge ZJ, Yu CX, Liu JB, Hu ZB. Expression quantitative trait loci for *PVT1* contributes to the prognosis of hepatocellular carcinoma. *Hepatoma Res* 2018;4:27. <http://dx.doi.org/10.20517/2394-5079.2018.24>

Received: 19 Mar 2018 **First Decision:** 29 May 2018 **Revised:** 22 Jun 2018 **Accepted:** 22 Jun 2018 **Published:** 29 Jun 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Aim: Plasmacytoma variant translocation 1 (*PVT1*), a long intergenic non-coding RNA, was overexpressed in liver cancer. A single nucleotide polymorphism (SNP) rs4733586 was identified as an expression quantitative trait loci (eQTL) for *PVT1* using bioinformatics analysis. This study was to assess the association of *PVT1* eQTL with hepatocellular carcinoma (HCC) prognosis.

Methods: A case-only study was performed to assess the association between SNP and HCC overall survival in 331 HCC patients with hepatitis B virus. Cox proportional hazard regression models were conducted for survival analysis with adjustment for age, gender, smoking status, drinking status, Barcelona-Clinic Liver Cancer stages, and chemotherapy or transcatheter hepatic arterial chemoembolization (TACE) status.

Results: The variant genotype C allele of rs4733586 was significantly associated with a higher death risk compared with T allele (adjusted hazard ratio = 1.26, 95% confidence intervals = 1.05-1.51, $P = 0.012$ in the additive model). By stepwise Cox proportional hazard analysis, four variables (age, drinking status, chemotherapy or TACE status, *PVT1* eQTL) were remained in the final regression model. In the stratified analysis, no heterogeneity was observed among different subgroups.

Conclusion: These findings suggest that eQTL SNP for *PVT1* may be susceptibility marker for the HCC overall survival.



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Keywords: Plasmacytoma variant translocation 1, expression quantitative trait loci, long non-coding RNA, hepatocellular carcinoma, survival

INTRODUCTION

Liver cancer is the second most common cause of cancer death in the world, of which hepatocellular carcinoma (HCC) is the predominant form^[1]. Ranked as the sixth most common form of cancer, HCC is also the third leading cause of cancer death^[2]. In previous study, 3-year survival rate among patients at intermediate stages was 50%, whereas among those at advanced stage was just 8%^[3]. Although several therapies including radiofrequency ablation, liver transplantation, tumor resection and some others are the potentially effective treatments for HCC, HCC still has a poor 5-year survival rate of about 7%^[4,5]. Due to different factors of disease and the poor survival outcomes of HCC patients, it is crucial to identify beneficial molecular biomarker to guide individualized treatment and to improve the prognosis of cancer patients.

Non-coding RNAs (ncRNAs) are emerging as novel regulatory factor in the cancer paradigm^[6]. Long noncoding RNAs (lncRNAs), longer than 200 nucleotides in length, are evolutionarily conserved non-protein coding RNAs^[7]. LncRNAs have been reported to play an important role in various biological processes related to cancer progressions, such as proliferation, apoptosis and invasion. Plasmacytoma variant translocation 1 (*PVT1*), a long intergenic non-coding RNA, is located in the chr8q24.21 region^[7]. Chromosome 8q24 contains a locus conferring an increased risk for multiple cancers^[8]. Recently, several studies have found that *PVT1* was functioned as an oncogene and was overexpressed in human tumors including cervical cancer, serous melanoma and prostate cancer^[9]. In addition, it was also reported that *PVT1* overexpression was associated with clinicopathological features and reduced patients' survival times^[9]. However, the potential function of *PVT1* expression quantitative trait loci (eQTL) in the prognosis of HCC has been rarely discussed.

In this study, we identified one single nucleotide polymorphism (SNP) (rs4733586) that may be the eQTL for *PVT1* (<http://www.regulomedb.org>) by using the bioinformatics analysis. Therefore, we thought that the SNP rs4733586 may be likely to regulate the expression of *PVT1*. Here, we assumed that *PVT1* eQTL may contribute to the development and progression of HCC. To verify our hypothesis, we examined the effect of the *PVT1* eQTL (rs4733586) on the HCC prognosis of 331 patients from Han population.

METHODS

Study subjects

This study was authorized by the local institutional review board at Nanjing Medical University. After approval by the ethics committees, all the participants were given written informed consent, and the registration of the participants was described before^[10,11]. In brief, all the patients were consecutively recruited from Nantong Tumor Hospital and the First Affiliated Hospital of Nanjing Medical University, Jiangsu, China^[12], and were face-to-face interviewed to collect the demographic information including age, gender, smoking and drinking status. We recruited patients with HCC with hepatitis B virus (HBV) and excluded those with hepatitis C virus (HCV). All the subjects were diagnosed as HCC by histopathological examination. To construct a relatively homogeneous population, our study was limited to HCC patients who have not undergone surgery in intermediate stage (B) or advanced stage (C) according to the Barcelona Clinic Liver Cancer (BCLC) staging system^[13]. Eventually, 331 of 414 intermediate or advanced HCC patients completed the follow-ups with the response rate of 80.0% and were performed the survival analysis. We followed up the study subjects every 3 months from the time of recruitment until the death or the last time of follow-up (January 2013).

Table 1. Information of primers for Sequenom MassARRAY iPLEX

SNP	Primer	Sequence (5'-3')
rs4733586	2nd-PCR Primer	ACGTTGGATGCAGATTGGAGAGTAGTGGCT
	1st-PCR Primer	ACGTTGGATGACATCCGCCCTGGGTGATTC
	Extend Primer	GTAGTGGCTCATCACA

SNP: single nucleotide polymorphism; PCR: polymerase chain reaction

Serological testing

As described in previous study^[11], HBsAg, anti-HBs, anti-HBc and anti-HCV were detected from every patient's collected serum by following the step of the enzyme-linked immunosorbent assay (Kehua Bio-engineering Co., Ltd., Shanghai, China).

SNP selection and genotyping

We found one common eQTL SNP (rs4733586) in the intron region of lncRNA *PVT1* based on the criteria of minor allele frequency (MAF) > 0.05 in Han Chinese from Regulome database. The genomic DNA was extracted from the leukocyte pellet by a series of treatments using conventional methods^[14]. Then, we use the Sequenom Mass ARRAY iPLEX platform (Sequenom Inc) to genotype the SNP rs4733586. The information of primers was shown in Table 1. To reduce the false positive rates and error rates, three blank (water) controls were detected in each 384-well plate during samples testing every time. To controlling the quality and yield a 100% concordance rate, more than 10% samples were randomly selected to repeat.

Statistical analysis

We calculated the median survival time (MST), and if the MST could not be calculated, then we use the mean survival time instead. Univariate and multivariable Cox proportional hazard regression analysis was performed to estimate the crude or adjusted hazard ratio (HR) and their 95% confidence intervals (CI), with adjustment of age, gender, smoking status, drinking status, BCLC stage, and chemotherapy or TACE (transcatheter hepatic arterial chemoembolization) status. The stepwise Cox regression model was also conducted to identify predictive factors of HCC prognosis, with a significance level set at $P < 0.050$ for entering and $P \geq 0.050$ for removing the respective explanatory variables. The heterogeneity between subgroups was evaluated using the chi-square-based Q-test. All the statistical analyses were carried out by the R software (Version 3.4.2, 2017-09-28; R Foundation for Statistical Computing, <http://www.cran.r-project.org/>).

RESULTS

The demographic characteristics and clinical features of the 331 HCC patients were summarized previously^[11,12]. Briefly, 258 of 331 HCC patients were deaths at the last time of follow-up. By univariate analysis, drinking status and chemotherapy or TACE status were significantly associated with the survival time (log-rank $P = 0.006$ and $P \leq 0.001$ respectively). Obviously, alcohol-drinking was a risk factor of death (HR = 1.43, 95%CI = 1.11-1.84), yet Chemotherapy or TACE was a protective factor (HR = 0.39, 95%CI = 0.29-0.51).

The polymorphisms of *PVT1* rs4733586 and its association with HCC survival in different genetic models (additive models, dominant model and recessive model) were examined by log-rank test and Cox regression analyses. As shown in Table 2, patients with variant genotype CC had a higher risk of death than those with homozygous wild-type TT (adjusted HR = 1.59, 95%CI = 1.13-2.26, $P = 0.008$) after adjusting for age, gender, smoking status, drinking status, BCLC stage, and chemotherapy or TACE status. Furthermore, the results of the additive model analysis were also significant (adjusted HR = 1.26, 95%CI = 1.05-1.51, $P = 0.012$). Kaplan-Meier plot of HCC-specific overall survival by rs4733586 genotypes was shown in Figure 1. The results showed that there was a statistical significance between genotype of rs4733586 and HCC survival (log-rank $P = 0.039$). Stepwise Cox proportional hazard analysis was then preformed to evaluate the effect of demographic characteristics, clinical features and rs4733586 on HCC survival [Table 3]. We found that four

Table 2. The association between polymorphisms of *PVT1* genes with HCC overall survival

Genotypes	Patients	Deaths	MST (month)	Crude HR (95% CI)	Adjusted HR (95% CI) ^a	P ^a
<i>PVT1</i> rs4733586						
TT	95	73	13.5	1.00	1.00	
TC	153	115	14.9	0.85 (0.63-1.14)	1.06 (0.78-1.44)	0.712
CC	77	67	12.6	1.25 (0.90-1.75)	1.59 (1.13-2.26)	0.008
Additive model				1.11 (0.93-1.33)	1.26 (1.05-1.51)	0.012
Dominant model						
TT	95	73	13.5	1.00	1.00	
TC/CC	230	182	14.3	0.96 (0.73-1.26)	1.21 (0.91-1.61)	0.191
Recessive model						
TT/TC	248	188	14.7	1.00	1.00	
CC	77	67	12.6	1.39 (1.05-1.84)	1.54 (1.15-2.05)	0.004

^aAdjusted for age, gender, smoking, drink, chemotherapy/TACE and BCLC stage. *PVT1*: plasmacytoma variant translocation 1; HCC: hepatocellular carcinoma; MST: median survival time; HR: hazard ratio; CI: confidence intervals; TT: wild-type allele; TC: heterozygous mutant allele; CC: homozygous mutant allele; TACE: transcatheter hepatic arterial chemoembolization; BCLC: Barcelona-Clinic Liver Cancer

Table 3. Stepwise Cox regression analysis on HCC overall survival

Variables	β	SE	HR	95% CI	P
Chemotherapy/TACE	-1.2246	0.1540	0.29	0.22-0.40	< 0.0001
Drinking (yes vs. no)	0.4423	0.1369	1.56	1.19-2.04	0.0012
Age (\leq 53 years vs. $>$ 53 years)	-0.4010	0.1348	0.67	0.51-0.87	0.0029
rs4733586 (additive model)	0.2263	0.0917	1.25	1.05-1.50	0.0136

β : the estimated parameter of the regression model; SE: the standard error of the regression model; HCC: hepatocellular carcinoma; TACE: transcatheter hepatic arterial chemoembolization; HR: hazard ratio; CI: confidence intervals

variables (age, drinking status, chemotherapy or TACE status, *PVT1* eQTL) remained in the final regression model, with a significant level of 0.050 for entering ($P < 0.0001$ for chemotherapy or TACE status, $P = 0.0012$, 0.0029 and 0.0136 for drinking status, age and rs4733586, respectively). However, in the stratified analysis [Table 4], no heterogeneity was noted among different age, gender, smoking status, drinking status, BCLC stage and chemotherapy or TACE status.

DISCUSSION

In this present case cohort study, we genotyped the *PVT1* eQTL (rs4733586) among 331 HCC patients and shed light on that the variants of SNP were significantly associated with poor prognosis in HCC.

Several studies have shown that some locus located in *PVT1* had potential risks to cancer. For example, one genome-wide association study identified a locus (rs1561927) at 8q24.21 that located 455 Kb telomeric of *PVT1* associated with pancreatic cancer risk^[15]. In a comprehensive genome-wide analysis, the authors identified lncRNA *PVT1* that may be involved in HCC cells metastasis by comparing lncRNAs expression profiles^[16]. Therefore, it is reasonable to believe that the key locus on the lncRNA *PVT1* may be associated with the progress of HCC.

Since thousands of new lncRNAs have been explored in the ENCODE project and RNA-seq analysis, the genetic variation and biological function of lncRNAs are becoming hot topics in cancer^[12]. SNP rs4733586 was identified as an eQTL for *PVT1* using bioinformatics analysis. *PVT1* oncogene encodes a long noncoding RNA and maps to chromosome 8q24.21^[17]. The well-characterized myelocytomatosis (*MYC*) oncogene also resides in the 8q24.21 region^[18], and *PVT1* is located downstream of *MYC* in this chromosomal region^[9]. Moreover, *PVT1* has been shown to be important for expression of *MYC* in tumors^[19]. *MYC* activation may influence cancer immunoediting through the suppression of immune surveillance against tumor

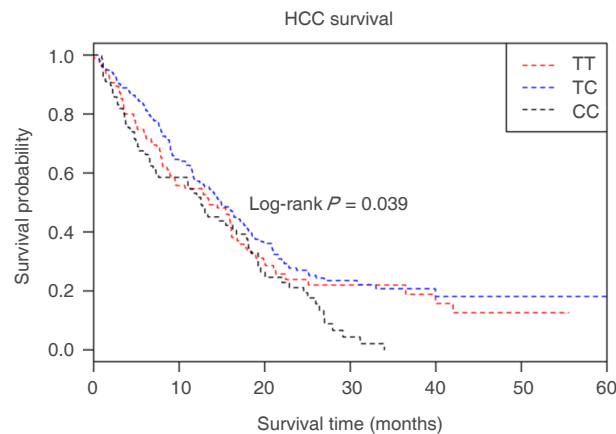


Figure 1. Kaplan-Meier plots of HCC-specific overall survival by *PVT1* eQTL rs4733586 genotypes, log-rank $P = 0.039$. X-axis: HCC patients' survival time (months); Y-axis: HCC patients' survival probability. "Red line" denotes patients carrying homozygous wild-type TT alleles; "blue line" denotes those with heterozygous TC alleles; "black line" denotes those with variant CC alleles. HCC: hepatocellular carcinoma; *PVT1*: plasmacytoma variant translocation 1; eQTL: expression quantitative trait loci; TT: wild-type allele; TC: heterozygous mutant allele; CC: homozygous mutant allele

Table 4. Stratification analysis of rs4733586 genotypes and HCC overall survival

Variables	rs4733586 (patients/deaths)			Adjusted HR (95% CI) ^a	P for heterogeneity
	TT	TC	CC		
Age, years					0.146
≤ 53	49/38	83/65	37/32	1.05 (0.82-1.33)	
> 53	46/35	70/50	40/35	1.37 (1.05-1.79)	
Gender					0.485
Male	77/59	138/103	65/56	1.18 (0.97-1.44)	
Female	18/14	15/12	12/11	1.40 (0.91-2.17)	
Smoking					0.721
Never	38/31	57/37	24/22	1.14 (0.85-1.56)	
Ever	57/42	96/78	53/45	1.23 (0.98-1.54)	
Drinking					0.634
Never	36/28	68/46	22/20	1.12 (0.80-1.55)	
Ever	59/45	85/69	55/47	1.23 (0.99-1.53)	
BCLC stage					0.071
Stage B	89/68	142/106	69/61	1.29 (1.07-1.55)	
Stage C	6/5	11/9	8/6	0.56 (0.23-1.36)	
Chemotherapy/TACE					0.135
No	33/26	32/26	25/25	1.27 (0.94-1.71)	
Yes	62/47	121/89	52/42	0.96 (0.77-1.19)	

^aAdjusted for age, gender, smoking, drink, Chemotherapy/TACE and BCLC stage. HCC: hepatocellular carcinoma; HR: hazard ratio; CI: confidence intervals; TACE: transcatheter hepatic arterial chemoembolization; BCLC: Barcelona-Clinic Liver Cancer; TT: wild-type allele; TC: heterozygous mutant allele; CC: homozygous mutant allele.

cells. During tumor progression, high *MYC* expression results in increased expression of *CD47* and *PD-L1*, suppressing both the innate and the adaptive immune response and favoring tumor growth^[20]. Previous studies had shown that there was a significant relationship between *PVT1* overexpression and poor overall survival of patients with gastric cancer, gynecology cancer and lung cancer^[7]. Ding *et al.*^[21] found that the relative expression levels of *PVT1* were significantly higher in cancerous tissues compared with the corresponding non-cancerous tissues. Other research group demonstrated that *PVT1* promotes cell proliferation, cell cycling, and the acquisition of stem cell-like properties in HCC cells by stabilizing NOP2 protein, and HCC patients with high *PVT1* expression had a poor prognosis^[22]. All these conclusions can be consistent with the results of this study.

However, there are several limitations of the study that need to be addressed in further studies. Firstly, the further verification needs to be conducted. A series of large-scale studies are needed to verify the associations between the eQTL in *PVT1* and the HCC prognosis. Secondly, there was few biological functional experiments conducted to provide additional evidence.

In conclusion, it was the first study to examine the association of *PVT1* eQTL with HCC prognosis. We found that rs4733586 might be served as a susceptibility marker for HCC survival.

DECLARATIONS

Authors' contributions

SNP selection and genotype: Ge ZJ , Yu CX

Data acquisition: Song C, Pu ZN

Statistical analysis: Tian T, Song C

Manuscript preparation: all authors

Critical revision and finalizing of the manuscript: Song C, Hu ZB

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Financial support and sponsorship

This work was supported by grants from the National Key Basic Research Program Grant (2013CB911400); Cheung Kong Scholars Programme of China; Jiangsu Specially-Appointed Professor project; the Priority Academic Program Development of Jiangsu Higher Education Institutions (Public Health and Preventive Medicine) and Top-notch Academic Programs Project of Jiangsu Higher Education Institutions (PPZY2015A067). The funders had no role in the study design, data collection, data analysis and interpretation, writing of the report, or the decision to submit the article for publication.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

This study was authorized by the local institutional review board at Nanjing Medical University. All the participants gave written informed consent.

Consent for publication

Not applicable.

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Review

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Pro-oncogenic role of SerpinB3 in hepatocellular carcinoma

Andrea Martini, Patrizia Pontisso

Department of Medicine, University of Padua, Padua 35128, Italy.

Correspondence to: Prof. Patrizia Pontisso, Department of Medicine, University of Padua, Via Giustiniani 2, Padua 35128, Italy.
E-mail: patrizia@unipd.it

How to cite this article: Martini A, Pontisso P. Pro-oncogenic role of SerpinB3 in hepatocellular carcinoma. *Hepatoma Res* 2018;4:28. <http://dx.doi.org/10.20517/2394-5079.2018.50>

Received: 4 May 2018 **First Decision:** 22 May 2018 **Revised:** 6 Jun 2018 **Accepted:** 7 Jun 2018 **Published:** 29 Jun 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Hepatocellular carcinoma (HCC) is one of the most relevant sanitary problems for its prevalence and poor prognosis. This tumor is characterized by highly heterogeneous features, both at clinical and molecular level. SerpinB3 (squamous cell carcinoma antigen-1 or SCCA1) is a serine-protease inhibitor that protects cells from oxidative stress conditions, but in chronic liver damage it may lead to HCC through different strategies, including inhibition of apoptosis, induction of epithelial to mesenchymal transition, cell proliferation and invasiveness. Mechanisms of tumor growth promotion induced by SerpinB3 encompass the inhibition of intratumor infiltration of natural killer cells and the up-regulation of Myc oncogene. Recently this serpin has also been identified as a Ras-responsive factor and modulator of metabolic pathways. In the liver SerpinB3 is undetectable in normal hepatocytes, but its expression progressively increases in chronic liver diseases, dysplastic nodules and hepatocellular carcinoma, especially in those with poor prognosis, in which it could also exert immunomodulatory effects. In serum SerpinB3/4 isoforms (or SCCA) circulate bound to IgMs (SCCA-IgM) in patients with HCC, and in patients with cirrhosis their levels have been found correlated to the risk of HCC development. Preliminary findings in patients with HCC revealed that SCCA-IgM levels are predictive of HCC prognosis.

Keywords: SerpinB3, chronic liver disease, hepatocellular carcinoma, chronic inflammation, SCCA-IgM

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and is ranked as the sixth most common neoplasm and the third leading cause of cancer death worldwide. This liver tumor has been recognised as a leading cause of death among patients with cirrhosis and its incidence is expected to increase in the next



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future^[1]. This increase in incidence, despite the control of hepatitis B virus and hepatitis C virus infections by vaccination and treatments, is expected by the rising levels of obesity and its metabolic complications^[2].

Despite intensive surveillance programs, considerable recent therapeutic advances and the use of potentially radical treatments, clinical outcome of HCC remains still poor, with the majority of patients presenting with advanced disease not eligible for curative therapy^[3]. These treatments are indeed applicable only for early stage tumors and include resection, liver transplantation and percutaneous ablation, while transarterial chemoembolization (TACE) and sorafenib are regarded as non-curative treatments, able to improve survival in intermediate and advanced stages, respectively^[1]. The identification of novel therapeutic targets is limited by the well-known intra-nodule and inter-nodule tumour heterogeneity and heterogeneity in tumour evolution^[4]. It is known indeed the each HCC is composed of a unique combination of somatic alterations, including genetic, epigenetic, transcriptomic and metabolic events that form its unique molecular fingerprint^[4]. The biological characteristics of the tumor are also enriched by the presence of a frequent underlying chronic liver disease that leads to a persistent exposure to chronic inflammation and oxidative stress by cirrhotic hepatocytes^[4]. In parallel to this pathological heterogeneity, gene expression profiling has allowed the establishment of several HCC transcriptomic classifications^[5-7]. One of these recently identified molecular subclasses (S1) of HCC, associated with poor prognosis, is characterized by aberrant activation of Wnt signaling and transforming growth factor-beta activation^[6]. This peculiar S1 signature is characterized by overexpression of genes associated to epithelial-to-mesenchymal transition (EMT), a process originally described for embryo development and now believed to be involved in tumor invasion and metastasis and known to be regulated by TGF-beta in HCC^[6]. It is interesting to note that high levels of SerpinB3 expression were identified recently only in this subclass^[8].

PHYSIOLOGICAL CHARACTERISTICS AND BIOLOGICAL FUNCTIONS OF SERPINB3

SerpinB3 (formerly known as squamous cell carcinoma antigen-1 or SCCA1) is a member of the family of serine-protease inhibitors (SERPINS). SerpinB3 and its highly homologous isoform SerpinB4 (formerly known as squamous cell carcinoma antigen-2 or SCCA2) were originally purified from a squamous cell carcinoma of the uterine cervix^[9]. They are encoded by two separate genes located on chromosome 18q21.3, which share a high degree of homology (up to 98%). The two encoded glycoproteins have a molecular weight of 45 kDa and are composed by 390 amino acids with up to 92% similar composition^[10]. SerpinB3 and SerpinB4 show distinct properties and substrate specificities: the former inhibits papain-like cysteine proteases^[11], whereas the latter inhibits both serine and cysteine proteases^[12]. The specific function or target depends mainly on the variety of the reactive-site loop (RSL), in which only 7 out of 13 amino acid residues (54%) are identical, and this reactive site is involved in the interaction with the protease, its recognition, and cleavage, resulting in its inhibition^[13]. SERPINB3/B4 are localized predominantly in the cytosol, however, they have also been detected in other subcellular compartments including lysosomes, mitochondria, the nucleus, and may function extracellularly^[10]. The localization of SerpinB3/B4 in the nucleus probably depends on physiological state of the cell. While these isoforms are detectable only in the cytoplasm at basal state in cell lines, they have been found in the nucleus in response to UV irradiation. In addition, in clinical samples, nuclear localization of SerpinB3/B4 has been commonly reported in various types of cancers, in psoriasis and in idiopathic pulmonary fibrosis^[10].

Regarding their tissue expression, SerpinB3/B4 are physiologically expressed in the basal and parabasal layers of normal squamous epithelium^[14], and they are overexpressed in neoplastic cells of epithelial origin^[15,16]. These proteins are frequently co-expressed in other organs, such as bladder, uterus, esophagus, lung, prostate, testis, thymus, and trachea, but the biological significance of SerpinB3/B4 in normal tissue development and function remains largely unknown^[10].

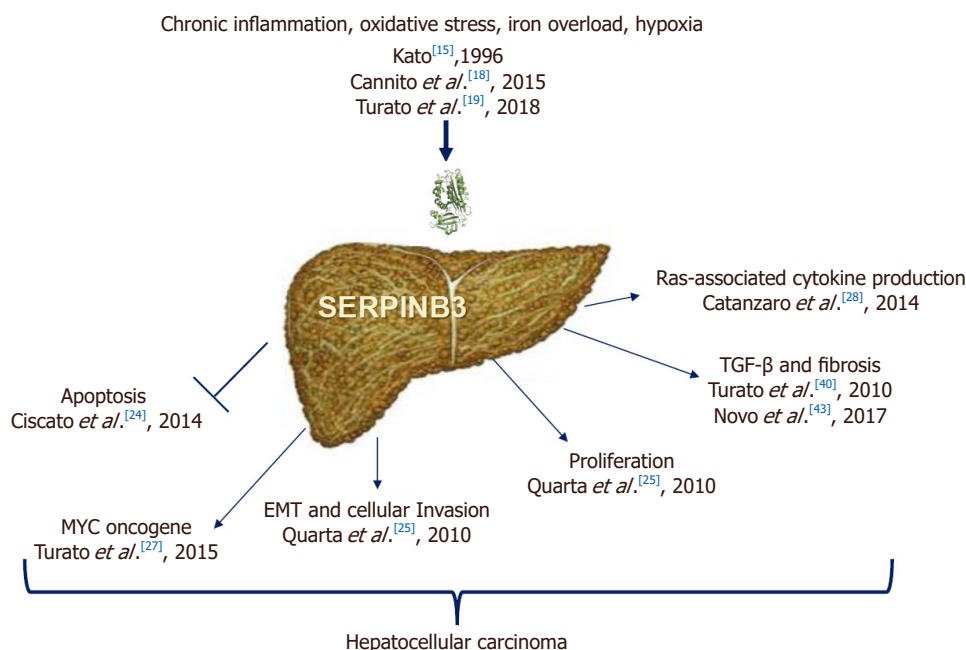


Figure 1. Schematic representation of factors involved in SerpinB3 induction and its pro-oncogenic properties described in the literature

SERPINB3 AND PRIMARY LIVER CANCER

Pro-oncogenic potential of SerpinB3

In recent years several data revealed new biological properties of SerpinB3 in the field of liver carcinogenesis [Figure 1]. The mechanisms that could lead to a dysregulation of SerpinB3 during hepatocarcinogenesis are still largely unknown. Initial studies indicate that this molecule can be upregulated by inflammatory cytokines, namely tumor necrosis factor- α , as anti-apoptotic cell death response^[17]. A novel mechanism involves a selective binding of HIF-2 α to SERPINB3 promoter^[18], induced by hypoxic and oxidative stress conditions, like iron overload^[18,19]. Somatic mutations affecting SerpinB3 repressor(s) cannot be excluded, however, further studies are required to explore this hypothesis.

Anti-apoptotic properties

Initial studies indicate that SerpinB3 has an anti-apoptotic effect, since in cancer cells it was found to confer resistance to drug-induced apoptosis by inhibiting lysosomal cathepsin proteases^[20] and consequent inhibition of the release of mitochondrial cytochrome c.

This serpin also displays a protective role under a variety of stress conditions, with an anti-apoptotic function unrelated to its proteinase inhibition activity^[21]. Indeed, SerpinB3 protects cells from exposure to radiation through an inhibitory effect either on the MAP family kinase JNK^[22] or p38^[23]. More recent findings have demonstrated a novel mechanism of action of SerpinB3, which could contribute to tumor cell resistance to anti-neoplastic drugs. This molecule was found located in the inner mitochondrial compartments, where its binding to the respiratory complex I protected cells from the toxicity of chemotherapeutic agents with a pro-oxidant action such as doxorubicin and cisplatin^[24]. This serpin reduced ROS generation induced by these compounds, a crucial step responsible for the opening of the mitochondrial permeability transition pore, shielding tumor cells from apoptotic death^[24].

Induction of epithelial-to-mesenchymal transition and cell proliferation

SerpinB3 induces cell proliferation (increasing β -catenin expression) and deregulation of adhesion processes as down regulation of E-cadherin and decrease of desmosomal junctions, leading to epithelial-to-mesenchymal transition (EMT) with increased cell invasiveness potential^[25]. Experimental studies have also reported that

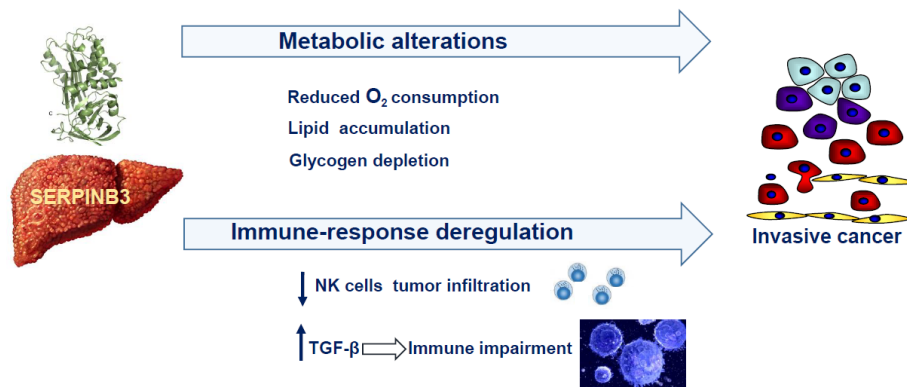


Figure 2. Biological activities determined by SerpinB3, with particular regard to metabolism and immune response modulation

mice transgenic for liver SerpinB3 showed higher liver regenerative ability compared to wild-type mice, supporting a role of this protein in promoting cell growth and proliferation^[26]. Other mechanisms of tumor growth promotion induced by SerpinB3 include the up-regulation of Myc oncogene transcription with two different strategies^[27]: the first mechanism is through the intracellular SerpinB3 antiprotease activity that blocks its cleavage exerted by Calpain, preventing the generation of the non-oncogenic cytoplasmic Myc-nick form and allowing nuclear translocation of Myc with pro-oncogenic activity. The second one consists in the transcriptional induction of Myc, through the increase of Yap pathway^[27]. Furthermore, recent findings indicate that SerpinB3/SerpinB4 isoforms are a Ras-responsive factor that plays an important role in Ras-associated cytokine production and tumorigenesis^[28].

Metabolic functions

Recent findings indicate that SerpinB3 can determine metabolic alterations in the liver through the induction of dipeptidyl peptidase-4 (DPPIV/CD26), a transmembrane glycoprotein, that is increased in various malignant tumors, including HCC, and the expression of these two molecules was found positively correlated in HCCs and in the surrounding cirrhotic tissue^[29]. Hepatoma cells overexpressing SerpinB3 showed increased DPPIV/CD26 levels and these features were associated with an increase in lipid droplet formation and with decreased glycogen deposition, typical features induced by DPPIV/CD26^[30-32] [Figure 2]. These results are in agreement with previous findings, reporting remarkable lipid accumulation and glycogen depletion in the liver of SerpinB3-transgenic mice^[33]. In addition, SerpinB3 was found overexpressed in human livers with NASH^[34], a condition at risk of HCC development. It is worth to note that SerpinB3 determined also a decreased oxygen consumption rate^[29], as a possible consequence of its physical interaction with mitochondrial respiratory complex I^[24].

SerpinB3 in liver cancer

In the liver SerpinB3 and its isoform SerpinB4, are undetectable in normal hepatocytes, but their expression progressively increases in chronic liver disease, in dysplastic nodules^[35] and in HCC^[36,37], suggesting their involvement in relatively early events of hepatocarcinogenesis^[35]. SerpinB3 has also been detected in hepatoblastoma, the embryonal tumor of the liver, especially in the most aggressive forms, where a direct correlation was observed between its gene expression, the up-regulation of Myc oncogene and tumor extension^[38].

The presence of SerpinB3 has been further described in liver stem/progenitor cells positive for the hepatic epithelial cell adhesion molecule (EpCAM), both in human fetal livers and in adult livers with cirrhosis, and these findings were corroborated by the induction of this serpin in a mouse model of liver stem/progenitor cell activation^[39]. Liver tumors with stemness signature are highly aggressive, and along this line the highest

levels of SerpinB3 have been found overexpressed, together with TGF- β 1, in the subset of aggressive forms of hepatocellular carcinoma, characterized by early tumor recurrence after surgical resection^[8].

The tight correlation between SerpinB3 and TGF- β , that requires the integrity of the RSL of SerpinB3, as documented by *in vitro* studies^[40], has been also confirmed in non-tumor cirrhotic livers. The alterations of the microenvironment, characterized by the presence of chronic inflammation associated with liver fibrosis, typical features of the cirrhosis status, have been identified as a hallmark of liver carcinogenesis. In fact, more than 80% of the cases of hepatocellular carcinoma arise in livers with cirrhosis, a condition which constitute a real precancerous stage^[41]. Activated hepatic stellate cells (HSC) represent key drivers of liver fibrosis and extracellular matrix (ECM) remodeling^[42], and a recent study has demonstrated that SerpinB3 is able to directly activate human HSC, resulting in a strong up-regulation of the expression of genes involved in fibrogenesis and angiogenesis^[43].

In recent years there is growing evidence that the impairment of immune surveillance plays a pivotal role in liver cancer development and progression^[44]. In this context, TGF- β is a key player, suppressing proper anti-tumor immune responses^[44], through the induction of regulatory T cells (Tregs) that have a profound ability to control immune responses^[45,46] and SerpinB3 might be also involved in the immune escape mechanism by enhancing TGF- β production. Other findings through which SerpinB3 seems to promote the immune impairment are its ability to inhibit the intratumor infiltration by natural killer cells^[47] and to reduce the inflammatory response in other experimental settings^[48].

Diagnostic and prognostic significance

One of the most important and yet unmet needs in clinical settings is the availability of serological markers to identify patients with cirrhosis at higher risk of HCC development. Since the incidence of hepatocellular carcinoma in individuals with cirrhosis is 3%-5% per year^[49], the identification of the subgroup of patients with possible HCC development within the next few years would allow the development of a personalized clinical management and more effective early therapeutic interventions. On the basis of the oncogenic potential of SerpinB3, and of the reported findings of the presence of SERPINB3/4 isoforms (or SCCA) in the vast majority of HCCs specimens^[36], in the last years ELISA assays have been developed to assess the presence of SCCA as free protein and/or bound to IgM as circulating immune complexes in serum^[50]. The occurrence of biomarker-IgM immune complexes has been described as the result of cancer immunoediting, in which natural IgMs are important players of the innate immune system preventing tumor formation^[51]. Free SCCA is barely detectable in serum of patients with advanced liver disease and primary liver cancer, while this molecule was found coupled to IgMs (SCCA-IgM) in the majority of patients with HCC, whereas in the healthy control population their levels were below the limit of detection^[50].

Patients with cirrhosis

The concentration of circulating SCCA-IgM has been found progressively increased at different stages of liver disease, from chronic hepatitis to cirrhosis and HCC, reflecting the extent of SCCA protein overexpression in the liver^[52]. In individual patients, the progressive increase of SCCA-IgM over time was remarkable in cirrhotic patients who developed HCC, and resulted unchanged in the majority of the cirrhotic patients without evidence of liver cancer during the same time interval^[52]. These data have been confirmed in another retrospective study^[53], where baseline values of serological SCCA-IgM were nearly 4-fold higher in patients who developed HCC than in those without HCC progression. In addition, SCCA-IgM values ≤ 200 AU/mL accurately identified patients at low risk of liver cancer in the subsequent year, with a negative predictive value of 97%^[53].

In agreement with these findings, the prognostic role of this biomarker was confirmed in a prospective study showing that, among patients matched for clinical stage of cirrhosis, those with baseline levels of

SCCA-IgM above the cut-off (200 AU/mL) developed more frequently HCC during follow-up than those negative for the biomarker^[54].

Patients with HCC

In a recent study that has retrospectively analyzed patients with cirrhosis and HCC, SCCA-IgM was proven efficient in the prediction of HCC prognosis, identifying HCC patients with long overall and progression-free survival^[55]. Median survival was indeed about two fold increased in patients with low levels of SCCA-IgM, compared to those with elevated SCCA-IgM levels. At multivariate analysis tumour size and SCCA-IgM levels were identified as the only independent predictors of survival. In addition, levels of this biomarker were correlated with overall response to treatment, with a median time to progression that was more than doubled in patients with low SCCA-IgM levels^[55]. The levels of the biomarker at four weeks were stable or increased in treated patients with stable disease or tumor, and were reduced in patients with complete response, while patients with partial response showed an intermediate behaviour. It is worth to note that in the same study, AFP was not able to predict complete response^[55]. Another recent study addressed the behaviour of SCCA-IgM in patients with HCC who underwent locoregional therapy. Among the enrolled patients with a new diagnosis of HCC, SCCA-IgM levels at basal time and after one month of treatment, resulted significantly lower in patients who responded to therapy compared to those who did not respond^[56]. These findings need to be confirmed in further studies, but are supported by a previous report that within the liver, HCCs with high SCCA-1 tissue expression have a poor prognosis and present higher rate of early recurrence after surgical resection^[8].

FUTURE PERSPECTIVES

Chronic inflammation and immune system play a crucial role in the development of dysplastic nodules and liver cancer^[41,44], as the pathogenesis of HCC has been associated with hepatocyte death, infiltration of inflammatory cells, and compensatory liver regeneration, which is dependent on the production of hepatic mitogenic cytokines produced by Kupffer cells, such as IL-6^[57]. A previous study documented a positive correlation between RAS mutation, enhanced SerpinB3 and interleukin-6 expression in samples of human colorectal and pancreatic tumors, reflecting an inflammatory response related to the nuclear factor kappa-light chain enhancer of activated B cell^[58]. Moreover, SerpinB3 was found physiologically expressed on the surface of CD27+ B lymphocytes^[59], and it has been detected in peripheral blood mononuclear cells at transcript level both in cultured and in primary monocytes^[60]. These findings suggest that SerpinB3 might play a role in the modulation of the immune response, favouring tumor development, but further studies are needed to clearly elucidate its role in this specific field.

DECLARATIONS

Authors' contributions

Drafted the article: Martini A

Revised the article and approved the version to be published: Pontisso P

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Both authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Understanding the inflammation-cancer transformation in the development of primary liver cancer

Hong-Jin Chen^{1,#}, Ming-Hua Hu^{2,#}, Fang-Gui Xu³, Hao-Jun Xu¹, Jun-Jun She⁴, Hong-Ping Xia^{1,4}

¹Department of Pathology, School of Basic Medical Sciences & The Affiliated Sir Run Run Hospital, Nanjing Medical University, Nanjing 21116, China.

²Department of Hepatobiliary Surgery, The Affiliated Yijishan Hospital of Wannan Medical College, Wuhu 241001, China.

³Department of General Surgery, The Affiliated Sir Run Run Hospital, Nanjing Medical University, Nanjing 21116, China.

⁴Department of General Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, China.

#Authors contribute equally to this work.

Correspondence to: Dr. Hong-Ping Xia, Department of Pathology, School of Basic Medical Sciences & The Affiliated Sir Run Run Hospital, Nanjing Medical University, Nanjing 21116, China. E-mail: xiahongping@njmu.edu.cn; Dr. Jun-Jun She, Department of General Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, China. E-mail: sjuns@sina.com

How to cite this article: Chen HJ, Hu MH, Xu FG, Xu HJ, She JJ, Xia HP. Understanding the inflammation-cancer transformation in the development of primary liver cancer. *Hepatoma Res* 2018;4:29. <http://dx.doi.org/10.20517/2394-5079.2018.18>

Received: 12 Mar 2018 **First Decision:** 10 May 2018 **Revised:** 11 Jun 2018 **Accepted:** 11 Jun 2018 **Published:** 5 Jul 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Primary liver cancer is one of the leading causes of cancer-related deaths worldwide. China has more than 55% liver cancer cases globally. The development of hepatocellular carcinoma (HCC) was caused by a variety of risks factors, including chronic inflammation by virus, alcohol consumption and non-alcoholic steatohepatitis. Emerging evidence has notarized inflammation as a critical component of HCC progression. The development of HCC is a multistep process which may originate from liver chronic injury and inflammation to subsequent fibrosis and/or cirrhosis and finally HCC. A large number of studies indicate that chemokines and cytokines are candidates linking molecules between inflammation and liver cancer. Here, we will describe a few of the key cytokines and chemokines and signal pathways which are involved in the inflammation of HCC. Inhibitors of inflammation for the prevention and overcoming antitumor immunity for treatment of liver cancer are promising candidates for the future management of patients with HCC.

Keywords: Inflammation, liver cancer, cytokines, chemokines, signaling pathways

INTRODUCTION

Primary liver cancer is one of the leading causes of cancer-related deaths and the fifth common largest tumor type worldwide (<http://globocan.iarc.fr>). It can be categorized into hepatocellular carcinoma (HCC),



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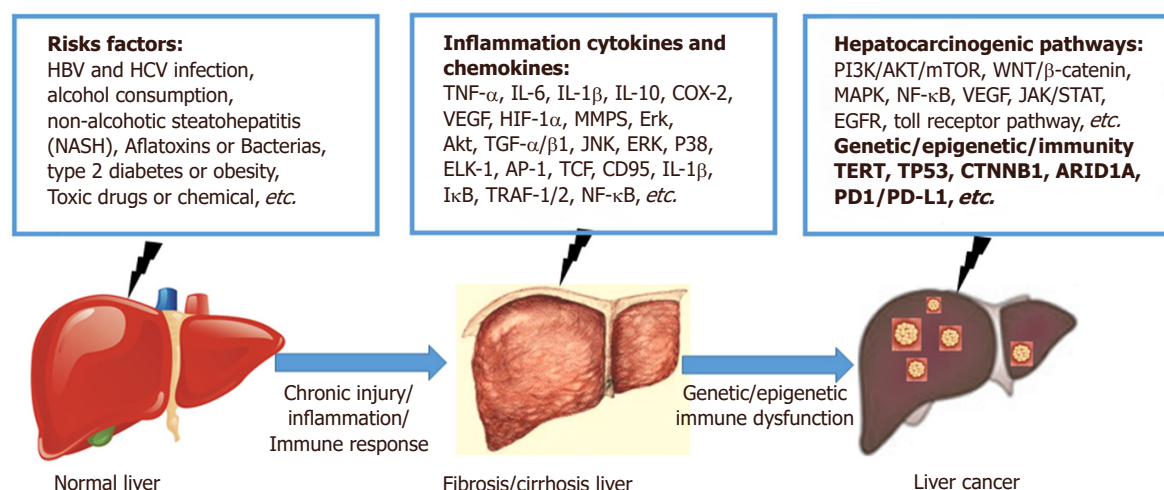


Figure 1. The multistep process of the development of liver cancer. The risk factors, inflammation cytokines and chemokines, and hepatocarcinogenic pathways are related to the inflammation-cancer transformation during the development of primary liver cancer

intrahepatic cholangiocarcinoma (iCCA), and other rare types such as hepatoblastoma and fibrolamellar carcinoma according to the pathological type. Tumor morphology can be divided into nodular, massive and diffuse types of liver cancer^[1]. It's worth noting that more than 500,000 people receive a diagnosis of HCC every year and the incidence is still increasing worldwide, by about 4% per year in men and 3% per year in women^[2]. The number of fatal cases accounts for about 5.4% of all malignancies each year globally. It is estimated that there will be over 1 million new cancer diagnoses of HCC each year by 2025^[3]. China has more than 55% liver cancer cases globally. HCC accounts for approximately 90% of primary liver cancer^[4]. Currently, it is very limited for HCC patients to choose the suitable treatment. Over the past 2 decades, the median survival time for advanced HCC patients is less than 1 year, and the 5-year relative survival rate is below 9%^[4]. Patients with well-preserved liver function will select surgical resection. The most effective way for HCC patients to improve the survival is liver transplantation. Unfortunately, those treatments often result in a poor prognosis, including a high risk of postoperative complications and recurrence of the tumor. Although there are various strategies, such as liver transplantation, surgical resection, target drugs (sorafenib, lenvatinib or regorafenib) and immunotherapy (nivolumab), to extend survival time of liver cancer patients, those treatments are not effective^[5]. It has been well recognized that HCC is a complex and heterogeneous malignancy, caused by a variety of risks factors, including chronic inflammation by virus, like hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol consumption, non-alcoholic steatohepatitis (NASH), bacteria, type 2 diabetes, smoking or chemical. Age and gender are also risk factors for HCC^[6]. HCC is more common in middle-aged men with a male to female ratio of up to (3-8):1. In China, the major component of the attributable risk is chronic hepatitis B^[7]. Based on those different causes, the molecular pathogenesis of HCC is very complicated. This review is intended to facilitate the understanding of the risk factors, inflammation cytokines and hepatocarcinogenic pathways related to the inflammation-cancer transformation during the development of primary liver cancer [Figure 1].

INFLAMMATION AND LIVER CANCER

Virchow postulated the connection between cancer and inflammation in 1863^[8]. It has been estimated that inflammation and chronic infection would lead to the development of about 15% human cancers^[9]. A large number of epidemiological investigations suggest that inflammation is one of the main factors leading to tumorigenesis or promoting tumor development^[10]. Recently, more and more data have notarized inflammation as a critical component of HCC progression. Direct evidences suggesting chronic inflammation, especially hepatitis B and hepatitis C, are the risk factor for HCC. Patients who have those diseases will get more risk to

develop HCC^[11]. The development of HCC is a multistep process. There is a common situation that HCC are originating from liver chronic injuries and inflammation, subsequent fibrosis and/or cirrhosis. When patients get liver injury or inflammation, the liver parenchymal cells will die and release signaling molecules of death, or cause inflammatory reaction. During chronic inflammatory hepatitis, the host's immune response to HBV or HCV is usually not strong enough to eradicate the infection and damage, eventually leading to the body's continued over-activation^[12-14]. Cirrhosis is a chronic, progressive diffuse change in the liver caused by a variety of factors. Long-term damage to liver cells will lead to degeneration and necrosis of liver cells. After a wide range of liver cell degeneration and necrosis, intrahepatic connective tissue is regenerating, then fibrous tissue is diffusing proliferation. This process helps promote the development and progression of liver cancer. The process involved many mechanisms such as oxidative stress, endoplasmic reticulum stress and mitochondrial damage, which will activate and promote the synthesis and secretion of tumor-related transcription factors and cytokines, resulting in DNA damage and further promoting tumorigenesis^[15-18].

INFLAMMATORY SIGNALING PATHWAYS OF HCC

The relationship between liver inflammation and HCC is strongly suggested by recent studies. A lot of evidences about inflammatory mediators and signaling pathways are reported in HCC^[4,9,19]. However, there is a lack of adequate clinical evidence to support the routine use of anti-inflammatory drugs to improve the prognosis of patients with liver cancer. So it is an interesting strategy to further investigate the anti-inflammatory treatment for liver cancer. A large number of studies indicated that chemokines and cytokines are candidates linking molecules between inflammation and cancer. The following parts will describe a few of the key cytokines and chemokines and signaling pathways which are involved in the inflammation and HCC.

Cytokines and chemokines

Inflammatory cytokines are critically important in the liver injury development. When liver tissue or cells stimulate with stimulants like alcohol and fatty acid, liver tissue synthesizes various types of cytokines to defend that^[20-25]. The well-studied cytokines include tumor necrosis factor- α (TNF- α)^[26,27], interleukin family (IL-6, IL-1 β)^[7,28,29], chemokines (VCAM-1, ICAM-1 and MCP-1)^[30-33], *etc.* TNF- α and IL-6 are two multifunctional cytokines in chronic hepatic inflammation. Kuffer cells will be activated during chronic hepatitis, including NASH, alcoholic hepatitis, and hepatic infections with HBV and HCV, and inflammatory cytokines. Following that, TNF- α and IL-6 will be synthesized and secreted in abundance. Elevated serum levels of TNF- α and IL-6 have been found in patients with chronic hepatitis B^[34,35]. Higher serum levels of TNF- α and IL-6 have been reported as the high risk factors for cirrhosis and HCC development in patients, especially with HBV and HCV infection. Several studies have shown that the hepatic tissue of DEN-treated rats or mice has increased IL-6 and TNF- α production levels compared with the control group^[36]. It has been certified that TNF- α causes DNA damage through the induction of reactive oxygen species (ROS)^[19,37]. IL-6 and IL-1 β can potentially promote autophagy in liver tumor cells^[25,38]. IL-1 β has been linked with inflammasome NLRP3 to promote the occurrence and development of chronic liver disease^[39]. Both IL-1 β and TNF- α are related to stimulate cancer cell proliferation during chronic inflammation situation^[40].

Chemokines induce chemotactic migration of targeting cells through their interaction with their receptors. A large number of studies have shown that chemokines are up-regulated in various liver injuries. In chronic liver inflammation, inflammatory factors, growth factors, oxygen stress and their products stimulate the expression of chemokines. High expression of chemokines can activate hepatic stellate cells (HSC) involved in the formation of liver fibrosis and even cirrhosis. It should be noted that IL-1 β and TNF- α can activate quiescent HSC to produce MCP-1, IL-8, indicating that inflammatory cytokines further accelerate the conversion of inflammation to cancer through chemokines^[41-43]. The CXC family is a family of chemokines that have attracted the most attention during the metastasis of cancer and this family can promote the migration of neutrophils, which often promote the development of inflammation. CXCL1, CXCL2, CXCL3 and CXCL have been reported to be highly expressed in hepatoma cells^[44,45]. It has been shown that CXCL12

activates expression of matrix metalloproteinase 10, which further stimulates migration of HCC cells^[46]. In summary, these evidences showed that inflammation contributes to cancer by supplying important and various molecules to the tumor microenvironment.

Signaling pathways

PI3K/AKT/mTOR pathway

In 1993, Kisen *et al.*^[47] observed the existence of autophagy in pre-cancerous hepatocytes induced by chemical carcinogens in rats, and proposed a new target that could potentially be used to treat HCC. Autophagy has the dual role of promoting and inhibiting the evolution of liver cancer^[48,49]. Autophagy often occurs in the reduction from the pre-cancer stage to the occurrence of cancer and the reduction of autophagy can lessen tumor cell autophagic death, so it plays a facilitating role in the growth of the tumor. However, before the formation of blood vessels, the tumor cells are in a state of low nutrition and hypoxia, which stimulate autophagy through the PI3K/Akt/mTOR pathway. Enhanced autophagy can provide nutrients to the hungry tumor cells by removing damaged proteins, organelles and macromolecules, thereby inhibiting apoptosis of tumor cell. The PI3K/Akt/mTOR pathway is of great importance to the development, progression and treatment of HCC. It has been reported that TNF- α and IL-6 induced VEGF expression and angiogenesis can be significantly inhibited by rapamycin, indicating that mTOR plays an important role in inflammation-induced angiogenesis^[50]. Inflammatory factors such as TNF- α , IL-6, IL-1 β and other secretions are also regulated by mTOR signaling pathway^[51]. In the case of sustained activation of the mTORC1 pathway, the expression of pro-inflammatory cytokines such as IL-6, TNF- α are decreased and the expression of the anti-inflammatory cytokine IL-10 is increased^[52].

The role of PI3K/Akt/mTOR pathway in HCC can be summarized as follows: (1) promoting the formation of tumor blood vessels: PI3K/Akt/mTOR pathway participates in the formation of blood vessels in tumor mainly through two ways: PI3K/Akt signaling pathway can activate the cyclooxygenase 2 (COX-2), which is a key enzyme that catalyzes the synthesis of prostaglandin-like substances, thereby promoting the angiogenesis of liver cancer^[53]. Hypoxia and growth factors can induce the expression of hypoxia inducible factor 1 α (HIF-1 α)^[54], the downstream blood vessels endothelial growth factor (VEGF) transcription^[55]. The PI3K/Akt pathway activation can up-regulate the expression of HIF-1 α and VEGF, thus making endothelial cell migrate to form new blood vessels; (2) promoting tumor invasion and metastasis: Johnson and Tee^[56] have shown that PI3K/Akt/mTOR activation can make the downstream molecules p70s6k phosphorylated to make actin filament remodel and to enhance the ability of tumor cells to move, thus increasing the invasion and metastasis of cancer cells. Activation of Akt increases the transcriptional activity of NF- κ B and the motor function of tumor cells, which facilitates invasion of cancer cells^[57]. The PI3K/Akt/mTOR pathway can up-regulate the expression of matrix metalloproteinase 2 (MMP-2) mRNA and protein, and degrade the extracellular matrix (ECM) to promote tumor cell metastasis^[58]; (3) promoting cell cycle progression: studies show that the PI3K/Akt/mTOR signaling pathway can transmit mitotic signals to p70s6k, and p70s6k can up-regulate major cell cycle proteins such as cyclin and CDK4^[59], while increasing the expression of CDK4 accelerates the cell cycle progression, thereby promoting cell proliferation and differentiation which both result in liver cancer^[60]. Unfortunately, the EVOLVE-1 randomized clinical trial showed that the MTOR inhibitor, everolimus, did not improve overall survival in patients with advanced HCC whose disease progressed during or after receiving sorafenib or who were intolerant of sorafenib^[61]. The molecular classification and predictive biomarkers may be necessary for further studies.

WNT/ β -catenin pathway

WNT signaling pathway plays a role in organogenesis, regeneration and differentiation, and maintaining the homeostasis of tissues and organs. In liver tissue, hepatocytes, hepatic stellate cells and Kupffer cells could express this pathway^[62]. There is growing evidence that WNT signaling pathway is involved in the development of HCC. The sequencing studies of HCC tissues have identified the frequently activating mutations of

CTNNB1 or inactivating mutations of AXIN1 and APC, which caused the activation of the WNT/ β -catenin pathway. The activating mutations of CTNNB1 results in the mutated β -catenin accumulation and migration into the nucleus to activate transcription from target genes, which will promote HCC cell proliferation and stemness. HBV-related HCCs showed less frequent activating mutations of CTNNB1 and more frequent inactivation mutation of AXIN1 compared to HCCs related to HCV infection, alcohol consumption, or NASH, which suggested that the mechanism of the WNT/ β -catenin signaling activation may be different in HCCs with different etiology^[63]. A recent interesting study showed that deletion of endogenous β -catenin in hepatocytes of mice aggravated HCC development driven by an oncogenic version of β -catenin together with MET. This hepatocarcinogenesis featured up-regulation of Erk, Akt and WNT/ β -catenin signaling and cyclin D1 expression. The transcriptomics analysis of these mice tumors showed similar transcriptomes to human HCCs with concomitant CTNNB1 mutations and MET overexpression. The β -catenin-deficient livers displayed many changes including increased DNA damage response, expanded Sox9+ cells, and up-regulation of pro-tumorigenic cytokines like IL-6 and TGF- β 1. This study together with previous studies suggested that both activating and inactivating mutations in CTNNB1, encoding β -catenin and activation of WNT- β -catenin pathway, play important roles in liver tumorigenesis in humans and mice^[64]. Recent researches show that abnormal expression of wnt signaling pathway is involved in the intrahepatic inflammation, abnormal lipid metabolism and oxidative stress, with resulting in the occurrence of chronic liver diseases such as non-alcoholic fatty liver disease and liver fibrosis^[65,66].

MAPK pathway

Ras/Raf/MEK/MAPK is one of the key signal transduction pathways in the HCC. Many growth factors could activate residual tyrosine of Ras/Raf/MEK/MAPK to phosphorylate itself including EGF, IGF, VEGF, PDGF, FGFs and HGF. MAPK signal transduction is a three-step kinase cascade way, first of all MAP-KKK is activated and phosphorylated by mitogen on the basis of the stimulation, the phosphorylated MAP-KKK turns to activate MAP-KK, after which, the activated MAP-KK is able to phosphorylate MAPK and finally translocate into the nucleus. The main pathways of MAPK signaling pathway are Ras-Raf-ERK, c-Jun N-terminal kinase (JNK), and p38- MAPK pathway^[67].

The Ras mutation on the oncogene activates Raf activation. The activated Raf activates it by phosphorylating the serine residues on the mitogen activated kinase kinase (MEK) loop, then MEK activates extracellular signal-regulated kinases (ERKs). ERK can also be activated by decreasing the level of dual specificity phosphatase (DUSP), when the Ras mutation rate is low in HCC patients. Activated ERK in turn phosphorylates a number of substrates that are linked to the cytoplasm and membrane, while also rapidly translocated into the nucleus to dephosphorylate and activate transcriptional molecules involved in proliferative responses such as ELK-1, AP-1, TCF, and the others, which regulate the expression of ETS, c-Jun, c-Fos, c-Myc, and cyclin D in HCC and affect the prognosis of HCC^[68]. In addition, activated ERK can regulate the phosphorylation of histone, the pro-apoptotic protein Bad and the transcription factor CREB by ribosomal S6 protein kinase-2 phosphorylation^[69]. Continuous activation of ERK results in the increase of phosphorylated ERK, which is the basis of hepatoma cell proliferation and invasion^[70].

JNK pathway maintains cell cycle continuity mainly through activation of JNK activating transcription factor c-Jun synergistic with ERK pathway^[69]. Activated JNK is not only bound to transcription factors ATF2 and c-Jun amino-terminal domain, phosphorylation of the active region of the transcription factor, activation of the transcription factor AP-1, up-regulating the expression of apoptotic precursors CD95 and TNF- α ^[71], but also regulates phosphorylation of Bcl-2 indirectly after being activated, through the mitochondrial pathway to diminish its anti-apoptotic ability. Studies have shown that JNK pathway can affect the invasion and metastasis of HCC cell line MHCC97H, and that JNK inhibitor can affect human HCC xenografts and increase chemically inducing murine liver cancer^[72,73].

p38 is mainly involved in cell inflammation and proliferation with four phenotypes of p38 α , p38 β , p38 γ and p38 δ . p38 α . The most important factor in the p38 MAPK pathway, regulates the release of inflammatory cytokines such as IL-1 β , IL-6 and TNF- α , and also increases ROS activity inducing hepatocyte apoptosis^[74]. The p38 pathway can down-regulate the expression of cyclin D1 and block the cell cycle in G1-S and G2-M, and can also affect the downstream gene GADD45A of the ring tumor suppressor gene p53 to regulate the development of early HCC^[75]. An interesting mouse study suggested that inhibiting p38 MAPK (MAPK14) could help treat the sorafenib resistant liver cancers. In a mouse model of sorafenib-resistant HCC, it was discovered through *in vivo* screening of a shRNA library that p38 MAPK knockdown contributed sensitivity to sorafenib. In other mouse models of HCC, sorafenib and a p38 MAPK shRNA or a small molecule MAPK14 inhibitor skepinone-L increased survival of mice with HCC compared with sorafenib alone^[76].

NF- κ B pathway

NF- κ B activation induces several pro-inflammatory cytokines which are prominent in supporting the progression of cancer^[77,78]. Chronic infection and inflammatory response are closely related to tumorigenesis. HCC is the result of chronic inflammatory response induced by hepatitis B/C^[79]. IKK/NF- κ B pathway plays an important role in hepatitis, liver fibrosis and HCC. NF- κ B is a class of nucleoprotein factors with multidirectional transcriptional regulation. The main endogenous inhibitory factor of NF- κ B is I κ B, which makes NF- κ B remain in the cytoplasm and inhibits its nuclear translocation. NF- κ B is phosphorylated by the I κ B protein kinase complex, which is then ubiquitinated and degraded. The released NF- κ B is transferred from the cytoplasm to the nucleus, and then regulates Inhibition of apoptosis proteins (IAPs), Bcl-2 family, TNFR-associated factor (TRAF-1, TRAF-2) and JNK in the cell^[80-82].

Continued abnormal activation of NF- κ B in hepatocytes results in the development of cholestatic hepatitis and HCC. Inhibition of NF- κ B can affect the normal apoptosis of hepatocytes^[82]. Blocking IKK/NF- κ B signal transduction pathway may reduce hepatic inflammation from chronic inflammation^[80]. It is possible to prevent the development of HCC by blocking the abnormal JNK pathway activation and scavenging ROS products and other ways to maintain the normal physiological level of NF- κ B in the liver^[83,84]. However, NF- κ B is often of over-abundant activation in liver cells to facilitate HCC transformation. Therefore, how to remove overactive NF- κ B and maintain it at normal physiological levels is the key to prevention and treatment of HCC.

VEGF pathway

VEGF is a multifunctional cytokine that promotes endothelial cell division, proliferation and angiogenesis, monocyte migration, and induction of inflammatory cytokines^[85,86]. Liver VEGF is mainly present in hepatocytes and endothelial cells with the VEGF receptors. Chronic liver disease includes hepatocyte atypical hyperplasia, adenoid hyperplasia, nodules and other regenerative processes. The expression of VEGF in cancer tissue initiates the neovascularization. In the early stage of liver disease, VEGF overexpression can cause the increase of blood VEGF. VEGF levels in HCC patients are higher than in patients with chronic hepatitis and cirrhosis. The increase of VEGF is negatively correlated with the prognosis of liver cancer^[87,88]. The current only FDA approved first line therapeutic drug for advanced HCC, sorafenib, is also a tyrosine kinase inhibitor (TKI) directed against the VEGF family. The ALICE-1 study suggested that the analysis of VEGF and VEGFR SNPs may represent a potential clinical tool for better selection of HCC patients who are more likely to benefit from sorafenib treatment. Currently, apatinib (YN968D1), a TKI that selectively inhibits the VEGFR2, is actively studied in advanced HCC alone or combination with TACE (NCT03046979, NCT03398122).

JAK/STAT pathway

In 1994, Darnell *et al.*^[89] found JAK-STAT pathway. It was a new extremely fast signaling pathway, which can transmit extracellular signals to the nucleus and through tyrosine kinase signaling and transcription

activator targets. The activation eventually leads to biological effects. JAKs family belongs to the non-receptor tyrosine kinase, so far there are 4 members of family: JAK1, JAK2, JAK3 and TYK2, with diverse sizes, molecular weight between 120-140 kDa and evolution degree conservative^[90]. STATs are a kind of cytoplasmic proteins, associating with target genes binding. STATs are downstream substrates for JAKs. After abnormal activation of JAK/STAT3 signaling pathway, p-STAT3 can bind to specific DNA in the nucleus and directly or indirectly up-regulate the expression of apoptotic genes and thus regulate cell proliferation and apoptosis^[91]. STAT is highly expressed in many human malignant tumor tissues and cell lines, especially STAT3, which is currently considered as an oncogene and may promote the occurrence and development of liver cancer by influencing cell growth, differentiation and apoptosis^[92,93]. Many evidence suggest possible interactions between STAT3 and NF- κ B signaling pathways. STAT3 can promote p65 into the nucleus, resulting in NF- κ B activation. JAK/STAT signaling pathway also play a part in pancreatic elastase-induced secretion of interleukin-18^[94]. Injection of JAK2 inhibitor AG490 significantly inhibited the activation of JAK2-STAT3 after hepatic ischemia, and decreased the activation of NF- κ B and TNF α ^[95]. A phase I/Ib study has been completed to assess the safety and anti-tumour activity of AZD9150 in patients with advanced/metastatic HCC (NCT01839604).

EGFR signaling pathway

The EGFR is a tyrosine kinase that contributes to the regulation of cellular homeostasis. It is a 170-KDa membrane protein that stimulates downstream cell proliferation, survival, and tumorigenesis^[96]. Members of the human ErbB/HER receptor family include EGFR (ErbB1/HER1), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4). EGFR encoded by the proto-oncogene *erbB1* with EGFR ligand family members of 10, such as EGF, transforming growth factor- α (TGF- α), amphiregulin, b cytokines, heparin-binding EGF and epidermal regulatory elements^[97]. EGFR activation can activate extracellular downstream signaling transduction pathways such as ERKs-MAPK and PI3K. It is involved in the regulation of cell division, differentiation and proliferation and promotes the repair of tissue injury. It is also closely related to tumor cell cycle progression, apoptosis inhibition, tumor angiogenesis and cell motility and invasion^[98,99]. EGFR is highly expressed in hepatoma cells stimulated by TGF- α or EGF. *In vitro* and *in vivo* experiments show that EGFR blockade can inhibit HCC proliferation and metastasis through the EGFR pathway, and have synergistic effect of HGF treatment with other growth factor signaling pathways^[100]. Currently, a pilot clinical trial studies the best dose of EGFR inhibitor erlotinib hydrochloride for preventing liver cancer in patients with scarring (cirrhosis) of the liver undergoing surgery. Erlotinib hydrochloride may help to prevent the development of fibrosis/cirrhosis and liver cancer in patients liver cirrhosis (NCT02273362).

TLR signaling pathway

TLR is a transmembrane protein present on the surface of human cells. TLR plays an important role in the innate immune response in the body as a major pattern recognition receptor. Currently, more than a dozen TLR have been found in the human body and they are widely distributed in various tissues with the specificity of cells and tissue distribution. TLR4, a natural receptor for LPS, plays an important role in the regulation of acute inflammatory responses, transduction of cell signals and apoptosis^[101]. During liver fibrosis in rats, the expression of TLR4 protein in liver showed that compared with the normal control group, the level of hydroxyproline in liver tissue began to increase significantly^[102], and the level of plasma endotoxin in model group increased gradually with a significant positive correlation between the content of hydroxyproline after CCl₄ treatment^[103]. In HCV patients, the severity of the disease is associated with the expression of TLR2/4 mRNA^[104]. Compared with the normal group, TLR2/4 mRNA expression of chronic hepatitis C patients were elevated. The study found that TLR4-MyD88-NF- κ B signaling pathway plays an important regulatory role in abnormal liver immune response, inflammatory response-triggered liver injury, activation of hepatic stellate cells and the progression of hepatic fibrosis^[105].

INHIBITORS OF INFLAMMATION FOR THE PREVENTION AND TREATMENT OF LIVER CANCER

As mentioned above, a large number of *in vitro* and *in vivo* experiments and some clinical studies have confirmed that chronic inflammation plays a key role in the development of HCC. Understanding the cytokines and signaling pathways required for the transmission of inflammation in HCC is more conducive to understanding the occurrence of HCC development, and can also provide some reference value for drug research and development. The following will discuss in detail some anti-inflammation agents in the potential of liver cancer treatment, which mainly was divided into two categories, natural medicine and synthetic drugs.

Natural anti-inflammatory agents

Natural medicine refers to modern medicine system that has a certain pharmacological activity of animal medicine, botanical medicine, mineral medicine, fruits, vegetables or spices. A large number of studies show that many natural anti-inflammatory drugs also have both cancer prevention and treatment potential. Many articles reported that natural products could be potentially used for the prevention and treatment of liver cancer through diet.

Curcumin, a chemical derived from the rhizomes of some plants from the family Zingiberaceae, is a diketone. Curcumin has a variety of pharmacological activities, including lipid-lowering, anti-tumor, anti-inflammatory, gallbladder, anti-oxidation and other effects^[106]. Existing research shows that curcumin can inhibit DEN-induced NF- κ B expression in liver tissue. Curcumin can also inhibit DEN-induced HCC by inhibiting the expression of IL-2 and IL-6 and promoting Gpx, GRE and SOD activities^[107]. El-Houseini *et al.*^[108] found that curcumin and taurine combination may be a new way to prevent liver cancer.

In 1940, resveratrol was first isolated from the roots of the leaf-walnut. In recent years, many studies have shown that resveratrol has anti-cancer, anti-cardiovascular disease, weight loss, anti-bacterial, anti-inflammatory, anti-oxidation pharmacological properties^[109]. *In vitro* experiments showed that the migration and invasion of HepG2 cells are inhibited by resveratrol, which decrease the expression of MMP-2, MMP-9 and NF- κ B nuclear transfer^[110,111]. Resveratrol was also been shown to inhibit SIRT1 mediated PI3K/AKT pathway, thereby down-regulating Bcl-2, caspase-3 and caspase-7 expression^[112]. DMU-212, a resveratrol analogue, has been reported to have effect on antioxidant status and apoptosis-related genes in rat model of hepatocarcinogenesis^[113].

N-acetylcysteine (NAC), a water soluble organosulfur compound present in garlic, is a classic anti-oxidant. Studies have shown that abnormal oxidative stress occurs in hepatocarcinoma. When NAC is used in hepatic stellate cells (HSCs), it can inhibit hepatic fibrosis and HCC development^[114]. Moreover, the use of NAC in HCC mice inhibited the high expression of GST, which may be related to insulin-like growth factor I (IGF-I) and iNOS^[115,116].

Gallic acid (GA) is an organic acid, which is the main component of many herbs such as dogwood and rhubarb. Experimental studies have shown that GA can inhibit the proliferation of HepG2 cells by inhibiting the expression of IL-8 and promoting the expression of IL-10 and IL-12^[117]. In the SMMC-7721 cells, GA induced caspase-3, caspase-9 and reactive oxygen species (ROS) activity^[118]. N-nitrosodiethylamine induces the high serum levels of alpha-fetoprotein, glypican-3, and STAT3. Those can all be inhibited by GA through activation of p38 in HepG2 to produce anti-oxidant effect^[119].

Flavonoids are a class of secondary metabolites of plants derived from a wide range of sources and can be derived from fruits, vegetables, roots, stems, flowers, beans, and daily intake of beverages such as tea and wine. Among them, baicalein, quercetin and genistein are three simpler and more studied agents. Baicalein is derived from plant *scutellaria baicalensis*. Studies have reported that baicalein can inhibit the proliferation

of Bel-7402 cells through periodic blockade^[120]. The process involves mainly involved in MAPK, Wnt, Hippo and PI3K-Akt/mTOR signaling pathways^[121]. Quercetin can enhance the antiproliferative effect of IFN- α in hepatocarcinoma cells by inhibiting SHP2 phosphatase activation of JAK/STAT signaling pathway^[122]. The results showed that quercetin nanoparticles could inhibit caspase/Cyto-c signaling pathway, inhibit AP-2 and NF- κ B, block Akt/ERK signaling pathway to play an antitumor effect^[123]. Genistein can induce HCC cell death by regulating the inhibition of aerobic glycolysis by HIF-1 α , and genistein can regulate gene products Cyclin D1, Bcl-xL, Bcl-2, c-myc and COX-2 by inhibiting NF- κ B and VEGF expression, and then alleviate the occurrence and development of HCC^[124].

Myrtenal is an important variety of spices, has been widely used in daily chemical, pharmaceutical, food and other industries. Studies have shown that, as a natural monoterpene, Myrtenal can inhibit DEN-induced high expression of TNF- α in HCC^[125]. Myrtenal also improves DEN-induced hepatocarcinogenesis by activating tumor suppressor protein p53 and modulating lysosomal and mitochondrial enzymes^[125].

Hesperidin is a chalcone compound. Many articles have reported that it has a good anti-liver cancer effect was shown through the Wnt pathway, ROS, ATP and calcium^[126-128]. More importantly, hesperidin on tumor cell invasion inhibition was realized mainly through inhibition of AP-1 and NF- κ B in human HCC cells^[129].

Synthetic anti-inflammatory agents

Aspirin is a clinical analgesic and antipyretic drug. Due to its long clinical application and high safety, research on its multiple clinical conditions is now receiving great attention. At present, studies have shown that aspirin has good anti-tumor activity^[130]. Among them, the application of liver cancer has been gradually reflected. A large number of clinical studies have shown that long-term use of aspirin in HCC patients can inhibit the expression of AMPK, mTOR and β -catenin and thus inhibit the progress of liver cancer^[131]. Our recent study suggested aspirin as a promising chemopreventive and chemotherapeutic agent for liver cancer. There is a current prospective randomized controlled trial registered in China to investigate the effect of sorafenib combined with aspirin in preventing patient risk for postoperative surgical recurrence of HCC (NCT02748304). We have demonstrated that by combining low-dose sorafenib and aspirin, the synergistic antitumor effects observed are related to the simultaneously silencing of ACSL4 and the induction of GADD45B expression. The clinical survival of HCC patients expressing ACSL4^{high}GADD45^{low} was significantly poorer compared to patients with ACSL4^{low}GADD45^{high} expression, thus demonstrating the potential clinical value of combining aspirin and sorafenib to treat HCC patients expressing ACSL4^{high}GADD45^{low}^[132].

There are so many special COX2-inhibitors, like celecoxib, etodolac, JTE-522 and nimesulide. Even though those are COX-2 inhibitors, their principles of pharmacological activity are different. Celecoxib inhibited the translocation of p65 to the nucleus from the cytoplasm^[133]. R-Etodolac (at physiological doses) and Celecoxib (at high concentrations) on HCC cells were accompanied by the down-regulation of β -catenin^[134]. CDAA model activated hepatic stellate cells and promoted CD45-positive inflammatory cells coming in the liver. JTE-522 can attenuate all the change^[135]. Nimesulide inhibits the proliferation of HepG2 by up-regulation of Smad4 and downregulation of HSP70 gene expression of SMMC-7721^[136]. Roxithromycin is a new generation of macrolide antibiotics. It inhibits constitutive activation of NF- κ B by diminishing oxidative stress or suppressing VEGF production in a rat model of HCC^[137]. Erlotinib, a special EGFR inhibitor, involves in development of HCC. It is reported that EGFR-ERK pathway has been inhibited by erlotinib in HCC model^[138]. However, due to extensive use of erlotinib, some patients with HCC in clinical trials have become resistant. Therefore, the study turned to combination therapy^[139-141]. In the literature, neurotensin regulation induces overexpression and activation of EGFR in HCC and restores response to erlotinib^[142].

CONCLUSION

Inflammation is one of the key factors to promote liver malignant transformation. A better understanding of the molecular processes of inflammation-cancer transformation in the development of primary liver cancer will be important to developing early detection for HCC and new drugs to efficiently prevent de novo hepatocarcinogenesis. It has been shown that inflammatory microenvironment constitutes different immune cells. Currently, overcoming antitumor immunity by immune checkpoint inhibitors represents one of the most promising therapeutic strategies for the treatment of many cancers including HCC^[143]. Some immune checkpoint inhibitors, such as anti-programmed death 1/programmed death-ligand 1 antibodies, have recently been reported in the promising clinical trial results. The Food and Drug Administration in United State has approved the nivolumab to be used for advanced HCC patients who fail to respond to first-line treatment^[144]. There are still no effective chemoprevention strategies in patients at high risk for HCC development besides the viral eradication in patients with viral hepatitis^[143]. We proposed that aspirin, a non-steroidal anti-inflammatory drug, may emerge as a promising chemopreventive and chemotherapeutic agent for HCC^[132]. There are great opportunities to further understand inflammation-cancer transformation and developing pharmacological strategies for preventing inflammation and HCC development and recurrence.

DECLARATIONS

Authors' contributions

Drafted the outline of this review: Chen HJ, Xia HP

Drafted the manuscript: Chen HJ, Hu MH, Xu FG, Xu HJ, Xia HP

Finalized the manuscript: She JJ, Xia HP

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by grants from National Young 1000 Talents Program of China, Jiangsu Province Education Department Grant, Southeast University-Nanjing Medical University Cooperative Research Project, Young Talent Support Plan of Xi'an Jiaotong University and Hundred Talents Program of Shaanxi.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Oncogenic Wnt3a: a promising specific biomarker in hepatocellular carcinoma

Min Yao^{1,2,#}, Miao Fang^{1,#}, Wen-Jie Zheng¹, Deng-Fu Yao¹

¹Research Center of Clinical Medicine, Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu, China.

²Department of Immunology, Medical School of Nantong University, Nantong 226001, Jiangsu, China.

#Authors contributed equally.

Correspondence to: Prof. Deng-Fu Yao, Research Center of Clinical Medicine, Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu, China. E-mail: yaodf@ahnmc.com

How to cite this article: Yao M, Fang M, Zheng WJ, Yao DF. Oncogenic Wnt3a: a promising specific biomarker in hepatocellular carcinoma. *Hepatoma Res* 2018;4:30. <http://dx.doi.org/10.20517/2394-5079.2018.32>

Received: 28 Mar 2018 **First Decision:** 22 May 2018 **Revised:** 11 Jun 2018 **Accepted:** 11 Jun 2018 **Published:** 6 Jul 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Hepatocellular carcinoma (HCC) is still one of the most common and rapidly fatal malignancies worldwide with a multi-factorial, multi-step, complex process, and poor prognosis. Early discovery and effective therapy of HCC are of utmost importance. Recent studies demonstrated that Wnt/ β -catenin pathway play important roles in occurrence and development of HCC including hepatocytes malignant transformation, metastasis, chemoresistance and liver cancer stem cells. Oncogenic wingless-type MMTV integration site family member 3a (Wnt3a) signaling is a promising biomarker in diagnosis and prognosis for HCC. This review presents current data on mechanisms of hepatocarcinogenesis involving participation of the Wnt canonical pathway, and focuses on the Wnt3a expression in HCC progression and its clinical application.

Keywords: Hepatocellular carcinoma, Wnt/ β -catenin pathway, signal molecules

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common or deathly human malignancy cancers worldwide^[1,2], especially in the areas along the Yangtze River. Recently, Chen *et al.*^[3] reported the observed survival and relative survival of leading cancer sites from a population-based cancer registry for 40 years. The main sites of the cancer types with a total of 92,780 incident cases in Qidong, China, HCC ranks the first based on the rank order of incidence among all malignancies (liver, stomach, lung, colon and rectum, oesophagus, breast, pancreas, leukaemia, brain and central nervous system, bladder, non-Hodgkin's



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Table 1. Chromosomal location of Wnt genes and tissue distribution

Gene	Location	Accession numbers	Tissues or tumors
<i>Wnt 1</i> ^[22]	12q13	X03072	Lipomas, myxoid liposarcomas, pleomorphic adenomas, myomas
<i>Wnt 2</i> ^[23]	7q31	X07876	Lung, heart
<i>Wnt 2b/13</i> ^[24]	1p13	XM052111, XM052112	Cervical cancer, gastric cancer
<i>Wnt 3</i> ^[25]	17q21	AY009397	Breast
<i>Wnt 3a</i> ^[26]	1q42.13	AB060284	Spinal cord, brain, liver
<i>Wnt 4</i> ^[27]	1p35	AY009398	Breast
<i>Wnt 5a</i> ^[28]	3p14-p21	L20861	Neonatal heart, lung, liver
<i>Wnt 5b</i> ^[29]	12p13.3	AB060966	Prostate, fetal brain & lung, kidney, liver, ovary, small intestine
<i>Wnt 6</i> ^[30]	2q35	AY009401	Kidney, placenta, spleen
<i>Wnt 7a</i> ^[31]	3p25	D83175	Placenta, kidney, testis, uterus, fetal lung, brain
<i>Wnt 7b</i> ^[32]	22q13.3	AB062766	Brain, kidney, prostate, lung, esophageal, gastric, pancreatic cancer
<i>Wnt 8a/d</i> ^[33]	5q31	AB057725, AY009402	Teratocarcinoma, mesoderm
<i>Wnt 8b</i> ^[34]	10q24	Y11094	Forebrain
<i>Wnt 10a</i> ^[30]	2q35	AB059569	Kidney, placenta, spleen, brain, liver
<i>Wnt 10b/12</i> ^[35]	12q13.1	U81787	Lung, uterus, thymus, spleen, breast
<i>Wnt 11</i> ^[36]	11q13.5	Y12692	Skeleton, lung
<i>Wnt 14</i> ^[37]	1q42	AB060283	Breast
<i>Wnt 15</i> ^[37]	17q21	AF028703	Breast
<i>Wnt 16</i> ^[38]	7q31	XM031374, XM00488	Spleen, appendix, lymph nodes

lymphoma, and cervix) and the poorest survival rate^[3]. The leading etiological factors of HCC include chronic hepatitis B or C virus (HBV^[4-6] or HCV^[7,8]) infection, aflatoxin contaminated food taken and non-alcohol fat liver diseases (NAFLD)^[9,10]. Chronic HBV carriers have a 5-15-fold increased risk of HCC compared with the general population. HBV-related proteins are known to take control of several cellular pathways like Wnt/ β -catenin, TGF- β , Raf/MAPK, and ROS for the virus's own replication^[11-13].

Carcinogenesis of HCC is a multi-factor, multi-step and complex process. Most of HCC patients died quickly because of the rapid tumor progression, and hepatic resection or transplantation is the only potential curative treatment for HCC patients^[14,15]. Activation of the Wnt/ β -catenin signaling pathway plays a significant role in the pathology and physiology of the liver and has been identified as a main factor in HCC because of hepatocytes malignant transformation with numerous genetic/epigenetic abnormalities, and affects cellular persistence, multiplication, migration, alteration and genomic instability^[16-18]. Abnormal expressions of Wnt signaling molecules were closely associated with the occurrence and progression of HCC. Recently, Pan *et al.*^[19,20] discovered and reported that the overexpression of oncogenic wingless-type MMTV integration site family member 3a (Wnt3a) could be a specific biomarker in diagnosis and prognosis of HCC. However, its exact underlying mechanisms in hepatocarcinogenesis still remain poorly understood. This review presents new advances of the underlying mechanisms of Wnt signaling, and focuses on expressions of hepatic or circulating Wnt3a, which serve as a promising molecular biomarker for HCC.

REGULATING MECHANISMS OF Wnt SIGNALINGS

Human Wnt genes encode a large family of secreted proteins that have been reported in many tissues^[21]. Total 19 Wnt proteins in human tissues or cancers are shown in Table 1. Proteins were identified that share 27% to 83% amino acid sequence identity, and evolutionarily conserved glycoproteins with 23 or 24 cysteine residues. Human Wnt proteins are all very similar in size, ranging in molecular weight from 39 kDa (Wnt7a) to 46 kDa (Wnt10a). Wnt protein folding may depend on the formation of multiple intramolecular disulfide

bonds. Analysis of the signaling activities of chimeric Wnt proteins has shown that the carboxy-terminal region of Wnt proteins may play a role in determining the specificity of responses to different Wnts. The amino-terminal region may mediate interactions with Wnt receptors but requires the carboxyl terminus to activate these receptors. The main regulating mechanisms of Wnt signaling are either through canonical pathway (Wnt1, Wnt2, Wnt3, Wnt3a, Wnt8a, Wnt8b, Wnt10a, and Wnt10b) characterized by the stabilization and subsequent nuclear transport of β -catenin resulting in the activation of transcriptional responses or via non-canonical pathway (Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, and Wnt11) with more diverse and several different signaling modes that regulate cell biological behaviors^[22-38].

The Wnt signaling molecules have been involved in liver tumorigenesis with activating liver cancer stem cells^[39]. In adults, Wnts function in homeostasis, and inappropriate activation of the Wnt pathway is implicated in a variety of cancers. Some signaling molecules in the Wnt pathway have been recognized to play an important role in the development and progression of tumors and regulate multiple cellular events such as cell proliferation, differentiation, and apoptosis through β -catenin-dependent canonical- or β -catenin-independent noncanonical pathway^[40]. Abnormal expression of some key molecules in the Wnt/ β -catenin pathway was associated with the development and progression of HCC. Wnt3a gene located on chromosome (1q42.13) has been regarded as an activator inducing β -catenin accumulation and activating the canonical Wnt signaling pathway. Studies on human Wnt3a have focused primarily on its key role in liver malignancy, and its high expression in cancerous tissues has been confirmed with a worse outcome^[20].

HBV INVOLVED IN Wnt ACTIVATION

HBV has a global distribution and is one of the leading causes of HCC. Its viral replication with several pathways like Wnt/ β -catenin, TGF- β , Raf/MAPK and ROS affects cellular persistence, multiplication, migration, alteration and genomic instability^[41,42]. The Wnt/FZD/ β -catenin pathway associated with HBV-related HCC development because of the progression of chronic liver diseases is known to be accompanied by disturbances in β -catenin expression (mainly overexpression)^[43,44], with its cytoplasmic or nuclear translocation. Viral proteins of HBV (HBx and HBsAg) can act as pathogenic factors that are involved in the modulation and induction of canonical Wnt signaling activation with aberration of adenomatous polyposis coli (APC), AXIN, secreted Frizzled related protein (SFRP) 1 and SFRP5.

The canonical Wnt signals are transduced through Frizzled receptors and LRP5/LRP6 co-receptors located on the cell membrane, initiating the β -catenin signaling cascade^[45,46]. This multi-protein destruction complex could target the proto-oncogene β -catenin for ubiquitin-mediated proteolysis, prevent glycogen synthase kinase 3 α (GSK-3 α)-mediated β -catenin degradation, leading to nuclear translocation of β -catenin, combine with T-cell factor/lymphoid enhancer factor, and thereby promote the transcription of downstream target genes, including *FGF20*, *DKK1*, *WISP1*, *MYC*, *CCND1*, and so on. Their interaction results in the enhancement of the pathway and leads to hepatocarcinogenesis^[47,48]. Thus, lack of Wnt secretion from hepatocytes did not affect overall injury, fibrosis or HCC burden although there were protein expression differences in tumor conformation^[49].

HCV PROVOKED Wnt SIGNALING

Epidemiological studies have validated the association between HCV infection and HCC. An increasing number of studies show that protein-protein interactions between HCV proteins and host proteins play a vital role in infection and mediate HCC progression^[50]. The role of nonstructural (NS5A) protein of HCV *in vivo* has been accentuated in induction of this pathway mainly to the canonical pathway. Interaction of Wnt signaling with HCV genome in hepatocarcinogenesis linked β -catenin phosphorylation and abnormalities in the E-cadherin-catenin unit function lead to loss of intercellular junctions, progression in liver fibrosis, and development of cirrhosis and HCC^[51,52]. Accumulating evidence indicates that HCV core or nonstructural

proteins provoke activation of the Wnt/ β -catenin signaling pathway, and the evidence supporting a role of Wnt/ β -catenin signaling in the onset and progression of HCC is compelling^[53,54].

Progression of HCV-related liver diseases is noted to be accompanied by disturbances in β -catenin overexpression, with its cytoplasmic or nuclear translocation and with lower expression of E-cadherin. More β -catenin mutations are manifested in HCV-associated than in HBV-related HCC. HCV proteins affect in a double manner expression of E-cadherin, including modulation of the Wnt pathway and reduction of E-cadherin expression at the transcriptional level. Alterations in cellular locations of β -catenin and E-cadherin in chronic HCV and HCC pointed to structural disturbances in intercellular junctions in livers and presence of the transcriptionally inactive form of β -catenin^[55,56]. Promoter hypermethylation of Wnt inhibitors was discovered in HCV-induced multistep hepatocarcinogenesis^[57], and the reduced expression of E-cadherin in long-lasting chronic HCV might represent an early indicator of the epithelial-mesenchymal transition^[58,59].

COUNTERACTIVE Wnt3a WITH Wnt5a IN HCC

Although accumulating clinical and basic evidences have suggested that the Wnt signaling is associated with the HCC progression^[60]. However, little research has been reported on the relationship between Wnt3a and HCC. Previous studies have found that Wnt3a showed higher expression in HCC than liver tissues, positively correlated with its target genes MMP 7 and c Myc. Intriguingly, their expressions are significantly correlated with Notch3 and Hes1 expression. Wnt3a was highly expressed in MHcc97H and SK Hep 1 cells *in vitro*^[61], as an important regulator of human HCC cell line growth, which could induce activation of the canonical Wnt pathway after binding with SULF2 and GPC-3. Also, it could increase cell proliferation in nude mouse xenografts *in vivo*^[60,61].

The expressions of hepatic Wnt3a were investigated in HCC tissues [Figure 1]. The positive Wnt3a with brown staining particles was mainly distributed in cytosol and membrane of hepatocytes in cancerous tissues and no or lower expression in their surrounding tissues. High Wnt3a expression like its down-stream disheveled 2, DKK1, and SFRP1 were all identified as independent predictive factors for poor HCC outcome^[20,62-64]. Compared with high hepatic Wnt3a in HCC tissues, the significant difference of Wnt5a intensity was found between low level in HCC tissues and high expression in their para-cancerous tissues. The intensity of Wnt5a expression was inversely correlated with Wnt3a level in cancerous tissues. Both decreasing Wnt5a and increasing Wnt3a expression in HCC tissues relation to the clinical staging from stage I to IV were confirmed as independent prognosis factors of HCC patients. The Kaplan-Meier survival curves demonstrated that HCC patients with high Wnt3a expression had a significantly lower survival rate compared to cases with lower Wnt3a [Figure 2], Wnt3a expression was associated with poorly-differentiated grade, liver cirrhosis, chronic HBV infection, and higher TNM stage, indicating that the abnormal Wnt3a expression could participate in promoting hepatocytes malignant transformation and progression of HCC^[65,66].

SERUM Wnt3a FOR HCC SPECIFIC DIAGNOSIS

Early diagnosis of HCC is of the utmost importance. Successful screening for HCC at early stage is challenging due to the lack of well characterized and specific biomarkers^[67,68]. Data of previous studies have confirmed that some Wnt signalings could modify HCC growth and invasive ability. However, achieving successful screening of abnormal Wnt3a signaling is critically important as early diagnosis could potentially provide an early monitoring opportunity. Along these lines, the Wnt pathway has been identified as contributing to the development and progression of HCC. Although serological AFP marker is commonly applied to HCC diagnosis, it has exhibited a low sensitivity and specificity with approximately 40% of negative patients. Although many biomarkers have been applied in diagnosis for HCC, only a few markers were confirmed with higher specificity or sensitivity for HCC, especially in early stage or small size HCC^[69-71].

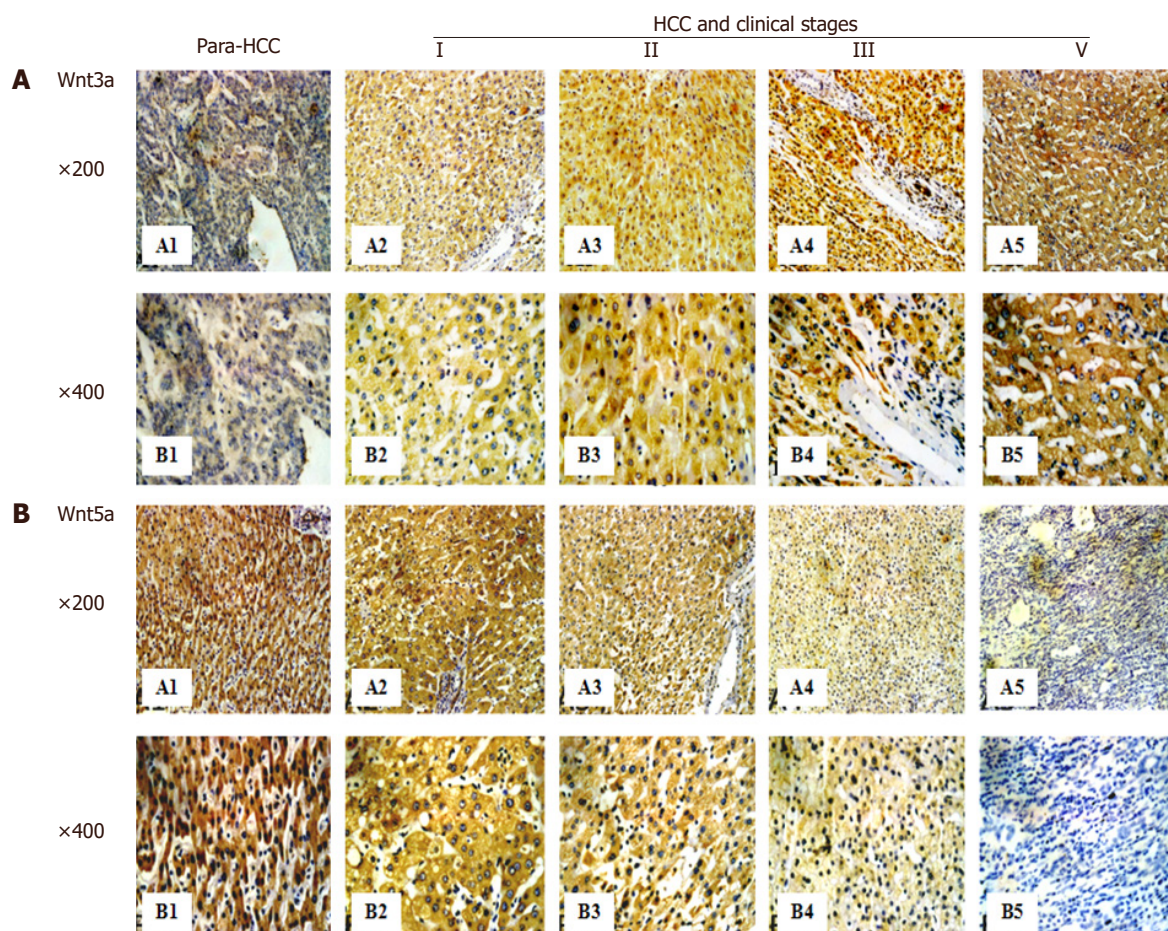


Figure 1. Immunohistochemistry of Wnt3a or Wnt5a expression in HCC tissues with different staging^[20,65]. (A) The Wnt3a expression in HCC tissues (SP, original magnification: A1-A5, $\times 200$; B1-B5, $\times 400$); (B) the Wnt5a expression in HCC tissue (SP, original magnification, A1-A5, $\times 200$; B1-B5, $\times 400$). In Wnt3a, A1 and B1, the low or without Wnt3a expression in the para-cancerous tissues, and A2-A5 and B2-B5, the brown staining of Wnt3a expression with gradually increasing from stage I, II to III-IV of HCC tissues; In Wnt5a, A1 and B1, the strongest Wnt3a expression in the para-cancerous tissues, and A2-A4 and B2-B4, the brown staining of Wnt3a expression with gradually decreasing from stage I to III of HCC tissues, and A5 and B5, the low or without Wnt5a expression were discovered in HCC tissues at stage IV. HCC: hepatocellular carcinoma tissues; Para-HCC: paracancerous tissues; Wnt3a: wingless-type MMTV integration site family member 3a; Wnt5a: wingless-type MMTV integration site family member 5a

Cancerous Wnt3a was over-expressed and could secrete into circulating blood. The incidence of serum Wnt3a level (> 800 ng/L) in HCC patients was 92.5% with significantly related to AFP level, liver cirrhosis, HBV infection, low differentiation degree, TNM staging, and extra-hepatic metastasis^[19]. According to the diagnostic specificity or the area under the receiver operating characteristic (ROC) curve, serological Wnt3a detection has been confirmed superior to AFP, HS-GGT^[72], and GPC-3^[73] with higher sensitivity and lower false-positive rate for HBV-related HCC patients [Table 2]. The combining of serum Wnt3a plus AFP detection has complemented diagnostic value and raised the sensitivity up to 96.3% for HCC diagnosis which was obviously higher in Wnt3a or AFP alone for distinguishing malignancy from benign liver lesions, suggesting that serum Wnt3a should be a novel specific marker for HCC diagnosis that was superior to routine AFP detection^[74] according to the specificity and the area under the ROC curve, especially in diagnosis of AFP-negative HCC.

Wnt3a SIGNALING WITH HCC TARGETED-THERAPY

Once HCC is advanced, there are multiple therapeutic venues, but most eventually fail. Effective treatment of HCC still is a challenging problem worldwide. Therefore, developing novel molecule-targeted therapies may

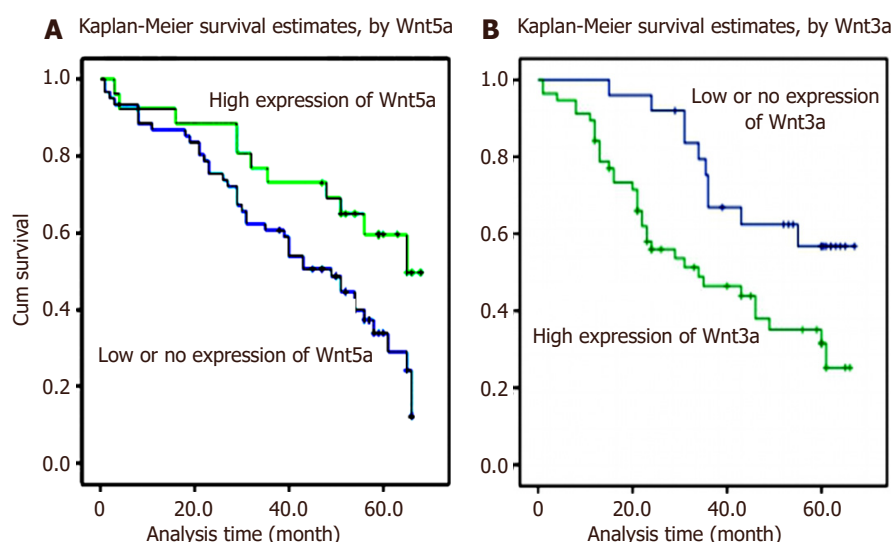


Figure 2. Overall survival curves of Wnt5a or Wnt3a expression in HCC^[20,65]. The hepatic Wnt5a or Wnt3a expression curves were calculated according to the Kaplan-Meier method. The accumulative survival curves of patients with HCC were made according to HCC tissues with low or high expression for Wnt5a or Wnt3a level (log-rank test, $P < 0.001$). (A) Wnt5a in HCC; (B) Wnt3a in HCC. HCC: hepatocellular carcinoma; Wnt3a: wingless-type MMTV integration site family member 3a; Wnt5a: wingless-type MMTV integration site family member 5a

Table 2. Comparative analysis of circulating Wnt3a, AFP, HS-GGT, and GPC-3 detection in diagnosis of HCC

	Wnt3a ^[19] (> 800 ng/L)	AFP ^[19] (> 50 ng/mL)	HS-GGT ^[17] (> 5.5 U/L)	GPC-3 ^[67] (positive)	Wnt3a ^[19] + AFP
Sensitivity (%)	92.50	61.25	85.70	52.84	96.25
Specificity (%)	94.34	69.81	97.24	99.58	62.26
Accuracy (%)	93.23	64.66	96.20	83.57	82.71
PPV (%)	96.10	75.38	89.70	98.48	79.38
NPV (%)	89.29	54.41	92.23	80.20	91.67

Wnt3a + AFP: combining detection of serum Wnt3a and AFP concentration; Wnt3a ($n = 80$), AFP ($n = 80$), HS-GGT ($n = 91$), and GPC-3 ($n = 123$). PPV: positive predictive value; NPV: negative predictive value; HCC: hepatocellular carcinoma; GPC-3: glypican-3; HS-GGT: HCC-specific gamma-glutamyl transferase; AFP: alpha fetoprotein

provide greater chance for effective therapies^[75] or overcoming resistance to sorafenib^[76]. Many mechanisms have been involved in the aberrant activation of Wnt signaling and regulating β -catenin activity^[77] or function by using small molecules (LGK974^[78], Celecoxib^[79], Genistein^[80]), specific antibodies (OMP-54F28, OTSA101)^[81] and small size peptide SAH-BCL-9^[82]. However, only a few of anti-cancer drugs that have been developed to target the related pathway of HCC formation or development have entered into pre-clinical trials, and none of these have advanced to the late clinical trial stage.

Oncogenic Wnt3a is involved in HCC development and increasing Wnt3a plays a crucial role in cell proliferation and metastasis, particularly in progression and mediated-oncogenesis involving signaling pathways, with brown granule-like staining localized in cancerous parts of atypical hyperplasia^[19,20]. Targeted oncogenic glypican-3 gene transcription of Wnt upstream inhibited the proliferation of human hepatoma cells by specific short hairpin RNA^[83]. Down-regulating Wnt3a expression inhibited cell viability and induced G0/G1 cell cycle arrest via decreased expression of cyclin D1 and c Myc, and increased expression of p21 and p27. In addition, deletion of Wnt3a significantly inhibited migration and invasion by down-regulating MMP 2/-7/-9 expression via the MAPK (p38, ERK1/2 and JNK) pathway^[61]. The abnormality of liver and circulating Wnt3a expression in HCC has provided initial evidence, and suggested that targeted-Wnt3a signaling could be a promising target or an effective target for HCC therapy.

PERSPECTIVES

In conclusion, molecular factors are involved in the process of HCC development and metastasis. HBx could integrate into human genome and this transcript could activate Wnt signaling as a long noncoding RNA^[84]. The associations between Wnt signaling and cancer initiation, tumor growth, metastasis, dormancy, immunity and tumor stem cell maintenance have been revealed, and Wnt signaling has exhibited numerous genetic abnormalities^[85,86] as well as epigenetic alterations including modulation of DNA methylation. The overexpression of Wnt3a in cancerous tissues has been discovered, and its higher level was only found in sera of HCC patients from a cohort study in chronic liver diseases, although it is the first time to report as a novel specific marker for HCC diagnosis and prognosis. Further studies will permit us to analyze Wnt3a role in hepatocarcinogenesis and explore its molecular-targeted for HCC therapy^[87,88].

DECLARATIONS

Authors' contributions

Conception and literature search: Yao M, Zheng WJ

Drafting the manuscript: Yao M, Fang M

Critical revision for intellectual content: Yao DF

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by the grants from the Projects of National Natural Science Foundation of China (81673241, 81702419), Jiangsu Medical Programs (KYCX17_1934, BE2016698), and Int. S&T Cooperation Program (2013DFA32150) of China.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Performance of different biomarkers for the management of hepatocellular carcinoma

Ángela Rojas¹, Yolanda Sánchez-Torrijos², Antonio Gil-Gómez¹, Chang-Hai Liu¹, Clara Rodríguez-Rivas¹, María Teresa Ferrer², Manuel Romero-Gómez¹

¹Traslational and Clinical Research Laboratory - Liver and Digestive Diseases - CIBERehd, Institute of Biomedicine of Seville, Virgen del Rocío University Hospital/CSIC/University of Seville, Seville 41013, Spain.

²Unit for the Clinical Management of Digestive Diseases, Virgen del Rocio University Hospital, University of Seville, Seville 41013, Spain.

Correspondence to: Prof. Manuel Romero-Gómez, Head of Unit for the Clinical Management of Digestive Diseases, CIBERehd, Institute of Biomedicine of Seville, Virgen del Rocío University Hospital/CSIC/University of Seville, Seville 41013, Spain.
E-mail: mromerogomez@us.es

How to cite this article: Rojas Á, Sánchez-Torrijos Y, Gil-Gómez A, Liu CH, Rodríguez-Rivas C, Ferrer MT, Romero-Gómez M. Performance of different biomarkers for the management of hepatocellular carcinoma. *Hepatoma Res* 2018;4:31.
<http://dx.doi.org/10.20517/2394-5079.2018.60>

Received: 15 May 2018 **First Decision:** 7 Jun 2018 **Revised:** 21 Jun 2018 **Accepted:** 22 Jun 2018 **Published:** 6 Jul 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the second cause of cancer related death due to latent liver disease, late diagnosis and non-available therapeutic treatment. Liver biopsy is still the gold standard in order to know the molecular biology of the tumor, its behaviour and invasive characteristics. Conventional diagnosis methods for HCC detection include imaging and serological tests with low sensitivity and specificity. In this review, we focus on the potential utility of certain serum biomarkers and a new approach, “liquid biopsy”, in the management of HCC patients.

Keywords: Hepatocellular carcinoma, liver biopsy, conventional diagnostic methods, management

INTRODUCTION

Liver cancer, known as well as hepatocellular carcinoma (HCC), is the fifth most common type of cancer worldwide and the second cause of cancer related death^[1,2]. Despite recent development of a diagnostic technics and treatment methods, the prognosis of HCC remains poor. Many patients are diagnosed when HCC is in advanced stages or due to an underlying liver disease, which results in less time for an appropriate treatment^[3].



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Table 1. Serum biomarkers for early diagnosis of hepatocellular carcinoma

Biomarker	Cut-off	Sensitivity % (95% CI)	Specificity % (95% CI)	AUC	Other utilities	Ref.
AFP	20	53 (46-59)	90 (87-93)	0.8	Prognosis	[9]
AFP-L3	10	28 (22-34)	97 (93-100)	0.66	Prognosis	[9]
DCP	150	61 (55-68)	70 (65-74)	0.72	Prognosis	[9]
GPC3	0-003-300	53 (0.49-0.57)	77 (0.74-0.81)	0.82	Treatment	[28,33]

AFP: alpha-fetoprotein; DCP: des-gamma-carboxy prothrombin; GPC3: glypican-3; AUC: area under the curve; CI: confidence interval

Conventional diagnosis methods for HCC detection include imaging and serological test with low sensitivity and specificity^[4]. In this review we provide a briefly outline of HCC serological biomarkers [Table 1] and highlight the recent development of circulating cancer byproducts detection: liquid biopsy.

CONVENTIONAL SERUM TUMOR MARKERS

Alpha-fetoprotein

Alpha-fetoprotein (AFP) is a glycoprotein that transports a great variety of molecules, and it is usually produced during fetal and neonatal development by the liver, yolk sac and gastrointestinal tract. Once it reaches its maximum concentration in the second trimester, its levels decrease until it is only detected in small amounts in serum^[5]. Elevated levels of AFP in adulthood can be related to malignant diseases, such as HCC and other gastrointestinal, pancreatic, biliary, nonseminomatous germ-cell testicular, and germ cell ovarian cancers^[6]. However, an increase of serum AFP levels can be expected in non-neoplastic conditions, such as pregnancy, cirrhosis (11%-47%) or acute hepatitis (30%-50%)^[7].

If we use a cut-off of 20 ng/mL, the sensitivity and specificity values of AFP 12 months prior to the time of HCC diagnosis are 47% and 75% respectively, while at the time of diagnosis, those values rise to 61% in the case of sensitivity and 81% in the specificity variable^[8]. However, if we increase the cut-off to 200 ng/mL, we improve the specificity to 100%, at the expense of decreasing sensitivity.

On the other hand, Marrero *et al.*^[9], carried out a case-control study among patients with compensated cirrhosis and patients with HCC [both hepatitis C virus (HCV+)], concluding that AFP had the best area under the receiver operating characteristic (ROC), curve [0.80, 95% confidence interval (CI): 0.77-0.84], with a cut-off of 10.9 ng/mL, for early stage HCC (BCLC stages 0 and A).

At present, AFP is used as a complementary biomarker to ultrasonography (US) for HCC surveillance, although clinical guidelines only recommend the last one^[10,11]. However, according to a recently published meta-analysis^[12], where 38 observational cohort studies that evaluated surveillance in patients with cirrhosis were included, it was observed that the use of US plus AFP improves detection of early-stage HCC compared with no surveillance [odds ratio (OR) = 2.16 (95% CI: 1.80-2.60)], while US alone had an OR of 2.04 (95% CI: 1.55-2.68); at the same time, US plus AFP had a risk ratio for improving survival of 1.86 (95% CI: 1.76-1.97), while US alone had a slightly lower risk ratio of 1.75 (95% CI: 1.56-1.98), although it was not statistically significant. There were no studies that directly compared US alone versus US plus AFP, and only 4 studies used US alone, while the rest of the studies relied on US and AFP at 6-month intervals.

Finally, in addition to early diagnosis, AFP can also predict the survival after liver transplantation (LT) in patients with HCC, as shown by She *et al.*^[13] in a study conducted in 250 patients, in which survival is less than 5 years post-LT if AFP levels are higher than 400 ng/mL [66% vs. 85% (AFP < 10 ng/mL), $P = 0.029$].

Serum AFP level is correlated with the tumor size. In fact, 80% of small HCC (< 2 cm) do not show high levels of serum AFP. In the other hand, AFP levels can be increased in patients with chronic liver disease with a degree of hepatocytes regeneration such as HCV-infection that shows a high level of AFP in absence

of malignancy^[14]. For these reasons, some additional biomarkers for the diagnosis of HCC are needed to improve the sensitivity of AFP and solve these issues.

AFP lectin fraction (AFP-L3)

AFP exists as three glycoforms, according to its binding capability to *Lens culinaris* agglutinin lectin (LCA): AFP-L1 (non-binding fraction), AFP-L2 (weak binding fraction), and AFP-L3 (binding fraction)^[15]. AFP-L1 is increased in chronic hepatitis and liver cirrhosis, whereas AFP-L3, that it's only produced by cancer cells, is specifically increased in HCC^[16].

Regarding the way of measurement, “bound” and “free” AFP isoforms are separated by affinity liquid chromatography. The concentration of bound AFP-L3 is determined fluorometrically, and results are reported as percentage ratio of AFP-L3 to total AFP^[17]. On the other hand, the cut-off used is 10%, observing values of 37%, 92%, 52% and 85% for sensitivity, specificity, positive and negative predictive value, respectively^[4]. These values increase when they are combined with AFP and des-gamma-carboxy prothrombin (DCP) to 77%, 59%, 32% and 91% respectively. However, according to a recently published study, ROC curve analysis showed that the highest specificity and sensitivity of the studied parameters are achieved at cut-offs of 15% as well as combining AFP-L3 and p53 improves sensitivity to 95.4% with a specificity of 85%^[18].

Given that the sensitivity is markedly decreased when total concentration of AFP was < 20 ng/mL (difficulty in detection), Oda *et al.*^[19] found a new way of measurement based on a microchip capillary electrophoresis and liquid-phase binding assay on a μ -ASWako i30 auto analyzer (Wako Pure Chemical Industries, Ltd., Osaka, Japan) that increased the sensitivity compared to the conventional measurement (12.5% *vs.* 44.6%), when using a cut-off value of 5%. Also, none of the benign liver disease patients with both serum AFP < 20 ng/mL and high sensibility-AFP-L3 < 5% developed HCC for a median follow-up of 35 months.

Finally, in a meta-analysis that included 12 studies that directly compared the diagnostic accuracy of serum AFP-L3 and AFP in the same population, it was found that, although the specificity for AFP-L3 (0.929) was increased *vs.* AFP (0.856), sensitivity also decreased significantly (0.48 *vs.* 0.62), with an area under the curve (AUC) of 0.756 *vs.* 0.863, respectively^[20].

In conclusion, AFP-L3 could be a complementary biomarker for the early diagnosis of HCC, but additional studies that really confirm its usefulness are needed.

Des- γ -carboxy prothrombin

Des- γ -carboxyprothrombin (DCP), also known as prothrombin induced by vitamin K absence II (PIVKA II), is a molecule produced during the process of hepatocytes malignant transformation due to the fact that the vitamin K-dependent carboxylase system becomes impaired, for which it is increased in patients with HCC^[21].

DCP sensitivity and specificity rate at the time of diagnosis were 74% and 86%, respectively, at a cut off of 40 mAU/mL and 43% and 100%, respectively, at a cut off of 150 mAU/mL; while for AFP it was 61% and 81% at a cut off of 20 ng/mL and 22% and 100% at a cut off of 200 ng/mL. Sensitivity and specificity were significantly reduced when determined 12 months before diagnosis, being 43% and 94%, respectively, for DCP and 47% and 75%, respectively, for AFP^[8].

In a case-control study, where controls were patients with compensated cirrhosis and patients with HCC, it was evaluated DCP and AFP-L3 as biomarkers for the early diagnosis of HCC. AUC for total AFP (0.83, 95% CI: 0.80-0.85) was similar to DCP (0.81, 95% CI: 0.78-0.84), but better than for AFP-L3 (0.72, 95% CI: 0.69-0.75).

However, in patients with early stage of HCC, AFP showed the best AUC (0.80, 95% CI: 0.77-0.84) followed by DCP (0.72, 95% CI: 0.68-0.77) and then AFP-L3 (0.66, 95% CI: 0.62-0.70). Intermediate-advanced stage of HCC compared to cirrhotic controls showed a highest AUC of DCP (0.89, 95% CI: 0.86-0.92) compared to total AFP (0.84, 95% CI: 0.81-0.88) ($P = 0.01$), indicating that DCP could be a more useful marker in advanced stages^[9]. The cut off points used were 20 ng/mL for AFP, 10% for AFPL3 and 150 mAU/mL for DCP.

Nevertheless, there are some important differences between DCP and AFP; DCP is more specific for HCC because the underlying liver disease (e.g., chronic hepatitis C) can lead to an elevation of AFP but not of DCP. The DCP-positive and AFP-negative tumors show greater aggressiveness, larger size, less differentiation and vascular invasion, and in short, an early recurrence after curative treatments^[22,23].

Along these lines, Hamamura *et al.*^[24] compared survival among four groups of similar patients diagnosed with HCC based on AFP and DCP levels (A: AFP below 100 ng/mL and DCP below 0.0625 AU/mL; B: AFP greater than 100 ng/mL and DCP below 0.0625 AU/mL; C: AFP below 100 ng/mL and DCP above 0.0625 AU/mL; D: AFP greater than 100 ng/mL and DCP above 0.0625 AU/mL). The survival rates obtained after 3 years were 73.4%, 48.3%, 42.7% and 0%, respectively, while those values at 5 years were 53.5%, 25.9%, 0% and 0%, respectively, in a statistically significant way. Therefore, it can be concluded that patients with high levels of AFP and DCP have a lower survival, as well as those with high DCP only have a worse prognosis than those who do not.

Several studies have suggested that DCP may be involved in cell proliferation of neoplastic cells by acting as a growth factor. This may have important prognostic implications in the future, especially if combined with AFP^[25,26].

Glypican-3

Glypican-3 (GPC3) is a member of the glypican family of glycosyl-phosphatidylinositol-anchored cell-surface heparan-sulfate proteoglycans. Its levels increase considerably in patients with HCC, while GPC3 is not detected in healthy liver tissue, so it has been identified as an useful tumor marker for HCC diagnosis^[27].

Thus, in a recently published meta-analysis, sensitivity and specificity observed were of 0.53 (95% CI: 0.49-0.57) and 0.77 (95% CI: 0.74-0.81), respectively, with an AUC of 0.82^[28]. In addition, it seems to have a higher sensitivity than AFP, with similar specificity^[29], whereas their combination notably increase both sensitivity and specificity (98.5% and 97.8%, respectively)^[30]. On the other hand, GPC3 is detectable in approximately one third of patients with HCC with normal AFP levels^[16].

With regard to early diagnosis, Libbrecht *et al.*^[31] studied the expression of GPC3 in histopathological samples of HCC with less than or equal to 3 cm of diameter present in the cirrhotic liver (also analysing non-lesional tissue), low-grade and high-grade dysplastic nodules, and focal nodules of hyperplasia.

Immunohistochemical studies and real time reverse transcriptase-polymerase chain reaction for GPC3 were performed. The expression of GPC3 by both techniques was much higher in small HCC than in cirrhosis and other types of small focal lesions, with a sensitivity and specificity for the diagnosis of HCC in small focal lesions of 0.77 and 0.96, respectively, in resected cases, and 0.83 and 1, respectively, for needle biopsies. This may be due to the stimulation of growth induced by GPC3, which upregulates the autocrine/paracrine canonical Wnt signaling, with a strong increase in its expression in the transition from premalignant lesions to small HCC^[32].

Since GPC3 acts as a growth factor in HCC, it could be a potential therapeutic target. Codrituzumab (GC33) is a recombinant, humanized monoclonal antibody that binds to human GPC3 with high affinity. The

mechanism of the GC33-induced tumor growth inhibition is an antibody-dependent cellular cytotoxicity^[33]. A phase I study have already shown its tolerability at doses of 20 mg/kg/week, with little response^[34,35]. In a randomized placebo-controlled phase II clinical trial conducted subsequently in patients with advanced HCC previously treated, codrituzumab showed no clinical benefit in this population^[36].

On the other hand, a GPC3 peptide vaccine that induces peptide-reactive cytotoxic T lymphocytes (CTLs) has also been tested, with a good response in mice, where it has shown to be able to induce a durable regression in GPC3⁺ tumors^[37]. It has proven to be adequately tolerated in humans, maintaining radiological stability in most patients. In addition, the overall survival was significantly longer (12.2 months, 95% CI: 6.5-18.0) in patients with high CTL specific frequencies of GPC3 than in those with low frequencies (8.5 months, 95% CI: 3.7-13.1; $P = 0.033$)^[38]. These results have been subsequently confirmed, but further studies are needed because of the small sample size of these trials^[39].

Therefore, new therapies with GPC3 are being developed as a therapeutic target as well as a diagnostic marker, and new studies with a larger sample size are necessary. Finally, biomarkers can also be used to establish new treatment strategies, such as GPC3, but more studies and clinical trials to validate their response and to improve the prognosis are required.

SERPINB3

SERPINB3 (formerly known as squamous cell carcinoma antigen-1) is a Clade B Serine Protease Inhibitor physiologically found in the spinous and granular layers of normal squamous epithelium, such as tongue, lungs, uterus and others, while become overexpressed by neoplastic cells of these organs^[40]. Recent studies showed that an aberrant expression of this protein also extends to cancers of other origin such as HCC^[41]. In fact, while it is not detected in normal hepatocytes, its expression progressively increases during the progression of chronic liver disease and hepatic carcinogenesis.

Furthermore, it was recently confirmed that its expression correlates with that of TGF- β 1 and that in fact, SERPINB3 contributes to TGF β 1 overexpression and release^[42]. So, far from its antiprotease activity, and its biomarker possibilities, SERPINB3 was suggested to be an oncoprotein in as much as it protects the cells from apoptosis and induces epithelial-mesenchymal transition, cell invasiveness and proliferation^[43]. Lastly, it has been found that its overexpression induces chronic unfolded protein response and as a consequence, activation of NF- κ B and production of IL-6^[44]. Besides, a knock-down of SERPINB3 produces an inhibition of tumor growth^[45].

In terms of circulating biomarker, it has been described that natural IgMs bind to several tumor antigens and create immunocomplexes, that in this case, showed a better diagnostic performance than the biomarker itself^[46]. Accordingly, levels of circulating SCCA-IgM have been recently found to increase over time, being predictive of fibrosis progression in patients with chronic hepatitis^[47]. Furthermore, it has been demonstrated that HCV-infected cirrhotic patients with low levels of serum SCCA-IgM have a decreased risk of developing HCC^[48]. Eventually, Biasiolo *et al.* reported that SCCA-IgM, instead of AFP, was associated with the prediction of HCC-free survival in a prospective cohort^[44].

LIQUID BIOPSY OF HCC

“Liquid biopsies” are based on the analysis of tumor components that are shed into the circulation, such as tumor-derived extracellular vesicles, circulating tumor cells (CTCs) or circulating tumor DNA (ctDNA) [Figure 1]^[49,50]. Numerous studies have shown the potential utility of circulating cancer byproducts detection from which we could extract molecular information about primary tumors^[51-54]. The liquid biopsy could be conducted in repeated samples providing accessible, accurate and dynamic information to evaluate the tumor status. These novel biomarkers are thought to have great potential and could provide individualized

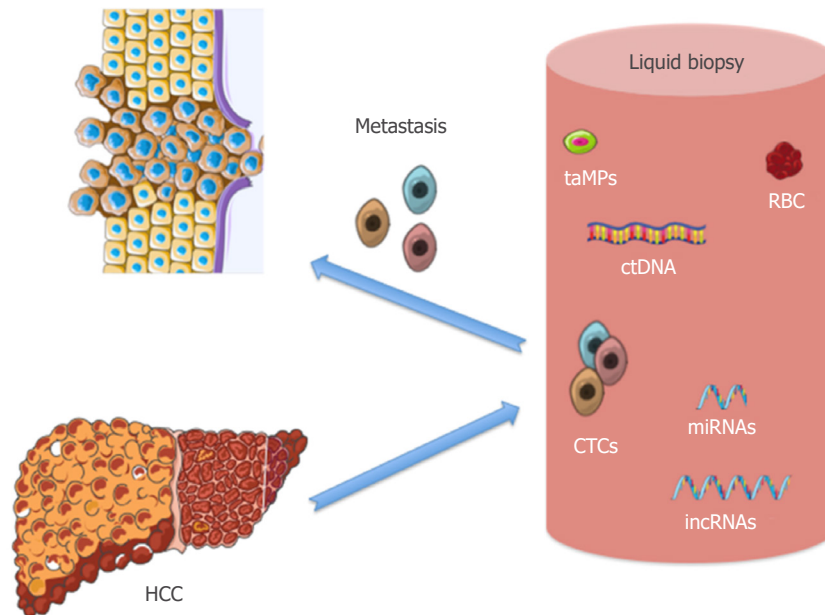


Figure 1. Liquid biopsy of hepatocellular carcinoma (HCC): circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), epigenetic non-coding RNA (miRNAs and lncRNA) and tumor-associated circulating microparticles (taMPs)

decision-making during HCC treatment, including the follow-up period; risk assessment, early cancer detection, treatment response or even prognostic outcome^[53-56].

CTCs

CTCs are spread by tumor malignant cells into peripheral blood in order to reach distal organs and eventually develop metastatic carcinoma^[54]. Several studies have analysed the role of CTCs as a marker to predict survival, recurrence or treatment response in different kinds of tumors^[53,57,58].

The presence of CTCs was reported for the first time in 1869 into the blood of a man with metastatic cancer^[59]. However, there are some limitations about the use of CTCs due to the incapacity in detecting these cells during the earlier stages of the disease (cells are proportional to tumor volume or aggressive biology behaviour; the larger the tumor, the higher the CTCs-positive rate in the peripheral blood). Unfortunately the frequency of finding CTC in blood is 1 to 10 in a background of millions of blood cells in patients with metastatic disease^[60]. In the last years, the major challenge for CTCs researchers has been to improve the sensitivity and specificity of CTCs purification in order to perform the molecular characterization of CTCs to ease the development of “accurate medicine”; a cancer management program.

The clinical relevance of CTCs detection in HCC patients has been deeply studied while CTCs isolation and enrichment technologies have emerged. The physical methods are based on the physical properties of CTCs such as size, density, migratory capacity and electric charge^[61]. The biological methods are focused on antigen-antibody binding against tumor specific biomarkers such as epithelial cell adhesion molecule (EpCAM), CD133, CD90 and human epidermal growth factor receptor2 (Her2) among others^[62,63]. EpCAM is the most common antigen used for the CTCs isolation; it is the only one clinically validated and approved by the FDA. However, its use has been controversial due to the epithelial-mesenchymal transition (EMT) process that is characterized by the decreasing of epithelial markers expression and the acquisition of mesenchymal profile^[64]. EpCAM^{mRNA+} CTCs enables to differentiate between HCC patients (advance and non-advance stage) and non-HCC with 42.6% sensitivity and 97% of specificity (AUC: 0.697). When combined with AFP level, the diagnostic value of CTCs was significantly improved and the AUC was 0.857 with a sensitivity of 73.0% and specificity of 93.4%^[65].

In addition, these authors showed that most of the patients with an elevated CTC level at the time of disease imaging reassessment showed disease progression after TACE or radiotherapy, whereas patients with stable or decreasing CTC levels showed tumor remission or stable disease^[65].

Due to this fact, researchers suggested a combination of antibodies against a variety of surface markers on CTCs in order to avoid the loss of CTCs during the isolation. For this reason, the CTC-chip, based on the microfluidic procedure with higher sensitivity and specificity in CTCs purification (99.1% and specificity 100% of the CTC-chip across all five cancers; metastatic lung, prostate, pancreatic, breast and colon cancer), is standing out intensely in this field^[66].

CTCs detection in HCC patients has been reported in several studies^[67,68]. The numbers of CTCs were closely correlated with portal vein thrombosis, tumor infiltration, prognosis and Child-Pugh grade^[61,64,69]. Most of the studies have shown that before liver resection or transplantation, tumor cells from the primary lesions were detached and threw into the blood being the early event of HCC metastases. Fan *et al*^[69,70] reported that the tumor recurrence after resection was associated with the number of CTCs detected, maybe because CTCs were still surviving into the blood^[70-72]. However, the role of CTCs and HCC recurrence require further investigations. The enumeration and characterization of CTCs may become an indispensable biomarker for monitoring the efficacy of HCC treatments however, clinical application of CTC assay in HCC remains in the initial stage, especially in the field of early diagnosis.

Cell-free tumor associated DNA

Circulating cell-free DNA (cfDNA) is defined as extracellular DNA present in plasma or serum samples. cfDNA is released into circulation from cells that undergo metabolic secretion, apoptosis or necrosis. Cells are phagocytized by macrophages releasing digested DNA into the circulating system. Tumor cells are considered to be the major source of tumor-related cfDNA in blood of cancer patients^[73,74]. cfDNA is also detected in healthy patients but in patients suffering from cancer cfDNA carries tumor-specific genetic or epigenetic alterations, such as mutations, copy number variations, chromosomal rearrangements or DNA methylations among others^[54]. Compared to tissue biopsy, circulating tumor DNA (ctDNA) may represent the entire molecular biology of the tumor and its qualitative and quantitative analysis might help to assess the biological characteristics of the tumor. Recently, several studies have demonstrated that ctDNA could be a non-invasive potential biomarker^[54,75]. ctDNA is highly specific and could be detected easier compared to CTCs purification, thus it could be an ideal source for the early diagnosis or as recurrence biomarker^[76,77].

To study the ctDNA in plasma or serum two strategies are implemented: (a) measuring the quantity or (b) detecting tumor - specific genetic aberrations.

Several studies have shown that HCC patients have large amounts of cfDNA being these associated with the degree of malignancy (poorer prognosis) and size of the tumor size^[77,78]. Huang *et al.*^[77] showed that plasma DNA detection was able to discriminate HCC from normal controls with 90.2% sensitivity and 90.3% specificity and AUC was 0.949 (95% CI) (measured by real-time quantitative PCR method). Moreover, plasma DNA and serum AFP revealed an elevated AUC of 0.974 with 95.1% sensitivity and 94.4% specificity in discriminating HCC from normal controls. Furthermore, the plasma DNA levels were positively associated with tumor size and vascular invasion ($P = 0.012$ and $P = 0.035$ respectively)^[77]. However further studies are needed due to the controversial results related to the methodology used.

Changes of DNA mutations could play an important role in the carcinogenesis process^[79-82]. By now, several studies confirmed that *TP53*, *EFGR*, *KRAS* and *APC* are genes with common tumor specific mutations. The proportion of HCC patients with detectable ctDNA varies wildly between studies. Tumor suppressive gene *TP53* mutations such as Ser249, were found present in 50% of HCC patients exposed to aflatoxin^[83]. However, Ser249 of *TP53*, one of the most reported mutations in HCC patients, was also detected in non-

cancer liver tissue of HCC, in plasma from healthy people, indicating that this mutation is not exclusive of HCC^[84,85]. In addition, rs894151 or rs12428080 were found significantly associated with a decreased overall survival and time to recurrence after liver transplantation^[86,87]. Using digital droplet PCR, ctDNA mutations in *TERT* promoter, *CTNNB1* and *TP53* could be detected in a higher rate (56%) compared to the use of NGS (20%). A recent pilot study of ultra-deep targeted sequencing of plasma DNA identifies driver mutations and it demonstrates how ultra-deep targeted sequencing of cfDNA in the plasma of HCC patients is a feasible, reliable and minimally invasive approach to interrogate HCC genetics and emerges as a promising tool for predictive biomarker development in HCC^[88].

DNA methylation is also an important epigenetic aberration found in ctDNA with a great application in the diagnosis, prognosis, and effective evaluation of HCC. Another tumor suppressor gene, Ras association domain family protein 1A (*RASSF1A*) was found hypermethylated in 93% HCC patients compared to healthy people being this frequency similar to the one found in *RASSF1A* hypermethylation in HCC tumor tissues^[89]. Moreover, the combination of several methylations has been postulated in order to improve the specificity and efficacy for the early diagnosis^[90]. The plasma methylation levels of *APC*, *GSTP1*, *RASSF1A*, and *SFRP1* were significantly higher in HCCs than those in normal or benign controls ($P < 0.05$). The combination of these four genes resulted in an increased AUC of 0.933 with 92.7% sensitivity and 81.9% specificity in discriminating HCC from normal control and *GSTP1* hypermethylation was significantly correlated with elevated serum AFP levels ($P = 0.026$).

Finally, the clinical significance of ctDNA as a diagnostic and predictive biomarker in HCC patients should be further evaluated. ctDNA may be quite low and therefore below the limit of detection, especially in early-stage and indolent tumors. The improvement of different technics such as digital PCR and sequencing technologies provide us an effective way for the discovery of additional ctDNA markers^[91,92].

Circulating non-coding RNA

In the context of liquid biopsy, non-coding RNA (ncRNA) is also included. The number of ncRNA genes is increasing due to the development of high-throughput RNA sequencing technology. They have a role in several physiological and pathological processes such as cancer. The main feature is their lack to codify for proteins. Depending on the length, ncRNAs could be classified into short or long ncRNAs with an arbitrary size cut-off at 200 bases of length, being the most known (a) the long-ncRNA (lncRNA) and (b) the microRNA (miRNA).

lncRNAs function take place by different molecular mechanisms such as interactions with DNA, RNA and proteins and can be classified into oncogenic or tumor suppressive genes^[93-98]. Besides lncRNAs, miRNAs are endogenous small RNAs molecules with 20-25 bases of length. They are involved in multiple activities of mammalian cells like lncRNA but its function is to regulate gene expression through their binding to the 3'UTR of mRNAs and consequently degradation or translational suppression of targeted gene transcript^[99]. Both lncRNAs and miRNAs are often deregulated in liver cancer and it has been reported the existence of circulating particle shape ncRNA in the peripheral blood. A group of ncRNA is packaged into small membrane vesicles called exosomes, binding to lipoprotein or other proteins in order to increase its stability^[100-103]. In fact, the possibility that circulating ncRNA could be useful as a biomarker in the context of HCC is raising. Interestingly, modified circulating levels of these RNAs were repeatedly found in HCC patients.

Circulating lncRNAs

lncRNAs have emerged as important regulators of gene expression in many types of cancer including HCC^[104,105]. Alterations in expression of several lncRNAs have been recently reported in HCC.

Serum levels of lncRNA-uc003wbd and lncRNA-AF085935 were found upregulated in HCC and HBV

patients compared to controls, showing that both lncRNAs could be potential biomarkers for HCC and HBV screening ($P < 0.001$). HCC patients compared with normal group showed an AUC value for lncRNA-uc003wbd: 0.86 (95% CI: 0.82-0.91) and for lncRNA-AF085935 was 0.96 (95% CI: 0.93-0.99). Authors suggest that both lncRNAs may serve as potential biomarkers for the detection of HCC and HBV^[106]. Long intergenic non-protein coding RNA 974 (Linc00974F-1) was increased in serum of HCC patients and it was useful as a tumor marker to improve the prognosis of HCC. The combination of Linc00974F-1 and CYFRA21-1 showed an AUC: 0.866, indicating a significant predictor of tumor growth and metastasis^[107]. In addition, SPRY4-IT1 expression was upregulated in the plasma from HCC patients suggesting this one to be a good diagnostic biomarker. Combination of SPRY4-IT1 and AFP (the cut-off value of AFP was at 200 ng/mL) possessed a moderate ability for discrimination between HCC patients and controls; the area was equal to 0.80^[108]. Highly upregulated in liver cancer (HULC) lncRNA has been implicated in the regulation of hepatoma cell proliferation, since it induces HCC cells to activate EMT and then promotes tumor progression and metastasis through the miR-200a/ZEB1 signalling pathway^[109]. Furthermore, HULC lncRNA was upregulated in the plasma of HCC patients compared to healthy controls (HULC was detected in 63% (19/30) of the HCC patients and 10% in the healthy control group (2/20) and with a positive correlation to Edmondson grades (the detection rates were 14%, 62%, and 100% for Edmondson grades I-II, II-III, and III-IV, respectively)^[110]. The lncRNA DANCR activates the Wnt pathway, one of the most important pathways responsible of HCC development^[111]. DANCR was up-regulated in tumor tissues and plasma of patients with HCC, and its expression was highly correlated with microvascular and liver capsule invasion of HCC. The results showed that AUC for plasma DANCR was 0.868 which was higher than that for AFP (AUC = 0.744) when differentiating patients with HCC from non-HCC patients^[112]. Besides these circulating lncRNAs, JPX, UCA1 and WRAP53 were found increased in HCC patients^[106,113,114] alone or in combination with other lncRNAs, miRNAs or serum biomarkers^[115]. Many reports have indicated that the deregulation of lncRNAs plays important roles in occurrence and progression of HCC however further studies are needed in order to use these as biomarkers.

Circulating miRNAs

Numerous studies have shown that circulating miRNAs are closely associated with tumor development and progression. In spite of these findings, miRNAs are considered good biomarkers for differentiating between HCC and healthy people.

For instance, miR-122 is a liver-specific miRNA whose role is to maintain the liver homeostasis. The loss of its expression contributes to the malignant phenotype of HCC cells and it has been described as the miRNA responsible to develop HCC in HCV infection^[116]. However, controversial results about miR-122 were reported due to the underlying aetiology and active ongoing necroinflammatory changes. miR-122 was found significantly downregulated in HBV-related HCC^[117] and Xu *et al.*^[118] found it increased in serum from patients with HCC and chronic hepatitis B together with miR-21 and miR-223. A positive linear correlation was present between serum ALT and serum miR-122 levels in mouse models of alcoholic liver disease ($r = 0.893$; $P < 0.001$)^[100] and it was postulated to be a key regulator of alpha-fetoprotein expression and it could influence the aggressiveness of the HCC in an *in vitro* model^[119]. Using panels of miRNAs may provide a high diagnostic accuracy of HCC regardless of the disease status, and it can also differentiate HCC from healthy controls and chronic liver injury^[120,121]. These were hsa-miR-206, hsa-miR-141-3p, hsa-miR-433-3p, hsa-miR-1228-5p, hsa-miR-199a-5p, hsa-miR-122-5p, hsa-miR-192-5p, and hsa-miR-26a-5p. The diagnostic accuracy using these miRNAs, as measured by AUC, was 0.665, 0.68, 0.607, 0.534, 0.609, 0.729, 0.69 and 0.677, respectively^[120]. Ali *et al.*^[121] showed that miR-122, miR-21 and miR-222 had the highest sensitivity and specificity, in discriminating HCC from healthy controls (miR-122: 94.3% and 92.9% respectively; miR-21: 80% and 92.9% respectively, and miR-222: 82.9% and 78.6%, respectively).

Another study demonstrated that serum miR-122, miR-885-5p, miR-221, miR-22 in association with AFP showed a high diagnostic accuracy for early detection of HCC in a cohort of cirrhotic patients (AUC = 0.982),

in the meantime that miR-122, miR-885-5p and miR-29b in association with AFP showed a high diagnostic accuracy for early detection of HCC in general population ($AUC = 1$)^[122]. The combination of lncRNA and miRNAs has been also studied. lncRNA-CTBP, miR-16-2, miR-21-5p and LAMP2 had high sensitivities (91%, 92.3%, 93.6% and 92.3% respectively) for discriminating HCC from healthy subjects and also from chronic hepatitis C patients (75%, 88.9%, 88.9% and 94.9% respectively)^[123]. miR-224 was highly expressed in HCC tissue and plasma, and after surgery the levels were normalized suggesting that miR-224 could reflect tumor dynamisms. There was an association between plasma miR-224 level, tumor size ($P = 0.0005$) and the incidence of recurrence ($P = 0.0027$). However no significant correlation were found with AFP serum levels^[124].

In addition, miR-21 was found upregulated in plasma from HCC patients compared to healthy volunteers. The combination of miR-21 and AFP increased its diagnostic value (more than 90%) suggesting its potential use as a biomarker of HCC diagnosis^[125]. A systematic review and meta-analysis concluded that circulating miRNAs, particularly miR-21 and miR-122 are promising biomarkers for the early diagnosis of HCC^[126]. In addition, miR-21 (oncogene) and miR-182 (tumor suppressor gene) were related with the development of metastasis^[127,128].

Due to the diversity showed in the results, numerous profiling studies are ongoing in order to report miRNA profiles based on sequencing microarrays to examine circulating miRNAs as HCC-associated biomarkers.

Tumor-associated circulating microparticles

Large cells membrane-derived extracellular vesicles (EVs), known as microparticles (MPs) and microvesicles (MVs), have been reported to play a role in the horizontal communication between cells^[129].

Hepatocytes secrete exosomes, MPs and MVs, and their production can change quantitatively and qualitatively in response to cellular stimulation and under different disease conditions^[130]. It was shown that tumors prepare their own tumor niches via the release of EVs including a possible suppression of the immune system and the activation of tumor neo-angiogenesis^[131]. MPs are between 100 and 1000 nm in size and bear on their surface the antigenic markers of the parent cell. They are formed and released during cellular activation or in early stages of apoptosis into the extracellular space. MPs can be isolated from whole blood, plasma and serum^[132].

Proteomic analysis revealed the presence of ~251 proteins in EVs derived from primary rat hepatocytes^[133]. Something that we have to be in account is that exosomes do not carry cell surface markers of their origin cells however MPs carry the surface signature of their cell of origin and the quantification of MP subsets using FACS sorting allows a non-invasive assessment of cell specific pathologies. Nowadays, there are many studies which focus is to identify the most efficient surface markers of tumor associated MPs (taMPs) and liver disease^[134-136].

A recent study showed that EpCAM and CD147 double positive taMPs could be a biomarker to compare colorectal carcinoma (CRC), non-small cell lung carcinoma (NSCLC) and pancreas carcinoma with healthy subjects. In all three types of tumor entities, EmCAM+CD147+ taMPs were found increased (AUROC: 0.8597, 0.8700 and 0.9000 respectively) indicating cancer presence. In addition, EpCAM+CD147+ taMPs were significantly correlating with CRC tumor volume ($r = 0.7288$, $P < 0.0001$). Furthermore, EpCAM+ taMPs were found decreased after tumor resection in serum of CRC patients suggesting a close dependence with tumor presence^[134]. They conclude that EpCAM+ and EpCAM+CD147+ taMPs might serve as an early indicator of cancer growth and monitor successful anti-tumour therapy and might be used as important liquid biopsy tool to differentiate between therapy responders and non-responders^[134].

Regarding HCC the role of circulating MPs as potential biomarkers is under intensive investigation. Abbate and colleagues showed that HepPar1-MPs are increased in the blood of subjects with HCC compared to

subjects with only liver cirrhosis or healthy livers ($P < 0.01$). An additional interesting finding of this study was the association between HepPar1+ MPs and the early recurrence of HCC after liver resection. HepPar1+ MPs, measured before liver resection, were significantly more numerous in the blood of subjects which displayed recurrence ($P = 0.021$)^[135].

Additionally, other study reported that MPs profiling for distinct MPs populations that are associated with chronic liver diseases robustly discriminates between chronic HCV infection and non-alcoholic fatty liver disease^[136]. Julich-Haertel *et al.*^[137] successfully differentiated HCC and cholangiocarcinoma (CCA) from chronic diseases without liver tumours base on MPs profile. AnnexinV+ EpCAM+ CD147+ taMPs were increased in HCC and CCA. Moreover, AnnexinV+ EpCAM+ ASGPR1+ taMPs allowed to differentiate between liver cancer (HCC or CCA) and cirrhosis from tumour-free individuals (sensitivity 75% and specificity 47%)^[137]. AnnexinV+ EpCAM+ ASGPR1+ taMPs were increased in liver cancer and decreased after liver resection indicating the powerful diagnostic accuracy ($P < 0.05$) and these MPs were correlated “moderately” with liver tumos diameters ($r = 0.56$, $P > 0.001$). However, no significant correlation between AFP levels, tumour diameter and AnnexinV+ EpCAM+ ASGPR1+ taMPs was found^[137].

The evidence about the hypothesis that taMPs populations could be used as a novel liquid biopsy tool to identify and discriminate liver tumours in patients with cirrhosis and their use as diagnostic and responder biomarkers need further studies.

CONCLUSIONS

In conclusion, nowadays the early diagnosis of HCC is difficult, despite being of vital importance for an adequate treatment and the consequent improvement of survival in these patients. However, no single biomarker represents an optimum sensitive and specific tool for this purpose.

Therefore, a study has been recently published in which several biomarkers (AFP, AFP-L3 and DCP) were combined to validate two statistical models for the early diagnosis and prognosis of HCC (GALAD and BALAD-2, respectively). Thus, GALAD discriminated patients with HCC from those with other hepatobiliary cancers with an area under the ROC curve (AUROC) value of 0.95, lower in case of small unifocal HCC (0.85-0.95). On the other hand, BALAD-2 established 4 different groups depending on the prognosis^[138]. In addition, there are many other biomarkers that are under study to check their utility in the management of this disease, such as golgi protein-73, osteopontin, soluble urokinase plasminogen receptor activator, *etc.*

The utility of the current blood molecular biomarkers included in the context of liquid biopsy, are promising as diagnostic, therapeutic and/or prognostic markers for HCC. Regarding this, a liquid biopsy could give us information about the genetics and epigenetics alterations present in the tumor showing great advantages compared to tissue biopsies; it is a non-invasive method to determine the molecular biology of the tumor as well as the feasibility of taking samples in order to monitorize the tumor state in real time. However, due to the lack of standardized technical approach, data is quite different among various studies. With the standardization of effective methods, liquid biopsy biomarkers alone or in combination with conventional serum biomarkers might serve as promising diagnostic, prognostic, therapeutic monitoring and risk assessment of HCC.

DECLARATIONS

Authors' contributions

Substantial contributions to the conception, the acquisition, analysis, and interpretation of data: Rojas Á, Sánchez-Torrijos Y, Gil-Gómez A, Liu CH, Rodríguez-Rivas C

Conception and design of the work: Rojas Á, Romero-Gómez M

Final approval of the version to be published: Ferrer MT, Romero-Gómez M

Availability of data and materials

Not applicable.

Financial support and sponsorship

The National Institutes of Health, Institute of Health Carlos III, ISCIII (PI16/01842 to Dr. Romero-Gómez); The Andalusian Health Service - Junta de Andalucía (PC-033-2017 to Dra. Rojas).

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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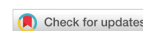
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Review

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The multifaceted oncogene SND1 in cancer: focus on hepatocellular carcinoma

Saranya Chidambaranathan-Reghupaty¹, Rachel Mendoza¹, Paul B. Fisher^{1,2,3}, Devanand Sarkar^{1,2,3}

¹Department of Human and Molecular Genetics, Virginia Commonwealth University, Richmond, VA 23298, USA.

²Massey Cancer Center, Virginia Commonwealth University, Richmond, VA 23298, USA.

³VCU Institute of Molecular Medicine, Virginia Commonwealth University, Richmond, VA 23298, USA.

Correspondence to: Dr. Devanand Sarkar, 1220 E Broad St, PO Box 980035, Richmond, VA 23298, USA.

E-mail: devanand.sarkar@vcuhealth.org

How to cite this article: Chidambaranathan-Reghupaty S, Mendoza R, Fisher PB, Sarkar D. The multifaceted oncogene SND1 in cancer: focus on hepatocellular carcinoma. *Hepatoma Res* 2018;4:32. <http://dx.doi.org/10.20517/2394-5079.2018.34>

Received: 2 Apr 2018 **First Decision:** 11 Jun 2018 **Revised:** 19 Jun 2018 **Accepted:** 19 Jun 2018 **Published:** 10 Jul 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Staphylococcal nuclease and tudor domain containing 1 (SND1) is a protein that regulates a complex array of functions. It controls gene expression through transcriptional activation, mRNA degradation, mRNA stabilization, ubiquitination and alternative splicing. More than two decades of research has accumulated evidence of the role of SND1 as an oncogene in various cancers. It is a promoter of cancer hallmarks like proliferation, invasion, migration, angiogenesis and metastasis. In addition to these functions, it has a role in lipid metabolism, inflammation and stress response. The participation of SND1 in such varied functions makes it distinct from most oncogenes that are relatively more focused in their role. This becomes important in the case of hepatocellular carcinoma (HCC) since in addition to typical cancer drivers, factors like lipid metabolism deregulation and chronic inflammation can predispose hepatocytes to HCC. The objective of this review is to provide a summary of the current knowledge available on SND1, specifically in relation to HCC and to shed light on its prospect as a therapeutic target.

Keywords: Staphylococcal nuclease and tudor domain containing 1, hepatocellular carcinoma, inflammation

INTRODUCTION

Hepatocellular carcinoma (HCC) is the primary liver malignancy arising from hepatocytes. It is the fifth common cancer in men and the ninth common cancer in women. It is the second leading cause of cancer-related deaths worldwide. A high mortality to incidence ratio of 0.95 reflects its poor prognosis and makes it an important public health burden (Globocan 2012). The main causes of HCC are viral infections like hepatitis B and hepatitis C, chronic alcoholism, obesity, liver cirrhosis and non-alcoholic steatohepatitis



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(NASH)^[1]. Treatment options are restricted to liver transplantation, surgical resection and ablation. Chemotherapy for HCC is not very promising. HCC incidence has almost tripled since the 1980s and it is the fastest rising cause of cancer related deaths in the US^[2]. Increase in rates of obesity and non-alcoholic fatty liver disease (NAFLD) is an important factor for this trend.

HCC is usually diagnosed at advanced stages. Unfortunately, patients with advanced HCC do not have the option of treatments like liver transplant or surgical resection since the liver is damaged beyond rescue at this stage. Advanced HCC is also resistant to standard chemo- and radiotherapy. Sorafenib, regorafenib and nivolumab are the three FDA approved chemotherapy drugs for advanced HCC. The multi-kinase inhibitor sorafenib was approved in 2007 and the SHARP trial showed that it increases overall survival of HCC patients from 7.9 to 10.7 months^[3]. Regorafenib, a sorafenib analog, was approved in 2016 and increases overall survival from 7.8 to 10.6 months^[4]. Nivolumab, an immune oncology agent that blocks programmed cell death 1 (PD1), a negative regulator of T-cell activation and response, thus allowing the immune system to attack the tumor, was approved in 2017 for patients who have been previously treated with sorafenib contingent on a successful phase III trial^[5]. Most of these drugs are expensive, effective in only a small percentage of treated patients, cause side effects and do not provide a promising increase in survival^[6]. Nivolumab increases overall survival to 13.2 months and has a more durable response^[7]. But, it is administered intravenously every two weeks and has the same demerits as the other chemotherapy drugs. The limitations of the current available treatment options mandate identification of new regulators of HCC that might be targeted to develop effective therapy.

STAPHYLOCOCCAL NUCLEASE AND TUDOR DOMAIN CONTAINING 1: A MULTIFUNCTIONAL ONCOGENE

Structure and activation

Human staphylococcal nuclease and tudor domain containing 1 (SND1) gene is located at chromosome 7q31.3 and codes for a protein of 910 amino acids with five highly conserved domains. It has a tandem repeat of four staphylococcal nuclease (SN) domains and a fifth fusion domain of a tudor and a partial SN domain [Figure 1A]. SND1 was first identified as a transcription co-activator that interacts with Epstein-Barr nuclear antigen 2 (EBNA2) in lymphocytes^[8]. It acts as a bridge between the subunits p56 and p34 of the general transcription factor TFIIE and the acidic domain of EBNA2^[9]. SND1 is an evolutionarily conserved protein in all eukaryotes from protozoa to humans except budding yeast *saccharomyces cerevisiae*^[10-12]. The upstream regulators of SND1 include the transcription factors NF- κ B, NF-Y, Sp1 and SREBP-2 [Figure 1B]. A CpG island with several Sp1 binding sites and an inverted CCAAT box binding to NF-Y regulate basal expression of SND1^[13-15]. NF- κ B binding site is located within the proximal 300 bp segment of SND1 promoter and confers TNF α -mediated induction of SND1^[13] [Figure 1B]. SREBP-2 binds to a proximal promoter region containing a serum response element and an enhancer box motif and induces SND1 expression upon cholesterol depletion^[16]. Activated Smad2 and Smad3 bind to SND1 promoter and confer TGF β -mediated induction of SND1 expression^[17] [Figure 1B].

Multifaceted properties

Staphylococcal nuclease protects bacteria from invading viruses by degrading viral nucleic acids. In higher organisms, repeats of SN domains and the addition of the tudor domain has created a multifunctional protein in SND1 especially with its ability to interact with a diverse array of proteins. SND1 is involved in regulating gene expression by transcriptional activation^[18-20], alternative splicing^[21], ubiquitination^[17], mRNA stabilization^[22] and RNA interference^[23]. These multifaceted properties allow SND1 to positively impact all hallmarks of cancer, notably sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis^[24,25].

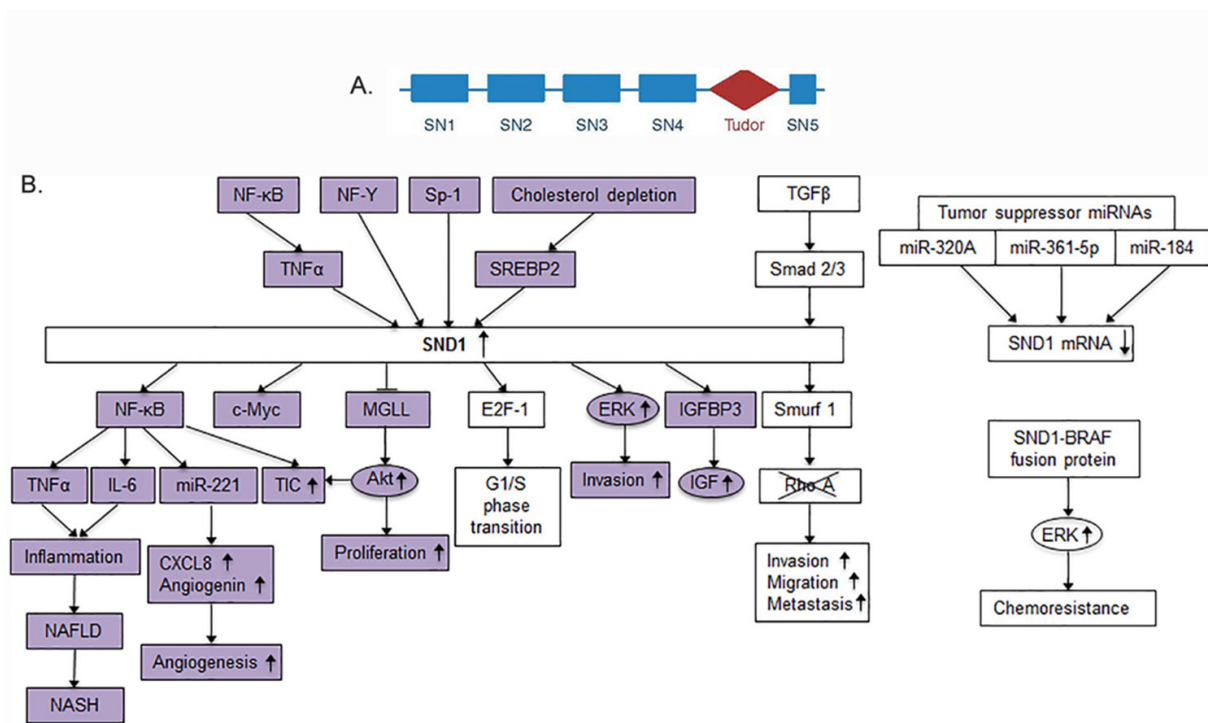


Figure 1. Upstream regulators of SND1 and downstream molecules involved in SND1 activity. A: Structure of SND1 protein; B: schematic overview of the upstream regulators of SND1 and downstream mediators of SND1 activity. Colored molecules indicate those that have been identified in HCC studies. SN: staphylococcal nuclease domains; SND1: staphylococcal nuclease and tudor domain containing 1; HCC: hepatocellular carcinoma

Downstream regulators and oncogenic mechanisms

SND1 interacts with and functions as a co-activator for a number of transcription factors that include signal transducer and activator of transcription 5 (STAT5)^[19], STAT6^[19,26], peroxisome proliferator activated receptor gamma (PPARγ)^[27] and c-Myb^[20]. It functions as a co-activator for the transcription factor E2F-1 facilitating G1/S phase transition^[28]. SND1 induces the E3 ubiquitin ligase Smurf1 resulting in ubiquitination and degradation of RhoA and promotion of invasion, migration and metastasis^[17]. SND1 interacts with the U5 spliceosomal RNA to assemble the spliceosome, affecting the levels of various splice variants, such as generation of a variable form of CD44 that promotes motility and invasiveness of prostate cancer cells^[21,29]. It is a subunit of the RNA-induced silencing complex (RISC) in *caenorhabditis elegans*, drosophila and mammals and functions in miRNA-directed mRNA degradation^[23]. SND1 is also involved in mature miRNA decay. Knocking out SND1 inhibits cell cycle progression by upregulating a cohort of miRNAs that downregulate mRNAs encoding proteins critical for the G1/S phase transition^[30]. In parallel to degrading mRNA or miRNA, SND1 shows the ability to bind to 3'-UTR of specific mRNA and increase its stability^[22]. Transcriptional activation of oncogenes, over-expression of oncogenic splice variants through alternative splicing, degradation of tumor suppressor proteins and silencing of tumor suppressor mRNAs are some of the means used by SND1 to contribute to tumorigenesis [Figure 2]. Given its role in regulating a wide variety of cellular properties, it comes as no surprise that SND1 functions as an oncogene in a variety of cancers, including breast, liver, lung, gastric, glial, prostate and colorectal cancer^[25]. Although the molecular mechanism by which SND1 is overexpressed in cancer is not clear, it has been identified as a target of a number of tumor suppressor miRNAs, such as microRNA-320a in lung cancer^[31], microRNA-361-5p in colorectal and gastric cancer^[32], and miRNA-184 in malignant glioma^[33] [Figure 1B]. SND1 can be activated by TGF-β1 and in turn activate Smurf1 to promote breast cancer metastasis^[34]. An SND1-BRAF fusion protein has been identified in gastric, pancreatic and lung cancers that results in activation of downstream MAPK signaling and confers resistance to chemotherapeutic drugs^[35-37] [Figure 1B].

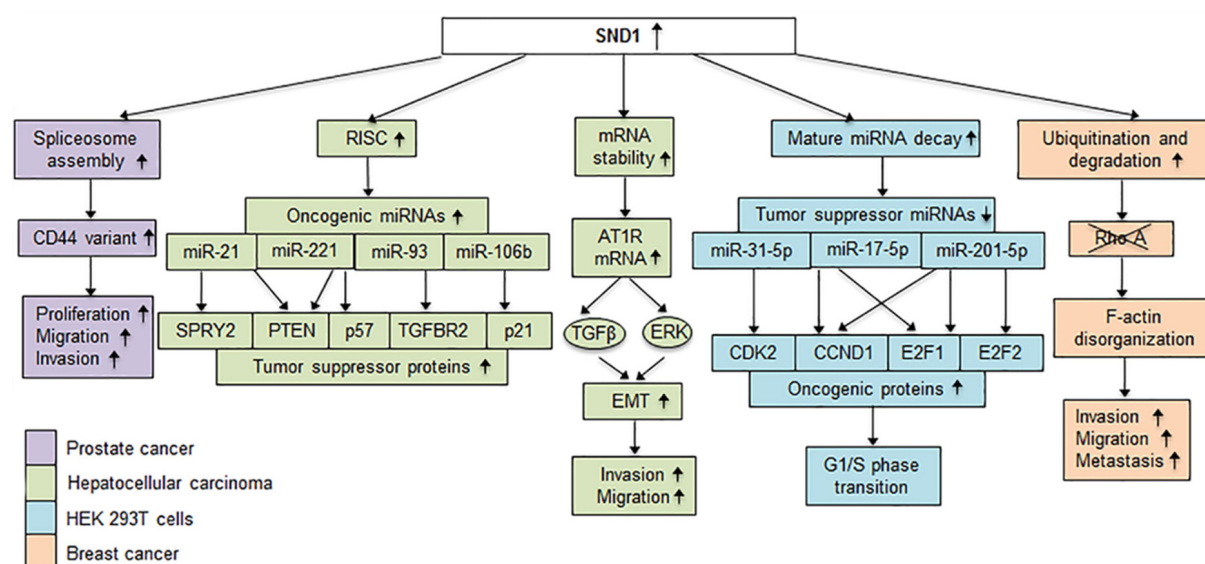


Figure 2. Mechanisms by which SND1 promotes oncogenesis. Downstream molecules that are upregulated, downregulated or degraded due to over expression of SND1 causing a variety of functions to go into disarray leading to tumorigenesis. Each color represents the specific cancer in which the mechanism has been studied. In prostate cancer regulation of spliceosome assembly by SND1 results in the production of an oncogenic variant of CD44 that promotes proliferation, motility and invasion. Tumor suppressor mRNAs that are targets of oncogenic miRNAs are degraded when SND1 over expression confers increased RISC activity in human HCC cells. SND1 increases AT1R mRNA stability, causing an increase in AT1R levels resulting in activation of ERK and TGF β signaling pathway, promoting EMT and migration and invasion by human HCC cells. SND1 mediates endonucleolytic decay of tumor suppressor miRNAs in HEK293T cells promoting upregulation of oncogenic proteins. In breast cancer cells, SND1 promotes expression of the E3 ubiquitin ligase Smurf1, leading to RhoA ubiquitination and degradation, disrupting F-actin cytoskeletal organization, increasing cell migration and invasion, and promoting metastasis. SND1: staphylococcal nuclease and tudor domain containing 1; HCC: hepatocellular carcinoma

SND1 AS AN ONCOGENE FOR HCC

In vitro and *in vivo* studies show that SND1 is an oncogene for HCC. Immunohistochemistry in tissue microarrays containing HCC and adjacent normal liver samples revealed that SND1 is over-expressed in a large percentage (~74%) of HCC patients^[38]. Chronic inflammation is a critical event in HCC pathogenesis and induction by inflammatory cytokines might underlie the overexpression of SND1 in human HCC patients. Overexpression and knockdown studies in human HCC cells have demonstrated that SND1 promotes proliferation, migration, invasion and *in vivo* tumorigenesis^[38-41]. As a component of the RISC in HCC cells, SND1 promotes oncogenic miRNA-mediated degradation of tumor suppressor mRNAs^[38] [Figure 2]. Some of the mRNAs degraded are PTEN, p57, p21, SPRY2 and TGFBR2 that are targets of miR-221 and miR-21, miR-221, miR-106b, miR-21 and miR-93, respectively^[38]. These miRNAs are known to be overexpressed in HCC and function as oncogenes. It should be noted that the primary nuclease in the RISC is the argonaute proteins and although a specific small molecule inhibitor of SND1 could partially block RISC activity, SND1 may not be the primary endonuclease in the RISC^[23]. However, when overexpressed, SND1 could significantly augment RISC activity in human HCC cells when compared to normal hepatocytes^[38].

By binding to and stabilizing angiotensin II type 1 receptor (AT1R) mRNA, SND1 activates TGF β and ERK signaling, thereby promoting epithelial-mesenchymal transition (EMT), *in vitro* migration and invasion by HCC cells^[39]. In HCC cells, SND1 activates NF- κ B, resulting in induction of miR-221 and angiogenic factors angiogenin and CXCL16 that promote tumor angiogenesis^[40]. Monoglyceride lipase (MGLL) inhibits Akt activation and SND1 interacts with and induces degradation of MGLL, resulting in activation of Akt and subsequent augmentation of cell proliferation and cell cycle progression by human HCC cells^[41]. SND1 downregulates IGFBP3 expression in human HCC cells that might result in activation of insulin-like growth factor (IGF) signaling, a frequent event in human hepatocarcinogenesis^[42].

In vivo studies with hepatocyte specific SND1 over-expressing mice (Alb/SND1) showed that transgenic animals have a higher incidence of spontaneous tumors, an increase in CD133+, CD44+ and EpCAM+ tumor initiating cells (TICs) and an increase in HCC drivers (c-Myc, TNF α and IL-6)^[43]. Upon treatment with a liver carcinogen, diethylnitrosamine (DEN), Alb/SND1 mice showed robustly aggressive tumor response and an increased expression of HCC (AFP and CD36), angiogenesis (CD31) and proliferation (PCNA) markers. Mechanistically, SND1 overexpression activates Akt, ERK, and NF- κ B signaling. Inhibitor studies unraveled roles of Akt and NF- κ B signaling in regulating SND1-induced increase in TIC while ERK pathway was shown to regulate SND1-induced invasion [Figure 2]. A small molecule inhibitor of SND1, 3', 5'-deoxythymidine bisphosphate (pdTp), significantly inhibited growth of orthotopic xenografts of human HCC cells in nude mice accompanied by decrease in markers of TIC and inflammation, thereby confirming SND1 as a potential therapeutic target for HCC and utility of pdTp as a therapeutic agent^[43].

SND1 AND INFLAMMATION

HCC initiation and progression are multistep processes. More than 90% of HCCs arise with hepatic injury and chronic inflammation in the background^[44]. Inflammation is also a hallmark of NASH, a growing public health concern and a major cause of HCC^[45,46]. Hepatic injury from viral infections, alcohol or high fat diet can cause cell death and the release of molecules called damage associated molecular patterns (DAMPs) that start the inflammatory cascade as a wound-healing response. The transcription factor NF- κ B, regulating a diverse array of pro-inflammatory cytokines, chemokines and adhesion molecules, is the single most important molecule causing inflammation. Overexpression of SND1, either in HCC cell lines or in Alb/SND1 mice, resulted in marked activation of NF- κ B, and Alb/SND1 mice presented with increased levels of pro-inflammatory cytokines, such as IL-6 and TNF α , thereby providing a link between SND1 and inflammation^[40,43]. On the other hand, SND1 itself is regulated by NF- κ B^[13]. Thus a vicious cycle exists where SND1 augments inflammation and the inflammatory process in turn induces SND1 that might cause predisposition to the development of HCC. As yet, the molecular mechanism by which SND1 activates NF- κ B remains to be determined. In primary hepatocytes, inhibition of SND1 activity by pdTp not only abrogated LPS-induced nuclear translocation of p65 subunit of NF- κ B but also reduced the level of total p65^[43]. This finding was also observed in human HCC xenografts in nude mice that were treated with pdTp^[43]. These findings suggest that as a transcriptional coactivator SND1 might be involved in regulating the expression of p65 itself, a hypothesis that needs to be interrogated.

SND1 AND STRESS RESPONSE

Under normal physiology, cells respond to stress by activating survival pathways to overcome stress or cell death pathways to eliminate damaged cells. A number of factors determine how cells choose between these two responses, and in the context of cancer a variety of proteins promote cell survival rather than cell death to augment tumorigenesis. SND1 seems to have a role in this stress-induced pro-survival signaling. Cells respond to conditions like oxidative stress, heat shock, viral infection, UV irradiation, DNA damage and hyperosmotic stress by forming stress granules (SGs) that are dense aggregations of translation-stalled mRNAs bound to messenger ribonucleoproteins (mRNPs) in the cytosol. Cancer cells use stress granules as a means to promote survival under adverse conditions of the tumor microenvironment. SND1 was identified as a component of cytoplasmic stress granules formed in response to oxidative stress^[47]. Ras GTPase activating protein SH3 domain binding protein (G3BP) is a phosphorylation dependent endoribonuclease that assembles stress granules and potentially degrades the SG mRNAs. Under oxidative stress, c-JNK phosphorylates SND1 at threonine 103, promoting the binding of its SN domain with G3BP to form stress granules^[48]. It is not yet clear if the role of SND1 is limited to assembling these SGs or extends beyond that where the endonuclease activity of SND1 participates in degrading SG mRNAs.

Unfolded protein response or endoplasmic reticulum (ER) stress plays an important role in regulating NASH and NASH-induced HCC^[49]. ER stress can be simulated *in vitro* by exposing cells to thapsigargin,

tunicamycin or ectopic expression of activating transcription factor 6 (ATF6), a crucial transcription factor in the unfolded protein response triggered by ER stress. Simulating ER stress in human liver cancer results in an increase in SND1 promoter activity showing that SND1 has a role in ER stress response^[50]. However, the functional consequence of this observation is yet to be elucidated. In response to DNA damage, SND1 is recruited to the damage site by Poly ADP-ribose polymerase 1 (PARP-1), a DNA damage sensor^[51]. The accumulated SND1 recruits to the damage site ATP-dependent chromatin remodeler (ARCA5) and histone acetyltransferase (GCN5), two enzymes that promote chromatin relaxation to enable access of DNA damage response related proteins to the damage site. This results in chromatin relaxation and consequent activation of ATM kinase and downstream DNA repair signaling pathways. Thus SND1 functions as a key determinant providing survival advantage under DNA damage stress.

ROLE OF SND1 IN LIPID METABOLISM

One of the most important metabolic alterations that occur during tumor development is the deregulation of lipid metabolism. Specifically, lipid biosynthesis rate is increased to provide a survival advantage for tumors. Lipids act as signaling molecules, disrupt normal tissue architecture, promote tumor migration and induce angiogenesis^[52]. Increased lipid synthesis causes steatogenesis or lipid accumulation, a common feature in carcinomas. In HCC, it is reflected by the formation of cytosolic organelles called lipid droplets (LDs) comprised of a core of neutral lipids coated by amphipathic lipids and associated proteins^[53]. The role of SND1 in lipid metabolism was evidenced when it was found on the surface of LDs originating from the ER in mammary epithelial cells and adipocytes^[54]. SND1 interacts with a lipoprotein part of the fatty acid synthase (FASN) complex to form LDs. Under steatogenic conditions, SND1 is targeted from cell compartments like the ER and golgi complex to low density LDs to facilitate their assembly^[55].

In addition to lipid storage, SND1 is involved in lipid transport. Once fatty acids are taken up from dietary sources or synthesized in the liver, they are transported to other locations in the body to serve energy demands. Hepatocytes use lipoproteins made up of a non-polar lipid core surrounded by apolipoproteins and amphipathic lipids like phospholipids and cholesterol for this purpose. Though they are structurally similar to LDs, their main function is lipid transport rather than storage. Overexpression of SND1 promotes the secretion of phospholipids that form a part of the lipoproteins in primary hepatocytes and facilitates the transfer of these phospholipids to apolipoproteins before their secretion from hepatocytes^[56]. Cholesterol is another component of the lipid core in both LDs and lipoproteins, the synthesis of which is regulated by SND1. Under conditions of cholesterol depletion, SREBP2, a regulator of cholesterol uptake and synthesis activates SND1^[16]. Overexpression of SND1 results in increased cholesterogenesis, metabolically coupled to cholesterol esterification, causing an increase in cholesteryl ester levels^[57].

Glycerolipids are lipids composed of mono, di- or tri- substituted glycerol moieties that are important constituents of biological membranes. Rapid synthesis of lipids is required for generation of biological membranes and facilitating cancer cell proliferation. SND1 induction with TNF α and subsequent profiling of SND1 promoter activity revealed that SND1 regulates a group of glycerolipid metabolic genes including CHPT1, LPGAT1, PTDSS1 and LPIN1 that are involved in biosynthesis of phosphatidylcholine, phosphatidylglycerol, phosphatidylserine and triacylglycerol respectively^[58]. SND1 interacts with and inhibits monoglycerolipid lipase (MGLL)^[41], a tumor suppressor that converts monoglycerolipids to glycerols and free fatty acids. Thus, SND1 causes an increase in glycerolipid levels in cells by causing an increase in their synthesis or preventing their catabolism in hepatocytes [Figure 3].

CONCLUSION

HCC is unique in having defined etiologies, all of which cause chronic inflammation. In addition, altered lipid metabolism in obesity-associated NASH is becoming a major driving force for HCC. It is intriguing

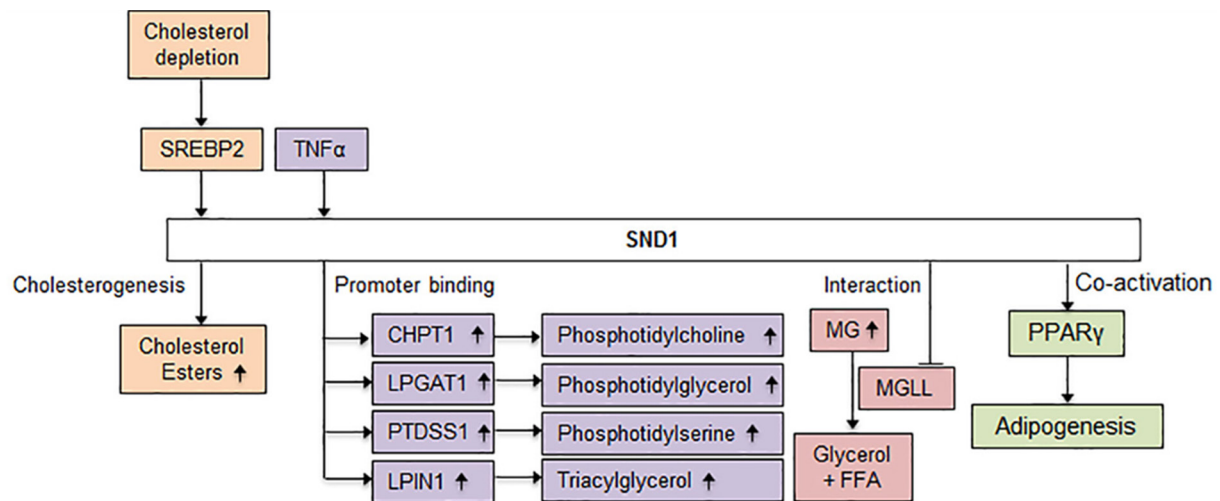


Figure 3. Possible role of SND1 in lipid metabolism. Exposure of human HCC cells to cholesterol-lowering drug or a lipoprotein-deficient medium triggers SREBP2 activation and increases SND1 promoter activity. Studies in rat hepatoma cells show that SND1 overexpression accumulates de novo synthesized cholesteryl esters. SND1 is induced by TNF α and subsequent profiling in human hepatoma cells revealed that SND1 binds to the promoter regions of a group of glycerolipid metabolic genes including CHPT1, LPGAT1, PTDSS1 and LPIN1 involved in the biosynthesis of phosphatidylcholine, phosphatidylglycerol, phosphatidylserine and triacylglycerol, respectively. As yet functional consequence of SND1 binding to the promoter of these genes has not been studied. In human HCC cells SND1 interacts with MGLL and results in ubiquitination and proteosomal degradation of MGLL. The increase in monoglyceride (MG) levels is predicted from the known role of MGLL. Studies in mouse adipocytes have shown that SND1 is a co-activator of PPAR γ in adipogenesis. SND1: staphylococcal nuclease and tudor domain containing 1; HCC: hepatocellular carcinoma

that SND1 plays a role in regulating both inflammation and lipid metabolism, and also the hallmarks of cancer by a variety of mechanisms, suggesting that targeting SND1 might be a viable option for HCC. This notion is strengthened by the observation that Alb/SND1 mice develop spontaneous HCC, thus establishing SND1 as a tumor driver^[43]. SND1 is the only eukaryotic protein with a tudor and SN domains and the quaternary fold can be employed to obtain specific small molecule inhibitors, such as pdTp. The efficacy of pdTp in inhibiting growth of HCC xenografts *in vivo* is exciting and promising. However, this inhibitor is required in high doses to inhibit SND1 and inhibits only the nuclease function and not the nucleic acid binding function. Thus, it is important to identify better analogs of pdTP and develop strategies that can achieve complete inhibition of SND1. Recent success of hepatocyte-specific nanoparticle-delivered siRNA targeting oncogenes in HCC opens up potential of such strategy to inhibit SND1. Genetic deletion studies *in vivo* would provide a clue to the effects such inhibitors could produce. Further in-depth studies using *in vitro* and *in vivo* models are required to better understand the functional attributes of this pleiotropic molecule so that it is efficiently targeted.

DECLARATIONS

Authors' contributions

Wrote the manuscript: Chidambaranathan-Reghupaty S, Sarkar D

Edited the manuscript: Mendoza R, Fisher PB

Availability of data and materials

Not applicable.

Financial support and sponsorship

The present study was supported in part by The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant 1R01DK107451-01A1 (D. Sarkar).

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Living donor liver transplantation for patients with hepatocellular carcinoma in Japan

Yasuhiko Sugawara, Hidekazu Yamamoto, Taizo Hibi

Department of Transplantation/Pediatric Surgery, Postgraduate School of Life Science, Kumamoto University, Kumamoto 8603-8556, Japan.

Correspondence to: Dr. Yasuhiko Sugawara, Department of Transplantation/Pediatric Surgery, Postgraduate School of Life Science, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto 8603-8556, Japan. E-mail: yasusuga-ty@umin.ac.jp

How to cite this article: Sugawara Y, Yamamoto H, Hibi T. Living donor liver transplantation for patients with hepatocellular carcinoma in Japan. *Hepatoma Res* 2018;4:33. <http://dx.doi.org/10.20517/2394-5079.2018.69>

Received: 25 May 2018 **First Decision:** 12 Jun 2018 **Revised:** 29 Jun 2018 **Accepted:** 3 Jul 2018 **Published:** 10 Jul 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Liver transplantation has now been an established treatment for hepatocellular carcinoma and cirrhosis. The Milan criteria have been accepted and applied widely in the world as an indication for deceased donor liver transplant. Due to the severe organ shortage, however, living donor liver transplant (LDLT) has accounted for the majority of transplantations in Japan and the other Asian countries/regions. LDLT cannot be limited by the restrictions imposed by the allocation system but depends on institutional criterion or case-by-case considerations. Accumulating data from a nationwide survey and each center experience have indicated that extending the Milan criteria is warranted.

Keywords: Liver transplantation, living donor, hepatocellular carcinoma

INTRODUCTION

Liver transplantation has now become a standard therapy for patients with hepatocellular carcinoma (HCC) in the early-stage^[1]. Liver transplantation can treat both the tumors and the underlying liver disease. Therefore patients who receive transplants theoretically have higher chance of cure than the other treatments for HCC^[2].

Early outcome^[3] following liver transplantation was poor, associated with high incidence of HCC recurrence after transplantation. However, Mazzaferro *et al.*^[4] proposed the criteria to restrict liver transplantation to only those patients with HCC of a single tumor ≤ 5 cm or two or three tumors ≤ 3 cm without major vessel invasion or extrahepatic tumor spread based on the imagings. They showed a 4-year patient survival of 75%



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and a recurrence-free survival of 83% of the patients who meet these criteria^[4]. Many centers worldwide have now adopted the criteria for deceased donor liver transplantation and also living donor liver transplantation (LDLT). However, the criteria have been sometimes estimated as being too strict to include many patients in the transplant list^[5].

In Asian countries/regions, unlike the Western countries, LDLT has accounted for the majority of transplantations^[6,7]. LDLT can be thought to be a private issue among the patients and the families. Therefore the selection criteria of the patients from the view point of tumor status can be considered on a case-by-case basis. Also the grafts are not always restricted by the system of the public organ allocation. It should be taken into account how high the recurrence rate and the chance of survival are and how firm the will to donate the part of the liver is. Many high-volume transplantation centers have performed LDLT for patients with HCC based on the criteria extending Milan^[8] to include patients with slightly larger tumors as transplant candidates and such an expansion of criteria did not result in a significantly higher rate of disease recurrence after transplantation. The review described the current status of liver transplantation for HCC in Japan and the other Asian countries/regions.

JAPANESE EXPERIENCE

In Japan, the serious shortage of deceased donor livers has still continued despite the approval of the Japanese Organ Transplantation Act in 1997 and its revision in 2006. According to a report from the Japanese Liver Transplantation Society Registry^[9], by the end of 2016, 378 liver transplantations were performed using deceased donor grafts while 8825 LDLTs were performed during the same period. Of these, 1598 were indicated for HCC. The 1-, 3-, 5-, 10-, 15- and 20-year survival rates of LDLT for HCC were 85%, 75%, 70%, 62%, 55%, and 54%, respectively.

The insuring system of the Japanese Ministry of Health, Labor, and Welfare covered the patients who undergo transplantation only when the tumor status is within the Milan criteria. The tumors should be diagnosed to be HCC by computed tomography or magnetic resonance imagings obtained within one month before transplantation. The tumors must be diagnosed on the dynamic computed tomography to be low density in plain, high in arterial phase, and low in portal phase. Local treatment for HCC must be done at least 3 months before transplantation is planned. Only the patients with tumors within the Milan criteria can be listed for and undergo deceased donor liver transplantation. In LDLT, however, many Japanese institutions have their own criteria beyond the Milan^[10].

A survey^[11] was done using a database consisting of the 653 patients who underwent LDLT for HCC in Japan between 1990 and 2005. On the preoperative imagings, 62% were within the Milan criteria while 38% were beyond. The overall patient survival was 83%, 73%, and 69%. The disease-free survival was 77%, 65%, and 61%, at 1, 3, and 5 years, respectively. The 5-year recurrence free survival was 90% and 61% for those within and beyond the Milan, respectively ($P < 0.001$). HCC recurred in the 92 (14%) recipients, with a rate at 1, 3, and 5 years of 9%, 20%, and 22%, respectively. The multivariate analysis revealed that preoperative alpha-feto-protein and des-gamma carboxyprothrombin (DCP) levels were independent factors for HCC recurrence.

Experience of each center

The Kyoto group^[12] proposed that the criteria should be “tumors ≤ 5 cm and the numbers are 10 or less than 10, and DCP levels < 400 mAU/mL”. One hundred ninety-eight patients underwent LDLT for HCC between 1999 and 2011. Of these, the 147 (76%) patients met the Milan criteria. The 5-year survival rate of those within the criteria was 82% and that of those beyond was 42% ($P < 0.001$). The 5-year recurrence rate for those within the Kyoto criteria was less than that for patients beyond them (4% vs. 51%, $P < 0.001$).

The principle criteria I have adopted for LDLT for HCC in University of Tokyo is “tumor numbers ≤ 5 cm and number of the tumors ≤ 5 ”^[13] (5-5 rule). Of the 125 HCC patients, 118 (94%) were within the 5-5 rule and 109 (87%) were within the Milan criteria. Overall survival was 88%, 82%, and 76% at 1, 3, and 5 years, respectively. Eleven patients (9%) developed the recurrence of HCC with a rate of 6%, 9%, and 11% at 1, 3, and 5 years, respectively. Multivariate analysis showed that the tumor status beyond the 5-5 rule, alpha-feto-protein level > 400 ng/mL, and DCP level > 200 mAU/mL were independent risk factors for recurrence of HCC.

The Kyushu University^[14] proposed the extended criteria which is “tumor size ≤ 5 cm (no restrictions on the numbers) and DCP level ≤ 300 mAU/mL”. One hundred nine HCC patients underwent LDLT. Of these, 103 patients (94%) were within the criteria while 55 (50%) met the Milan criteria. The 5-year recurrence free survival of the patients who met the criteria was 71%, while all the 6 patients beyond the criteria developed recurrence of HCC within 2 years after transplantation. Totally 90 patients within the criteria were prospectively analyzed^[15]. The 5-year recurrence-free survival of the within-Milan and that of beyond were 90% and 80%, respectively with no significant difference ($P = 0.22$).

LDLT FOR HCC IN ASIAN COUNTRIES OTHER THAN JAPAN

In Asian countries/regions other than Japan, the majority of liver transplantation for HCC patients are also LDLT^[6]. Apart from the predominance of hepatitis B related HCC^[8,16], therefore, the situation in other Asian countries/regions is similar to that in Japan. The Taiwan group adopted the Milan criteria in LDLT^[17]. The 1-, 3-, and 5-year survivals were 98%, 96%, and 90%, respectively. The Asan medical center in South Korea^[18], like Japanese institutions, advocates their own criteria, stressing “the tumor numbers ≤ 6 and the maximum diameter of the tumor size ≤ 5 cm”. The overall 5-year patient survival rates were 76.3%. The Hong Kong group^[19] has changed the criteria. Before 2002, the radiological Milan criteria were used. From 2002 till 2005, the selection criteria were expanded to match the radiological University of California, San Francisco criteria (1 tumor ≤ 6.5 cm, or 2-3 tumors ≤ 4.5 cm and total tumor diameter ≤ 8 cm). From 2006 onwards, the selected patients with more advanced HCC were enrolled for LDLT according to the following exclusion criteria: (1) no evidence of gross vascular tumor invasion, (2) no evidence of distant metastases and (3) no evidence of diffuse HCC.

Notably, most expanded criteria in the Asian countries/regions restrict the tumor ≤ 5 cm as the indication for LDLT. In contrast there is a large discrepancy regarding the limitation for the numbers. Previous studies^[20] indicated that tumors > 5 cm have a high recurrence rate after transplantation. There may be an association between tumor size, vascular invasion and poor differentiation. Microscopic vascular invasion was present in the 20% of tumors ≤ 2 cm, 30% to 60% of those of 2-5 cm, and up to 60% to 90% for those > 5 cm^[21].

ANTIVIRAL THERAPY

In Japan, the incidence of hepatitis-associated HCC is high (~90%) and the antiviral therapy for patients undergoing liver transplantation for hepatocellular carcinoma is mandatory. The combination of long-term antiviral and low-dose hepatitis B immune globulin can effectively prevent hepatitis B virus recurrence in more than 90% of transplant recipients^[22]. As to hepatitis C, now direct antiviral agents (DAA) have enabled us effective treatment for patients who underwent liver transplantation for hepatitis C virus related cirrhosis^[23]. The currently available direct antiviral agents achieve a satisfactory sustained viral response in post-liver transplantation patients^[24]. Optimal timing of the DAA treatment is not yet established, but it may be appropriate to consider DAA treatment after the patients' condition and graft function become stable.

CONCLUSION

As the number of the deceased donors was scarce in Japan, unique indications and strategies in liver transplantation have been developed. LDLT will continue to be a mainstay treatment for patients with

HCC and cirrhosis. The indication of transplantation in patients with HCC still continues to be under debate in Japan.

DECLARATIONS

Authors' contributions

Designed the study: Yamamoto H, Hibi T

Wrote the draft of the manuscript: Sugawara Y

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Somatostatin in hepatocellular carcinoma: experimental and therapeutic implications

Elias Kouroumalis¹, Demetrius Samonakis^{1,2}, George Notas³

¹Department of Gastroenterology, University of Crete Medical School, Heraklion 71003, Greece.

²Department of Gastroenterology, University Hospital, Heraklion 71100, Greece.

³Department of Experimental Medicine, University of Crete Medical School, Heraklion 71003, Greece.

Correspondence to: Dr. Elias Kouroumalis, Department of Gastroenterology, University of Crete Medical School, Heraklion 71003, Greece. E-mail: kouroumi@uoc.gr

How to cite this article: Kouroumalis E, Samonakis D, Notas G. Somatostatin in hepatocellular carcinoma: experimental and therapeutic implications. *Hepatoma Res* 2018;4:34. <http://dx.doi.org/10.20517/2394-5079.2018.33>

Received: 31 Mar 2018 **First Decision:** 28 May 2018 **Revised:** 19 Jun 2018 **Accepted:** 19 Jun 2018 **Published:** 11 Jul 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

The neuropeptide somatostatin has been shown to control the secretion of several hormones and growth factors, but also to inhibit the proliferation of several tumor cells. Hepatocellular carcinoma (HCC) is a leading cause of death all over the world due to very limited treatment modalities. Early reports showed that somatostatin may influence HCC growth, making somatostatin a potential therapeutic candidate. The introduction of somatostatin analogues with long half-lives has made this prospect feasible. In this review, experimental data regarding the presence of somatostatin receptors and their functional significance in HCC are presented. Potential mechanisms of direct anti-tumoral activity of somatostatin, including effects on tumor cell proliferation and apoptosis, inhibition of various trophic factors and angiogenesis are also reviewed, as well as indirect actions affecting liver fibrosis, inflammation and macrophage-associated innate immunity. Data on the use of somatostatin analogues for the treatment of induced HCC in experimental animals are presented and human studies of somatostatin treatment of advanced HCC are critically analyzed. Reasons and pitfalls for treatment failures are identified and indications for the proper use of somatostatin, either alone or as an adjunct to other modalities in future trials are proposed.

Keywords: Somatostatin analogues, hepatocellular carcinoma, somatostatin receptors, action mechanisms, treatment

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer among men and the seventh among women, with approximately 600,000 annual deaths worldwide. It is the third cause of cancer-associated



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death, after cancers of stomach and lung. HCC global incidence varies usually following the distribution of hepatitis B or C viruses. It is highest in China, eastern Asia and Africa (20-35 per 100,000 population) and low (< 5 per 100,000) in Northern Europe and the USA. Mediterranean and eastern European countries have an intermediate rate of 10-20 per 100,000 population^[1-3].

In 1968, a hormone secretion inhibitory molecule was described, later cloned and named somatostatin^[4]. The somatostatin (SST) protein has two active forms created by alternative cleavage of a single pre-protein: the 14 amino acids SST14 and the 28 amino acids SST28, different only in potency but not in function^[5]. It soon became obvious that SST had many potential therapeutic implications but the natural molecules had the inherent drawback of a very short half-life (less than 3 min) that made *in vitro* and *in vivo* applications very difficult. Therefore somatostatin analogues (SSA), namely octreotide, vapreotide, lanreotide and pasireotide were later synthesized to overcome the difficulty^[6-8].

Extensive research resulted in identification and cloning of five somatostatin receptor (SSTR) subtypes (SSTR1- SSTR5) with two splice variants (SSTR2A and SSTR2B) for SST2. They are a family of transmembrane G-protein-coupled receptors and are encoded by separate genes on different chromosomes. All five receptors bind natural SST14 and SST28 with a high affinity. The synthetic analogues bind to some but not all receptors with varying affinity. Octreotide and Lanreotide have a binding affinity only for SST2 and SST5 while pasireotide binds to all receptors with the exception of SST4^[9,10].

Several intracellular pathways are activated after binding SST or its analogues to the receptors leading to down-stream signaling and modulation of adenylyl cyclase (AC) (SSTRs 1-5), phosphotyrosine phosphatases (PTPs) (SSTRs 1-3) and mitogen activated protein kinase (MAPK) (SSTR4), as well as calcium and potassium channels and the sodium-proton antiporter^[6,9,11-13].

Research data have made clear that somatostatin has several antineoplastic actions and could be used in clinical applications in various human cancers^[14], including HCC. An extensive review has been recently published^[10]. The present report will therefore summarize both experimental and clinical data on the use of SST and SSA in HCC.

EXPERIMENTAL DATA

There are many reports providing strong evidence that somatostatin may have an effect on HCC. Research is focused on the variability of SSTRs present in isolated cells and liver tissue, but also on functional aspects of the activation of these receptors.

SST receptors in liver cells

Hepatoma cells

Hep G2 cells are the most widely used human hepatoma cell line in liver research. The presence of mRNA of only 2, 3 and 4 SSTR subtypes was demonstrated in these cells by Northern blotting^[15] but inconsistent results have been reported.

Another report found that cultured HepG2 cells expressed all five SSTRs, at both the protein and mRNA levels, while HuH7 hepatoma cells lack SSTR3^[16]. Using immunohistochemical staining, HepG2 cells were reported to display weak expression of SSTR2 and moderate levels of SSTR5. Hep3B cells showed weak expression of SSTR3 and strong SSTR2 and SSTR5 staining while HuH7 cells additionally stained positive also for SSTR1, but not SSTR3^[17].

Our laboratory reported that HepG2 cells were found to express SSTR2, SSTR3 and SSTR5 receptors by RT-PCR. All these SSTRs were shown to have a mainly intracellular distribution with different individual

distribution patterns. Membrane binding sites for SST were mainly of the SSTR3 and SSTR5 types, with a weak SSTR2 binding^[18]. Later, we demonstrated the presence of SSTR2 and SSTR5 in another hepatoma cell line, the Hep3B cells^[19]. Importantly we have also demonstrated that HepG2 cells express cortistatin and we attributed the SSTRs internalization to the endogenous production of cortistatin^[18]. Cortistatin is a 17-aminoacid peptide with high affinity to all somatostatin receptor subtypes^[20]. Internalization of SSTR2 receptors after octreotide administration has also been reported in neuroendocrine tumors^[21].

Liver stellate cells (HSCs)

Activated rat hepatic stellate cells were reported to express SSTR subtypes 1, 2, and 3^[22], while another report found all five SSTRs in HSCs, at both the protein and mRNA level^[16]. Using a different approach, the expression of SSTR2, SSTR3 and SSTR5, but not SSTR1 and SSTR4, was demonstrated by confocal microscopy in rat HSCs. The mRNA expression level of SSTR2 was much higher than the other subtypes^[23].

We have recently shown that quiescent HSCs (day 0 or day 3) do not express SSTRs by immunocytochemistry or western blot. However at day 7, SSTRs 1, 2A, 2B, 3 and 4 started to appear in some cells activated by adherence to plastic but only after day 10, all cells were positive for SSTRs 1, 2A, 2B, 3 and 4. Therefore, whatever the effect of somatostatin might be on these cells, it is not evident from the beginning of any experiment^[24].

Kupffer cells

In contrast to HSCs, quiescent rat Kupffer cells were shown to express mRNA of SSTR 1-4. However immunocytochemistry identified only the presence of internalized SSTR 3 and SSTR4 receptors. Western blotting on the other hand detected SSTR2 and SSTR2a. Thus it seems that in quiescent cells the detection of SSTRs depends on the method used. Moreover Kupffer cells were found to express both somatostatin and cortistatin, a finding that may explain the internalized receptors. Stimulation of the cells with lipopolysaccharide activated the expression of SSTR2, SSTR3 and SSTR4^[25,26].

SST receptors in HCC tissue

Somatostatin receptors were identified in 41% of HCC in an earlier report on the presence of regulatory peptides receptors in HCC. These receptors showed high affinity for both natural somatostatin and octreotide^[27]. This observation was verified and further extended. Cirrhotic livers and HCC expressed all five SSTRs both at the protein and mRNA levels, but normal livers were negative for all SSTRs^[16]. Moreover, it seems that all HCCs do not display similar expression patterns for SSTRs. Expression rates as high as 75% for SSTR5 and as low as 41% for SSTR2 were demonstrated while SSTR4 was absent. There was no correlation between SSTR expression and tumor stage or underlying liver disease^[28]. Higher overall rates of all SST receptors were reported in both HCC and cirrhosis in a report from China. In contrast with the previous study, high expression of SSTR4 was also identified. The protein levels of receptors were markedly higher in HCC than in cirrhosis. Moreover there was a strong correlation of all receptors with serum AFP levels^[29]. A high 67% expression of SSTR2 was also shown but there was no correlation with tumor molecular characteristics including tumor suppressor genes^[30]. Very high expressions of SSTR1 and SSTR5 were also reported in a recent study of 41 liver biopsies^[31].

On the contrary, a recent report from Germany found very low overall rates (8%-15%) of weak SSTRs expression in the tissue of patients with either cirrhosis or HCC. It should be stressed however that all but two of their patients had alcohol-related disease. This is important when therapeutic implications are concerned^[17].

Thus, the available literature indicates variable expression of SSTR subtypes in both hepatoma cell lines and liver tissue from cirrhotic and HCC patients. This may be due to different methodologies, different etiologies of cirrhosis and HCC or different molecular events leading to HCC. Nonetheless SSTRs are expressed in a significant proportion of HCC and may therefore be a potential therapeutic target. This is further supported by functional data.

Functional data

Early preclinical studies have demonstrated that both natural SST and its synthetic analogues exert an anti-proliferative effect in hepatoma cell lines^[32,33]. In addition to proliferation, SSAs were shown not only to decrease cells in the S-phase but most importantly to induce apoptosis in a dose-dependent manner in HepG2 cells^[15]. These effects on proliferation and apoptosis were verified and correlated with the presence of SST receptors in HCC cell lines. Apoptosis was significantly lower in normal hepatocytes^[34].

In contrast with these studies, no influence of SSAs on either proliferation or apoptosis could be identified in another study. However the migration of hepatoma cells (HepG2 and HuH7) was significantly reduced after incubation with a selective SSTR1 agonist in Boyden invasion chambers. These findings may indicate a reduced invasive capability of hepatoma cells attributable to the presence of SSTR1^[16].

Negative results on proliferation and apoptosis were also verified using a different cell line. Short-term octreotide treatment of Bel-7402 cells did not affect cell proliferation and apoptosis. The SSTR2 protein level was significantly decreased after exposure to octreotide^[35]. Different results were very recently obtained using the same Bel-7402 cells. All SSAs tested increased cellular apoptosis but had no effect on cellular proliferation while the effect on SSTRs expression was variable^[36].

However *in vivo* experimental data have demonstrated that SST significantly inhibits tumor proliferation. The same investigators, have convincingly shown that long-term SSA treatment effectively inhibited the development and growth of HCC and improved survival rates, possibly through resensitization and upregulation of SSTR2 and SSTR5^[35,36].

A very interesting observation was reported by Xie *et al.*^[37]. While octreotide significantly enhanced apoptosis on HepG2, no such response was observed in HepG2 cells transfected with the HBV X gene. Moreover the expression of SSTR2 and SSTR5 was reduced in these cells. This may have therapeutic implications.

The role of HSCs/myofibroblasts in HCC has not been extensively investigated but early studies indicate that they favor tumor progression producing hepatocyte growth factor^[38,39]. SST or its SSAs have been reported to influence hepatic stellate cells and indirectly the progress of HCC. Thus SST at nanomolar concentrations was found to decrease rat HSC proliferation and increase apoptosis^[40].

SST caused a significant decrease of collagens I and III production by activated rat HSCs without reduction of cell proliferation thus implicating a direct action of somatostatin on HSC^[41].

The effect of octreotide on cellular proliferation of isolated rat hepatic stellate cells was recently investigated in our lab. The drug had no effect on proliferation but strongly inhibited procollagen production from activated stellate cells. It also inhibited PDGF and TGFb1 dependent procollagen production probably through activation of phosphotyrosine phosphatase (PTP) and phosphoserine-phosphothreonine phosphatase (STP)^[24].

Mode of action

Octreotide is effective in inhibiting growth of HCC *in vivo* and *in vitro*^[42]. There are several potential mechanisms through which SST and SSAs might inhibit HCC progress.

Cell proliferation and apoptosis

Despite the negative results mentioned before it is accepted today that SST and SSAs have a direct antiproliferative effect on cancer cells via specific SSTRs. SSTRs 1, 4 and 5 modulate the MAP kinase pathway and induce G1 cell cycle arrest^[43]. However, the cell cycle arrest mechanisms depend on the SSTR

subtypes involved and are not similar to all cell types. SSTR1 acts through the stimulation of the tyrosine phosphatase SHP-2, activation of the MAP kinase ERK pathway and induction of the p21Waf1:Cip1^[44], while the SSTR5 acts through inhibition of guanylate cyclase, and MAP kinase ERK^[45]. The cytostatic role of the SSTR2 has been connected to the modulation of ERK1/2 signaling pathway^[46] and the activation of the phosphotyrosine phosphatases (PTPs) SHP-1, SHP-2 and PTP η . SHP-1 induces proapoptotic caspase-mediated signals and also causes apoptosis by activation of the NF- κ B leading to the inhibition of the JNK anti-apoptotic effects. Activation of PTP η , dephosphorylates intracellular effectors such as the ERK and the PI3K/Akt pathways leading to upregulation of the cyclin kinase inhibitors p21cip1/waf1 and p27kip1. Cells are therefore accumulated in the G1 phase and cell proliferation is blocked^[8,47]. pERK1/2 was inhibited in response to natural SST while receptor-specific agonist treatment caused a dual effect: inhibition at lower concentrations and activation at higher concentrations^[48].

Earlier studies also pointed out that SSTR2, but not SSTR3, mediated induction of cyclin-dependent kinase inhibitors p21 and p27Kip1 leading to cell cycle arrest^[49]. However, a recent report has shown that SSTR2 and SSTR3 co-expression strongly induced p21 and p27Kip1 expression and therefore had a cytostatic effect^[48].

Inherent to the anti-proliferative effect of SST is the induction of apoptosis whether dependent or independent of p53^[34,50,51]. Apoptosis induction is mediated by either the SSTR2 activation or the co-expression and heterodimerization of SSTR2 and SSTR3^[48,52].

Caspase-mediated signaling pathways of octreotide antitumor activity in HepG2 cells were also reported from our lab. We have observed an interesting phenomenon that may have therapeutic implications. Measuring activities of various caspases and apoptosis in HepG2 cells we found that octreotide decreased proliferation only at concentrations of 10^{-8} mol/L, while lower concentrations increased proliferation, indicating that measurements of serum octreotide levels may be important, at least in clinical trials, to verify optimal therapeutic drug concentrations^[53].

There are additional molecular pathways through which SST and SSAs increase apoptosis in a time and dose dependent manner in human hepatoma cells. Thus, they were found to increase expression rates of the Fas-Fas ligand system leading to apoptosis^[54].

Another intriguing mechanism is the facilitation of apoptosis by endogenous opioids. We have demonstrated in HepG2 cells that opioids inhibit proliferation and induce apoptosis. Since functional opioid receptors were not found on HepG2 cells we demonstrated that opioids bind to somatostatin receptors activating a PTP signaling cascade^[55]. Interestingly, a native functional endogenous opioid system was recently described. Opioid growth factor (OGF) and its receptor were identified in hepatoma cell lines and in specimens from HCC. OGF inhibited tumor cell replication by inhibition of DNA synthesis without interfering with apoptosis^[56].

Direct or indirect inhibition of various trophic factors associated with the progress of HCC

One of the most important systems involved in tumor progression is the growth hormone-insulin-like growth factor-somatostatin (GH-IGF-SST) system. Several components of this system have been shown to be regulators of hepatocarcinogenesis^[57-59]. In particular over-expression of IGF1 receptor and decrease of IGF-binding proteins have been described in patients with HCC and hepatoma cell lines. Interestingly an increase of cathepsin D, an acid serum protease that cleaves IGF binding proteins, has also been described in HCC^[60,61]. Many studies have evaluated the relation between increased levels of IGF1 receptors and liver diseases and the oncogenic role of IGF2 and its implication in angiogenesis, migration and, consequently, in tumor progression^[62].

Pasireotide, a somatostatin analogue with high affinity for all SSTRs except SSTR4, is a more potent inhibitor of IGF1 than octreotide^[63]. It is noteworthy that the GH-IGF system is connected with the important role of Raf/MEK/ERK, one of the signaling cascades stimulated by IGF1R in experimentally induced apoptosis of hepatoma cell lines and possibly explains why the Ras gene is activated in 30% of HCCs^[64] while its substrate RAF kinase is over-expressed in many HCCs^[65]. The same pathway is activated by other growth factors known to be over-expressed in HCC like PDGF, EGF and TGFa^[66,67].

SST also inhibits the secretion of other hormones (gastrin, glucagon, insulin) which have been shown to be trophic factors for cancer cells but their significance in hepatocellular carcinoma evolution has not been elucidated^[7,68].

Direct inhibition in vivo and in vitro of angiogenesis

Neo-angiogenesis is a vital process allowing tumors to grow and metastasize^[69]. The SSA octreotide was able to inhibit angiogenesis induced by HCC *in vivo*^[70]. In nude mice with an implanted hepatocellular carcinoma, octreotide showed a strong anti-angiogenic activity^[71]. Available evidence suggests that SSAs inhibit angiogenesis either directly through somatostatin receptors on endothelial cells or indirectly through the inhibition of vascular endothelial growth factor (VEGF) or via inhibition of adenylyl cyclase^[7,72,73]. Recently, a combination of celecoxib and octreotide was found to have a potent anti-angiogenetic activity by decreasing the phosphorylation of the integrated signaling pathways of p-ERK kinase-HIF-1a (hypoxia-inducible factor-1a)-VEGF^[74]. This combination has been tried in hepatocellular carcinoma as analyzed in the relevant section.

Antineoplastic effect via immune modification - innate immunity

SST and SSAs may exert an anti-tumor activity through modulation of immune pathways. More data are required in this field^[75-77]. Many studies have been focused on the effects of somatostatin on the innate component of immunity and in particular on inflammation and oxidative stress. Reduced secretion of reactive oxygen species by macrophages after incubation with SST has been reported^[78]. More specifically for the liver, the amount of hydrogen peroxide released by Kupffer cells treated with SST was reduced compared to controls. Moreover SST also reduced production of nitric oxide and TNFa by Kupffer cells^[79].

We have verified that octreotide reduces TNFa and NO production by Kupffer cells decreasing iNOS activity probably through an interference with phosphatidylinositol 3-kinase pathways. Like most, if not all cancers, HCC has an inflammatory component. SST may therefore inhibit the growth of HCC by reducing inflammation. In this respect we showed that rat Kupffer cells treated with octreotide produced reduced amounts of the pro-inflammatory cytokine IL-12 and increased amounts of the anti-inflammatory IL-13^[80].

Macrophages are deeply involved in HCC pathogenesis through other mechanisms as well. Myeloid cells, including tumor-associated macrophages (TAMs) have been identified in large numbers in HCC microenvironment and are often associated with poor prognosis^[81,82].

During induction of HCC, there is an increased production of IL-6 and TGFb1 by macrophages leading to activation of STAT3 and progression of the tumor^[83]. At the same time, predominant activation of STAT3 leads to an M2 macrophage polarization^[84]. M2 cells are involved in polarized Th2 responses and to tumor progression and immunoregulation^[82]. TGFb1 production by Kupffer cells is reduced by octreotide *in vitro*, therefore the polarization of liver resident macrophages towards the M2 phenotype may be reduced as well^[85]. We have also proposed that the antitumor effect of octreotide in HCC may in part be explained by its antiapoptotic effect on Kupffer cells. Using caspase3 mRNA as an index of apoptosis, we measured pro- and antiapoptotic molecules in Kupffer cells after incubation with octreotide. The increased apoptosis of cultured

Kupffer cells was reversed by octreotide as a down-regulation of pro-apoptotic and an early increase of anti-apoptotic molecules were demonstrated^[85].

Another important function of liver associated macrophages is mediated through the production of chemokines and their actions on their receptors. The co-operation of CCR2 bearing macrophages and T cells results in the clearance of senescent hepatocytes, thus preventing HCC development. In case of established HCC, however, recruitment of CCR2 positive macrophages leads to accumulation of suppressive TAMs resulting in tumor progression due to the inhibition of CD8 T lymphocytes and natural killer cells^[86]. CCL2 is highly expressed and is a prognostic factor in HCC. Inhibition of CCL2/CCR2 signaling suppressed liver tumor in experimental animals through activation of T cell anti-tumor response as expected^[87].

CC chemokines and particularly CCL2 (MCP-1) are also involved in the progression of liver fibrosis^[88]. Kupffer cells were shown to secrete large amounts of CC chemokines (MCP-1, Rantes) and CXC chemokines (IL-8, MIP-2) after LPS stimulation. Octreotide inhibited only CC chemokines but not CXC chemokine secretion, an effect mediated by PI3-kinase. Therefore inhibition by octreotide of CC chemokines and specifically MCP-1 will lead to reduced HCC growth both directly inhibiting the accumulation of tumor suppressive macrophages and indirectly reducing fibrosis. Whether modifications of macrophage micro-environment influence HCC progression remains to be elucidated^[89].

Strictly speaking, although hepatic stellate cells are not members of the innate immune system, they may participate in inflammation producing pro-inflammatory molecules^[90,91]. Somatostatin inhibited the secretion of the pro-inflammatory cytokines IL-1 β and IL-8 from rat liver stellate cells^[92].

Indirect anti-neoplastic effect through modulation of fibrosis

Most HCCs are developed in a cirrhotic background. As mentioned before, SST has a profound effect on hepatic stellate cells reducing collagen I and III production and also procollagen production through activation of phosphotyrosine (PTP) and phosphoserine-phosphothreonine (STP) phosphatases without affecting stellate cell proliferation. A direct action of SST on stellate cells has been proposed^[16,21].

Moreover SST may influence fibrosis through its action on Kupffer cells augmenting matrix degradation. Kupffer cells produce large amounts of MMP1 (the enzyme responsible for native collagen degradation), and lipopolysaccharide activation induces a significant early increased production of MMP1. Octreotide had a synergistic effect with lipopolysaccharide on MMP1 secretion. In addition lipopolysaccharide and octreotide, alone or in combination, induced a significant inhibition of the large amounts of TGF- β 1 produced by unstimulated Kupffer cells. Inhibition of TGF β 1 implied that SST may also indirectly influence stellate cells and liver fibrosis^[93]. Some of the anti-tumoral actions of SST have been reviewed^[94,95]. **Figure 1** summarizes the cellular pathways of SST actions in HCC.

In vivo animal data

In an earlier report, HCCs were developed after implantation of Morris hepatoma cells in rats. Partial hepatectomy enhanced tumor progress, but treatment with octreotide inhibited the growth of the tumor^[96]. Similarly octreotide was shown to inhibit liver regeneration after partial hepatectomy^[97].

Subsequent studies from China have corroborated these results using the nude mice HCC xenograft model and octreotide administration. Tumor weights were significantly reduced, the growth was inhibited and secondary primaries and lung metastases were also decreased. More importantly, survival of the treated animals was significantly prolonged^[98,99]. Recent studies reported on the effect of a combination of a COX 2 inhibitor with an SSA. They have demonstrated that the combination had an anti proliferative effect but most importantly it suppressed the metastasis of HCC in nude mice^[100]. Moreover the same combination

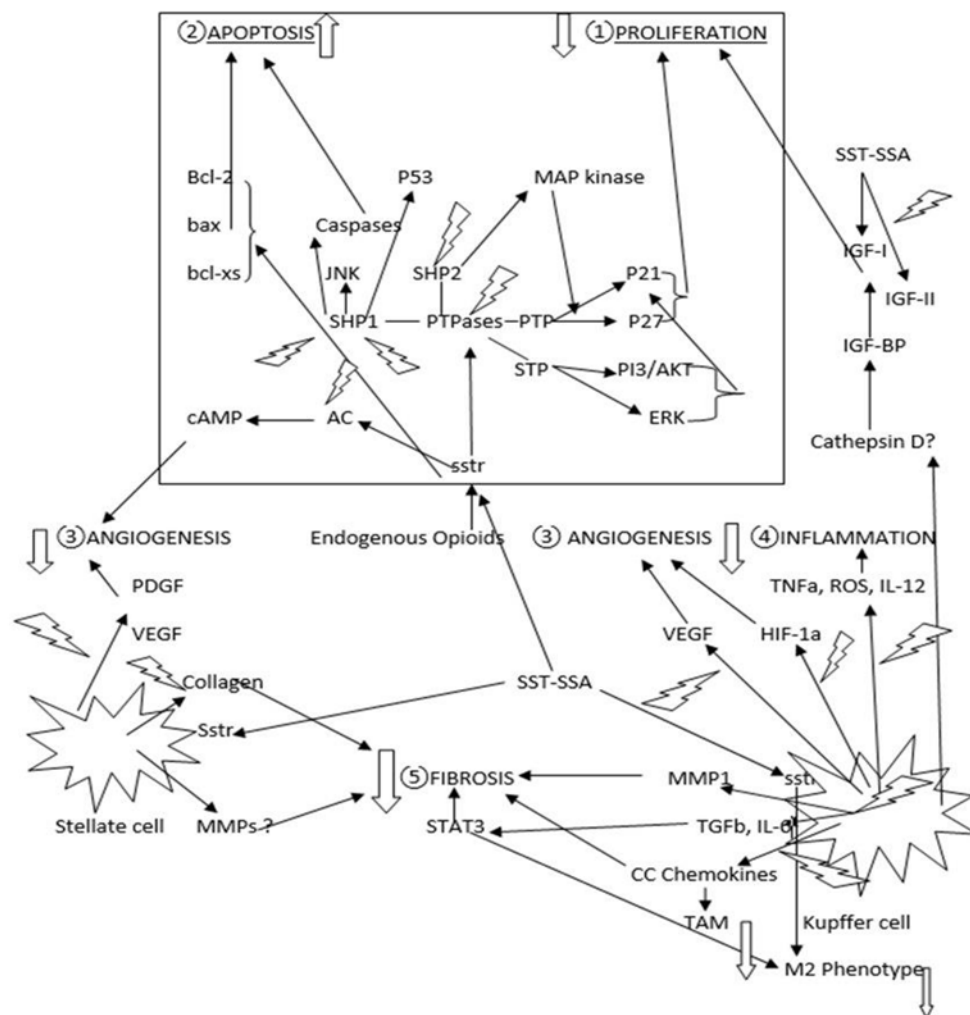


Figure 1. Anti-tumoral effect of somatostatin is achieved through various cellular pathways leading to inhibition of cell proliferation, inflammation, fibrosis and angiogenesis. Apoptosis is increased. It seems that a reduction of tumor associated macrophages (TAM) and a swift from M2 polarization in Kupffer cells may also help in the final effect

significantly prolonged the survival of rabbits with experimental liver cancer previously treated with tumor arterial embolization (TAE)^[101]. Treatment with octreotide and celecoxib after TAE, synergistically inhibits hepatic allograft metastasis by promoting tumor encapsulation and inhibition of angiogenesis^[102].

Lanreotide, a long acting SSA, was used as a cancer chemopreventive agent in a series of animal experiments. Thus, lanreotide was able to decrease the size of diethyl- nitrosamine induced liver preneoplastic foci by inhibiting cell proliferation and increasing apoptosis. This was associated with a decrease of cyclin D1 and an increase of p27kip1^[103]. Lanreotide also reduced the number of chemically induced HCCs and significantly decreased fibrosis and the level of angiogenic factors^[104,105]. In another animal model, albino mice developed HCC by injection with diethyl-nitrosamine. The administration of octreotide alone or in combination with a plant extract prevented malignant transformation. This effect was associated with a substantial reduction of oxidative stress observed in the control animals^[106].

A rather intriguing finding was recently reported in rats with a high fat diet induced obesity. Octreotide increased hepatic glucogenesis associated with increased glucose synthase and decreased fasting blood

glucose. More importantly, octreotide significantly reduced liver steatosis in obese rats. If confirmed, these results may justify the use of octreotide as a preventive measure of HCC in non alcoholic fatty liver disease^[107].

These experimental preclinical data indicating anti-proliferative and anti-metastatic effects of SSAs in HCC, supported the initiation of clinical studies in patients with HCC.

CLINICAL DATA

Favorable data

For the first time octreotide was used for HCC treatment by our group in a randomized controlled trial of 58 mostly Okuda II and III patients. Subcutaneous octreotide almost doubled survival while treated patients clearly had a lower hazard of death (0.383), in the multivariate analysis.

We confirmed these results later in a non randomized trial with long-acting analogues where the relative risk of death of the untreated patients was 2.7 (95% CI: 1.4-5.3) compared to the treated patients. Approximately 40% of tumors either regressed (10%) or remained stable (30%), a figure similar to the overall reported expression of somatostatin receptors as mentioned before. Moreover patients retained their appetite, a satisfactory body weight and sense of well being even if tumors were radiologically progressing. The etiology of HCC in our group was related to viral hepatitis in over 90% of cases^[108,109]. We have pointed out that somatostatin is not a rescue drug and the survival benefit is significant in the Kaplan-Meier survival curves only after 6 months of treatment. Moreover we observed that HCCs whose etiology was alcoholic cirrhosis were less responsive, particularly in patients who continued drinking^[18].

In an uncontrolled study of 21 patients, lanreotide caused a 43% response, similar to ours (one tumour regressed and 8 were stable, despite the fact that no patient had SSTRs on octreotide scintigraphy). Five patients (24%) had a decrease in serum-AFP levels by at least 30%^[15]. A similar uncontrolled study of mostly viral HCC cases reported that octreotide improved survival time in non-cirrhotic patients. It should be noted however that 40% of the cirrhotics were Child Pugh C stage and that most of them died before 6 months^[110].

Another Greek group also reported that octreotide doubled survival in a randomized trial of patients with HBV or HCV related HCC who had detectable SSTRs on ¹¹¹Indium octreotide scintigraphy. By contrast SSTRs negative patients had no survival benefit. Again the Kaplan-Meier curve showed that the benefit was significant after approximately 6 months of treatment^[111].

In a controlled trial from China, a combination of tamoxifen and octreotide was compared to conventional chemotherapy. In the octreotide arm, a complete response or partial response was found in 43% of patients and survival was also doubled compared to chemotherapy^[112].

A controlled study from Pakistan in reported tumor regression in 45.4% of patients with HCV related HCC, while alpha fetoprotein reduction was noticed in 50%. Significant survival benefit and improvement of quality of life were also found^[113]. A seemingly negative small observational study on patients with advanced HCC has been reported from the USA. The median survival was only 4.5 months. However, 6/22 patients (27%) survived for more than 10 months and most interestingly these were patients of Asian descent with a history of HBV infection^[114].

In a retrospective controlled study of 95 patients on octreotide (57% viral etiology and 43% alcoholics), survival rates of patients with Barcelona classification stage B were significantly higher (22.4 months),

compared to patients who received palliative care only (2.9 months). Patients with BCLC stage A had also higher survival (31.4 months) compared to palliative care (15.1 months) but this was not significant due to small number of patients in this group^[115].

In addition to these data there have been two case reports of HCC patients of viral etiology who responded with complete regression of the tumor with either lanreotide or long-acting octreotide^[116,117]. Recently a case of HBV-associated HCC with SSTR2 overexpression and metastases in the lung and mediastinal lymph nodes detected 17 months after left hepatectomy was described. Treatment with lanreotide 30 mg twice a month resulted in a significant size reduction of the mediastinal nodes and complete disappearance of the lung nodes. This objective response lasted for 42 months^[118].

A randomized study of fairly advanced HCC compared treatment with either octreotide alone or in combination with rofecoxib. Survival in both groups was significantly associated with baseline serum VEGF and IGF1 levels^[119].

Two large recent trials from China highlighted the significance of the presence of SSTR2 and SSTR5 for the response to SSAs. Importantly these were studies on early-stage HCC and treatment was administered after resection of the primary tumor. In the study by Li *et al.*^[120], 76 patients with operable HBV-related HCC were divided into two groups according to SSTR2 and 5 expression profiles. The mean survival time was longer in the high SSTR2/5 expression group. Similar results were reported in another study of 99 HBV-related HCC. Recurrence rate and survival were significantly higher in patients with high expression of SSTR2^[121]. Both studies concluded that the expression profile of SSTRs can be used as an independent prognostic factor.

There have been interesting results when SSAs were compared to transarterial chemoembolization (TACE) or radiofrequency ablation (RAF) or were given in combination with TACE or sorafenib.

In an earlier report of a prospective non-randomized study from Germany, 41 patients were treated with octreotide and compared for survival to another group of patients treated with TACE. A median survival of 571 days was found in the octreotide group, similar to the TACE group^[122]. This was confirmed later in a larger randomized trial where, octreotide treatment had a similar outcome compared to patients who received TACE or multimodal therapy^[115].

In an observational study, a combined approach of RAF followed by octreotide was adopted for treatment of viral-associated HCCs, mostly Child A and Child B (60% and 34% respectively). All patients had multiple liver HCC nodules; 14% had complete or partial tumor regression and a clinical benefit was evident in 80%. Mean survival was 31.4 months. Serum VEGF was significantly correlated with response^[123].

In a different setting, 147 patients diagnosed with HCC suitable for TACE received 2-4 TACE procedures; 84 patients received an additional heparin plus octreotide combination and 63 patients were given only heparin and served as the controls without randomization. They reported a significant reduction in the incidence of tumor metastasis within a year of follow-up post-TACE, in the combination treatment^[124].

In a recent randomized study from China, 71 patients with mostly viral associated HCC, BCLC stages B and C were assigned to either TACE ($n = 35$) or TACE plus celecoxib plus octreotide ($n = 36$) and were followed up for 3 years. The median overall survival of the TACE + C + L group of 15.0 months was twice as much compared to that of the TACE group (7.5 months) and the survival benefit was very significant for both BCLC stage B or C. Equally significant was the improvement in the quality of life in favor of octreotide. Post-embolization syndrome was also significantly lower in the octreotide group^[125].

The results of the combination of octreotide plus sorafenib were reported in a prospective non controlled phase II study of advanced viral associated HCC (mostly HCV), Child-Pugh A or B; 10% of patients achieved partial response and 66% had stable disease with a median survival of 12 months. The combination was well tolerated^[126].

Further work from the same group has shown that responders had a significant decrease of reactive oxygen species in the peripheral blood mononuclear cells and this reduction was enhanced when octreotide was added to sorafenib. A 50% pERK activity reduction was observed in responders compared to an 80% increase in non responders. Sorafenib induced a 40% increase in serum NO and this was further increased after octreotide^[127]. Whether SSAs offer any advantage as an addition to sorafenib remains to be established.

Unfavorable data

A retrospective observational non controlled study found no evidence of survival benefit in 63 patients (40% alcoholics)^[128]. The first negative randomized controlled study was reported by Yuen *et al.*^[129]. It has been heavily criticized by us and many others, because the selected patients had a very short survival of 1.9 months in the control group ($n = 35$) vs. 2 months in the octreotide group ($n = 35$) indicating that most patients belonged to BCLC stage D. In fact 21/35 patients received either none or just one long-acting octreotide injection^[130].

A non-randomized subsequent study found limited beneficial response after octreotide administration. However, 4 patients (6%) did not receive any octreotide because their disease progressed so rapidly they were unable to start treatment. These patients were included in the survival analysis; 5% received 1 dose, 19% 2 doses, 16% 3 doses and 16% 4 doses. Additionally, from the 30 patients surveyed, 6 were not enrolled due to intolerance to the test dose. The selection of patients also raises some questions. A significant number (50%) had vascular thrombosis (extent is not specified) and 13% had metastatic disease. It should be noted that among the 14 patients who received treatment of more than 3 months, 50% were judged to be stable, which is in accordance to virtually all previous results^[131,132].

Another open-label study of 63 patients (22% alcoholics) reported little anti-cancer activity and a median survival of 8 months. However, the reason for stopping treatment was disease progression or toxicity and therefore assessment of survival was not really feasible^[133].

A randomized controlled study compared the effect of tamoxifen (control group) with tamoxifen plus octreotide in 109 patients (52.4% alcoholics) and reported no survival benefit. Again the median survival of the treatment group was only 3 months and 44% of patients received only 1-3 injections. Moreover the median survival in Child-Pugh A patients was only 6 months^[134].

The HECTOR study, a randomized double-blind placebo-controlled multicenter trial of 120 patients, showed no survival benefit for octreotide compared to placebo, with a median survival of 4.7 and 5.3 months respectively. Quality of life was also unaffected. However 52% of the treatment group had alcoholic cirrhosis and at 6 months the survival rate was only 40%^[135].

Similarly negative were the results from another multicenter randomized placebo controlled study. But again 50% of the randomized patients had alcoholic cirrhosis^[136]. A recent everolimus plus pasireotide open-label study of 26 patients (BCLC stage C 88%, and > 60% alcoholics) also gave negative results with a median survival of 6.7 months. However the reason for treatment discontinuation was disease progression and not death. Treatment was administered for only a median of two 28-day cycles. Yet, 10/22 evaluable patients had stable disease as best response^[137].

A seemingly not favorable open label trial of twenty patients (all HBV or HCV) treated with pasireotide was recently reported. 90% had prior therapy, 75% had BCLC stage C, and 55% had metastatic disease. Despite this, a stable disease in 9 patients was demonstrated (45%), and the median survival was 9 months^[138].

The situation is possibly clarified from a Chinese meta-analysis of approximately 800 patients from 9 trials. The 6- and 12-month survival rates in the octreotide group were significantly higher than those of the control group (6-month: RR 1.41, 95% CI: 1.12-1.77, $P = 0.003$; 12-month: RR 2.66, 95% CI: 1.30-5.44, $P = 0.008$) but this was not the case when only western studies were analyzed^[139]. This meta-analysis vividly describes that there is a discrepancy in results between China (and in that regard Greece) and Western countries. This is also evident from the analysis presented in this review.

One possible explanation for the negative results is the tachyphylaxis through which SSTRs are internalized upon prolonged exposure of tumors to somatostatin analogues. However there is evidence that resensitization may occur^[135]. In addition the expression profile of receptors is variable among tumors. Also production of endogenous cortistatin may further affect the expression and internalization of the receptors^[95].

Serum levels of octreotide may also be a critical parameter in HCC response. As mentioned before octreotide decreased proliferation only at concentrations of 10^{-8} mol/L, while lower concentrations increased proliferation, making drug serum levels an important parameter at least in clinical trials^[53]. However, these possible resistance mechanisms cannot explain the differences between the East (and Greece) and the West.

A critical evaluation of the reported studies offers potential explanations for the discrepancies. First, as we pointed out, the survival benefit is evident only in patients that live long enough to have a treatment period of more than 6 months. Somatostatin is not a magic bullet and the potential molecular pathways of its action require some time to produce measurable results. The second explanation is very important. Practically all negative western studies recruited a large number of alcoholic cirrhosis (between 25% and 60%) reflecting the etiological background of their population. In contrast, Chinese studies have almost exclusively recruited viral cirrhosis in accordance with cirrhosis etiology in their population. The same was true in the original Greek studies. It was our impression that our few alcoholics did not respond equally well to somatostatin particular those that do not abstain from alcohol. This critical point is not mentioned in any of the negative papers. As mentioned before, a very recent report from Germany found very low rates of weak expression of SSTRs in liver tissue from alcohol related HCC patients^[17]. Only two of their patients had virus-related HCC.

CONCLUSION

Selection of patients is critical in any study of HCC treatment^[18,130,132]. It seems that SST is suitable for patients with viral cirrhosis ideally after identification of the expression on the tumor of SSTR2 and 5, either with scintigraphy or even better by immunofluorescence after a liver biopsy. Eligible patients are those classified as BCLC stage B or C^[140] which is the same indication with TACE and possibly sorafenib. Patients with alcoholic cirrhosis-related HCC may be treated as those in BCLC stage B, but the response will be limited. In that respect it is tempting to use SST as an adjunct to TACE.

Finally, it would be interesting in future to see if there are additional differences between viral and alcoholic related HCC like differential expression of receptors or production of trophic factors. In a recent study an increase of serum IGF2 level was reported to be associated with the occurrence of HCC metastasis after TACE and octreotide, as metastatic foci were found in 97% when IGF2 was increasing in contrast to only 13.6% of patients with an IGF2 decrease^[141]. In the context of the previous discussion, it should be noted that the expression of IGF2 in HCC was strongly associated with HBV infection^[142].

DECLARATIONS

Authors' contributions

Devised the review, supervised the project and prepared the final draft: Kouroumalis E

Revised the clinical data and participated in the preliminary draft: Samonakis D

Revised experimental data and participated in the preliminary draft: Notas G

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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[¹⁸F]FDG PET imaging evaluation on non-alcoholic fatty liver disease and hepatocellular carcinoma model treated with sorafenib

Fernando Gomes de Barros Costa¹, José Tadeu Stefano¹, Daniele de Paula Faria², Caio de Souza Levy¹, Maria Cristina Chammas³, Camila de Godoi Carneiro², Isabel Veloso Alves Pereira⁴, Bruno Cogliati⁴, Flair José Carrilho¹, Claudia P. Oliveira¹

¹Department of Gastroenterology (LIM-07), University of São Paulo School of Medicine, São Paulo 01246-903, Brazil.

²Nuclear Medicine Laboratory (LIM-43), Radiology and Oncology Department, University of São Paulo School of Medicine, São Paulo 01246-903, Brazil.

³Radiology and Oncology Department, University of São Paulo School of Medicine, São Paulo, São Paulo 01246-903, Brazil.

⁴Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo 05808-010, Brazil.

Correspondence to: Dr. Fernando Gomes de Barros Costa, Department of Gastroenterology (LIM-07), University of São Paulo School of Medicine, Lim-07 Ave. Dr. Arnaldo, São Paulo 01246-903, Brazil. E-mail: fgbcosta85@gmail.com; Dr. Claudia P. Oliveira, Department of Gastroenterology (LIM-07), University of São Paulo School of Medicine, Lim-07 Ave. Dr. Arnaldo, São Paulo 01246-903, Brazil. E-mail: claudia.oliveira220@fm.usp.br

How to cite this article: Costa FGB, Stefano JT, Faria DP, Levy CS, Chammas MC, Carneiro CG, Pereira IVA, Cogliati B, Carrilho FJ, Oliveira CP. [¹⁸F]FDG PET imaging evaluation on non-alcoholic fatty liver disease and hepatocellular carcinoma model treated with sorafenib. *Hepatoma Res* 2018;4:35. <http://dx.doi.org/10.20517/2394-5079.2018.06>

Received: 8 Feb 2018 **First Decision:** 9 Mar 2018 **Revised:** 11 May 2018 **Accepted:** 11 May 2018 **Published:** 12 Jul 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Aim: Evaluate the effect of sorafenib in a rat model of non-alcoholic fatty liver disease (NAFLD) related to hepatocellular carcinoma (HCC) by quantifying the correlation between changes in glucose metabolism on PET imaging and degree of tumor differentiation.

Methods: NAFLD related HCC was induced by the combination of high fat and choline deficient diet with diethylnitrosamine (100 mg/L) for 16 weeks. Then carcinogenic stimuli were suspended, liver nodules were identified by abdominal ultrasound and two groups were randomized: control ($n = 10$) and sorafenib ($n = 20$). Rats received daily gavage administration of 1 mL saline or sorafenib (5 mg/kg/day) for more 3 weeks. After treatment, [¹⁸F]FDG PET scan was performed on animals.

Results: [¹⁸F]FDG uptake was lower in the sorafenib group than that in the control group (3.3 ± 0.48 vs. 5.5 ± 1.5 , $P = 0.01$). Direct correlation was found between poorly-differentiated HCC and TumorSUVmax/MuscleSUVmax ratio ($R^2 = 0.54$, $P = 0.006$). Treatment was associated with significantly more residual tumors that were well differentiated (Grades I/II) than in the untreated control group (39% vs. 5%, respectively, $P = 0.01$).



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Conclusion: Sorafenib shows promise as a treatment for reducing the aggressiveness of HCC as demonstrated by [^{18}F] FDG PET and immunohistochemistry.

Keywords: Animal model, hepatocellular carcinoma, liver steatosis, non-alcoholic fatty liver disease, positron emission tomography, sorafenib

INTRODUCTION

Non-alcoholic, fatty liver disease (NAFLD) is associated with obesity and known to progress to non-alcoholic, steatohepatitis (NASH), cirrhosis and then hepatocellular carcinoma (HCC), or directly progress from NASH to HCC^[1-3]. Liver cancer is the 16th cause of global mortality and HCC accounts for up to 90% of all primary liver cancers^[4,5]. Observational studies showed that diabetes, obesity, and iron overload are risk factors for development of HCC and NAFLD^[2].

Animal models are crucial to elucidate the physiopathology of HCC and to test potential therapeutic targets^[6,7]. The ideal model of NAFLD-related HCC should replicate human HCC development, given a high caloric diet, obesity, insulin resistance, dyslipidemia, similar hepatic markers, natural evolution of HCC, genetic aspects and activation of the same signaling pathways^[8,9]. There are many animal models for HCC that include genetically altered animals and orthotopic tumor implantation, however they are not ideal to replicate NAFLD as they do not exhibit liver and metabolic changes^[7,10]. Previous work used a mixed experimental model of NAFLD-related HCC with fat and choline deficient diets together with diethylnitrosamine (DEN) in drinking water to achieve HCC development within 16 weeks; a shorter period than usual^[7,11]. DEN has been used as a carcinogen and is capable of inducing HCC with intra- and inter-tumor variability as it occurs in humans^[12-14].

Earlier studies performed with HCC patients, treated with sorafenib, demonstrated that higher values of 2-deoxy-2- ^{18}F -fluoro-D-glucose (^{18}F FDG) uptake (expressed as SUVmax) were correlated with lower overall survival and more advanced HCC^[15-21]. In HCC cell lines, the absence of p53 expression is indicative of a worse prognosis, as is the increased ^{18}F FDG uptake^[22]. A recent metabolic study of HCC by positron emission tomography (^{18}F FDG PET) showed that this imaging technique could be a prognostic tool, as results correlate with long-term survival and early recurrence of HCC after liver transplantation^[21,23]. This tool can be used to identify the most undifferentiated and aggressive tumors to select patients who should undergo liver transplantation^[17].

Sorafenib was the first drug approved by the FDA for treatment of advanced HCC (BCLC C)^[24,25]. The usual dose for humans is 800 mg/day, providing an average of 127 $\mu\text{mol/L/h}$ of plasmatic concentration ($\text{AUC}_{0-12\text{h}}$), while the equivalent dosage in rats is 5 mg/kg/day^[26-28]. It had been showed that sorafenib (2.5 mg/kg/day) has the capacity to prevent hepatic fibrosis, mitochondrial dysfunction, and reduce inflammation (interleukines 6 and 10) in NAFLD animal models^[29]. Furthermore, Yang *et al.*^[30] tested it for 8 weeks (5 mg/kg/day) and demonstrated that sorafenib improved hepatic venous dysregulation, inhibited recruitment and activation of leukocytes, and reduced splanchnic vasodilatation and ascites.

This study aimed to evaluate the effect of sorafenib in a rat model of NAFLD related to HCC by using [^{18}F] FDG PET imaging as a tool to quantitate the degree of HCC differentiation *in vivo*.

METHODS

This study was approved by the ethical committee for animal use of the University of Sao Paulo Medical School (protocol 108/14), following the current standards of small animal care.

Thirty male Sprague-Dawley rats weighing 300-400 g at 8 weeks of age were used. HCC secondary to NAFLD was induced by high fat and choline deficient diets [35% of total fat, enriched with 54% of trans-fatty acids (Rhostrer Ltd., BR)], with a DEN (Sigma-Aldrich Chemical, St. Louis, MO, USA) dose of 100 mg/L in the drinking water ad libitum for 16 weeks. After this period stimuli were suspended and the animals were randomly split into 2 groups. The control group ($n = 10$) received 1 mL of saline solution (0.9%) daily by gavage for 3 more weeks. The sorafenib group ($n = 20$) received 5 mg/kg/day of sorafenib (Bayer Healthcare Pharmaceuticals, Cologne, GY) by gavage for 3 more weeks.

At 16 weeks, rats were anesthetized with ketamine 80 mg/kg (Cristalia, BR) and xylazine 10 mg/kg (Bayer, BR) intraperitoneally and then submitted to abdominal ultrasound (US) to quantify, measure, and localize hepatic nodules^[31]. Board-certificated radiologist (M.C.C) with 24 years of experience performed the procedure using a Philips Ultrasound IU 22 system (Bothell, WA, USA) with a VL13-5 transducer. Only nodules larger than 0.2 cm were considered and catalogued in the distribution of parenchyma: left lobe, right lobe, medium lobe, and caudate lobe.

PET images with [¹⁸F]FDG were acquired at the end of treatment (3 weeks after initial sorafenib) in a small animal PET scanner (LabPET4 Gamma Medica-Ideas, Northridge, CA, USA), using methods similar to those outlined in Park *et al.*^[15]. Animals were anesthetized with isoflurane 5% in oxygen 100% for induction and 2%-3% for maintenance. [¹⁸F]FDG was injected in the penile vein (37.7 ± 6.29 MBq) and the animals were allowed to wake up after injection for better tracer distribution. After 45 min of tracer injection the animals were anesthetized again and the image acquired for 30 min. Computed tomography (CT) images were also obtained with 65 kVp, 165 μ A in 512 projections and magnification of 1.3 for anatomic correlation.

PET images were reconstructed by the ordered subsets expectation-maximization 3D (OSEM-3D) method^[32] with 20 interactions, 4 subsets, a transverse field of view of 100 mm, and a matrix of 240×240 , for pixel resolution of $0.42 \text{ mm} \times 0.42 \text{ mm}$. The CT images were reconstructed with a filtered back projection method, a matrix of 512×512 , and pixel resolution of $0.17 \text{ mm} \times 0.17 \text{ mm}$. CT images were used for attenuation correction of the PET images.

Images were analyzed by the PMOD™ software (PMOD Technologies Ltd., Zurich, CH) to obtain quantitative measures of [¹⁸F]FDG uptake in defined regions of interest in the rats in each group. The visualization interface in the software allowed regions of interest (ROI) to be drawn specifically and entirely within tumor lesions, liver tissue and muscle. The uptake values were expressed as using the dose-normalized parameter standardized uptake value (SUV). $\text{SUV} = \text{radioactivity concentration (kBq/mL)} / [\text{injected dose (kBq)} / \text{animal weight (g)}]$. The maximum value of the SUV within a region of interest is expressed as SUVmax.

Three days after the PET scan (week 19), the rats were euthanized with dextroketamine (Cristalia, BR) 120 mg/kg and xylazine (Bayer, BR) 10 mg/kg intraperitoneally. Liver samples of the right and left lobes and the larger tumors evidenced in PET/CT were collected for histological analysis. The liver specimens were fixed in 4% formaldehyde and stained with hematoxylin-eosin (HE). These samples were blindly scored by a veterinary hepatopathologist (B.C) with 12 years of experience, using a modified classification standardized by Kleiner *et al.*^[33]. The variables analyzed were steatosis (0-3), lobular inflammatory changes (0-3), hepatocyte ballooning (0-2), fibrosis (0-4), and ductular reaction (0-3) through the NALFD activity score (NAS)^[33]. HCC was diagnosed with characteristics defined by Thoolen *et al.*^[34] for rats and then classified by Schlageter *et al.*^[35]. Histological classifications are considered the gold standard for assessment of HCC and are used in this study to evaluate the performance of the non-invasive characterization using ¹⁸F-FDG PET.

Immunohistochemical analysis was performed for protein glutamine synthetase (GS), hepatocyte specific antigen (HEP-PAR-1), and cytokeratin 19 (CK-19). HCC was considered for nodules with positive results for

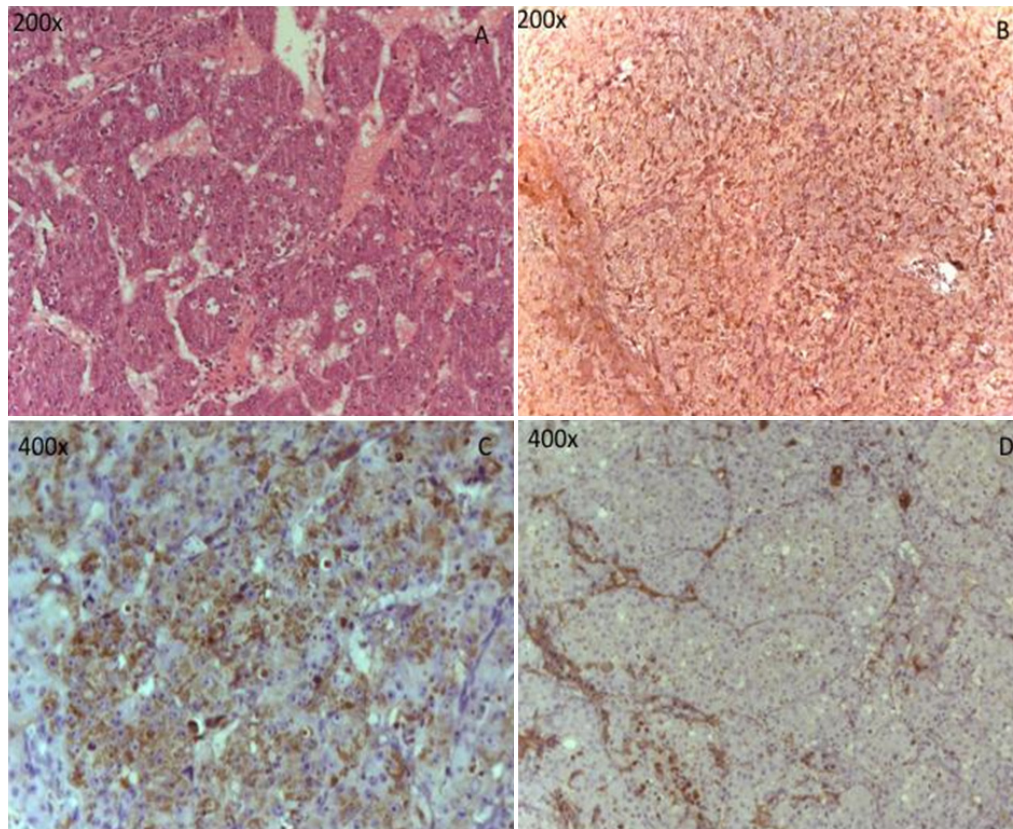


Figure 1. Illustrations of histologic and immunohistochemical view of the HCC Grade III Edmondson-Steiner. (A) Hematoxylin-eosin, $\times 200$; (B) glutamine synthetase, $\times 200$; (C) HEP-PAR-1, $\times 400$; (D) CK-19, $\times 400$

GS or HEP-PAR-1, and negative for CK-19 [Figure 1]. Antibody dilution was GS 1:3000, HEP-PAR-1 1:500 and CK-19 1:200. The method used involved immunoperoxidase with antigenic recovery by humid heat.

Statistical analysis was done with Excel® and GraphPad Prism® 7.0. The Student *t*-test was used for Gaussian distribution variables and the Mann-Whitney test was used for non-Gaussian distribution variables. Calculation of descriptive statistics: mean, median, and standard deviation, was performed using the appropriate form of the statistic for the distribution pattern of variables. To compare histological findings with PET findings, chi-square, linear regression, Kruskal-Wallis and Tukey *post-hoc* tests were used. Only *P* values less than 0.05 were considered statistically relevant.

RESULTS

All animals completed the first 16 weeks, but between the 16th and 19th week, when the treatment began, animals' mortality reached 60% in both groups. The main causes of death were pneumonia, hemorrhagic ascites due to tumor rupture, included during anesthesia induction for PET scan. Mean survival in the sorafenib group was 130 ± 4.9 days with a median of 133 days, and the control group was 126.3 ± 8.5 days with a median of 130 days ($P = 0.07$). The animals' body weight did not show a statistical difference between groups during the study, however it was different at time of euthanasia [Table 1].

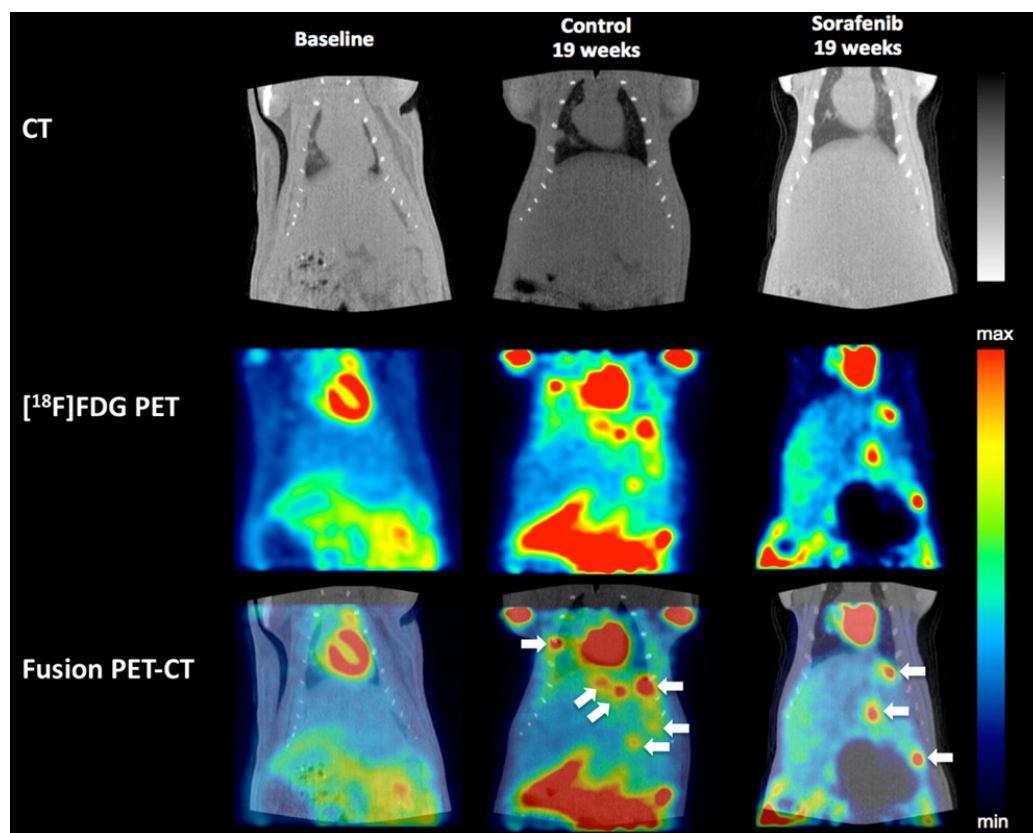
US findings showed no difference between groups regarding the average liver nodules distribution per animal (4.88 ± 2.75 in the control group vs. 4.95 ± 3.11 in the sorafenib group, $P = 0.48$) or major nodule size [Table 2]. The average number of nodules per animal detected by PET was 4.37 ± 1.59 in the sorafenib group and 8.5 ± 3.7 in the control group ($P = 0.006$) [Figure 2].

Table 1. Weight evolution according to studied groups

Weight (g)	Control average (n = 10)	Sorafenib average (n = 20)	P value
16th week	479.5 ± 45.4	463 ± 46.2	0.28
19th week	440.5 ± 67	420 ± 34.4	0.24
Euthanasia	486.3 ± 38	394 ± 48.5	0.003
Liver weight	35 ± 4.6	27.5 ± 11.6	0.13

Table 2. The sonographic findings at the 16th week of experimentation before treatment with sorafenib or placebo

Liver US 16th week	Control (n = 10)	Sorafenib (n = 20)	P value
Major nodule (cm)	1.04 ± 0.69	0.72 ± 0.92	0.34
Median of nodules per animal	5	5	
Average of nodules per animal	4.88 ± 2.75	4.95 ± 3.11	0.48
Quantity of nodules	44	99	
Percent of nodules in the left/medium lobes	75	61	0.14
Percent of nodules in the right/caudate lobes	25	39	0.22
Percent of ascites	11	10	0.46

**Figure 2.** Illustrative images of the CT, PET with [^{18}F]FDG, and fusion PET/CT. Note that the sorafenib group shows 3 high uptake lesions in the liver, while the control group shows 5 high uptake lesions in the liver and 1 lesion in the right lung (indicated by white arrows in the fusion image)

[^{18}F]FDG uptake (expressed in SUVmax) was different between the two groups: 2.4 ± 1.98 in the sorafenib group and 3.8 ± 1.74 in the control group ($P = 0.01$) [Figure 3]. According to HCC Edmondson-Steiner classification, SUVmax had this distribution: grade II, median 2.1 (1.72-4.93); grade III, median 3.86 (1.63-11.3); grade IV, median 4.87 (4.34-5.91); $P = 0.008$ [Figure 4]. Significant differences were seen between grade II vs. III ($P = 0.023$) and grade II vs. IV ($P = 0.013$), but between grade III and IV the differences were not significant ($P = 0.449$).

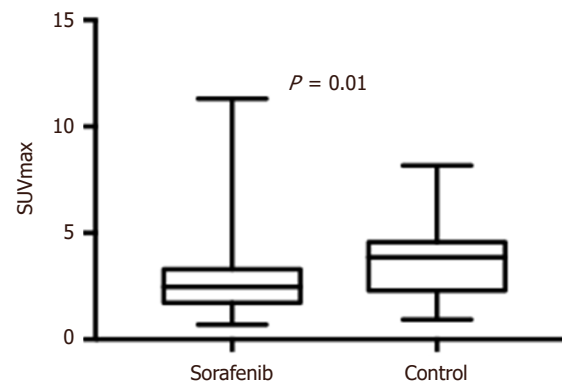


Figure 3. Comparison of SUVmax values between nodules of the control and sorafenib groups

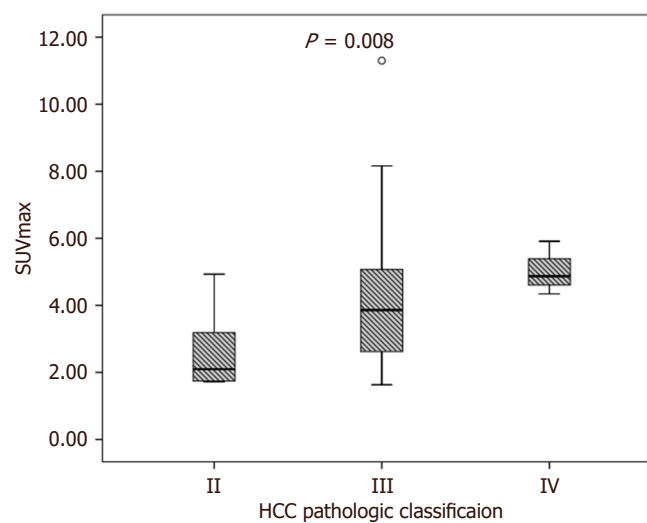


Figure 4. Comparison between SUVmax and HCC pathological classification

Correlation between less differentiated/undifferentiated HCC (Grades III/IV) and the highest values of [^{18}F] FDG uptake, presented as tumor SUVmax ($R^2 = 0.44$, $P = 0.01$) or as a tumor ratio, either Tumor SUVmax/Liver SUVmax ratio ($R^2 = 0.42$, $P = 0.02$) or Tumor SUVmax/SUVmax muscle ratio ($R^2 = 0.54$, $P = 0.006$) was found [Figure 5].

The pathology results showed that the sorafenib-treated group had more well-differentiated HCC (39% vs. 5%, respectively I/II vs. III/IV, $P = 0.01$), and less poorly-differentiated HCC (52% vs. 81%, respectively I/II vs. III/IV, $P = 0.003$). There was no difference between the two groups for necroinflammatory activity, degree of hepatic fibrosis, vascular invasion, intra-nodule hemorrhage, nodule necrosis, and low-grade dysplastic nodules [Table 3].

DISCUSSION

Our study is the first to evaluate the effect of sorafenib in a mixed experimental model of advanced HCC secondary to NAFLD using PET imaging with [^{18}F]FDG for quantitation of tumor growth. The decreased HCC nodules per animal in the treated group suggests the positive effect of sorafenib treatment, which is affirmed by the higher proportion of well-differentiated lesions (Edmondson-Steiner Grades I/II) in the treated group. The PET findings showing fewer lesions with high uptake per animal in the sorafenib group

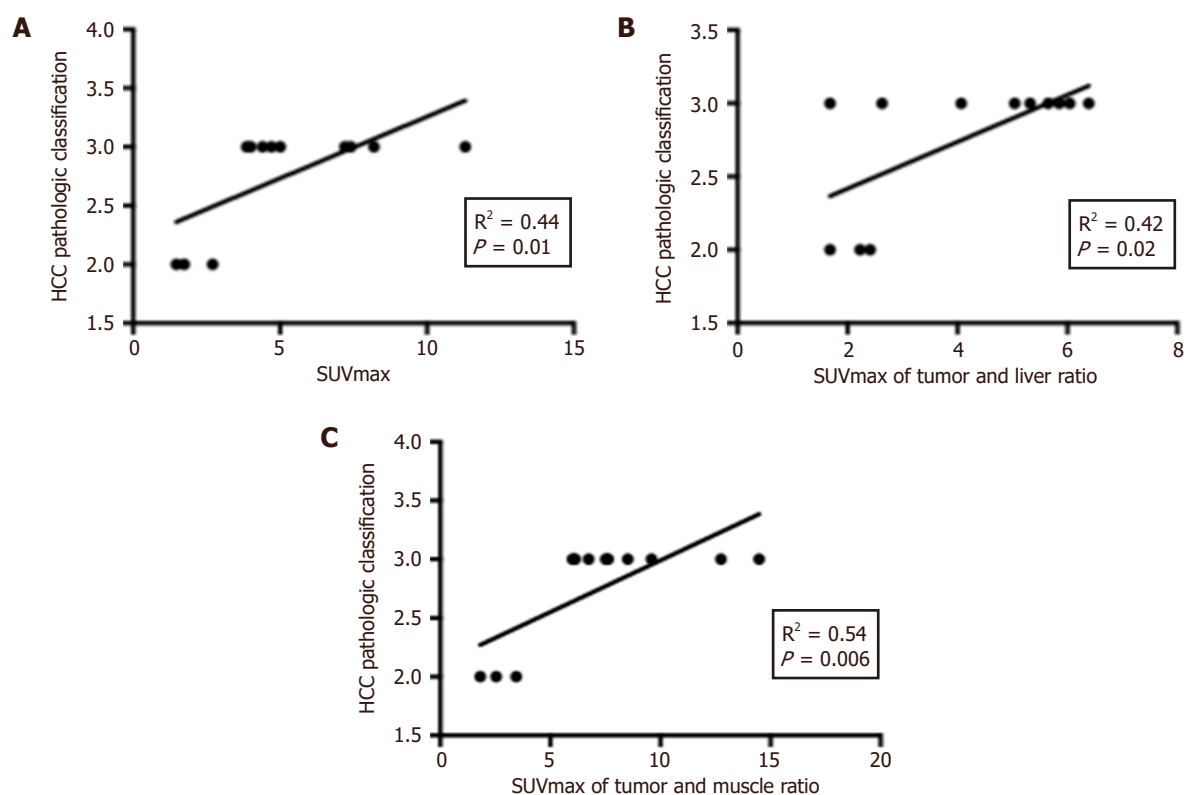


Figure 5. Dispersion graphs. (A) Plot of HCC Grade of differentiation and SUVmax taken by lesions; (B) plot of HCC Grade of differentiation and relation between tumor and liver tissue SUVmax; (C) plot of HCC Grade of differentiation and relation of tumor and muscle SUVmax. Correlation and P values are noted on the graphs

Table 3. Histological findings at the end of the study, showing differences and similarities between treated and control groups

Histological finding	Control	Sorafenib	P value
NAS 4	100% (4/4)	75% (6/8)	0.23
NAS 6	0% (0/4)	25% (2/8)	
Liver fibrosis stage 3	0% (0/4)	25% (2/8)	0.23
Liver fibrosis stage 4	100% (4/4)	75% (6/8)	
Vascular invasion	75%	43%	0.30
Intranodular hemorrhage	31% (7/22)	43% (10/23)	
Intranodular necrosis	41% (9/22)	47% (11/23)	0.51
Low grade dysplastic lesions	14% (3/22)	9% (2/23)	
Grade I/II HCC lesions	5% (1/22)	39% (9/23)	0.01
Grade III/IV HCC lesions	81% (18/22)	52% (12/23)	

than in the control group ($P = 0.006$) also suggest the positive effect of sorafenib for decreasing undifferentiated lesions (Grades III/IV of Edmondson-Steiner).

Mattina *et al.*^[36] published a meta-analysis summarizing the antitumor efficacy of sorafenib in preclinical studies. They found that 95% of the models used human xenotransplants to assess effectiveness of the drug. Although most cell lines show robust action of sorafenib in mice, others like McA-RH7777 did not respond^[12]. In our study, we observed a decrease in the mean number of HCC lesions per animal, and a decrease in lesion aggressiveness; without a complete cure.

Groß *et al.*^[12] compared differences in the response of sorafenib between the model with isolated use of DEN in water for 8 weeks and a model inoculated with cancerous cells in the liver by injecting them in the portal vein. The DEN model had intra- and inter-tumoral variability, as it does in humans, while the cancer cells

were more homogeneous. In addition, sorafenib only acted on the DEN model, decreasing tumor growth and perfusion^[12]. Similar findings were demonstrated in another study that observed a low objective response (3%) by RECIST criteria^[37]. This cytotoxic response pattern was also observed in another clinical study, in which approximately 42% of patients were stable or had a minor/partial response^[38].

In the current study, we found the presence of advanced fibrosis or cirrhosis in all animals, as well as advanced HCC with vascular invasion in 75% of control animals ($n = 4$) and 43% of animals in the treated group ($n = 8$). These findings highlight the clinical relevance of our model, as most preclinical studies used younger animals with less advanced disease, thus not reflecting the hepatic microenvironment observed in humans^[36].

Despite treatment with sorafenib, the mortality rate (60%) was similar in both groups, resulting from the severity of liver cirrhosis and advanced HCC; sometimes with decompensation in ascites (about 10% in both groups) and pulmonary metastasis. In humans, the prognosis of advanced HCC is bleak, with a median survival of 6 months or 25% in 1 year^[24]. Park *et al.*^[15] used PET/CT with [¹⁸F]FDG to evaluate rats exposed only to intraperitoneal DEN, administered once a week for 16 weeks. They reported a mortality rate similar to our study, or about 65% in week 19. A more precise evaluation of the effect of sorafenib on survival was not possible because all animals were euthanized 3 days after the last PET scan to minimize risk of losing more animals prior to the endpoint of the study.

The development of a biochemical marker or diagnostic tool to identify the most undifferentiated and aggressive tumors has been studied in an attempt to better select patients for curative treatment^[17,24]. [¹⁸F]FDG PET appears to be a potential tool, because it has been shown that higher values of [¹⁸F]FDG uptake correlates with lower overall survival, advanced HCC, undifferentiated histology (loss of p53 expression), and increased liver transplantation recurrence^[17,19,22,39]. Lee *et al.*^[23] retrospectively evaluated patients who underwent liver transplantation for HCC and had [¹⁸F]FDG PET prior to surgery. They found that HCC patients with lower [¹⁸F]FDG uptake had more highly differentiated HCC (Grades I/II Edmondson-Steiner classification), with lower rates of microvascular invasion and no recurrence of HCC after a 3-year follow-up^[23]; results that are similar to the results found in our work.

Kim *et al.*^[17] showed that the [¹⁸F]FDG uptake calculated as ratio between tumor and liver adjacent tissue was more accurate than tumor uptake in predicting HCC post-transplant recurrence (SUVmax Tumor/SUVmax Liver 0.869 *vs.* tumor SUVmax 0.762). In our study, the best correlation of [¹⁸F]FDG uptake and HCC Grades at III/IV was found with SUVmax Tumor/SUVmax Muscle ($R^2 = 0.54$, $P = 0.006$), followed by SUVmax tumor ($R^2 = 0.44$, $P = 0.01$) and Tumor SUVmax/Liver SUVmax ratio ($R^2 = 0.42$, $P = 0.02$); somewhat lower than what was reported by Kim *et al.*^[17].

The absence of a pretreatment (16th week) [¹⁸F]FDG PET scan is one limitation of our study, as we could not follow the evolution of the same node with one diagnostic tool throughout the experiment. This decision was based on two reasons: (1) high probability of increasing mortality rate on additional anesthesia during image acquisition (animals were already weak at that point) and (2) at pretreatment time point it was necessary to ensure the presence of lesions that could be detected by PET (> 1 mm) at a later point in time; therefore, a method with higher spatial resolution was chosen (US) to check homogeneity of lesion count and size between groups. US was able to show that the groups were homogeneous and that comparison between groups with PET at the end of the experiment was feasible. Although US is more sensitive in the detection of small lesions and offers good localization, it is not able to provide detailed information about tumor grade. Therefore we chose to use PET as a correlate for tumor aggression.

We showed that sorafenib could be responsible for reduced number of nodules and aggressiveness of HCC (more Grade I/II lesions than III/IV), as well as reduced tumor [¹⁸F]FDG uptake. [¹⁸F]FDG PET could be used

as a diagnostic tool for *in vivo* assessment of the degree of histological differentiation of HCC, since higher values of tracer uptake were correlated with more poorly differentiated HCC. Our methods and animal model, NAFLD, which exhibited progression to advanced HCC, will be useful for further studies of hepatic carcinogenesis.

DECLARATIONS

Acknowledgments

We would like to thank Mariana Maciel Torres and Edson Pereira da Costa for assistance with handling animals during this research. We want to thank Bayer Healthcare Pharmaceuticals for the donation of sorafenib.

Authors' contributions

Concept and design: Oliveira CP, Stefano JT, Carrilho FJ

Data acquisition: Costa FGB, Levy CS

Ultrasound work: Chammas MC

Pathology work: Pereira IVA, Cogliati B

Nuclear medicine work: Carneiro CG, Faria DP

Data analysis: Costa FGB, Stefano JT, Oliveira CP, Faria DP

Statistical analysis, literature search and manuscript preparation: Costa FGB

Manuscript editing: Costa FGB, Levy CS, Stefano JT, Oliveira CP, Faria DP

Manuscript review: Costa FGB, Stefano JT, Oliveira CP, Faria DP

Availability of data and materials

The data and materials could be obtained from the corresponding author.

Financial support and sponsorship

This work was supported by bursaries of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) to Fernando Gomes de Barros Costa.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

This study was approved by the ethical committee for animal use of the University of Sao Paulo Medical School (protocol 108/14).

Consent for publication

Not applicable.

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Review

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Hepatitis C related hepatocellular carcinoma in the era of direct-acting antivirals

Akshata Moghe, Obaid S. Shaikh

Veterans Affairs Pittsburgh Healthcare System and Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh School of Medicine, Pittsburgh, PA 15240, USA.

Correspondence to: Dr. Obaid S. Shaikh, Veterans Affairs Pittsburgh Healthcare System and Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh School of Medicine, University Drive C, FU #112, Pittsburgh, PA 15240, USA. E-mail: obaid@pitt.edu

How to cite this article: Moghe A, Shaikh OS. Hepatitis C related hepatocellular carcinoma in the era of direct-acting antivirals. *Hepatoma Res* 2018;4:36. <http://dx.doi.org/10.20517/2394-5079.2018.54>

Received: 7 May 2018 **First Decision:** 8 Jun 2018 **Revised:** 20 Jun 2018 **Accepted:** 26 Jun 2018 **Published:** 18 Jul 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Globally, hepatocellular carcinoma (HCC) is the second leading cause of cancer related death. Hepatitis C virus infected patients with cirrhosis or bridging fibrosis are particularly at risk. The risk is reduced among patients who achieve viral clearance with interferon-based regimens. Direct-acting antivirals (DAA) have revolutionized the management of HCV as the treatment is well tolerated, convenient to administer and is highly effective. Earlier studies showed conflicting results in the effect of DAA induced sustained virologic response (SVR) on the subsequent development or recurrence of HCC, with some studies showing an increased risk. More recently, two large retrospective studies provided convincing evidence that DAA induced SVR reduces the risk of HCC development. Irrespective of viral clearance, patients with cirrhosis and advanced fibrosis and those with treated HCC continue to be at increased risk requiring long-term surveillance studies.

Keywords: Antiviral agents, viral clearance, hepatoma, hepatitis C virus, cancer surveillance

HCC INCIDENCE - USA AND GLOBAL

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver, accounting for nearly three fourths of all liver cancers^[1]. In the last decade, it has been the seventh most common cancer in the United States^[2]. Yet, with its high lethality and limited effective therapeutic options, it has risen to be the second-leading cause of cancer-associated mortality world-wide^[3]. In the United States, the incidence of HCC has quadrupled in the last four decades, from 1.5 cases per 100,000 in 1973 to 6.2 cases per 100,000



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in 2011^[4]. However, a recent epidemiological study observed that the rate of increase in HCC incidence has slowed down in recent years^[5]. The study included data acquired from the surveillance, epidemiology, and end results (SEER) program and noted that although HCC incidence increased by 4.5% per year from 2000 to 2009, it only increased by 0.7% annually from 2010 to 2012^[5]. Variations in HCC incidence by gender, age, ethnicity, race and geographical location were also noted. Men had a higher average annual percentage increase of 3.7% compared to the 2.7% increase in women. In spite of the overall plateauing of HCC incidence, the incidence in some sub-groups such as men aged 55-64 years continued to rise, corresponding to the baby boomer population with peak rates of hepatitis C virus (HCV) infection. By 2012, the rate of incidence in Hispanics was higher than that among Asians within the United States. Amongst the states included in the SEER database, Texas had the highest age-adjusted HCC incidence^[5]. An earlier study based on SEER data showed similar results^[6]. Thus, for the first time in four decades, there was no significant increase in the incidence or incidence-based overall mortality of HCC. The studies indicated a deceleration of HCC incidence around the year 2006^[4].

Worldwide, HCC is still amongst the top three leading causes of cancer-related deaths. In the Asia-Pacific region, HCC incidence remains high because of the high prevalence of hepatitis B virus (HBV) infection^[7]. Except for Japan, Australia, Singapore and New Zealand, where HCV is more prevalent, HBV accounts for almost 80% of HCC in this region^[7]. HBV and HCV are also the most common risk factors for HCC in China that leads the world by accounting for more than half of the HCC cases world-wide^[7].

ETIOLOGY: ROLE OF HCV IN THE DISEASE BURDEN OF HCC

Hepatitis C virus is a single-stranded RNA virus from the family *Flaviviridae*^[8]. Along with HBV, it accounts for more than 70% of the HCC cases worldwide^[9]. However, it is quite distinct from HBV with regards to its mechanisms of hepatocarcinogenesis^[9,10]. Being a DNA virus, HBV has the ability to incorporate into the host genome and to intrinsically affect DNA replication and induce carcinogenesis. In contrast, HCV cannot integrate within the host genome and uses other mechanisms to promote carcinogenesis. Those mechanistic pathways invariably stem from chronic inflammation, which is the hallmark of HCV infection. HCV proteins have been implicated to play a role in hepatocarcinogenesis and some of the proposed mechanisms include induction of oxidative stress, modulation of cell regulation pathways and interaction with tumor suppressor proteins. In addition to the HCV core protein, other proteins such as E2, NS3 and NS5A have also been studied for their potential role in carcinogenesis^[11]. The high rate of replication errors in the HCV RNA leads to the formation of quasispecies which are adept at evading the immune system and in establishing chronic infection^[10]. Hepatitis C viral infection thus results in chronic hepatitis in nearly 80% of cases in comparison to 5% of HBV-infected patients who develop chronic disease^[10]. Chronic hepatitis C progresses to liver fibrosis in 60%-70% of patients, cirrhosis in 10%-20% and eventually HCC in 1%-5% within two decades of harboring the virus. The ability of HCV to promote cirrhosis is 10- to 20-fold higher than HBV^[10]. Therefore, unlike HBV, almost all HCV-infected persons who develop HCC have underlying cirrhosis^[12,13]. An additional important factor for the high burden of HCV-induced HCC is the lack of a preventative vaccine like the HBV vaccine which has been instrumental in reducing the global incidence of HBV^[9].

HCV PREVALENCE - USA AND GLOBAL

The World Health Organization (WHO) estimated the global prevalence of HCV to be around 3%, amounting to more than 170 million people worldwide^[14-16]. There is a lot of geographic variation in the prevalence of HCV, with African and the Middle Eastern countries such as Egypt, Cameroon, Saudi Arabia, Iraq and Syria topping the list^[15]. Egypt has the highest prevalence of HCV in the world with endemic levels of infection, and that is reflected in the high incidence of HCC^[17]. Along with Egypt, the Asian countries of China, India, Pakistan and Indonesia also carry a heavy burden of HCV and together make up half of the global HCV population^[14,15].

Table 1. Timeline of drug approvals for hepatitis C (USA)

Approval date	Anti-viral agent	Trade name
Feb 26, 1991	Interferon alfa-2b	Intron-A
1996	Interferon alfa-2a	Roferon
Sep 10, 1997	Interferon alfacon-1	Infergen
Aug 7, 2001	Peginterferon alfa-2b	Peg-Intron
Oct 16, 2002	Peginterferon alfa-2a	Pegasys
May 13, 2011	Boceprevir	Victrelis
May 23, 2011	Telaprevir	Incivek
Nov 24, 2013	Simeprevir	Olysio
Dec 6, 2013	Sofosbuvir	Sovaldi
Oct 10, 2014	Sofosbuvir/ledipasvir	Harvoni
Dec 19, 2014	Ombitasvir/paritaprevir/ritonavir/dasabuvir	Viekira Pak
Jul 24, 2015	Daclatasvir	Daklinza
Jul 24, 2015	Ombitasvir/paritaprevir/ritonavir	Technivie
Jan 28, 2016	Elbasvir/grazoprevir	Zepatier
Jun 28, 2016	Sofosbuvir/velpatasvir	Epclusa
Jul 18, 2017	Sofosbuvir/velpatasvir/voxilaprevir	Vosevi
Aug 3, 2017	Glecaprevir/pibrentasvir	Mavyret

Developed countries have typically demonstrated lower prevalence of HCV infection compared to developing countries. In the United States, HCV seroprevalence is 1.6% to 1.8%, amounting to 5-7 million individuals^[15,18]. The populations at risk are intravenous drug users, incarcerated and homeless persons, and those born in the “baby boomer” years between 1945 and 1965. During those years, extensive illicit intravenous drug use in social settings and the use of contaminated blood products led to the spread of HCV. Since the establishment of standard screening practices for blood products and organs, a noticeable decline of incident HCV cases has been noted^[18-20]. This is also true for other developed countries including Australia, Japan and parts of Europe^[15]. Currently, the major risk factor for transmission in those countries is the sharing of infected needles by intravenous drug users^[15].

As HCV and HIV have similar routes of transmission, co-infection is common especially in countries such as Thailand, Malaysia and China, where intravenous drug abuse and addiction are major problems^[21]. Of the 40 million known HIV infected persons in the world, approximately 4.5 million are co-infected with HCV^[22]. Unfortunately, HIV-induced immunosuppression leads to accelerated progression of HCV disease, resulting in cirrhosis within 5-10 years of infection rather than the usual 10-20 years^[21]. Alcohol abuse also accelerates HCV disease progression.

HCV TREATMENT - EVOLUTION FROM INTERFERONS TO DIRECT-ACTING ANTIVIRALS

Before the turn of the century, standard treatment for chronic hepatitis C consisted of the combination of interferon-alfa administered three times a week with ribavirin daily for 24 or 48 weeks^[23,24]. Subsequent introduction of pegylated interferons allowed for once a week injections and improved response rates. Still, treatment was associated with considerable side-effects limiting its applicability particularly among patients with comorbidities and organ transplant status other than liver transplantation.

The introduction of direct-acting antivirals (DAA), telaprevir and boceprevir, in 2011 dawned a new era in the management of HCV infection^[25] [Table 1]. Both drugs were NS3/4A protease inhibitors, and were used in combination with peg-interferons and ribavirin to avoid the emergence of resistant variants^[26]. Those agents improved SVR rates but did not improve the side-effect profile. Thus, the use of triple therapy came with its own challenges particularly with regard to compliance and monitoring^[15]. Simeprevir was another protease inhibitor that was approved to be used in combination with peginterferon and ribavirin with similar effects. Those three drugs constituted the first generation of DAAs.

It was the approval of sofosbuvir (nucleotide analog NS5B polymerase inhibitor) in 2013 that heralded the advent of all-oral regimens and a change in treatment landscape once again^[25] [Table 1]. The next two years saw the introduction of several other DAA - sofosbuvir in combination with ledipasvir (NS5A inhibitor), and combination of ombitasvir (NS5A inhibitor), paritaprevir (NS3/4A protease inhibitor), ritonavir (CYP3A inhibitor) and dasabuvir (non-nucleoside NS5B polymerase inhibitor), and daclatasvir (NS5A inhibitor)^[25]. The latter was approved for treatment of HCV genotype 3 infection in combination with sofosbuvir. The regimens could be used in both non-cirrhotic and well compensated cirrhotic patients who were either treatment naïve or treatment experienced, and they achieved high SVR rates with reduced duration of treatment and better tolerability^[25]. Elbasvir (NS5A inhibitor) and grazoprevir (NS3/4A protease inhibitor) fixed dose combination was approved for treatment naïve or treatment experienced patients infected with genotype 1 or 4, with or without cirrhosis. The regimen was contraindicated in patients with Child's B or C cirrhosis; however, it could be used in patients with advanced renal failure without dose adjustment. All regimens had overall SVR rates of greater than 95%. That was the second generation of DAAs.

The approval of sofosbuvir and velpatasvir (NS5A inhibitor) as a fixed dose combination initiated the third generation of DAAs [Table 1]. Whereas the response rate to previous regimens was HCV genotype dependent, this combination was pan-genotypic and could be used in patients with or without cirrhosis. It also had approval to be used in decompensated cirrhosis in combination with ribavirin; however, it was not recommended to be used in patients with severe renal impairment as defined by an eGFR of < 30 mL/min. Two other fixed dose combinations were more recently introduced to this pan-genotypic armamentarium of antivirals. Sofosbuvir, velpatasvir, and voxilaprevir (NS3/4A protease inhibitor) fixed dose combination was approved for patients without cirrhosis or those with compensated cirrhosis, and without severe renal impairment. The combination was indicated for patients previously treated with an HCV regimen containing an NS5A inhibitor, or those with genotype 1a or 3 who were previously treated with a regimen containing sofosbuvir without an NS5A inhibitor. Glecaprevir and pibrentasvir (NS5A inhibitor) fixed dose combination was approved for patients without cirrhosis or those with compensated cirrhosis. The combination could also be used in adult patients with genotype 1 infection, who were previously treated with a regimen containing an HCV NS5A inhibitor or an NS3/4A protease inhibitor, but not both. Hepatitis B reactivation during HCV treatment has been reported among coinfecting patients resulting in fulminant hepatic failure and death. It is therefore recommended to test all patients for current or prior HBV infection before initiation of HCV treatment, and to monitor all coinfecting patients for HBV reactivation during therapy and during post-treatment follow up.

IMPACT OF HCV TREATMENT ON DISEASE BURDEN OF CIRRHOSIS/BRIDGING FIBROSIS

In view of the etiologic role of HCV in the progression of hepatic fibrosis and hepatocarcinogenesis, viral clearance would be expected to cause cessation of fibrosis progression or potentially regression of fibrosis. Similarly, it may also reduce the risk of HCC development. This issue was evaluated among patients treated with interferon and ribavirin. A meta-analysis of four key randomized trials assessed the effects of HCV treatment on histologic features^[27]. The pooled studies included 3010 treatment naïve patients who underwent liver biopsies before and after treatment. Treatment regimens involved unmodified interferon or pegylated interferon, in combination with ribavirin. To be deemed as an improvement in fibrosis, at least one-point reduction in METAVIR fibrosis stage from baseline was required. Conversely, an increase by one or more points was considered fibrosis progression. In addition to improvement in necrosis and inflammation, the analysis showed a significant improvement in fibrosis progression with all treatment regimens. In 49% (75/153) there was reversal of cirrhosis; however, fibrosis worsened in 8% to 23%. Factors independently associated with lack of fibrosis progression included low HCV RNA level (< 3.5 million copies/mL), minimal to no baseline inflammatory activity, healthier body mass index (< 27 kg/m²), younger age (< 40 years), achievement of SVR and a lower pre-treatment fibrosis stage^[27]. In another study, the effect of combination

therapy with thrice weekly interferon alfa-2b and daily ribavirin for 24-48 weeks was assessed^[28]. Among 90 treatment naïve patients enrolled, 34 patients underwent a liver biopsy following completion of 48 weeks of therapy. Compared to the pre-treatment biopsy, fibrosis stage improved in 32% (11/34), and all three patients with cirrhosis had regression of fibrosis. Improvement in fibrosis progression was independently associated with younger age and low pre-treatment HCV RNA level. In another study of 933 patients with HCV who achieved SVR with interferon-based therapies, non-invasive markers (Fibrotest, Fibroscan) or liver biopsy were used to assess severity of fibrosis. Among the patients who achieved SVR (29%), 56% (24/53) of the patients with cirrhosis had regression of cirrhosis noted at a median follow-up of 6.3 years. However, during that period 12% of the patients with SVR developed new cirrhosis suggesting that the net reduction in cirrhosis was a meager 5%^[29]. Those findings led to the suggestion that HCV therapy should ideally be initiated in earlier stages of fibrosis to achieve the benefit of cirrhosis prevention^[30].

With the dawn of DAA era, the assessment of hepatic fibrosis incidentally shifted from liver biopsy to non-invasive modalities principally transient elastography (TE). One limitation of TE is that a change in liver stiffness (LS) following SVR may not entirely reflect a reduction in fibrosis as inflammatory component contributes to stiffness and it resolves quickly with SVR^[30]. This limitation needs to be considered while inferring from studies that examined the effect of DAA on hepatic fibrosis by using LS as a surrogate marker. In a study of 392 patients treated with DAA, an average reduction in LS from 12.65 kPa pre-therapy to 8.55 kPa 40 weeks after achieving SVR was noted, suggesting a 32% reduction in LS. That correlated with a significant reduction in the FIB-4 and APRI fibrosis scores^[31]. Another study demonstrated a progressive reduction in LS among 255 patients who achieved SVR with DAA - from average score of 26.4 kPa prior to therapy to 23.5 kPa at the end of therapy and subsequently to 21.3 kPa at 12 weeks following completion of treatment, indicating a 20% reduction in fibrosis^[32]. In a Japanese study of 210 patients who achieved SVR with daclatasvir and asunaprevir (NS3/4A protease inhibitor) combination, there was significant reduction in LS values, progressively from baseline to end-of-treatment to 24 weeks following treatment completion^[33].

EFFECT OF DAA ON THE INCIDENCE OF HCC

Several studies examined the effect of SVR from interferon-based therapies for hepatitis C on subsequent development of HCC. A meta-analysis established with moderate level of certainty that SVR achieved with interferon-containing regimens reduced all-cause mortality and decreased the risk of HCC at any stage of fibrosis^[34]. In fact, the estimated risk reduction of HCC after achieving SVR with interferon-based therapies in patients with HCV-induced fibrosis/cirrhosis was an impressive 76%^[34]. However, the reduction in HCC risk was not uniform as risk persisted in some patients despite viral clearance, particularly among those older than 65 years and those with advanced fibrosis or cirrhosis^[35].

Viral clearance induced by DAA has been shown to reduce liver and non-liver related critical events and overall mortality. In a retrospective review of 467 patients (409 with decompensated cirrhosis) treated with DAA, viral clearance was achieved in 381 (82%) patients^[36]. MELD scores improved in treated patients while they worsened in untreated patients. The authors concluded that viral clearance was associated with improvement in liver functions within 6 months compared to untreated patients. In a prospective study of patients with compensated cirrhosis, the effects of SVR on patient outcomes was studied^[37]. Patients were treated with interferon or with DAA. Among 1323 patients included, 668 (50%) achieved SVR after a median follow up of 58 months. Patients with SVR had reduced incidence of HCC and hepatic decompensation. In addition, SVR was associated with reduced mortality and risk of death from liver and non-liver related causes.

However, several reports cast doubt on the beneficial effect of DAA induced SVR on the development of HCC. In a study of 103 patients, 58 with treated HCC and complete radiologic response had DAA induced SVR or HCV RNA negativity^[38]. At a median follow-up of 5.7 months, 3 patients died and 16 (28%) developed

radiologic tumor recurrence. Those results implied that DAA therapy increased the recurrence rate of treated HCC. In another study of 344 cirrhotic patients without HCC (59 with treated HCC and complete response) who received DAA, 91% achieved SVR^[39]. During a follow-up period of 24 weeks, 26 (8%) were noted to have HCC - 17/59 (29%) with previous HCC and 9/285 (3%) without previously diagnosed HCC. The rate of HCC recurrence was higher compared to historical controls. In a retrospective study of HCV patients with cirrhosis and treatment with DAA, the development of *de novo* HCC was examined^[40]. *De novo* HCC was noted in 9% of the patients during or within 6 months of DAA therapy with new indeterminate lesions in another 3%. The authors concluded that as this rate exceeded the previously reported rate of 3% within 6 months of completing treatment, DAA appeared to increase the risk of *de novo* HCC development. In contrast, an analysis of data from three French prospective multicenter cohorts did not show an increased risk of HCC recurrence following DAA therapy^[41]. The cohorts included more than 6000 patients treated with DAA. Among patients with previously treated HCC, the rate of HCC recurrence was similar in patients who received DAA *vs.* those who did not receive DAA.

More recently, two large retrospective studies provided more definitive evidence of the effect of SVR induced by DAA on the development of HCC. In a study of 22,500 patients treated with DAA at any of the 129 US Veterans Health Administration (VHA) hospitals, 39% were noted to have cirrhosis^[42]. New HCCs were noted in 271 patients including 183 with SVR. Overall, annual HCC incidence was 1.19/100 person-years with significant reduced risk of HCC among patients with SVR compared to those without SVR (0.9 *vs.* 3.45/100 person-years). Although, patients with cirrhosis had the highest annual incidence of HCC after SVR (1.82 *vs.* 0.34 in patients without cirrhosis), the protective effect of SVR was similar among patients with or without cirrhosis. The second study was conducted by our group at Veterans Affairs Healthcare System Pittsburgh using the “Electronically Retrieved Cohort of HCV Infected Veterans (ERCHIVES)” database that is populated with a wide range of clinical information pertaining to veterans seropositive for HCV^[43]. We identified 17,836 patients without prior HCC - 3534 received interferon-based therapy, 5734 received DAA and 8468 patients constituted the control untreated group. SVR was achieved by 67% of the interferon treated group and 96% of the DAA treated group. Among patients with cirrhosis who achieved SVR, HCC-free survival was similar in the interferon treated and DAA treated groups. Both groups had improved HCC-free survival compared to the untreated group. The two studies established that DAA induced SVR reduced the risk of HCC development; however, absolute HCC risk remained high among patients with cirrhosis.

The divergence of conclusions reached in the studies is intriguing. Earlier reports of increased HCC recurrence and *de novo* HCC following DAA induced viral clearance were based on smaller cohorts which were likely affected by selection bias. The reported increase in HCC was explained on the basis of changes in hepatic microenvironment. It was postulated that rapid viral clearance induced by DAA stunned immune surveillance that was characteristic of chronic hepatitis C. The resulting disruption in immunomodulation allowed niches of dormant neoplastic cells to proliferate unchecked. A direct effect of DAA on cancer cell growth was also proposed^[44]. In contradistinction to those studies, the two VHA studies reported beneficial effects of DAA induced SVR. Despite the limitations of retrospective design, the considerable size of the study cohorts provided a more definitive evidence of the beneficial effect of DAA induced SVR on development of HCC.

SUMMARY

Patients with advanced hepatic fibrosis or cirrhosis due to hepatitis C are at considerable risk of HCC. Viral clearance induced by interferons was noted to effect significant risk reduction for HCC development. In contrast, an increase in *de novo* HCC and HCC recurrence was reported following SVR achieved with DAA. More recently, two large studies provided convincing evidence for the beneficial effect of DAA induced SVR

on subsequent development or recurrence of HCC; however, absolute risk of HCC remained high among such patients. Patients with advanced fibrosis or cirrhosis therefore require continued HCC surveillance irrespective of SVR.

DECLARATIONS

Authors' contributions

Critically reviewed available information: Moghe A, Shaikh OS

Wrote the manuscript: Moghe A

Planned, reviewed and finalized the manuscript: Shaikh OS

Availability of data and materials

Not applicable.

Financial support and sponsorship

Obaid S. Shaikh has research grant support from Gilead Sciences, Pfizer, Mallinckrodt Pharmaceuticals and Merit Medical Systems.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Aberrant pre-mRNA splicing regulation in the development of hepatocellular carcinoma

Supriya Sen

Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, CA 90095, USA.

Correspondence to: Dr. Supriya Sen, Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, 611 Charles E Young Drive E, Los Angeles, CA 90095, USA. E-mail: ssen@ucla.edu

How to cite this article: Sen S. Aberrant pre-mRNA splicing regulation in the development of hepatocellular carcinoma. *Hepatoma Res* 2018;4:37. <http://dx.doi.org/10.20517/2394-5079.2018.39>

Received: 12 Apr 2018 **First Decision:** 9 Jun 2018 **Revised:** 21 Jun 2018 **Accepted:** 22 Jun 2018 **Published:** 18 Jul 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Alternative splicing is a highly regulated process that plays a critical role in diversification of the transcriptome and proteome in the cell. Several diseases, including different types of cancers, have been associated with aberrant regulation of alternative splicing. Thus, correcting alternative splicing is an attractive strategy to restore normal cell physiology in patients with cancer including hepatocellular carcinoma (HCC). This review summarizes the role of alternative splicing events related to HCC and potential therapeutic applications for it.

Keywords: Alternative splicing, hepatocellular carcinoma, splicing factors

HEPATOCELLULAR CARCINOMA: THE CAUSE OF DISEASE AND MORTALITY

Liver cancer is the fifth most leading cause of cancer death worldwide^[1]. More than 700,000 people are diagnosed with this cancer and 600,000 people die each year throughout the world. Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer, accounting 70%-85% of all liver cancer in adults, primarily caused by chronic liver injury and inflammation, e.g., viral hepatitis or alcoholic and non-alcoholic cirrhosis and nonalcoholic fatty liver disease (NAFLD)^[2,3].

From the molecular point of view, HCCs are complex tumors^[4]. The prognosis of HCC is unsatisfactory due to lack of reliable early diagnostic and screening tests and effective treatment options. Seventy percent of HCCs have been detected in an advanced stage at diagnosis. The molecular pathogenesis of the disease has also remained poorly understood. Therefore, a better understanding of HCC biology and identification of the prognostic molecular markers with benefits for HCC risk assessment and development of novel therapeutic



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approaches is urgently required.

REGULATION OF ALTERNATIVE PRE-MRNA SPLICING

Alternative splicing (AS) is a process by which multiple messenger RNAs (mRNAs) are generated from a single pre-mRNA, resulting in functionally distinct protein products that may have different or even opposing roles^[5]. Genome-wide studies showed that nearly all multi-exon genes in human undergo alternative splicing and produce multiple mRNA isoforms from a single pre-mRNA in a tissue or developmental stage-specific manner^[6,7]. Thus, AS is an important mechanism to vastly expand transcriptomic and proteomic diversity from a finite genome^[8]. This is accomplished by the differential recognition of splice sites by RNA binding splicing factors in the pre-mRNA^[9]. The different types of AS are shown schematically in Figure 1. The most common type of AS consists of a single cassette exon that is either included or skipped in the mRNA. Other forms of AS include alternative selection of 5' and 3' splice sites, selection of mutually exclusive exon, and intron retention. Different *cis*-regulatory elements in the pre-mRNA play a critical role in alternative selection of splice sites by binding to splicing regulatory proteins. Based on the location of binding in the pre-mRNA and function, there are four *cis*-regulatory elements: exonic splicing enhancers, exonic splicing silencers, intronic splicing enhancers and intronic splicing silencers. These *cis*-regulatory elements which are present within the alternative exon itself or upstream/downstream intron sequences bind *trans*-regulatory splicing factors and either promote or inhibit the usage of the alternative exon(s). Though there are a number of RNA-binding proteins that regulate alternative pre-mRNA splicing, two of the well-studied families are serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs)^[10]. Other less common families include the CELF/BRUNOL family, and the RBM family^[10-12]. Both SR proteins and hnRNPs can promote or inhibit exon recognition depending on location of the binding and sequence context^[10].

ALTERNATIVE SPLICING AND HCC

Alternative splicing is a major post-transcriptional regulatory event that can modulate key aspects of cancer cell biology including cell proliferation, metabolism, apoptosis, survival, invasiveness, angiogenesis, drug-resistance, and metastasis^[13-15], thus playing a very critical role in the development and progression of cancers. In case of HCC, splicing alterations of genes such as *DNA methyltransferase 3b (DNMT3b)*, *Aurora kinase B (AURKB)*, *E3 ubiquitin ligase (MDM2)*, *TENSIN2*, *MAD1*, *SVH*, *TP53*, and *Fibronectin1 (FN1)*^[16] have long been reported. Recent studies have shown that the list of tumor-specific aberrantly spliced mRNAs is increasing and implicated in HCC^[17].

Alternative splicing facilitates the development of HCC either by generating oncogenic variants or by inactivating the tumor suppressors. For example, an alternative POLDIP3 transcript promotes HCC progression^[18]. POLDIP3 is a target of ribosomal protein S6 kinase 1, and regulates DNA replication and mRNA translation. The alternative POLDIP3 transcript (POLDIP3-β), which lacks exon 3, was found to be significantly up-regulated in clinical HCC tissue compared to paired adjacent noncancerous hepatic tissue. This POLDIP3-β isoform has been shown to increase HCC cell proliferation, inhibit HCC cell apoptosis, enhance HCC cell migration, and promote xenograft growth. Another example is the cell fate determinant protein, Numb, which is aberrantly spliced in HCC and produces an isoform that contains a long proline-rich region (PRRL)^[19]. In HCC cell lines, PRRL generally promotes and PRRS (short proline-rich region) suppresses proliferation, migration, invasion, and colony formation. PRRL-Numb expression has been shown to increase in HCC and be associated with early recurrence and thus reduces overall survival after surgery^[19].

It was observed that, in HCC cell lines and tumors, insulin receptor (IR) is aberrantly spliced and promotes expression of the mitogenic isoform of insulin receptor (IR-A) that is generally expressed in the embryonic tissues but not in the adult liver. In contrary to the isoform IR-B that is normally expressed in the adult liver and promotes metabolic effects of insulin, IR-A signals proliferative effects via binding to insulin-like

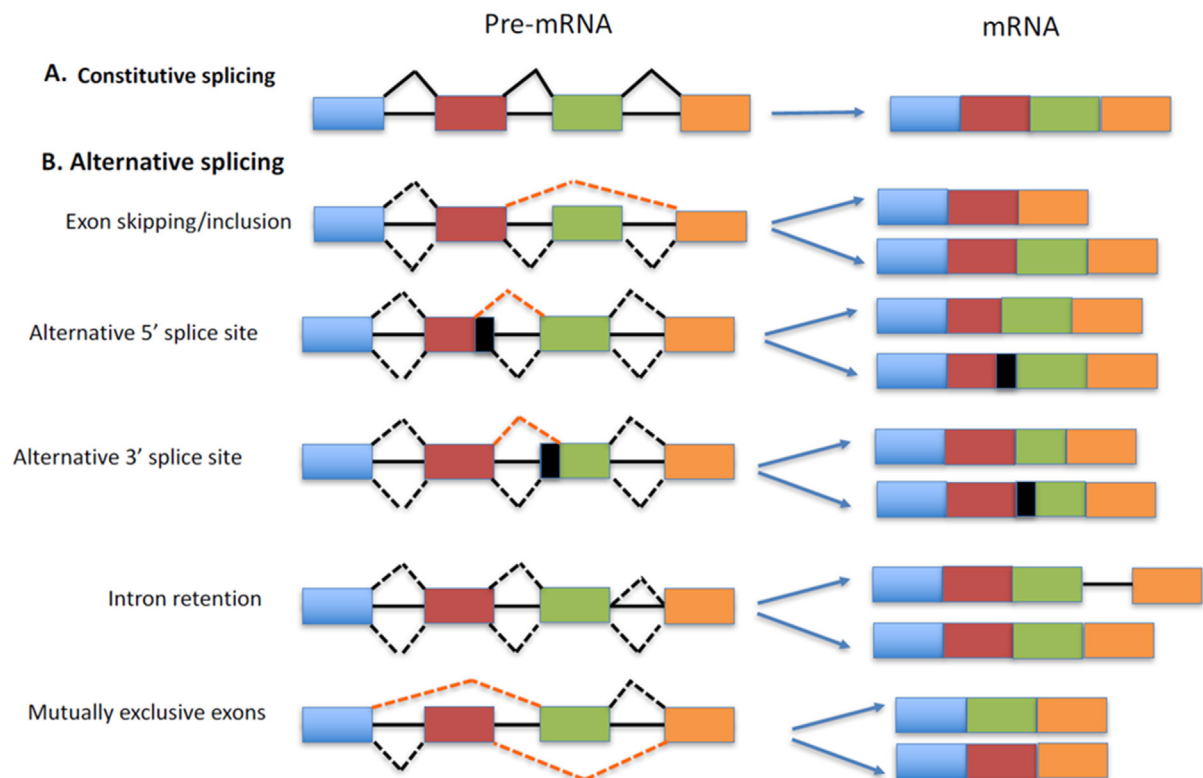


Figure 1. Different types of splicing events are shown schematically. (A) In constitutive splicing, all introns are spliced out and all exons are joined together to produce mRNA. (B) By alternative splicing, pre-mRNA can encode more than one mRNA isoform. Different isoforms can be generated by exon skipping/inclusion of alternative exons, the selection of alternative 5' or 3' splice sites, the retention of intron(s) or selection of the mutually exclusive exon(s). Exons and mRNAs are illustrated as boxes, while introns are represented by solid lines

growth factor II (IGF-II). An IR-B to IR-A switch has been frequently observed in HCC tumors regardless of tumor etiology^[20].

Another study demonstrated the oncogenic role of the truncated isoform of estrogen receptor α (ER- $\alpha 36$) in primary HCC^[21]. In contrast to wild-type ER (WT-ER α), the major variant in normal liver tissue, the ER $\alpha 36$ splice variant has the opposite function in primary HCC, and that ER $\alpha 36$ increases in primary HCC tissue. Also, the high levels of WT-ER α mRNA appear to predict better survival of patients with HCC though the mechanism is yet to be explored.

Similarly, variants 5 and 6 of trans-membrane protein CD44^[22], the variants a and b of extracellular matrix protein osteopontin (OPN)^[23], and variant J of the transcription factor7-like 2 (TCF7L2), also known as T-cell factor 4 (TCF-4)^[24], are some oncogenic isoforms that contribute to the development of HCC. Thus, selectively targeting these oncogenic isoforms would be a promising therapeutic strategy for HCC.

Regarding tumor suppressors, aberrant splicing of *Hugl-1* transcripts has been identified in HCC specimens. The majority of these aberrant *Hugl-1* transcripts encode truncated proteins lacking one or more conserved WD-40 repeat motifs that resulted from skipping part of and/or entire exon or insertion of intron sequences. Two truncated *Hugl-1* proteins were found exclusively in HCC tissues. Aberrant *Hugl-1* transcripts (78.3%, 20 of 23) had a short “direct repeat” sequence flanking their deleted regions. Over-expression of two representative HCC-derived aberrant *Hugl-1* variants was shown to promote HCC cell migration, invasion, and tumorigenicity in nude mice. Moreover, the abnormal *Hugl-1* was significantly correlated with poor differentiation and large tumor size of HCC. This suggests that *Hugl-1* mRNA is frequently mutated by aberrant splicing in HCC, which may be involved in HCC^[25,26].

Some tumor suppressors are also self-inactivated in HCC by alternative splicing. The tumor suppressor isoform of *TP73* gene is *TAp73*, which promotes apoptosis and limits the anchorage-independent growth of tumor cells. Truncated isoforms ($\Delta\text{Ex}2\text{p}73$, $\Delta\text{Ex}2/3\text{p}73$, and $\Delta\text{N}^{\text{p}73}$) of *TP73* are generated by aberrant splicing and serve as dominant negative inhibitors of *TAp73* and inhibit its tumor suppressor activity^[27]. Several studies have shown that these isoforms are over-expressed in HCC compared to normal liver and correlated with poor patient prognosis^[28]. In HCC, $\Delta\text{Ex}2\text{p}73$ expression is correlated with activation of the epidermal growth factor receptor (EGFR) and the down-regulation of the mRNA splicing factor *Slu7*. From a mechanistic perspective, activation of EGFR by its ligand amphiregulin (AR), whose expression is up-regulated in HCC, and c-Jun N-terminal kinase-1 activity facilitates *TAp73* alternative splicing and $\Delta\text{Ex}2\text{p}73$ production^[27].

Tumor suppressor KLF6 that regulates many genes involved in cell cycle, apoptosis and differentiation also inactivated by its dominant-negative SV1 isoform in HCC^[29]. Studies found that SV1 isoform of KLF6 is over-expressed in HCC that promotes cellular proliferation and KLF6 full form is decreased in HCC tissue. The oncogenic activation of the Ras/PI3K/Akt pathway and subsequent down regulation of splice regulatory protein ASF/SF2 or SRSF1 leads to this aberrant splicing of KLF6 in HCC. Also, upstream of Ras, the EGFR tyrosine kinase activity could potentially trigger KLF6 SV1 generation^[29]. These findings suggest potential antagonistic functions of the two isoforms in HCC and relative abundance of the isoforms might dictate the cellular fate. Thus, unraveling the regulatory mechanisms that promote these aberrant splicing might provide effective molecular targets for HCC therapy.

ABERRANT REGULATION OF SPLICING FACTORS AND ONCOFETAL TRANSFORMATION IN HCC

Aberrant expression or activity of splicing factors is a major cause of splicing deregulation; thus, it is quite expected that, increased or decreased expression of crucial splicing factors leads to disease. Indeed, deregulation of splicing regulators such as SRSF1, SRSF10, RBFOX2, MBNL1/2, and QKI proteins has been observed and accounts for hundreds of altered alternative splicing events present in multiple cancer types^[30-32]. In HCC, the splicing dysregulation may be influenced by down-regulation of splicing factors ESRP2, CELF2 and SRSF5 and up-regulation of splicing factors SRSF1 or SF2/ASF, SRSF2, hnRNPA1, hnRNPA2B1, hnRNPH and CUGBP1. In multiple HCC samples, decreased expression of ESRP2, CELF2 and SRSF5 were observed^[17], whereas, a significant correlation was found between the increased expression of IR-A and up-regulation of splicing factors SRSF1, hnRNPA1, hnRNPA2B1, hnRNPH, and CUGBP1^[20]. This observation is in agreement with the previous *in vitro* studies that showed, SRSF1, CUGBP1 and hnRNPA1 promote IR-A expression in hepatoma cell-lines^[33,34]. Studies showed that overexpression of the SR proteins SRSF1 and SRSF3 promote tumor growth in nude mice and these proteins are elevated in certain cancers^[35]. Interestingly, in the mouse model, hepatocyte-specific deletion of SRSF3 caused spontaneous HCC with aging^[36], suggesting that the function of individual splicing factor depends on the cellular context. It was observed that the splicing factors that play important roles in the maturation of liver, down-regulation of those factors promoting HCC. Studies have shown that embryonic liver development and HCC share similar alterations in many genetic programs, and HCC patients with gene expression profiles similar to embryonic stem cells had a worse prognosis^[37,38]. Also, in case of HCC, it has been observed that different mRNA isoforms that are developmentally regulated and not generally expressed in the adult liver, are often expressed in cancer tissue. However, little is known about the mechanisms driving hepatocellular dedifferentiation during chronic liver diseases and tumor development.

Expression of splicing factor *Esrp2* is increased in the adult liver as this splicing factor plays an important role in mesenchymal to epithelial transformation (MET) that is the opposite of epithelial to mesenchymal transformation (EMT), observed in cancer tissues. Studies showed that, homozygous knockout of *Esrp2*^[39] led to impaired adult splicing patterns in the liver in the mouse model, suggesting the role of this splic-

ing factor in the fetal to adult transition in hepatocytes. Consistent with these findings, knockdown of pre-mRNA splicing regulator SLU7 in human liver cells and mouse liver resulted in profound changes in pre-mRNA splicing of genes essential for hepatocellular differentiation and reversion to a fetal-like gene expression pattern^[40]. Moreover, *Slu7* expression has been found to be significantly compromised in chronic liver diseases and in HCC^[27] suggesting a role of SLU7 down-regulation in the progression of liver pathogenesis. Interestingly, SLU7 also preserves survival of HCC cells and other solid tumors via oncogenic miR-17-92 cluster expression^[41] indicating a complex regulatory role of this splicing factor in pathogenesis of liver diseases.

Hepatocyte-specific deletion of SRSF3 caused impaired hepatocyte maturation and also glucose and lipid metabolism in early adult life^[42]. Loss of SRSF3 facilitates expression of the mitogenic isoform of insulin receptor (IR-A) that is generally not expressed in adult liver allowing aberrant activation of mitogenic signaling. Loss of SRSF3 in hepatocytes also promotes aberrant splicing and expression of EMT genes and activates Wnt/beta-catenin signaling leading to c-Myc induction. Additionally, loss of SRSF3 promotes inclusion of the profibrogenic EDA exon in fibronectin gene (FN1) and expression of the short isoform of XBP1 (XBP1s) in hepatocytes and SRSF3 knock-out mice developed spontaneous HCC with aging^[36]. In support of this, SRSF3 has also been found to be reduced or mislocalized in human HCC^[40], suggesting a potential preventive role of SRSF3 in HCC. Interestingly, a recent report suggests XBP1s as a newly discovered molecule involved in the HCC progression by promoting EMT^[43] by enhancing the expression of Twist and Snail. Pathological analysis showed that the expression of XBP1s was closely correlated with distant metastasis.

Recently, Yuan *et al.*^[44] identified an important oncofetal protein, MBNL3, and an oncofetal splicing event, inclusion or skipping of lncRNA-PXN-AS1 exon 4, both of which play vital roles in hepatocarcinogenesis and serve as prognostic biomarkers and therapeutic targets for HCC. This suggests that identifying the common molecular events between embryonic liver development and HCC would promote the understanding of molecular pathogenesis of HCC and the development of more effective targeted therapies.

SUMMARY AND PERSPECTIVES

The findings reviewed here, though handful, are sufficient to show that the AS plays a very critical role in regulating HCC progression and diagnostic. Thus, understanding the mechanisms of alternative pre-mRNA splicing for HCC related genes are important for the development of new therapeutic strategies such as targeting HCC specific isoform as biomarkers and targeting oncogenic isoform.

With the fast development of technologies, next generation sequencing provides a powerful way to study the transcriptome to uncover the aberrant splicing events in different cancers including HCC. For example, analyzing the ultra-deep transcriptome landscape of human liver cancer, Lin *et al.*^[45] identified potential biomarkers for HCC, including *ALG1L*, *SERPINA11*, *TMEM82* and *DUNQU1* and the AS event of *FGFR2*. Using antisense oligonucleotides or splicing switch oligonucleotides that can complementarily bind to a target site in pre-mRNAs and regulate the splicing could be used to selectively target specific isoforms of RNA with oncogenic potential^[46,47]. Targeting specific isoforms of RNA and protein has the potential to improve drug efficacy and reduce side effects. In summary, we are hoping that the integration of pre-mRNA alternative splicing in the pathogenesis of HCC will contribute to the better understanding of the disease and development of new therapies.

DECLARATIONS

Authors' contributions

The author contributed solely to the review.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

The author declares that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Fat and hepatocellular carcinoma

Clara Balsano^{1,2}, Cristiana Porcu^{1,2}, Silvia Sideri^{1,2}, Simona Tavoraro^{1,2}

¹MESVA Department, University of L'Aquila, L'Aquila 67100, Italy.

²Francesco Balsano Foundation, Rome 00198, Italy.

Correspondence to: Prof. Clara Balsano, MESVA Department, University of L'Aquila, L'Aquila 67100, Italy.

E-mail: clara.balsano@cc.univaq.it

How to cite this article: Balsano C, Porcu C, Sideri S, Tavoraro S. Fat and hepatocellular carcinoma. *Hepatoma Res* 2018;4:38.

<http://dx.doi.org/10.20517/2394-5079.2018.51>

Received: 28 May 2018 **First Decision:** 25 Jun 2018 **Revised:** 7 Jul 2018 **Accepted:** 8 Jul 2018 **Published:** 25 Jul 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Obesity and diabetes are associated with the onset of hepatocellular carcinoma (HCC). These two illnesses correlate also with the development of non-alcoholic fatty liver disease (NAFLD). Currently, NAFL is considered the leading form of chronic liver disease in the Western industrialized countries. Insulin resistance is the common pathogenic factor among these three pathologies. NAFL is characterized by fat accumulation in the liver that involves greater than 5% of the liver parenchyma with no evidence of hepatocyte injury. However, NAFL may progress toward non-alcoholic steatohepatitis that in turn may lead to advanced fibrosis, cirrhosis and HCC. It is alarming that NAFLD related HCC has been, at present, considered as a growing burden worldwide, and its prevalence is tending to further increase together with the increasing incidence of obesity and diabetes. Worthy of note is that in the presence of chronic accumulation of fat in the liver it has been reported the emergence of HCC during chronic liver disease in absence of liver cirrhosis, usually the major risk factor for the development of HCC. Thus, in the future NAFLD related HCCs will place a growing strain on health-care systems from the need for their management. Unfortunately, most of the NAFLD related HCC patients are diagnosed at advanced stages and are characterized by a poor prognosis, because they are ineligible to radical treatments. Thus, it is urgent to boost up new screening policies to make early diagnoses, as well as to develop preventive-therapeutic strategies.

Keywords: Hepatocellular carcinoma, obesity, non-alcoholic fatty liver, non-alcoholic steatohepatitis, copper

INTRODUCTION

In Western countries the growing epidemics of obesity and type 2 diabetes are associated with increasing incidence of hepatocellular carcinoma (HCC)^[1]. These two conditions are strictly associated with the development of non-alcoholic fatty liver disease (NAFLD) and considered the leading forms of chronic liver



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disease in the Western industrialized countries^[2]. NAFLD has a wide geographic distribution to take on epidemic proportions: prevalence of NAFLD has been reported to be 25% worldwide. The highest prevalence is reported from South America (31%) and Middle East (32%), followed by Asia (27%) and USA (24%), while the prevalence is lowest in Africa (14%). In Europe the median prevalence is 23%-26% with variations in different European populations^[3].

NAFLD is caused by an insulin resistance and, as reported above, often occurs with the presence of diabetes, obesity, and metabolic syndrome; therefore, liver can be considered the alarm bell of all of these pathologies^[4]. The progressive form of NAFLD is non-alcoholic steatohepatitis (NASH) that can lead to advanced fibrosis, cirrhosis and HCC. It is worrying that NAFLD related HCC has been at present recognized as a growing burden worldwide, and its impact is expected to further grow together with the increasing incidence of obesity and diabetes^[4].

Of particular interest is the emergence of HCC during chronic liver disease in absence of liver cirrhosis, that is known as the major risk factor for HCC development^[5,6].

HCC development requires decades and is characterized by a gradual transition through a dysplastic to transformed liver tissue^[7]. Liver transformation is the result of uncontrolled cell growth that results in the accumulation of genomic alterations occurring during cell division thus becoming the driving force for tumorigenesis. Five mechanisms are involved in maintaining genomic stability during cell division: (1) high-fidelity of DNA replication in S-phase; (2) precise distribution of chromosomes in daughter cells during mitosis; (3) DNA repair throughout the cell cycle; (4) cell cycle checkpoints; and (5) induction of apoptosis or senescence in case of genomic instability^[8]. On the other hand, there are multiple oncogenic mechanisms that participate in genomic instability: alterations in the DNA-damage-response pathways, telomere erosion, chromosome segregation defects^[9].

Even if several pathogenic mechanisms, such as obesity-mediated chronic inflammation and diabetes, have been described to be involved in NAFLD related HCC till now, as extensively reported below, we do not have clear ideas on the pathogenic mechanisms driving transformation of the cell during NAFLD^[10,11]. In this context, low grading chronic inflammation has indubitably a crucial role in NAFLD disease progression toward HCC^[12]. During low grade inflammation, the overproduction of reactive oxygen species (ROS) induces the output of advanced glycation end-products (AGEs), advanced lipoxidation end-products (ALEs) and protein oxidation products (PrOPs) in tissues^[13,14], inducing pro-inflammatory cascades and increasing the risk of liver tissue transformation. Thus, to better understand the pathogenic mechanisms underlying NAFLD related HCC, first of all, we should better know all the biological factors involved in promoting inflammation that consequently participate in hepatocarcinogenesis.

Further studies should be performed to highlight new insights in the pathogenesis of HCC during NAFLD. However, scientific consensus exists on the concept that the progression of NAFLD toward HCC is surely linked not only to environmental but also genetic factors. Accordingly, genome-wide association studies highlighted several single nucleotide polymorphisms (SNPs) associated with the pathology of NAFLD [Table 1]. Furthermore, induction of epigenetic alterations due to unhealthy diet and/or other environmental factors are surely involved in NAFLD related HCC.

Perspective studies are needed to implement screening strategies and preventive approaches for NAFLD-related HCC development, particularly in the non-cirrhotic population. The notions reported in this review, describing several NAFLD-related molecular target pathways, will be useful to clinicians to outline diagnostic and prognostic profiles of these complex and heterogeneous patients.

Table 1. Single nucleotide polymorphisms associated with the pathology of nonalcoholic fatty liver disease

Gene	SNP	Region	Location	Functional class	Total allele frequency (Gnomad)
PNPLA3	rs738409-G	22q13.31	22:43928847	missense_variant	0.2709
PNPLA3	rs2896019-G	22q13.31	22:43937814	intron_variant	0.1981
SAMM50	rs738491-T	22q13.31	22:43958231	intron_variant	0.356
SAMM50	rs2143571-A	22q13.31	22:43995806	intron_variant	0.2495
GCKR	rs1260326-T	2p23.3	2:27508073	missense_variant	0.6381
GCKR	rs780094-T	2p23.3	2:27518370	intron_variant	0.6702
GATAD2A	rs4808199-A	19p13.11	19:19434290	intron_variant	0.1817
COL13A1	rs1227756-G	10q22.1	10:69828748	intron_variant	0.4676
FDFT1	rs2645424-A	8p23.1	8:11826954	intron_variant	0.5508
CRACR2A	rs887304-A	12p13.32	12:3648382	3_prime_UTR_variant	0.764
SAMM50 - PARVB	rs2073080-T	22q13.31	22:43998522	intron_variant	0.2017
EHBPIL1	rs6591182-A	11q13.1	11:65582285	missense_variant	0.4756
KLRG1	rs6487679-G	12p13.31	12:9218736	intergenic_variant	0.8025
ZNF512	rs1881396-T	2p23.3	2:27621734	3_prime_UTR_variant	0.2063
MUM1	rs2668423-T	19p13.3	19:1370527	intron_variant	0.7159
ACTR5	rs6128907-C	20q11.23	20:38759219	intron_variant	0.1645
KHDRBS3 - RNU1-35P	rs4243849-G	8q24.23	8:135700894	intergenic_variant	0.3522
FARP1	rs9584805-G	13q32.2	13:98341776	intron_variant	0.3288
LOC643381 - CNTN5	rs4237591-G	11q22.1	11:98595538	intergenic_variant	0.3955
SLC38A8	rs11864146-A	16q23.3	16:84013110	intron_variant	0.169
SLC9A9	rs2800-G	3q24	3:143705980	intron_variant	0.6618
FDFT1	rs2645424-A	8p23.1	8:11826954	intron_variant	0.5508
LCP1	rs7324845-A	13q14.13	13:46129007	intron_variant	0.8398
ST8SIA1	rs2216228-G	12p12.1	12:22212901	intron_variant	0.1949
SLC9A9	rs7632299-A	3q24	3:143337625	intron_variant	0.2716
ETS1	rs3935794-G	11q24.3	11:128520782	intron_variant	0.07114
RNA5SP489 - RPL13AP7	rs9977253-G	21q21.2	21:25272769	intron_variant	0.7688
EEF1A1P20 - MTCYBP22	rs10067427-G	5q21.1	5:100006343	intergenic_variant	0.4205
YIPF1	rs11206226-A	1p32.3	1:53854664	intron_variant	0.03217
SDK1	rs688020-C	7p22.2	7:4188921	intron_variant	0.4197
MACROD2	rs6079395-A	20p12.1	20:14347253	intron_variant	0.5135
CACNA2D1	rs10954668-A	7q21.11	7:82218335	intron_variant	0.2566
COL13A1	rs7077164-A	10q22.1	10:69823442	intron_variant	0.35
TEX36	rs10510146-A	10q26.13	10:125607576	intron_variant	-
SEL1L3	rs959903-A	4p15.2	4:25808474	intron_variant	0.2551
NGF - TCEB1P20	rs7552722-A	1p13.2	1:115378734	intergenic_variant	0.6805
CDH2 - ARIH2P1	rs11083271-A	18q12.1	18:28346095	intergenic_variant	0.2673
SDR42E1P5 - IL18RAP	rs11465670-C	2q12.1	2:102417980	upstream_gene_variant	0.1239
SLC46A3	rs1305088-A	13q12.3	13:28704313	non_coding_transcript_exon_variant	0.854
RAB37	rs12942311-C	17q25.1	17:74714657	intron_variant	0.2134

EPIDEMIOLOGY

HCC causes more than 700,000 deaths/year worldwide and accounts for 70%-85% of cases of liver cancers. HCC is the fourth most often diagnosed cancer in males (70% occur over age 50) and the seventh in females^[15]; moreover, it represents the overall second cause of cancer deaths^[16,17]. These statistics reflect the poor prognosis of liver cancer worldwide.

About 80% of HCC cases occur in less developed countries and are typically associated with alcohol, chronic hepatitis B (HBV) and C (HCV) infections: importantly, the incidence in these countries is decreasing^[18,19]. On the other hand, in western countries the HCC incidence is increasing, ranging from 2.4% over 7 years to 12.8% over 3.2 years of median follow-up period, following the geographic distribution of obesity^[4]. In particular, 10 year annual cumulative risks of HCC in alcohol, HCV or NAFLD are 1.1%, 2.9% and 3.1%,

respectively^[20]. Accordingly, an increasing number of HCC has been reported in the setting of obesity and diabetes^[15,21] and it has been associated with an increased relative risk of dying for HCC^[22].

Unfortunately, even if consistent epidemiological data concerning viral and alcoholic hepatitis have been reported, there is a lack of strong epidemiological results regarding the incidence and prevalence of NAFLD-related HCC. The problem is mainly due to the absence of a correct and clear definition of NAFL/NAFLD/NASH. Thus, so far we cannot evaluate the real dimension of NAFLD-related HCC and how to lower and prevent its appearance.

A few longitudinal outcome studies reveal that the cumulative mortality in NAFL/NASH, in a follow-up period between 5.6 and 21 years, vary from 0% to 3%^[23], but we have to take into account that there are 400,000 and 40,000-80,000 new cases/year of NAFL and NASH, respectively.

Finally, the unquestionable evidence showing the increased risk of HCC in patients with NAFLD, and mainly its appearance in non-cirrhotic patients, is in close association with the alarming and more rapidly increasing indication for liver transplantation in respect to any other liver disease^[24].

PATHOGENESIS

The aberrant activation of immune response and inflammation signaling observed in NAFLD have a key role in the pathogenesis and progression of this liver disease.

The accumulation of lipids in patients with NAFLD may induce an intracellular chronic status of oxidative stress that, in turn, leads to the activation of low-grade inflammation. The enlargement of adipocytes may lead over time to the rupture of these cells. As a consequence, macrophages are recruited in the site of inflammation and M1/M2 macrophage polarization is induced. The activation of macrophages stimulates the production of adipose tissue related adipocytokines, that, once released in the systemic circulation, reach different organs, including liver^[25,26]. The inversion of M1/M2 ratio is due to the increase of M1 macrophages and reduction of M2 macrophages^[26]. The higher number of M1 cells cause an over production of several pro-inflammatory cytokines, such as IL-1 β , IL-6, IL-8, IL-12, and TNF- α .

Consequently, the serum of NAFLD patients is characterized by the presence of high levels of TNF- α and IL6, that in turn are correlated with a higher risk of progression to NASH^[27-30]. Moreover, higher levels of TNF- α induce insulin resistance^[31-33] and contribute to exacerbate the liver damage through the activation of nuclear factor-kappa-B (NF κ B) inflammatory pathways^[34]. Furthermore, NF κ B protein has been recently found to be involved in the regulatory feedback of two important chemokine receptors: C-X-C chemokine receptor type 4 and 7 (CXCR4/7)^[35]. The NF κ B-CXCR4/7 axis mediates the signaling of toll-like receptors, TLR3 and TLR4, promote, in this way, the progression of NASH towards HCC^[36].

Thus, deeply understanding the role of chronic inflammation as underlying the cause of liver transformation will improve the prevention and cure of this cancer.

An incorrect lifestyle is currently considered the main predisposing factor of NAFLD-related HCC. In fact, the development of HCC in NAFLD includes low-grade chronic inflammatory response (NASH) associated with genetic alterations, oxidative stress, obesity, insulin resistance and alteration of gut microbiota [Figure 1]. The pathogenic mechanisms involved in the progression of NAFL toward NASH are characterized by two hits: excess accumulation of triglyceride (TG) in the hepatocyte and, in a second moment, induction of oxidative stress and inflammation by several factors, such as free radicals^[37]. In line with these findings, more and more researchers are recognizing the central role of low-grade inflammation in inducing all of the

Table 2. Summary of miRNAs significantly associated with NAFLD, NASH and HCC patients

Disease	Upregulated miRNAs	Downregulated miRNAs	References
NAFLD	miR-21, miR-34a, miR-122 (serum), miR-146b-5p (tissue), miR-181b, miR-451	miR-29a, miR-139-5p, miR-30b-5p, miR-122-5p (tissue), miR-155, miR-422a, miR-181d, miR-99a, miR-197, miR-146b (serum)	[49-56]
NASH	miR-21, miR-33a, miR-34a, miR-122, miR-144, miR-192, pri-miR-7-1, pri-miR-26a-1/2	miR-125b, miR-451	[50,51,57-60]
HCC	miR-10a, miR-21 (tissue), miR-23a, miR-31, miR-34a-5p, miR-93-5p, miR-122, miR-155, miR-183, miR-221-3p, miR-222-3p, miR-375, miR-423	let-7f, miR-16, miR-21 (serum), miR-24, miR-30e, miR-99a, miR-106b, miR-125b, miR-145, miR-146a, miR-148a, miR-155, miR-183, miR-199a, miR-199a3p, miR-200c, miR-215, miR-223, miR-229, miR-7706	[50,61-67]

HCC: hepatocellular carcinoma; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis

NAFLD-related comorbidities, such as insulin resistance, diabetes, and cardiovascular disease. Accordingly, NASH is a recognized cause of cirrhosis and is associated with development of HCC^[38].

In this context, it is worth mentioning that multiple additional mechanisms may be implicated in the progression from NAFLD to NASH and HCC. In fact, keeping in mind that there are a growing number of patients who can progress from NAFLD to advanced fibrosis in the absence of significant inflammation, the alterations in immunologic, endocrine and metabolic pathways have a key role in the progression of NASH toward HCC.

Accordingly, despite the few data on NAFLD-related hepatocarcinogenesis, it has been highlighted that the phosphoinositide 3-kinase (PI3K)-AKT-mTOR pathway, implicated in the control of cellular energetic homeostasis, is deregulated in over 50% of NAFLD-related HCCs^[39].

The β -catenin/WNT signaling, that has a crucial role in cell proliferation, stem cell self-renewal and cell migration, was found affected by somatic mutation in > 37% of NAFLD-related HCC^[39].

Below we reported a detailed description of some factors involved in HCC development in patients with NAFLD.

Regarding the genetic factors involved in the progression from NAFLD to HCC, recent genome-wide studies have highlighted genetic heterogeneity of liver cancers. Of note, some SNPs, such as Patatin-like phospholipase domain-containing 3 (PNPLA3) gene variant I148M, have been related to the development and progression of NAFLD, NASH and NAFLD-related HCC, whereas others, such as the transmembrane 6 superfamily member 2 (TM6SF2) gene variant E167K, have been mainly correlated with the development of cardiovascular diseases^[5,40,41]. In this context, the most recent findings from genomic profiling let us better understand that different pathways are involved in the initiation and progression of liver cancer^[42], as shown in [Figure 1](#).

In addition, altered transcriptional gene expression might be linked to inappropriate microRNAs (miRNAs)-guided transcriptional control. The human genome is envisaged to encode approximately 1000 miRNAs^[43], which are a perfect class of blood-based biomarkers for cancer detection^[44]. MiRNAs are endogenous 19-24 nucleotides noncoding single-stranded RNAs, which control, at post-transcriptional level, many complementary target mRNAs implicated in several pathophysiological processes, such as cell proliferation, differentiation, metabolism, apoptosis and cancer^[45]. Lack of miRNA processing enzymes in cancer cells promotes tumor invasiveness and more aggressive phenotypes, revealing their main role in controlling tumor- and metastasis-initiating events^[46-48]. Accordingly, different sets of miRNAs have been specifically correlated with NAFLD, NASH and HCC [\[Table 2\]](#)^[49-67]. Among the miRNAs recently identified in NAFLD patients, it is worth mentioning the up-regulation of miR-146b-5p, miR-181b and miR-375, and the down-regulation of miR-29a, miR-30b-5p, miR-122-5p, miR-139-5p, miR-155 and miR-422a^[49,53-56]. In addition, in NASH it has

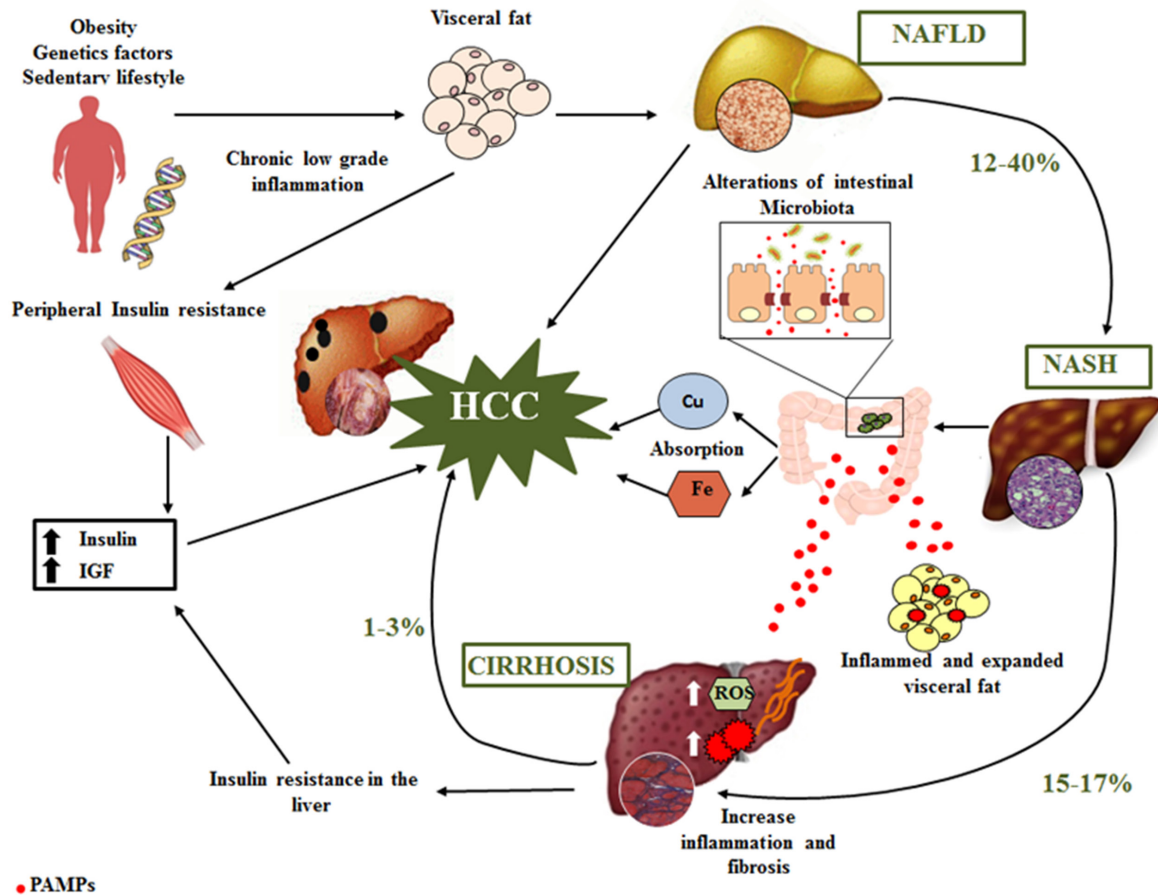


Figure 1. Pathogenic mechanisms involved in the development of HCC. HCC: hepatocellular carcinoma; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; IGF: insulin-like growth factor; ROS: reactive oxygen species; PAMPs: pathogen-associated molecular patterns

been reported the up-regulation of miR-33a and miR-144 and the down-regulation of miR-451^[58,60]. Finally, in NAFLD-related HCC the up-regulation of miR-10a, miR-33a, miR-144, miR-155, miR-183, miR-375 and miR-423 and the down-regulation of miR-229 and miR-7706 were found^[65-67].

MiRNA analyses, in combination with other clinical parameters and standard liver examinations, may be extremely useful to predict the possible progression of NAFLD toward HCC, and for monitoring the response to treatments^[50]. However, despite the association between definite miRNA signatures and pathogenesis of NAFLD-related HCC, the expression levels of specific hepatic miRNAs during liver tissue transformation are still controversial. Further studies are needed to shed light on their function in the context of NAFLD-related HCC.

Regarding oxidative stress, reactive oxygen species (ROS) have a central role in HCC onset. ROS, in fact, are noticeable factors playing an essential role in regulating cell homeostasis. In this regard, our group has recently highlighted the role of altered systemic biometals distribution in NAFL/NASH patients and the associated increasing levels of ROS^[68]. Accordingly, toxic biometal accumulation is a common feature in many cancers. Moreover, perturbations of mechanisms that control transcripts encoding proteins that regulate biometals have been described in cancer cells, including differences in epigenetic control (methylation and acetylation), miRNAs expression and protein activities^[68,69].

In particular, biometals have the ability to catalyze oxidation-reduction reactions, which can lead to the production of ROS, thus their tight homeostatic regulation should be always present in the body. Accordingly, although the mechanisms are at present still unclear, a contribution of iron (Fe), copper (Cu) and zinc (Zn), in the development of HCC, has been often suggested^[68,70-72]. In fact, dysregulation of Fe, Cu or Zn homeostasis stimulate proliferation, modulate the expression of epithelial mesenchymal transition (EMT) related proteins, glycolysis, and antioxidant molecules, such as SOD1 and 2, HIF1, GSH, in various cancer cells and human tumors^[68,69,73-75].

Accordingly, NASH and NAFL patients display higher iron absorption after the administration of an oral iron absorption test and its deposition has been related to HCC development in NAFLD-cirrhosis^[76]. The underlying mechanisms are not already clear, but might be related to oxidative DNA damage^[77]. Interestingly, a recent meta-analysis highlighted that HFE mutations C282Y and H63D, associated in homozygosity with hemochromatosis, were characterized by a higher risk of HCC in NAFLD patients^[78]. In addition, our group highlighted that a statistical significant enhancement of serum copper levels has been reported in NAFLD-cirrhotic patients and the altered homeostasis of this biometal was even more evident in HCC patients. In the presence of higher concentrations of extracellular copper liver cells are sensitized to transformation. The pathogenic copper-related pro-oncogenic mechanism seems to be, at least in part, managed by MYC, which is able to directly bind a specific region of the CTR1 promoter, regulating its transcription^[68]. In this regard, it is really interesting the recent study reporting that Golgi protein 73 (GP73) is an effective and reliable serological marker for the diagnosis of advanced fibrosis and prediction of appearance of cirrhosis^[79]. The awareness that copper serves as a limiting factor for multiple aspects of tumor progression, including growth, angiogenesis and metastasis suggests more attention to be paid to the potential and undiscovered role of copper-specific chelators as effective therapeutic agents against HCC.

The prevalence of obesity is increasing worldwide as well as the link between obesity and cancer, becoming an important and accepted risk factor for the development of HCC. As reported above, it is currently accepted that NAFLD is caused by an insulin resistance and often appears in the presence of obesity. The relationship between obesity and HCC was supported by a cohort study in Italy. In this work the odds ratio progressively increased in the patients who have associated metabolic syndrome factors^[80]. Obesity is characterized by the excess of adipose tissue and the altered secretion of adipocytokines that correlate with the occurrence of HCC and liver-related death in patients with cirrhosis^[81]. In the last decade, it has become evident that obesity-related metabolic inflammation is involved in different aspects of HCC progression and metastatic dissemination, among which: neural regulation, innate immune responses, intestinal immune system and endocrinal regulation. Unfortunately, only few studies have been focusing on long-term mechanisms involved in obesity related HCC development^[82], thus prospective studies are needed.

Finally, alterations in intestinal microbiota (or dysbiosis, defined as any change in the composition of the microbiota commonly found in healthy conditions), creating a pro-inflammatory microenvironment in the liver, seem to play a main role in the development of NAFLD-related HCC^[83]. Dysbiosis, beyond the known risk factors for NAFLD, promotes the development of chronic liver diseases and HCC, independent of body mass index (BMI) and insulin resistance, producing a large amount of bioactive molecules, which deeply affect physiological and pathological body status^[84]. Interestingly, in a mouse model, drugs able to modify the microbiome (e.g., rifaximin) may prevent HCC development. Rifaximin may additionally improve portal hypertension, spontaneous bacterial infection (SBP) risk, liver fibrosis and hepatic encephalopathy^[85]. Actually, metabolic alterations have been associated with dysbiosis: ob-ob mice (homozygous for the obese mutation) have an imbalance of the intestinal microbiota with a decrease of Bacteroides and an increase in Firmicutes. This pattern of intestinal bacteria has the increased capacity to harvest energy from diet^[86], as well as the microbiota composition described in NAFLD^[57]. The altered microbioma (the genetic information genomes of gut microbiota) is characterized by the ability to produce alcohol, which in turn will be increased in the

blood promoting hepatic oxidative stress and liver inflammation^[87]. As demonstrated in obese patients, the equilibrium can be restored in case of a fat restriction diet^[83].

Target/biomarker discovery and “Omic” approaches will help in finding new pro-oncogenic and oncosuppressor to be used as novel biomarkers. The new knowledge on HCC pathogenesis will open new avenues in the diagnosis and design of patient-tailored therapies.

HCC IN NAFLD CHRONIC HEPATITIS

NAFLD has a proportion of HCC, occurring in the absence of cirrhosis, higher than other chronic liver diseases. HCC in NAFLD generally lacks encapsulation and is well differentiated and characterized by large dimensions^[88]. Multiple studies described a significant proportion of HCC (from 51% to 65%) that have stage 0-2 fibrosis^[89-91], highlighting a specific dangerous behavior of NAFLD chronic hepatitis. Given the high number of patients with non cirrhotic NAFLD, screening for HCC in this population is not practicable^[15]. Interestingly, the features of NAFLD-related HCC are similar to those of HCC of obese patients and of non-cirrhotic HCC, independently of the etiology^[92,93]. Accordingly, it has been reported that obese patients have a relative risk of liver cancer of 189% relative to the 117% of overweight subjects^[94]. Thus, the pathogenic mechanisms of hepatocarcinogenesis in steatosis might be different from the classic mechanisms involved in cirrhosis^[95]. In fact, all the NAFLD-related HCC pathogenic mechanisms are independent from fibrosis and this might explain the particular epidemiology of HCC in NASH, where non-cirrhotic HCC is quite frequent relative to other etiological factors.

In the light of what has been reported above, pathophysiological studies are needed to better understand the underlying mechanisms involved in NAFLD-related HCC development. In this context, it is important to note that the EASL evidence based clinical practice guidelines should be improved because the up-to-date version does not exhaustively represent this specific problem.

HCC IN NAFLD CIRRHOSIS

Cirrhosis in NAFLD modifies prognosis and management. Increasing age, obesity and diabetes are considered as risk factors for the progression of NAFLD to cirrhosis^[96]. Thus, it is well known that a subset of individuals with NAFLD may progress to liver cirrhosis, which in turn could be complicated by liver failure or even HCC, requiring liver transplantation (LT), resection, or loco-regional therapies^[97].

However, although NAFLD has begun the most common cause of chronic liver disease worldwide^[3,98], even today, a significant amount of patients with NAFLD are already incidentally diagnosed with cirrhotic. Unfortunately, NAFLD patients are asymptomatic, thus, the diagnosis of cirrhosis often occurs incidentally (70%) because it is done during clinical assessments for the investigation of different medical conditions unrelated to liver disease or an unexpected surgical finding. Accordingly, about the 15% of NAFLD patients selected for biopsy have cirrhosis, confirming that the prevalence of cirrhosis in patients with NAFLD is higher than expected^[99]. In the presence of liver cirrhosis, the main problem is the occurrence of important complications, such as: liver decompensation, thrombocytopenia, splenomegaly or, sometimes, HCC related with a poor survival^[100,101]. Late diagnosis increases the risk to find a late stage HCC, no longer curable with the available treatments, whereas the diagnosis of HCC, if done at the early stage, is associated with better results.

Cirrhosis has to be seen as a prognostic factor predicting negative outcomes in patients. Accordingly, in recent studies, it has been reported in NAFLD cirrhotic patients an overall mortality of 80% and a liver-related mortality of 55%, after 12 years^[99].

Early recognition of NAFLD patients with cirrhosis, who have a higher risk of progression toward HCC, is the first crucial aim to reduce NAFLD-related morbidity and mortality. Thus, in patients with NAFLD, an improvement of diagnostic approach alertness is required for underrating the prevalence and the important clinical condition of NAFLD. Clinicians have developed adequate screening^[102]. Finally, it is important to underline that ultrasonography (US) is likely inadequate in several subgroups of patients (obese, Child Pugh B or C, alcohol and NASH related cirrhotic) and does not permit the exclusion of the presence of HCC^[103].

DECLARATIONS

Authors' contributions

Wrote the manuscript: Balsano C

Contributed critical revisions, edited the manuscript, and read and approved the final version of the manuscript: Balsano C, Porcu C, Sideri S, Tavolaro S

Availability of data and materials

Not applicable.

Financial support and sponsorship

We thank the Francesco Balsano Foundation for financial support.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Oxidative stress and hepatocarcinogenesis

Ying Fu¹, Fung-Lung Chung²

¹Laboratory of Molecular Biology, Center for Cancer Research, NCI, Bethesda, MD 20892, USA.

²Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC 20007, USA.

Correspondence to: Dr. Ying Fu, Laboratory of Molecular Biology, Center for Cancer Research, NCI, Bethesda, MD 20892, USA. E-mail: ying.fu@nih.gov; Dr. Fung-Lung Chung, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC 20007, USA. E-mail: flc6@georgetown.edu

How to cite this article: Fu Y, Chung FL. Oxidative stress and hepatocarcinogenesis. *Hepatoma Res* 2018;4:39. <http://dx.doi.org/10.20517/2394-5079.2018.29>

Received: 24 Mar 2018 **First Decision:** 5 Jul 2018 **Revised:** 12 Jul 2018 **Accepted:** 13 Jul 2018 **Published:** 1 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide. There are two major challenges for HCC, the first being that early detection is generally not applicable, and secondly, it is usually fatal within several months after diagnosis. HCC is an inflammation-induced cancer. It is known that chronic inflammation leads to oxidative/nitrosative stress and lipid peroxidation, generating excess oxidative stress, together with aldehydes which can react with DNA bases to form promutagenic DNA adducts. In this review, the evidence between oxidative stress and liver carcinogenesis is summarized. We focused on the potential of using DNA adducts as oxidative stress biomarkers for liver carcinogenesis.

Keywords: Oxidative stress, DNA adduct, hepatocellular carcinoma, prevention, hepatocarcinogenesis

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide, because of late diagnosis and poor therapeutic outcome^[1-4]. HCC accounts for 5.5% of all cancer cases globally, and particularly the incidence of HCC has been increasing in the US since the 1980s^[5,6]. The incidence of HCC strongly correlates with liver inflammation from exposure to one or several risk factors including hepatitis B virus (HBV), hepatitis C virus (HCV), inherited metabolic diseases, heavy alcohol exposure, obesity, type 2 diabetes and aflatoxins^[7-13].

In this review, we will mainly discuss the role of oxidative stress in hepatocarcinogenesis. The search for reliable biomarkers for liver cancer has been executed in different areas: DNA methylation, genomics, pro-



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teomics, microRNA and liquid biopsy^[14-20]. We want to highlight that promutagenic DNA adducts is a new field which need further investigations in the search of biomarkers for HCC.

HEPATOCARCINOGENESIS AND OXIDATIVE STRESS

More than 90% of HCCs arise in the context of hepatic inflammation^[21-29]. Chronic liver inflammation leads to oxidative/nitrosative stress and lipid peroxidation (LPO), generating excess reactive oxygen species (ROS) and reactive nitrogen species (RNS), together with aldehydes which can react with DNA bases to form promutagenic DNA adducts through either endogenous or exogenous insults^[30]. Oxidative stress has been demonstrated as an important factor to carcinogenesis since the first experiment on ROS-induced transformation of mouse fibroblast cells in the 1980s^[31]. It has emerged as an important player in the development and progression of liver carcinogenesis for different etiologies (e.g., HBV- and HCV- induced liver diseases)^[32]. HCC incidences in the USA are largely associated with HCV-related cirrhosis, but changes observed by epidemiological studies have attributed obesity and diabetes as risk factors as well^[33]. The increased oxidative stress in obesity and diabetes may play a crucial role in hepatocarcinogenesis^[34,35]. Because oxidative stress drives genomic damage and genetic instability to cause mutations, and mutations play a crucial role in carcinogenesis. This notion is supported by the chemopreventive effect demonstrated in a large number of epidemiology studies on the relationship of high fruit and, vegetable consumption with low cancer incidences, among which, antioxidants effects and maintenance of normal DNA repair capacity are indicated to be two crucial mechanisms of actions^[36,37]. The same concept was illustrated when knocking out antioxidant defenses significantly increased the rate of liver cancer, e.g., knock-out mice lacking CuZnSOD (copper-zinc superoxide dismutase) are found to increase liver carcinogenesis^[38]. Another mouse model showed that knocking out nuclear respiratory factor-1 (Nrf1), an essential transcription for mediating oxidative stress, induces steatosis, fibrosis and liver cancer, eventually^[39].

The notion that oxidative stress induces HCC is also supported by studies on hemochromatosis. A positive correlation between mild/excess iron deposition and HCC in patients with hemochromatosis suggests a possible carcinogenic role for oxidative stress induced by iron through Fenton reactions^[40,41]. In the iron-nitrilotriacetic acid rat model of hemochromatosis, elevated genotoxic products from oxidative stress, 4-hydroxyl-2-nonenal (HNE) and malondialdehyde (MDA), are found^[42]. This increase is also accompanied by damaged cellular defense system, for instance, vitamin E level, GSH/GSSG ratio and superoxide dismutase are all decreased. HNE has the potential to damage genomic DNA and cause mutations, e.g., HNE adduct has been demonstrated to cause p53 mutations which are associated with more than 50% of HCC incidences^[43]. A more important link was discovered in patients with hemochromatosis who suffered iron overload and p53 mutations following HCC development^[41,44-46]; it suggests that oxidative stress is an underlying mechanism of HCC carcinogenesis^[44]. The role of oxidative stress in liver carcinogenesis is also supported by the result of a multicenter study: using tissue microarray screening, cytochrome P450 1A2 (CYP1A2) oxidase in non-cancerous tissue is found and validated as the only predictive factor for HCC recurrence^[47].

Oxidative stress is a crucial factor in the initiation and progression of HCC under various pathological conditions^[48]. Oxidative stress can be induced by ROS produced in the mitochondria in non-alcoholic fatty liver disease, which damages hepatocytes, promotes pathologic polyploidization, triggers inflammation, and contributes to insulin resistance^[49-53]. Additionally, oxidative stress is also involved in migration, invasion, and metastasis of HCC^[54-56]. In that, biomarkers of oxidative stress can predict HCC risk and also the recurrence of HCC. Quantitative methods for the evaluation of oxidative stress can be divided into three categories: (1) determination of compounds modified by oxidative stress; (2) determination of the activity of antioxidant enzymes; and (3) determination of oxidative stress indicators containing transcription factors. Serum quantification of derivatives of reactive oxygen metabolites (d-ROM) level, a simple method for measuring hydrogen peroxide, is found to predict the risk of HCC recurrence after surgical resection or radiofrequency

ablation (RFA)^[57]. Since cancer is a genetic disease, we think that mutagenic DNA adducts that arise from oxidative stress have the potential to serve as more direct and precise biomarkers to predict HCC risk and recurrence. A major oxidative stress and promutagenic DNA adduct, 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxo-dG), was found to be increased during hepatocarcinogenesis. It suggests a role of mutagenic DNA lesions in HCC formation^[58,59]. In an HCV/HCC clinical trial, the result supports the hypothesis that HCV induces inflammation that causes oxidative DNA damage (increase of 8-oxo-dG, a DNA lesion), and promotes hepatocarcinogenesis.

LPO induced DNA adducts, including various propano- and etheno- adducts, have been investigated as potential lead markers for various types of inflammatory/oxidative stress cancer-prone diseases (e.g., chronic pancreatitis, Crohn's disease, ulcerative colitis, alcohol related hepatitis, *H. pylori* infection) and cancer initiation/promotion^[60,61]. It is also known that the propano DNA adducts [e.g., γ -hydroxy-1, N^2 -propanodeoxyguanosine (γ -OHPdG)] arisen from lipid peroxidation are mutagenic and associated with liver carcinogenesis^[62]. The levels of propano DNA lesions are the balance of oxidative stress induced LPO and DNA repair. Nucleotide excision repair (NER) pathway is mainly responsible for repairing these bulky DNA adducts^[43,63,64]. Patients with HBV may exhibit inefficiency of removing bulky DNA adducts because HBx protein has been shown to inhibit NER pathways through suppressing XPB and XPD helicases [transcription factor IIIH (TFIIH)]^[65]. We reason that DNA adducts possibly play a role of causing mutations by HBV, but further testing should be done to prove this hypothesis.

γ -OHPdG is an endogenous product of acrolein, a reactive aldehyde generated by LPO^[66]. γ -OHPdG is known to cause G to T and G to A mutations that may involve critical genes such as *p53*^[67-70]. Our recent studies demonstrated an association of the levels of γ -OHPdG with HCC development in a NER deficient mouse model with spontaneous HCC development. It is also found that antioxidants can suppress γ -OHPdG and prevent liver cancer significantly^[71,72]. Further analysis found that GC>TA mutation is the dominant alteration, accounting for approximately 90% of mutations. The high GC>TA mutation frequency implies that γ -OHPdG may play a role in the mutagenesis of HCC development^[71,72]. Understanding the role of DNA adducts of lipid peroxidation and the repair pathways involved may shed light onto mutagenesis during HCC development, and this knowledge will help us to find a way to its prevention^[73]. To our knowledge, there is still no clinical data regarding LPO-derived DNA adducts as a predictive biomarker for HCC risk, we hope the ongoing interventional multi-center clinical trial "defined green tea catechin extract in preventing liver cancer in patients with cirrhosis (NCT03278925)" will shed some light on γ -OHPdG as a biomarker for liver carcinogenesis.

Thanks to recent advances in imaging modalities and the prevalence of a surveillance method for HCC, an increasing proportion of patients now receive local ablation therapy or curable resection. However, the high annual recurrence rate (approximately 20%) is still a huge hurdle before achieving long-term disease-free survival^[74]. Neoadjuvant and adjuvant therapy for resectable HCC is still a difficult challenge. There are two major postoperative recurrence mechanisms: *de novo* carcinogenesis (usually late recurrence) and metastatic recurrence (usually occurs within one year and is related to intrahepatic metastasis)^[75]. Precise prevention strategies are needed to target these mechanisms^[76]. Three major strategies have been developed to address this issue^[77]. The first one is a virus eradication method using interferon. But this method is not going to rescue the hepatocytes which have been damaged by hepatitis virus^[78]. The second strategy is the use of anticancer drugs. Difficulties have been reported in the STROM trial (sorafenib as adjuvant treatment in the prevention of recurrence of hepatocellular carcinoma) and with the use of UFT (Tegafur-uracil)^[79]. The last strategy is to induce differentiation of liver cancer cells. For example, using Pertinoin, an acyclic retinoid which can induce apoptosis and differentiation of cancer cells. This method has shown promising survival beneficial effects in a clinical phase II trial. Other than these strategies, branched chain amino-acid supplementation, vitamin K2 and acyclic retinoid have also been examined^[80]. The reality is that no chemopreven-

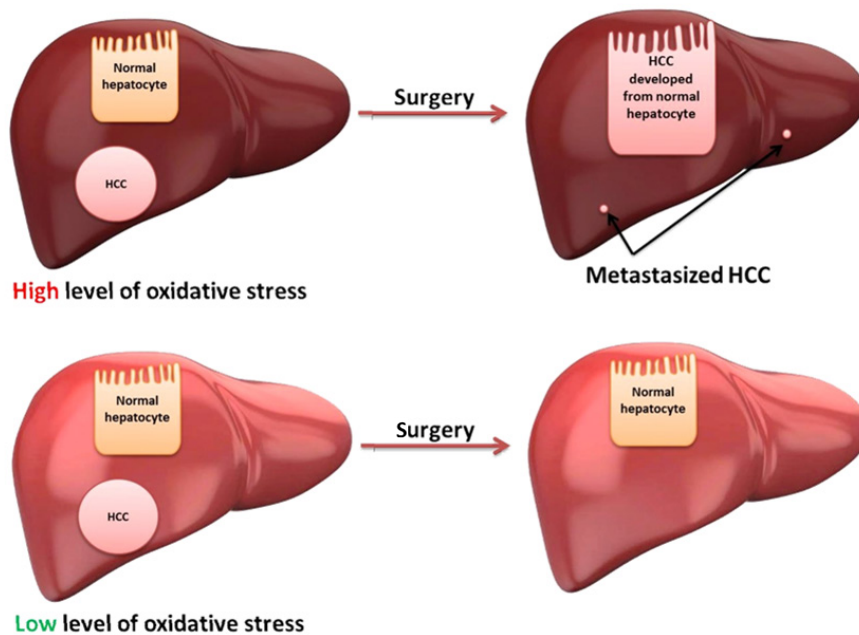


Figure 1. Oxidative stress and liver recurrence after surgery. HCC: hepatocellular carcinoma

tive agent has been approved by FDA against HCC recurrence. There is still a lot of effort to be made to win this war against HCC recurrence. Future design may require focus on combination therapy. For instance, vitamin K2 and angiotensin-converting enzyme inhibitor have shown suppression effect on cumulative recurrence of HCC after curative therapy partially through reducing VEGF-mediated neovascularization^[81].

FUTURE PERSPECTIVES

Clinical trials using oxidative stress biomarkers for HCC and predicting HCC recurrence after curable surgery have been conducted [Figure 1]. Multi-center trials should be carried out to prove this application. The link between oxidative stress, DNA adducts, mutations, and cancer needs to be systematically studied; it is an area of study that can be accelerated by emerging technologies (e.g., next generation sequencing, Chip-seq, and SMART sequencing^[82]). New technologies are needed to demonstrate in real-time link between exact DNA lesion sites (from normal tissue) and mutations (from tumor tissue). The idea of using antioxidants to prevent HCC recurrence has yet to be fully tested^[83-85]. Use of oxidative stress markers to guide these trials warrants future investigation.

DECLARATIONS

Acknowledgments

We thank Dr. Aiwu Ruth He for discussions when preparing this review. The authors also thank Cindy Clark, NIH Library Writing Center, for manuscript editing assistance.

Authors' contributions

Design of the review: Fu Y, Chung FL

Literature review and manuscript writing: Fu Y, Chung FL

Manuscript revision: Fu Y, Chung FL

Availability of data and materials

Not applicable.

Financial support and sponsorship

National Cancer Institute (R01-CA-190678, to Chung FL), the NCI Director's Challenge Innovation Award (to Fu Y) and ONYX Pharmaceuticals Research Award (to Fu Y).

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Immunotherapy: a new era for hepatocellular carcinoma

Ya-Jing He¹, Ya-Bing Guo¹, Wei Zhu¹, Yu-Kai He^{2,3}, Jin-Lin Hou¹

¹State Key Laboratory of Organ Failure Research, Guangdong Provincial Key Laboratory of Viral Hepatitis Research, Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China.

²Georgia Cancer Center, Medical College of Georgia, Augusta University, Augusta, GA 30912, USA.

³Department of Medicine, Medical College of Georgia, Augusta University, Augusta, GA 30912, USA.

Correspondence to: Dr. Ya-Jing He, State Key Laboratory of Organ Failure Research, Guangdong Provincial Key Laboratory of Viral Hepatitis Research, Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China. E-mail: heyajing529@126.com

How to cite this article: He YJ, Guo YB, Zhu W, He YK, Hou JL. Immunotherapy: a new era for hepatocellular carcinoma. *Hepatoma Res* 2018;4:40. <http://dx.doi.org/10.20517/2394-5079.2018.45>

Received: 29 Apr 2018 **First Decision:** 20 Jun 2018 **Revised:** 28 Jun 2018 **Accepted:** 28 Jun 2018 **Published:** 6 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Cancer is a major disease threatening human health. The overall prognosis for hepatocellular carcinoma (HCC) patients is poor, with a dismal 5-year survival rate of approximately 5%-30%. The dysfunction of immune system plays a pivotal role in the development of cancer, which has attracted attention of several researchers. Recent advances in immunotherapy have led to various inspired achievements and refreshed our concepts about cancer treatments. In this article, several types of immune-based therapies for treating HCC are reviewed. Their underlying mechanisms, preclinical and clinical study results, potential prospects, and deficiencies are discussed, and an outline for future research directions is proposed.

Keywords: Hepatocellular carcinoma, immunotherapy, cancer treatments

INTRODUCTION

Cancer is one of the primary diseases that threaten human health. Nearly 14.1 million new cases of cancer and 8.2 million cancer-related deaths worldwide were estimated in 2012. Moreover, 782,000 new cases of liver cancer have been recorded, with nearly half of these cases reported in China alone^[1]. Liver cancer is the second most common cause of cancer deaths among adult men worldwide. Nearly 746,000 deaths (9.1% of the total) were caused by liver cancer in 2012. Hepatocellular carcinoma (HCC) is the most primary,



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common, malignant liver cancer. The overall prognosis for HCC patients is poor, with a dismal 5-year survival rate of approximately 5%-30%^[1,2].

The most common progression of liver cancer is from chronic inflammation to cirrhosis and eventually developing to HCC through a prolonged period leading to multiple function disorders. Immunosuppression may be one of the most important reasons. T cell dysfunction, also known as T cell exhaustion, occurs in chronic infections and cancers. Various cell populations, including infiltrating immune cells and tumor cells, stroma cells with related cytokines and metabolites, cause T cell dysfunction in the tumor microenvironments. Exhausted T cells lack robust effector functions and express multiple inhibitor receptors that reduce efficient immunological surveillance of tumor^[3,4]. Tumor recurrence and relapse-free survival (RFS) are correlated to CD3+, CD8+ immune cells or tumor infiltrating lymphocytes (TILs), as well as the inhibitory receptors such as programmed cell death protein 1 (PD-1) and its ligand^[5]. The potential immunosuppressive mechanism involves the hepatoma-intrinsic cell cycle-related kinase (CCRK) signaling stimulated by the expansion of the polymorphonuclear (PMN) myeloid-derived suppressor cells (MDSCs), which have been correlated to potent T cell suppression and poor prognosis of patients^[6,7]. Expression of ectonucleoside triphosphate diphosphohydrolase 2 (ENTPD2) on the surface of cancer cells is induced by hypoxia, which elevates extracellular 5'-AMP and prevents the differentiation of MDSCs, consequently, contributing to the maintenance of MDSCs^[8].

Recently, cancer immunotherapy has emerged from being an adjacent to a frontline therapy and has demonstrated positive outcomes involving various cancers. Antagonistic antibodies for the PD-1 and cytotoxic T cell lymphocyte antigen-4 (CTLA-4) pathways have been approved by the Food and Drug Administration (FDA) for use in a growing number of cancers, including Hodgkin's lymphoma (HL), melanoma, bladder, non-small-cell lung and kidney cancers^[9,10]. Tumor tissue deep sequencing has advanced the neoantigen-based vaccines^[11,12] and neoantigen-specific T cells^[13-16] to clinical trials and resulted in discovering significant antitumor effects that will make individualized immunotherapy become a reality. In 2017, 2 kinds of chimeric antigen receptor (CAR) T cells that target CD19 have received FDA approval for treatment of diffuse large B-cell lymphoma (DLBCL) and acute lymphoblastic leukemia (ALL), respectively^[17-19]. Here, we provide an overview of current preclinical and clinical immunotherapeutic approaches for HCC.

IMMUNOTHERAPY APPROACHES

Cancer vaccine

Promoting tumor specific immune responses, especially the cytotoxic CD8+ T cells is the main goal of cancer vaccines. In colorectal cancer (CRC), breast cancer and ovarian cancer, the reduced frequency of tumoral cytotoxic CD8+ T cells is correlated with poor disease prognosis^[20-23]. On the other hand, it is a positive prognostic factor in that TILs are present in tumor deposits. The investigation of vaccines that target specific mutated antigens is being encouraged due to the technological developments in the recent few years. Several kinds of cancer vaccines are being tested, for instance proteins, peptides, tumor cells, antigen presenting cells (APC), and viral vectors.

Vaccination with antigens

The first step toward DC vaccine production is loading tumor antigens on the immature dendritic cells (DCs). Tumor antigen candidates could be mutated genes, neoantigens, viral genes, tissue-specific genes, whole proteins, deoxycholate citrate sugar (DCA) constructs and tumor lysates of autologous or allogeneic tumor cells or tumor cell lines, which belong to either tumor-associated antigens (TAAs) or tumor-specific antigens.

Alpha-fetoprotein (AFP) is a fetal serum protein produced in the liver and is normally synthesized only during fetal development until shortly after birth, while it is produced again in instances of HCC. Specific

cytotoxic T lymphocytes (CTLs) targeting against this antigen have been shown to exist in the T cell repertoire, without being peripherally or centrally deleted, which suggests AFP is a promising target antigen for HCC immunotherapy^[24,25]. Human T cell repertoire could effectively respond to the AFP self-antigen in the context of major histocompatibility complex (MHC) class I or after the administration of AFP peptide-pulsed DC^[26,27]. Previous studies using DCs or T cells pulsed with AFP-derived peptides suggest that AFP-derived peptides are suitable epitopes as immunotherapy targets. However, because of the self-nature of AFP, the vaccine-activated immune responses were weak. Thus, not surprisingly, the clinical results were not satisfactory^[27,28] except that a recent phase 1 clinical trial in HLA-A24 patients showed that immunization with AFP-derived peptides resulted in immune responses in 33% (5 of 15) of patients, of whom one patient had complete response^[29]. To enhance the AFP-specific immune responses, investigators mutated the AFP epitope to create epitope-optimized vaccines. They recently found that epitope-optimization of AFP antigen together with genetic immunization can activate potent AFP-specific CD8 responses^[30]. The activated CD8+ T cells in mice could not only cross-recognize short synthetic wild-type AFP peptides, but also identify and kill the tumor cells expressing wild-type AFP, which successfully prevents the immunized mice from developing carcinogen-induced autochthonous HCC. Further studies show that the antitumor effects of vaccine-activated AFP-specific CD8 T cells are correlated to optimal T cell receptor (TCR) signaling strength and induction of stem-like memory T cells^[31,32].

Conversely, cancer vaccine development benefits from deep sequencing and rapid identification of neoepitopes in the tumor lesions^[33], a modern technology has emerged for designing a personalized immunotherapy approach by using neoantigens -mutated antigens generated in the tumor mass that are unique to each patient's cancer to elevate the immune function and kill cancer cells. The identification of personalized somatic mutations can be conducted by whole-exome sequencing and matching DNA from normal cell with tumor cell from each patient. Mutated peptides are then synthesized to create a new vaccine that has a high likelihood to bind to the autologous human leukocyte antigen (HLA)-A or HLA-B proteins. In a phase I clinical study for melanoma with neoantigen-based vaccines, 15 (16%) and 58 (60%) of the 97 unique neoantigens could be targeted by vaccine-induced polyfunctional CD8+ and CD4+ T cells, respectively. No recurrence of tumor was noticed in 4 of the 6 vaccinated patients for up to 25 months after vaccination.

Vaccination with APC

Antigen presenting cell, including dendritic cells, activated B cells, and peripheral blood mononuclear cells, have been widely investigated as candidates for tumor vaccine. In the innate immune system, the most efficient APCs are the DCs^[34,35]. They are well-known to be the most potent APCs for inducing antigen-specific T cell responses. They acquire and present tumor antigens to T lymphocytes, promote the generation of CTLs and helper T cells^[36], decrease the proportion of CD4+CD25+ regulatory T cells^[37] and induce anti-tumor immune response^[38]. After the first DC-based vaccine for the treatment for prostate cancer, the study of DCs is continuously growing internationally. DC-based therapies are increasingly investigated and used to treat many kinds of patients with cancer or other diseases. In terms of manufacturing the DC vaccines, it is important to select proper tumor antigens and choose the appropriate method for loading the tumor antigens onto the DCs. Tumor antigen-pulsed DC vaccines can effectively develop mature DCs (mDCs) and enhance T cell stimulation to generate potent CTLs.

There are 3 generations of DC vaccines according to the development of different subsets. First-generation DC vaccines are not fully matured, consisting of patient-derived natural DCs or monocyte-derived DCs (mo-DCs). Antigens, such as tumor cell lysates or recombinant/synthetic antigenic peptides, are loaded onto the DCs *ex vivo* and then reinjected in the patients. The first-generation DC vaccines provided satisfactory outcomes in terms of safety and feasibility but not of expected clinical efficacy^[39-41]. The second-generation DC vaccines consisted of mo-DCs matured via maturation cocktails. Such vaccines are widely used in the clinics because of its minimal immunogenic side effects and better clinical responses^[42]. Nowadays, the clinical progress on DC vaccines has reached a new era: next-generation DC vaccines. Many defined DC subsets

(including patient-derived and mo-DCs) confer the next-generation DC vaccines superior functionalities for presenting MHC-I/II antigen and eliciting CTL responses^[43]. Pulsing DCs with CD44 and epithelial cell adhesion molecule (EpCAM) peptides can activate cancer stem-like cells (CSCs) peptide-specific immune responses leading to better clinical outcomes when combined with standard chemotherapy for advanced carcinomas^[44]. Cytoplasmic transduction peptide (CTP), a novel antigen delivery tool, can transduce tumor antigen such as the forkhead box protein M1 (FoxM1) into the cytosol of DCs^[45].

Vaccination approaches

Many studies have focused on pre-conditioning the DC-based vaccine sites and have already reported some interesting discoveries. The lymph node homing and immune function of tumor antigen-specific DCs can be significantly improved by pre-conditioning the vaccine site with a potent recall antigen, such as tetanus/diphtheria (Td) toxoid. A significant increase in both PFS and overall survival (OS) in Td-treated patients compared with DC-treated patients has been approved for clinical trials^[46]. Furthermore, RNA-lipoplexes (RNA-LPX) encoding endogenous self-antigens or mutant neo-antigens or viral can enable precise and effective targeting of DCs and perform effectively *in vivo*, as well as induce strong effector and memory T cell responses. This could result in a universally applicable vaccine type for DC based cancer immunotherapy^[47]. Exosomes derived from AFP-expressing DCs (DEX_{AFP}), another type of vaccine for cancer immunotherapy elicits strong antigen-specific immune responses and restructures the microenvironment in tumor^[48]. DCs can also be loaded via RNA transfection^[49] or recombinant viral transduction^[50].

Immune checkpoints-specific antibodies

The interactions between an APC and a T cell through the TCR-antigen/MHC complex simultaneously trigger both co-stimulatory and co-inhibitory signals. The balance between these signals determines the overall activation and function of T cells. Several co-inhibitory molecules (PD-1, CTLA-4, BTLA-4, LAG-3, TIM-3 and CD160) expressed on the surface of T cells are the targets of antibodies^[51-55]. Checkpoint blocking antibodies have been approved by the FDA since 2014 for patients with lung cancer, melanoma, and other tumors. For HCC, CTLA-4 and PD-1 antibodies have been intensely investigated and are both advancing to the clinical trial stage.

CTLA-4

Blocking CTLA-4 induces a strong antitumor immune response^[56], and research on CTLA-4 is ongoing^[57,58]. CTLA-4 blockers were mainly ipilimumab and tremelimumab. In 2011, FDA approved ipilimumab for the treatment of melanoma. However, for the CTLA-4 molecular targeted therapy, only tremelimumab is currently undergoing clinical trials related to liver cancer. In a phase II clinical trial of tremelimumab^[59], median OS was 8.2 months and median TTP was 6.48 months among all 21 patients enrolled. Among the 17 patients continuously treated with tremelimumab, no complete remission (CR) was observed, while 3 patients (17.6%) had confirmed partial remission (PR) that was maintained up to 3.6, 9.2 and 15.8 months, respectively. Overall, a good safety profile was recorded and no treatment-related death occurred. The feasibility and safety of tremelimumab combined with ablation (chemoablation or radiofrequency ablation) in patients with advanced HCC was assessed in another clinical trial^[60]. Among the 19 patients evaluated, 5 patients (26%) achieved confirmed PR. The median OS was 12.3 months and median TTP was 7.4 months with a median potential follow-up of 18.8 months for the total study population ($n = 28$). Tremelimumab was well tolerated across the different dose cohorts and no dose-limiting toxicities (DLT) was encountered. Recently, ipilimumab, another drug combined with the fully humanized anti-CTLA-4 IgG1 antibody, has been investigated in several clinical trials. These results have not been published.

PD-1/PD-L1

PD-1 is expressed on T cells binding with its ligand (PD-L1, PD-L2)^[61,62]. PD-L1 is expressed on APC^[63] and negatively regulates downstream signals of T cell receptor stimulation to reduce T cell activation and cytokine

production, while decreasing tumor-killing ability^[64,65]. PD-1, lymphocyte-activation gene-3 (LAG3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3) and CTLA4 are expressed on CD4+ and CD8+ T cells, as well as B cells and natural killer (NK) cells^[66]. The expression of TIM-3, PD-1, CTLA4, and LAG3 was significantly higher in CD4+/CD8+ T cells and TAA-specific CD8+ TILs in the HCC tissue than in the control tissue or blood. Blocking these immune checkpoints may increase *ex vivo* proliferation and effector cytokine production of tumor-infiltrating T cells^[67]. Thus, the anti-tumor immune response of immune cells can be enhanced, and tumor growth controlled^[64,68]. Nivolumab, a fully human IgG4 monoclonal antibody PD-1 inhibitor was investigated in a multiple ascending-dose, phase I/II study in HCC patients. In 39 patients whose response could be evaluated, 2 CR (5%), and 7 PR (18%) cases were reported. Response duration was 14-17 or more months for CR, less than 1-8 or more months for PR, 1.5-17 or more months for stable disease, and an OS of 72% at 6 months. The toxicity profile has been well managed^[69]. Subsequently, another randomized, multi-center clinical trial comparing the efficacy with nivolumab *vs.* sorafenib is ongoing (NCT02576509). Besides, the overall expression of PD-L1 on tumor cells is negatively correlated with tumor recurrence and survival in HCC patients. It can be used as an independent prognostic factor for the disease-free survival of patients with liver cancer^[70,71]. Currently, plenty of early clinical trials of PD-1/PD-L1 blockers alone or in combination with CTLA-4 blockers for liver cancer are ongoing. At present, the FDA has already approved 5 PD-1/PD-L1 checkpoint blocking antibodies for non-HCC tumors, 2 are PD-1 antibodies: nivolumab, pembrolizumab; 3 are PD-L1 antibodies: durvalumab, atezolizumab, and avelumab. Furthermore, nivolumab has been approved by FDA for HCC patients who received sorafenib treatment in the USA in September 2017. The clinical efficacy of each drug in controlling HCC will be worth anticipating.

Adoptive cell therapy

Adoptive cell therapies (ACTs) that expand certain cells *ex vivo* and then infuse them back to patients have in recent years gained attention for the clinical treatment of tumors. These modified cells are able to transfer to the site of tumor and mediate its destruction^[72]. Modified strategies are mainly focused on T cells especially the CD8+ T cells that perform specific tumor killing function^[15].

CIK/DC-CIK immunotherapy

CIK/DC-CIK is one of the ACTs that can expand autogenous T lymphocytes *ex vivo* and are stimulated by many kinds of cytokines co-cultured with DC pulsed by tumor antigens alternatively^[73]. After culturing, CIK cells would comprise of CD3+CD56+ cells, CD3+CD56- cytotoxic T cells, and CD3-CD56+ NK cells. These heterogeneous cells are characterized by dual functions, acting both as NK-like and CD8+ specific effector T cells^[74]. At the same time, CD8+ specific effector T cells can specifically be activated by DC loaded with tumor antigens. A multicenter, randomized, open-label, phase III trial on the efficacy and safety of adjuvant immunotherapy with activated CIK cells showed that the median time of recurrence-free survival (RFS) was 44 months in the immunotherapy group and 30 months in the control group of patients with HCC when subjected to curative treatment^[75]. Given that the efficacy of immunotherapy is primary influenced by the complex immune microenvironment in HCC patients, immune factors should be considered for and may represent additional prognostic parameters for predicting survival benefits of immunotherapy. In addition, adoptive CD8+ T cells cannot be replicated *in vivo* after infusion, though it can be expanded abundantly *ex vivo*. Therefore, CIK/DC-CIK need to be transfused repeatedly to achieve better clinical efficacy. In a retrospective study of 448 HCC patients that received complete hepatectomy combined with/without CIK cell immunotherapy, the prognosis was significantly improved in the CIK treatment group compared with the surgery only group. Higher PD-L1 expression predicts better OS and RFS, especially in the subgroup with high hepatitis B viral load^[76]. However, another clinical trial reported no significant differences in DFS and OS between the patients who received CIK (*n* = 100) and who did not (*n* = 100) after curative hepatectomy^[77]. The clinical efficacy of CIK/DC-CIK treatment needs to be further demonstrated.

Genetically engineered T cells

As a pivotal role in killing tumor cell, the function of T cells has always been the focus of investigation. With the development of modern genetic techniques, T cells can be genetically engineered for enhanced anticancer immune functions. These engineered T cell therapy has been first applied in hematological malignancy^[78,79] and then gradually introduced to treat solid tumors such as glioblastoma^[80], prostate cancer^[81] and sarcoma^[82]. Recent studies on modified T cells expressing engineered TCRs and CARs show encouraging results to advance from basic to clinical research.

TCR engineered T cells

Endogenous TCRs recognize the peptide segments submitted by MHC-I and MHC-II on the cell surface with a heterodimer consisting α - and β -chains. Each TCR is a heterodimer that determines the TCR antigen-specificity. TCR-T was genetically modified with TCR chains for targeting specific antigens expressed on tumor cells to cure specific diseases. As the peptides were processed and submitted by MHC, they present various antigens as an expanded pool of potential targets. For this reason, TCR-T can target more antigens in comparison to CAR-T^[83]. It was the first successful application of ACT when 17 patients with metastatic melanoma were treated using autologous T cells transduced with TCR recognizing the MART-1 melanoma-melanocyte differentiation antigen^[84]. Although objective cancer regressions were observed in mice and expanded clinical trials, severe “on-target, off-tumor” toxicity occurred in the skin, eyes, and ears of patients because of the expression of antigenic targets in these organs^[85,86]. AFP and GPC3 are commonly expressed in the HCC. These two specific antigens are good targets for engineered T cell therapy. Peptide GPC3₃₆₇ is a predominant peptide identified on HLA-A2 positive hepatoma cells. CD8(+) T cells that express GPC3₃₆₇-specific T cell receptor can recognize and kill GPC3-positive hepatoma cells and reduce growth of HCC xenograft tumors in mice^[87]. In a recent study, novel AFP-specific murine TCR genes have been identified that can redirect human T cells to specifically recognize and kill HCC tumor cells^[32]. AFP-specific murine TCR genes were identified in another study. These TCR-T cells specifically recognize HLA-A*02:01+/AFP⁺ HCC tumor cells and produce effector cytokines to kill them *in vitro*. Adoptive transfer of TCR-T cells prevent and regress HepG2 tumor outgrowth in NSG mice, irrespective of CD4 or CD8 TCR-T cells. Though tumor developed in one of the TCR-T-treated mice, it was eradicated 3 weeks after transfer^[32]. HBV infection is one of the most common causes of HCC tumorigenesis. In one case report, a patient seems to have developed HCC relapse 10 years after liver transplantation for HBV+ HCC. At the time of HCC relapse, HBsAg (but not HBV DNA) was detected in the blood analysis, while HBsAg, HBcAg and HBV DNA were negative in liver biopsies for the transplanted liver. Subsequently, HCC autologous T cells genetically modified to express an HBsAg specific T cell receptor were transferred to this patient. The results show reduced levels of HBsAg without exacerbation of liver inflammation or other toxicity, while clinical efficacy could not be established. This leads to a novel strategy of personalized immunotherapy targeting specific peptides in the treatment of HBV associated HCC^[88].

TCR-T therapy has got into clinical trial of multiple myeloma (MM), metastatic melanoma and esophageal cancer, while the safety reports differ from each other. In a phase I trial of MAGE-A4 T cell receptor gene-transduced lymphocytes in patients with recurrent esophageal cancer, none of 10 patients experienced any adverse events for the first 14 days after T cell transfer^[89]. However, the safety is not optimistic in other 2 trials. Seven of 20 patients with MM had SAEs after infusion of NY-ESO-1 specific TCR engineered T cells^[90]. While 2 of 14 patients had serious adverse events (SAEs) of acute respiratory distress requiring intubation associated with patchy pulmonary infiltrates within 1 week of cell infusion with MART-1 T cell receptor transgenic lymphocytes and dendritic cell vaccination in patients with metastatic melanoma^[91]. Therefore, the toxicity may bring new challenges to the development of TCR-T therapy. Recently, 4 TCR-T therapies in HCC have started phase I/II clinical trial (NCT02686372, NCT02719782, NCT03441100, NCT03132792). The safety of these trials needs to be paid significant attention as well as clinical efficacy.

Chimeric antigen receptor engineered T cells

Unlike TCRs, CARs are formed by a combination of antibody-derived or ligand-derived domains and TCR domains. Due to engineering specific antigens, CAR-T specifically expresses a receptor to direct the T cells to target and destroy cancer cells. Therefore, CAR-T therapy represents specific recognition and lethality. Meanwhile, specificity enhancement of CAR-T cells can make them activate at very low level of target on non-malignant tissue so that prevent off-tumor toxicity. Based on the different engineered chains of CAR, CAR-T has developed from the first generation to the second and third generation^[83,92]. With the development of CAR-T therapy, CAR-T cells have longer survival times, better functional properties, and less toxicity. These characteristics make CARs “living drugs” that exert both immediate and long-term therapeutic benefits. The third generation of GPC3-CAR-T cells are able to efficiently kill GPC3-positive HCC cells, while suppressing the growth of HCC xenografts. The cytotoxic effects were positively correlated to the GPC3 expression levels in the target^[93,94]. A phase I clinical trial for anti-GPC3 CAR T has been sponsored in 2015 to evaluate the safety and effectiveness for patients with relapse or refractory HCC. Thirteen patients were enrolled in this trial, and the results are eagerly anticipated (NCT02395250). A different CAR-T approach towards HCC was recently developed by utilizing the antibody against HLA-A2/AFP₁₅₈ peptide complex^[95]. If successful, this approach may expand CAR-T therapy to the intracellular tumor antigen. T cells expressing ET1402L1-CAR (AFP-CAR) could selectively lyse liver cancer cells that were HLA-A*02:01/AFP*. Under *in vivo* conditions, both intratumoral infection and intravenous administration of AFP-CAR T cells significantly inhibit tumor growth in mice. The robust antitumor activity was attempted in an established intraperitoneal liver cancer xenograft model. The phase I clinical trial of an ET1402L1-CAR started only in 2017 (NCT03349255). Autologous CAR-modified T cell directed CD133 (CART-133) is another therapy targeting for CD133, which has developed into a phase I trial for HCC and pancreatic carcinomas and colorectal carcinomas. The results showed 3 PR and 14 stable disease in all 23 patients. For safety, the reduction of hemoglobin, lymphocytes, and thrombocytes occurred in nearly all the patients. Lymphopenia presented in all the non-HCC patients with grade 2-4 and all HCC patients with grade 2^[96]. So far, most clinical results of CAR-T have come from the treatment of hematologic diseases. The clinical trial of CAR-T for solid tumors is just beginning. However, cytokine releasing syndrome and on-target/off-tumor toxicity are still very important side effects which should be solved in either hematologic diseases or solid tumors.

Due to the complexity of the immune system post-infusion, ACT is more complex than other types of immunotherapy. For expressing the different antigens and the varied microenvironments in different patients, several biotechnology companies are turning their efforts to develop personalized approaches. This is being attempted by screening personalized tumor antigens and expanding personalized lymphocytes in the individuals. Although multiple commercial models have been proposed, the effectiveness and safety need further investigation.

SUMMARY AND FUTURE RESEARCH DIRECTION

Although the number of HCC related deaths is high, its prognosis remains poor and available treatment options are limited. Over the past decades, immunology has evolved from the basic to the clinical realm, which has contributed to many immunotherapies entering the clinics, which is encouraging and offers new treatment prospects for HCC. Strategies including immune checkpoint blockers, genetically engineered T cells (TCR-T and CAR-T) have already secured FDA approval for many types of cancer treatments. The screening and identification of HCC neoantigens have reinvigorated the relevance of immunotherapy, and precisely, pushing the personalized treatment into a reality. The progress in the field of cancer treatment is obvious, yet, tumor is still a dreadful disease with limited options to cure. Making the treatment more accurate and effective for HCC remains a huge challenge. To better understand tumor, further research of the tumorigenesis mechanism is needed. With immune suppression in tumor microenvironments, further research should likely focus on alleviating inhibition of immune suppression and restoring normal immune functions. Various immune functions also need to be further investigated including tumor antigen

presentation for APC, recognition and killing of tumor by immune cells, and function restoration of immune cells. For each of the immunotherapy strategies outlined, precision, accuracy, efficiency, thoroughness, and safety must be considered. Clinical trials and experiments should be thoroughly designed to derive real value of clinical testing. Target patients, method of administration, treatment strategy are additional factors for consideration. In addition, novel drugs and approaches are still expected to be introduced. In conclusion, there is no doubt that a new era is beginning for HCC treatment, which shines the light of hope in our quest to conquer cancer.

DECLARATIONS

Authors' contributions

Designed and drafted the manuscript: He YJ

Reviewed and modified the manuscript: Guo YB, Zhu W, He YK, Hou JL

Read and approved the final manuscript: all authors

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Heat shock reduces HCV replication via regulation of ribosomal L22 in Alu-RNA molecule dependent manner

Hamada Farghaly, Adel A. Guirgis, Hany Khalil

Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City 79, Egypt.

Correspondence to: Dr. Hany Khalil, Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City 79, Egypt. E-mail: hkhali74@gmail.com

How to cite this article: Farghaly H, Guirgis AA, Khalil H. Heat shock reduces HCV replication via regulation of ribosomal L22 in Alu-RNA molecule dependent manner. *Hepatoma Res* 2018;4:41. <http://dx.doi.org/10.20517/2394-5079.2018.30>

Received: 26 Mar 2018 **First Decision:** 10 May 2018 **Revised:** 29 Jun 2018 **Accepted:** 29 Jun 2018 **Published:** 7 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Aim: Hepatitis C virus (HCV) infection is a global health problem that affects more than 180 million people worldwide. HCV is associated with several hepatic and other hepatic disorders including malignancies. HCV is a small enveloped positive-single strand RNA virus that belongs to *Hepacivirus* in the family *Flaviviridae*. Here we aim to provide a new therapeutic strategy via treatment of infected HepG2 cells with heat shock (HS).

Methods: The potential inhibitory effect of HS on HCV replication was assessed by the relative gene expression of NS5A and its corresponding protein by flowcytometry which has been additionally used to monitor other cellular factors.

Results: HS treatment of infected HepG2 cells has the ability to disturb HCV replication possibly via stimulation of the *Alu* non-coding element which inhibits gene expression of ribosomal L22. Ribosomal protein L22 (RPL22) is one of the abundant RNA-binding proteins that are known to facilitate synthesis and translation of viral RNA and to participate in balancing the protein components of the ribosome itself.

Conclusion: HS treatment of infected cells leads to up-regulation of long RNA-Alu molecule that regulates the expression of RPL22 and subsequently reduces HCV replication in HepG2 cells.

Keywords: Hepatitis C virus, *Alu* non-coding gene, heat shock treatment



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INTRODUCTION

Hepatitis C virus (HCV) infection is the most common cause of chronic liver disease that is considered as the common sign of liver transplantation in United States, Australia and European countries. Almost 3% of global population are infected with HCV which mean approximately 180 million people worldwide. HCV belongs to the family *Flaviviridae* which replicates in the cytoplasm of liver hepatocytes^[1,2]. HCV acute infection is most often asymptomatic leading to chronic infection of about 75% patients. The manifestation of chronic HCV is directly alternated from asymptomatic state to cirrhosis and hepatocellular carcinoma (HCC) phase. Indeed, HCV infection is slowly progressed without clinical appearance in the liver of many patients. Therefore, approximately 20%-30% of infected individuals may develop liver cirrhosis over 20 to 30 years period of infection^[3]. HCV genome encodes a large open reading frame (ORF) which have translated into polypeptide chain with approximately 3000 amino acids that have cleaved into ten proteins. Three structural proteins including the core, E1 and E2, in addition to five non structural proteins contain NS3 (helicase/protease), NS4A, NS4B, NS5A, and NS5B (RNA-dependent RNA polymerase)^[4]. HCV entry to the host involves a complex series of interactions including attachment, fusion and entry. Attachment of HCV with host receptor and co-receptors is facilitated by heparin sulfate proteoglycans that are expressed on hepatocytes surface. Meanwhile, LDL receptor (LDLR) binds to HCV and promotes its virion entry in pH-dependent by clathrin-mediated endocytosis^[5-7]. During entry process, many cellular factors have been identified, including the scavenger receptor class B type I (SRB1), CD81, tight junction proteins, claudin-1 (CLDN1) and occludin (OCLN)^[8,9]. SRB1 and CD81 have been identified as binding partners of HCV that are highly expressed in the liver and increase the selective uptake of HDL cholesterol esters into hepatocytes^[9,10]. Interestingly, targeting of these receptors and others cellular factors provides potential avenues to prevent HCV infection and suggests that modulation of their physiological role does not lead to significant toxicity on host cells. Generally, treatment of HCV contains several drugs that directly interact with viral proteins such as symeprevir, grazoprevir and asunaprevir (NS3 inhibitors) which inhibit HCV-NS3 proteases^[11,12]. Several drugs target HCV-NS5B polymerases (NS5B inhibitors) such as sofosbuvir and dasabuvir, while others interact as NS5A inhibitors such as daclatasivir, elbasvir and ledipasvir^[13].

Importantly, over-use of antiviral drugs is considered as a factor for development of viral-escape mutation, which leads to rapid HCV resistance. Recently, genome wide RNAi screening revealed many host cell factors that are essential for the replication of HCV^[14]. These factors are attractive candidates for potential antiviral medications as it is less likely that HCV will develop resistance rapidly to drugs that target host cell factors. Several studies have been reported on the essential role of heat shock treatment and its associated proteins (HSP) during viral replication including HSP27, HSP70 and HSPB8^[15-17]. Such stress proteins have crucial impact in viral entry, activation, life cycle and assembly of human immunodeficiency virus (HIV)^[15]. HSPB8 showed competitive antiviral activity through direct interaction with HCV-NS4B protein^[17]. Further, HSPs is able to prevent the inflammatory damage and promote the production of anti-inflammatory cytokines indicating the potential immunoregulatory role of HSPs^[18]. Interestingly, one of HS response properties is the activation of non-coding RNA-Alu repeats which interact as inhibitory elements of transcription process^[19]. A variety of long non-coding RNAs molecules (lnc-RNAs) are transcribed in mammalian cells to post-transcriptionally regulate gene expression. Lnc-RNAs play crucial roles in modulating mRNA stability, regulating mRNA translation and mediating protein modifications. Alu non-coding element is the most abundant repetitive RNA elements in the human genome. Recently, several studies demonstrated that Alu molecules modulate gene expression at the post-transcriptional level^[20,21]. On the other hand, ribosomal proteins (RPs) highly contain RNA-binding sites with auxiliary functions, particularly by the viruses, which are so adept at usurping the cellular machinery^[22]. Ribonucleoproteins are responsible for synthesis of new proteins beside other critical functions including the fundamental three-dimensional structures of small and large RNA molecules in ribosomal subunits^[23]. One of these ribosomal proteins is RPL22 which has the ability to interact and support HCV-RNA translation^[24]. In the current work, we investigated the potential up-regulation of the long RNA molecule, Alu, in response to HS in HepG2 cells that were pre-infected with

HCV genotype 4. The potential targeted gene by activated Alu molecule has been detected using qRT-PCR and flowcytometry. HCV replication in treated cells has been monitored to figure out the inhibitory effects of HS on viral replication.

METHODS

HepG2 cell line

HepG2 cells were obtained from VACSERA, Giza, Egypt and were propagated in order to obtain increasing numbers of cells for further investigations. Propagation was done using RPMI media which supplemented with 1% L-glutamine, 10% bovine serum albumin (BSA) and 1% penicillin/streptomycin at 37 °C at CO₂ incubator.

HCV infection

Blood sample from a patient with HCV genotype 4 was identified and provided from Ain Shames Specialized Hospital, Egypt. For infection, HepG2 cells were incubated for three days with the serum of derived sample in multiplicity of infection (MOI) of 0.5^[25].

HS treatment and virus infection

To figure out the effect of HS on HCV replication, HepG2 cells were seeded in 6-well plates (2 × 10⁵ cells per well). Cells were infected for 3 days with HCV (MOI = 0.5), other cells were incubated without infection. All cells were then stimulated by HS using warm media (45 °C for 5 min). The infectious media was collected and stored at -80 °C for LDH detection as an indicator for cytotoxic effect of heat shock.

Cytotoxic effect and metabolic activity of host cells

To determine the time cytotoxic 50% (TC₅₀) of HS, HepG2 cells were seeded in 96-well plates in a density of 5 × 10⁴ cells per well. The cells were then treated for different time point (0-10 min) with warm media (45 °C). After each incubation period, the cytotoxic effect was monitored by using water-soluble tetrazolium salt (Cell proliferation reagent WST-1, Sigma, USA) according to the manufacture protocol. The number of living cells was calculated and cell survival was investigated by using inverted microscope and detection of lactate dehydrogenase (LDH) level using LDH detection kit (Abcam, ab102526). According to the manufacture procedures, equal amounts of infections medium and LDH buffer (40 µL) were incubated with LDH substrate for 1 h then the relative LDH production was calculated according to the standard curve. Cells that were treated with 50 and 100 µL of Triton x-100 served as a positive control^[26].

RNA isolation and quantitative real time-PCR

Total RNAs were isolated from treated and untreated cells using TriZol (Invitrogen, USA) and chloroform methods. Isolated RNA was dissolved in RNase free water and the concentration of all samples was adjusted to final concentration of 100 ng/µL. Then 10 µL from each isolated and purified total RNA was used to generate cDNA using cDNA synthesis kit (Qiagen, USA). According to the manufacturer protocol, total RNA was incubated with reverse transcriptase and poly (dT)s primers at 45 °C for 1 h followed by 5 min incubation at 95 °C. The cDNA was then incubated at -20 °C until used^[27,28]. q RT- PCR was used to detect the relative expression of viral NS5A, non-coding Alu gene and L22 ribosomal gene in infected HepG2 cells upon heat shock treatment compared to control, the qRT-PCR was performed by using SYBR green and the following oligonucleotides specific for NS5A, Alu and L22 genes; NS5A-For-5'-ATTTCGTTCTAGTGGGATCCA-3', NS5A-Rev-5'-AAGAGTCCAGTATTATCACCTT-3', Alu-for-5'-AAAACGGTCAAACCCCGT-3', Alu-rev-5'-TATGTGCCAGGCACTTTT-3' and L22-for-5'-GAATTCGCACCGACTCGTAC-3' and L22-rev-3'-GGTGTTCGCAAAGGTGCTGTCCC-5'. Levels of GAPDH, as internal control, were amplified using specific oligonucleotides GAPDH-for 5'-TGGCATTGTGGAAGGGCTCA-3' and GAPDH-rev-5'-TGGATGCAGGGATGATGTTCT-5'. The following parameters have been used in qRT-PCR program, 94 °C for 3 min, 40 cycles (94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s) and finally 72 °C for 10 min^[19,26].

Flowcytometry analysis

HepG2 cells were seeded in 6-well plates in concentration of 2×10^5 cells per well, 25 μ L serum of infected patient with HCV genotype 4 that contains 1×10^5 virus (MOI = 0.5) was added to each well, and incubated for 3 days at CO₂ incubator. The old media was removed and fresh warm media (45 °C) was added for infected cells for 5 min. Then the media has been removed and cells were washed using PBS, and trypsinized by using trypsin. Finally the cells were collected in PBS and centrifuged at room temperature at 5000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in PBS that contains Triton-X-100 (0.01%) for permeabilization, then cells were centrifuged as previously described. The supernatant was removed and the pellet was resuspended in PBS that contains 1% BSA and 1:1000 diluted rabbit monoclonal antibodies for either NS5A or RPL22 protein (Promega, USA) followed by 1 h incubation at room temperature. The cells were centrifuged as previously described and were washed using PBS for three times. The cells were then incubated for 1 h in the dark with secondary antibody goat anti-rabbit (Promega, USA) in dilution of 1:100. Finally, the stained cells were centrifuged and washed by PBS and were collected in 500 μ L PBS for flowcytometry (Becton Dickinson Facscalibur).

Prediction tools

To investigate the possible interaction and potential binding site between Alu-repeated sequence and RPL22 sequences, IntaRNA software has been used^[29-31].

Statistical analysis

A student's two-tailed test was used to determine significance values of relative gene expression in treated and non-treated cells. SDS 2-2.2 software was used to analyze the Ct values of the q RT-PCR and to drive and calculate the relative gene expression using $\Delta\Delta$ Ct equations^[32].

RESULTS

Heat shock has no cytotoxic effect on cell viability rate

HS is the consequences for subjecting the cells to higher temperature than the optimal temperature range for biological functions. The influence of HS on cells' viability rate was monitored dependent on cell imaging, number of living cells and TC₅₀ following incubation. Additionally, lactate dehydrogenase (LDH) production from treated cells was measured as an indicator for systemic toxic effect of HS. HepG2 cells were seeded in either 6-well plates or 96-well plates and were incubated overnight. Next, the cells were infected with HCV by adding the patient-derived serum to fresh media (MOI = 0.5) followed by 3 days of incubation. The infected cells were subjected to warm media (45 °C) for the indicated time points. Cell viability rate dependent HS-time course was detected by using WST-1 assay which revealed that the TC₅₀ is greater than 10 min [Figure 1A]. This result indicates that the cytotoxic effect of HS is initiated upon 10 min of treatment. Furthermore, cells imaging and living cells upon 5 min of HS treatment showed no detrimental influence on treated cells in comparison with cells that were left without treatment (NT) [Figure 1B and C]. The relative LDH production showed negligible differences between 5 min-HS-treated cells, non-treated cells (NT) and mock in comparison with cells that were treated with Triton-X 100 as detergent agent [Figure 1D]. These data reveal that treatment with HS (45 °C) for 5 min has no cytotoxic effect in HepG2 cells.

HS treatment disturbs HCV replication via regulation of viral NS5A gene

In order to investigate whether the HS treatment has an influence on HCV replication, the relative gene expression of viral NS5A and its corresponding protein level have been detected in HepG2 cells following HS treatment. NS5A is a zinc-binding and proline-rich hydrophilic nonstructural protein that plays a crucial role in HCV-RNA replication. NS5A has the ability to modify NS5B polymerase activity and modulate multiple aspects in cellular immune response^[33]. Thus, the expression of NS5A reveals the capability of viral replication in infected cells. Here, the expression of HCV-NS5A has been detected at both RNA and protein

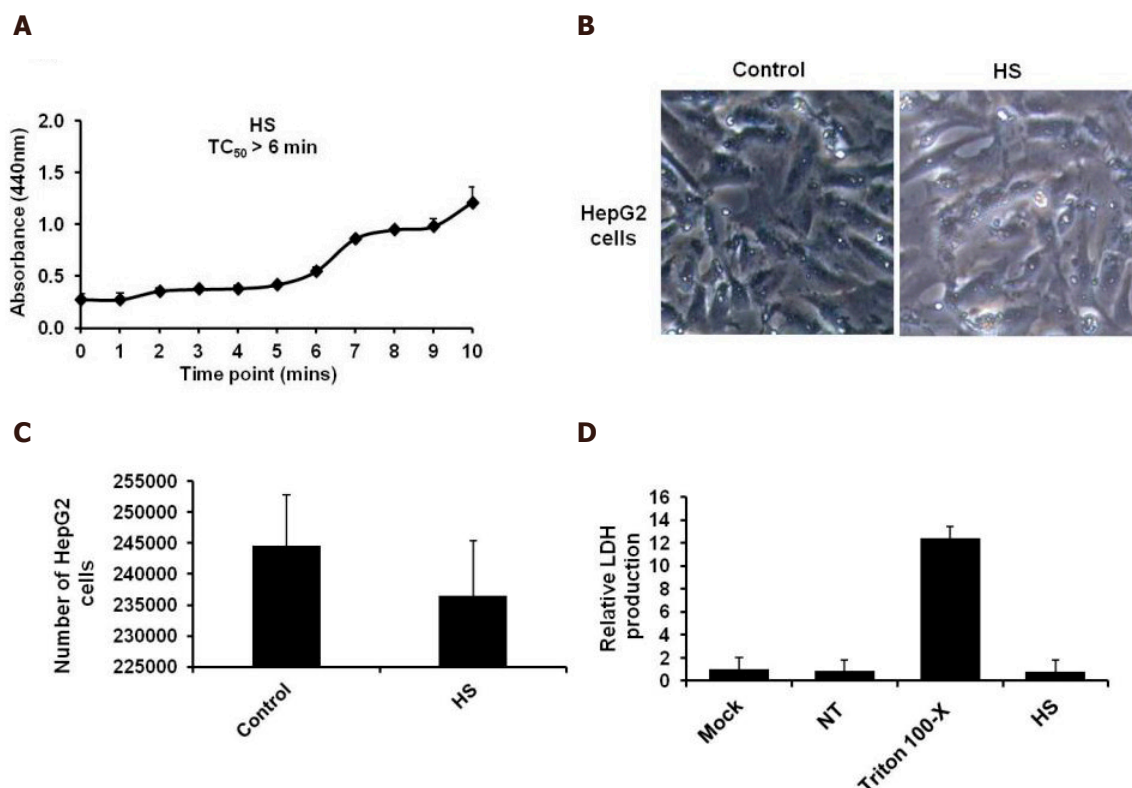


Figure 1. Cell viability and toxic effect of heat shock (HS). (A) Time cytotoxicity 50% (TC₅₀) in HepG2 cells that were subjected to different time point of HS (0-10 min); (B) cells images reveal cell viability of HepG2 cells that are treated with heat shock in comparison with non-treated cells (NT); (C) number of living HepG2 cells that have been treated with heat shock in comparison with NT cells; (D) relative LDH production of treated cells with heat shock in comparison with NT cells, Triton x-100 and mock. Error bars indicate the standard deviation of two independent experiments

levels using qRT-PCR and flowcytometry, respectively. Our results showed that the relative expression of NS5A was decreased in HepG2 cells that were pre-treated with HS in comparison with control infected cells indicated by qRT-PCR [Figure 2A]. Further, the statistical analysis of mean values calculated from cycles threshold (CTs) revealed a significant differences of NS5A expression in HS treated cells compared to control infected cells ($P > 0.05$). Moreover, the expression of NS5A protein in HepG2 cells has been detected in 30% of total cells that were pre-treated HS. While 70% of total control infected cells revealed normal level of NS5A that indicated by flowcytometry [Figure 2B]. These results indicated that HS stress could prevent HCV replication in HepG2 cells via depletion of its NS5A expression at both RNA and protein levels.

HS inhibits RPL22 gene expression via stimulation of Alu-RNA in HepG2

To investigate the effect of HS on Alu-RNA elements and its potential targeted gene RPL22, the relative expression of *Alu* and *RPL22* genes were detected in HepG2 treated cells compared to control infected cells using qRT-PCR. Our findings showed that the relative expression of *Alu* molecule has been significantly accumulated in response to HS treatment in HepG2 infected cells in comparison with control infected cells ($P = 0.009$) [Figure 3A]. Meanwhile, the relative gene expression of ribosomal RPL22 was significantly reduced in infected HepG2 cells that were subjected to HS ($P = 0.001$) [Figure 3B]. These data suggest that HS stress leads to activation and accumulation of Alu-RNA elements that may regulate the expression of ribosomal RPL22 gene in infected cells. In order to investigate the potential binding site of RPL22 by Alu-repeat sequences, IntaRNA software was used. The docking interaction indicates a seeding region on target location (11-26) by the query location (173-186) [Figure 3C]. These findings indicate the possible regulation of RPL22 messenger RNA (mRNA) by Alu elements in response to HS treatment.

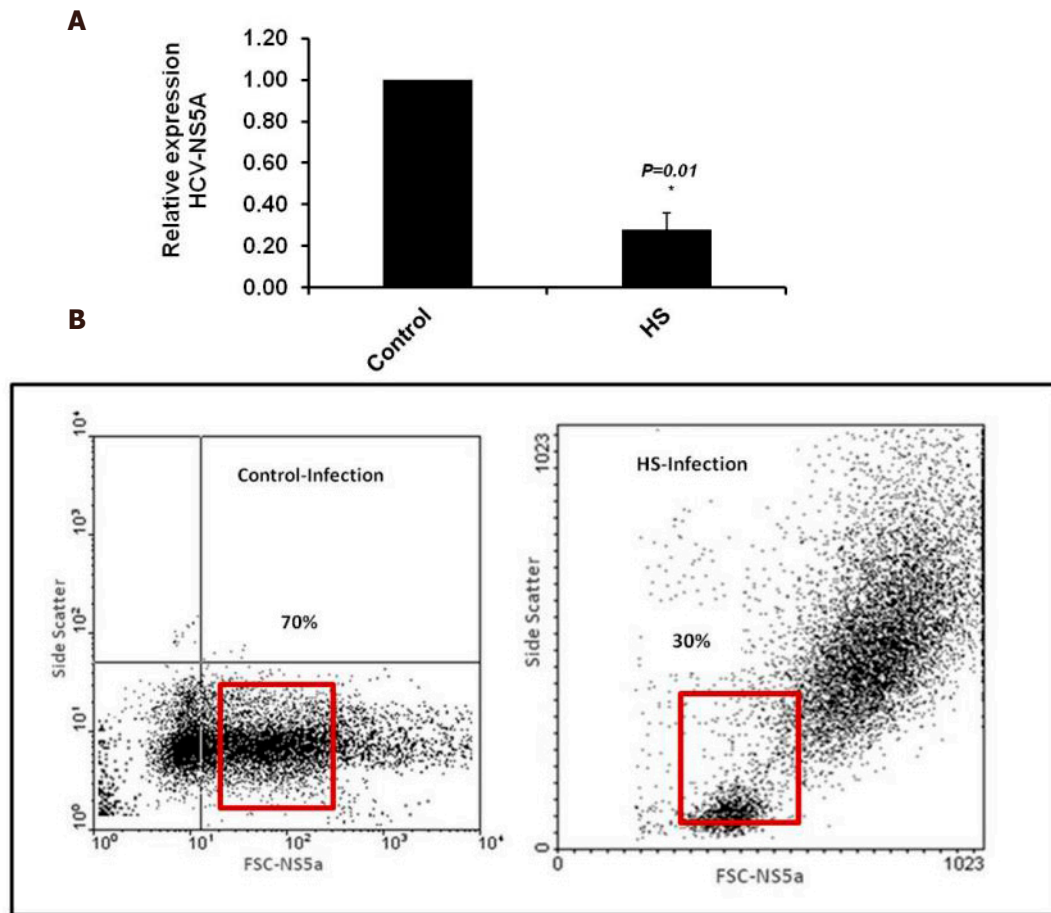


Figure 2. Heat shock (HS) reduces HCV-NS5A gene expression profile. (A) Relative gene expression of HCV-NS5A in HepG2 cells that were stressed with HS for 5 min in comparison with non-treated and infected cells (control-infection); (B) the expression of NS5A corresponding protein in HS treated cells (HS-infection) compared to control-infected cells indicated by Flowcytometry. Error bars indicate standard deviation of two independent experiments

L22 ribosomal protein is regulated by HS stress condition

To assess the inhibitory effect of HS on RPL22 protein, the protein profile of RPL22 has been detected in HepG2 cells that were infected with HCV and subjected to 5 min HS using flowcytometry. The result showed that approximately 25% of total HepG2 cells were positively expressed RPL22 in response to HS treatment, while 40% of total cells were positive to RPL22 in control-infected cells [Figure 4]. These findings indicate that few minutes of HS stress lead to obvious depletion of RPL22 in HepG2 cells. Together, our data demonstrate that HS treatment is an environmental stress leading to accumulation of Alu-RNA element that post-translationally regulates RPL22 and subsequently disturbs HCV replication in HepG2 cells.

DISCUSSION

Our findings provide a new therapeutic strategy against HCV infection without detectable toxic effect on cell viability. Treatment with HS (45 °C for 5 min) is able to decrease virus replication indicated by viral NS5A expression at RNA and protein levels. This interruption in viral replication may be due to up-regulation of *Alu* repeats element as a response to HS. *Alu* molecule is non-coding RNA that is present at elevated levels in stress condition. Consequently, *Alu* repeats are increasingly being associated with the physiological stress response^[19]. *Alu* sequences are the most abundant short interspersed repeated elements in the human genome. The accumulation of *Alu*-RNA molecules has been observed in variety of cancer cells in association with cellular microRNA^[33]. However, the exact molecular function of *Alu*-RNA element is still not completely

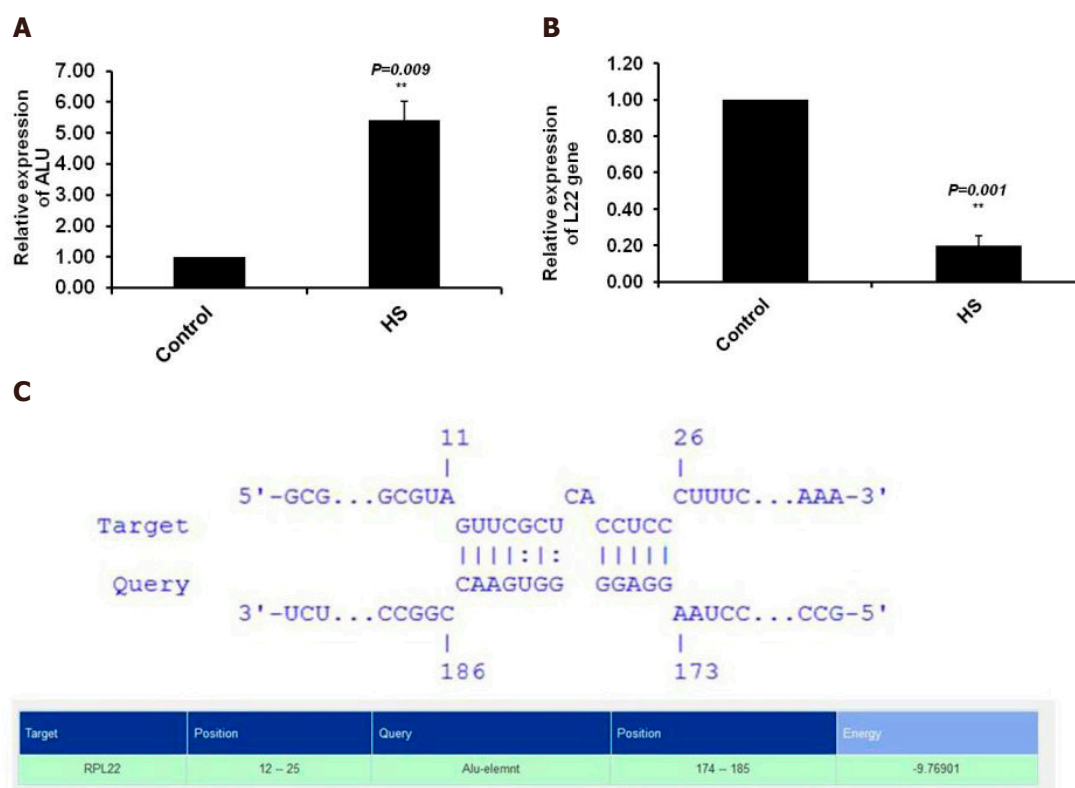


Figure 3. Down-regulation of L22 in Alu-RNA element dependent manner. (A) Relative expression of *Alu* non-coding gene in cells that were treated with heat shock (HS) in comparison with non-treated cells (control) using qRT-PCR; (B) relative expression of L22 gene in cells that were treated with HS in comparison with non-treated cells (control) using qRT-PCR. Error bars indicate the standard deviation of two independent experiments; (C) the possible binding site and seeding region of Alu repeated sequences (query) and RPL22 sequences (target) indicated by IntaRNA software

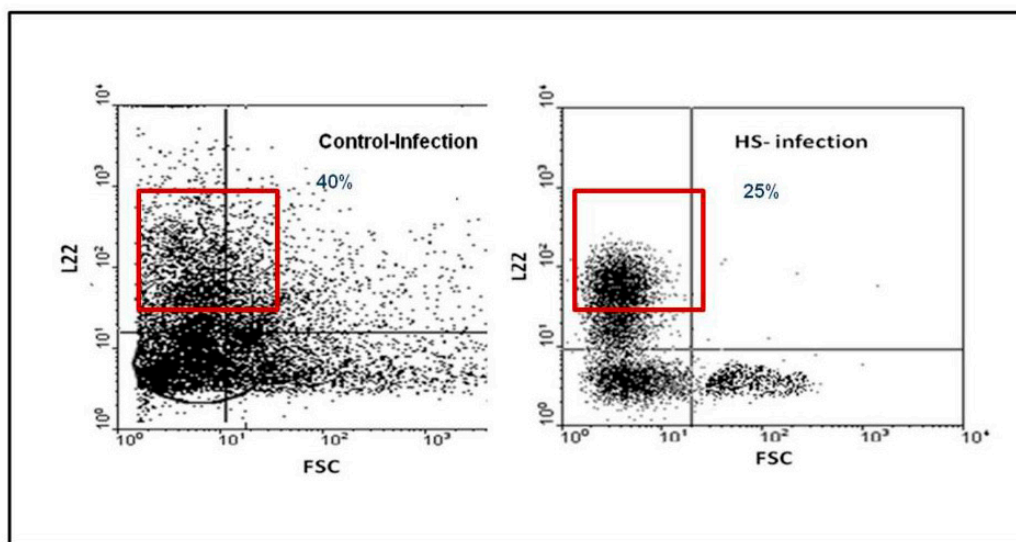


Figure 4. Interruption of L22 corresponding protein in response to heat shock (HS). The expression protein level of RPL22 elevated by percentage of positive HepG2 cells that were treated with HS and infected with HCV compared to control infected cells using flowcytometry

understood. Recent studies have demonstrated that Alu RNA plays a major role in post transcriptional regulation of gene expression for example by affecting alternative splicing, mRNA stability and protein synthesis^[33,34]. One of the recent identified targets that is modulated by Alu-RNA is the RPL22-mRNA^[20]. Thus, here our findings further confirm the regulatory effect of Alu-RNA molecules on gene expression of RPL22 that activated upon HS treatment. Interestingly, regulation of RPL22 in Alu-RNA dependent manner leads to significant interruption of HCV replication in HepG2 cells. Therefore, the current data provide a new technique that can prevent HCV replication in host cells without a harmful effect on treated cells as compared with non-treated cells. HS refers to cellular exposure to rapid stress changes such as temperature, toxins, oxidative stress, heavy metals, and pathogenic infections. Specifically temperature induced HS, even of a few degrees, has the ability to disturb protein folding. Other cellular damages have been reported in response to HS stress including rearrangement of cytoskeleton, alternation of organelle location, decreasing of ATP production, decreasing of proteins translation, changes in RNA splicing and gene silencing^[35]. The present data indicate the possible regulation of RPL22 expression in infected HepG2 cells that were subjected to HS. RPL22 is an RNA-binding protein with 60S large ribosomal subunit that plays a crucial role of macrolide resistance in bacteria^[36]. In vertebrates, RPL22 mutation might increase the proliferation of cells and then increase cancer risk. However, RPL22 has not been implicated in any lung diseases, especially in lung cancer^[36,37]. Other study demonstrated that human RPL22 protein interact with HCV-NS5A and support viral RNA translation^[38]. NS5A protein is the most common HCV research regarding its potential regulation of cellular immune response following infection. NS5A protein is translated from HCV genome as one of a large number of ploy-proteins that processed by NS3 protease^[39]. NS5A protein modulates host interferon signaling via direct interaction with the cellular factor retinoic acid-inducible gene-I (RIG-I) protein resulted in blocking of interferon signaling in infected cells^[40]. Additionally, NS5A plays the key role during HCV replication cycle and viral particles assembling through interaction with several viral and host proteins to insure viral replication. Several evidences indicate that NS5A is localized in certain modified cytoplasmic membrane during HCV replication that facilitates its significant role in HCV replication complex and replicase^[41,42]. Here, the relative expression of NS5A has been detected by q-RT-PCR using newly designed specific oligonucleotides. Our results showed that NS5A relative expression was significantly reduced in infected cells that were subjected to HS in comparison with control infected cells. On the other hand, flowcytometry has been used to investigate the clearance status of NS5A protein in HepG2 cells that were treated with HS. Interestingly, in comparison with control infected cells, our findings reveal that the percentage of NS5A positive cells was 30% of infected cells that were treated HS. Meanwhile, the percentage of NS5A positive cells was up to 70% regarding the control infected cells. These data demonstrate that HCV replication is potentially interrupted in HepG2 cells that were subjected to 5 min of HS. Taken together, these data provide an evidence for the possible inhibition of HCV infection via HS treatment affecting the expression of RPL22 through activation of Alu non-coding repeated element in HepG2 cells.

DECLARATIONS

Acknowledgments

The authors thank the chemist Rofida Refaai, Ain Shames Specialized Hospital for providing the blood samples derived from patient infected with HCV genotype 4. Special thanks to Prof. Dr. Mahmud Elmetean, Faculty of Medicine, Ain Shames University, for his medical supervision.

Authors' contributions

Planned and designed the study: Khalil H

Did the experiments assessing and developments: Farghaly H, Guirgis AA, Khalil H

Wrote the manuscript: Khalil H

Availability of data and materials

The data and materials are obtained from the corresponding author.

Financial support and sponsorship

Working at Dr. Khalil laboratory is supported by Science and Technology Development Fund (STDF), Egypt; Project ID: 4694.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

This study was approved by the ethical committee for post graduate studies of the University of Sadat City.

Consent for publication

Not applicable.

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Review

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Molecular mechanism of hepatocellular carcinoma

Seung Kew Yoon

Division of Hepato-Gastroenterology, Department of Internal Medicine, College of Medicine, The Catholic University of Korea; The Catholic University Liver Research Center & WHO Collaborating Center of Viral Hepatitis, Seoul, South Korea.

Correspondence to: Prof. Seung Kew Yoon, Division of Hepato-Gastroenterology, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, #222, Banpo-daero, Seocho-gu, Seoul 137-701, South Korea. E-mail: yoonsk@catholic.ac.kr

How to cite this article: Yoon SK. Molecular mechanism of hepatocellular carcinoma. *Hepatoma Res* 2018;4:42.
<http://dx.doi.org/10.20517/2394-5079.2018.23>

Received: 19 Mar 2018 **First Decision:** 19 Apr 2018 **Revised:** 12 Jul 2018 **Accepted:** 17 Jul 2018 **Published:** 8 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Development of hepatocellular carcinoma (HCC) is very complex and occurs through a multistep biological process of malignant transformation of normal hepatocytes in which various factors, including genetic and epigenetic alterations, regulation of oxidative stress, inflammation, and immunity are involved. To date, numerous studies have described the molecular pathogenesis of HCC, but the precise molecular mechanisms of HCC development remain unclear. Emerging single-cell transcriptome analysis technology is a powerful tool for defining sub-populations within heterogeneous bulk tumor tissue and allows molecular characterization of each cell. This breakthrough method can unveil the molecular mechanisms of HCC. In this article, I discuss recent advances in the molecular pathogenesis of HCC through this newly emerging concept of single-cell analysis.

Keywords: Hepatocellular carcinoma, molecular mechanism, pathogenesis, characterization

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer in men, the seventh most common in women and the third leading cause of cancer-related deaths worldwide^[1,2]. HCC accounts for approximately 85% of liver cancers^[1] and is characterized by a highly heterogeneous pathogenesis with an aggressive clinical course leading to poor survival. The risk factors for HCC are relatively well defined compared with those for other cancers. The risk factors include chronic hepatitis B virus (HBV), hepatitis C virus (HCV) infection, chronically heavy alcohol consumption, aflatoxin B₁ (AFB₁) exposure and nonalcoholic fatty liver disease (NAFLD)^[2]. The incidence of HCC is considered to be significantly higher in eastern Asia and sub-Saharan Africa, which are endemic areas of HBV infection, but the incidence of HCC is rising in Western countries due to increases in HCV infection, chronic alcoholic intake and NAFLD^[3,4].



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In an era of precision medicine for cancer treatment, it is essential to investigate the molecular mechanisms of carcinogenesis and tumor progression. In addition to array-based comparative analyses, genome-wide association studies (GWAS), next-generation sequencing (NGS), and RNA sequencing analyses for cancer research, studies on host immune mechanisms associated with immune evasion of cancer are also required to develop tumor immunotherapy.

It is generally accepted that hepatocarcinogenesis is very complex and occurs through a multistep biological process during malignant transformation of normal hepatocytes in which various factors, including genetic and epigenetic alterations, are involved. Specifically, recent advances in NGS technologies have facilitated a more profound understanding of the molecular mechanisms of HCC, which have contributed to the development of targeted therapies for cancers by identifying genes and associated signaling involved in carcinogenesis and tumor progression. Despite these advances, it remains difficult to effectively treat advanced HCC because most advanced cases are accompanied by poor liver function and liver cirrhosis. Surgical approaches, including resection and liver transplantation, are not available in these cases, so molecular targeted therapy combined with immunotherapy has become an alternative strategy to prolong patients' survival. To this end, further investigation of the molecular pathways involved in hepatocarcinogenesis and tumor progression is indispensable.

In this article, I discuss recent advances in molecular pathogenesis based on major etiologic factors for the development of HCC.

MOLECULAR MECHANISMS OF HBV-RELATED HCC

Among the major risk factors for HCC, HBV is the most common causative agent that increases the incidence of HCC in East Asia and sub-Saharan Africa. The HBV genome contains four genes (C, S, X and P), which encode the core protein, envelope protein, X protein and a polymerase. Among them, hepatitis B X protein (HBx) is known to have a critical role in the development of HCC. Accumulating evidence reveals that HBx has multifunctional activities including interruption of apoptosis in hepatocytes^[5] and DNA repair mechanisms through transcriptional regulations of p53^[6], facilitation of cellular signal transduction, cell cycle progression, and maintenance of genetic stability of HBx through interactions with different host factors^[7].

Chronic HBV infection enables viral DNA to integrate into the host genome, leading to an oncogenic transformation. A recent NGS study revealed that HBV integration was found in more than 80% of HBV positive HCC and was more extensive in tumor tissue compared with surrounding non-tumor tissue^[8]. In particular, three cancer-associated genes, telomerase reverse transcriptase (TERT), mixed-lineage leukemia 4 (MLL4) and cyclin E1 (CCNE1) were observed at frequent integration sites in HBV positive tumors. These findings suggest a significant association between HBV integration and hepatocarcinogenesis. Moreover, mutations in TERT promoter are found in more than 50% of HCC tissue^[9]. Although the mechanism by which TERT is activated in cancer is not clearly understood, and a recent study revealed that the GA-binding protein transcription factor (GABP), a member of the E-twenty six (ETS) transcription factor family, is selectively recruited to the mutated TERT promoter and activates TERT expression^[10].

Accumulating evidence has shown that HBx plays important roles in hepatocarcinogenesis. Several mechanisms by which HBx may function at the molecular and cellular levels are as follows: (1) transactivation of promoters of cAMP response element binding protein (CREB) response element (CRE)-containing genes, including the oncogene Yes-associated protein (YAP)^[11]; (2) alteration of the DNA specificity of CREB and activating transcription factor 2 (ATF-2), resulting in binding and activation of the HBV enhancer^[12]; (3) modulation of the DNA binding specificity of the p53 tumor suppressor, resulting in altered expression of

its target genes^[13]; and (4) regulation of cellular signaling pathways, such as activation of the Ras-Raf-MAPK pathway, Src-dependent pathway, PI3K-Akt pathway, inflammation-associated NF- κ B/STAT-3 pathways, and wnt/ β -catenin pathway^[14-18]. In addition, HBx affects epigenetic alterations through hyper- or hypomethylation of oncogenes and tumor suppressor genes, promoting histone acetylation and de-acetylation of tumor related genes as well as alterations of several microRNAs^[19-21].

MOLECULAR MECHANISMS OF HCV-RELATED HCC

HCV is a single stranded RNA virus with a 9.6-kb genome that encodes a large polyprotein that is cleaved at multiple sites to produce at least 10 proteins, including structural proteins [core, envelope (E)1 and E2] and non-structural (NS) proteins (proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). Epidemiologic studies worldwide have provided evidence that HCV is the major risk factor of HCC, and that chronic HCV infection induces liver fibrosis and cirrhosis, ultimately resulting in HCC. Historically, after identification of HCV, it has been very difficult to study its pathogenesis and development into chronic liver diseases and HCC due to the lack of *in vitro* culture systems. To overcome this limitation, transgenic animal models expressing single or multiple HCV viral proteins were developed^[22]. Despite these models, evidence for HCV playing a direct role in hepatocarcinogenesis remains controversial^[23]. Because of revolutionary studies on viral replication in cell culture^[24,25], studies investigating hepatocarcinogenesis by HCV have been actively performed. HCV that replicates only in the cytoplasm of a hepatocyte has not yet integrated into the host genome. Integration of viral elements into the host genome leads to direct oncogenic transformation of hepatocytes. Several studies have provided evidence for a direct role of HCV in the pathogenesis of HCC. Previous studies have described the role of the HCV core protein related to the development of HCC. The HCV core protein activates STAT3 via an IL-6 autocrine pathway^[26] and enhances telomerases activity^[27], which can induce oncogenic transformational changes in hepatocytes. In addition, NS3/4A enhances cellular proliferation by activating phosphorylation of extracellular signal-regulated kinases (ERKs) and inhibiting p53-mediated apoptosis and p21 promoter activity^[28,29]. In addition, chronic HCV infection induces oncogenic transformation in several ways: vigorous and continuous inflammation via NF- κ B^[30]; oxidative stress, inducing DNA mutagenesis^[31]; alteration of tumor suppressor genes^[32]; direct alteration of the wnt/ β -catenin pathway by NS5A; and blocking of TGF- β signaling through an interaction between TGF- β receptor I (T β R-I) and NS5A^[33]. Moreover, a recent study demonstrated that a lack of microRNA-122 resulted in a high incidence of tumors in a mouse model, but the mechanism by which this occurs has not been elucidated^[34].

Patients coinfecting with HBV and hepatitis delta virus (HDV) have been reported to have rapid and serious disease progression^[35]. However, little is known about whether and how co-infection of HBV and HDV can accelerate hepatocarcinogenesis. Recent studies suggest that marked liver inflammation, dysregulation of nuclear signaling pathways, increased oxidative stress, and epigenetic changes through HDV replication can enhance malignant transformation of hepatocytes, resulting in accelerated HCC development^[36].

MOLECULAR MECHANISMS OF ALCOHOL-RELATED HCC

Alcohol consumption, particularly over-consumption, is a serious global health problem. In general, heavy alcohol consumption leads to fatty liver, alcoholic steatohepatitis (ASH), cirrhosis, and eventually, HCC. ASH has been reported to progress to HCC at a rate of 3%-10% annually^[37]. Though the pathogenetic mechanisms underlying alcohol-induced tumor initiation have been well defined, the alcohol-related signaling pathways involved in tumor promotion and progression are poorly understood.

Induction of Cytochrome p450 2E1 (CYP2E1), a member of the cytochrome p450 mixed-function oxidase system, by chronic alcohol consumption induces various biologic effects, such as increases in alcohol metabolism, enhanced oxidative stress, increased hepatotoxicity and interactions with various drugs, xenobiotics and carcinogens^[38]. In particular, acetaldehyde produced by alcohol metabolism strongly induces oxidative

stress, exacerbating liver diseases.

Recent studies have described the association between CYP2E1 polymorphisms and alcohol-related disorders, including alcoholic cirrhosis^[39,40], but no significant association was found between CYP2E1 Pst I/Rsa I polymorphism and HCC in a recent meta-analysis^[41]. The molecular mechanisms for the direct role of alcohol on hepatocarcinogenesis remain unclear. However, a recent large-scale study using exome sequencing analysis of 243 liver tumors identified mutational signatures associated with specific risk factors and demonstrated that the Catenin beta 1 (CTNNB1) cluster was significantly related to alcohol as a risk factor for HCC^[42].

Sirtuins (SIRT1), nicotinamide adenine dinucleotide+ (NAD⁺)-dependent class III histone deacetylases, are linked to histone deacetylation and suppression of gene transcription, as well as the aging process^[43]. A previous study illustrated that alcohol reduced hepatic SIRT1 expression, suggesting that loss of SIRT1 activity may initiate alcoholic liver disease^[44]. However, the role of SIRT1 in the development and progression of tumors remains controversial. Numerous studies have demonstrated that SIRT1 acts to inhibit cell transformation and tumor progression, but other studies have suggested tumor promoting roles for SIRT1^[45-47]. In the context of HCC, a recent study by Jang *et al.*^[48] demonstrated the existence of positive feedback regulation between c-myc and SIRT1 that promotes tumor cell proliferation and predicts poor survival in human HCC. More recently, Mercer *et al.*^[49] performed *in vivo* experiments using ethanol feeding for long periods following injection of diethylnitrosamine (DEN). Their results suggest that chronic ethanol consumption activates Wnt/ β -catenin signaling, leading to increased hepatocyte proliferation and promotion of tumorigenesis following an initiating insult to the liver.

MOLECULAR MECHANISM OF NASH-RELATED HCC

NAFLD comprises a spectrum of liver disorders from simple fatty liver to NASH, hepatic fibrosis/cirrhosis and HCC. Individuals with NASH progress to HCC at a rate of 0.5% annually^[50]. The risk factors for NAFLD include metabolic syndrome, visceral adiposity, extreme dieting and type 2 diabetes. Additional factors accelerating the transition from simple fatty liver (SFL) towards NASH and HCC include the gut microbiota, adipose-related inflammation, and excessive intake of lipids^[51]. NASH associated with end-stage liver disease (ESLD) and HCC have become the second leading causes of liver transplantation in the USA^[52]. Generally, SFL is reversible through weight control by exercise and calorie restriction. However, once SFL has progressed to NASH, medical attention is required because of its progression to ESLD or HCC. To date, numerous animal models have been established to investigate NASH-associated HCC, but these models have limitations for elucidating cause-and-effect relationships in the development of HCC. Nonetheless, these animal models have provided crucial evidence for pathogenic mechanisms in NASH-associated HCC. The process of liver injury occurs through activation of oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, autophagy and intrahepatic NKT and CD8⁺ T cells^[53]. During the inflammatory process in NASH, several cytokines, adipokines and lymphokines contribute to hepatic fibrogenesis via the regenerative process of hepatocytes^[54]. In addition, recent studies have demonstrated that up-regulation of the insulin-like growth factor 1 (IGF1)/insulin substrate 1 pathway by hyperinsulinemia^[55] and enhancement of IL-6 and TNF levels by obesity^[56] contribute to hepatocarcinogenesis. Interestingly, a study by Yoshimoto *et al.*^[57] suggested that obesity-induced gut microbial metabolites promote liver cancer through the senescence secretome. In summary, the hepatic microenvironment of NASH, which is considered to be a proinflammatory milieu, plays an important role in the development and progression of HCC.

Genetic factors as well as environmental factors have also been considered to be risk factors for NAFLD-associated HCC. The nucleotide polymorphisms rs738409 C/G, which results in an isoleucine to methionine substitution at residue 148 (I148M) in human patatinlike phospholipase domain containing 3, leads to an alteration of TAG remodeling in lipid droplets. This variant has been linked to an increased risk for liver

fibrosis and NAFLD-related HCC^[58]. Additionally, transmembrane 6 superfamily 2 (TM6SF2) E167K and glucokinase regulator (GCKR) rs780094 gene variants have been reported to be associated with a higher risk for fatty liver and liver fibrosis^[59]. Although numerous factors that contribute to HCC from NAFLD have been revealed, there remain several unsolved issues for the molecular mechanism of HCC in the context of NAFLD, including the direct role of the gut microbiome, epigenetic regulation, identification of metabolomics profiles, and function of cancer stem cells linked to lipid metabolism.

MOLECULAR MECHANISMS OF HCC BY SINGLE-CELL TRANSCRIPTOMIC ANALYSIS

Recent advances in NGS technologies have facilitated deeper insights into the molecular mechanisms of tumor development and progression, thereby opening the way for a new era of personalized medicine. In particular, NGS-based transcriptome analysis (RNA-seq) has become a powerful tool for both characterizing the transcriptomes of each cell and profiling alternative splicing variants associated with cell function^[60]. To date, almost all genomic studies have been carried out using bulk samples. However, RNA-seq using bulk tissue samples comprising various cell populations is inappropriate for comprehensively investigating transcriptomic profiling because each cell in the tumor is constantly differentiating, proliferating, and heterogeneous. Thus, the newly developed single-cell RNA sequencing (scRNA-seq) technology is a powerful approach to dynamically analyze the genetic and cytologic heterogeneity of each cell in specific tumor tissue, providing a more comprehensive understanding of the molecular mechanism of carcinogenesis and the process of cancer evolution. The heterogeneity of single cells is diversely manifested in morphologic and phenotypic characteristics, genomics, and proteomics. Proper targets that can be used to analyze the heterogeneity of cancer cell using scRNA-seq include cancer stem cells (CSCs), circulating tumor DNA (ctDNA) and cell-free DNA (cfDNA)^[61-63].

Recently, RNA-seq-based transcriptome analyses using tumor and non-tumor tissue from 10 HBV-related HCCs were first reported by Huang *et al.*^[64]. Differentially expressed genes (DEGs; 1378) and differentially expressed exons (DEEs; 24,338) were identified in their study. Comprehensive functional analyses demonstrated that DEGs were most significantly enriched in cell growth-related, metabolism-related and immune-related pathways, suggesting a very complicated mechanism for hepatocarcinogenesis. Furthermore, RNA-seq data analyses at the exon level revealed a highly complex landscape of transcript-specific differential expression in HCC. In particular, a novel, highly up-regulated exon-exon junction was detected in the ATAD2 gene. This is the first study dealing with transcriptome profiles, including exon level expression changes and novel splicing variants using RNA-seq, and represents the most comprehensive characterization of HBV-related HCC transcriptomes as well as provides important clues for understanding the molecular mechanisms of HCC pathogenesis at system-wide levels. More recently, to further explore the dynamic mechanisms that simultaneously occur in genetic and epigenetic regulation on gene expression associated with heterogeneity at the single cell level in cancer, single-cell triple omics sequencing (scTrio-seq) techniques, including the genome, epigenome and transcriptome, have been developed^[65]. Recently, Hou *et al.*^[66] using scTrio-seq technology, have demonstrated correlations between genomic (copy-number variations, CNVs), transcriptomic, and methylomic data analyzed in the same individual cells in HCC. In addition, they revealed that changes in the gene dosage of certain regions due to CNVs proportionally affect the RNA expression levels of those corresponding regions^[66].

Although few studies have reported on the heterogeneity of liver CSCs at the single-cell level in HCC, a recent study showed that different CSC subpopulations contain distinct molecular signatures, suggesting that CSC heterogeneity may contribute to the molecular and biological diversity of HCC cell groups and, consequently, patient prognosis^[67]. Therefore, heterogeneity at the single cell level of liver CSCs may be critical for tumor progression and prognosis in HCC and might be important for the development of targeted agents for HCC.

It is generally accepted that tumor development and progression are closely linked to the failure of immune surveillance, which includes elimination of tumor cells at the initial stage or immune defense to prevent immune escape^[68]. In general, activated CD8+ T cells are essential in anti-cancer immunity, while regulatory T cells (Tregs) mediate significant immune dysfunction against cancer^[69]. The main reason for the decline of anti-cancer immunity is T cell dysfunction or exhaustion. Several factors responsible for this phenomenon have been proposed, including abnormal increases in check point inhibitors, such as programmed cell death protein 1 (PD1), cytotoxic T lymphocyte antigen 4 (CTLA4), lymphocyte activation gene 3 protein (LAG3), and killer cell lectin-like receptor G1 (KLRG1)^[70]. Moreover, tumor-infiltrating lymphocytes (TILs) surrounding cancer play an important role in host immunosurveillance associated with tumor biology. TILs present in HCC are composed of intratumoral CD8+ T cells and peritumoral CD4+ T cells independent of histogenetic origin^[71] but their roles in tumor killing are not clearly understood. More recently, Zheng *et al.*^[72] carried out deep scRNA-seq on 5063 single T cells isolated from peripheral blood, tumor, and surrounding non-tumoral tissue from 6 HCC patients. Analyzing the transcriptional profiles of these individual cells coupled with assembled T cell receptor (TCR) sequences, 11 functional T cell subsets were identified based on their molecular and functional properties. Specific subsets, such as exhausted CD8+ T cells and Tregs, are preferentially enriched and potentially clonally expanded in HCC. FOXP3, CTLA4, TNFRSF18, TNFRSF4 and CCR8 were highly expressed in tumor-infiltrating Tregs, while MYO7A, WARS, and CXCL13 LAYN, PHLDA1, and SNAP47 were identified in tumor-infiltrating exhausted CD8+ T cells. In particular, high expression of PHLDA1 and SNAP47 was significantly associated with poor prognosis in HCC patients. In addition, it was demonstrated that LYAN was highly expressed in both tumor Tregs and exhausted CD8+ T cells from tumor tissue of HCC and shown that LYAN is up-regulated on activated CD8+ T cells and Tregs, repressing the CD8+ T cell function *in vitro*^[72]. These data are crucial for understanding hepatocarcinogenesis and developing targeted immunotherapies in HCC.

CONCLUSION

The pathogenesis of HCC is very complicated and depends on the specific etiologic factors involved. HCC pathogenesis is a multistep process that involves diverse molecular and cellular signaling pathways. For this reason, patients with HCC should be managed with multiple therapeutic modalities by multidisciplinary teams rather than a single treatment approach to achieve better clinical outcomes. The major risk factors for HCC include hepatitis B and C virus infection, alcohol, NAFLD, chemical toxins and hereditary disorders. During hepatocarcinogenesis, numerous factors, such as oxidative stress, inflammation, hormone systems, hypoxia and immunity, are dysregulated, leading to the development of HCC. Nonetheless, the precise molecular mechanisms defining the development of HCC have not been entirely elucidated. Emerging scRNA-seq technology is a powerful tool for defining sub-populations of cells within a heterogeneous bulk of tumor tissue and has been a breakthrough that has the potential to unveil the molecular mechanisms of HCC. In addition, single-cell genome analysis can be applied to monitor circulating tumor cells and cell-free DNA to evaluate tumor recurrence. Moreover, analysis of the transcriptome heterogeneity and characterization of the heterogeneous molecular signatures in HCC will lead to development of novel therapeutic target agents and ultimately help tailor individual cancer therapy.

DECLARATIONS

Authors' contributions

The author contributed solely to the paper.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This research was supported by grants of Global High-tech Biomedicine Technology Development Program

of the National Research Foundation (NRF) & Korea Health Industry Development Institute (KHIDI) funded by the Korean government (MSIP&MOHW) (No. 2015M3D6A1065146).

Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Factors predicting hepatocellular carcinoma in hepatitis C infection

Zaigham Abbas¹, Minaam Abbas²

¹Department of Hepatogastroenterology, Dr. Ziauddin University Hospital, Clifton, Karachi 75500, Pakistan.

²Wellcome/Cancer Research UK Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK.

Correspondence to: Dr. Zaigham Abbas, Department of Hepatogastroenterology, Dr. Ziauddin University Hospital, Clifton, Karachi 75500, Pakistan. E-mail: drzabbas@gmail.com

How to cite this article: Abbas Z, Abbas M. Factors predicting hepatocellular carcinoma in hepatitis C infection. *Hepatoma Res* 2018;4:43. <http://dx.doi.org/10.20517/2394-5079.2018.26>

Received: 20 Mar 2018 **First Decision:** 12 Jun 2018 **Revised:** 19 Jun 2018 **Accepted:** 20 Jun 2018 **Published:** 8 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Hepatitis C virus (HCV) has emerged as a leading cause of hepatocellular carcinoma (HCC). In most cases, the virus causes HCC in the presence of chronic hepatic inflammation, advanced fibrosis, and cirrhosis. A combination of viral, environmental, and genetic factors are likely to determine the host immune response to the infection as well as the progression to HCC. Clinical and epidemiologic studies have identified many of the risk factors associated with HCC development in patients with chronic hepatitis C. Male sex and older age are considered as independent risk factors for HCC, while alcohol consumption accelerates fibrosis, increasing the risk for progression to HCC. Obesity, diabetes mellitus, nonalcoholic fatty liver disease, aflatoxin exposure and occult hepatitis B infection, all contribute to a higher HCC risk. HCV patients infected with HCV genotype 3 are also more likely to develop HCC and genetic variations such as single nucleotide polymorphisms, which may also alter the risk. Sustained virological response to the antiviral therapy results in significantly more favorable long-term outcomes. The incidence of HCC after HCV eradication is similar between patients treated with peginterferon plus ribavirin and direct-acting antiviral therapy.

Keywords: Hepatitis C, hepatocellular carcinoma, risk factors, alcohol, cirrhosis, diabetes, nonalcoholic fatty liver disease, directly acting antiviral agents

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer in men and ranks seventh among women. It is also the third leading cause of cancer-related deaths in the world^[1,2]. Hepatitis C virus (HCV) has emerged as the foremost cause of HCC in many countries and has surpassed hepatitis B virus (HBV)



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as a significant risk factor for the disease^[3]. In the majority of cases, HCC in hepatitis C occurs following persistent liver insult in the form of chronic hepatic inflammation, advanced fibrosis and cirrhosis^[4,5]. Recently, studies have also shown a direct role for HCV in cancer promotion with various HCV proteins demonstrating oncogenic properties^[6]. Overall, a combination of viral, genetic, host and environmental factors likely influence HCC carcinogenesis^[7]. Factors that thus affect or modify the likelihood of HCC development in patients with chronic hepatitis C, have been identified by clinical and epidemiologic studies. This review seeks to identify and analyze these diverse factors.

AGE AND GENDER

Male sex and older age are independent risk factors for HCC in chronic hepatitis C patients^[1,8-12]. In one study investigating a Chinese cohort, age > 55, and male sex were associated with an increased risk of developing HCC^[13]. Multivariate analyses of another study showed that older age, truncal obesity, and diabetes were significant predictors of advanced disease and HCC^[14,15]. Furthermore, a study on patients with transfusion-acquired HCV infection concluded that age at transfusion > 36 affected the risk for hepatic decompensation and was an independent risk factor for HCC development, alongside gender^[16].

Interestingly, multiple pregnancies may also increase the risk of HCV-related HCC. This raises questions about the role of estrogens and other pregnancy-related hormones in the modulation of HCV infection and its progression to HCC in female patients^[17].

ALCOHOL ABUSE

Patients with a history of alcohol abuse have a significantly higher prevalence of HCV infection than the general population^[18]. Furthermore, alcohol consumption in patients with chronic hepatitis C accelerates the process of fibrosis with an increased risk for progression to cirrhosis and HCC. Indeed, a study of 2235 patients with chronic hepatitis C, daily alcohol consumption of 50 g or greater was associated with a 34% increase in the rate of fibrosis progression^[19]. A meta-analysis of 20 articles (published between 1995 and 2004), involving more than 15,000 HCV chronically infected persons, illustrated that the pooled relative risk of cirrhosis associated with heavy alcohol intake (defined in the range of at least 210-560 gram per week) was 2.33 [95% confidence interval (CI), 1.67-3.26]^[20].

Alcohol abuse has been shown to be a key independent predictor of progression to HCC^[21,22]. The exact amount of alcohol that increases the risk of HCC in patients with HCV is unknown but it appears that even modest alcohol use can accelerate fibrosis and so the risk for HCC^[23]. Indeed, a case-control study to evaluate the risk of HCC for HCV infection found that the odds ratio (OR) of HCC development in HCV RNA positive patients was 26.1 (95% CI: 12.6-54.0) among subjects with alcohol intake of 0-40 g/day and increased to 62.6 (23.3-168) and 126 (42.8-373) with an alcohol intake of 41-80 and greater than 80 g/day, respectively^[24].

The progression to HCC may be the direct result of an increase in HCV replication and an attenuation of the antiviral action of interferon due to alcohol^[25]. Impaired host cellular immunity (due to dendritic cell dysfunction)^[26] and increased oxidative stress and mitochondrial injury^[27] due to alcohol consumption, all contribute to the development of HCC.

DIABETES AND NON-ALCOHOLIC FATTY LIVER DISEASE

Hepatitis C patients with obesity, diabetes mellitus, and/or non-alcoholic fatty liver disease (NAFLD) have a higher risk of developing HCC^[28,29]. In fact, five of seven studies analyzing diabetes demonstrated significantly increased HCC risk associated with concurrent diabetes with effect sizes ranging from HR 1.73 (95% CI: 1.30-2.30) to RR 3.52 (95% CI: 1.29-9.24). Additionally, insulin resistance, as measured by HOMA-IR, was

also found to be significantly associated with HCV-related HCC^[30]. Diabetes not only increases the risk of HCC in treatment-naïve chronic hepatitis C patients^[31] but also in patients with eradicated HCV^[1,9,12,32].

Meanwhile, one of three studies analyzing body mass index demonstrated a significant association with HCC risk (BMI ≥ 30.0 vs. BMI < 23 ; RR 4.13, 95% CI: 1.38-12.40) and two of the three studies analyzing steatosis demonstrated the significantly higher risk of HCC associated with steatosis^[28]. Indeed, HCV patients in the US were found to progress more rapidly to HCC than their counterparts in China and the underlying fatty liver disease was found to be a major contributor to this difference^[15].

HEPATITIS B CORE ANTIBODY POSITIVITY

The risk of HCC increases in patients with hepatitis C who have occult hepatitis B infection or are hepatitis B core antibody positive^[14,33]. In one study, the presence of hepatitis B core antigen was one of the independent predictors associated with the occurrence of HCC in HCV patients without advanced fibrosis^[34]. On the other hand, HCV sero-status (positive vs. negative among patients with chronic hepatitis B may also increase the risk of HCC, independent of HBV viral load, with a HR of 2.5 (95% CI: 1.7-3.6)^[35].

AFLATOXIN

Significant contamination of food by aflatoxin is an additional risk factor for HCC in some parts of Asia^[36,37]. While studies have shown synergism between aflatoxin and HBV in causing HCC, much less is known about whether aflatoxin and HCV synergize in a similar fashion. It is interesting to note that HCV prevalence itself is much higher in areas where aflatoxin exposure is also high^[38].

ADVANCED FIBROSIS AND CIRRHOSIS

HCC develops in hepatitis C patients mostly in the setting of advanced fibrosis and liver cirrhosis^[13]. For patients without pre-existing cirrhosis, a higher Fibrosis-4 (FIB-4) index translates to a higher risk of HCC^[39]. Untreated patients with cirrhosis have a significantly higher HCC incidence rate (45.3 per 1000 person-years) compared to those treated with either IFN or DAAs^[40,41]. Moreover, liver cirrhosis, high AST to platelet ratio index (APRI) levels, and IL28B rs12979860 at baseline are all associated with HCC development in patients without sustained virological response (SVR) after peg-IFN combination therapy^[42]. Even with SVR, the absolute risk of HCC is high in patients with established cirrhosis^[1,8,9,12,43-46].

HCV GENOTYPE

HCV patients infected with HCV genotype 3 are at higher risk for end-stage liver disease, HCC, and liver-related death compared to other genotypes^[11,43]. This association is independent of patients' age, diabetes, body mass index, or antiviral treatment^[43]. The risk of HCC remains high even after eradication of genotype 3 HCV^[1,46-48]. This genotype may have a particular oncogenic mechanism, leading to HCC development even in non-cirrhotic patients^[49]. Certain polymorphisms of the core, NS3, and NS5A proteins of HCV genotype 1b may be associated with the development of HCC^[50].

SINGLE NUCLEOTIDE POLYMORPHISMS

Genetic variations, such as single nucleotide polymorphisms (SNPs), may alter disease risk and thus may be used as predictive markers of disease outcome. A genome-wide association study found a strong association between the SNP rs17047200, located within the intron of the toll-like 1 gene (TLL1) on chromosome 4, and the development of HCC in patients who achieved an SVR after treatment for chronic HCV infection^[9]. Additionally, the association of variants in patatin-like phospholipase domain containing 3 (PNPLA3) and the unfolded protein response regulator GRP78, with the risk of developing HCC, has been described in Italian

HCV patients^[51]. Moreover, the reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) gene, a novel transformation suppressor gene, has also been linked to HCC amongst several other malignancies. However, a study conducted on an Egyptian cohort concluded that the RECK gene rs10814325 TT genotype could not be considered a risk factor for HCC development in hepatitis C patients, but may be related to the disease progression and metastasis^[52].

Furthermore, the GG and GG + GA genotypes of IL17A gene may also serve as a risk factor for HCC development by increasing IL17 and IgE levels^[53]. WT IL-23R GG^[54], transforming growth factor- β 1 (TGF- β 1)-509 and tumor necrosis factor- α (TNF- α)-308 genes polymorphisms may also serve as risk factors for cirrhosis and HCC in chronic hepatitis C patients^[55].

NON-RESPONSE TO THE THERAPY

Antiviral therapy reduces the development of HCC and complications of cirrhosis in patients with chronic hepatitis C^[56]. A risk scoring system has been developed to predict HCC development for HCV patients following antiviral therapies. The system includes age, gender, platelets count, alpha-fetoprotein levels, fibrotic stage, HCV genotype and response to the antiviral therapy^[10].

The cumulative risk of HCC development is higher in subjects with high HCV RNA titer than subjects with low titer^[45]. SVR results in significantly more favorable long-term outcomes, and decreased risk of progression to cirrhosis and HCC^[13,57]. Indeed, a meta-analysis showed that SVR after treatment at any stage of fibrosis is associated with reduced HCC risk^[58]. The risk of developing HCC diminishes significantly 2 years after SVR^[44].

The risk of HCC after HCV eradication, though considerably reduced, remains relatively high at 0.33% per year^[47]. Compared to subjects with spontaneous viral clearance, subjects with antiviral treatment-induced HCV viral clearance are at higher risk for HCC development, especially if they have significant hepatic fibrosis^[12].

Antiviral therapy for patients with normal ALT levels can also lower the HCC incidence in responders, particularly for elderly and male patients^[59]. Moreover, even in patients who have developed HCC within the Milan criteria and have undergone curative treatment for HCC, elimination of HCV and SVR inhibits recurrence and contributes to a preferential prognosis^[60].

DIRECTLY ACTING ANTIVIRAL AGENTS

The role of DAAs (used in the treatment of HCV) in the development of HCC is controversial, with several early studies demonstrating a tenuous link. However, a retrospective population-based cohort study of 17,836 patients treated with either an interferon-based regimen or DAA, showed that the risk of HCC was the same in both groups^[40]. A meta-analysis of 41 studies further clarified the issue and concluded that the risks of HCC development after HCV eradication were similar between patients treated with peginterferon plus ribavirin and direct-acting antiviral therapy and that there was no evidence to suggest that DAAs promoted HCC^[8,61]. The seemingly higher incidence of HCC following SVR with DAA therapy was related to baseline risk factors and patient selection, and not the use of interferon-free therapy *per se*. The cohort of patients treated with DAAs in earlier studies included older patients and patients with more advanced cirrhosis who were already predisposed to a higher risk for HCC at baseline. In a cohort study of 857 patients, individuals receiving interferon-free therapy were more likely to be older, of white ethnicity, Child-Turcotte-Pugh B/C vs. Child-Turcotte-Pugh A; thrombocytopenic, non-genotype 3, and treatment experienced. HCC occurrence was observed in 46 individuals during follow-up. In univariate analysis, IFN-free therapy was associated with a significantly increased risk of HCC (HR: 2.48; $P = 0.021$). However, after multivariate adjustment for baseline factors, no significant risk attributable to interferon-free therapy persisted^[41].

Among patients treated with DAA, SVR is associated with a considerable reduction in the risk of HCC. However, in patients with SVR, the absolute risk of HCC remains high in patients with established cirrhosis^[62].

CONCLUSION

Hepatitis C accounts for the majority of the cases of HCC in many parts of the world. HCC typically occurs in patients with advanced hepatic fibrosis or cirrhosis in the setting of chronic inflammatory state induced by HCV. Clinical and epidemiologic studies have identified host and viral factors associated with HCC development in patients with HCV infection. Direct-acting antiviral drugs do not increase the risk of developing HCC. Sustained virological response to the antiviral therapy results in significantly more favorable long-term outcomes.

DECLARATIONS

Authors' contributions

Both authors contributed by literature review and manuscript writing, editing and review.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Exosome-based liquid biopsy in the management of hepatocellular carcinoma

Aparna Jayachandran^{1,2}, Sasidhar Venkata Manda³, Ritu Shrestha^{1,2}, Kim R. Bridle^{1,2}, Prashanth Prithviraj⁴, Darrell H. G. Crawford^{1,2}

¹School of Clinical Medicine, The University of Queensland, Brisbane, QLD 4072, Australia.

²Gallipoli Medical Research Institute, Greenslopes Private Hospital, Brisbane, QLD 4120, Australia.

³Apollo Hospitals Educational and Research Foundation, Hyderabad, Telangana 500096, India.

⁴Fiona Elsey Cancer Research Institute, Ballarat, VIC 3350, Australia.

Correspondence to: Dr. Aparna Jayachandran, Gallipoli Medical Research Institute, Greenslopes Private Hospital, Brisbane, QLD 4120, Australia. E-mail: a.jayachandran@uq.edu.au

How to cite this article: Jayachandran A, Manda SV, Shrestha R, Bridle KR, Prithviraj P, Crawford DHG. Exosome-based liquid biopsy in the management of hepatocellular carcinoma. *Hepatoma Res* 2018;4:44. <http://dx.doi.org/10.20517/2394-5079.2018.59>

Received: 14 May 2018 **First Decision:** 3 Jul 2018 **Revised:** 11 Jul 2018 **Accepted:** 12 Jul 2018 **Published:** 13 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Hepatocellular carcinoma (HCC) commonly presents at an advanced stage due to the lack of efficient early screening tools. Early, non-invasive biomarkers useful in the diagnosis and prognosis of HCC would be of significant benefit for HCC management. Development of exosome-based liquid biopsy as a non-invasive method for the management of HCC has gained much traction. Exosomes are small membranous vesicles secreted by most cell types including HCC cells. Exosomes serve as couriers for the intercellular transfer of important biomolecules, including, protein, nucleic acids and lipids to nearby and distant cells in the body. The molecular cargos carried by exosome have been described to play significant roles in cancer progression. Herein, we will dissect how HCC-derived exosomes confer aggressive traits such as tumour growth, invasion, immune remodelling and drug resistance to HCC cells. We review the current literature concerning exosomes as biomarkers in a diagnostic setting, evaluating their prognostic, predictive and monitoring capabilities. This review will highlight and discuss emerging research in the utility of exosome-based liquid biopsies therapeutic tools in HCC management. Here we will also focus on advances in exosome biology in preclinical studies.

Keywords: Hepatocellular carcinoma, liquid biopsy, exosomes, microRNA, epithelial-to-mesenchymal transition, cancer stem cell

INTRODUCTION

Hepatocellular carcinoma (HCC), a fatal primary malignancy of hepatocytes remains a global challenge due to its high mortality rates and high frequency of recurrence^[1-3]. Surgical resection, chemotherapeutic or



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radiotherapeutic interventions are intensively used in the clinic; however, the survival benefit is limited^[4]. The poor prognosis of advanced HCC is, in part, related to the lack of reliable biomarkers for early diagnosis and the lack of effective therapeutic agents for unresectable tumours. Currently, the clinical diagnosis of HCC relies on serum alpha-fetoprotein (AFP) levels and imaging examination, including ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and invasive tumour biopsy^[5]. However, it is widely known that these screening methods have low sensitivity, high false negative rates and cannot predict post-therapy recurrence and also fail to monitor real time disease and therapy^[6,7]. Furthermore, conventional tumour biopsies provide a small sample size and may fail to reflect the entire tumour heterogeneity that is essential in treatment procedures and prescribing a targeting therapy based on the genotype. Therefore, to improve the prognosis and survival of HCC patients, there is an urgent need for more sensitive and effective tools for early detection, screening, real time monitoring of the disease and prognosticating risk of relapse.

EXOSOME-BASED LIQUID BIOPSY

Liquid biopsy, as a minimally invasive and cost effective method for sampling of genetic, proteomic and metabolic material from different types of cancers, has drawn much attention in recent years^[8]. Recently, the discovery that exosome-based liquid biopsy may have diagnostic and therapeutic applications has garnered considerable interest^[9]. Exosomes are small membranous cell-derived extracellular vesicles of endocytic origin with 50-100 nm in diameter^[10]. These nano-vesicles are secreted by most type of cells and can be detected and isolated from various body fluids such as serum, urine, plasma, saliva, milk and malignant ascites^[9].

The exact function of exosomes remains largely unknown. Initially, exosomes were considered to function as cellular garbage bags for the disposal of excess or non-functional cellular constituents^[9,11]. Emerging studies have revealed that exosomes serve as an intercellular courier of important functional biomolecules including protein, lipid, DNA, messenger RNA, and microRNA^[12]. Exosomes have a unique function in modulating intercellular communication among both nearby and distant cells in the body and thereby influencing both pathological and physiological processes. Exosomes interact with their target cells by fusion of membranes and transfer their content to regulate cellular activities in target cells^[13]. Additionally, proteins on the surface of exosomes have been known to interact with cell surface receptors on target cells to mediate intracellular signalling^[13].

In cancers, the production and composition of exosomes are markedly altered. For instance, it is estimated that approximately 2000 trillion exosomes are contained in normal human blood and the number of exosomes increase to approximately 4000 trillion in blood of cancer patients^[9,14]. The underlying cause for enhanced levels of exosome production remains unclear. Cancer cell-derived exosomes function in an autocrine or paracrine manner to modulate the tumour microenvironment^[15]. Moreover, the cargo shuttled by tumour-derived exosomes determines their effect on target cells, and the exosomes play important roles in their ability to influence tumour growth and progression. The role of exosomes in the areas of diagnosis, prognosis and treatment of tumours have been intensively investigated in many cancers, including HCC^[13,16]. Tumour cell-derived exosomes were shown to carry and transfer oncogenes, pathogens and microRNAs^[17]. Understanding the role of exosomes and their relevance to HCC offers the potential for new biomarkers for diagnosis and new druggable targets for treatment.

BENEFITS OF EXOSOME-BASED LIQUID BIOPSIES

Exosome-based liquid biopsies have several advantages over traditional biopsies. First, due to its minimally invasive nature, multiple samples of exosomes can be collected at different time points during treatment. Whereas, the deeply located tumours are often not accessible to be monitored during treatment and obtaining multiple tumour biopsies is difficult in clinical settings^[18]. Second, similar to cells, the cargo of exosomes reflect the metabolic state of cells they originate from, in real time. Exosomes also express specific markers seen in their cells of origin, making it easier to track the origin of exosomes^[9]. Third, they

are distinguishable by their size and morphology (cup-shaped appearance) through electron microscopy. Moreover, exosome surface profiling through flow cytometry and ELISAs allow classification of these subcellular vesicles to an extent^[19]. Fourth, many detection and isolation techniques have been developed for exosomes in research and therapy. Many commercial kits are available for high efficiency exosomes isolation from small amounts of body fluids^[10]. Fifth, the lipo-proteinous architecture of exosomes also protects the exosomal constituents from degradation. For example, microRNAs (miRs) within the exosomes are resistant to RNases and are stable in the circulation and may be promising candidates as novel biomarkers of cancers^[20]. Sixth, the routinely used serum HCC markers such as AFP and des-gamma-carboxyprothrombin (DCP) are not accurate for the early detection of HCC as they lack adequate sensitivity and specificity for effective HCC surveillance^[21]. Furthermore, several factors unrelated to HCC such as obstructive jaundice, vitamin K deficiency, alcohol intake, or warfarin treatment may elevate the serum DCP levels^[22]. A recent study has highlighted that exosomal serum miRs are promising biomarkers to improve sensitivity, specificity, early detection and prognostic prediction of HCC^[20]. Thus, exosome-based diagnostics may improve the detection of early HCC and prove to be more superior to the frequently used HCC biomarkers such as AFP and DCP.

Finally, exosome-based liquid biopsy is preferred as a robust standalone diagnostic and prognostic method compared with other liquid biopsy-based biomarkers such as circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs). Both ctDNA and CTCs have limitations biologically and technically and appear unsuited for clinical practice at the present moment. ctDNA is a single stranded or double stranded DNA, shed by either living or dying tumor into the blood^[23]. Clinical use of ctDNA levels alone as cancer biomarker is currently not recommended as it is not a cancer-specific biomarker and elevated ctDNA levels have been detected in healthy controls with infections. Moreover, increased ctDNA levels are associated with pathological conditions unrelated to cancer such as chronic inflammation and autoimmune disease^[24]. ctDNA are less stable as they have a short half-life^[25].

CTCs are cancer cells that have detached from tumor tissue and are present in the bloodstream. They have the potential to seed the cancer to other sites^[26]. CTC application is confronted with many challenges. A major challenge with CTCs is to obtain tumor cells in adequate numbers for evaluation, as CTCs are rare in blood (1-10 CTCs per 10 mL). CTCs also lack cancer-specific surface markers, making detection and isolation difficult^[25]. In summary, the many benefits of exosome-based liquid biopsy application render it a useful method for both diagnosis and prognosis of cancers including HCC and it also appears promising in providing a new dimension to personalized cancer care.

ISOLATION AND IDENTIFICATION METHODS OF HCC-DERIVED EXOSOMES

Differential ultracentrifugation is considered the gold standard method for purification of exosomes and most of the studies have applied this technique for isolating HCC-derived exosomes^[27,28]. However, depending on the starting material and downstream applications, other methods for the purification and enrichment of HCC-derived exosomes have also been used such as ultrafiltration, size exclusion chromatography (SEC) or the ExoQuick TC method^[19,29].

Majority of HCC-derived exosomes have been identified by a combination of different methods including, round or cup shaped morphology by transmission electron microscopy, size of 50-100 nm in diameter by nanoparticle tracking analysis and exosomal surface profiling for markers such the tetraspanins (CD9, CD63, and CD81), heat shock proteins (HSP90 and HSP60), Alix and Tsg101 by immunoblot and flow cytometry^[19,20,29-32]. Studies have demonstrated high expression of glypican-3 (GPC-3) and AFP, traditional markers of HCC, within the HCC-derived exosomes, thereby confirming the hepatoma-based origin of these exosomes^[31].

Table 1. HCC exosome-derived liquid biopsy-based biomarkers

Exosome-derived biomarker	Region	Patient cohort	Samples	References
Exosomal RNA biomarkers				
miR-21	China	HCC ($n = 30$) and chronic hepatitis B ($n = 30$)	Serum exosome	[27]
miR-178	Japan	HCC patients before surgery ($n = 6$) and HCC patients who underwent LDLT ($n = 59$)	Serum exosome	[20]
miRs-221, 191, 181a, 26a, let-7a	China	HCC ($n = 50$), Hepatitis B patients ($n = 50$) and healthy subjects ($n = 50$)	Serum exosome	[42]
miRs-18a, 221, 222 and 224	Korea	HCC ($n = 20$), cirrhosis ($n = 20$) and Hepatitis B ($n = 20$)	Serum exosome	[43]
miRs-21, 519d and 494	Italy	HCC patients ($n = 118$)	Serum and tissue exosome	[17]
miR-320a	China	HCC patients ($n = 6$)	CAFs and PAFs	[44]
miR-125b	China	HCC ($n = 30$ and $n = 128$), CHB ($n = 30$), cirrhosis ($n = 30$)	Serum exosome	[39]
miR-665	China	HCC ($n = 30$), healthy ($n = 10$)	Serum exosome	[40]
miR-638	China	HCC ($n = 126$), healthy ($n = 21$)	Serum exosome	[41]
miR-1247-3p	China	HCC without lung metastasis ($n = 90$), HCC with lung metastasis ($n = 20$), healthy ($n = 25$)	Serum exosome	[19]
Xist	China	206 females including HVs, CHB, cirrhosis and HCC	Peripheral blood exosomes	[45]
Exosomal protein biomarkers				
LG3BP and PIGR	Spain and Poland	HCC ($n = 29$), healthy individuals ($n = 32$), CCA ($n = 43$), PSC ($n = 30$)	Serum extracellular vesicles	[30]

CLINICAL UTILITY OF EXOSOMES IN THE MANAGEMENT OF HCC

Exosomes have been extensively studied for diagnostic purposes and as drug delivery vehicles^[33]. Notably, it was demonstrated that engineered cells can produce exosomes capable of preferentially binding to tumour cells^[34]. In this section, we will highlight the studies that address the utility of exosomes as biomarkers and therapeutic tools in the management of HCC.

Exosomes as biomarkers of HCC

The contents of exosomes may serve as novel specific diagnostic biomarkers for detection of early stage and advanced HCC, summarised in Table 1. Exosomes may help discriminate HCC patients with high risk of recurrence and poor prognosis and guide timely comprehensive therapy for these patients.

Exosomal RNA biomarkers

Serum exosomal miRNAs have received considerable attention as potential non-invasive biomarkers for diagnosing cancers. miRNAs are non-coding RNAs that are 22 nucleotides long and target mRNAs for cleavage or translational repression, thus modulating a variety of biological processes^[35,36]. Wang *et al.*^[27] found enriched miR-21 in serum exosomes from 30 patients with HCC and negligible amounts in chronic hepatitis B patients or healthy volunteers. These authors also reported that miR-21 enrichment in serum exosomes provided increased sensitivity of detection than whole serum. Conversely, another study described that miR-21 expression was much lower in patients with HCC^[37]. In line with this study, Qi *et al.*^[38] confirmed low expression of miR-21 in HCC patients. Reasons for this conflicting data may be due to differences in detection techniques, as well as, differences in patient cohorts.

The content of serum exosomes has been associated with aggressiveness, prognosis and survival of HCC patients. For instance, downregulation of miR-718 in serum exosomes was associated with the recurrence of HCC after liver transplantation in 59 HCC patients. *HOXB8* was identified as a potential target gene of miR-718, such that the downregulation of miR-718 resulted in the overexpression of *HOXB8* in HCC patients. High expression of *HOXB8* plays an important role in the progression and recurrence of HCC^[20]. Exosomes extracted from serum samples collected from two cohorts of HCC patients showed high levels of miR-125b which was an independent predictive factor for postoperative recurrence and overall survival of HCC patients^[39]. Serum exosomal miR-665 levels were significantly higher in HCC patients than those in healthy subjects. Additionally, exosomal miR-665 levels were elevated in larger tumours with local invasion

and at an advanced clinical stage (stage III/IV) of HCC^[40]. Another study found decreased expression of serum exosomal miR-638 in HCC patients^[41]. High miR-1247-3p in serum exosomal levels correlated with lung metastasis, poor overall survival and poor disease-free survival in HCC patients^[19].

A study identified a panel of miRs including miR-221, miR-191, let-7a, miR-181a, and miR-26a to be an optimal gene reference set for normalising the expression of liver-specific miRNAs^[42]. The serum levels of a panel of exosomal miRs including miR-18a, miR-221, miR-222 and miR-224 were significantly higher in patients with HCC than those with Hepatitis B and cirrhosis^[43]. The serum levels of exosomal miR-101, miR-106b, miR-122 and miR-195 were lower in patients with HCC than in patients with hepatitis B^[43]. Circulating miRNAs, miR-939, miR-595, miR-519d and miR-494 could identify cirrhotic patients with HCC. Upon comparison of serum and tissue miR levels in 14 patients surgically treated for HCC, a correlation between circulating and tissue levels of miR-519d, miR-494 and miR-21 was found in HCC patients^[17]. A whole micro-RNAome microarray analysis was applied to explore dysregulated expression of miRNAs in patients with cirrhosis, early, intermediate and advanced HCC. This study identified exosome-mediated dysregulation of circulatory miRNAs, miR-519d, miR-21, miR-221 and miR-1228^[17]. A significant reduction in miR-320a level was detected by miRNA sequencing of exosomes derived from cancer-associated stromal fibroblasts (CAFs) when compared to corresponding paracancer fibroblasts (PAFs) of 6 HCC patients^[44]. By using nanoparticle tracking analysis, the serum exosome concentration in HCC patients was found to be higher than in cholangiocarcinoma (CCA) and primary sclerosing cholangitis (PSC) patients^[30]. Long noncoding ribonucleic acid (lncRNA) X-inactive-specific transcript (Xist) was upregulated in peripheral blood of HCC patients^[45].

Exosomal protein biomarkers

Furthermore, when the protein content of serum exosomes was characterised in 29 HCC patients and 32 healthy individuals, Galectin-3-binding protein (LG3BP) and polymeric immunoglobulin receptor (PIGR) was found to be abundant in HCC patients. In particular LG3BP could distinguish patients with HCC from CCA and PSC patients^[30]. Together these studies suggest exosomal miRNAs, lncRNA and proteins may serve as novel diagnostic and prognostic biomarkers of HCC.

Exosomes as delivery vehicles for HCC therapeutics

Emerging studies demonstrate the importance of exosomes as potential targets for therapeutic intervention. Exosomes can be used as biological delivery vehicles for incorporating specific cargo into target cells. One study used exosomes to horizontally transfer therapeutic miRNAs into HCC cells^[46]. A recent study demonstrated the inhibitory effects of mesenchymal stem cells on HCC. In this study, rats models of HCC treated with adipose-derived mesenchymal stem cell (ADMSC) exosomes harboured significantly smaller tumours and more intratumoural invariant (CD8α+) natural killer T (NKT)-cells and low-grade HCC than the controls^[47]. As ADMSCs produce large amounts of exosomes, these cells are well suited for the mass production of exosomes^[48]. Another study utilised ADMSCs derived exosomes for miR-122 delivery into HCC xenograft models^[49]. This study also demonstrated that miR-122 promoted chemosensitivity of HCC cells^[49]. Furthermore, exosomes isolated from human hepatic stellate (LX2) cells were loaded with miR-335-5p and these exosomes were taken up by HCC cells *in vitro* and *in vivo*. This preclinical study showed an inhibition of HCC cell proliferation and invasion *in vitro* and also demonstrated HCC tumour shrinkage *in vivo* upon uptake of these engineered exosomes^[50]. There are several advantages of using exosome-based therapy, as exosomes show low immunogenicity, toxicity and are stable in tissue and in circulation. Together, this information suggests that exosomes have great translational potential as therapeutics or delivery vehicles for targeted therapy. Therefore, further studies must identify the optimal delivery method of exosomes to HCC patients.

HCC-derived exosome functions in preclinical studies

HCC-derived exosomes have pleiotropic biological functions, including roles in tumour growth, metastasis, immune response, intercellular communication, and drug resistance. In this section, we will dissect

Table 2. Preclinical studies demonstrating function of exosomes in HCC

Process	HCC cell lines	Effect	References
mRNA surveillance	HepG2, Hep3B	Nup98 prevents p21 mRNA degradation by the exosome	[52]
Intercellular communication, microRNA-based communication	Hep3B, HepG2 and PLC/PRF/5	Modulate the constitutive expression and downstream signalling of TAK1	[54]
Long noncoding RNA-based communication	Hep3B and PLC/PRF/5	Transfer of TUC339 to regulate HCC growth	[55]
Tumour growth and metastasis	SMMC-7721	Self-derived exosomes promote growth and motility	[15]
	HKCI-C3, HKCI-8, MHCC97L and MIHA	Motile cell-derived exosomes induced motility in non-motile cells	[56]
	MHCC97-H and SMMC-7721	miR-320a suppresses HCC cell migration	[44]
	Hep3B cell, 97H and LM3	Motile HCC cells secrete more sugar metabolism regulatory proteins	[28]
	HepG2 and Hep3B	miR-490 rich mast cell-derived exosomes blocked motility of HCC cells	[29]
	MHCC97-H and MHCC97-L	Enriched adenylyl cyclase associated protein 1 in motile HCC cells	[57]
	CSQT-2, HCC-LM3, HepG2 and MHCC-97L	miR-1247-3p promotes lung metastasis	[19]
Immune modulation	PBTC, MHCC97H, SMCC-7721	14-3-3 ζ promotes anti-tumour immune response	[32]
	DC2.4, Hepa1-6	Induces immune response to suppress tumour growth	[4]
	Hepa1-6	Induces anti-tumour response by decreasing T regulatory cells	[31]
Chemoresistance	HepG2, Hep3B, PLC/PRF-5 and Huh-7, MzChA1 cells	Exposure of HCC cells to diverse anti-cancer agents increased exosomal linc-VLDLR expression	[59]
	HepG2 and Hep3B	miR-122 delivered via exosomes sensitised cells to doxorubicin and sorafenib	[49]
	MHCC-97 L, MHCC-97H and LO2	Larger tumours formed in mice treated with sorafenib and invasive cell-derived exosomes	[60]
	MHCC97H, MHCC97L, HepG2, Huh7, LX2	Conditioned media from activated fibroblast with high miR-1247-3p conferred sorafenib resistance	[19]
	HepG2 and PLC/PRF/5	linc-RoR and TGF- β modulated stemness	[59]
Cancer stem cells	SMMC-7721	miR-1247-3p enhanced stemness	[19]
EMT	MHCC97-H	Overexpression of miR-320a induces an EMT	[44]

the diverse functions of exosomes derived from HCC cells. These functions are summarised in Table 2. Collectively, these data may provide the foundation for further studies into the regulatory roles of exosomes in the development and progression of HCC.

mRNA surveillance

Exosomes have been known to participate in control mechanisms that remove aberrant RNAs in the nucleus and the cytoplasm^[51]. In HCC cell lines, HepG2 and Hep3B, the exosomes recognise and degrade p21mRNA upon Nup98 depletion as a process of mRNA surveillance related either to impaired export or defects in RNA protein complex formation in the 3'UTR region^[52].

Intercellular communication

Exosomes have emerged as important mediators of intercellular communication that can shuttle protein and RNA to recipient cells and can elicit a potent overall effect on transformed cell tumours^[13,53]. For example, Hep3B, HepG2 and PLC/PRF/5 cell-derived exosomes can modulate the expression of transforming growth factor- β activated kinase-1(TAK1) and associated downstream signalling and enhance transformed cell growth in recipient cells^[54]. Furthermore, vacuolar protein sortin 4 homolog A (VPS4A) regulates exosome-mediated aberrant miRNA expression in HCC cells^[15]. The potential of exosomes to transfer lncRNA is increasingly recognised. Kogure *et al.*^[55] first demonstrated that lncRNA with highly conserved sequences ultraconserved RNAs (ucRNAs) influences intercellular signalling. In HCC cell lines PLC/PRF/5 and Hep3B, the intercellular transfer of ucRNA TUC339 by exosomes represents a unique signalling mechanism by

which tumour cells can promote HCC growth and spread^[55]. Thus, the use of exosomes as biological delivery vehicles is of considerable interest.

Modulation of HCC tumour growth and metastasis

Exosomes are considered to serve essential roles in tumour growth and metastasis by regulating complex interactions between tumour cells and their microenvironment. Several studies addressed whether HCC cell-derived exosomes can influence the biological behaviour of the parental HCC cells. A study revealed that incubation of SMMC-7721 cells with self-derived exosomes caused a notable increase in cell growth, migration, and invasion^[15]. Another study described a comprehensive RNA and protein profiling of exosomes derived from motile and non-motile HCC cell lines. Exosomes derived from metastatic HCC cell lines HKCI-C3, HKCI-8 and MHCC97L were enriched in protumorigenic RNAs and proteins, such as MET protooncogene, S100 family members and the caveolins. Of interest, exosomes from motile HCC cell lines could significantly enhance the migratory and invasive abilities of non-motile immortalised hepatocyte line MIHA. Motile behaviour in MIHA cells was triggered by activation of PI3K/AKT and MAPK signalling pathways which in turn increased secretion of active matrix metalloproteinase, MMP-2 and MMP-9^[56]. A comparative proteome analysis of exosomes from the non-motile Hep3B cell, and the motile 97H and LM3 cells found the motile HCC cells to secrete more sugar metabolism regulatory proteins via exosomes in the tumour microenvironment^[28]. The ability of exosomes to modulate the motile ability of tumour cells was tested by comparing protein profiles of cell lines with distinct metastatic potential. Among these, adenylyl cyclase associated protein 1, a protein implicated in HCC metastasis, was significantly enriched in exosomes from cells with high motile ability. Moreover, incubating low motile MHCC97 L cells with highly motile MHCC97 H cell-derived exosomes, enhanced the motile ability of MHCC97-L cells^[57]. Thus, it is conceivable that highly motile HCC cell-derived exosomes could modify normal hepatocytes and less motile HCC cells in their microenvironment to facilitate tumour growth, invasion and metastases.

Alteration in exosomal miRs also influences tumour behaviour. For instance, HCC cell-derived exosomes have been shown to activate the MAPK/ERK pathway through miR-665 and further promote the proliferation of tumour cells^[40]. Whereas, the expression of miR-320a was significantly downregulated in HCC cell lines. miR-320a binds to its direct downstream target and suppresses HCC cell proliferation, migration and metastasis^[44]. Previous studies have shown that the increase of mast cells (MCs) usually indicates a poor prognosis of HCC patients^[58]. MC-derived exosomes showed increased expression of miR-490 and the transfection of HepG2 and Hep3B cells with these exosomes inhibited migration and invasion in both the HCC cell lines^[29]. Exosomes derived from high-metastatic cancer cells contribute to fibroblast activation to foster lung metastasis of liver cancer via transfer of miR-1247-3p^[19].

Immune modulation

Emerging evidence suggests that HCC-derived exosomes can mediate dialogue between cancer cells and immune cells to promote antitumor immune responses for tumour growth. For instance, Wang *et al.*^[32] demonstrated that 14-3-3 ζ , also called 14-3-3 protein zeta was transmitted from HCC cells to T cells via exosomes and resulted in the inhibition of anti-tumour functions of tumour-infiltrating T cells in the HCC microenvironment. HCC-derived exosomes have been shown to be enriched in HCC antigens, which in turn can prime cytotoxic T lymphocytes and elicit a stronger immune response *in vitro* and *in vivo* compared with cell lysates^[4]. Exosomes from HCC cells can also present tumour antigens to versatile mediators of the immune system, the dendritic cells to induce a strong immune response and to suppress tumour growth^[4]. Similarly, another study combined tumour-derived exosome-pulsed dendritic cells and PD-1 antibody with sorafenib and observed the effects on tumours in mice with orthotopic HCC. This treatment combination induced antitumor responses and changed the tumour microenvironment by decreasing T regulatory cell accumulation in tumour tissue after sorafenib treatment^[31].

Chemoresistance

Cell behaviour can be modulated by the content within exosomes during chemotherapeutic treatment in HCC cells. For example, exposure of HCC cells to diverse anti-cancer agents such as sorafenib, camptothecin, and doxorubicin increased the lncRNA, linc-VLDRL within the exosomes released from these treated cells and promoted chemotherapeutic resistance^[59]. Another study explored how transforming growth factor (TGF)- β selectively enriched lncRNA linc-RoR within exosomes and thereby facilitated chemoresistance^[59]. Treating HepG2 and Hep3B cells with miR-122 loaded exosomes derived from adipose tissue derived mesenchymal stem cells (ADMCs) sensitised these cells to doxorubicin and sorafenib^[49]. Furthermore, a study found that exosomes derived from HCC cells can block the therapeutic effects of sorafenib and promote tumor growth^[60]. This study demonstrated that exosomes derived from highly invasive hepatoma cells, MHCC-97 L and MHCC-97H had greater efficacy than that of exosomes derived from less invasive cells, LO2. Notably, combined treatment with sorafenib and exosomes derived from highly invasive hepatoma cells resulted in the formation of larger tumours in mice than those in mice treated with sorafenib alone or sorafenib plus exosomes derived from less invasive cells^[60]. After treatment with conditioned media collected from fibroblasts pre-treated by exosomes derived from high-metastatic cancer, tumour cells showed increased spheroid formation ability, motility, and resistance ability to sorafenib^[19]. Thus, exosomes can modulate chemoresistance in recipient cells that incorporate these exosomes. Understanding how exosomes confer resistance to cellular stress will enable us to develop more effective treatments for HCC.

Cancer stem cells

Accumulating evidence implicates cancer stem cells (CSCs) in the growth and spread of HCC^[61]. CSCs have the capacity for self-renewal and ability to differentiate, and have been identified to confer resistance to chemotherapy. Exosomes have been implicated in promoting stemness in HCC. For example, TGF- β treatment enhanced the growth of CD133+ CSCs in HepG2 cells. Both stemness and chemoresistant phenotype of CSCs were modulated by lncRNA linc-RoR within the exosomes derived from TGF- β treated cells^[59]. SMMC-7721 cells treated with miR-12473p revealed increased sphere formation with elevated expression in CSC marker genes such as CD133, lrg5, Oct4, nanog and CD90^[19].

Epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is a cellular process during which epithelial cells undergo a phenotypic switch to become more aggressive and motile mesenchymal cells^[62]. The process of EMT is also a major contributor for metastasis and drug resistance^[61]. More recently, exosomes have been described to mediate EMT in many cancers^[63]. In HCC cell lines, the expression of miR-320a was significantly downregulated and induced EMT as evidenced by changes in EMT marker expression. miR-320a simultaneously enhanced the expression of mesenchymal marker, N-cadherin and suppressed the expression of epithelial marker E-cadherin, thereby eliciting EMT^[44].

DISCUSSION

Numerous studies suggest clinical usefulness of exosomes as minimally invasive biomarkers for the detection, diagnosis and prognosis of different cancers^[9,10]. In HCC, there is poor consensus regarding the use of exosome-derived miRs as diagnostics. This could be ascribed to diversity of study designs, analytical conditions, choice of internal control genes, choice of body fluid used such as serum or plasma, choice of control and patient populations and sample size^[17]. Furthermore, most of the data reported in HCC literature have been obtained on eastern patients, whose tumour biology might not match that of western patients. Indeed, obtaining exosome-based liquid biopsies might prove to be beneficial in cases where obtaining tumour biopsies is difficult in clinical settings. However, it is still necessary to standardise methods for exosome isolation and characterisation by using guidelines proposed by the EV-TRACK Consortium^[64]. Furthermore, the potential use of exosomes as delivery vector needs more critical evaluation.

Tumour-derived exosomes have been described as regulators of metabolic reprogramming in various tumour microenvironments^[65]. Metabolic reprogramming is a process whereby tumours increase their glucose availability by suppressing uptake of glucose by non-tumour cells^[66]. However, their role in HCC remains to be elucidated. Although a few studies have explored the role of exosomes in EMT and cancer stem cells, further studies are warranted in these areas. Another relevant aspect is angiogenesis, a major process which regulates nutrient availability of fast growing solid tumours^[10]. The role of exosomes in facilitating angiogenesis and its consequence on HCC metastasis remain unexplored. Collectively, these phenomena impose major challenges on cancer treatment and both *in vitro* and *in vivo* studies in these areas will lay the foundation for future clinical trials.

CONCLUSION

Exosomes are biologically active nanovesicles that can transfer information to recipient cells to mediate local as well as distant cell-cell communication. In summary, increasing number of studies has shown that HCC-derived exosomes are potent mediators of tumor growth, proliferation and motility. They also play a pivotal role in moulding the host immune response. Other relevant aspects influenced by HCC-derived exosomes are chemoresistance, EMT and CSCs. The ease of isolating exosomes and their content from different body fluids may provide a new source of biomarkers with application in diagnosis, prognosis and in monitoring disease progression during and after treatment. Moreover, exosomes have shown great potential as drug delivery systems for the treatment of HCC. Overall, exosomes show a tremendous potential for better cancer care and effective treatment outcomes for HCC.

DECLARATIONS

Authors' contributions

Conception and manuscript writing, provision of study materials, collection and assembly of data, and final approval of manuscripts: Jayachandran A, Manda SV, Shrestha R, Bridle KR, Prithviraj P, Crawford DHG

Availability of data and materials

Not applicable.

Financial support and sponsorship

This manuscript publication is funded by Gallipoli Medical Research Foundation.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Opinion

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Elimination of hepatitis from Pakistan by 2030: is it possible?

Yasir Waheed¹, Masood Siddiq²

¹Foundation University Medical College, Foundation University Islamabad, Islamabad 44000, Pakistan.

²Jinnah Memorial Hospital, 2-Civil Lines, Rawalpindi 46000, Pakistan.

Correspondence to: Dr. Yasir Waheed, Foundation University Medical College, Foundation University Islamabad, Islamabad 44000, Pakistan. E-mail: yasir_waheed_199@hotmail.com; Prof. Masood Siddiq, Jinnah Memorial Hospital, 2-Civil Lines, Rawalpindi 46000, Pakistan. E-mail: dr.masood.siddiq@gmail.com

How to cite this article: Waheed Y, Siddiq M. Elimination of hepatitis from Pakistan by 2030: is it possible? *Hepatoma Res* 2018;4:45. <http://dx.doi.org/10.20517/2394-5079.2018.58>

Received: 14 May 2018 **First Decision:** 20 Jun 2018 **Revised:** 17 Jul 2018 **Accepted:** 30 Jul 2018 **Published:** 14 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Globally 71 million people are living with hepatitis C virus (HCV) out of which 7.1 million (10%) are present in Pakistan. Genotype 3 is the most common HCV type in the country. World Health Organization is working with health authorities in different countries for effective control of HCV, to reduce its incidence by 90% and to reduce hepatitis related mortality by 65% by the year 2030. There are several challenges that hinder elimination of HCV from Pakistan including the lack of patient awareness about the causes and transmission of disease, lack of affordability for investigations and drug treatment and lack of experienced healthcare professionals. Other major contributors to achieve HCV elimination are lack of effective drugs and delayed regulatory approvals combined with compromised monitoring by health authorities and lack of robust epidemiological data. Efforts are needed to educate the public about the modes of transmission and prevention of HCV infection, and massively upscale screening along with treatment. There is a dire need to prevent more than 200,000 new infections that occur each year in Pakistan. Given the scale of the problem, it is very unlikely that the government alone can handle it.

Keywords: Hepatitis C virus, global health sector strategy, hepatitis elimination, national hepatitis strategic framework, punjab hepatitis ordinance, hepatitis diagnosis, screening

OPINION

Viral hepatitis caused 1.4 million deaths in 2015, which is comparable with the annual deaths from tuberculosis and higher than the annual deaths from HIV^[1]. The hepatitis epidemic remained neglected for



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many years until 2015, the global burden of disease figure came out^[2] and hepatitis is considered as the 7th leading cause of deaths worldwide. After that hepatitis is included in the Sustainable Development Goals by United Nations^[2]. World Health Organization (WHO) has developed a Global Health Sector Strategy (GHSS) to eliminate viral hepatitis by 2030. The major goals of GHSS on viral hepatitis are to reduce hepatitis incidence by 90% and to reduce hepatitis mortality by 65% by 2030^[3]. Globally, 71 million people were living with hepatitis C virus (HCV) in 2015 and 75% of them were living in lower and middle-income countries. According to the Polaris Observatory, 7.1 million hepatitis C cases are present in Pakistan, which covers about 10% of the global HCV burden^[4,5].

WHO is working with health authorities in different countries to develop effective hepatitis control programs, and to achieve hepatitis elimination by 2030. As of November 2017, 84 countries have developed national plans and strategies to control hepatitis^[5]. Nine countries (Iceland, Qatar, Australia, Georgia, Japan, Netherlands, Egypt, France, and Germany) are on track to achieve HCV elimination targets by 2030, 22 countries are working towards elimination and Pakistan is among the countries in which HCV elimination is un-achievable with its present policy^[6].

The government of Pakistan has launched the National Hepatitis Strategic Framework (2017-2021) in October, 2017. Effective implementation of NHSF depends on the concerted Federal and Provincial actions from all stake holder in the health and other sectors to respond to viral hepatitis^[7]. The major routes of hepatitis transmission in Pakistan are unscreened blood transfusions, shaving from barbers, reuse of needles and syringes and reuse of the same dental and surgical instruments for different patients^[8]. Pakistan is the country with the highest number of therapeutic injections per person per year. The most dominant genotype of hepatitis C in Pakistan is 3^[8]. The conventional and Pegylated Interferon based therapy also showed good results in Pakistani patients in the last decade as compared with genotype 1 patients^[9-11]. The Sofosbuvir based therapy showed excellent response in Pakistani Hepatitis C patients^[12]. There is a dire need to speed up the registration and availability of new direct acting antivirals for Hepatitis in Pakistani market.

There is a strong need for early diagnosis and treatment of HCV in Pakistan. According to WHO's progress report on access to hepatitis C treatment, 161,000 HCV patients got treatment in Pakistan in the year 2016 (mostly through the private sector)^[5]. A recent modelling study suggests that Pakistan needs to scale up its HCV treatment number (up to 880,000 treatments per year), to achieve the GHSS targets on viral hepatitis. The treatment number can be minimized (to 525,000 per year) by targeting the treatment to people who inject drugs and people living with cirrhosis and through scaling up prevention interventions^[13]. Recently, Punjab provincial government has promulgated the Punjab Hepatitis Ordinance 2017. Hopefully this ordinance will play an important role in controlling hepatitis in the province.

Pakistan also needs to improve its HCV surveillance system. According to a national survey conducted in 2007, 4.8% of the Pakistani population was living with HCV^[14], which according to current population estimates (207 million) constitutes about 9.9 million HCV cases, while the Center for Disease Analysis estimates suggests the presence of 7.1 million HCV cases in the country^[4].

There is a strong need to speed up the HCV diagnosis and find the missing millions living with HCV. Globally, only about one in five people affected with HCV in 2016 had been diagnosed^[5]. Non-governmental organizations (NGOs) are playing a significant role in the fight against hepatitis across the globe. There is no funding specifically allocated for the NGOs' work on hepatitis elimination in Pakistan. The prevalence of Hepatitis is very high in high-risk population groups including people who inject drugs, thalassemia patients and refugees^[15,16]. There is a dire need to start HCV micro-elimination projects in high-risk population groups including people who inject drugs, transgender population, and homeless people.

Egypt, a lower middle-income country, showed excellent commitments in the fight against hepatitis. By September 2017, a cumulative total of 1.5 million people had received HCV treatment in the country^[5]. The Ministry of Health and Population of Egypt is planning to screen 15 million Egyptians for the presence of HCV in 2018. World Bank offered to lend the ministry \$200 million to assist its plan to screen 15 million Egyptians for the presence of HCV^[17].

Pakistan also needs to show strong political and financial commitments in the fight against hepatitis. Modelling techniques suggest that HCV can become a rare disease in the next 20-25 years, with a significant financial commitment^[18]. Extensive HCV treatment and preventive measures are required in Pakistan to achieve the HCV elimination targets in WHO's GHSS on viral hepatitis, without which Pakistan's HCV burden will increase markedly.

The elimination of Hepatitis from Pakistan by 2030 seems impossible with the current initiatives. It will be a significant impact if the country succeeded in controlling the hepatitis from the country and reduced the annual hepatitis deaths from 200,000 to less than 25,000. The control of hepatitis epidemics requires political will, financial investment and support from pharmaceutical, medical and civil societies around the globe^[19].

DECLARATIONS

Authors' contributions

Design: Waheed Y, Siddiq M

Literature research: Waheed Y, Siddiq M

Data analysis: Waheed Y, Siddiq M

Manuscript writing: Waheed Y, Siddiq M

Manuscript editing: Waheed Y, Siddiq M

Manuscript revision: Waheed Y, Siddiq M

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Screening for hepatocellular carcinoma: summary of current guidelines up to 2018

Nevin Yilmaz^{1,2}, Ugur Eser Yilmaz³, Kaya Suer⁴, Vedat Goral⁵, Nedim Cakir⁴

¹Gastroenterology Section, Research and Trainee Hospital, Mugla Sitki Kocman University, Mugla 48000, Turkey.

²Gastroenterology/Hepatology Department, Near East University School of Medicine, Nicosia, Mersin 10, Northern Cyprus.

³Koc University School of Medicine, Istanbul 34010, Turkey.

⁴Department of Infectious Diseases and Clinical Microbiology, Near East University School of Medicine, Nicosia, Mersin 10, Northern Cyprus.

⁵Department of Gastroenterology, Medipol University School of Medicine, Istanbul 34214, Turkey.

Correspondence to: Prof. Nevin Yilmaz, Gastroenterology/Hepatology Department, Near East University School of Medicine, Near East Boulevard, Nicosia, Mersin 10, Northern Cyprus. E-mail: nevinylmz@yahoo.com

How to cite this article: Yilmaz N, Yilmaz UE, Suer K, Goral V, Cakir N. Screening for hepatocellular carcinoma: summary of current guidelines up to 2018. *Hepatoma Res* 2018;4:46. <http://dx.doi.org/10.20517/2394-5079.2018.49>

Received: 2 May 2018 **First Decision:** 8 Jun 2018 **Revised:** 24 Jun 2018 **Accepted:** 29 Jun 2018 **Published:** 16 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer related to worldwide death with a great geographical variation. To be eligible for curative therapy at the time of diagnosis is important. However, the majority of cases are diagnosed at late stages. This can be achieved with applicable screening modalities. Until now, many organizations around the world have developed guidelines according to their own evidence-based data for screening of HCC. The purpose of this article is to review the screening modalities of HCC to assist gastroenterologists and providers involved in the management of HCC.

Keywords: Hepatocellular carcinoma, screening, guidelines, surveillance

INTRODUCTION

As emphasized in publications, liver cancer is the second most common cause of worldwide cancer deaths with the fifth most common cancer in men and the ninth in women in 2012^[1]. Hepatocellular carcinoma (HCC) represents the major histological sub-type up to 90% of primary liver cancers^[2-5].

The first HCC cases in hepatitis-associated cirrhosis have been reported in the 1940s^[6]. Following the dis-



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covery of alpha-fetoprotein (AFP) in HCC by the Russian scientist, screening HCC is widely recommended for patients who are under risk for more than 40 years^[7,8].

Over the time, the underlying etiologies, incidence, and HCC outcomes are changed according to the countries. While the incidence of HCC is rising in the west, attributed to the past HCV epidemic (baby-boomers) and trends of metabolic disorders, it is decreasing in the East^[9-11]. In despite of receiving regular HCC surveillance, nearly 40% of patients still died in 5 years^[12,13]. These changes are accommodating the new research on and development of new guidelines for HCC management.

Guidelines mean “rules or instructions about the best way to do something”^[14]. They assist health care providers in the decision-making process according to evidence-based data, with guiding clinical practice in circumstances where all possible resources and therapies are available^[15]. International scientific societies have issued recommendations for establishing a common standardized approach in the management of HCC.

Although these organizations are international, the recommendation-guidelines mostly directed to their own cases. It is essential for gastroenterologist to be familiar with these organizations and their proposed guidelines. As recommended in the guidelines, it is more appropriate to follow the guidelines but to adapt on the patient basis. In this article, you will find a summary of the current screening guidelines for HCC of three different continents.

CURRENT GUIDELINES

The success of the screening is influenced by the availability of effective treatment with the identification of the target population and the selection of appropriate screening tests. The cost-effectiveness should also be taken into consideration. In this review, the target group is divided into cirrhotic and non-cirrhotic patient group.

Screening recommendations for cirrhotic adults

Cirrhosis is the strongest predisposing factor for HCC formation. Nearly 85%-95% of HCC is developed on the cirrhotic liver^[16-18]. These patients have a lifetime risk of developing HCC by 30% with leading cause of liver related death in compensated cirrhosis^[2,19,20]. The risk varies with the underlying condition; the highest 5-year cumulative risks are reported in HCV cirrhosis (17% in the west, 30% in Japan), hemochromatosis (21%), HBV cirrhosis (10% in high endemic areas, 15% in the west), alcoholic cirrhosis (8%-12%), and biliary cirrhosis (4%). Also, the presence of co-infection (HCV/HBV or HBV/HCV) or alcohol abuse increases the risk by at least 200%^[21]. In addition to underlying etiology, other patient-related factors influence the risk of HCC. In general, low platelet count of less than $100 \times 10^9/L$, presence of esophageal varices in addition to older age and male gender correlate with development of HCC among patients with cirrhosis^[22-24]. However, current guidelines do not incorporate with the risk of stratification models (RSM) for cirrhotic that may be useful in the future for excluding some patients from screening.

Screening modalities consist of the periodic application of diagnostic tools with cost effectiveness which is generally taken into consideration based on the gain of life expectancy and guidelines indicating that an incidence of $\geq 1.5\%$ year would warrant surveillance of HCC in cirrhosis^[25,26]. Guidelines including the last updated screening section with data-supported recommendations were selected for the review; recommendations are as follows.

From North America

The American Association for the Study of Liver Diseases (AASLD-2017): routine screening is recommended for HCC in adults with cirrhosis. The initial screening is performed with ultrasound (US) with or without

alpha-fetoprotein (AFP) every 6 months. AASLD does not suggest performing surveillance of patients with Child-Pugh class C cirrhosis unless they are on the transplant waiting list, given the low anticipated survival for these patients. They pointed out some technical remarks regarding screening modalities (US alone or plus AFP), interval (4-8 months) and modification in screening strategy based on etiology of liver diseases or risk stratification models^[26]. In the previous guideline (AASLD-2011), ultrasound scanning alone was recommended^[27].

The Canadian Association for the Study of the Liver (CASL 2014): this report is from consensus conference updated of the existing consensus - CASL 2011. The current statements for cirrhosis are similar with AASLD except they recommend US alone in every 6 months. The committee does not recommend AFP either alone or combined with US due to less sensitivity of AFP (67% sensitivity). They also do not recommend other biomarkers (AFP) lectin fraction (AFP-L3) and des-gamma-carboxy prothrombin (DCP) due to less validation^[28,29].

From Asia

The Asian Pacific Association for the Study of the Liver (APASL-2017): their recommendation is using combination of US and serum AFP measurement in every 6 months. The cut-off value of AFP should be set at 200 ng/mL for the cirrhotics. They do not suggest screening to cirrhotics not ineligible for treatments due to severe liver disease or other comorbidities which is similar with North America groups^[30].

CHINESE-2017: updated from 2011. Their recommendation for cirrhosis is identical with APASL. The only difference is that there is no excluding criteria for severe liver diseases^[31].

The Japan Society of Hepatology (JSH-2015): updated from 2013. Modalities and screening intervals mostly differ from the other countries and Asia. Besides AFP, a protein induced by vitamin K absence or antagonist-II (PIVKA-II) and AFP-L3 measurements are also recommended by the JSH to increase sensitivity. The JSH evidence-based clinical practice guidelines for HCC divided patients into an extremely high-risk group (hepatitis B or C cirrhosis) and a high-risk group (patients with chronic hepatitis B, chronic hepatitis C, or non-viral cirrhosis). Their recommendations for extremely high-risk patients are periodic imaging screening by US every 3-4 months along with three tumour markers (AFP, PIVKA-II and AFP-L3). Additionally, they recommend multi-detector computed tomography (MDCT) or MRI examinations in every 6-12 months as the first step of screening (optional) method even there is no evidence of tumour on US, because of poor visualization capability^[32,33]. The recommendations for the high-risk group cirrhosis are more cost effective and included periodic screening by US along with three tumour markers, every 6 months. MDCT and MRI are not recommended for high-risk patients^[32,33].

Japan Society of Hepatology- Liver Cancer Study Group (JSH-LCSG 2014): consensus-based guidelines. The JSH-LCSG practice guidelines use identical definitions for the extremely high-risk group and high-risk group. However, JSH-LCSG recommends EOB-MRI (gadolinium-ethoxybenzyl-diethylenetriamine penta-acetic acid-enhanced magnetic resonance imaging) instead of dynamic MDCT which has higher detection sensitivity than CT, as the first-line modality for surveillance every 6-12 months, even if no tumour is detected on US^[33,34].

From Europe

The European Association for the Study of the Liver (EASL-2018): the guideline is in press, their screening recommendations for Child-Pugh stage A and B patients are used by abdominal ultrasound every six months. AFP or other tumour biomarkers (AFP, AFP-L3 and DCP) are not recommended due to less accuracy for early detection of HCC. Stage C cirrhosis is excluded from screening except for transplant candidates^[2].

Table 1. Recommendations for cirrhotic adults

Continent	Guidelines	Modality	Time interval (months)	Exceptions
North America	AASLD-2017	US with or without AFP	6	Child-Pugh stage C unless awaiting liver transplantation
Asia	CASL-2014	US	6	Same as AASLD
	APASL-2017	US and AFP	6	Severe liver diseases/other co-morbidities (ineligible for curative therapy)
	CHINESE-2017	US and AFP	6	NS
	JSH-2015*	Extremely-high risk patients: (HBV/HCV cirrhosis) - US and three Tm markers (AFP/PIVKA-II/ AFP-L3) - CT or MRI (optional) High risk patients: (cirrhosis of another etiology) US and three tumor markers (AFP/PIVKA-II, AFP-L3)	3-4 6-12 6	NS
Europe	JSH-LCSG-2014	Recommend EOB-MRI instead of CT or MR	Same as JSH	NS
	EASL-2018**	US	6	Same as AASLD
	SPANISH-2016 (AEEH, SEOM, SERAM, SERVEI and SETH)	US	6	NS
	SEOM-2015	US	6	Same as AASLD
	ESMO-ESDO-2012	US	6	NS

*3rd JSH-HCC guidelines, 2013 update; **in press. HBV: hepatitis B virus; HCV: hepatitis C virus; AFP: alpha-fetoprotein; NS: not specified; US: ultrasound; PIVKA-II: proteins induced by vitamin K absence; CT: computed tomography; MRI: magnetic resonance imaging; EOP-MRI: gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOBDTPA)-enhanced magnetic resonance imaging

Selected guidelines from Spain (consensus document from The Spanish Association for the Study of the Liver (AEEH), Spanish Society of Medical Oncology (SEOM), The Spanish Society of Medical Radiology (SERAM), The Spanish Society of Vascular and International Radiology (SERVEI), The Spanish Society of Liver Transplantation (SETH)-2016 and European Society for Medical Oncology (ESMO)/European Society of Digestive Oncology (ESDO)-2012 recommend every 6 months US examination to patients with or without cirrhosis, and specify the theory behind this recommendation^[35,36]. However; SEOM-2015 guideline excluded Child C patients from screening (unless awaiting for liver transplantation) like EASL^[37].

This section was summarized in [Table 1](#).

Recommendations for non-cirrhotic adults

A small proportion of patients with HCC is diagnosed in the non-cirrhotic liver (NCL) with the risk of being less than 1% annually in patients with chronic hepatitis without significant fibrosis, in contrast to 3%-7% annually when the patient develops cirrhosis^[26,35,38]. HCC in NCL ranges widely from 7% to 54% according to the etiology of the liver disease and varies of the geographic areas^[39]. While viral hepatitis is pre-screened with decrease in the east as known, metabolic causes predominate in the west.

As compared to cirrhotic HCC, it has lower prevalence of the three main risk factors (hepatitis B and C virus infections and alcohol abuse), with an increased prevalence of other etiological factors, such as non-alcoholic fatty liver diseases, obesity and type 2 diabetes mellitus, exposure to genotoxic substances-aflatoxin, tobacco, sex hormones, inherited diseases and genetic mutations^[2,3,11,26,30,36,38-40].

In contrast to cirrhotic, NCL-HCC are more suitable for surgical treatments even in more advanced tumour stage at the time of diagnosis, since it is generally detected at a symptomatic stage due to unsettled scheduled screening program in these groups^[2,38,39].

There is also a risk stratification model for non-cirrhotic HCCs. PAGE-B (platelet, age, gender, hepatitis B) that is developed for HBV is recommended for non-cirrhotic HBV patients by EASL-2018^[2,24,41].

Recommendations from guidelines are as follows.

From North America

AASLD-2017: there is no proposal for non-cirrhotic patients in the current guideline. The previous AASLD guideline (2010), described the high-risk HBV carriers for HCC [Table 2] and the recommendation for screening was US in every 6 months^[26,27].

CASL-2014: identical with AASLD-2010, the CASL recommends HCC screening for the following high-risk groups by using US in every 6 months: Asian male hepatitis B carriers over the age of 40, Asian female hepatitis B carriers over the age of 50, hepatitis B carriers with a family history of HCC, Africans and African Americans with hepatitis B^[29].

From Asia

APASL-2017: recommendations for non-cirrhotic group similar to CASL. Differently, they recommend screening in Africans older than 20 years old. The surveillance strategy is combination of US and serum AFP, every 6 months, recommending that the cut-off value of AFP can be set at a lower value in a population with hepatitis virus suppression or eradication^[30].

CHINESE-2017: recommendations for non-cirrhotic-chronic liver diseases (any etiology) are AFP with ultrasonography in every 6 months for surveillance^[31].

JSH-2015 and JSH-LCSG 2014: for the high-risk non-cirrhotic (patients with chronic hepatitis B, chronic hepatitis C), they recommend an US examination along with measurement of three tumour markers (AFP/PIVKA-II, AFP-L3) in every 6 months [Table 2]^[32-34].

From Europe

EASL-2018: categorized the non-cirrhotic HBV patients at intermediate or high risk of HCC according to PAGE-B classes for Caucasian subjects, respectively 10-17 and ≥ 18 score points^[2,41].

To this group and non-cirrhotic F3 patients, regardless of etiology screening based on an individual, risk assessment is recommended for patients in the low HCC risk class (PAGE-B score ≤ 9), who do not reach the 0.2%/year threshold for starting screening. The PAGE-B score has not yet been validated in Asia due to Caucasian subjects. They recommend an US examination in every 6 months^[2].

The consensus document from the AEEH, SEOM, SERAM, SERVEI and SETH -2016 has not specified the screening for non-cirrhotic subjects^[35]. However, SEOM-2015 recommended screening for high-risk HBV chronic hepatitis patients (higher viral load, viral genotype or Asian or African ancestry) and non-cirrhotic patients with chronic hepatitis C and advanced fibrosis (F3)^[37].

ESMO-ESDO-2012 recommendations are similar to SEOM-2015, which suggests to non-cirrhotic HBV carriers with high viral load (> 10.000 copy/mL) and non-cirrhotic patients with chronic hepatitis C and advanced cirrhosis^[36].

This section was summarized in Table 2.

Table 2. Recommendations for non-cirrhotic adults

Continent	Guidelines	Target population	Modality	Time interval (months)
North America	AASLD-2017	No recommendation for surveillance of non-cirrhotics at this time		
	AASLD-2010	HBV carriers: - Asian female > 50 years - Asian male > 40 years - Family history of HCC - African/North American Blacks	US	6
Asia	CASL-2014	Same as AASLD-2010	US	6
	APASL-2017	Non-cirrhotic (HBsAg positive): - Asian females > 50 years - Asian males > 40 years - Africans aged > 20 years - History of HCC in the family	US and AFP	6
	CHINESE-2017	Chronic liver diseases of any etiology	US and AFP	6
	JSH-2015 and JSH-LCSG-2014	High risk patients (chronic hepatitis B or C)	US and three tumor markers AFP/PIVKA-II/AFP-L3	6
	EASL-2018	Non-cirrhotic HBV patients at intermediate or high risk of HCC* Non-cirrhotic F3 patients regardless of etiology**	US	6
	SPANISH-2016 (AEEH, SEOM, SERAM, SERVEI and SETH)	No recommendation for surveillance of non-cirrhotics at this time		
Europe	SEOM-2015	High-risk HBV chronic hepatitis patients (higher viral load, viral genotype or Asian or African ancestry) Non-cirrhotic patients with chronic hepatitis C and advanced fibrosis (F3)	US	6
	ESMO-ESDO-2012	Non-cirrhotic HBV carriers with high viral load (> 10,000 copy/mL) Non-cirrhotic patients with chronic hepatitis C and advanced fibrosis (F3)	US	6

*According to PAGE-B classes for Caucasian subjects, intermediate or high risk of HCC (10-17 and U 18 score points, respectively;

**considered for surveillance based on an individual risk assessment. HCC: hepatocellular carcinoma; F3: bridging fibrosis; HBV: hepatitis B virus; AFP: alpha-fetoprotein; US: ultrasound

REMARKS FROM GUIDELINES & COMMENTS

All three continents propose a 6-month screening interval using ultrasonography with or without AFP, regardless of cirrhosis, except Japan. Japanese guidelines suggest a shorter interval (3-4 months) for extremely high-risk cirrhotic patients, the three tumour markers (AFP/PIVKA-II, /AFP-L3) along with ultrasound and EOB-MRI with 6-12 months interval, or dynamic CT.

Based on the tumour doubling time (range 29 to 398 days), the 6-month interval represents a reasonable choice^[42], since shorter interval detects more small lesions, but does not improve detection of small HCC^[43]. The incidence of HCC in the target population and available facilities may affect the screening interval. However, there is still a question about optimal interval for screening ranging from 4 to 8 months^[2,26,30].

Sensitivity of ultra-sonogram is ranging from 58% to 89% with specificity greater than 90% when used as a screening test before they presented clinically, other than that it seems to be less effective for detecting early-stage HCC (sensitivity of only 63%)^[2,26,44,45].

AFP is not recommended along with ultrasound in North America and Europe because the present studies were not directed to determine an improvement in survival. AFP is usually elevated in cirrhosis intermittently, but markedly elevation in small tumour is rare^[2,16,26,30]. Therefore, APASL suggests cut-off value (set at 200 ng/mL) of AFP for screening programs when used in combination with US. Combined with US, AFP provides additional detection in 6%-8% of cases not previously identified by US, confirmed more recently^[2,46].

Serological tests that are under investigation for early diagnosis of HCC include (PIVKA II) AFP-L3, alpha-fucosidase, and glypican. These markers have been tested mostly for diagnosis and prognosis, but need to be

studied in screening set-up^[2,26,30].

As imaging modality, Japanese guidelines recommend EOB-MRI or dynamic CT to be performed in every 6-12 months for screening for extremely high-risk of cirrhotic patients since small nodules may not be detected on ultrasound alone^[32,34].

HCC risk stratification models for cirrhotics have not yet been included in the guidelines and the majority of the presented guidelines exclude Child C cirrhosis from screening protocols unless they are eligible for curative therapy.

Screening guidelines for non-cirrhotics differ from countries, mainly in selection of the target population. Whereas AASLD-2017 does not specify, APASL-2017 and EASL-2018 describe the target population in the guidelines.

EASL-2018 made a breakthrough and used the PAGE B score system for non-cirrhotic HBV patients. The score system is intended to determine unnecessary screening for Caucasian patients with chronic HBV. However, the PAGE-B score has not yet been validated in Asia^[2].

Overall F3 (bridging fibrosis) patients regardless of aetiology were also included in the screening protocol at first time by EASL-2018 developers.

CHINESE-2017 recommends screening for patients with chronic liver diseases regardless of aetiology. In contrast, Japan guidelines suggest screening for patients with only chronic HBV and HCV.

In general, the screening modalities for non-cirrhotics are almost identical with cirrhotics except Japan guidelines. The Japan guidelines recommend the three tumour markers additional to ultra-sonogram, for every 6 months.

Final question is: do the screening modalities really work? Japan and Hong Kong HCC screening methods were compared in that particular context. In Hong Kong, where there was no formal surveillance program, 20% of HCC were detected only in the pre-symptomatic period with low survival rate (17.8 months) whereas in Japan over 75% of cases were detected by surveillance. The median survival was 52 months in Japan and the stage of HCC at presentation was the most important factor influencing survival according to the cohort^[47].

CONCLUSION

Recommendations from the three continents are mostly influenced in the prevalence of HCC and availability of resources. It may be necessary to modify the screening methods according to the condition of patients. This situation is more evident in those countries with no national guidelines and/or heterogeneous patient population. Hence, developing countries should be encouraged to issue their own guidelines. The common point is that, cost-effectiveness is universal and screening modality is one of the factors that influence the variation in survival.

DECLARATIONS

Acknowledgments

Special thanks to Burhan Gurtunca, PhD for his contributions on editing.

Authors' contributions

Design: Yilmaz N

Literature review: Yilmaz N, Suer K, Goral V, Cakir N

Manuscript writing: Yilmaz N

Manuscript review and editing: Yilmaz UE

Approved the final article: all authors

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Laparoscopic liver resection for hepatocellular carcinoma

Massimo Giacca, Daniel Cherqui

Hepatobiliary Center, Paul Brousse Hospital-Assistance Publique-Hopitaux de Paris, Université Paris Sud, Villejuif 94800, France.

Correspondence to: Dr. Massimo Giacca, Hepatobiliary Center, Paul Brousse Hospital-Assistance Publique-Hopitaux de Paris, Université Paris Sud, Villejuif 94800, France. E-mail: massimogiacca4@gmail.com

How to cite this article: Giacca M, Cherqui D. Laparoscopic liver resection for hepatocellular carcinoma. *Hepatoma Res* 2018;4:47. <http://dx.doi.org/10.20517/2394-5079.2018.79>

Received: 16 Jun 2018 **First Decision:** 9 Jul 2018 **Revised:** 18 Jul 2018 **Accepted:** 22 Jul 2018 **Published:** 17 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Hepatocellular carcinoma (HCC) represents the most common indication of laparoscopic liver resection (LLR). It must be acknowledged that most series concern minor hepatectomies for peripheral lesions located in favorable segments, and such procedures are now performed in the majority of HPB centers. However, there are growing reports concerning major hepatectomies (i.e., 3 segments or more) and complex resections such as anatomical resections in difficult segments (i.e., postero-superior). Retrospective comparative studies, including some with propensity score matching, and meta-analyses showed that LLR is associated with short-term benefits including reduced blood loss, length of stay and morbidity with identical oncological results and survival rates. In addition, laparoscopy leads to less post-operative abdominal adhesions, improving operative outcomes in case of repeat hepatectomy or secondary liver transplantation. Despite the lack of results of randomized-controlled trials in HCC, a consensus exists that the laparoscopic approach can improve the outcome of major liver resections, provided it is performed in experienced centers. This requires specific high-quality training.

Keywords: Laparoscopy, hepatocellular carcinoma, cirrhosis, hepatectomy, liver resection

INTRODUCTION

Since 2000, when the first case-series was published^[1], laparoscopic liver resection (LLR) has represented a growing challenge. The number of resections and the extension to major hepatectomies and difficult locations have increased worldwide over the last 10 years. In contrast with other procedures, liver resections address



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various diseases including primary and secondary liver cancer on normal or diseased underlying liver. Furthermore, different types of resections including major and minor hepatectomies can be performed, which include various procedures according to tumor type and location in the liver segments. The only available Randomized Control Trial (RCT) concerns LLR for colorectal liver metastases^[2]. Results from this RCT confirmed previous retrospective reviews and meta-analyses by demonstrating benefits of LLR compared to open resection, such as reduced blood loss, morbidity and hospital stay. Two international consensus conferences on laparoscopic liver surgery were held in Louisville (USA) in 2008 and in Morioka (Japan) in 2014^[3,4]. The conclusions of the Morioka meeting validated minor LLRs as standard practice in surgery, while complex anatomical resections and major LLRs were still in an exploratory phase. The Morioka consensus also focused on underlining how major LLRs require high-level skills and emphasizing that a structured training should be performed, together with the establishment of a scoring system to evaluate difficulty before surgery. Currently, a laparoscopic approach seems applicable in 20%-50% of liver resections, certainly depending on local experience and skills^[5]. Authors of the largest review and meta-analysis published so far^[6], with data from 9000 patients, propose LLRs as a feasible alternative to open liver resection (OLR) mainly in patients undergoing a minor resection or in those undergoing a major liver resection without biliary or vascular reconstruction. At present, LLR is accepted worldwide, with favorable outcomes compared to OLR, mainly in terms of length of stay, blood loss and post-operative complications, with comparable oncological and survival outcomes.

Almost 90% of HCCs evolve from chronic liver disease, with different prevalent etiologies in the Eastern and Western world. Several medical and surgical approaches or, more often, combinations of these, are used to treat HCC, but surgical resection and liver transplant play the main role. Sixty-five percent of LLRs are performed for malignant disease, with HCC remaining the main indication. This is in part attributable to the large contribution of Asian literature where HCC resection is very common, and also the accurate surveillance and screening programs which allow detection, in a growing number of cases, of small single tumors which are the best candidates for LLR^[5].

OPERATIVE AND POST-OPERATIVE OUTCOMES

The first series of LLRs for HCC on cirrhosis studying both short-term outcomes and survival rates was published in 2006^[7]. It concluded that LLR in selected patients with peripheral HCC on chronic liver disease was a safe procedure with good midterm results. More recent studies confirmed these results especially in cirrhotic patients^[8-14]. Meta-analyses proved that patients with HCC undergoing LLR have reduced intra-operative blood loss and length of stay when compared to those undergoing OLR^[15,16]. A systematic review and meta-analysis on LLR vs. OLR for HCC was published in 2013 by Yin *et al.*^[11] This study included 1238 patients from 15 studies, all requiring left lateral or right peripheral resection. Together with reduced intra-operative blood loss, it showed a lower rate of post-operative morbidity in patients undergoing laparoscopic resections. There was no significant difference in terms of survival, both overall survival (OS) and disease-free survival (DFS). Two studies comparing laparoscopic and open resections for HCC using the propensity score were published in 2015^[17,18]. The one by Han *et al.*^[17] showed no inferiority of LLR, with similar 1, 3 and 5-year OS and DFS rates, lower post-operative morbidity and post-operative transient liver failure. These groups of patients had comparable operative times. A study by Takahara *et al.*^[18] showed similar results with reduced blood loss, post-operative morbidity, ascites and liver failure in patients who underwent LLR. In this group of patients operative time was longer and oncological results comparable.

MAJOR HEPATECTOMIES

In recent years the laparoscopic approach has extended to major hepatectomies. In 2017, Yoon *et al.*^[13] in a propensity-score analysis comparing patients who had laparoscopic and open right hepatectomy for HCC

on cirrhosis, demonstrated better results in the laparoscopic group for length of hospital stay, level of post-operative pain and ascites. Rate of incisional hernia was also lower in this group. These authors used the comprehensive complication index (CCI) to prove a significantly less severe overall morbidity. They showed no significant difference in terms of intra-operative blood loss. None of the patients in both groups required transfusions. In the Yoon's cohort of patients, operative time was significantly shorter in the open group. The main limits of this study are that it is not an RCT and that the great majority of patients (more than 90%) had HBV-related cirrhosis. We are of the opinion that patients with HCC on chronic hepatitis B may offer a less challenging setting for resection and less post-operative complications when compared to other etiologies of cirrhosis^[19].

Another recent propensity-score study by Xu *et al.*^[20] compared the laparoscopic and open approaches for major hepatectomies to treat HCC on cirrhosis. This study, which included 103 patients, confirmed a lower occurrence of post-operative ascites and showed no difference in all other medical and surgical post-operative complications. A lower post-operative occurrence of ascites had already been observed by other authors and described in meta-analyses^[15,16,21]. Also in the Xu's series, the open group had significantly lower operative and Pringle times, while the laparoscopic group showed a significantly shorter length of stay and a higher overall cost of hospitalization.

A study that aimed at comparing laparoscopic and OLR for HCC following sequential trans-arterial chemoembolization (TACE)-portal vein embolization (PVE) was published by Goumard *et al.*^[22] The results from this study showed no difference in oncological radicality in terms of R0 resections and tumor margins. LLRs were proven to offer shorter length of stay and fewer grade B post-operative liver failures.

Results from the first Asia Pacific consensus meeting of LLR for HCC were published in 2018^[23]. The meeting of experts produced 22 recommendations, concluding that minor LLRs should be performed in experienced centers and major LLRs in centers of excellence. In these selected centers LLR with portal vein reconstruction is also possible if vascular involvement only targets the left lateral branches. The meeting's conclusions also mention some of the new frontiers of LLRs, such as the use of indocyanine green fluorescence and robotic resection, which could become high-quality tools to optimize surgery in the near future.

CONVERSION RATES

Another main-point of interest in evaluating the feasibility of LLRs for HCC is conversion rate. Goumard *et al.*^[22] had a higher conversion rate compared to the other studies, reaching 25% but never in an emergency setting. These authors defined conversion criteria as: significant bleeding, failure to accurately recognize the biliary anatomy and poor exposure leading to failure or slow progression during parenchymal transection. Work from other authors showed conversion rates ranging from 5% to 13%^[14,17,24]. The largest available case series in all LLRs for HCC is a retrospective analysis by Dagher *et al.*^[25], which presented a conversion rate of 10%. A recent retrospective analysis of 2861 cases of LLRs by Halls *et al.*^[26] showed a conversion rate of 7.8%, in which bleeding was the most common cause. Almost 19% of conversions were due to adhesions. In this series, 11.5% of patients had cirrhosis and a conversion rate of 11.1%, which turned out to be statistically significant when compared to the conversion rate of 7.3% in non-cirrhotic patients.

ONCOLOGICAL OUTCOMES

In all studies, there was no evidence of inferiority of LLR in terms of oncological results and survival rates, both OS and DFS^[13,14,17,18,20,27]. Moreover, the work by Han *et al.*^[17] compared the laparoscopic and open groups in terms of pathological liver status, tumor size and satellites, microvascular and capsular invasion, tumor grade and stage. No significant difference was found. Recently, a retrospective study by

Woo-Hyoung *et al.*^[28] analyzed 234 patients undergoing anatomical LLR for HCC: DFS was 67.5% and 55.3%, OS was 91.7% and 87.1% at 3 and 5 years respectively. In this work anatomical resection emerged as a good prognostic factor for HCC recurrence, but had no impact on the OS. Another recent study by Guro *et al.*^[29] considered retrospectively 177 patients who underwent major LLR or OLR, finding the early (< 1 year) recurrence rate to be significantly higher in the open group, with similar OS and DFS rates. Population in this study also showed a larger tumor size in the open group, which could explain the better results in the laparoscopic one.

FEASIBILITY OF LLR

Although postero-superior segments (1, 4a, 7 and 8) are known to be the less accessible ones, recent literature leans toward the concept that tumor location should no longer be a criteria for patient selection in laparoscopic surgery^[30,31]. Already in 2010, Yoon *et al.*^[32] published a retrospective study comparing postero-superior (PS) and antero-lateral (AL) resections for HCC. The study concluded that PS patients had longer operative time and length of hospital stay, but no significant difference in terms of post-operative morbidity, recurrence or survival. A non-significant tendency towards a higher rate of conversion was shown in PS patients.

In 2012, Ishizawa *et al.*^[33] analyzed 62 patients who had resections in all segments, confirming that PS resections require longer operative time and are also affected by higher blood loss. The authors proved accurate LLR to be feasible in all segments, but considered PS resections as “difficult segmentectomies” which should be performed by surgeons with advanced open and laparoscopic experience.

Last, the laparoscopic approach reduces the formation of post-operative adhesions. This appeared, in the case of repeat hepatectomy, to reduce operative time and difficulty of the adhesiolysis which could impact on peri-operative morbidity in terms of bleeding and bowel or other organ injuries^[13,34]. This suggests that LLRs should be preferred, when feasible, considering the risk of recurrence and especially in potential candidates for liver transplant^[35].

LLR VS. ABLATION

Regarding single small HCCs, several authors have debated whether to perform laparoscopic resection or local ablation. OLR was shown to be associated to higher rate of complications, greater blood loss and longer hospital stay compared to radiofrequency ablation (RFA)^[23,36,37]. These disadvantages are likely to be reduced in laparoscopic resections. LLR seems to have better oncological results, in terms of lower recurrence rates, when compared to RFA for the treatment of small (< 3 cm) HCCs^[23,38-40]. OS in the two procedures do not differ significantly^[39,41].

The main limitations of this study are that it was a single-center non-systematic review.

CONCLUSION

In conclusion, data have been accumulated in the recent literature in favor of safety and reliability of LLR for HCC, especially in a cirrhotic setting. Currently, while LLR is the standard practice for patients requiring minor hepatectomies, evidence regarding the feasibility of major LLRs is growing. Several studies also show short-term benefits of LLR for major hepatectomies, with identical oncological results. A particular advantage in the cirrhotic patient is a lower risk of postoperative decompensation and ascites. Still, these operations are mainly performed in experienced centers. The next challenge will be the dispatch and training of surgeons in accordance to these procedures, in order to achieve a meaningful improvement in patient care and clinical outcomes.

DECLARATIONS

Authors' contributions

Concept and design of study or acquisition of data or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; final approval of the version to be published: Giacca M, Cherqui D

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Chitinase-3-like protein 1 as a predictor for the progression or regression of liver fibrosis

Biaoyang Lin^{1,2,3}, Shengjun Wu⁴, Yunhua Liu⁵, Longgen Liu⁶, Saadiya Mushtaq⁷

¹Zhejiang-California International Nanosystems Institute (ZCNI) Proprium Research Center, Zhejiang University, Hangzhou 310058, Zhejiang Province, China.

²Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310058, Zhejiang Province, China.

³University of Washington School of Medicine, Seattle, WA 98195, USA.

⁴Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310020, Zhejiang Province, China.

⁵Department of Liver Diseases, The Second Hospital of Yunnan Province, Kunming 650000, Yunnan Province, China.

⁶The Third People's Hospital of Changzhou, Changzhou 213001, Jiangsu Province, China.

⁷Virology Department, Armed Forces Institute of Pathology, Block E-16, New Cob Lane, Javed Sultan Shaheed Colony, Rawalpindi Cantt, Rawalpindi, Pakistan.

Correspondence to: Dr. Biaoyang Lin, Zhejiang-California International Nanosystems Institute (ZCNI) Proprium Research Center, Zhejiang University, Hangzhou 310058, Zhejiang Province, China. E-mail: biaoylin@gmail.com

How to cite this article: Lin B, Wu S, Liu Y, Liu L, Saadiya M. Chitinase 3-like protein 1 as a predictor for the progression or regression of liver fibrosis. *Hepatoma Res* 2018;4:48. <http://dx.doi.org/10.20517/2394-5079.2018.19>

Received: Mar 12 2018 **First Decision:** Jun 22 2018 **Revised:** Jul 18 2018 **Accepted:** Jul 22 2018 **Published:** 17 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Huan-Liang Wu **Production Editor:** Cai-Hong Wang

Abstract

Liver fibrosis is a wound-healing response of liver cells to chronic injuries caused by viral infections, including hepatitis B virus (HBV), hepatitis C virus (HCV), toxins, and alcohol abuse. The ability to stage diseases for treatment naïve patients to initiate proper medical procedures and predict the clinical causes of the disease or the treatment response is important given the increased prevalence of liver fibrosis caused by HBV, HCV and fatty liver diseases. CHI3L1 (chitinase-3-like protein 1, also known as YKL-40), which belongs to the chitinase family but lacks chitinolytic activity and is highly expressed in the liver, seems to fulfill this role. CHI3L1 is a non-invasive staging marker for liver fibrosis caused by HBV, HCV and non-alcoholic fatty liver disease as well as a predictor of the clinical causes and fibrotic changes after treatments. CHI3L1 predicts histological progression of liver fibrosis and fibrosis progression rate (fibrosis unit/year), rapid fibrosis progression after liver transplantation and response to interferon and recent direct acting antiviral therapy in chronic HCV patients. CHI3L1 also predicts response to antiviral therapy in chronic HBV patients.

Keywords: CHI3L1, liver fibrosis, progression, regression, hepatitis B virus, hepatitis C virus, treatment response



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INTRODUCTION

Liver fibrosis is a wound-healing response of liver cells to chronic injuries caused by viral infections, toxins, alcohol abuse and other causes. Liver fibrosis is accompanied by a constant process of destruction and repair of the hepatic parenchyma that is caused by inflammation and often results in serious complications, including portal hypertension and liver failure. Liver fibrosis can also give rise to hepatocellular carcinoma. Liver fibrosis can lead to cirrhosis, which is defined as the end stage of liver fibrosis^[1]. In China, hepatitis B is the major cause of inflammation leading to liver fibrosis and cirrhosis^[2,3]. Cirrhosis is an important factor in the development of hepatocellular carcinoma (HCC) because the cumulative 5-year risk of developing HCC in patients with cirrhosis ranges from 5% to 30%, depending on several factors, including the presence and stage of underlying liver disease, ethnicity, age, sex and the duration of exposure to primary hepatotropic viruses. To reduce the burden of the end stage liver diseases (cirrhosis and HCC), it is critical to identify liver fibrosis at its early stage, predict the direction and speed of the progression, and finally to monitor and predict the treatments responses (antiviral or anti-fibrotic treatments).

Although many biomarkers (e.g., APRI, FIB4, fibrometer, fibrotest, *etc.*) and imaging methods (e.g., Fibroscan, ARFI, MRE) have been widely proposed for staging liver fibrosis, their abilities in predicting liver fibrosis progression are very limited. Given that fibrosis is a very slow process, it often takes years to progress or recede from one pathological stage to the next. Therefore, a biomarker that can fulfill this role is most desirable. A search for such a biomarker would require an understanding of the mechanism of liver fibrosis and the key molecules involved in the process.

CHI3L1 (also known as YKL-40) belongs to the chitinase family but lacks chitinolytic activity, which is highly enriched in the liver^[4]. CHI3L1 acts as a growth factor for fibroblasts and is involved in matrix remodeling^[5]. Serum CHI3L1 levels are associated with the severity of liver fibrosis caused by non-alcoholic fatty liver disease^[6], schistosomiasis^[7,8], hepatitis C virus (HCV)^[9,10] and hepatitis B virus (HBV)^[11].

CHI3L1 PREDICTS HISTOLOGICAL PROGRESSION OF LIVER FIBROSIS IN CHRONIC HCV PATIENTS

Fontana *et al.*^[12] analyzed the association of serum fibrosis marker levels with the risk of clinical and histological disease progression in a large cohort of patients with chronic hepatitis C consisting of 462 prior non-responders to peg-interferon and ribavirin enrolled in the randomized phase of the Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) trial. They performed pretreatment liver biopsy and follow-up biopsies at years 2 and 4 and defined histological progression as a ≥ 2 -point increase in the Ishak fibrosis score in patients without cirrhosis. Clinical outcomes included development of decompensation, hepatocellular cancer, death or an increase in the Child-Turcotte-Pugh score to ≥ 7 . They collected and compared serial YKL-40 levels in patients who progressed clinically to the levels in patients who did not progress using random effects modeling. YKL-40 levels increased in both groups of patients over time ($P = 0.0026$) and were significantly increased in the progressors ($P < 0.0001$).

CHI3L1 PREDICTS RESPONSE TO INTERFERON THERAPY IN CHRONIC HCV PATIENTS

Saitou *et al.*^[10] analyzed noninvasive markers as predictors of interferon responses with HCV-associated diseases. A total of 109 patients with HCV-associated liver disease were enrolled, and 88 patients underwent liver biopsy. In total, 67 of 109 patients received interferon therapy. YKL-40 was superior to other fibrosis markers for predicting severe fibrosis (F2-F4) from mild fibrosis (F0-F1) (YKL-40, AUC = 0.809; HA, AUC = 0.805). They also evaluated the changes of the levels of fibrosis markers before and after interferon (IFN) therapy. After IFN therapy, only the concentration of serum YKL-40 significantly decreased in the responder group and the non-responder group ($P = 0.03$). No changes were noted among type IV collagen, amino-terminal peptide

of type III procollagen, hyaluronic acid (HA). They concluded that YKL-40 might be a useful non-invasive serum marker to evaluate the efficacy of IFN therapies in patients with HCV-associated liver disease.

CHI3L1 PREDICTS RESPONSE TO ANTIVIRAL THERAPY IN CHRONIC HBV PATIENTS

Wang *et al.*^[13] compared serum CHI3L1 levels with liver tissue collagen proportionate area (CPA) and liver stiffness measurement (LSM) in a cohort of 131 CHB patients before treatment and after receiving entecavir-based antiviral therapy for 78 weeks. Before treatment, correlation analysis revealed positive correlations between CHI3L1 levels and the CPA ($r = 0.351$, $P < 0.001$) and between CHI3L1 and LSM ($r = 0.412$, $P < 0.001$). After 78 weeks of treatment, serum CHI3L1 levels decreased compared with baseline (87.8 vs. 69.6 ng/mL, $P < 0.001$). Furthermore, the changes in CHI3L1 are correlated with changes in CPA ($r = 0.366$, $P < 0.001$) and the changes in LSM ($r = 0.438$, $P < 0.001$) before and after antiviral treatments. They concluded that CHI3L1 is a useful non-invasive marker for the assessment of liver fibrosis in CHB patients before treatment and a potential useful marker for monitoring the change in liver fibrosis during therapy. More interestingly, in many cases, CHI3L1 concentrations decreased after 78 weeks of antiviral therapies, whereas histological stages based on biopsy did not change. However, upon closer examination of the histological images, they found that many samples exhibited improvement in fibrosis as demonstrated by thinning of the septa and reduction in the numbers of the septa. However, the Ishak histological stage remains the same based on the classification standards (personal communication).

CHI3L1 PREDICTS FIBROSIS PROGRESSION RATE (FIBROSIS UNIT/YEAR) IN CHRONIC HCV PATIENTS

Kamal *et al.*^[7] conducted serial liver biopsies in a 10-year longitudinal cohort study consisting of patients with HCV alone or HCV and schistosomiasis. Two liver biopsies were performed for patients at the time of acute HCV infection and at the end of the follow-up to calculate the fibrosis progression rate/year. In addition, CHI3L1 serum concentrations were measured yearly and at the end of the follow-up. The serum CHI3L1 change rate (difference between baseline and follow-up values) was compared with the fibrosis progression rate/year. Kamal *et al.*^[7] reported that the CHI3L1 change rate had a very high linear correlation with the fibrosis progression rate/year ($r = 0.892$, $P < 0.001$). Furthermore, the CHI3L1 increase rate increases from years 4 to 8 compared with years 1 to 4 for HCV mono-infected patients, and the increase was noted at year 2 instead of at year 4 in HCV and schistosomiasis co-infected patients. Using data from the table of Kamal *et al.*^[7], we generated a scatter plot of CHI3L1 concentration and the fibrosis progression rate per year (increase in histological stages per year) [Figure 1]. As noted, no fibrosis progression is noted when the CHI3L1 concentration is 53 ng/mL. As the CHI3L1 concentration increases, the speed of fibrosis progression increases. When the CHI3L1 concentration is 110 ng/mL, the speed of fibrosis progression is at 0.8 histological stages per year [Figure 1].

CHI3L1 PREDICTS RAPID FIBROSIS PROGRESSION AFTER LIVER TRANSPLANTATION FOR HCV PATIENTS

Pungpapong *et al.*^[14] obtained serum and liver biopsy samples from 46 liver transplantation (LT) recipients at two time points: time point 1, means of 5 ± 2 (biopsy 1) months; time point 2, means of 39 ± 6 (biopsy 2) months post-LT. Rapid fibrosis progression (RFP) was defined as an increase in the fibrosis score ≥ 2 from biopsy 1 to biopsy 2 (a mean interval of 33 ± 6 months). They analyzed the ability of parameters, including serum CHI3L1 and hyaluronic acid (HA), histological assessment, and hepatic stellate cell activity (HSCA) at biopsy 1, to predict RFP. They found that serum HA and YKL-40 performed significantly better than conventional parameters and HSCA in predicting RFP post-LT. Furthermore, CHI3L1 (cutoff ≥ 200 $\mu\text{g/L}$) exhibited 96% accuracy and performed better than serum HA (cutoff ≥ 90 $\mu\text{g/L}$) in predicting RFP at biopsy 1 with 80% accuracy.

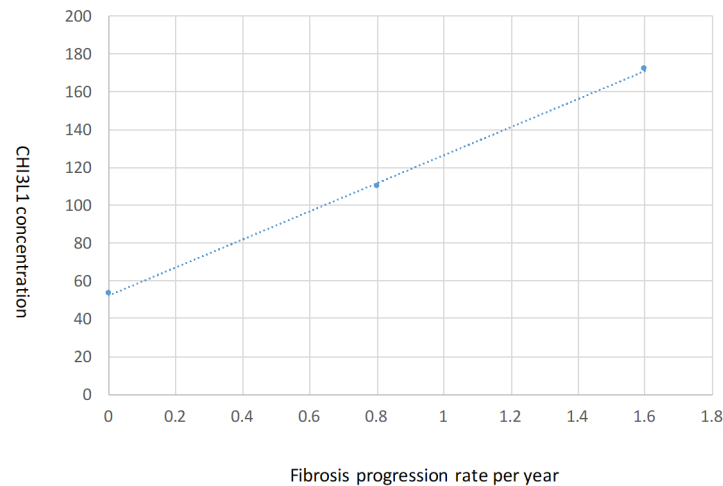


Figure 1. Scatter plot demonstrating the slope of CHI3L1 concentration and fibrosis progression rate per year

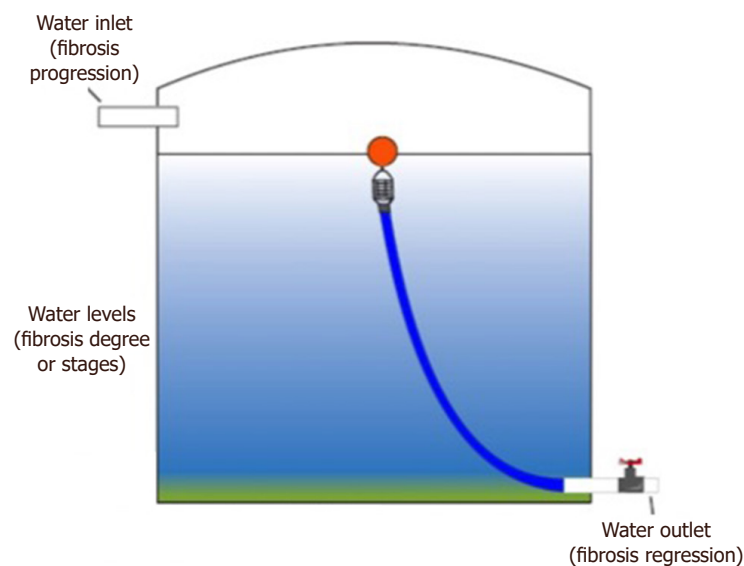


Figure 2. A water tank model to describe the relationship between the progression or regression of liver fibrosis and CHI3L1

CONCLUSION

CHI3L1 is not only a staging marker for fibrosis in treatment naïve HBV- or HCV-infected patients and NAFLD patients. CHI3L1 is also predictive of progression or regression of fibrosis. These abilities are likely due to the fact that CHI3L1 is actively involved in the process of liver fibrosis. Johansen *et al.*^[15] used immunohistochemical analysis to demonstrate that CHI3L1 is expressed in areas with fibrosis, particularly leading edges/areas with active fibrogenesis. CHI3L1 staining was not observed in hepatocytes but was expressed in Kupffer cells^[6] and potentially hepatic stellate cells (HSC)^[15]. He *et al.*^[16] demonstrated that CHI3L1 binds to interleukin-13 receptor $\alpha 2$ (IL-13R $\alpha 2$), activates MAPK (macrophage mitogen-activated protein kinase), protein kinase B/AKT, and Wnt/ β -catenin signaling, and regulates TGF- $\beta 1$ production via IL-13R $\alpha 2$ -dependent mechanisms. CHI3L1 also promotes HSC activation and proliferation^[4].

Here, we present a water tank model [Figure 2] to explain the relationship between the progression or regression of liver fibrosis and the concentration and increasing speed of CHI3L1. The inlet of water represents the parameters of CHI3L1, and the girth of the inlet pipe represents the absolute concentration of

CHI3L1. The water pressure (inlet water speed) represents the speed of the increase of CHI3L1 concentration in liver. The outlet represents the natural ability of the liver to repair the fibrosis damage (e.g., degradation of the extracellular matrix). The height of the water tank represents the degree (stages) of liver fibrosis. For example, if the water intake is greater than the water outflow, then the height of the water tank (degree of the fibrosis) would increase after a period of time, thus representing a model of chronic liver fibrosis similar to that observed in chronic HBV patients. If treatment, such as antiviral treatment of HBV, was initiated, the water intake would decrease (measured by a reduction in CHI3L1 concentration). Thus, over time, the height of the water tank (degree of fibrosis) would decrease due to natural recovery properties of the liver.

DECLARATIONS

Authors' contributions

Drafted the manuscript: Lin B

Edited and approved the manuscript: Wu S, Liu Y, Liu L, Saadiya M

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

The author declares that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Hypofractionated ablative radiation therapy for hepatocellular carcinoma: practical considerations and review of the literature

Marsha Reyngold¹, Eugene J. Koay², Christopher H. Crane¹

¹Department of Radiation Oncology, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA.

²Department of Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA.

Correspondence to: Dr. Christopher H. Crane, Department of Radiation Oncology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10065, USA. E-mail: cranecl@MSKCC.org

How to cite this article: Reyngold M, Koay EJ, Crane CH. Hypofractionated ablative radiation therapy for hepatocellular carcinoma: practical considerations and review of the literature. *Hepatoma Res* 2018;4:49. <http://dx.doi.org/10.20517/2394-5079.2018.84>

Received: 2 Jul 2018 **First Decision:** 19 Jul 2018 **Revised:** 1 Aug 2018 **Accepted:** 3 Aug 2018 **Published:** 20 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Huan-Liang Wu

Abstract

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy. The prognosis for patients who present with inoperable primary liver tumors is poor with median survival times of 12 months or less. Tumor-related liver failure is a common cause of mortality, underscoring the importance of local control. Recent advancements in external beam radiation therapy delivery techniques have enabled dose escalation that in turn has significantly improved local control and has allowed radiation therapy to emerge as an effective modality in this setting. In this review, we outline the critical practical aspects of treating liver tumors with radiation including choice of fractionation, motion management, image guidance and use of intensity-modulated radiation therapy vs. proton beam therapy. We review our approach to ablative radiation therapy for HCC with consideration of underlying cirrhosis and provide a brief overview of the current literature.

Keywords: Hypofractionated ablative radiation therapy, stereotactic ablative radiotherapy, radiation, large hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and is the second leading cause of cancer-related death worldwide. While surgical resection and/or transplantation represent well established curative options for early stage cancer in patients with compensated liver disease, other local treatments play an important role in more advanced patients, including patients with large locally unresect-



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able tumors, liver dysfunction and extrahepatic disease. It is important to note that death in patients with unresectable HCC is often related to liver failure as a direct consequence of local tumor progression. The mechanisms of liver failure include functional liver parenchymal loss, biliary obstruction, portal venous obstruction, and hepatic venous outflow obstruction resulting in ischemia (Budd Chiari). Some of these may occur even with small tumors that are located near hilum or the confluence of the hepatic veins and inferior vena cava. Although not well studied in patients with HCC, data on direct causes of death from another primary liver tumor, intrahepatic cholangiocarcinoma, treated with radiation at The University of Texas MD Anderson Cancer Center demonstrated that death resulted from tumor-related liver failure in 89% of patients whose cause of death could be determined. Half of those deaths were from biliary obstruction and the other half from vascular compromise or a combination^[1]. This underscores the importance of local therapies even for patients with advanced disease and suggests that effective local control may translate into a major survival benefit.

Current practice includes many options for liver directed-therapy in inoperable patients. Percutaneous image-guided ablative options (radiofrequency, microwave, cryoablation or percutaneous ethanol injection) are preferred for small peripheral tumors located away from segmental and main bile ducts, the liver surface, and major vessels. Additional options include arterially directed options such as bland transarterial embolization, transarterial chemoembolization or radioembolization with yttrium-90 beads. Radiation therapy is a complementary option for patients with liver tumors and is a preferred option for tumors near the biliary tree, hilum of the liver, main portal vein, or inferior vena cava. For large liver tumors, radiation therapy may be the most effective local therapy available.

Effective radiation therapy for liver tumors such as HCC is predicated on the ability to deliver ablative doses with minimal risk of injury to the surrounding normal structures including liver parenchyma, which is often compromised in this patient population-as well as the bile ducts, chest wall, stomach, duodenum and colon. A number of treatment related factors can improve the therapeutic ratio of liver radiation therapy (RT), including increasing the number of fractions, controlling respiratory motion, using soft tissue image guidance, and using proton therapy to spare liver. In the following sections we examine how these factors enable the delivery of ablative RT for HCC.

LIVER TOLERANCE

Historically, radiation therapy to the liver was thought to be unsafe based on the inability of the whole liver to tolerate doses exceeding 30 Gy^[2]. Investigators from the University of Michigan subsequently showed that partial liver volumes can tolerate high focal doses of radiation, defined the radiation dose-response relationship for liver tumors, and described objective parameters to evaluate dose-volume relationships of ablative liver treatments^[3,4].

Notably, radiation-related liver toxicities may have distinct mechanisms and presentations in patients with cirrhosis and without cirrhosis. Radiation induced liver disease (RILD) is now classified as either classic (triad of anicteric hepatomegaly, elevated alkaline phosphatase and ascites) or non-classic (jaundice and markedly elevated serum transaminases). Several reports have noted that patients with advanced cirrhosis are at a higher risk of non-classic radiation-induced liver disease^[5-7]. Most recently, it has been recognized that patients who undergo radioembolization with ⁹⁰Y are susceptible to radioembolization-induced liver disease^[8], which presents with jaundice and ascites in the absence of tumor progression. The mechanisms underlying these different presentations of radiation-related liver toxicities remain subjects of ongoing research; but it is clear that the dose-volume relationship is altered in the presence of limited liver reserve^[5-7,9]. In addition to cirrhosis, other common reasons for limited liver reserve include limited normal-liver volume due to previous resection or hepatotoxic chemotherapy, and tumor-related dysfunction due to biliary or vascular

compromise. Indeed, owing to the presence of underlying liver disease in many patients with primary liver tumors such as HCC, the tolerance dose of the liver has been shown to be different for patients with primary versus metastatic liver cancers^[3]. Thus, evaluation of liver reserve/function is an important aspect of planning liver RT.

The most commonly used classification of liver function is the Child-Pugh score, which accounts for the presence or absence of ascites and encephalopathy and measurements of bilirubin, albumin, and prothrombin, the latter as an international normalized ratio. Although developed in a different context, Child-Pugh score has been used to evaluate patients for RT. In general, patients with Child-Pugh Class A and B7 cirrhosis can safely receive radiation, but patients with Class B8 or above are not considered candidates. Medical management of cirrhosis or other liver disease is always optimized before radiation therapy is begun.

In addition, several imaging modalities allow functional liver assessment. Indocyanin green (ICG) enables assessment of overall hepatic metabolic function and Sulfur colloid Technetium 99m SPECT/CT can define the spatial distribution of functional and cirrhotic liver parenchyma. ICG measurements correlate with development of RILD and mortality^[10-12]. Furthermore, subsequent effort showed that ICG measurements can help guide RT: a 5-fraction RT regimen was risk adapted based on ICG measurements at baseline and after 3 initial fractions^[13]. Results for 90 patients with HCC and liver metastases showed 2-year local control of 95%, with only an 8% risk of change in CP score > 2 ^[14]. Further work will be needed in patients with larger tumors and more advanced cirrhosis.

While cirrhosis is a major challenge to delivering radiation safely, surgical resection of the liver can reduce hepatic reserve through the removal of functional healthy liver. Although hepatic regenerative capacity can mitigate this problem, large resections can nonetheless substantially limit hepatic reserve. For example, 20%-25% of patients with bilobar liver metastases with planned two-stage hepatectomy cannot undergo the second stage owing to inadequate liver hypertrophy after portal vein embolization and a predicted inadequate liver remnant^[15,16]. The role of radiotherapy for patients with small liver remnants ($< 1000 \text{ cm}^3$) remains to be defined.

Biliary obstruction often occurs in patients with HCC. Ursodiol is helpful for partial biliary obstruction with stent placement reserved for complete obstruction.

Another aspect of HCC that can directly impact liver function is its predilection to vein invasion. Portal and hepatic venous tumor thrombosis may complicate RT delivery due to liver decompensation caused by the thrombus, the larger radiation volumes needed to cover the thrombus, and the presence of ascites resulting from portal hypertension. Importantly such tumors may represent even a greater management challenge for other local modalities^[17]. Studies of radiation alone or in combination with transarterial approaches for tumors with portal vein tumor thrombosis have shown that efficacy of radiation in this setting is not influenced by the location of the tumor thrombus in the same way that transarterial options are and suggested that radiation should be considered in this setting [Figure 1]^[18-20].

FRACTIONATION

Development of stereotactic ablative radiotherapy (SABR) has revolutionized our approach to patients with liver tumors. Studies of lung^[21,22] and liver^[23] cancer have shown it to effectively ablate small tumors, defined as local control rates of approximately 90% at 2 years or longer. For these organs consisting of parallel functional subunits, overall organ function depends on preserving a minimum number of these subunits and can otherwise tolerate destructive doses of radiation to small parts of its volume. However, SABR in 3 to 6 fractions is challenging or impossible when the tumors are near critical organs at risk (OARs) whose func-

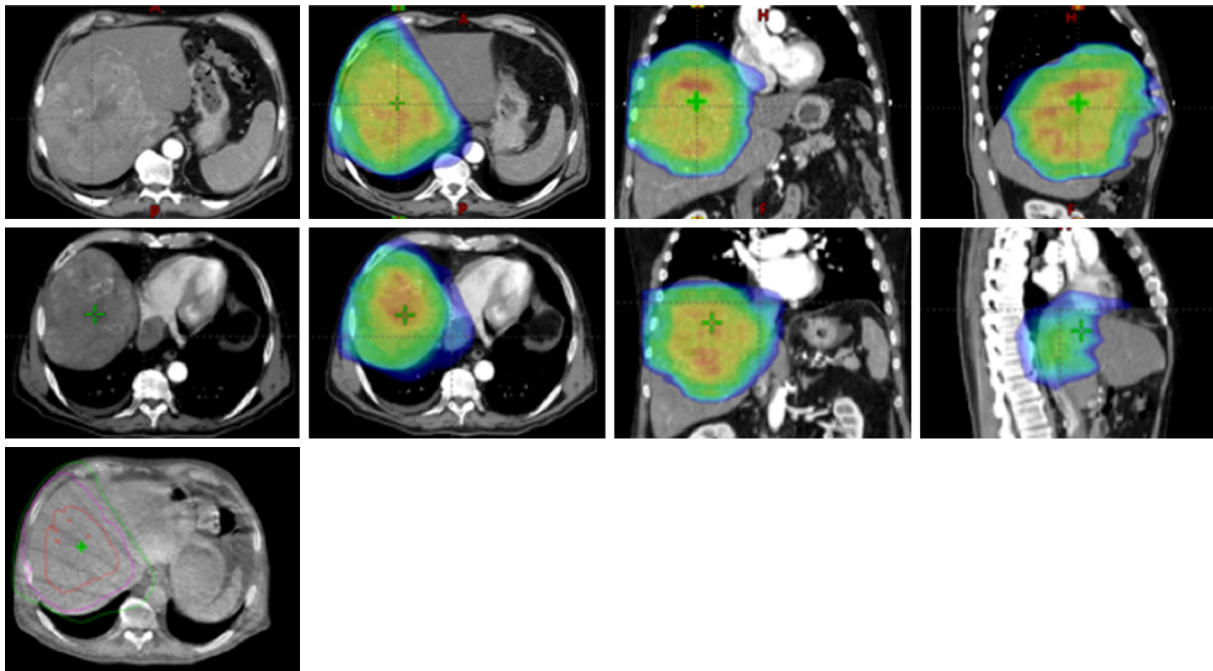


Figure 1. Treatment plan of a patient with an 18-cm hepatocellular carcinoma with extension into the hepatic veins, inferior vena cava and right atrium. Deep inspiration breath-hold was used for motion management. Representative arterial phase images from the simulation computed tomography (CT) are shown in the first column at the level of mid-liver (first row) and at the level of the right atrium (second row), with corresponding dose color wash distributions in all planes immediately to the left. Lowest dose displayed in deep blue is 45 Gy (in 25 fractions) and central hotspot is 75 Gy. Intracardiac extension was treated to 50 Gy. A representative cone beam CT image is shown in the third row

tional subunits are arranged in series, such as the spinal cord and the gastrointestinal (GI) tract or when the tumors are large making it difficult to spare enough liver parenchyma below a certain dose. Typical doses to achieve an ablative effect for HCC given in 3-6 fractions is 54 Gy or biologically equivalent doses (BEDs) of approximately 100 Gy. However, for tumors located near the GI tract these doses cannot be delivered using 3-6 fractions because even a small radiation hotspot can impair organ function. In these cases, the total dose to the tumor is often reduced by 20% to 50% to meet normal tissue constraints, which directly reduces the efficacy of the treatment. Similar dose reductions may be necessary to protect a sufficient amount of liver or to remain within tolerance for the biliary tree^[24]. Yet, ablative doses with BED of 100 Gy can be safely delivered to these tumors when a more protracted fractionation is used.

For example, in sequential phase I and II trials of SABR given in 6 fractions to 102 patients with large HCCs with median tumor size 7 cm, the median radiation dose was only 36 Gy in 6 fractions in order to maintain a low risk of RILD. The locoregional control rate at 1 year was good (87%), but inferior to some of the results with protons where more protracted fractionation schemes were used (outlined in a later section), and the rate of grade ≥ 3 toxicity was high (30%) with 7 patients dying of treatment-related causes^[25].

These results emphasize that the key to successfully controlling large liver tumors is achieving an ablative dose while staying within tolerance of the organs at risk (OARs), which can often be accomplished by increasing the number of fractions beyond typical 3-6 SABR fractionation schemes [Figure 1].

MOTION MANAGEMENT

Due to the proximity of organs at risk, controlling organ motion is a critical component of treating HCC with ablative radiation. Both intrafraction and interfraction motion need to be considered. Intrafraction

motion is primarily due to respiration (and/or patient movement), while interfraction motion is primarily impacted by the change in location/shape of the liver and luminal organs day-to-day.

The amplitude of liver movement with respiration varies significantly from patient to patient and depends on the location of the tumor within the liver. The range of motion generally is greatest in the cranio-caudal direction, with amplitude exceeding 2 cm in some patients^[19]. Further, although breathing amplitudes can be different during four-dimensional computed tomography (4D CT)-based treatment planning versus during radiation delivery, the direction of variability seems to be predictable^[20]. With regard to the effect of tumor location within the liver, the closer a tumor is to the center of the hemidiaphragm, the greater the motion. The liver also deforms throughout the respiratory cycle, especially in elderly patients with diminished abdominal wall muscle tone. Chest versus abdominal breathing also affects liver shape, and must remain consistent from the simulation to the treatment delivery. It is important to note that organ motion of the diaphragm is perhaps even more important for proton therapy than for photon therapy because the dose delivery is significantly more affected by tissue density of the surrounding organs in the case of proton therapy.

Intrafraction organ motion due to breathing can either be addressed with respiratory motion control coupled with image guidance or by accounting for the range of motion of the tumor with an internal target volume (ITV). It is often not advisable to use the latter option for liver tumors because of the proximity of organs at risk and the larger normal liver volume that needs to be included. The addition of abdominal compression is an effective way to reduce the ITV. Several commercial devices are available for this application. The most common technique uses an abdominal compression plate that is placed 3 to 4 cm below the costal margin. The plate is connected to a load cell that can measure how much force is being applied to the abdomen. This device is usually used when the superior-inferior movement of the tumor exceeds 1 cm, but it may also be needed for tumors within 1 cm of the GI tract^[26]. Because compression plates can cause variable deformation of the liver, an alternative solution for liver tumors is the use of a pneumatic compression belt. This option has been reported to reduce respiratory motion to less than 5 mm^[27]. Notably, compression only minimizes rather than eliminates motion, and does necessitate the use of an ITV approach.

Motion management can very efficiently be accomplished with respiratory gating. Options include inspiratory or expiratory breath hold including the Varian RPM system or the Active Breathing Control system. Interfractional variations in breath hold position can exceed 4 mm^[28,29], and so a breath hold technique is usually coupled with image guidance to verify the target position with each fraction. Image guidance can be achieved by using 2D image sets or with 3D images obtained in the breath hold position.

Day-to-day differences in bowel position and shape are other uncertainties that must be accounted for and monitored to ensure safe treatment. The extent to which the luminal GI organs affect accurate proton delivery has not been well described and may not be predictable. Filling of the stomach can vary substantially from day to day, depending on the amount of air, liquid, and solid present within it. This variation can lead to an increase in the range of the proton beam, but not the photon beam. This is a relatively minor problem to deal with if during the planning process beams are designed such that they don't traverse the gastrointestinal tract. The left lobe of the liver is susceptible to deformation caused by stomach filing, whereas the right lobe is less affected by the surrounding organs. Generally, we instruct our patients to ingest nothing for at least 3 h before radiation sessions in an attempt to reduce the variability of stomach filling and enhance the tendency of the stomach to pull away from the left lobe of the liver. The amount of solid, liquid and gas in the ascending, transverse, and descending colon can vary from day to day. This variability should be monitored and assessed for position changes near the tumor. We use simethicone for patients who have significant amounts of gas in the large bowel. Reduction in bowel gas can often increase the separation between the tumor and colon.

IMAGE GUIDANCE

As described in the previous paragraphs, image guidance is a critical component of treatment with ablative doses. Some options for image guidance include fiducial-based kilovoltage X-ray solutions that can be used for tumor tracking, deep inspiration breath hold, end inspiration breath hold, and free-breathing gating techniques such as end-expiratory gating and abdominal compression. Another option, soft tissue imaging via CT-on-rails or cone beam CT (CBCT) have the advantage of being able to visualize the interface of the liver with the GI tract, and, most of the time, the tumor within the liver.

Because cone beam CT images are acquired over 40 to 60 s, motion artifact is significant. This can be substantially reduced with a deep inspiration breath hold image acquisition. Most patients can hold their breath for that duration if the image is acquired during deep inspiration. This technique produces images that are clear enough to assess the interface between the stomach and the liver, which can vary from day to day. A gated cone-beam CT is another option but is currently still an emerging technology. For photon therapy, magnetic resonance imaging equipped linear accelerators may offer the best soft tissue definition. This capability will become more widely available in the future.

Most small liver tumors can be treated with a free-breathing ITV that accounts for respiratory motion and setting up to bony landmarks. For larger tumors, or tumors near the GI tract, we recommend a deep inspiration breath-hold technique. Metallic fiducials or surgical clips that have been placed from prior surgery can be used for initial set up. Alternatively, it is possible to use a soft tissue set-up to the liver shape obtained with a breath-hold cone-beam CT.

ROLE OF PROTON BEAM THERAPY

Proton therapy is a form of external beam radiation therapy that utilizes accelerated protons as particles to deliver therapeutic radiation. The benefit of protons derives from the lack of exit dose, resulting in lower integral doses to normal tissues compared to intensity modulated radiation therapy. Theoretically, when using a dosing schema based on meeting a particular mean liver dose threshold, the lack of exit dose may allow for a potentially greater dose of radiation delivered to the tumor. However, the use of protons is also associated with unique challenges that must be taken into account when planning and delivering a treatment.

Proton beam range is highly dependent on the electron density of tissues it transverses. This is one of the reasons for range uncertainty that must be accounted for when creating PTV margins in addition to margins needed for setup uncertainties, and target motion. For liver treatments specifically, the presence of different amounts of air in the luminal organs day to day and diaphragm motion that moves the interphase between lung and soft tissue can significantly impact delivered doses to target and surrounding structures, and must be accounted for when treating with protons. Another important disadvantage of protons is their wider penumbra due to lateral scatter, which results in less conformality. Therefore, PTV coverage for tumors close to the sensitive GI structures is best achieved with IMRT. Dosimetrically the greatest advantage for PBT over photons may in treatment of very large liver tumors with small healthy liver remnants located far from luminal GI tract. NRG-GI003 is a recently opened US multi-institutional phase III trial that randomizes patients with unresectable HCC to photon *vs.* proton based hypofractionated SBRT will determine whether PBT may confer an OS advantage compared to photons. Both a 5 and 15-fraction regimens are allowed at the discretion of the treating physician.

CLINICAL OUTCOME DATA OVERVIEW

Historically, the majority of the ablative radiation therapy experience has come from Japan, where HCC is endemic and quite common. Protons have been largely used as they allowed larger treatment volumes to be treated to larger doses per fraction. Results from hypofractionated regimens (16-25 fractions) to ablative

Table 1. Select studies of proton beam therapy for HCC

Study details			Tumor characteristics				Outcomes				GI toxicity
Study	Fractionation scheme	Number	Median size, cm (range)	Child-Pugh A	Multiple tumors	Prior local therapy	2Y LC	2Y OS	3Y OS	Median survival, months	Grade 3 ^{**}
Kawashima <i>et al.</i> ^[12]	76 GyE in 20	30	4.5 (2.5-8.2)	67%	10%	37%	96%	66%	62%	41*	6
Mizumoto <i>et al.</i> ^[31]	66 GyE in 10 72.6 GyE in 22 77 GyE in 35	266 104 95 60	3.4 (0.6-13)	76%	53%	63%	(3y) 87%	-	61%	51	6
Bush <i>et al.</i> ^[33]	63 Gy in 15	76	5.5 [§]	30%	14%	-	(5y) 80% [†]	-	70% (Tx) 10%* (no Tx)	34 (CP A) 13 (CP B) 12 (CP C)	0
Hong <i>et al.</i> ^[32]	67.5 GyE in 15 58.05 GyE in 15	44	5.0 (1.9-12)	79.5%	27.30%	20%	95%	63%	-	50	0
Chadha <i>et al.</i> ^[34]	75.9 Gy in 15	37	5.2	85%	24%	30%	86%	54%	-	25	6

*Estimated from Kaplan-Meier curve; **no grade 4 or 5 toxicity was reported; §mean; †Crude rate. CP: Child-Pugh; LC: local control; OS: overall survival; GI: gastrointestinal; Tx: transplant

doses for large tumors are similar to those after surgical resection, with 5-year local tumor control rates of 90% and overall survival (OS) rates of 50% among some patients^[12,30,31].

Representative studies of ablative proton beam therapy for HCC from Japan and early experience in the US are summarized in Table 1^[12,31-33]. Several fractionation schemes have been successfully used with higher doses per fraction reserved for peripheral tumors located > 2 cm away from the hilum or sensitive GI structures. Like most studies of patients with HCC, these studies have very heterogeneous inclusion. While overall survival is dependent on patient and tumor characteristics, including liver function, tumor size, multifocality and the presence tumor vascular thrombosis, local tumor control has been in the 90% range when ablative doses have been delivered. Mizumoto *et al.*^[31] reported on 266 patients treated with three protocols developed at the Proton Medical Research Center in Tsukuba, including 66 GyE in 10 fractions for tumors > 2 cm away from the portal region, 72.6 GyE in 22 fractions for tumors within 2 cm of the hilum and further reduction to 77 GyE in 35 fractions for tumors adjacent to the GI tract. The majority of the tumors were less than 5 cm. The average 3-year local control and OS were 87% and 61%, respectively. Interestingly, there were no significant differences in local control among the three different fractionation schemes used.

In the US, a recent multi-institutional phase II study of high-dose hypofractionated proton beam therapy for liver tumors included 44 HCC patients with median tumor size of 5.0 cm and tumor vascular thrombosis present in 29.5%. Planned dose was 67.5 GyE in 15 fractions for peripheral tumors and 58.05 GyE in 15 fractions for central tumors. Dose de-escalation was allowed for meeting liver constraints. Median dose delivered was 58 GyE (range 40.5-67.5). LC and OS for this group at 2 years were 94.8% and 63.2%, respectively^[32]. Importantly, very few grade 3 toxicities and no grade 4-5 toxicities were observed. Worsening Child-Pugh score (all A to B) was noted in 3.6%.

There are no definitive data on the optimal dose for control of HCC, but collectively these studies suggest that dose escalation above BED of 100 Gy [approximately 80 Gy in 2 Gy equivalents (EQD)] is associated with excellent outcomes and can be safely accomplished using proton beam therapy. Although to date the majority of published experience on the use of ablative doses for large liver tumors requiring 10 or more fractions has been using protons [Table 1], largely due to the greater liver parenchyma sparing they offer over photons, when the same liver constraints are adhered to with photon-based plans using the principles described in this review, the clinical outcomes are similar [Figure 1] (our unpublished data). With greater availability of photon-based therapy and some of the dosimetric advantages IMRT offers over protons, IMRT-

based hypofractionated ablative treatments will most certainly become more frequently used in the future.

In summary, while patients with relatively small, isolated tumors with well-compensated cirrhosis represent ideal candidates for ablative dose escalation, this approach may also be used for select candidates with larger tumors or Child-Pugh class B/C liver disease.

CONCLUSIONS

Radiation therapy is an important local modality for large unresectable HCC. Small tumors can be treated with straight forward approaches that may not require respiratory motion management or soft tissue image guidance. However, large liver tumors are among the most challenging cases to treat with radiation because of the sensitivity of the liver parenchyma, the presence of underlying liver disease, the proximity of the duodenum, colon, stomach, and main bile ducts. Respiratory motion and interfraction motion of the surrounding bowel complicate sparing these organs. These challenges can be overcome by adhering to the following principles which apply to both photon and proton beam therapy. In general, proton therapy spares liver parenchyma better and IMRT spares GI luminal structures better.

- (1) Evaluation and optimization of liver function prior to RT. Child-Pugh Class A and B7 are most appropriate candidates for ablative RT.
- (2) Selection of fractionation scheme that allows the delivery of ablative radiation doses of 100 Gy BED (80 Gy EQD2) while sparing sensitive normal structures. For most large central tumors, this requires the use of 15-25 fractions with an SBRT technique in order to stay within the tolerance of the OARs.
- (3) Respiratory motion management. Breath hold or gating is preferred for large tumors because it minimizes the liver volume that is treated, help to spare the GI tract, and minimizes motion artifact on cone beam images.
- (4) Use of soft tissue image guidance. When tumors are located near the GI tract, soft tissue guidance is most important. CBCT allows for verification of the position of the GI tract as well the liver shape.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: all authors

Performed data acquisition, as well as provided administrative, technical, and material support: all authors

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Hepatic resection for hepatocellular carcinoma

Shun Yamaguchi[#], Taichiro Kosaka[#], Susumu Eguchi

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 8528102, Japan.

[#]Authors contributed equally.

Correspondence to: Dr. Susumu Eguchi, Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto1-7-1, Nagasaki 8528102, Japan. E-mail: sueguchi@nagasaki-u.ac.jp

How to cite this article: Yamaguchi S, Kosaka T, Eguchi S. Hepatic resection for hepatocellular carcinoma. *Hepatoma Res* 2018;4:50. <http://dx.doi.org/10.20517/2394-5079.2018.68>

Received: 25 May 2018 **First Decision:** 16 Jul 2018 **Revised:** 31 Jul 2018 **Accepted:** 1 Aug 2018 **Published:** 22 Aug 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Hepatic resection has become the standard treatment of primary liver cancer. Indications for hepatic resection in patients with hepatocellular carcinoma (HCC) vary greatly between Japan and other countries because the clinical practice guidelines for HCC defined by the Japan Society of Hepatology differ from the EASL-EORTC clinical practice guidelines. Hepatic resection is not recommended as a treatment for the patients at Barcelona Clinic Liver Cancer (BCLC) stage B. Otherwise, there are many surgeons/clinicians who believe that not all HCC patients at BCLC stage B should be excluded from an indication for hepatectomy because many reports showed good prognosis after hepatic resection for HCC patients over BCLC stage B. The survival rate is expected to increase with better outcomes of hepatectomy in the future. This paper has described indications for hepatectomy for patients with HCC through comparison of domestic guidelines with overseas guidelines, focusing on their differences.

Keywords: Hepatic resection, hepatocellular carcinoma, guidelines

INTRODUCTION

Indications for surgical resection in patients with hepatocellular carcinoma (HCC) vary greatly between Japan and other countries. This is because many Japanese medical institutions decide on the indication based on the clinical practice guidelines for HCC defined by the Japan Society of Hepatology^[1], which differs from the EASL-EORTC clinical practice guidelines^[2] in terms of the HCC stage, and the hepatic reserve as an indication for hepatectomy. This paper compares both guidelines in terms of surgical resection for hepatocellular carcinoma.



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EVALUATION OF PREOPERATIVE HEPATIC RESERVE

Liver carcinoma is often caused by viral hepatitis, alcoholic hepatitis, or non-alcoholic steatohepatitis (NASH), and when hepatectomy is performed it is necessary to pay attention to postoperative decrease in residual liver function as well as curability of the cancer.

The Child-Pugh score^[3] is used worldwide to assess preoperative hepatic reserve. The EASL-EORTC clinical practice guidelines^[2] usually exclude Child-Pugh B and C patients and even Child-Pugh A patients with increased portal blood pressure or high levels of bilirubin from indications for hepatectomy. The Japanese guidelines also recommend a treatment decision based on the Child-Pugh score, and hepatectomy in Child-Pugh A and B patients and liver transplant in Child-Pugh C patients have shown favorable results^[4,5]. Importantly, an indication for liver transplant in Child-Pugh C patients is because liver transplants performed in Japan are usually living-donor liver transplantation due to the scarcity of brain-dead donors, and patients undergoing liver transplantation have developed liver cancer mostly caused by decompensated cirrhosis.

The evaluation scale often used in Japan for hepatectomy is assessment of liver damage under the general rules for the clinical and pathological study of primary liver cancer calculated by an indocyanine green retention rate after 15 min (ICG15), ascites, serum bilirubin level, serum albumin level, and prothrombin activity^[6]. Actually, many reports showed that the ICG load test was a significant predictor of postoperative death^[7,8], and the Makuuchi criteria^[9] for safe hepatic resection, which are used as a reference for hepatectomy in many institutions, also base the advisability of hepatectomy on bilirubin level, ICG15, and ascites as well as the resectable limits. There was little mortality in patients undergoing hepatectomy in compliance with these criteria. Based on what was mentioned above, the ICG load test is considered likely to be important for decision-making concerning indications for hepatectomy.

Some reports showed that technetium-99m-galactosyl human serum albumin (99mTc-GSA) liver scintigraphy was more useful than ICG15 retention rate in the assessment of histological hepatic damage^[10,11] and more effective in the prediction of complications and operative death in patients with hepatic disorders^[12]. However, 99mTc-GSA scintigraphy using nuclides is performed only at a limited number of institutions and is not common worldwide.

INDICATIONS FOR HEPATECTOMY

Indicators for surgery other than hepatic reserve include tumor diameter, the number of tumors, presence of vascular invasion and extrahepatic metastasis. Looking at the stage classification, the EASL-EORTC-guidelines^[2] recommend hepatectomy as a treatment option for HCC patients at Barcelona Clinic Liver Cancer (BCLC) stage 0 or BCLC stage A and with normal portal blood pressure and bilirubin level. Transarterial chemoembolization (TACE) is recommended as a treatment for the patients at BCLC stage B [Figure 1]. However, studies showed the 5-year survival rate and perioperative mortality rate in HCC patients at BCLC stage B and undergoing hepatectomy were 30% to 57% and 2.6% to 5.4% respectively^[13-16], and the prognosis of the patients with solitary hepatocellular carcinoma and undergoing hepatectomy was much more favorable than those undergoing TACE. There are many surgeons/clinicians who believe that not all HCC patients at BCLC stage B should be excluded from an indication for hepatectomy.

In terms of the number of HCC tumors, a better prognosis was reported in patients with a solitary tumor than in patients with multiple tumors^[17]. Hepatectomy was more useful than local ethanol injection treatment in patients with liver damage A or B under the general rules for the clinical and pathological study of primary liver cancer. The treatment plan may change depending on if the HCC tumor size is larger or smaller than 3 cm. Hasegawa *et al.*^[18] reported that hepatectomy showed more favorable outcomes than radiofrequency ablation (RFA) in patients with a solitary tumor smaller than 3 cm. As written above, hepatectomy is recommended as the first treatment option for patients with solitary HCC, and RFA is reported for patients with HCC smaller than 3 cm as the second treatment option equivalent to hepatectomy [Figure 2].

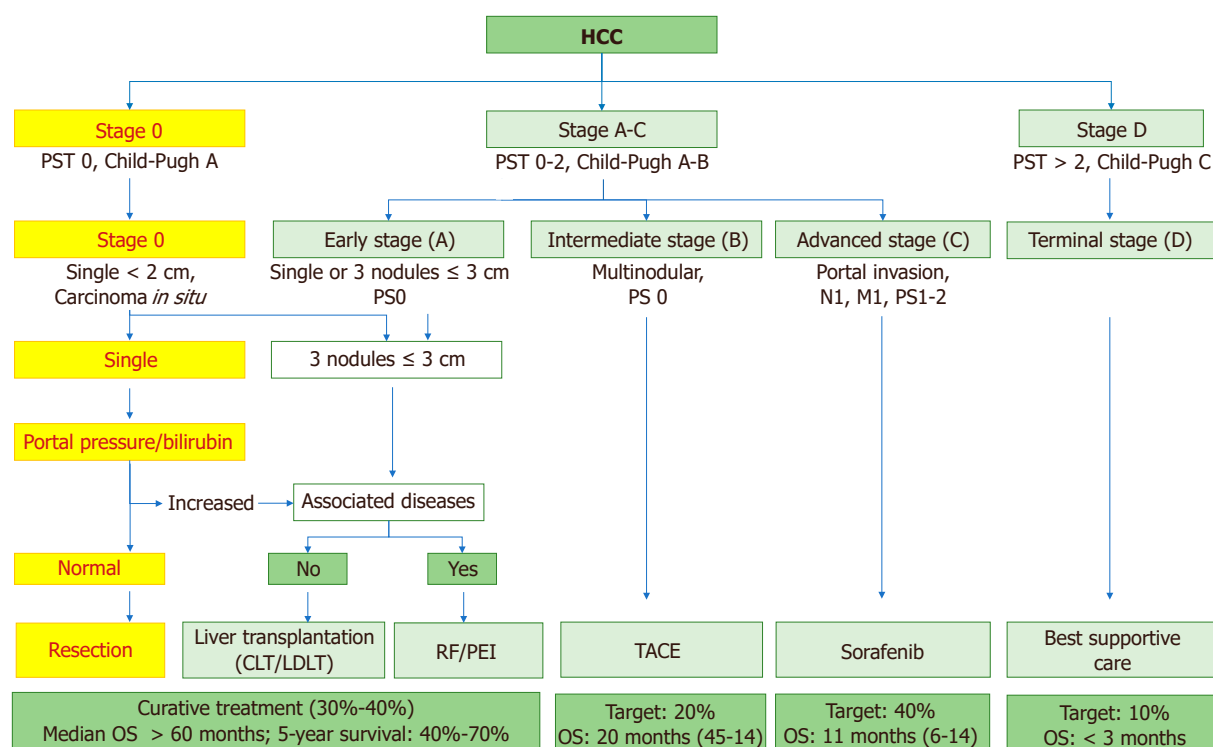


Figure 1. Updated BCLC staging system and treatment strategy: EASL-EORTC Clinical Practice Guidelines^[2]. HCC: hepatocellular carcinoma; PST: performance status; CLT: cadaveric liver transplantation; LDLT: living donor liver transplantation; RF: radiofrequency; PEI: percutaneous ethanol injection; TACE: transcatheter arterial chemoembolization; OS: overall survival

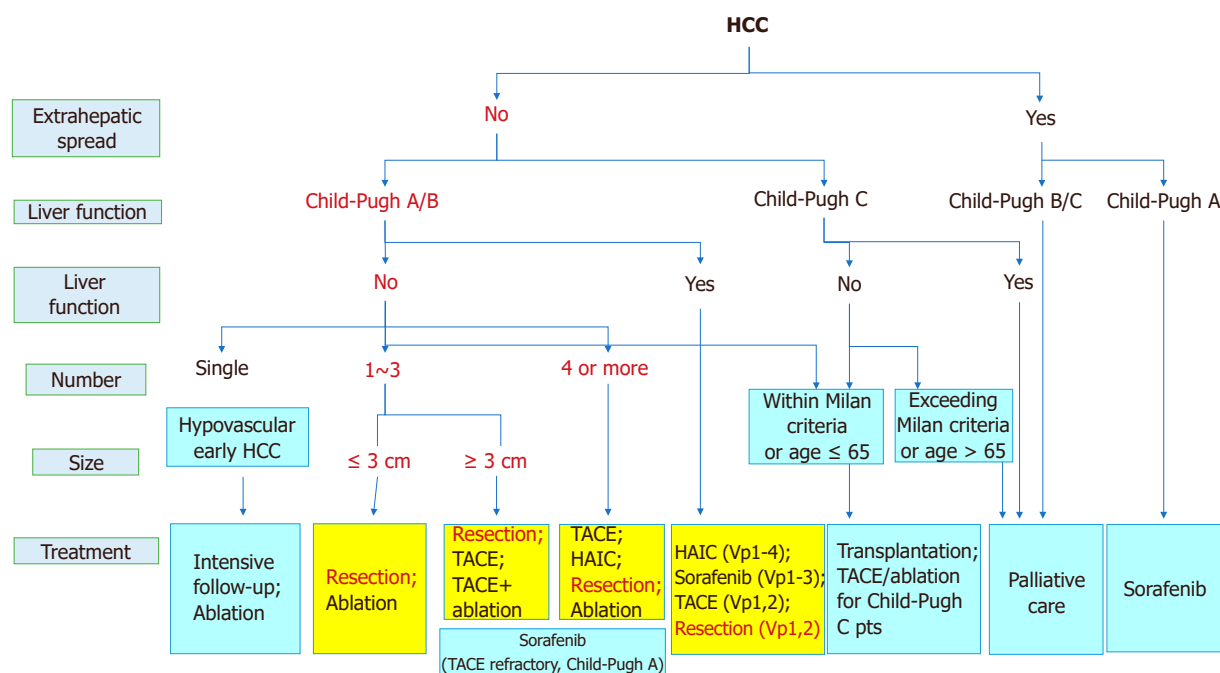


Figure 2. JSH-LCSGJ consensus-based treatment algorithm for hepatocellular carcinoma (HCC) revised in 2014^[1]. TACE: transcatheter arterial chemoembolization; HAIC: hepatic arterial infusion chemotherapy; JSH: Japan Society of Hepatology; LCSGJ: Liver Cancer Study Group of Japan

Hepatectomy or RFA is recommended for the patients with 2 or 3 tumors 3 cm or smaller, based on the data examined by Hasegawa *et al.*^[18]. Huang *et al.*^[19] compared hepatectomy and RFA for HCC patients under the Milan criteria and showed a better survival rate in patients undergoing hepatectomy. However, since the patient characteristics in their study were very different from those in Japan, a randomized controlled trial (SURF trial, UMIN000001795) comparing hepatectomy and RFA in Japanese HCC patients under the Milan criteria has been conducted in Japan. The trial has not reported a high level of evidence for surgical resection and RFA in HCC patients with 4 or more tumors, and recommends transcatheter embolization/chemoembolization (TAE/TACE) as the first treatment option and hepatic arterial infusion chemotherapy and molecular targeted drug therapy as the second treatment option for those patients.

Indications for hepatectomy (vascular invasion)

The indications for surgery for HCC with vascular invasion are described here. The 5-year survival rate for HCC patients with portal vein invasion and undergoing hepatectomy was found to be 1% to 38%, which showed a survival benefit^[20,21]. Kokudo *et al.*^[21] reported that the prognoses of patients with Child-Pugh score A and undergoing hepatectomy were strongly favorable and that hepatectomy was effective in patients with localized invasion in the first branch of the portal vein. TACE, molecular targeted drug therapy, and hepatic arterial infusion chemotherapy for HCC patients with vascular invasion were also reported^[22-24], but a consensus on these treatments has not yet been reached in Japan. Therefore, hepatectomy, embolization therapy, hepatic arterial infusion chemotherapy, and molecular targeted drug therapy are recommended equally at present in Japan as treatments for HCC patients with vascular invasion.

The AASLD^[25] guidelines suggest that adults with Child-Pugh class A cirrhosis and resectable T1 or T2 HCC undergo resection rather than radiofrequency ablation. These patients are indicated for resection. Most studies define patients with resectable HCC as those: (A) with one to three unilobar lesions, with an upper size limit of 5 cm for single lesions and 3 cm for more than one lesion; (B) without radiographic evidence of extrahepatic disease of macrovascular invasion; and (C) occurring in the setting of minimal or no portal hypertension and in the absence of synthetic dysfunction. It is different from Japanese guidelines. The Chinese guidelines^[26] similarly define general surgical indication for cases with less than three tumors. But, it is different from AASLD guidelines at the point about including resection of portal vein tumor thrombus (PVTT) and concomitant splenectomy for cases with portal hypertension.

In Europe and the US, the use of molecular targeted drug therapy is recommended for HCC patients with vascular invasion at BCLC stage C.

HEPATECTOMY PROCEDURE

Since HCC is known to spread through veins into the liver, systematic removal of the tumor-bearing portal territory is advisable, if possible. Some recent literature has reported that patients undergoing systematic resection had better prognoses than those undergoing nonsystematic resection (segmental resection)^[27-31]. However, many patients develop HCC in the background of chronic liver diseases, and some of them may not undergo systematic resection at present due to poor hepatic reserve. Therefore, the indication for surgery and the surgical procedure are often determined upon consideration of the balance between tumor conditions and liver function conditions. While systemic resection is anatomic resection of the tumor-bearing portal territory with consideration to HCC development through the portal vein, nonsystematic resection is resection of the tumor with some surgical margin regardless of the anatomy of the vessels. Some studies have reported that a comparison of surgical outcomes between systematic resection and nonsystematic resection showed no significant difference in cumulative survival rate and relapse-free survival rate^[32-34]. It is recommended in Japan to choose either a small range of systematic resection or nonsystematic resection as

reductive surgery, depending on hepatic function, for patients with small HCC (5 cm or smaller) and major resection of at least 2 segments for patients with large HCC.

The procedure for resection of the right hepatic vein at its root with preservation of the inferior right vein^[35], the procedure for systematic resection of the HCC-bearing portal territory with dye infusion under ultrasound guidance^[36], and the procedure for systematic resection of the identified tumor-bearing territory with transection of Glisson's sheath^[37,38] are reported as the surgical procedures preserving the liver parenchyma. The procedure for resection of segment 3 and 4 with preservation of segment 2^[39] is also included.

The surgical procedure for HCC in the caudate lobe generally removes the ventral liver parenchyma also, which has raised the question of impaired hepatic functions. Surgical procedures such as dorsal resection of the caudate lobe isolated and identified using the counterstaining technique^[40,41] and isolated resection of the caudate lobe after parenchymal transection along the middle hepatic vein^[42] currently have been designed.

Reports on laparoscopic hepatectomy for HCC are increasing lately. It is reported that laparoscopic hepatectomy is superior to open hepatectomy due to the magnifying effect of the area being operated on and allows less hemorrhage from the hepatic veins due to the hemorrhagic reduction effect of the pneumoperitoneum^[43-45]. It is also reported that laparoscopic hepatectomy has a lower incidence of complications such as ascites than open hepatectomy^[46-48]. Laparoscopic hepatectomy for HCC has been reported to have long-term outcomes equivalent to those of open hepatectomy and superior to radiofrequency ablation in local control for small HCC located at the liver surface. In Japan, laparoscopic hepatectomy is currently recommended based on the judgments of the International Consensus Conference on Laparoscopic Liver Resection that laparoscopic hepatectomy could be performed on patients with hepatic reserve sufficient to undergo open hepatectomy and is advisable for partial resection or lateral segmental resection for solitary tumor with a maximum diameter no more than 5 cm located in the anterior inferior segments (segments 2 to 6)^[49]. In Europe and the US, some reports have described laparoscopic hepatectomy but have not made a clear recommendation for it. In any case, it is considered that laparoscopic hepatectomy should be done by a team with well-experienced surgeons and only at a well-equipped medical institution providing adequate intensive care during the perioperative period due to insufficient accumulation of evidence about safety in laparoscopic hepatectomy.

PROGNOSIS OF PATIENTS UNDERGOING HEPATECTOMY

The studies have reported that there was no significant difference in postoperative relapse rate between patients with resection margin of at least 1 cm and patients with resection margin of less than 1 cm^[50-52] and comparison of the prognosis in patients with resection margin of at least 5 mm and less than 5 mm also showed no significant difference in survival rate^[53,54]. Based on these results, a minimum distance of resection margin is allowed for hepatectomy for HCC in Japan. In contrast, Hu *et al.*^[55] reported that the prognosis was favorable in patients with Milan criteria-compliant HCC with resection margin of at least 1 cm. Another study showed that patients with a resection margin of at least 2 cm had a more favorable prognosis than patients with a margin of 1 cm^[56]. It is thought that the distance of the resection margin may affect prognosis.

Well-known predictors of poor prognosis after hepatectomy also include tumor diameter of at least 5 cm, multiple tumors, no capsular formation, positive vascular invasion, impaired liver function, TNM classification stage 3 or 4, and AFP level of at least 32 ng/mL^[57,58]. Some research has indicated that tumor size is not a prognostic predictor^[59,60].

Tumor markers such as PIVKA-II and AFP are reported as predictors of recurrence after hepatectomy for HCC. HCC patients with a thrombus in the main portal vein or the first branch of the portal vein are considered

Table 1. The result of hepatic resection for HCC

Author	HCC characteristics	1-year OS (%)	3-year OS (%)	5-year OS (%)
Garancini <i>et al.</i> ^[65]	BCLC A/B	95/83.3	61.1/50	46.2/41.2
Wu <i>et al.</i> ^[66]	BCLC O-A	95.9	85.3	67.6
Jiang <i>et al.</i> ^[67]	BCLC A, multifocal	96	71.7	36.3
Li <i>et al.</i> ^[68]	BCLC A or B, ruptured	66.3	23.4	10.1
Xu <i>et al.</i> ^[69]	BCLC B or C	81.4	48.5	28.2
Wang <i>et al.</i> ^[70]	Small tumors	92.6	83.3	73
Shrager <i>et al.</i> ^[71]	Large HCC (> 10 cm)	57	30	19
Lee <i>et al.</i> ^[72]	Large HCC (> 10 cm)	66	44	31
Shah <i>et al.</i> ^[73]	Large HCC	69	63	54
Pandey <i>et al.</i> ^[74]	Large HCC	63	35	28.6
Ng <i>et al.</i> ^[75]	Large or multinodular	74	50	39
Roayaie <i>et al.</i> ^[76]	Macroscopic vascular invasion	52	22	14
Pawilk <i>et al.</i> ^[20]	Portal or hepatic vein invasion	45	17	10
Ban <i>et al.</i> ^[77]	Portal vein thrombosis	70	37	22
Vitale <i>et al.</i> ^[78]	BCLC-C	55	44	0

HCC: hepatocellular carcinoma; OS: overall survival; BCLC: Barcelona Clinic Liver Cancer

to have a poor prognosis. The Glasgow Prognostic Score (GPS) is one of the important predictors and is believed to make the prognoses of HCC patients clearer^[61]. In addition, some studies have shown that the preoperative neutrophil-lymph node ratio (NLR) is a predictor of poor prognosis^[62]. Sarcopenia is also considered to be a predictor of poor prognosis^[63]. Japanese study showed that patients with non-B non-C HCC had a better prognosis and a lower risk of recurrence than those with hepatitis C virus (HCV)-related HCC^[64].

We investigated the outcomes of HCC after hepatic resection^[65-78]. There are no significant difference mortality of HCC patients between BCLC A and B [Table 1]. Garancini said that surgical treatment of HCC in BCLC stage B should not be considered contraindicated for such patients. HCC patients with vascular invasions had higher mortality rate than single large HCC. We should pay attention to vascular invasions more than tumor size for good surgical prognosis.

At last, we showed outcomes of hepatic resection in Japan. The Liver Cancer Study Group of Japan determined that the cumulative survival rate^[64] at all HCC stages was 90.2% at 1 year, 81.3% at 2 years, and 56.8% at 5 years. Looking at the 5-year survival rate by tumor diameter, survival rate was 73.9% in patients with tumor size of less than 2 cm ($n = 4168$), 63.1% in patients with tumor size of 2 to 3 cm ($n = 7212$), 59.7% in patients with tumor size of 3 to 5 cm ($n = 6022$), and 52.4% in patients with tumor size of 5 to 10 cm ($n = 3869$). The 5-year survival rate of patients with tumor size of 10 cm and bigger was 45.4%. Thus, patients with increasing tumor size have a worse prognosis. Looking at survival rate by the number of tumors, while the 1-year survival rate and the 5-year survival rate were 90% and 50% to 60% respectively in patients with one or two tumors, the 5-year survival rate declined to 37% in patients with more than three tumors [Table 2]. Looking at the 5-year survival rate by stage, survival rate was 82.8%, 70%, 52%, 31%, and 26.8% in patients at stage I, II, III, IVA, and IVB, respectively. However, the 5-year survival rate has been increasing steadily in recent years. While it was 12.5% in the 1980s, it steadily increased to 44% in the 2000s.

The incision criteria are different in each guideline. But, expansion of criteria for resection is progressing. The survival rate of HCC after hepatic resection is expected to increase with better outcomes of hepatectomy in the future.

CONCLUSION

This paper has described indications for hepatectomy for patients with HCC through comparison of domestic guidelines with overseas guidelines, focusing on their differences.

Table 2. Cumulative survival rates (%) of hepatocellular carcinoma patients in Japan^[61] treated with hepatic resection

Years		Number	Cumulative survival rates (%)				
			1	3	5	7	10
Tumor number	1	16,531	93.7	80.8	67.0	54.7	39.0
	2	3494	90.0	71.0	54.8	40.4	27.2
	≥ 3	2717	81.1	55.8	37.9	28.1	20.4
Portal vein invasion	Vp0	19,075	94.4	80.5	65.5	52.4	36.8
	Vp1	1908	84.9	62.4	48.2	39.0	28.9
	Vp2	714	69.1	42.2	29.2	22.5	17.3
	≥ Vp3	852	59.8	34.3	25.0	20.5	15.6
TNM stage by LCSGJ	I	2339	97.8	90.0	74.3	61.4	42.5
	II	9755	94.1	78.0	62.5	50.1	35.5
	III	3902	85.6	61.8	43.5	33.5	23.3
	IVA	1208	69.4	38.9	25.9	20.3	15.4
	IVB	2118	56.5	28.0	18.7	14.5	14.5

LCSGJ: Liver Cancer Study Group of Japan

DECLARATIONS**Authors' contributions**

Mainly edited the manuscript: Yamaguchi S, Kosaka T

Qualified the manuscript: Eguchi S

Read and approved the manuscript: Yamaguchi S, Kosaka T, Eguchi S

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Cancer immunotherapy for hepatocellular carcinoma

Joo-Ho Lee¹, Soo-Yeon Oh², Jin Yong Kim², Naoshi Nishida³

¹Department of Gastroenterology and Hepatology, CHA Bundang Medical Center, CHA University School of Medicine, Seongnam 13496, South Korea.

²Department of Gastroenterology, Chaum Life Center, CHA University School of Medicine, Seoul 06062, South Korea.

³Department of Gastroenterology and Hepatology, Kindai University Faculty of Medicine, Osaka-Sayama 589-8511, Japan.

Correspondence to: Dr. Naoshi Nishida, Department of Gastroenterology and Hepatology, Kindai University Faculty of Medicine, Osaka-Sayama 589-8511, Japan. E-mail: naoshi@med.kindai.ac.jp

How to cite this article: Lee JH, Oh SY, Kim JY, Nishida N. Cancer immunotherapy for hepatocellular carcinoma. *Hepatoma Res* 2018;4:51. <http://dx.doi.org/10.20517/2394-5079.2018.78>

Received: 13 Jun 2018 **First Decision:** 11 Jul 2018 **Revised:** 31 Jul 2018 **Accepted:** 1 Aug 2018 **Published:** 10 Sep 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Cai-Hong Wang

Abstract

Most hepatocellular carcinomas (HCCs) arise on a background of chronically inflamed liver, and thus are considered typical immunogenic cancers. Although there have been advances in treatment options for HCC, many patients still struggle with a limited chance of survival requiring further innovative approach. Especially for the advanced HCC, many other molecular targeted therapies had been evaluated without success. Based on the immunological mechanisms thought to be acting during HCC development, the effects of diverse immunomodulatory regimens such as therapeutic vaccination, immune checkpoint inhibitors, and adoptive cellular immunotherapy have been investigated. Notably, many strategies have been developed in adoptive cellular immunotherapy, including dendritic cells, cytotoxic T cells, natural killer cells, cytokine-induced killer (CIK) cells, and genetically engineered T cells. In recent clinical trials, adjuvant CIK cell immunotherapy increased progression free survival after curative treatment of HCC. Most recently, new immunomodulatory agents were introduced for oncological treatment, eventually leading to the clinical breakthrough of checkpoint inhibitors targeting cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death-1 (PD-1). To date, very promising published evidence with checkpoint inhibitors in HCC has been reported in the clinical trials with anti-CTLA-4 agent tremelimumab and a large phase II trial with anti-PD-1 agent nivolumab. Further investigations of immuno-oncology potentially popularized the applications of immunotherapy in the various stages of HCCs, and thus immune-based therapies are the promising innovative approach for patients with HCC. Hopefully, the immuno-oncology will bring about a paradigm shift of anti-cancer treatment for HCC.

Keywords: Hepatocellular carcinoma, adoptive immunotherapy, cytotoxic T lymphocyte associated antigen-4, programmed cell death 1 protein



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INTRODUCTION

Hepatocellular carcinoma (HCC) ranks fifth as the most common cancer in the world and the second most common cause of cancer-related death, accounting for 70%-85% of primary liver cancers^[1-3]. The current standard treatments for HCC offer a fair chance of survival but there are still many patients who struggle with only a limited chance of survival^[4]. A majority of patients present with disease too advanced to be treated with curative modalities such as surgical resection, transplantation, or radiofrequency ablation (RFA)^[2]. Although the Barcelona Clinic Liver Cancer (BCLC) guideline recommends sorafenib in advanced HCC, which proved a survival benefit of 2.8 months compared to the placebo group^[5], many liver cancer centers still select multimodality approaches including transarterial chemoembolization (TACE), radiotherapy (RT), and hepatic arterial infusion chemotherapy (HAIC)^[6,7].

Most HCCs arise on a background of chronically inflamed liver, and thus are considered typical immunogenic cancers^[8]. Based on the immunological mechanisms thought to be acting during HCC development, the effects of diverse immunomodulatory regimens such as therapeutic vaccination, immune checkpoint inhibitors, and transfer of adoptive cellular immunotherapy, have been investigated^[8,9]. In the 21st century, cell-based therapies developed to bolster human anti-tumor immunity represent a growing component of cancer therapeutics^[10,11]. Of note, adoptive cellular immunotherapies have employed several types of immune cells, including dendritic cells (DCs), cytotoxic T lymphocytes (CTLs), lymphokine-activated killer (LAK) cells, cytokine-induced killer (CIK) cells, and natural killer (NK) cells^[12]. In addition, therapeutic cancer vaccines utilizing tumor antigens with or without DCs have been investigated.

Immune suppressor cells comprising tumor-associated macrophages (TAMs), regulatory T cells (Tregs), or myeloid-derived suppressive cells (MDSCs) in the HCC tumor microenvironment, could disturb the immune surveillance resulting in cancer immune evasion or immune escape^[13]. It is well known that the interactions of HCC cells with the immune cells and their factors of immune system play a major role in its progression^[14,15]. Inadequate co-stimulation, failure of tumor-associated antigens (TAAs) processing and presentation by antigen-presenting cells (APCs), along with suppression of effector cells are proposed mechanisms that result in weakened immune response in HCC patients^[15,16]. To complement these immunosuppressive tumor microenvironment of HCC, previous cancer immunotherapy has aimed so far to enhance immune cell activity to kill the HCC tumor cells.

In this regard, cancer vaccines help the immune system recognize and attack cancer cells^[17]. Unlike preventive vaccine, which prevents a development of a certain disease in advance, therapeutic cancer vaccine aims to treat the existing cancer. DCs are professional APCs that serve as a key player for inducing and activating the effector anti-tumor CTLs. There is ample evidence to justify therapeutic DC vaccines in HCC^[18]. Decreased function of peripheral blood DCs in patients with HCC is well established^[19]. Up to date, although DC vaccines are used in various stages of clinical trials of HCC, unfortunately, no therapeutic cancer vaccine has been approved for HCC^[19]. Meanwhile, the failure of these approaches for boosting immune responses by cancer vaccine using peptides or DCs, could be associated with the brake function in immunity (i.e., immune checkpoints)^[20]. It is now clear that tumors modulate immune checkpoints as one of the mechanisms to escape anti-cancer immune surveillance.

These immune checkpoints are known to regulate different stages and signaling processes of the immune response^[21]. At the initial stage of “priming” of naïve T cell activation, cytotoxic T lymphocyte associated antigen-4 (CTLA-4):B7 binding blocks stimulatory signals, and stops the development of potentially autoreactive T cells^[22]. Compared to CTLA-4, the major role of programmed cell death 1 protein (PD-1) and its ligand, PD-L1, is related to regulate previously activated CTLs at the later “effector” stage of immune response^[23]. In the tumor microenvironment, antigen-specific T cells induce PD-1 expression on reactive CTLs and upregulate PD-L1 in cancer cells^[8,23].

The above immune checkpoint molecules are highly expressed in HCCs that are recognized as immunogenic tumors^[24]. Also, the hepatitis B (HBV) and hepatitis C virus (HCV) infections, two major pathogens of HCC, have been shown to interfere with antiviral immunity via the immune checkpoint pathways^[25-27]. Blocking these immune checkpoint molecules restores T cell function, which release the brakes on the anti-tumor immune surveillance, allowing the immune system to more effectively detect and kill the HCC tumor cells^[2,20,28].

As “cancer immunotherapy comes of age”^[29] in this era, the topic of “immuno-oncology in HCC” could be a timely one. In this review, we focus on the human clinical immunotherapy trials in HCC, according to the four major categories: (1) adoptive immunotherapies using CIK, NK and engineered T cells; (2) therapeutic cancer vaccine; (3) immune checkpoint blockades; and (4) combination of immunotherapies with other cancer treatments.

ADOPTIVE CELLULAR IMMUNOTHERAPY

Adoptive cellular immunotherapy is a form of passive immunization in which autologous effector cells are *ex vivo* sensitized and or expanded and then given back to the cancer patients^[30]. To date, adoptive immunotherapy is one stone in the pillar of cancer immunotherapy, which relies on the various lymphocytes including tumor-infiltrating lymphocytes (TILs), CD8+ CTLs, CD56+ NK cells, LAK cells, CIK cells, and engineering T cells. As one of main immunotherapeutic strategies, adoptive immunotherapy is widely used in the current cancer clinical trials. A sizable portion of immunotherapy clinical trials for HCCs are adoptive cellular immunotherapies [Table 1]^[30].

In 1989, regression of tumor size in ten HCC patients was shown after treatment with LAK cells combined with interleukin-2 (IL-2)^[31]. Later, two separate, but similar, clinical trials combining adriamycin chemotherapy with LAK cells after hepatoma resection were performed in 1991 and 1995^[32,33]. The former study showed a decrease in postoperative recurrence rate of HCC^[32]. However, in the latter study in 1995, there was no statistically significant difference between the two groups in the survival rate^[33].

Another source of adjuvant immunotherapy is TILs^[34]. TILs acquired from patients with hepatic malignancies, activated by IL-2 and anti-CD3 antibody and labeled with indium-111 were found to move to the tumor sites preferentially^[34]. This might augment the antitumor effects of adoptive immunotherapy. In 1997, TILs isolated from resected tumors of 12 patients with HCC were activated and expanded *in vitro* by IL-2, and then infused to the patients^[35]. In this study, TIL infusion as an adjuvant immunotherapy for HCC patients significantly decreased recurrence rate at 6 and 12 months compared to the control group.

Another promising cellular immunotherapy as the adjuvant setting for HCC involves CIK cell immunotherapies. Also, the recent clinical trials from many Asian-Pacific countries reported that adjuvant CIK cell immunotherapy increased progression free survival (PFS) after curative treatment for HCC^[30,36,37].

Adoptive immunotherapy using CIK cells

CIK cells are heterogeneous cell population consisting of CD8+ CTLs, CD56+ NK cells and both CD3+CD56+ NK like T (NKT) cells that were first discovered in the 1990s^[11,37]. CIK cells display both anti-tumor ability of antigen specific CD8+ CTLs and non-major histocompatibility complex (MHC) restricted cancer cell killing capacity of NK cells [Figure 1]^[38]. Earlier clinical studies have shown a potent antitumor activity of CIK cells against various types of tumors^[36].

In 2000, CIK cell immunotherapy is demonstrated to be a safe and feasible treatment that can lower recurrence rate and improve PFS after curative resection of HCC^[37]. In this randomized trial, CIK cells were infused 5 times during the first 6 postoperative months. During the median follow-up of 4.4 years, recurrence rate reduced remarkably by 18% in the CIK cell treatment group (59%, 45/76) compared with that

Table 1. Selected clinical trials with adoptive cellular immunotherapy for HCC

Registered No.	Recruitment status	Start year	Phase	Immunotherapy	Included patients of HCC
NCT00161187	Completed	2001	I	Therapeutic allogeneic lymphocytes: irradiated lymphocytes from a donor	Unresectable or metastatic disease
NCT01828762	Completed	2005	I	Autologous immune killer cell	Locally advanced or metastatic HCC
NCT00699816	Completed	2008	III	Immuncell-LC	Stage I/II, after curative treatment
NCT01749865	Completed	2008	III	CIK	After radical resection
NCT00769106	Completed	2008	III	CIK	After radical resection
NCT01024530	Unknown	2009	II/III	Autologous immune killer cells with TACE	BCLC stage B/C
NCT01212341	Completed	2010	I	MG4101: allogeneic NK cells	Solid tumors
NCT01147380	Completed	2010	I	Liver NK cell inoculation with liver transplantation	Liver transplant recipient
NCT01174121	Recruiting	2010	II	Autologous TILs and IL-2 with cyclophosphamide, fludarabine and pembrolizumab	Metastatic HCC who has received sorafenib
NCT01218867	Completed	2010	I/II	Anti-VEGFR2 CAR CD8 and PBL with cyclophosphamide, IL-2 and fludarabine	Metastatic cancer
NCT01462903	Unknown	2011	I	Autologous TILs and IL-2	Metastatic HCC after primary operation, radiotherapy and chemotherapy
NCT01758679	Recruiting	2012	IV	CIK and Licartin	Postoperative HCC
NCT01801852	Recruiting	2013	I	Autologous NKT cell infusion	Refractory to conventional treatment
NCT01897610	Recruiting	2013	II	Immuncell-LC with sorafenib	Stage III/IV
NCT02008929	Recruiting	2014	II	MG4101: allogeneic NK cell	After curative resection
NCT01914263	Recruiting	2014	I	Cord blood-derived CIKs	After radical resection
NCT02587689	Recruiting	2015	I/II	Anti-MUC1 CAR T cells	MUC1+ malignancies
NCT02959151	Recruiting	2015	I/II	GPC3-CAR T cell	HCC with GPC3 high expression
NCT02725996	Not yet recruiting	2016	II	Autologous NK cells	Stage I/II, after curative treatment
NCT02856815	Not yet recruiting	2016	II	Immuncell-LC	BCLC stage B, tumor removal has been confirmed after TACE
NCT02715362	Recruiting	2016	I/II	GPC3-CAR T cells with transcatheter arterial infusion (TAI)	Persistent cancer after at least one prior standard of care chemotherapy
NCT02839954	Recruiting	2016	I/II	Anti-MUC1 CAR-pNK cells	MUC1+ malignancies
NCT02959151	Recruiting	2016	I/II	GPC3-CAR T cell	HCC with GPC3 expression
NCT02854839	Recruiting	2016	IIA	MG4101: allogeneic NK cells	Complete remission after TACE
NCT03175679	Recruiting	2017	I	iNKT cells and IL-2 with 5-fluorouracil	Relapsed/advanced HCC, BCLC stage C
NCT03199807	Not yet recruiting	2017	IB/II	Personalized new antigen reactive immune cells (NRT), radiotherapy	Advanced HCC, unresectable and no chemotherapy before
NCT03130712	Recruiting	2017	I/II	GPC3-CAR T cells intratumor injection	Advanced HCC, persistent cancer after at least one prior standard of chemotherapy or surgery
NCT03132792	Recruiting	2017	I	Autologous genetically modified AFP ³³² T cells: genetically changed T cells that target alpha-fetoprotein	Positive for HLA-A*02:01 or HLA-A*02:642 allele
NCT03302403	Not yet recruiting	2017	N/A	Autologous T cells transduced with CAR recognizing CD19, BCMA, GPC3 and Claudin18.2	Advanced HCC with previous ablation or resection in the last 4 to 12 weeks
NCT02905188	Not yet recruiting	2018	I	GPC3-CAR T cells with fludarabine and cytoxan	BCLC stage A/B/C
NCT03441100	Not yet recruiting	2018	I	IMA202 Product (CAR T cell) with fludarabine and cyclophosphamide	HCC not amenable to treatments with curative intent

HCC: hepatocellular carcinoma; CIK: cytokine induced killer; TACE: transarterial chemoembolization; BCLC: Barcelona clinic liver cancer; NK: natural killer; TIL: tumor infiltrating lymphocyte; IL-2: interleukin-2; VEGFR: vascular endothelial growth factor receptor; CAR: chimeric antigen receptor; PBL: peripheral blood lymphocyte; NKT: natural killer T; MUC1: mucin1; GPC3: glypican-3; AFP: alpha-fetoprotein; HLA: human leukocyte antigen; BCMA: B-cell maturation antigen; N/A: not applicable

in the control group (77%, 57/74). Moreover, the PFS was significantly improved in the CIK treatment group ($P = 0.01$). All of the adverse events (AEs) were grade I or II and self-limiting. AEs associated with treatment were fever (47%), headache (4%), nausea (4%), dizziness (1%), itching (1%) and tachycardia (1%).

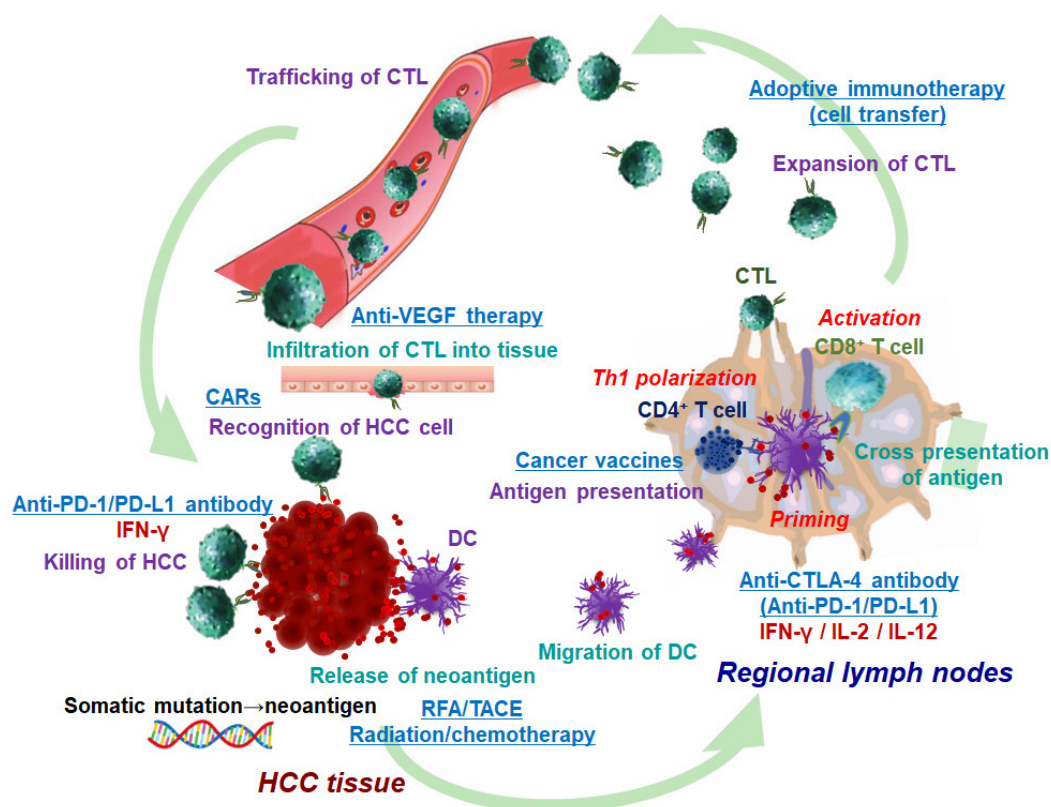


Figure 1. Cancer-immunity cycle and targets of immune therapies. Hepatocellular carcinoma (HCC) cells produce various tumor-associated antigens (TAAs) and neoantigens; the latter derive cancer-specific somatic mutations. The initial steps of anti-tumor immune response include uptake of TAAs and neoantigens by dendritic cells (DCs). After that, the DCs migrate into regional lymph nodes and present processed antigen to CD4⁺ T cells. Antigen recognition leads to proliferation of CD4⁺ T cells and induction of interferon (IFN)- γ in the presence of IL-12 and type I IFN (Th1 polarization). The cross-presentation of antigenic peptide to CD8⁺ T cells by DCs facilitates the development of antigen-specific CD8⁺ cytotoxic T lymphocytes (CTLs). After the trafficking of CTLs to HCC tissues, the antigen-specific CTLs exert anti-tumor effector function through release of humoral factors, such as granzyme B and perforin, and interaction with death receptors on tumor cells. Locoregional therapies and systemic chemotherapies should enhance the release of neoantigens and TAAs through HCC cell death. Cancer vaccines can promote the antigen presentation; anti-CTLA-4 antibody mainly acts in priming phase and facilitates the Th1 polarization and activation of CD8⁺ T cells. Adoptive immune therapies (immune cell transfer) increase the peripheral anti-tumor immune cells; chimeric antigen receptor (CAR) T cells can more directly targets cancer cells compared to conventional adoptive immunotherapies. Anti-vascular endothelial growth factor (VEGF) potentially induce infiltration of T cell into tumor tissues. Anti-PD-1/PD-L1 antibodies block the co-inhibitory signal of CD8⁺ T cells and induce cancer cell killing. CTLA-4: cytotoxic T lymphocyte associated antigen-4; PD-1: programmed cell death 1 protein; PD-L1: PD-1 ligand

In 2004, the influence of autologous CIK cells was investigated in terms of phenotypes of CIK effector cells, peripheral T lymphocyte subsets and DC subsets in 13 HCC patients who had liver cirrhosis and chronic HBV infection^[39]. Peripheral blood mononuclear cells (PBMCs) were collected by a blood cell separator, and then expanded by priming them with interferon-gamma (IFN- γ), monoclonal antibody against CD3 and IL-2. After two weeks of *in vitro* incubation, the percentages of CD8⁺ CTL and CD3⁺CD56⁺ NKT cells increased significantly from 33.5% and 7.7% to 36.6% and 18.9%, respectively. CIK cell therapy increased the proportions of type I DC and type II DC from 0.59% and 0.26% to 0.85% and 0.43%, respectively (all $P < 0.01$). These results indicated that autologous CIK cells could efficiently improve the immunological status in HCC patients.

In 2009, a randomized trial was conducted to investigate the impact of postoperative adjuvant CIK immunotherapy on the prognosis^[40]. In 127 HCC patients who underwent radical hepatic resection, CIK cell therapy significantly increased the disease-free survival rate compared with the control group. However, the overall survival (OS) was not significantly different^[40].

In 2010, the impact of adjuvant CIK therapy after TACE combined with sequential RFA on tumor recurrence was demonstrated in relation to serum AFP level^[41]. After curative TACE plus RFA therapy, 83 patients with AFP level less than 37.5 ng/mL (1.5 times the normal range) were randomly assigned for CIK immunotherapy or for best supportive treatments. CIK cell infusions were given either intravenously or via common hepatic arteries every week for at least 4 times. During the follow-up of 12 months, AFP levels in the CIK group but not in the control group gradually decreased from the baseline levels, and those reduced levels were maintained. Furthermore, the reduced AFP levels of the CIK group were lower than the AFP levels of the control group with statistical significance both in 1 month ($P < 0.05$) and in 3 months ($P < 0.05$) after treatment. The 1-year recurrence rate was 7.1% for the CIK study group and 23.1% for the control group ($P = 0.04$). In addition, the authors showed that HBV DNA titer decreased after CIK cell therapy. They concluded that the adjuvant CIK cell therapy can reduce the serum AFP and HBV DNA levels and decrease the 1-year recurrence rate of patients with HCC after curative TACE plus RFA^[41].

The most recent clinical trial, reported in 2015, demonstrated that adjuvant CIK cell immunotherapy after curative treatment for HCC increased not only the PFS but also the OS^[36]. In this study, 230 patients with HCC who were treated by surgical resection, RFA, or percutaneous ethanol injection were included. Patients were assigned randomly to receive adjuvant CIK cell immunotherapy 16 times during 60 weeks or no adjuvant therapy. The median time of PFS was 44.0 months in the CIK cell therapy group and 30.0 months in the control group ($P = 0.01$). Hazard ratios (HR) of all-cause death (0.21; 95% CI, 0.06-0.75; $P = 0.008$) and HR of cancer-related death (0.19; 95% CI, 0.04-0.87; $P = 0.02$) were significantly lower in the CIK cell immunotherapy group compared with the control group. This study proved that adjuvant immunotherapy with activated CIK cells increase PFS as well as OS of HCC patients after the curative treatments including surgery and RFA^[36]. However, the efficacy of CIK immunotherapy for HCC needs to be further validated, by extending the sample size and follow up duration of the HCC research cohort.

NK cell based immunotherapy

Human NK cell, recognized as a CD3-CD56+ lymphocyte, is a very important part of innate immune system. It provides surveillance toward tumor cells eliminating those when detected. Thus, NK cell was suggested to be used for cancer therapy^[12]. NK cells are characterized by an inborn receptor diversity which allows NK cells to recognize and to respond to different pathogens including virus-infected cells and neoplastic cells without prior sensitization or acquired receptor rearrangement^[17]. It is well known that NK cells can be long-lived, remember past exposures, and interact with MHC class I molecules to acquire full function. NK cell function is tightly regulated by signals from natural cytotoxicity receptors, CD16 receptor for antibody-dependent cellular cytotoxicity (ADCC), C-type lectins, and killer cell immunoglobulin-like receptors (KIR).

Recently, there have been advances in *ex vivo* techniques of NK cell activation and expansion^[17]. Autologous cytokine-stimulated NK cell therapy has been tried with multiple tumors such as renal cell carcinoma, glioblastoma and myeloma^[42]. On the other hand, allogeneic NK cell therapy is particularly beneficial because it can enhance the anti-cancer efficacy of NK cells via donor-recipient incompatibility in terms of KIRs on donor NK cells and MHC class I on recipient tissues^[43]. Thus, the use of allogeneic NK cell therapy is being actively investigated in hematologic malignancies with or without hematopoietic stem cell transplantation^[12]. In these settings, HLA-haploidentical NK cells have been used mostly.

In HCC patients, impaired functions of DC and NK cell were observed in relation to elevated level of serum MHC class I-related chain A (MICA), an inhibitory ligand for NKG2D^[44]. Increase of Tregs and MDSCs were also known to contribute to the functional impairment of NK cells and in turn the reduced anti-tumor immune response^[30,45]. In contrast, increased number of NK cells in peripheral blood and tumor tissues accompanied by an upregulation of related chemokines was an immune-gene signature which determines a long-term survival in resectable HCC^[46].

A group in the University of Miami suggested that NK cells extracted from donor liver graft perfusate could be used as a source of a treatment to reduce recurrence rate after liver transplantation (LT)^[47]. When the NK cells acquired from donor graft was activated with IL-2, activation markers and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is critical for NK cell mediated cancer cell death, were greatly upregulated. The authors concluded that the adoptive transfer of IL-2 stimulated NK cells from deceased donor liver graft could be a promising treatment for LT patients with HCC^[47]. Moreover, the cytokines and chemokines released by activated NK cells may stimulate both innate and adaptive immune responses toward cancer.

In this regard, a phase I clinical trial was conducted to evaluate the feasibility and safety of the adoptive transfer of activated NK cells extracted from cadaveric donor liver graft perfusate after LT (NCT01147380). According to preliminary results posted on the website clinicaltrials.gov, there seemed to be no side effects or serious adverse events. There are also ongoing clinical trials on adoptive NK cell therapy. A phase II clinical trial (NCT02008929) initiated in August 2014 aims to evaluate the safety and efficacy of *ex vivo* expanded allogeneic NK cells (MG4101) as a secondary treatment after curative liver resection on advanced HCC patients with a high risk of recurrence. Adoptive cell transfer of allogeneic NK cells that came from a totally unrelated donor had been demonstrated to be safe without any significant side effects [Figure 1]^[30]. Notably, another multi-center, open label, phase IIA clinical trial (NCT02854839) with a purpose to evaluate the safety and efficacy of allogeneic NK cell (MG4101) therapy for intermediate-stage HCC patients after TACE started in September, 2016.

Adoptive immunotherapy using genetically engineered T cell receptor or chimeric antigen receptors

The emergence of immuno-oncology as the first broadly successful strategy for metastatic cancer will require clinicians to integrate this new pillar of medicine with the pillars of already established therapeutic methods such as chemotherapy, RT and targeted small molecule compounds^[48]. Chimeric antigen receptor (CAR) T cell therapy combines adoptive cellular immunotherapy with targeted molecular therapy, and it has proven that engineered immune cells can serve as a powerful new class of cancer therapeutics [Figure 1]. Adoptive immunotherapy retargeting T cells to CD19 via CAR is an investigational treatment capable of inducing complete tumor regression of B-cell malignancies^[49]. The major hurdle in developing CAR T cell therapy is the on-target off-tumor toxicity as was shown in a metastatic colon cancer patient who died 5 days after infusion of ErbB2 targeting CAR T cell^[50]. Expression of ErbB2 on lung epithelium even with a low level brought a detrimental result. Therefore, finding a target antigen which is effective enough for cancer-killing and at the same time safe enough for the normal tissue is a key requirement in the development of CAR T cell therapy for HCC^[51].

It has been demonstrated that HBV antigens can serve as a tumor specific antigen in HBV related HCC and can be targeted by adoptively transferred HBV-specific T cell receptor (TCR) redirected T cells in preclinical models^[52,53]. Recently, Qasim *et al.*^[54] have reported the clinical results of immunotherapy for HCC metastases with autologous TCR redirected T cells, targeting HBV surface antigen (HBsAg) in a liver transplant patient. Autologous T cells genetically modified to express an HBsAg specific TCR were infused with no immediate infusion-related toxicities despite the patient's frail condition. The authors confirmed that HBV antigens were expressed in metastatic lesions of HCC and demonstrated that tumor cells were recognized *in vivo* by the engineered T lymphocytes. Furthermore, the engineered T cells successfully survived, expanded, and mediated a reduction in HBsAg levels without exacerbation of liver inflammation or other toxicity. Although the clinical efficacy in this patient was not established with end-stage metastatic HCC, these results confirm the feasibility of autologous CAR T cell immunotherapy targeting HBsAg in HBV associated HCC^[54].

In 2010, Food and Drug Administration (FDA) approved a phase I/II study of CAR T cell immunotherapy targeting vascular endothelial growth factor receptor (VEGFR)-2 (NCT01218867), where HCC patients without hepatitis B and C were included^[55]. The result of this study is still awaited. Recently, phase I/II

Table 2. Selected clinical trials with therapeutic cancer vaccine immunotherapy for HCC

Registered No.	Recruitment status	Start year	Phase	Immunotherapy	Included patients of HCC
NCT00004604	Completed	1997	I	CEA RNA-pulsed DC cancer vaccine	Metastatic adenocarcinoma expressing CEA that has failed conventional therapy
NCT00019331	Completed	1997	II	Ras peptides and IL-2 or GM-CSF	Solid tumors potentially expressing mutant Ras
NCT00005629	Completed	1999	I/II	AFP gene HCC vaccine	HLA-A*0201 positive, serum AFP levels > 2 times above the upper limit of normality
NCT00022334	Completed	2001	I/II	AFP peptide-pulsed autologous DC	HLA-A*0201 positive, HCC with a serum AFP determination > 30 ng/mL
NCT00028496	Completed	2001	I	Recombinant fowlpox-CEA(6D)/TRICOM vaccine, sargramostim and recombinant fowlpox GM-CSF vaccine adjuvant	Failed standard curative options and no standard palliative options required within the next 8 weeks
NCT00027534	Completed	2002	I	TRICOM-CEA(6D)	Histologically confirmed advanced or metastatic malignancy expressing CEA
NCT00629759	Completed	2006	I	JX-594: recombinant vaccinia virus (TK-deletion plus GM-CSF)	Progressing HCC
NCT00610389	Unknown	2008	II	DC with PEG-IFN alfa and GM-CSF	HCC not amenable of curative treatment with Child's stage A or B
NCT01266707	Unknown	2010	I	VEGFR1 and VEGFR2 specific epitope vaccine	Unresectable or treatment-resistant HCC
NCT01828762	Completed	2012	N/A	DC incubated with irradiated autologous tumor stem cells in GM-CSF	BCLC stage A/B, after resection and TACE
NCT01974661	Completed	2013	I	COMBIG-DC (ilixadencel): allogenic dendrite-cell based therapeutic vaccine	BCLC stage B/C, not eligible for curative treatment or TACE
NCT02232490	Recruiting	2014	III	Hepcortespensilisimut-L (V5)	HCC with AFP serum test higher or equal to 30 IU/mL
NCT02409524	Recruiting	2016	II	AlloVax, AlloStim and CRCL	Unresectable HCC with minimum 90 days of sorafenib treatment
NCT03203005	Recruiting	2017	I/II	A new cancer vaccine called IMA970A combined with CV8102 with cyclophosphamide	BLCL stage 0/A/B following any standard treatment

HCC: hepatocellular carcinoma; CEA: carcinoembryonic antigen; RNA: ribonucleic acid; DC: dendritic cell; GM-CSF: granulocyte-macrophage colony-stimulating factor; AFP: alpha-fetoprotein; HLA: human leukocyte antigen; TK: thymidine kinase; TRICOM: triad of costimulatory molecules (B7-1, ICAM-1 and LFA-3); PEG-IFN: pegylated interferon; VEGFR: vascular endothelial growth factor receptor; BCLC: Barcelona clinic liver cancer; TACE: transarterial chemoembolization; COMBIG: combined toll-like receptor interferon-gamma; CRCL: chaperone rich cell lysate; N/A: not applicable

clinical trials with CAR T cells targeting glypian-3 (GPC3), alpha-fetoprotein (AFP), and mucin 1 (MUC1) are being conducted [Table 1]^[56,57]. Moreover, a CAR NK cell immunotherapy targeting MUC1 is being conducted (NCT02839954)^[58].

Taken together, adoptive cellular immunotherapy in HCC is a safe and feasible treatment. However, its efficacy in preventing recurrence and prolonging survival in advanced HCC patients remains controversial^[59]. Indeed, cellular immunotherapy seems to be more effective in patients with low burden of micrometastases^[36]. The current situation lacking sufficiently effective cellular immunotherapy for advanced stages of HCC calls for further improvement in immunotherapeutic strategies and additional approaches with immune checkpoints modulators.

THERAPEUTIC CANCER VACCINES

Therapeutic cancer vaccine is an important part of cancer immunotherapy. Vaccination with cancer antigens or peptides is believed to help the immune system to recognize cancer cells and attack them more easily [Tables 2 and 3]. In therapeutic cancer vaccine, DC is an important component. As professional APCs, DCs serve as an essential link between innate and adaptive immune systems^[17]. Two functional states of DC are described, as immature or mature DCs. Several factors can induce maturation of DCs. Mature DCs are specialized APCs, which express high levels of surface MHC I and MHC II class, as well as the appropriate

Table 3. Current trials on combinational immunotherapy strategies in HCC

Registered No.	Recruitment status	Start year	Phase	Immunotherapy	Included patients of HCC
NCT01522820	Completed	2012	I	DEC-205/NY-ESO-1 fusion protein CDX-1401 with sirolimus	After resection or TACE
NCT01853618	Completed	2013	I/II	Tremelimumab with TACE, RFA, SBRT or Cryoablation	BCLC stage B/C
NCT01821482	Recruiting	2013	II	DC-CIK	After complete resection or TACE
NCT02562755	Recruiting	2015	III	Pexastimogene devacirepvec (Pexa Vec) with sorafenib	Advanced HCC (BCLC-C or AASLD-B)
NCT02487017	Recruiting	2015	II	DC-CIK with TACE	After TACE treatment
NCT02432963	Active, not recruiting	2015	I	Modified vaccinia virus Ankara vaccine expressing p53 and pembrolizumab	Advanced HCC, confirmed p53 involvement, failed to or refusal to standard therapy
NCT02821754	Recruiting	2016	II	Durvalumab and tremelimumab with RFA, cryotherapy or TACE	Multiple HCC technically amenable to ablative therapy
NCT02837029	Recruiting	2016	I	Nivolumab with Yttrium Y 90 glass microspheres	Stage III/IV
NCT02795429	Recruiting	2016	I/II	PDR001 with or without INC280	Advanced, recurrent or metastatic HCC
NCT02886897	Recruiting	2016	I/II	DC-CIK and anti-PD-1 antibody	Advanced HCC
NCT03259867	Recruiting	2017	IIA	Nivolumab or pembrolizumab with trans-arterial tirapazamine embolization	Advanced HCC (BCLC-C), progressive disease (PD) on, intolerant of or refusing sorafenib
NCT03380130	Recruiting	2017	II	Nivolumab with selective internal radiation therapy	Candidates for locoregional therapy using selective internal radiation-spheres
NCT03277352	Recruiting	2017	I/II	INCAGN01876, pembrolizumab and epacadostat	Locally advanced or metastatic disease
NCT03241173	Recruiting	2017	I/II	INCAGN01949, nivolumab and/or ipilimumab	Locally advanced or metastatic disease
NCT03126110	Recruiting	2017	I/II	INCAGN01876, nivolumab and/or ipilimumab	Locally advanced or metastatic disease
NCT03095781	Recruiting	2017	I	Hsp90 inhibitor XL888 and pembrolizumab	Stage IV or locally advanced unresectable gastrointestinal adenocarcinomas
NCT03203005	Recruiting	2017	I/II	A new cancer vaccine called IMA970A and CV8102 with cyclophosphamide	BCLC stage O/A/B following any standard treatment
NCT03067493	Recruiting	2017	II	Neo-MASCT (antigen-pulsed DC, autologous specific cytotoxic T-cells)	Primary HCC with previous RFA or resection
NCT03071094	Recruiting	2017	I/IIA	Pexastimogene devacirepvec (Pexa Vec) and nivolumab	Advanced HCC per EASL-EORTC
NCT03482102	Recruiting	2018	II	Tremelimumab and durvalumab with radiation	Locally advanced/unresectable or metastatic disease
NCT03439891	Recruiting	2018	II	Nivolumab with sorafenib	Unresectable, locally advanced and/or metastatic HCC
NCT03511222	Not yet recruiting	2018	I	Vorolanib and pembrolizumab	A solid tumor that can be treated with either pembrolizumab or nivolumab as part of standard of care

HCC: hepatocellular carcinoma; GM-CSF: granulocyte-macrophage colony-stimulating factor; BCLC: Barcelona clinic liver cancer; PD-1: programmed cell death 1 protein; TACE: transarterial chemoembolization; RFA: radiofrequency ablation; AASLD: American association for the study of liver diseases; SBRT: stereotactic body radiotherapy; DC: dendritic cells; CIK: cytokine-induced killer; Hsp: heat shock protein; Neo-MASCT: neoantigen multiple target antigen stimulating cell therapy; EASL: European association for the study of the liver; EORTC: European organisation for research and treatment of cancer

costimulatory molecules required for T-cell activation. One of the most important functions of mature DCs is the rapid production of high amounts of type I IFN, especially in response to virus-derived nucleic acids through activation of Toll-like receptors (TLRs), both TLRs 7 and 9.

Although immunotherapy is not recommended for the clinical management of HCC patients under current guidelines, several different immunotherapy vaccine strategies have been investigated in the last decade for HCC^[15]. Moreover, significantly lower numbers of CD83+ DCs (mature and activated DCs) have been found in liver tissue of patients with HCC compared with liver cirrhosis patients^[60].

Many of the HCC clinical studies on therapeutic cancer vaccines have focused on AFP-based vaccinations since the majority of human HCCs overexpress AFP^[15]. CD8+ T cell epitopes derived from AFP peptides

were used to carry on the first HCC vaccine clinical trial. AFP positive HCC patients received three biweekly intradermal injections of the AFP peptides. All of the patients ($n = 6$) developed the AFP-specific T cell responses, clearly proving the immunogenicity of AFP even in the environment of high circulating levels of AFP in HCC patients^[61]. Subsequently, the authors conducted another phase I/II trial. This time, they immunized AFP positive HCC patients with autologous DCs *ex vivo* pulsed by AFP epitopes. DCs were prepared from PBMCs cultured with granulocyte-macrophage colony-stimulating factor and IL-4 for 7 days^[62]. In this study, AFP-specific T cell response and increased IFN- γ production were shown. Despite this immune response, clinical response was not observed. The authors found the reason for it in a subsequent study that CD4+ T cell help was lacking, which resulted in non-functional AFP-specific CD8+ T cells^[63]. Unfortunately, a limited number of clinical trials for HCC have been conducted based on therapeutic vaccine immunotherapy.

Meanwhile, the bioactivity and beneficial effects of DC infusion were evaluated in HCC patients following trans-catheter hepatic arterial embolization (TAE). In this study, tumor recurrence was not completely prevented in patients with TAE and DC infusion than in those with TAE alone. However, TAE with DC infusion enhanced the tumor-specific immune responses more effectively than TAE alone. The authors demonstrated that combination therapy using TAE together with DC infusion is safe for patients with cirrhosis and HCC^[64].

In another phase II study, the safety and efficacy of vaccination with mature autologous DCs pulsed with a liver tumor cell line lysate (HepG2) have been investigated in patients with advanced HCC and not suitable for radical or loco-regional therapies^[65]. The authors showed that autologous DC vaccination in patients with HCC is safe and well tolerated with evidence of antitumor efficacy with generation of antigen-specific immune responses in some cases. More recent study, reported in 2013, also showed similar results. The safety and efficacy of the autologous pulsed DC vaccine was compared to supportive treatment in advanced HCC patients. They showed that autologous DC vaccination in advanced HCC patients was safe and well tolerated. Additionally, both CD8+ CTL and serum IFN- γ were elevated after DC vaccine^[66].

Actually, to date, no vaccine has been approved so far for HCC treatment^[19]. Further investigations and improvements of therapeutic cancer vaccines will be required to achieve better efficacies in HCC patients.

IMMUNE CHECKPOINT BLOCKADES IN HCC

During the last decade, new immuno-oncological treatments were introduced for diverse cancers, eventually leading to the clinical breakthrough of immune checkpoint blockades targeting CTLA-4, PD-1, PD-L1 and PD-L2^[67,68]. Under physiological conditions these checkpoint molecules resolve T cell activation to maintain inflammatory homeostasis, also limit collateral tissue damage and prevent unwanted auto-immunity, as observed in response to chronic viral hepatitis^[26,27]. Meta-analysis data on solid tumors have suggested that overexpression of PD-L1 in tumor cells, as well as in APCs of tumor microenvironment, is associated with poor prognosis in patients with malignant tumors including HCC^[21,69]. The subsequent PD-1/PD-L1 interaction results in T-cell exhaustion and immune evasion by cancer cells^[70]. The inhibitory effects of the PD-1/PD-L1 pathway on T cell-mediated antitumor immunity are commonly reported regarding HCC carcinogenesis, and the PD-L1 is over-activated in HCC^[9,71]. Also the PD-1/PD-L1 interaction is known to be associated with persistent HBV and HCV viremia, or the progression of HCC, by suppressing specific T-cell immunity and thereby inducing immune tolerance or immune escape of cancer cells^[8,27].

Notably, immune checkpoint inhibitors have proven effective in patients who are refractory to tyrosine kinase inhibitors (TKIs) such as sorafenib, and recently several blocking antibodies targeting PD-1 or CTLA-4 have shown promising results in advanced HCC patients who received previous treatment with sorafenib^[20,28,72]. Compared to TKIs, immunotherapy has several advantages for the treatment of cancer, as its effects are

not hampered by common mutations or neoantigen heterogeneity of tumor cells^[28,73]. Therefore, immuno-oncology agent is effective regardless of the response to prior therapies, and also a durable response can be expected due to adaptive immunity to the cancer cells^[74]. However, the profile of AEs is completely different from those of other cytotoxic and molecular targeting agents^[28]. The tolerability of immuno-oncology agents generally depends on the severity of immune-related AEs (irAEs), although the majority of irAEs are mild and manageable^[20,75].

Different clinical trials are currently underway to investigate the safety and efficacy of checkpoint inhibitors for HCC immunotherapy as in monotherapy or in combination [Table 3]^[28].

The PD-1/PD-L1 pathway

Higher intra-tumoral expression of PD-1/PD-L1 had been associated with significantly poorer PFS and OS after hepatectomy as well as postoperative recurrence in HCC^[76]. It was shown that PD-1 immune checkpoint inhibitor therapies have a strong therapeutic effect on patients with high levels of PD-L1 expression^[77]. This could be due to the ability of the PD-1/PD-L1 pathway to act as an anti-apoptotic receptor on cancer cells [Figure 1]^[23,69].

To date, two kinds of anti-PD-1 (nivolumab and pembrolizumab) and anti-PD-L1 (durvalumab, avelumab) antibodies have been applied for clinical trials in HCC and nivolumab, pembrolizumab, and avelumab are in development as monotherapy^[20,28]. Two phase III studies are currently ongoing: a comparison of nivolumab and sorafenib in the first line setting for advanced HCC (CheckMate 459), and a comparison of pembrolizumab and a placebo in the second line setting for patients with advanced HCC who progressed on sorafenib (KEYNOTE 240)^[20,78].

Nivolumab, a fully human IgG4 anti-PD-1 monoclonal antibody, was granted accelerated approval from U.S. FDA on September 2017 for treatment of HCC patients who were previously treated with sorafenib. Approval was based on findings in a phase I/II, open-label, non-comparative, dose escalation and expansion trial (CheckMate 040) consisting of patients with HCC and Child-Pugh A cirrhosis^[78]. Between November 2012 and August 2016, 262 eligible patients were treated (48 patients in the dose-escalation phase and 214 in the dose-expansion phase). At the American Society of Clinical Oncology (ASCO) meeting in 2015, results of the dose-escalation trial of CheckMate 040 were presented; 68% of patients had drug-related AEs, the complete response (CR) rate was 5%, and the partial response (PR) rate 14%. The safety profile of nivolumab is generally consistent with what was previously-reported in other tumor types. Twelve (25%) of 48 patients in the dose-escalation phase had grade 3/4 treatment-related AEs. Autoimmune disease and hepatic dysfunction, which were the AEs of initial concern, were not observed^[20]. In the 2017 ASCO meeting, final results of the phase I/II CheckMate 040 study with nivolumab in advanced HCC showed favorable results with objective response rate (ORR) 20% and disease control rate (DCR) 64%^[78]. The OS rate of the fixed dose of 3 mg/kg nivolumab group at 12 months was 62%. Considering that a high proportion (66%) progressed on sorafenib treatment, these outcomes appear to be extremely good. In addition, nivolumab was effective regardless of prior sorafenib administration and viral status, indicating that nivolumab could be effective even in cases refractory to sorafenib^[20,28,78]. However, the ORR of HBV-positive cases was lower (14%) compared to non-HBV cases (20%-23%)^[28,78]. There was no significant association between PD-L1 expression in HCC and the response to nivolumab^[20,78].

Another anti-PD-1 antibody, pembrolizumab, was associated with PR and prolongation of survival in a patient with progressive metastatic HCC while being treated with sorafenib^[79]. The randomized, placebo-controlled phase III KEYNOTE 240 study (NCT02702401) to compare the efficacy and safety of the pembrolizumab with best supportive care for the treatment of advanced HCC after failure to sorafenib is ongoing. Recently, findings from the KEYNOTE 224 study (NCT02702414), open-label phase II trial

investigating pembrolizumab monotherapy in patients with advanced HCC who were previously treated with sorafenib, were presented at the 2018 Gastrointestinal Cancers Symposium. Results showed the ORR of 16.3% (95% CI, 9.8%-24.9%; $n = 17/104$) with CR of 1% (95% CI, 0.0%-5.2%) and PR of 15.4% (95% CI, 9.1%-23.8%). The DCR was 61.5% (95% CI, 51.5%-70.9%; $n = 64/104$) and median PFS time was 4.8 months (95% CI, 3.4-6.6 months), with a 6-month PFS rate of 43% and 6-month OS rate of 78%.

Furthermore, a clinical trial of monotherapy agents targeting PD-L1, such as avelumab, has also been conducted in advanced HCC patients (NCT03389126)^[28].

The CTLA-4 pathway

Tremelimumab is an IgG2 type anti-CTLA-4 antibody that was evaluated in a phase II clinical trial (NCT01008358) investigating the tremelimumab monotherapy in 21 patients with HCV-related HCC^[80]. This study with tremelimumab in HCV infected HCC patients has shown a good safety profile along with a promising PR rate of 17.6% and a time-to-progression (TTP) of 6.5 months^[80]. In this CTLA-4 trial, a transient complete virologic response or decrease in HCV viral load was also observed in most patients with the DCR of 76.4%^[28,80]. The trial demonstrated efficacy of tremelimumab monotherapy in HCC patients and the anti-tumoral and antiviral effects that warrant further investigation.

Notably, the feasibility of combined locoregional therapies and tremelimumab administration was investigated in patients with liver cirrhosis and HCC^[81]. The use of tremelimumab plus RFA, cryoablation, or TACE in patients with BCLC B or C HCC was associated with ORR of 26.3% in areas outside of the ablation zone, and median TTP was 7.4 months. The combination of tremelimumab with local tumor ablation is a smart synergistic mechanism, because, in patients responding to local ablative therapy, prolonged TTP gives time for immunotherapy to unfold^[72]. In addition, local tumor ablation releases TAA from apoptotic or necrotic HCC tissue, which in turn accelerates tumor specific APCs and CTLs activation, resulting in immunological synergy evolving from the combination of both treatment modalities [Figure 1]. Also, in this study, 12 of 14 patients with quantifiable HCV experienced a marked reduction in viral load, especially in the patients with PR. Studies have shown that tremelimumab in combination with tumor ablation is a potential new treatment for patients with advanced HCC^[81]. Particularly, the positive antiviral immune responses may act as a surrogate for disease control in HCC immunotherapy^[72].

In summary, immune checkpoint blockade therapy (anti-PD-1 and anti-CTLA-4) had a favorable safety profile in patients with HCC^[20]. It can be used safely in patients with HBV and HCV infection, and its high ORR was a great achievement compared to the rates achievable with other types of immunotherapy^[28].

Other immune checkpoint pathways

Although anti-PD-1/PD-L1 antibody is a promising agent for the treatment of HCC, a considerable percentage of HCC patients could not attain satisfactory tumor control, likely due to the immune suppressive cellular components, humoral mediators, and diverse inhibitory checkpoint molecules^[28,82]. Their crosstalk becomes more complex during tumor progression. Also, the continuous production of cytokines and chemokines in the inflamed liver and solid immunosuppressive stroma of HCC could induce the production of many types of suppressive checkpoint molecules^[83,84].

Cellular components including MDSCs, TAMs, Tregs and type 2 helper T cells might facilitate the immune evasion of HCC tumor cells^[20,83]. MDSCs also produce transforming growth factor (TGF)- β and IL-10 that lead to the suppression of CD56+ NK cell and CD8+ CTL activities^[85]. TGF- β from MDSCs induces the expression of T-cell immunoglobulin and mucin-containing protein-3 (TIM-3) on TAMs, which is associated with galectin-9 and further facilitates the M2 polarization of macrophages in tumors^[86]. Galectin-9, which

is a ligand of TIM-3, also induces Treg stimulation and T cell exhaustion^[83]. The TIM-3/galectin-9 signaling pathway reportedly mediates T-cell dysfunction in HBV-associated HCC, which might explain the poor ORR of HBV-associated HCC compared with that of non-HBV-associated HCC during the anti-PD-1 antibody administration^[78,83,87].

Galectin-3 interacts with lymphocyte activation gene-3 (LAG-3) and inhibits CD8+ T cell and NK cell functions^[83]. LAG-3 expression on TILs, along with PD-L1 on tumor cells, is also reported in HCC^[88]. As in the PD-1/PD-L1 in HCC tumor, the galectin-3/LAG-3 expression is also associated with a poor prognosis in HCC patients^[89]. Another report also showed up-regulated LAG-3 expression and impaired effector function of CD8+ CTLs in HBV-positive HCC patients^[90]. Taken together, TIM-3, LAG-3, and PD-1 act synergistically and facilitate the HCC immune evasion resulting in worse prognosis^[88]. According to these findings, TIM-3 and LAG-3, checkpoint molecules expressed on the effector T cell, could mediate resistance to the PD-1/PD-L1 blockade^[86,88]. Given the fact that there are multiple players in the establishment of immune escape in HCC, anti-PD-1/PD-L1 therapy is being paired with agents targeting TIM-3 (NCT03099109) and LAG-3 (NCT01968109), respectively^[83].

HCC IMMUNOTHERAPIES IN COMBINATION WITH OTHER CANCER TREATMENTS

While the present adoptive immunotherapy has been restricted to the patients with small tumor burdens so far, treatments using these engineered immune cells have generated some remarkable responses in patients with advanced cancer by combinational immunotherapy^[91]. Ongoing investigations of adoptive immunotherapy combined with traditional HCC treatments, including surgery, locoregional interventions, and systemic chemotherapy, may achieve the best objective responses in various stages of HCCs^[92,93]. In 2013, a retrospective study was conducted in 174 HCC patients from January 1999 to April 2012. Among them, 85 patients were given CIK cell infusion after treatment with TACE and RFA alone. The results demonstrated that CIK cell infusion significantly prolonged the PFS in patients compared to TACE or RFA monotherapy^[94]. A different approach is pretreatment of HCC with TACE, RFA, or RT to induce inflammation of cancer cells, thereby creating conditions that favor tumor neoantigen generation prior to the initiation of immunotherapy^[84].

Although the efficacy of immune checkpoint inhibitors in HCC is promising, the majority of the patients remain refractory, due to the immunosuppressive mechanisms of HCC comprising multiple humoral mediators and suppressive checkpoint molecules^[82,83]. To enhance the anti-tumor activity, several studies on combined immune checkpoint blockades are being conducted [Table 3]. The most relevant combination is a CTLA-4 and PD-1/PD-L1 blockade^[28]. The rationale of this strategy is based on the idea that if CD8+ CTL do not exist in cancer tissue, blockade of the PD-1/PD-L1 pathway cannot be expected to be efficacious. Therefore, blocking CTLA-4 may be an effective strategy to increase the number of activated effector T cells that infiltrate the tumor tissue^[20]. Durvalumab, a monoclonal antibody to PD-L1, is currently evaluated in combination with an anti-CTLA-4 antibody (tremelimumab) for sorafenib-experienced HCC patients in a phase II trial (NCT02519348)^[83]. Another anti-CTLA-4 antibody, ipilimumab, is also being analyzed in combination with the anti-PD-1 antibody, nivolumab, for evaluation of the safety and efficacy in HCC patients (NCT01658878, NCT03222076)^[20].

Given the fact that molecular target agents could collectively block the signaling from various growth factors and affect immune effectors and the vasculature, the combination of TKIs and immune checkpoint inhibitors could reactivate the immune response to HCC^[28,84]. Several early phase studies are currently underway to explore the safety and tolerability of TKIs such as sorafenib (NCT03211416, NCT01658878, NCT02988440), lenvatinib (NCT03418922, NCT03006926), cabozantinib (NCT03299946, NCT01658878), axitinib (NCT03289533), and capmatinib (NCT02795429) in combination with immune checkpoint

inhibitors^[83]. Of note, current clinical trials are focusing on how immunosuppressive conditions in HCC might be overcome using immune checkpoint inhibitors in combination with different types of immune checkpoint blockades, TKIs, and other conventional treatments^[83]. To improve the HCC immunotherapy strategies as well as immune stimulatory approaches, identification of TAAs and neoantigens specific to HCC and testing the potential benefits of combinatorial immunotherapies will achieve the most beneficial effect for HCC patients^[59].

CONCLUSIONS AND FUTURE PERSPECTIVES

The journal *Science* selected cancer immunotherapy as its “Breakthrough of the Year” in 2013, and especially the use of immune checkpoint blockade in cancer therapy is making a paradigm shift in cancer treatment^[20,95]. Of note, immunotherapy has the potential to achieve complete, long-lasting remissions and cancer cures, representing the most promising new cancer treatment approach with few side effects^[74]. Although disease progression is sometimes observed immediately after initiation of immunotherapy, some responders require longer duration of immunotherapy to achieve tumor response^[20]. Therefore, the biomarkers of immunotherapy to predict response are urgently needed, both from the perspective of the effective use of medical resources and to prevent adverse effects caused by unnecessary treatment^[84]. There are several highly promising candidate predictors of the cancer immunotherapy: PD-L1 expression in tumor tissue, TAA related mutanome analyses including next-generation sequencing, and the immunome analyses, which employ T cell repertoire analysis and proteomic analysis^[96]. Also, additional questions still remain regarding the most effective combination of therapeutic modalities and biomarkers to predict long term treatment outcomes in HCC immuno-oncology.

Notably, to date, very promising published evidence with checkpoint inhibitors in HCC has been reported in the clinical trials of anti-CTLA-4 agent tremelimumab and a large phase II trial with anti-PD-1 agent nivolumab^[56,78,80]. Further investigations of immuno-oncology potentially spread the applications of immunotherapy in the various stages of HCCs, and thus immune-based therapies will bring about a paradigm shift of anti-cancer treatment for HCC. We hope the immunotherapy will play a key role in HCC treatment in the near future.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Lee JH, Nishida N

Performed data acquisition, as well as provided administrative, technical, and material support: Oh SY, Kim JY

Availability of data and materials

Not applicable.

Financial support and sponsorship

This study was supported by a grant of the Korea Healthcare technology R&D project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (HI13C1398).

Conflicts of interest

The authors declare there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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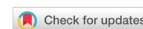
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Review

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The fibroblast growth factor receptor pathway in hepatocellular carcinoma

Joycelyn Jie Xin Lee, Su Pin Choo

Division of Medical Oncology, National Cancer Centre Singapore, 11 Hospital Drive, Singapore 169610, Singapore.

Correspondence to: Dr. Su Pin Choo, Division of Medical Oncology, National Cancer Centre Singapore, 11 Hospital Drive, Singapore 169610, Singapore. E-mail: choo.su.pin@singhealth.com.sg

How to cite this article: Lee JJX, Choo SP. The fibroblast growth factor receptor pathway in hepatocellular carcinoma. *Hepatoma Res* 2018;4:52. <http://dx.doi.org/10.20517/2394-5079.2018.42>

Received: 25 Apr 2018 **First Decision:** 19 Jun 2018 **Revised:** 2 Aug 2018 **Accepted:** 10 Aug 2018 **Published:** 13 Sep 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cai-Hong Wang **Production Editor:** Huan-Liang Wu

Abstract

Hepatocellular carcinoma is the third most common cause of cancer-related death globally and portends a poor prognosis. The fibroblast growth factor receptor (FGFR) pathway is increasingly acknowledged to play a role in the pathogenesis of hepatocellular carcinoma (HCC) and is postulated to be upregulated as a mechanism of resistance to anti-VEGF treatment. We attempt to review the importance of the FGFR pathway in HCC oncogenesis, as well as the current clinical evidence on the efficacy and safety of FGFR pathway inhibitors in HCC.

Keywords: Hepatocellular carcinoma, targeted therapy, fibroblast growth factor

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death globally^[1]. Most patients have advanced disease on diagnosis. In unresectable advanced disease, sorafenib used to be the only available systemic therapy option available and prognosis was poor with a one-year survival rate of less than 50%^[2].

HCC tumours harbour an average of 30-40 mutations, of which 20% may be driver mutations^[3]. The molecular complexity and heterogeneity of HCC likely underlies the reason for failure of multiple phase III trials of targeted agents over the years. With improving technologies, we have been able to learn more about the molecular mechanisms underlying the oncogenesis of HCC, and in recent past have seen breakthroughs with several new drugs being added to our armamentarium both in the front-line and second-line setting^[4], and many more compounds showing great promise on the horizon^[5].



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One signaling pathway that is increasingly recognized to play a role in the carcinogenesis of HCC is the fibroblast growth factor (FGF)/fibroblast growth factor receptor (FGFR) pathway, which has roles in oncogenesis, mediating cell proliferation and neo-angiogenesis^[6,7]. Preclinical models suggest that inhibition of the FGFR pathway is a feasible therapeutic strategy^[7] and many clinical trials using FGF/FGFR pathway inhibitors have since been conducted or are ongoing in hepatocellular carcinoma.

We attempt to review the importance of the FGF/FGFR pathway and current clinical evidence to date for use of the pathway inhibitors in HCC.

FGF/FGFR PATHWAY AND ITS ABERRATIONS IN CANCER

The human FGF family consists of 22 structurally related molecules that interact with four FGFRs. Each FGFR comprises three components, an extracellular domain which interacts with the FGF ligand, a trans-membrane domain, and an intracellular domain. FGFs act as ligands which can bind to more than one kind of FGFR, causing downstream activation of several pathways including the mitogen-activated protein kinase pathway regulating cellular proliferation, and the phosphoinositide-3 kinase-Akt pathway controlling cellular survival^[8]. FGF/FGFR signaling is involved in normal embryonic development of the liver and lungs^[9] as well as adult wound healing and angiogenesis^[10].

FGFRs are widely expressed in adult tissue, although their relative levels differ in the various organ systems. Under normal conditions, hepatocytes express high levels of FGFR3 and FGFR4 and have lower levels of FGFR1 and FGFR2^[11].

FGFR signaling has significant effects on tumour neo-angiogenesis, both via the direct promotion of endothelial cell proliferation through effects on the tumour microenvironment^[12], as well as indirectly via interactions and synergism with the vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) pathways^[13].

FGFR pathway activation has also been shown to be an important resistance mechanism in response to therapeutic pressure with use of anti-VEGF therapy^[6,14]. In both the preclinical^[15] and clinical^[16] settings, tumours progressing on anti-VEGF treatment have been shown to have a higher level of expression of FGF2. As such, upfront dual inhibition of VEGFR and FGFR, or introduction of FGFR inhibition after progression on a VEGF pathway inhibitor^[17] can potentially result in greater clinical benefit compared to inhibition of the VEGF pathway alone.

FGFR aberrations occur in approximately 7% of all solid tumours and in almost every tumour type, though the frequency and type of aberration differ^[18]. Pathway aberrations identified include^[19]: (1) gene amplification, or post-transcriptional changes giving rise to receptor overexpression; (2) gene mutations, resulting in constitutively activated receptors or receptors that have a reduced dependence of ligand binding for activation; (3) translocations, resulting in expression of FGFR-fusion proteins with constitutive FGFR kinase activity; (4) alternative splicing of FGFR and isoform switching, changing ligand specificity and increasing the range of FGFs that can activate the FGFR; (5) upregulation of FGF ligand expression.

Overall the most common aberration seen in solid tumours is FGFR gene amplification, most commonly in FGFR1. FGFR mutations in cancer differ from those seen in hereditary disorders in that they are not limited to the kinase domains, but may occur in any part of the gene^[19].

RELEVANCE OF THE FGF PATHWAY IN HCC

The importance of the FGF/FGFR pathway in HCC can be seen in the fact that more than 80% of HCCs

overexpress at least one FGF and/or FGFR^[20]. The main FGFRs expressed in liver tissue are FGFR3^[21] and FGFR4^[22].

Whilst healthy hepatocytes express minimal levels of FGF1 or FGF2, these levels increase when there is cirrhosis and increasing levels correlate with the progression of cirrhosis into HCC. Higher levels of FGF1 and FGF2 are also seen in more advanced tumour stages^[23]. There is hence interest in using FGF1 and FGF2 expression levels as a prognostic marker^[24], though its utility as a diagnostic marker or for follow-up of HCC patients is limited by its non-specificity^[25].

In preclinical models, FGF1 and FGF2 were shown to stimulate proliferation of HCC cell lines^[26] through the activation of tumour invasion and angiogenesis resulting in an increase in capillarised sinusoids^[27]. There is however substantial redundancy in FGF1- and FGF2-mediated signaling, suggesting that direct targeting of these ligands may have limited therapeutic efficacy^[28].

The FGF8 subfamily, comprising FGFs 8, 17 and 18, also promotes oncogenesis through stimulating hepatocyte proliferation. At least one member of the FGF8 subfamily or its corresponding receptors FGFR2, FGFR3 and FGFR4 is upregulated in more than 50% of HCCs^[20]. The use of small interfering RNA (siRNA) targeting FGF18 has been shown to reduce the viability and proliferation of HCC cells^[20].

The FGF19 subfamily, comprising FGFs 19, 21 and 23, act as endocrine factors mediating metabolic effects through FGFR signaling. FGF19, which comes mainly from the ileum, plays a role in the physiological regulation of bile acid and cholesterol metabolism as well as insulin sensitivity. FGF19 binds exclusively to FGFR4 with the co-receptor β -Klotho (KLB) stabilising the interaction. FGF19/FGFR4 signaling is thought to be of particular importance in the carcinogenesis of HCC^[29], with FGF19 expression increased, through focal amplification of 11q, in approximately 6%-12% of HCC cases^[30]. FGFR4 expression is also upregulated in almost half of HCCs^[31]. In addition, FGF19 levels may be prognostic, with higher expression in resected HCC specimens being associated with larger tumour size and stage and higher risk of recurrence after hepatectomy^[32].

In vitro studies show that FGF19 induces HCC cell proliferation^[29] and inhibits apoptosis^[33]. Mice models also confirm that the ectopic expression of FGF19 promotes hepatocyte proliferation, dysplastic change and precipitates the formation of HCC^[34]. Similarly, FGFR4 knockout mice showed increased hepatocyte injury when challenged with the hepato-toxin carbon tetrachloride^[35]. Targeting the FGF19/FGFR4 interaction through various approaches appears to be effective in inhibiting hepatocarcinogenesis and HCC growth in preclinical models, be it through the use of a neutralizing antibody against FGF19^[36], through genetic knockdown^[30], or through siRNA^[33]. Using siRNA to knockdown FGFR4 also showed similar results in mice models, which had impaired regeneration and increased liver injury after partial hepatectomy^[37].

As previously mentioned, the FGF/FGFR pathway has been shown to be upregulated after initial blockade of the anti-VEGF pathway^[38], and may be an important resistance mechanism to anti-VEGF therapy including that of sorafenib. For a long time, sorafenib was the only systemic treatment option for advanced HCC, having demonstrated an improvement in overall survival of 2-3 months in two large phase III trials^[39,40]. Whilst having inhibitory effects on multiple targets including VEGFR, PDGFR and Raf kinases, sorafenib has no anti-FGFR activity^[41]. Concomitant dual blockade of FGF/FGFR and VEGF pathways are hence a potentially attractive approach in the efforts to overcome this resistance^[38].

OVERVIEW OF FGF/FGFR PATHWAY INHIBITORS AND THEIR TOXICITIES

Current available inhibitors against the FGF/FGFR pathway can be classified into [Figure 1](#): (1) monoclonal antibodies which competitively inhibit FGF binding to the FGFR extracellular domain; (2) FGF-ligand traps; and (3) small molecule tyrosine kinase inhibitors (FGFR TKIs).

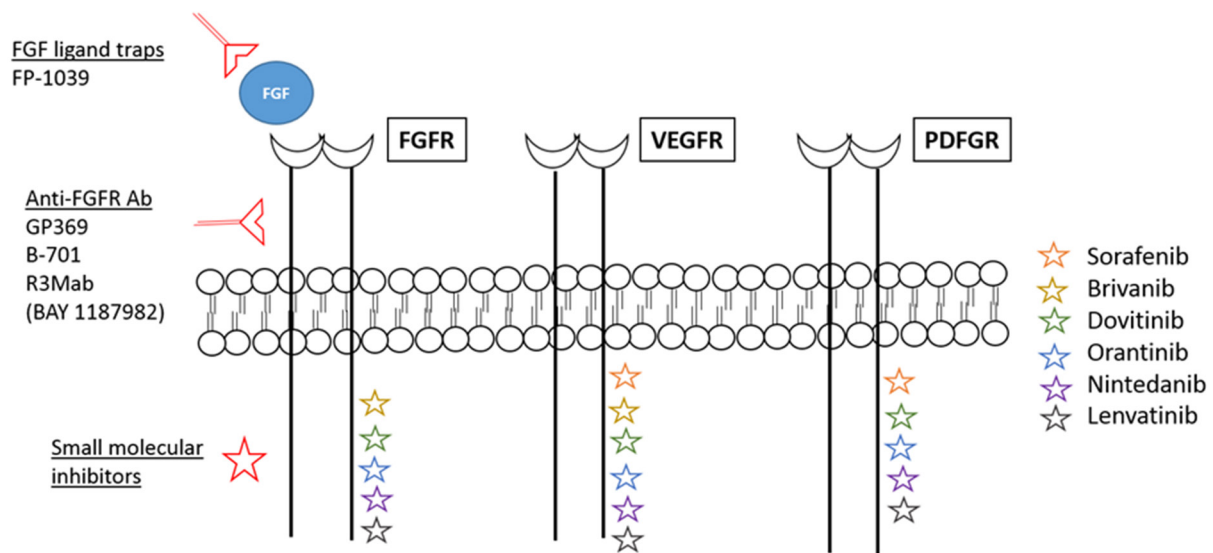


Figure 1. Overview of FGFR pathway inhibitors (adapted from^[31] and Sandhu *et al.*^[28]). FGFR: fibroblast growth factor receptor; VEGFR: vascular endothelial growth factor receptor

Most of the multi-kinase inhibitors have inhibitory effects on both VEGFR and FGFR because of the structural similarities in the kinase domains of both receptors, though they may vary in their relative potency for inhibition for the two groups of receptors, with the majority having a higher potency for VEGFR than FGFR. Whilst multi-kinase inhibitors may potentially increase therapeutic efficacy by simultaneously disrupting resistance pathways, toxicity and off-target effects inevitably increase, which may limit the ability to achieve doses required for effective FGFR inhibition^[19,42].

Selective FGFR inhibitors on the other hand, may have unique on-target dose-limiting toxicities. Preclinical models with selective FGFR TKIS caused hyperphosphataemia-mediated tissue calcification through the inhibition of FGF23 signaling in the kidney and bone, where it plays a critical role in vitamin D and phosphate homeostasis^[43,44]. This was replicated in the clinical setting with 83% of patients treated at the maximum tolerated dose in the BGJ398 phase I trial developing hyperphosphataemia^[45]. This resulted in repeated dose interruptions and reductions, and ultimately prompted trial sponsors to explore an alternative intermittent dosing schedule^[45]. An increase in serum FGF23, phosphate and vitamin D levels is being studied as potential on-target biomarkers for effective FGFR inhibition^[46]. Other mechanism-based toxicities observed in preclinical models and clinical studies include cutaneous toxicities such as nail toxicities, xerostomia, stomatitis, as well as dose-dependent keratopathy and retinal pigment epithelial detachment. Although multitargeted VEGFR/FGFR inhibitors may cause hypertension and proteinuria, these problems seem to occur with a lesser frequency with selective FGFR inhibitors.

COMPLETED CLINICAL STUDIES OF FGF/FGFR PATHWAY INHIBITORS IN HCC

An overview of the completed clinical studies of FGF/FGFR pathway inhibitors in HCC is given below [Table 1].

Brivanib

Brivanib is a selective inhibitor of VEGFR2 and FGFR1. In preclinical studies, it attenuated hepatic fibrosis *in vivo*^[47] and hence was postulated to be useful in slowing the progression of cirrhosis to HCC^[48]. In a single-

Table 1. Summary of completed clinical trials of FGFR multikinase inhibitors in hepatocellular carcinoma (adapted and updated from^[76])

Trial		Endpoints
Brivanib	PII: 1L systemic therapy in advanced HCC ^[49] <i>n</i> = 55 NCT00355238	6m PFS 18.2% mPFS 2.7m mOS 10 m
	PII: 2L systemic therapy in advanced HCC <i>n</i> = 41 NCT00355238	mTTP 2 m
	PIII: 1L systemic therapy in advanced HCC (non-inferiority trial) ^[50] <i>n</i> = 1155 NCT00858871	mOS 9.5 m (brivanib) <i>vs.</i> 9.9 m (sorafenib)
	PIII: 2L systemic therapy in advanced HCC <i>n</i> = 295 NCT00825955	mOS 9.4 m (brivanib) <i>vs.</i> 8.2 m (placebo) (NS) mTTP 4.2 m (brivanib) <i>vs.</i> 2.8 m (placebo) (SS) ORR 10% (brivanib) <i>vs.</i> 2% (placebo) (SS)
	PIII: in combination with TACE as adjuvant ^[52] NCT00908752	mOS 26.4 m (TACE/brivanib) <i>vs.</i> 26.1 m (TACE/placebo)
	PIII: 1L systemic therapy in advanced HCC in Asia-Pacific population <i>n</i> = 165 NCT01232296	mOS 8.0 m (dovitinib) <i>vs.</i> 8.4 m (sorafenib) mTTP 4.1 m (dovitinib) <i>vs.</i> 4.1 m (sorafenib)
Orantinib (TSU-68)	PI/II: any line systemic therapy advanced HCC ^[56] <i>n</i> = 12 (PI) <i>n</i> = 35 (PII) NCT00784290	ORR: 2.9% CR, 5.7% PR, 42.8% SD mTTP 2.1 m, mOS 13.1 m
	PIII: in combination with TACE as adjuvant ^[58] <i>n</i> = 889 NCT01465464	mOS 31.1 m (TACE/orantinib) <i>vs.</i> 32.3 m (TACE/placebo)
Nintedanib (BIBF 1120)	PI/RPII: 1L systemic therapy in advanced HCC in Western population ^[60] <i>n</i> = 93 (PII) NCT01004003	mTTP 5.5 m (nintedanib) <i>vs.</i> 4.6 m (sorafenib) mOS 11.9 m (nintedanib) <i>vs.</i> 11.4 m (sorafenib) mPFS 5.3m (nintedanib) <i>vs.</i> 3.9m (sorafenib) G3 or higher AE 68% (nintedanib) <i>vs.</i> 90% (sorafenib)
	PI/RPII: 1L systemic therapy in advanced HCC in Asian patients <i>n</i> = 95 (RPII) ^[61] NCT00987935	mTTP 2.8 m (nintedanib) <i>vs.</i> 3.0 m (sorafenib) mOS 10.2 m (nintedanib) <i>vs.</i> 10.7 m (sorafenib) G3 or higher AE 56% (nintedanib) <i>vs.</i> 84% (sorafenib)
Lenvatinib (E7080)	PII: 1L systemic therapy in advanced HCC in Asian patients ^[63] <i>n</i> = 46 NCT00946153	mTTP 7.4 m mOS 18.7 m ORR 37% DCR 78%
	RPIII: 1L systemic therapy in advanced HCC (non-inferiority trial) ^[64] <i>n</i> = 954 NCT01761266	mOS 13.6 m (lenvatinib) <i>vs.</i> 12.3 m (sorafenib)

HCC: hepatocellular carcinoma.

arm phase II study in advanced HCC, brivanib was shown to have anti-tumour activity in both the frontline and second-line setting, reporting a 6-month progression free survival rate of 18% when used as first line treatment^[49]. The registration phase III trial (BRISK-FL) however was a negative trial, with brivanib failing to demonstrate non-inferiority to sorafenib in the first-line setting, though it had similar anti-tumour activity albeit a less well-tolerated safety profile with higher rates of drug discontinuation^[50]. A second-line phase III study of brivanib against placebo after sorafenib failure or intolerance (BRISK-PS) also failed to show an overall survival advantage though it had a better improved time to progression and overall response rate^[51]. Following the results of these two trials, the phase III trial of brivanib as adjuvant therapy to transarterial chemoembolization (TACE) was prematurely terminated though analysis similarly suggested no improvement in survival with brivanib use^[52].

Dovitinib

Dovitinib is a non-selective FGFR inhibitor which also has effects on VEGFR, PDGFR, FGFR, c-KIT and other targets. In HCC xenograft models, dovitinib inhibited tumour growth and angiogenesis, and reduced the development of metastases and prolonged mouse survival^[53]. In other preclinical work, it also induced apoptosis in sorafenib-resistant cell lines^[54]. When translated to the clinical setting however, the randomized phase II study comparing dovitinib versus sorafenib as first-line treatment in advanced HCC in Asian-Pacific

ic patients failed to show improved overall survival and anti-tumour activity with dovitinib. Of note though, subgroup analysis showed that the subset of patients with higher baseline plasma soluble VEGFR1 (sVEGFR1) levels had longer median overall survival^[55], and although inconclusive, it suggests that the enrichment of a patient population through biomarker selection may be a feasible approach for future studies. No phase III trials were or are being conducted using dovitinib for the indication of HCC.

Orantinib (TSU-68)

Orantinib, a multi-kinase inhibitor of FGFR, VEGFR and PDGFR, showed promising efficacy in pretreated patients with advanced HCC, with 51% of patients achieving disease control, and a good safety profile in phase I/II HCC studies^[56]. Following a similarly designed phase II study suggesting prolonged progression free survival^[57], a randomized phase III trial was conducted in Asia in patients with unresectable HCC studying either orantinib or placebo after TACE. This study was however terminated early for futility after interim analysis showed no improvement in overall survival with the use of orantinib^[58].

Nintedanib (BIBF 1120)

Nintedanib, a multikinase VEGFR/PDGFR/FGFR inhibitor, showed inhibition of HCC cell line growth *in vitro* and decreased tumour growth and angiogenesis in a xenograft mouse model of HCC^[59]. Two phase I/ randomized phase II trials comparing nintedanib and sorafenib in patients with unresectable HCC were performed in the Western population^[60] and the Asian population^[61] with similar results. Both trials reported similar overall survival and time to progression results with both drugs, with fewer serious drug-related adverse events but higher drug discontinuation rates. We await further studies of this compound in patients with advanced HCC.

Lenvantinib (E7080)

Lenvantinib is a multi-kinase inhibitor with inhibitory effects against VEGFR, FGFR1 - 4, KIT and RET. Although higher doses have been tested in other solid tumour types, a lower dose of 12 mg was tested in a phase I trial of lenvatinib in HCC patients^[62], and used subsequently in a Phase II trial conducted in Japan and South Korea^[63]. This led to the phase III study comparing lenvatinib and sorafenib in patients with unresectable HCC (REFLECT), showing non-inferiority of lenvatinib in terms of overall survival, and improvements in secondary endpoints of progressive free survival and objective response rate with lenvatinib^[64]. Following this study, further studies of lenvatinib in advanced HCC are being conducted or planned, such as a trial studying the combination of lenvatinib and anti-programmed death 1 (anti-PD1) inhibitors in the first line setting (NCT03418922, NCT03006926), as well as a trial studying the safety and efficacy of subsequently second-line treatment after initial lenvatinib use (NCT03433703).

ONGOING CLINICAL STUDIES OF OTHER FGF/FGFR INHIBITORS IN HCC

Although most of the completed clinical studies in HCC used multi-kinase inhibitors, several ongoing clinical studies are being conducted with promising selective FGFR inhibitors.

Erdafinitib (JNJ-4276493)

Erdafinitib is an oral selective pan-FGFR inhibitor which has shown a manageable safety profile in a phase I study in advanced or refractory solid tumours. Common drug-related adverse events encountered in the phase I study included hyperphosphataemia, nausea, stomatitis and dysgeusia, with one dose-limiting toxicity of bilateral retinal pigment epithelium detachment necessitating treatment discontinuation^[65]. An ongoing phase I/IIa study is currently recruiting targeting Asian patients with advanced HCC with FGF19 amplification (NCT02421185). Phase II and III trials are also being conducted with the drug in other tumour types, and notably, the drug received FDA breakthrough therapy designation in the treatment of FGFR-alteration positive urothelial cancer recently, following promising results in a phase II clinical trial^[66].

BLU-554

BLU-554, a selective and potent inhibitor of FGFR4, was derived from an earlier compound BLU9931 which suppressed proliferation in HCC tumour xenograft models with an activated FGFR4 signaling pathway^[67]. A

phase I first-in-human study of BLU-554 in patients with HCC (NCT02508467) is ongoing, and preliminary results reported suggest promising clinical activity in FGF19 immunohistochemistry positive (IHC+) patients who have failed prior systemic therapy^[68].

OTHER PROMISING FGF/FGFR INHIBITORS IN CLINICAL STUDIES

BGJ398

BGJ398 is a selective and potent pan-FGFR inhibitor which has shown to have preliminary clinical activity in a variety of solid tumours including FGFR3-mutant bladder and urothelial cancers, FGFR1-dependent squamous lung and head and neck cancers^[45] as well as FGFR-altered cholangiocarcinoma^[69]. Ongoing clinical trials are being conducted and/or planned in the above tumour types.

AZD4547

AZD4547 is a selective FGFR1 - 3 inhibitor with activity in FGFR2-amplified gastric cancer models^[70] as well as FGFR1-amplified NSCLC models^[71]. The randomised phase II trial in FGFR2-amplified gastric cancer did not show an improved progression free survival for AZD4547 compared to paclitaxel though exploratory biomarker analyses suggests that marked intratumoural heterogeneity of FGF2 amplification could have contributed to the negative results^[72]. The phase II/III study of AZD4547 as second-line therapy in treating FGFR-positive patients with stage IV squamous cell lung cancer is ongoing (NCT02965378).

Anti-FGFR antibodies

GP369, a monoclonal antibody against the extracellular domain of the FGFR2-IIIB receptor has shown potent anti-tumour activity in breast and gastric cancer cell lines with FGFR2 amplification^[73]. MFGR1877S (R3Mab) (NCT01363024) and B-701, both monoclonal antibodies targeting FGFR3, show promise in urothelial cancers, with the latter compound being tested in combination with pembrolizumab in the second line-setting (NCT03123055).

On the other hand, the auristatin-based antibody drug conjugate BAY 1187982 also shows significant tumour growth inhibition in models of FGF2 amplified human gastric and breast cancers^[74], which led to a phase I dose-escalation trial in FGFR2-expressing solid tumours (NCT02368951) though the trial had to be terminated early due to concerns over toxicity.

FGF-ligand traps

FP-1039 comprises of a soluble fusion protein consisting of extracellular FGFR1-IIIC fused to the Fc domain of IgG1 hence acting as a ligand trap of FGF1, FGF2 and FGF4. A phase II trial is currently recruiting to study FP-1039 alone and in combination with chemotherapy (docetaxel or paclitaxel and carboplatin) in solid tumours (NCT01868022).

CONCLUSION

Although the majority of clinical studies with FGF/FGFR pathway inhibitors have been negative in hepatocellular carcinoma aside from REFLECT, the results suggest that these compounds do have anti-tumoural activity and better biomarker-based enrichment of a target population is likely the key in planning more successful future trials^[75]. Several ongoing clinical trials of FGF/FGFR pathway inhibitors in a biomarker-enriched population are ongoing and we await the results of these promising studies.

DECLARATIONS

Authors' contributions

The two authors are responsible for all the work of this article.

Availability of data and materials

The data were strictly obtained from medical records according to the privacy policy and ethics code of our institute.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Thermal ablation of large unresectable hepatocellular carcinoma in cirrhotic patients

Giovan Giuseppe Di Costanzo, Raffaella Tortora, Anna Opramolla, Marco Guarracino

Transplantation Department, Liver Unit, A Cardarelli Hospital, Naples 80131, Italy.

Correspondence to: Dr. Giovan Giuseppe Di Costanzo, Transplantation Department, Liver Unit, A Cardarelli Hospital, Via A. Cardarelli 9, Naples 80131, Italy. E-mail: ggdicostanzo@libero.it

How to cite this article: Di Costanzo GG, Tortora R, Opramolla A, Guarracino M. Thermal ablation of large unresectable hepatocellular carcinoma in cirrhotic patients. *Hepatoma Res* 2018;4:53. <http://dx.doi.org/10.20517/2394-5079.2018.56>

Received: 11 May 2018 **First Decision:** 19 Jun 2018 **Revised:** Jul 10 2018 **Accepted:** Jul 10 2018 **Published:** 17 Sep 2018

Science Editors: Guang-Wen Cao **Copy Editor:** Huan-Liang Wu **Production Editor:** Zhong-Yu Guo

Abstract

Hepatocellular carcinoma (HCC) is one of the most common and lethal malignancies worldwide. Surgery is the mainstay of treatment, but less than 20%-30% of patients are good candidates. Actually, thermal ablation is considered the best treatment with curative intent for cirrhotic patients with unresectable HCC ≤ 3 cm. Unfortunately, radio frequency efficacy in obtaining the complete ablation of HCC nodules diminishes with increasing tumor size and local tumor progression is more frequent in larger nodules. To overcome these problems, higher-powered generators, different devices and techniques have been attempted. Furthermore, microwave ablation has been introduced with the promise of a large ablative capacity. The aim of this review is to describe the role of thermal ablation for the treatment of large unresectable HCC.

Keywords: Hepatocellular carcinoma, hypertermic ablation, radiofrequency, microwave ablation

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and lethal malignancies worldwide. Surgery is the mainstay of treatment, but less than 20%-30% of patients are good candidates mainly due to cancer multifocality, position of nodules, liver insufficiency, and severe portal hypertension^[1]. When feasible, resection ensures better local control of cancer and longer disease-free survival, but it carries a higher rate of complication as compared to local ablation^[2,3]. In early 1990's, thermal ablation with radiofrequency (RFA) has been introduced for the treatment of HCC. This technique has become increasingly popular and a large amount of studies have been published confirming its efficacy. Actually, thermal ablation is considered the best treatment with curative intent for cirrhotic patients with unresectable HCC ≤ 3 cm^[4-8].



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In nodules up to 2 cm in size, RFA allows the complete ablation in more than 90% of cases and may obtain results comparable to surgery^[9-11]. Randomized studies have shown higher efficacy of RFA as compared to chemical ablation with ethanol achieving the complete necrosis of HCC nodules ≤ 3 cm with fewer sessions and reducing the rate of local cancer progression^[12-14]. Unfortunately, RFA efficacy in obtaining the complete ablation of HCC nodules diminishes with increasing tumor size and local tumor progression is more frequent in larger nodules^[15,16]. The lower efficacy of ablation in nodules larger than 3 cm is also due to a more aggressive biological behaviour of large cancers, as high levels of biomarkers, poor histological grade or capsule invasion^[17]. Viable tumor cells after partial ablation may develop “resistance” to heat and may exhibit a more aggressive growth^[18]. Furthermore, the position of nodules and the amount of blood flow inside and at periphery of nodules may affect the ablative effect of RFA (heat-sink effect)^[19,20]. To overcome these problems, higher-powered generators, different devices and techniques have been attempted. Furthermore, microwave ablation (MWA) has been introduced with the promise of a large ablative capacity. The treatment of large HCC lesions represents a great challenge for clinicians because the late diagnosis of such cancer is not rare despite the use of surveillance. A careful multidisciplinary evaluation of liver function, cancer characteristics, and patient status is needed to establish the best treatment in the single case.

The aim of this review is to describe the role of thermal ablation for the treatment of large unresectable HCC.

RADIO FREQUENCY ABLATION

In a seminal study by Livraghi and Coll, 114 patients with 126 nodules larger than 3.0 cm were treated with single or triple cluster of cool-tip monopolar electrodes. Complete ablation was achieved in 61% of nodules in the size range 3.1-5.0 cm, and only in 24% of nodules 5.1-9.5 cm^[21]. To improve these results, a protocol derived from a mathematic model was applied to calculate preoperatively the site and the number of needle insertions^[22]. The model was based on the analysis of how many overlapping ablation spheres were needed to cover the HCC nodule. To ablate nodules sized 3.6-7.0 cm, 1-13 electrode placements were performed. The success rate in 121 nodules was 87%. A limitation of the application of such protocol was the difficulty in determining the accurate placement of needles in larger lesions. Using an open approach and single or cluster cool-tip needles a complete ablation rate of 91% may be achieved in nodules of 3.5-8.0 cm^[23]. “Surgical RFA” as compared to percutaneous RFA showed similar efficacy in small nodules, but was associated to better survival rates in patients with larger HCC^[24]. However, with this approach the rate of complications and post-RFA liver impairment was higher as compared to patients treated percutaneously. The highest rate of complete ablation using cool-tip needles has been reported in a large Asian cohort. The authors achieved a complete necrosis in 98.9% of 360 treatments for HCC 3.1-5.0 cm, and in 97.7% of 44 treatments for tumors > 5.0 cm^[25]. These results have never been reproduced in a Western study. In order to increase the ablation area bipolar and multipolar electrodes have been attempted. The use of bipolar devices may allow a better distribution of temperature inside the tissue^[26]. In a small prospective study including 26 patients with 27 tumors 5.0-8.5 cm, three separate bipolar internally cooled electrodes achieved the complete ablation in 22 among 27 nodules (81%), including three tumors that showed segmental portal vein invasion^[27]. However, multipolar electrodes resulted more effective than monopolar devices in obtaining the complete necrosis of nodules up to 4.5 cm, but in larger tumors the efficacy was comparable^[28]. Another way to increase the ablation area is the use of expandable electrodes and interstitial saline infusion that may create lesions significantly larger than not cooled needles^[29]. However, in small HCC internally cooled electrodes compared to expandable electrodes had similar effectiveness^[30]. A strategy to increase the efficacy of RFA in larger nodules is the insertion of multiple needles inside the tumor that may be alternatively activated using a switching algorithm^[31,32]. However, with the devices actually available, RFA ablative capacity in nodules > 5 cm is scarce. To overcome the limited efficacy of RFA in larger nodules, combination treatments of RFA plus percutaneous ethanol injection or plus transarterial chemoembolization (TACE) have been attempted. In Eastern studies, in combining these treatments, a higher rate of cancer ablation and a better overall and recurrence-free survival than RFA alone have been reported^[33-41].

Another possibility may be the combined treatment with sorafenib and RFA or triple combination also with TACE, with the aim of increasing the necrosis and reducing the rate of recurrence^[42-44]. Regarding complications of RFA ablation, in a large survey 6 deaths (0.3%) were observed. Five of these patients had large HCC complicating cirrhosis, in 3 patients the cancer nodules were located in risky areas and two had Child-Pugh B cirrhosis^[45]. Therefore, caution should be observed in such cases. Major and minor complications occurred in 2% and 5%, respectively. Similar rates have been observed in more recent studies^[46,47]. A pre-RFA value of bilirubin > 2.5 mg/dL may predict liver decompensation after treatment^[48].

MICROWAVE ABLATION

Due to the advancement of microwave technology and the development of cooled electrodes, percutaneous microwave ablation (MWA) is actually considered a safe and effective alternative to RFA for thermal ablation of HCC^[49,50]. As compared to RFA, MWA has theoretical advantages including the shorter procedural time, very rapid increase in tissue temperature, and it is less affected by tissue impedance and the heat-sink effect^[51]. Both in ex vivo and in vivo porcine liver model, MWA produced larger coagulation zones than bipolar RFA^[52].

Two metaanalyses comparing the two techniques have been published^[53,54]. Chinnaratha *et al.*^[54] analyzing three studies including 450 patients with HCC nodules > 5.0 cm or more than 3 nodules found a benefit for MWA as compared to RFA with a pooled OR of 1.88^[55-57]. Furthermore, MWA treatment was associated with a lower rate of local tumor progression in large HCC as compared to RFA. The evaluation of studies including larger HCC and the metaanalysis of Facciorusso *et al* confirmed that MWA was significantly more effective than RFA in inducing the complete necrosis of tumours^[58,59]. Also other authors confirmed MWA is safe and effective in the treatment of large HCC^[60,61] and subcapsular lesion^[62].

A recent randomised controlled trial did not show superiority of MWA over RFA in terms of efficacy, major complications and local tumour progression at 2 years of follow-up in patients with hepatocellular carcinoma lesions of 4 cm or smaller^[63], confirming a previous study published in 2002^[64]. Chong *et al.*^[65] suggested to apply ALBI score for the selection of patients in order to identify the cases with worse liver function in whom to prefer MWA to surgery.

More than overlapping insertions, the placement of multiple antennas may obtain larger ablation areas, mainly when simultaneous activation is used^[66]. This is an advantage as compared to RFA that do not allow the simultaneous activation of multiple electrodes. Another approach is the insertion of electrode under laparoscopic guidance. This technique resulted effective in small nodules, but it might be useful for the treatment of large nodules with an exophytic growth^[67]. A study in 14 centers on 736 patients treated with MWA using the AMICA system found 22 (2.9%) major complications, 54 (7.3%) minor complication, and no deaths^[47].

LASER ABLATION

Laser ablation (LA) is the less popular technique for performing thermal ablation and there is only one case-control study designed to evaluate the efficacy of this treatment in large HCC. This study compared LA with the multifiber technique and TACE for the treatment of solitary large HCC with a diameter of 4.0-7.5 cm^[68]. LA approach resulted more effective than TACE in inducing complete tumour necrosis. Overall, 26 (63.4%) patients from the LA group and 8 (19.5%) from the TACE group showed a complete response to treatment ($P < 0.001$). In univariate analyses, baseline predictors of complete response were Child-Pugh class A and treatment modality with LA. Furthermore, the rate of local cancer progression was observed in 19.5% of LA successfully treated patients and in 75% of TACE treated ($P < 0.001$). In nodules with a median diameter of 5.2 cm (3.1-9.6 cm), combined treatment with LA performed before TACE obtained the complete ablation in 90% of 45 tumours in 30 patients^[69]. In our Unit, a study evaluating the use of sorafenib as neoadjuvant

therapy in patients with large HCC who receive LA is ongoing. A multicenter Italian study evaluated the rate and type of complications after LA with the multifiber technique. Among 520 patients and 1004 sessions, 4 deaths (0.8%), 15 major complications (1.5%), and 62 minor complications (6.2%) were observed. All deceased patients had intermediate or large tumours and 2 of these were in Child-Pugh C class^[70].

CONCLUSION

Thermal ablation is a very popular technique for the treatment of unresectable HCC in patients with cirrhosis. In small HCC sized < 3.0 cm, RFA may achieve good results that in some cases are comparable to that of surgical resection. Compared to surgery, local ablation features, mini-invasive approaches, with less impact on liver function less morbidity and hospital stay and less costs. The applicability of thermal ablation in nodules > 3 cm which constitutes the objective of this review, is still a matter of debate. During the last 25 years, technical advances have increased the efficacy of such technique, expanding the range of its application. However, the level of evidence is poor due to the scarcity of appropriated designed randomized studies. A main problem in inducing the complete necrosis of large HCC nodules is the lack of very experienced operators. In fact it is evident that a single needle insertion is insufficient in inducing the complete necrosis of large tumors. The increase in the potency emitted by a single source may be insufficient to ablate the periphery of large nodules and may be risky causing unwanted complications. In this setting, overlapping electrode placements and multiple needle insertions are the better way to increase the treatment effectiveness. The correct placement of electrodes inside the nodules is crucial for obtaining the therapeutic success. The simultaneous activation of inserted needles seems more effective than alternate activation in inducing larger and confluent coagulation areas. Therefore, theoretically MWA and LA might be favored as compared to RFA in the treatment of large tumors. A strategy frequently used in clinical practice is the use of combined treatments, mainly percutaneous ablation and TACE. The sequential use of such treatments seems to achieve a better local tumor control, but randomized studies are awaited to define its applicability.

DECLARATIONS

Authors' contributions

Concept and design of study, drafting the article, final approval of the version to be published: Di Costanzo GG

Acquisition of data or analysis and interpretation of data; drafting the article, final approval of the version to be published: Tortora R

Acquisition of data or analysis and interpretation of data; revising it critically for important intellectual content; final approval of the version to be published: Opramolla A

Concept and design of study; revising it critically for important intellectual content; final approval of the version to be published: Guarracino M

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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HCV-discovery to elimination, “myth or reality”

Wasim Jafri, Basit Siddiqui, Safia Awan

Aga Khan University Department of Medicine, Section of Gastroenterology, Karachi 75500, Pakistan.

Correspondence to: Dr. Wasim Jafri. Aga Khan University, Stadium Road, Karachi 75500, Pakistan. Email: wasim.jafri@aku.edu

How to cite this article: Jafri W, Siddiqui B, Awan S. HCV-discovery to elimination, “myth or reality”. *Hepatoma Res* 2018;4:54. <http://dx.doi.org/10.20517/2394-5079.2018.36>

Received: 10 Apr 2018 **First Decision:** 28 Jun 2018 **Revised:** 30 Jul 2018 **Accepted:** 1 Aug 2018 **Published:** 20 Sep 2018

Science Editors: Guang-Wen Cao **Copy Editor:** Yuan-Li Wang **Production Editor:** Zhong-Yu Guo

Abstract

Hepatitis C virus (HCV) was discovered in 1989, before that it was commonly known as transfusion associated non A non B hepatitis. It rapidly assumed the role of leading cause of cirrhosis and liver cancer and a leading indication for liver transplant globally. For over two decades the treatment was suboptimal with the use of pegylated interferon and ribavirin combination across all genotypes. The vaccine development also failed for over two decades. However a major breakthrough happened in December 2013 when the Food and Drug Administration (FDA) approved the first pan genotypic oral directly acting drug Sofosbuvir. Since then many new directly acting drugs have been approved through fast track by the FDA. Today we have directly acting antiviral agents for all HCV patients providing cure rates of over 90%. Looking into this success the World Health Organization has set targets for 2030 for HCV elimination. There are several countries which have formed strategies to achieve this goal and others are still thinking to develop their own strategies. The availability of generics have reduced the prices substantially, however the problem is so gigantic that unless proper operational strategies for elimination are developed by the developing world especially by China and Pakistan, the two countries having the largest existing pool of HCV patients, the goals of elimination may not come true.

Keywords: Hepatitis C virus, elimination, directly acting antiviral agents, Pakistan

INTRODUCTION

Year 2015 has seen the major developments on the fronts of global reawakening for curtailing down the huge burden of viral hepatitis by the World Health Organization (WHO) adopting the 2030 Agenda for Sustainable Development goals, which called on the global integrated efforts to combat viral hepatitis. It was not long when the burden of health care related to the human immunodeficiency virus (HIV) was



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enormous, but sustainable achievement of goals and target oriented approach has given this viral pandemic a breakpoint and we are seeing a decline in the global prevalence of HIV. WHO on similar grounds and experiences has acquired a target oriented approach on the issue of ever increasing burden of viral hepatitis. At the beginning it seems like a myth, but it can be made a reality with advent of new frontiers in the management of chronic viral hepatitis.

History

Viral hepatitis is one of the biggest health problems and discovery of causative viruses is one of the most significant breakthrough scientific achievements in this era. Previously identified as “Australian antigen” and later on described, as hepatitis B virus surface antigen (HBsAg), is often regarded as the initial discoveries in the history of viral hepatitis^[1]. A literature search of military records of first and second world wars revealed that “campaign jaundice” caused significant health related problem of the troops and caused a significant impact on war strategies^[2].

Discovery of non A non B hepatitis (NANBH) as hepatitis C virus (HCV) in 1988 was a milestone, a collaborative work of Centre for Disease Control (CDC) and California biotechnology company^[3]. Although the actual virus was identified later, this effort brought a novel molecular method for viral genome identification. Houghton and colleagues, the pioneers of this method, identified the unique cDNA using sera of chimpanzees and humans with NANBH that crossed with a single stranded RNA which was only extracted from NANBH patient^[4]. From the next year antibodies to HCV were measured, which laid down the foundation of blood screening for HCV in 1990^[5,6]. Major hurdle after identification of these viruses was to bring about the therapeutic measures into existence, to control their spread and eliminate them. Further developments in the form of therapeutic hepatitis vaccines and oral agents became one of the major advancements on the frontiers of treatment.

HCV characteristics

HCV has viral and genetic characteristics in common with the Flaviviridae family^[5]. The HCV surrounds its RNA with a protective coat known as the capsid, which is built from proteins. This enveloped, spherical virus of approximately 50 nm in diameter^[5], has an estimated half-life of 2.7 h^[6]. Daily, 10 trillion (i.e., 10¹²) virions are produced and cleared in an untreated individual with HCV infection^[6]. Structurally, the HCV genome is an unsegmented, linear single strand of RNA of positive sense^[5]. The genome is approximately 9.6 kilobase (kb) in length, comprising a polyprotein of about 3000 amino acid residues^[7].

HCV is a considerably heterogeneous family of viruses, with at least 6 known genotypes (genotypes 1 to 6) and numerous (> 80) subtypes^[8]. Additionally, in an individual, HCV can form heterogeneous populations of viral genomes that are closely related but different viruses^[8]. The quasispecies nature of HCV and the envelope structure of the virus may be promoting factors in its rapid mutation by allowing it to escape immune surveillance of the host^[9].

Global burden

Viral hepatitis is a global health burden and a leading cause of death worldwide, according to WHO 1.34 million death was estimated to occur in 2015 as a result of HCV infection. These numbers are comparable to or exceed the number of deaths caused by tuberculosis and HIV. Mortality due to viral hepatitis has seen new peaks in the recent years and most deaths in the context of viral hepatitis in 2015 were due to chronic liver disease (720,000 deaths due sequelae of decompensated cirrhosis) and primary liver cancer (470,000 deaths due to hepatoma). During the same year, approximately 257 million people were chronically infected with hepatitis B virus, and chronic hepatitis C (CHC) infection was responsible for 71 million infections^[10]. The ongoing HCV epidemic is affecting all regions with major differences between and within countries. The WHO 2015 report has described Eastern Mediterranean Region and the European Region as showing the

rising reported prevalence of HCV. After the population shifts and migrant crisis due to current geopolitical scenario in the northern African countries and Arab world, reported prevalence in the bordering areas is again increasing^[10]. On the other hand China and Pakistan are placed in the 2017 WHO report as the areas of world with highest prevalence of CHC, which claims 350,000 lives every year in the world. The global burden of chronic hepatitis B is around 350 millions, killing around 600,000 people yearly^[11].

With the advent of highly successful therapy (> 90% success rates) for CHC as directly acting antiviral agents (DAAs), the treatment duration has shrunk to 8-12 weeks for most of the time, despite this major advancement, as of 2015 out of 71 million people infected with CHC, only 7% had access to this therapy^[12].

For United States of America, CDC described that 3 to 4 million people are infected currently with HCV. While in Egypt, the situation was very grave till early 2015 when Egypt was ranked as the country with highest prevalence, with a prevalence rate well above 10%. The prevalence of infection is greater than 10% in certain parts of Asia with high rates found in certain geographic regions of Taiwan, Japan and Italy. However, there are a number of countries/regions where data are not available^[13,14].

Hepatitis C epidemic in Pakistan

Situation in Pakistan is grave, as it's been placed among highly prevalent countries. Recently Pakistani researchers have increased their focus on studying endemic patterns of HCV infection and genotype distribution leading to publication of eighty six relevant studies^[15]. This data on increasing prevalence have been comprehensively reviewed previously^[15-17]. Pakistan has the second largest burden of hepatitis C^[11], prevalence data published locally in last seven years has shown alarming figures with an almost 40% increase in HCV antibody detection rates in general population as per the recent review published in 2016 by Umer *et al.*^[15]. This all translated into high nosocomial transmission rates, highest burden in economically disadvantaged areas and in marginalized communities. A shift in relative distribution of genotypes in Sindh and Khyber Pakhtunkhwa provinces is seen, which the predominant areas are dealing with migrant crises and internally displaced peoples (IDPs). A nationwide survey on prevalence of hepatitis B and C was done in 2007-2008 which estimated that approximately 8 million people are exposed to HCV^[18], and 2010 saw a landmark step as the establishment of hepatitis sentinel sites nationally and surveillance system located in provincial and federal capitals^[19]. Despite these landmark developments, still they are far behind than what was expected from these centres in terms of their clear task of bringing about an integrated service model for identifying what is beneath the tip of the iceberg of HCV epidemic in Pakistan, as more recent estimates suggests that Pakistan is home to one-tenth of the global HCV burden^[20].

Between 2016 and 2030, it is estimated that Pakistani population will be around 250 million, and prevalence of HCV will rise from 3.9% to 5.1%, with a disturbing figure of 1.4 million deaths among those over 20 years of age. Burden of this endemic infection will continue to rise with 12.6 million prevalence of CHC and a projected 1.1 million new infections with each passing year^[21].

HCV-elimination strategies

Global hepatitis strategy by WHO defined a goal to eliminate viral hepatitis by 2030 has been adopted, which can be achieved by reducing incidence by 90% and mortality by 65%, calling for integrated and collaborative work and dedication by the policy makers and health care providers.

The World Health Assembly endorsed a Global Health Sector Strategy (GHSS) on viral hepatitis 2016-2021, in May of 2016. This will translate into the elimination of viral hepatitis by 2030.

Five key pillars of global health sector strategy include strategic information, interventions equity, financing and innovation. These key elements are devised for facilitation of progress monitoring globally, regionally

and nationally. This will enhance the methodological calculation of impact of different interventions and tools used to reduce rates of new infections and saving precious human life between 2016 and 2030. These strategic parameters are aligned with plans and strategies of other relevant programs including those for sexually transmitted infections, HIV, blood safety, safe injections, vaccines, tuberculosis and non-communicable diseases. This integrated model will give an end to viral hepatitis and net result on elimination of the disease.

Hepatitis C treatment

Risk of disease progression in patients living with CHC can be prevented by effective screening and diagnostic modalities and by proper implementation of care linkage, and provision of highly effective anti viral therapy. HCV patients with risk behaviors should be targeted and effectively engaged in the linkage to care and should be offered sound counseling, this will help reduce further spread of infection. This is the key element that has been stressed by different hepatology society guidelines. Engaging communities in screening activities and linking counseling with care and treatment strategies are needed for combating HCV epidemic. Treatment affordability as a major barrier for successful strategy is another concern apart from other barriers.

With the advent of DAAs, cure rates exceeding 90% even with newer 8 weeks pangenotypic regimens have been reported in large trials. Despite this astonishing success in the therapeutic armamentarium of CHC the low and middle income countries are not able to handle the problem with success as the national strategic plan for the elimination of Hepatitis C by 2030 for most of the countries is not developed.

Economics

A few impressive calculations determined that treating patients annually with a number exceeding 328,000 persons by 2018 could reduce the prevalence of HCV by 94% and liver-related mortality by 75% by 2030. Calculations regarding disability-adjusted life year (DALY) with or without cirrhosis also given this therapy high cost effectiveness, while taking into consideration the indirect costs, this intervention is again cost saving^[22]. Requirements for meeting the WHO targets include removal of restrictions for treatment by treating all the patients, providing access to everyone and screening at mass levels, so that 80% of infected persons will be diagnosed by 2030 and 260,000 patients would continue to be treated per year. This methodological approach will curtail down infections by 90% and prevent nearly a quarter million mortality in next 13 years. While challenges have been encountered persistently in the developing world due to poor and reliable data management mechanisms and quality of hepatitis services provided and a limited timely intervening capacity. Apart from these, safe and necessary injection practices and disposal of waste in effective manner are also among the major barriers.

Strong government commitment to new treatments is necessary to ensure universal coverage. According to the European Liver Patients Association (2017), national plans must be developed and include forecasting and budgeting to expedite unrestricted access to treatment, in order to succeed in eliminating HCV.

Global strive towards elimination of HCV

After WHO's 2030 elimination goal was laid down in 2015, till now only 9 countries are on the track of achieving this goal. While 22 countries are working towards the direction of achieving on-track policies, the rest of the world is still far behind the laid down parameters^[11].

A glimpse of global policy making; European and Australia: Universal access to DAAs

Only a few of the countries reviewed, have granted universal coverage for DAAs. Australia, Portugal, Germany, and since 2017, France and Italy, offer access to DAAs for all patients, regardless of their level of fibrosis. Scotland and England do not have fibrosis requirements, but have limits on numbers of patients

who can be treated each year, so usually only patients with higher fibrosis levels receive treatment. Other countries such as Spain, Belgium, and Switzerland only provide treatment for patients with a certain fibrosis level by prioritizing severe cases. Some of these countries are already considering broadening access to additional fibrosis levels. For instance, in Spain access has been broadened to all fibrosis levels in some regions and commitment to broaden it at national level has been recently announced; and in Belgium access was extended to second stage fibrosis (F2) patients in January 2017, and full access is expected by 2019.

Patients who inject drugs, a European and Australian approach

The European Monitoring Center For Drugs and Drug Addiction (EMCDDA) has been working since the era of HIV. It has published different policies on its website and those were heavily cited and were taken into consideration by major health providers. It has recently been presenting its data describing insights for policy making to halt the HCV prevalence in the EU and Turkey but also the strategies for meeting the 2030 elimination goal.

According to EMCDDA report 2017^[23], 14 EU countries plus Norway have HCV policies in place, which are very effective. Thirteen of them were adopted recently between 2013 and 2017. Nine EU countries have clinical guidelines limiting treatment access to people who use drugs.

After effectively bringing down the prevalence in general population of EU countries, and providing treatment to all patients who are chronically infected with the exception of a few EU countries. Now the focus is on patients who inject drugs (PWIDs), which includes lifting the ban from those who are actively injecting and treating this population apart from providing them more syringes to break the transmission chain^[24].

The European Liver Patients' Association (ELPA) is also providing a platform for policy making as is reflected by the Hep-Core 2017^[25] study, which is acting as a benchmark for monitoring changes in European policy landscape.

As per the current policies, EU and Australia will achieve the WHO target much earlier than 2030.

Egyptian model, an eye opener for the middle to low income countries

In the middle and low income countries the Egyptian model is the best strategy for combating HCV. Egypt was regarded as one of the countries with the highest prevalence till 2015 according to WHO report^[11]. This was the outcome of mass treatment with unsterilized syringes for schistosomiasis during 1960s to 1980s, which has represented the largest ever iatrogenic spread of blood borne infection in the history^[26]. After this mammoth burden with an estimated 10% seroprevalence^[27], the agreement of Gilead Sciences, than Bristol-Meyers-Squibb and Ministry of Health, brings down cost of Sofosbuvir and Daclatasvir and making local brands in much cheaper cost. Then with continuous efforts of opening up of health care provision, treatment centres and web based appointment system, Egypt is regarded as being on the right path as per latest estimates from local and international audits^[28]. Egyptian model is not only cost efficient but also easily acquirable. Egypt is the only low- and middle-income country, among nine of countries which are on the track of WHO 2030 elimination program.

Situation in Pakistan

According to latest estimates^[11] Pakistan has the second largest viremic pool of HCV patients after China, with ever increasing morbidity and mortality due to this highly prevalent infection in the country. With the advent of DAAs, like the Egyptian strategy of getting Sofosbuvir in low cost, Gilead Sciences gave the similar licensure to Ministry of Health, Pakistan, by this effort and now with the availability of the generic

Sofosbuvir and Daclatasvir, generic brand is available here in Pakistan in < than \$50/month of therapy, while generic Sofosbuvir with ribavirin is reaching below \$20/28 day therapy^[24]. Due to lack of continuous health surveys in a country of 220 million population, the estimates available only show the tip of the iceberg^[28]. Health care system is still in the phase of continuous evolution and ongoing acquisition of web based system, striving to achieve smooth data collection and management which is the key element in assessing the population at risk and marginalized communities. As per Polaris observatory^[11] Pakistan is among those countries which are not on the track yet.

The training in emerging advances in the management of CHC infection for healthcare professionals in Pakistan (Teach-Pak) project

The Teach-Pak has started a large scale physician training module system for management of liver disease. Every year two to three batches of physicians based all over Pakistan are been trained via this program and this has started showing its impact. These trained physicians then educate the physician community in their respective area of practice^[29].

The concept of micro-elimination of HCV, a Pakistani perspective

There are multiple welfare and philanthropic organizations, which are working extensively on the marginalized communities, i.e., prisoners, trans genders, PWIDs, for promoting health care seeking behavior, awareness of health risk practices and exposures and treating them free of cost with latest available options in the developing world for both hepatitis B and C.

Similarly Pakistan has seen the major disruption of its health care system after the historical migration of Afghans during the era of 1980s, and again during 2000s and till now IDPs, that is the main driving force of disruption of health care provision and substantial additional burden on economics of already resource-poor setup. This community is a challenge; it is extremely difficult to engage them in the health care assessment due to persistently changing dynamics, lack of education and religious and cultural taboos.

Despite all the hurdles, Pakistan has developed strategic HCV elimination program as per WHO guidelines in October 2017, but the country is striving to come up with a proper implementation of healthcare policies providing free of cost DAAs in government hospitals. It will be evident from the upcoming national survey that how far the goal of WHO elimination program is from current strategies observed in Pakistan.

CONCLUSION

With the discovery HCV in 1989 and development of all oral hepatitis C therapy 2014, the world is looking at the rise and fall of a virus, which became a global epidemic. It played a major havoc globally especially in the low and middle income countries. With appropriate provision of health care delivery, early detection, universal treatment of all chronically infected, close follow up and special attention towards marginalized communities, the WHO 2030 elimination goal will not be regarded as a myth but a reality.

DECLARATIONS

Authors' contributions

Concept and design, critical revision and finalizing of the manuscript: Jafri W

Data acquisition: Siddiqui B

Data analysis: Awan S

Manuscript preparation: Awan S, Siddiqui B

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Mechanisms and clinical behavior of hepatocellular carcinoma in HBV and HCV infection and alcoholic and non-alcoholic fatty liver disease

Riccardo Nevola, Luca Rinaldi, Mauro Giordano, Aldo Marrone, Luigi Elio Adinolfi

Department of Medical, Surgical, Neurological, Metabolic, and Geriatric Sciences, University of Campania "L. Vanvitelli", Division of Internal Medicine, Piazza Miraglia, Naples 80138, Italy.

Correspondence to: Prof. Luigi Elio Adinolfi, Department of Medical, Surgical, Neurological, Metabolic, and Geriatric Sciences, University of Campania "L. Vanvitelli", Division of Internal Medicine, Piazza Miraglia, Naples 80138, Italy. E-mail: luigi.elio.adinolfi@unicampania.it

How to cite this article: Nevola R, Rinaldi L, Giordano M, Marrone A, Adinolfi LE. Mechanisms and clinical behavior of hepatocellular carcinoma in HBV and HCV infection and alcoholic and non-alcoholic fatty liver disease. *Hepatoma Res* 2018;4:55. <http://dx.doi.org/10.20517/2394-5079.2018.38>

Received: 10 Apr 2018 **First Decision:** 8 Jul 2018 **Revised:** 15 Jul 2018 **Accepted:** 16 Jul 2018 **Published:** 20 Sep 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Huan-Liang Wu **Production Editor:** Zhong-Yu Guo

Abstract

Hepatocellular carcinoma (HCC) is the main tumor of the liver and is the sixth most frequently diagnosed tumor in the world. It is the evolution of chronic hepatic injury secondary to different etiologies. Chronic hepatitis B virus and hepatitis C virus infection, chronic alcoholic hepatitis, as well as non-alcoholic fatty liver disease are the most common causes behind the development of HCC. The introduction of effective prophylaxis and treatment against hepatitis B, the recent use of highly effective hepatitis C treatments, as well as lifestyle changes observed in recent decades in the general population causing an increase in obesity and metabolic syndrome have led to significant epidemiological change in HCC in relation to the changed etiologic prevalence of liver injury. Increasing evidence was emerging, emphasizing how the development of HCC is a complex and multifactorial process. The knowledge of the molecular mechanisms involved is important for the understanding of the basic factors of the development of hepatocarcinogenesis and of possible therapeutic approaches. Several pathogenic mechanisms and clinical expression of HCC occur in relation to the different etiologies of the underlying liver disease. The different clinical behavior of HCC often makes diagnosis difficult at an early stage, that is necessary for an effective therapeutic approach. This review analyzes the possible different pathogenic mechanisms involved in the development of HCC and emphasizes the different epidemiological and clinical aspects of HCC observed in the most common forms of liver diseases of viral and non-viral origin.

Keywords: Hepatocellular carcinoma, hepatitis C virus, hepatitis B virus, alcohol hepatitis, non-alcoholic fatty liver disease



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INTRODUCTION

Liver cancer is the fourth leading cause of cancer-related death in the world, with about 810,000 deaths annually^[1]. It has a high incidence rate and is the fifth most commonly diagnosed cancer in males and the ninth in females^[2]. In addition, unlike other more common neoplasms that have a downward trend in incidence, the rate of incidence of liver cancer appears to be increasing^[3]. Hepatocellular carcinoma (HCC) is the most common liver cancer histotype, accounting for 80% of the liver cancers^[4].

At present the main causes of HCC are viral infections such as hepatitis B virus (HBV) and hepatitis C virus (HCV) and alcohol abuse, but the obesity and the metabolic syndrome epidemic that is occurring in Western countries is leading to a significant increase in HCC secondary to non-alcoholic fatty liver disease (NAFLD)^[1,5]. Chronic HBV infection is associated with about 33% of total deaths observed for HCC, while 30% is associated with alcohol abuse, 21% with chronic HCV infection and 16% with the remaining etiologies, including the ever-increasing metabolic etiology^[1]. In fact, widespread lifestyle changes and the pandemic of the metabolic syndrome are causing a significant increase of about 9% per year in the incidence rates of NAFLD-associated HCC. Looking to the future, particularly in industrialized countries, this last condition could become the main factor of HCC causing an important epoch-making change between metabolic and viral forms^[6].

The pathogenic mechanisms underlying the development of HCC, as well as the epidemiology, the clinics and the underlying diseases from each etiology are extremely dissimilar and explain the heterogeneous clinical impact of HCC. The different clinical behavior of HCC often makes diagnosis difficult at an early stage that is necessary for an effective therapeutic approach. Increasing evidence is emerging, emphasizing how the development of HCC is a complex and multifactorial process. The comprehension of the molecular mechanisms involved is important for the understanding of the basic factors of the development of hepatocarcinogenesis and of possible therapeutic approaches.

This review aims to define an updated clinical picture of HCC, its epidemiological changes and, above all, to highlight the differences in the pathogenic mechanisms related to each single etiology associated with HCC. [Table 1](#) summarizes the main characteristics of HCC in relation to the different etiological association.

The common denominator in the pathogenesis of HCC

Regardless of etiology, any chronic hepatitis can alter the balance of the immune system causing a low-grade chronic inflammation that leads to the creation of reactive oxygen species (ROS), as well as the induction of cell proliferation and the onset and progression of liver fibrosis. A high turnover of hepatocytes exposes the patient to a higher rate of genetic alteration, such as point mutations, chromosomal abnormalities or epigenetic alterations, whose accumulation represents the first phase of hepatocarcinogenesis. In addition, there are specific risk factors of the host such as diabetes mellitus and the male sex, and factors related to the etiological agent that increase the oncogenic potential of the inflammatory liver disease, thus causing the development of HCC, either in the presence or absence of significant hepatic fibrosis^[7].

Irrespective of etiology, cirrhosis of the liver is an already pre-malignant condition that promotes the development of genetic aberrations and cellular transformations. In fact, the chronic hepatic inflammatory state and the accelerate hepatocyte turnover observed in cirrhosis promote the accumulation of genic mutations. The subsequent uncontrolled proliferation and the high rate of genetic errors will lead to the development of HCC.

HCC and HBV chronic infection

Clinical and epidemiological factors affecting development of HBV-related HCC

Currently, chronic HBV infection is responsible for about half of all observed HCC cases^[8-11]. It has been estimated that HCC occurs 10-25 times more frequently in patients with positivity for HBV than uninfected

Table 1. Main features of HCC in relation to different etiologies

HCC Features	Related to			
	HBV	HCV	ALCOHOL	NAFLD
Annual Incidence	Non-Cirrhotic: 0.3%-0.6% Cirrhotic: 2.2%-3.7% Low Increasing	3%-7% Increasing	1.2%-5% Stable	NAFLD: 0.044% NASH: 0.529% Increasing
Gender Prevalent	Male	Male	Female	Indifferent
Onset stage of liver disease	Chronic hepatitis Cirrhosis	Cirrhosis	Cirrhosis	Chronic Hepatitis Cirrhosis
Carcinogenic factors	Viral factors	Viral factors (Genotype-related)	Direct (dose-related)	Inflammation
	Inflammation	Inflammation	Inflammation	Oxidative stress
	Oxidative stress	Oxidative stress	Oxidative stress	Lipid peroxidation
	Cirrhosis	Cirrhosis	Endotoxemia	Mitochondrial damage
	Immunologic		Low Vitamin A levels	Endotoxemia
	Genetic polymorphisms (DCL1, TGF- β 1, STAT4, TPTE2, CTL-4, MDM2, among Asians)		Cirrhosis	Cirrhosis
Co-factors	Viral co-infection (HIV, HDV, HCV)		Genotoxicity	
	Diabetes	Viral co-infection (HBV, HIV)	Diabetes	Diabetes
	Alcohol	Diabetes	Obesity	Metabolic syndrome
	Tobacco	Metabolic syndrome	Genetic polymorphisms (PNLA3, TM6SF2)	Leptin/adiponectin imbalance
		Obesity		Genetic polymorphism (PNLA3)
		Alcohol		
Related-death of total	33%	21%	30%	16%
Effect of anti-viral treatment on incidence	Reduced	Reduced		

individuals^[12]. HBV-related HCC is predominantly observed among males (203,000 new cases in 2015 versus 70,000 in females), with a male-to-female ratio of about 3 to 1 and is also responsible for one-third of HCC-related deaths^[1]. In addition to epidemiological factors, the significant disparity in incidence between the two sexes is also determined by the different hormonal pathways. Androgens stimulate virus replication and transcription in males causing a higher viral load which is associated with an increased risk of occurrence of HCC^[13]. In fact, high HBV-DNA serum levels have been reported to be associated with a nonlinear dose-response to a higher incidence and recurrence of HCC^[14]. Furthermore, it has been reported that estrogens appear to act as protectors towards the development of HCC^[15].

HBV-related HCC shows a tendency to occur at all stages of the natural history of chronic HBV hepatitis and not only in cirrhosis as in most cases seen during chronic HCV and alcoholic hepatitis. Accordingly, up to a third of patients develop HCC on a non-cirrhotic liver^[12].

An additional factor associated with an increased risk of developing HCC is the duration of the disease^[16]. The chronic inflammatory state and hepatic oxidative stress induced by chronic HBV infection accelerate cell senescence processes. The expression of aging processes is expressed at the genomic level in the form of shortening of telomeres, whose length is inversely proportional to the degree of fibrosis and reaches the lowest values in HCC^[17]. Although cellular senescence is a protective mechanism itself, since it limits proliferation and reduces the risk of carcinogenic transformation, it has been shown that telomerase activity persists at high levels in 80%-90% of HCC cases thus emphasizing the development of escape mechanisms from the protective phenomena of cellular aging^[12,18].

It has been shown that some co-factors associated with chronic HBV infection such as diabetes mellitus, alcohol consumption or tobacco use, as well as exposure to carcinogens (e.g., aflatoxin) may act in synergy with the virus in determining an early onset and a more rapid progression of HCC^[19,20]. In particular, the

risk of developing HCC has been reported to be 6-, 5- and 4-fold higher in the case of concomitant use of alcohol, tobacco and in the presence of obesity, respectively^[21].

The presence of HBV coinfection with HIV or HDV or HCV causes a more rapid progression of liver disease and a significant higher occurrence of HCC even in the early stages of the disease^[22,23].

HBV genotype appears to significantly influence the appearance of HCC. In particular, it has been reported that HBV genotype C, which has a high prevalence in Southeast Asia, is associated with a higher risk of HCC compared to other genotypes^[24,25]. It has been shown that HBV genotype C most frequently causes double helix breaks in the host genome, induces a greater stress of the endoplasmic reticulum through the accumulation of ROS and causes a greater number of chromosomal rearrangements that can promote carcinogenesis as better described below^[26].

As mentioned above, high viral loads, as well as a seropositive status of HBe antigen (HBeAg) are associated with a higher risk of developing HCC^[14]. It has been reported that HBeAg seropositive patients has a relative risk of HCC of 60.2 compared to 9.6 of those seronegative^[27]. It has also been shown that high levels of HBV-DNA are closely linked to a high probability of developing HCC^[28].

A genetic predisposition to the development of HCC during HBV infection has been reported among South-Asians. Single nucleotide polymorphisms (SNPs) at the level of several genes, e.g. DCL1, TGF- β 1, STAT4, TPTE2, CTL-4, MDM2 have been associated with the development of HCC^[12,29-33]. It is unknown, however, if these data may be extended to other ethnicities.

Pathogenic mechanisms of HBV-related HCC

HBV can cause the onset of HCC through direct and indirect mechanisms. The direct carcinogenic effect of HBV derives from its ability to integrate its own genome into that of the host, altering chromosomal stability and triggering various oncogenic mechanisms. In the early stages of the natural history of infection, HBV-DNA is converted into a covalently closed circular DNA form (cccDNA)^[34] that allows the virus to persist in the infected cell nucleus and acts as a reserve for viral genome replication^[12]. Although viral integration is more likely to occur randomly, whenever it occurs at the level of specific sites in the host genome, which are either close to the genes involved in cell cycle regulation and proliferation or those involved in cell survival mechanisms, can allow the clonal expansion of cells^[35,36]. This mechanism is also responsible for the constant expression of viral oncogenic proteins such as HBx or preS/S polypeptides, which over time may lead to alterations in the control of cell transcription and proliferation^[12]. The fact that such integration is more commonly seen in cancer tissue than in adjacent liver tissue (86.4% and 30.7%, respectively) seems to be an evidence of the central role played by viral genome integration in the development of HCC^[37]. In this context, the transcription of a chimeric gene (viral/human) called HBxLINE1 has recently been identified and has been found in about a quarter of patients with HCC and associated with a worse prognosis^[38].

In addition to the integration mechanisms of the viral genome, specific mutations in the *X regions*, *pre-core*, *core-promoter* and *pre-S* may increase the risk of developing HCC^[39,40]. Among the most frequent mutations at the nuclear promoter level, the double mutation *A1762T/G1764A* is closely related to the probability of developing HCC. The presence of this mutation may represent a potentially risk-predictive biomarker for HCC as its presence may be evident many years before the development of HCC^[41]. Several other potentially oncogenic mutations have been identified in the *pre-S* region. The combination of *pre-S C1653T*, *C1653T* + *T1753V* mutations and the aforementioned *A1762T/G1764A* have a specificity greater than 80% in the prediction of HCC development^[42]. HBV-infected patients with mutations in the *pre-S* region have a 3.8-fold higher risk of developing HCC than non-mutated virus infection. These mutations are present in about 60% of cases of HCC and may alter the protein expression of the viral envelope. The accumulation of surface

proteins mutated at the endoplasmic reticulum may be able to induce the formation of ROS, with consequent oxidative stress to the host DNA and induction of the hepatocyte transformation^[12,43-45]. Mutations in the *S* region can also contribute to the development of HCC. In this regard, it has recently been shown that a non-sense mutation at position 172 or 182 of viral genome can contribute significantly to the progression of liver disease, and in particular the sW182 mutation was found to be related to the development of HCC^[46].

In addition to the structural proteins, the viral genome also codes for HBx proteins that is involved in the transcription mechanisms of cccDNA and viral replication and seems closely related to the oncogenicity of HBV^[35,47,48]. HBx appears to be able to cause chromosomal instability affecting the mitotic checkpoints, cell proliferation through stimulation of CREB genes, inhibition of apoptosis through interaction with p53, promotion of neoangiogenesis through stimulation of vascular endothelial growth factor and angiopoietin 2 (ANG2) and induction of cell migration phenomena inducing the matrix metalloproteinase 3 and 9 expression^[49,50]. Accordingly, HBx appears to be a crucial point of the oncogenic power of the virus, as well as in promoting invasiveness and the ability to metastasize of HCC^[35]. HBx, among other things, inhibits senescence mechanisms through inhibition of p53 and inactivation of the suppression factors of cancer^[51].

A great research interest is growing on the effects of viral protein expression such as wild type and HBx mutant, envelope and core proteins on different transcription and signaling pathways such as Wnt/ β -catenin, TGF- β , NFkB, Raf/MAPK, P53 and ROS involved in the pathogenesis of HCC related to HBV. The beta-catenin pathway regulates multiple cellular processes and plays an important role in hepatocarcinogenesis and in progression from chronic inflammation to HCC^[52]. Mutations in the *CTNNB1* gene (catenin beta 1) may activate the Wnt/ β -catenin pathway and lead to the accumulation of β -catenin in HCC. The Wnt/ β -catenin pathway is a potential promising target for future molecular HCC therapies^[53].

Effect of Immune-tolerance phase of HBV infection and occult HBV on development of HCC

The immune tolerance phase of HBV infection is characterized by a high level of viral replication in the absence of significant cytolytic activity. These patients have been defined to be at low risk of disease progression, so, at present, there is no indication for antiviral treatment^[54,55]. However, recent studies have questioned this principle by demonstrating high levels of chromosomal integration and clonal expansion of the viral genome and hepatocytes, emphasizing that carcinogenesis may also occur at this stage and in the absence of cytolytic activity^[56]. In addition, a prospective study showed that the estimated cumulative incidence of HCC over a 10-year follow-up period is significantly higher in the immune tolerant group than the active immune group (12.7% vs. 6.1%, respectively)^[57]. Furthermore, data from a recent study have demonstrated the benefits in terms of clinical outcomes such as the development of cirrhosis and HCC of antiviral treatment even during the immune tolerance phase^[58]. Therefore, it was hypothesized that HBV positive patients not treated in the immune tolerance phase may be at a higher long-term risk of HCC^[57]. On this basis it was suggested that the immune tolerance phase should not be more considered a “benign” condition and that the levels of HBV-DNA rather than alanine aminotransferase (ALT) values should be considered when estimating the risk of occurrence of HCC^[58,59]. Further study and consensus will be needed to define this important aspect.

A special mention must be made for the so-called occult HBV infection (OBI), a condition in which HBV-DNA is detectable in the liver and possibly in the serum at low levels in the absence of HBsAg in the serum. Several studies point out that the OBI can be a hazardous condition for the development of HCC^[60,61]. In these patients many of the above-described oncogenic mechanisms associated with HBV remain active. Recent evidence has shown that an OBI condition was present in 75% of HBsAg negative HCC cases, underlining the possible OBI role in HCC genesis^[62]. Furthermore, a condition of occult infection that increases the risk of developing HCC seems to persist longer in the neoplastic tissue itself than in adjacent tissue^[61,63]. It has also been shown that the risk of developing HCC is significantly higher in patients with

HCV-related cirrhosis and concomitant OBI carrier status than in negative OBI patients^[64]. Therefore, regardless of the etiology, in patients with chronic hepatitis the presence of OBI may represent a significant co-factor for the development of HCC.

Effect of HBV treatment on development of HCC

It is well known that the long-term suppression of viral replication through the nucleos(t)ide analogues reduces but does not eliminate the risk of HCC. Several large patient case studies have shown that HCC incidence rates have significantly decreased in patients undergoing treatment^[65,66]. It has recently been confirmed that the reduced incidence of HCC in patients receiving antiviral treatment is independent of age, sex, HBeAg status, cytolysis level and the presence of cirrhosis^[67]. Treatment does not appear to have a significant clinical impact on patients with low levels of viremia (HBV-DNA < 2000 IU/mL)^[67]. A large retrospective study of non-cirrhotic positive HBV patients showed that the incidence of HCC is significantly lower in patients receiving antiviral therapy regardless of the levels of ALT^[68]. In addition, the required number of patients to be treated (NNT) to prevent 1 case of HCC 10 years after initiation of treatment was found to be similar both in the group of patients with ALT < 2 ULN (NNT = 14) and in those with ALT ≥ 2 ULN (NNT = 15)^[68]. These data appear to confirm that hyper-ALT should not be considered a necessary requirement for antiviral treatment in patients with HBV-DNA > 2000 IU/mL^[69].

HCC and HCV chronic infection

Clinical and epidemiological factors affecting development of HCV-related HCC

Chronic HCV infection is the third leading cause of HCC and accounts for about one-third of total incidence rates and one-fifth of HCC-related deaths^[1]. In recent years, the incidence of HCV-related HCC has undergone the greatest increase compared to that associated with other etiologies^[1]. The risk of developing HCC in the course of chronic HCV infection increases in proportion to the degree of hepatic fibrosis. In fact, most cases of HCV-related HCC occur during an established cirrhosis, suggesting that cirrhosis-mediated carcinogenesis may play a primary role in the development of HCC^[70]. In patients with HCV-associated cirrhosis it is estimated that the annual incidence rate of HCC is between 3% and 7%^[71,72]. The incidence of HCC is significantly higher among elderly patients (> 60 years) perhaps also due to the fact that the progression of fibrosis is related to the duration of the disease^[73,74]. Compared to HBV infection or NAFLD, HCC related to HCV infection shows a tendency to appear in a more advanced phase of liver disease^[75]. In addition to cirrhosis, other factors such as diabetes mellitus, metabolic syndrome, fatty liver disease and obesity are associated with a higher risk of developing HCC^[76-78]. The presence of HBV or HIV coinfection, alcohol abuse or iron overload are additional risk factors for hepatocarcinogenesis in HCV-induced cirrhosis^[9,22,79].

The role played by serum HCV-RNA levels in the development of HCC is controversial^[80-82]. Lee *et al.*^[80] in a large series reported that elevated serum HCV RNA levels were associated with a significant increase in the incidence of HCC. Furthermore, they demonstrated that the presence of elevated levels of hepatic cytolysis and HCV genotype 1 (12.6% vs. 4.5% for non-genotype 1) were associated with a higher HCC rate^[80]. Other studies have confirmed the oncogenic potential of the HCV genotype 1^[74,83]. A meta-analysis of 57 papers showed that patients with HCV genotype 1b have twice the risk of developing HCC compared to patients with non-1 genotype^[83]. Therefore, in patients with HCV infection the presence of high viremia, high levels of ALT and genotype 1 appear to be risk factors for HCC.

Pathogenic mechanisms of HCV-related HCC

HCV is unable to integrate its genome into host cells and requires a constant replication process to maintain chronic infection^[84]. Therefore, its oncogenic potential appears to be mostly indirect and mediated by the development of significant hepatic fibrosis. HCV infection causes a chronic inflammatory state, induces hepatocyte necrosis, as well as collagen production and accumulation, which will eventually lead to an

alteration of the structure of the hepatic parenchyma. The increase in hepatocyte turnover due to the continuous processes of cell death and regeneration, as well as the progression of fibrosis, lead to a high probability of genetic alterations, whose accumulation leads to the formation and proliferation of cell clones that favour the development of HCC^[85]. Furthermore, apoptosis of hepatocytes can amplify the fibrogenic signal, thus stimulating the activation of stellate cells and causing the progression of hepatic fibrosis towards cirrhosis which is a pre-malignant condition^[86]. HCV infection is also able to modify the intracellular signalling pathways of transforming growth factor beta (TGF- β) signalling, thus accelerating the progression of liver injury and increasing the risk of HCC^[87].

Although the pathogenesis of HCV-related HCC is mostly due to the development of cirrhosis and cell regeneration mechanisms, different alterations in gene expression and signal transduction pathways involved in cell proliferation and in the neoplastic transformation of hepatocytes have been described in chronic HCV infection^[88]. In this regard, there are various demonstrations, mostly obtained on animal models, which suggest that different viral proteins may play a direct role in hepatocarcinogenesis^[85,89-92]. The NS3 non-structural protein is a serine protease that appears to be involved in the neoplastic transformation process by inducing the acquisition by the hepatocyte clones of a proliferative condition, as well as the escape from the host cell surveillance mechanisms^[89]. In combination with the NS4A factor, it interacts with the ATM kinase and alters DNA cell repair mechanisms^[93]. Similarly, the NS5A phosphoprotein appears to be able to alter the cell growth mechanisms and the physiological replication cycle of the host cell through interaction with the CDK1/2-cyclin kinase-dependent complex^[90]. The HCV core protein and the E2 envelope protein have been shown to stimulate cell growth and heteroplastic degeneration^[91,92]. The HCV core protein in particular seems to play a key role in the pathogenesis of HCC. Its oncogenic potential appears to be considerably high, as it causes oxidative stress on one side and alters the intracellular signalling cascade of the protein kinase on the other, resulting in a dysregulation of cell growth control^[85]. In particular, the HCV core-protein is able to provoke an overproduction of ROS by increasing the lipid peroxidation and a mitochondrial dysfunction through the rearrangement of the lipoprotein double layer of the mitochondrial membrane^[94,95]. The oxidative stress induced by the HCV core-protein leads to damages in the genome of the host cell with accumulation of genetic aberrations that predispose the evolution towards cancer^[85,95,96]. Furthermore, the presence of insulin resistance and hepatic steatosis, which are associated with a high frequency to HCV infection, exacerbates the production of ROS^[95]. In addition, the HCV core-protein is able to inhibit DNA repair mechanisms damaged by oxidative stress and alter various intracellular antioxidant systems^[95,97]. At the same time, this protein is able to directly alter gene expression and intracellular regulation mechanisms. In this regard, a greater expression of tumor necrosis factor- α (TNF- α) and interleukin- β (IL- β) was observed, together with an higher activity of the relative downstream effectors c-Jun N-terminal kinase and activator protein-1, and a stimulation in the mitogen-activated protein kinase (MAPK) cascade^[98,99]. HCV core-protein is also capable of inhibiting the tumor suppression genes RB1, TP53 and TP73 as well as cell-cycle modulators such as CDKN1A^[98,100]. Cytokines overexpression and gene expression alterations may represent the mechanisms through which HCV core-proteins modulate the apoptotic signalling pathways and mechanisms of defence and proliferation of the hepatocytes. Histologically, transgenic mice carrying the core gene develop an early hepatic steatosis, similarly to what happens in men during chronic HCV infection. These mice show progressively the onset of hepatocellular adenomas characterised by the presence of numerous intracytoplasmic fat drops, which then evolve towards the formation of HCC more or less rich in lipid drops, depending on the stage of differentiation^[101]. These data highlight the key role played by HCV core-protein in the process of carcinogenesis.

HCV is able to cause alterations in the glucose and lipid metabolism, another important factor in the development of HCC^[85]. HCV, in fact, stimulates the activation of insulin-like growth factor (IGF), a cell growth regulator, through the induction of proliferative and anti-apoptotic mechanisms^[102]. Through the degradation of insulin receptor substrate 1 and 2 (IRS-1 and IRS-2)^[103], the virus is also able to interfere with

insulin signalling and induce insulin-resistance, which in turn is responsible for the activation of hepatic stellate cells and subsequent fibrosis^[104,105]. The presence of insulin resistance or diabetes mellitus represent independent risk factors for the progression of the liver disease and the development of HCC in patients with chronic HCV infection^[106]. Some evidence suggests that somatic mutations of the leptin receptor (LEPR) gene may increase the susceptibility to hepatocyte cancer transformation^[107].

Finally, virus-induced immune alterations can also help create an ideal environment for HCC development. The HCV, in fact, is able to inhibit the production of interferon type 1 and to alter the immune response of both T cells CD8+ and natural killer^[105,108]. In combination with the aforementioned cytokine alterations and oxidative stress, these immune alterations contribute to the persistence of chronic inflammatory hepatic disease, which provides fertile soil for malignant degeneration.

Effect of treatment on development of HCV-related HCC

The recent introduction of direct-action antivirals (DAAs) for the treatment of HCV infection that causes a sustained virologic response (SVR) in more than 95% of cases appears to induce a significant decrease in HCC cases associated with this infection. As already demonstrated for interferon-based therapeutic regimens^[109,110], several studies seem to demonstrate that achieving SVR using DAAs reduces the risk of HCC^[111]. The incidence rate of HCC in cirrhotic patients with SVR may decrease up to 1% per year, although a lower rate of reduction is observed in patients with concomitant metabolic syndrome. However, because the risk of HCC in patients with cirrhosis persists even after HCV elimination, a reasonable time frame will be required before significant epidemiological changes can be observed^[109]. Although it is quite clear that the achievement of SVR reduces the long-term risk of HCC, the possible role of DAAs in increasing the risk of HCC de novo and recurrence of HCC successfully treated is still a matter of debate^[112]. In a meta-analysis of 26 studies including 11,523 patients^[113] it was found that there were no significant differences in incidence rates of de novo HCC between treatment regimens based on DAAs and IFN. Ioannou *et al.*^[114] identified a 71% HCC risk reduction after achieving SVR through DAAs on a cohort of approximately 62,000 patients and confirming that there are no substantial differences in the HCC rate between patients treated with DAA and those treated with IFN. Kanwal *et al.*^[115] show that achieving SVR in patients treated with DAA is associated with a 76% reduction in the risk of HCC.

For what concerns the impact of DAAs on the recurrence of HCC previously treated with curative intent, the data available are still controversial and further studies may be necessary for a correct evaluation of the impact of DAAs therapeutic regimens on HCC recurrence risk.

HCC and alcohol abuse

Clinical and epidemiological factors affecting development of HCC secondary to alcohol abuse

Consumption of alcohol is the second leading cause of HCC worldwide, as it is responsible for around a third of cases^[1]. Europe and Latin America are the areas with the highest incidence rates of HCC secondary to alcohol abuse, accounting for about half of the total. It has been estimated that chronic alcohol consumption is associated with an approximately 2-fold increase in the odds ratio for the development of HCC, but this risk increases up to 5-7 times if consumption exceeds 80 g/day for a time period of more than 10 years, thus underlining the close dose/risk correlation^[116,117]. Although there is no absolute “threshold” dose that can be applied as a parameter to all people, as the risk of alcohol-related damage is individual, an average chronic consumption of ≥ 2 drink/day in females and ≥ 3 drink/day in males for longer than a 10-year time span is associated to the onset of alcoholic liver disease (ALD), which encompasses a wide spectrum of clinical pictures ranging from steatosis to steatohepatitis to the development of liver cirrhosis. From the time when cirrhosis is established, the risk of occurrence of HCC is 1%-2.5% per year^[118,119]. Compared to other etiologies, the risk of neoplastic transformation appears to be lower among ALD patients. In fact, a recent observational study showed that the cumulative incidence rates of HCC after 10 years of observation were lower in ALD cases (8.4%) than in cases of chronic HCV infection (22%) and NAFLD (23,7%), with an annual incidence

rate of 1.1%, 2.9% and 3.1% respectively^[119]. In this regard, however, the data in the literature seem to be conflicting.

The amount and duration of the alcohol consumption are directly related to the stage of the liver disease and the risk of HCC^[116]. In particular, the cumulative lifetime amount of alcohol assumed acts as a major determinant of oncologic risk^[120]. A ≥ 3 drink/day consumption is strongly associated with the incidence of HCC and liver-related death^[121]. Furthermore, alcohol consumption is also closely related to the more rapid increase in cancer growth once it has developed^[122]. This highlights the importance of the dose-response relation between alcohol consumption and HCC.

The female gender has an approximately 5-fold higher risk of developing liver cirrhosis and/or HCC for lower doses of alcohol than males^[121]. In addition, females appear to show a faster progression of the damage towards cirrhosis in comparison with males^[123].

A meta-analysis^[124] evaluated the occurrence of HCC after cessation of alcohol use. An annual reduction of 6%-7% of the risk of developing HCC after cessation of alcohol consumption was estimated and an average period of 23 years because the risk is comparable to that of an ever ethylist.

Although the cumulative amount of alcohol during the lifetime is the main predictor of the risk of HCC, not all alcohol users are destined to develop cirrhosis and/or HCC. Indeed, a number of both genetic and clinical cofactors are also implicated in modulating the risk of ALD evolution to HCC^[125]. Several SNPs have been reported to increase the risk of HCC, in particular those able to interfere with the metabolism of ethanol and lipids (PNPLA3, TM6SF2), as well as hepatic iron accumulation^[126-128].

Different co-morbidities can modulate cancer risk. Obesity is an important co-factor in the development of alcohol-induced HCC^[129,130]. The risk of developing HCC is three times higher among alcohol users with a BMI ≥ 30 kg/m² compared to those not taking alcohol and with a lower BMI^[131]. This synergy is also recognized to exist between alcohol and other co-factors of liver injury^[132]. The coexistence of diabetes mellitus and chronic alcohol consumption leads to a significant increase in the risk of developing HCC^[129,133,134].

A study of patients with alcoholic cirrhosis showed that the incidence of HCC among patients with and without diabetes mellitus was 32.7% and 3.2% after 5 years, 32.7% and 20.2% at 10 years, 66.3% and 20.2% at 15 years, respectively^[135]. It has been reported that the risk of HCC among patients with diabetes mellitus who consume more than 4 drinks/day has increased by 4.2 times^[136].

The high consumption of alcohol in cirrhotic patients with concomitant HBV infection increases the risk of HCC by about 10% per year, apart from the progression of its onset at an earlier age^[132,137,138]. Similarly, data are reported for OBI or previous HBV infection^[139,140]. Several studies conducted on alcoholic subjects show the synergistic effect with chronic HCV infection^[141,142] or hemochromatosis^[143] on the incidence of HCC. Simultaneous exposure to alcohol and tobacco also appears to increase the risk of HCC^[129].

Pathogenic mechanisms of HCC secondary to alcohol abuse

The presence of cirrhosis is the major mechanism related to the development of HCC. However, alcohol is able to directly induce carcinogenesis causing oxidative stress, inflammation and endotoxemia.

Ethanol is first converted to acetaldehyde and then to acetate by alcohol-dehydrogenase (ADH) and acetaldehyde-dehydrogenase (ALDH) respectively, within a process that increases the NADH/NAD⁺ ratio^[144]. This condition, in turn, causes a drastic change in the mitochondrial redox balance, which leads

to an increase in the oxidation of fatty acids, as well as of lipogenesis, thus inducing the development of steatosis^[145]. Ethanol is able to inhibit hepatocyte β -oxidation, increasing the synthesis and the uptake of fatty acids as well as promoting liver steatosis and inflammation^[146,147]. Acetaldehyde, in addition to being toxic, is also highly oncogenic. The highest levels of ADH activity in tumor cells, when compared to ALDH, indicate that they have a high oxidation capacity of ethanol but a low ability to remove acetaldehyde^[148]. Chronic alcohol consumption acts as an activator of cytochrome CYP2E1, which in turn increases the hepatic production of acetaldehyde. Its accumulation is responsible for the production of ROS that gives rise to oxidative stress induced by alcohol. The latter is responsible for mitochondrial damage, which in turn increases the production of ROS, thus creating a vicious circle that maximizes oxidative stress in the hepatocytes^[149]. The accumulation of iron in the liver^[150] and the low oxygen tension of the tissue induced by alcohol are also responsible for the production of ROS. Furthermore, accumulation of intracytoplasmic lipid droplets in ethanol-induced steatosis (as well as in NAFLD) can make hepatocytes more susceptible to toxic or other insults^[151]. It follows that the subsequent generation of ROS, in combination with the accumulation of damaged proteins and the increased susceptibility acquired by hepatocytes to damage of other nature, is able to induce lipid peroxidation, enzymatic inactivation and mutations of DNA, which can cause cell damage and inhibit apoptosis^[149]. In particular, alterations in cellular DNA methylation processes (especially at levels of the oncosuppressor genes such as *RASSF1A* and/or *DOK1*) represent one of the most frequent genotoxic effects of chronic alcohol consumption^[152]. All this causes serious abnormalities in the proliferation of hepatocytes, which may eventually lead to the development of HCC.

Alcohol induces alteration of the microbiota and may contribute to the development of liver injury and HCC. Damage to the tight junctions of the intestinal epithelium following the chronic abuse of ethanol increases the permeability of the intestinal barrier and promotes the migration of bacteria and endotoxins from the intestine to the portal system, thus fuelling the previously caused liver inflammatory status^[153]. Bacterial endotoxin interacts with the toll-like receptors (TLR) present at the Kupffer's cells, stimulating the production of pro-inflammatory cytokines that contribute to the progression of alcohol-induced liver injury^[153]. The alteration of TLR4 following the translocation of intestinal bacteria is able to induce carcinogenesis by interacting with cancer-initiating stem-like cells^[154-156]. The TLR4/intestinal microbiota interaction through processes of cellular proliferation stimulation and apoptosis inhibition play a role in the progression of HCC but it is not required for the induction of HCC^[156].

As for other etiologies, alteration in the length of the chromosomal telomere occurs also in the course of ALD. It has been shown that the telomeres of individuals taking > 4 drinks/day are shorter than those of subjects who take ≤ 4 drinks/day^[157] and that telomerase reactivation is closely related to the mechanisms of induction of hepatocarcinogenesis through uncontrolled hepatocyte replication^[148,158].

A further mechanism of induction of HCC secondary to ALD is represented by the impact of alcohol on the homeostasis of vitamin A, whose hepatic level decreases in chronic alcohol consumption^[159]. Alcohol acts as a competitive inhibitor of vitamin A, inducer of its catabolism, through CYP2E1 and is a promoter of its mobilization from the liver to peripheral tissues^[160]. Reduced levels of vitamin A in the liver can contribute to the development of HCC by altering the mechanisms of cell proliferation and apoptosis^[149,161].

From the above it is clear that ethanol abuse plays an important role as a promoter rather than an inducer of cancer development through a process in which oxidative stress is the basis of alcohol-induced cytotoxicity.

HCC and NAFLD

Clinical and epidemiological factors affecting development of NAFLD-related HCC

Due to the recent obesity and metabolic syndrome epidemic, NAFLD is currently the fastest growing chronic liver disease worldwide. The overage prevalence of NAFLD is currently estimated at 25%^[5]. It is

characterized by the intrahepatic accumulation of triglycerides and includes a spectrum of diseases ranging from simple steatosis to steatohepatitis (NASH) and to cirrhosis of the liver. The annual incidence of HCC in patients with NAFLD is reported to be 0.44 in 1000 patients, while the annual incidence in patients who have already developed NASH is 5.29 cases in 1,000 patients^[5]. It has been estimated that NAFLD is responsible for about 14% of HCC cases in the United States, with an annual rate of increase of 9%^[6].

A recent meta-analysis of cases of liver cancer diagnosed in the United States between 2004 and 2009 showed that the prevalence of HCC secondary to NAFLD is about 14%^[6]. The estimated annual cumulative incidence of HCC in cirrhosis by NAFLD is 2.6%^[5,73,160]. A recent study of our group found an annual rate of incidence of 3.5% of HCC in patients with cirrhosis from NAFLD, this incidence is slightly lower than that observed in cirrhosis secondary to HCV (4.5%)^[75]. Similar annual incidence rates of HCC were also observed by Ascha *et al.*^[162] (2.6% and 4.0% in patients with metabolic cirrhosis and HCV-related cirrhosis, respectively).

However, as already mentioned, increasing evidence suggests that NAFLD may cause the development of HCC even in non-cirrhotic patients with mild or absent fibrosis^[133,163-168]. There are conflicting data on the true prevalence of HCC on non-cirrhotic steatotic liver^[169]. A recent review of data on 61 studies published between 1992 and 2011 shows that the risk of HCC in non-cirrhotic patients with NAFLD appears to be extremely low^[166]. On the other hand, there are several studies that support the opposite hypothesis^[163,164,167,168,170]. In a group of 31 patients with NAFLD and HCC, Paradis *et al.*^[164] observed that, 65% of cases were in a F0-F2 fibrosis stage, whereas in the control group with liver disease of another etiology, only 26% of HCC were in the F0-F2 fibrosis stage. Mittal *et al.*^[167], in a cohort of 107 patients with HCC and NAFLD, 34.6% of liver cancer cases occurred in the absence of cirrhosis. Piscaglia *et al.*^[168] have recently observed a high incidence rate (70%) of HCC in non-cirrhotic patients with NAFLD, although histology was only available for one third of patients.

The evaluation of further co-factors appears to be fundamental for the individual assessment of the risk of HCC. Obesity and diabetes mellitus in particular are by now well-known independent risk factors for HCC. Calle *et al.*^[78] have shown, in a large cohort of patients, how obesity increases the risk of HCC by 2-4 times. In comparison with individuals with normal weight, Larsson *et al.*^[171] estimated the risk of HCC in normal weight and obese subjects by establishing a relative risk of 1.17 and 1.89, respectively.

It has been shown that the presence of diabetes mellitus increases the risk of HCC in patients with NAFLD^[19,172,173]. A recent study of 480 patients with NAFLD or ALD showed that the prevalence of HCC among diabetic patients was statistically higher compared to normoglycemic patients (8% and 3%, respectively) and the incidence rate of HCC during 3 years follow-up was almost three times higher (27% and 10% respectively)^[172]. Davila *et al.*^[173] confirm that the risk of HCC is three times greater in the presence of diabetes mellitus. Furthermore, diabetes mellitus and obesity can act in synergy. An Italian study has observed that the presence of one of the two factors leads to 3.5 odds ratio (OR) of HCC, while the OR increases to 11.8 in the presence of both, compared to normal weight and normo-glycemic subjects^[174]. Therefore, an obese and diabetic patient with NAFLD is the most classic patient phenotype that shows a high probability of developing HCC, particularly when co-factors are associated with an existing hepatic damage. In fact, when obesity is accompanied by chronic alcohol consumption or by HCV or HBV infection, the risk of developing HCC shows a tendency to increase exponentially, thus observing the synergistic action of these co-factors of hepatic injury^[130,175,176].

The general clinical picture of HCC occurring in NAFLD shows peculiar characteristics. In this regard, Younossi *et al.*^[6] showed how the development of HCC on NAFLD involves an older average population with a higher prevalence of cardiovascular disease. Weinmann *et al.*^[177] confirm a high average age (67.6 years) of patients with HCC in NAFLD, a higher prevalence among males, a higher incidence of

myocardial infarction and ischemic stroke, as well as a higher prevalence of obesity and diabetes mellitus. NAFLD-HCC patients show lower mean ALT levels and a higher platelet count than HCV-related HCC patients^[75,178].

The onset of HCC in NAFLD is generally an early event in the natural history of liver disease^[75,177]. In fact, the incidence of HCC on metabolic cirrhosis in a Child-Pugh A score appears to be 1.8 times higher than that observed in HCV-related cirrhosis^[75]. In a recent Italian study the diagnosis of HCC was placed at an early stage (Child-Pugh A) in 82.3% of patients with NAFLD, compared to 68.1% of patients with chronic HCV infection^[168]. The model for end-stage liver disease scoring (MELD) is also significantly lower for HCC in NAFLD than in other etiologies^[177].

Contrary to the stage of cirrhosis of the liver, the stage of the diagnosis of the neoplasia is generally more advanced for the HCC related to the NAFLD than for other etiologies. Compared to patients with HCV infection, HCC in patients with NAFLD often presents greater dimensions at diagnosis and more frequently shows infiltration (21% in patients with NAFLD versus 4% in patients with HCV) or multifocal lesions^[168,170,178].

The diagnosis in the advanced stage of neoplasia is not due only to the pathogenic mechanisms and epidemiological factors mentioned above but is mainly due to the lower attention to follow-up and screening of NAFLD^[179,180]. The diagnosis of HCC in patients with NAFLD is often incidental, outside the surveillance protocols and in any case late, as it is dependent on the appearance of symptoms^[168,178]. Patients with NAFLD seem to have the highest rate of cirrhosis undiagnosed before evidence of HCC compared to other etiologies, resulting in a decrease in attention to ultrasound surveillance and subsequent delay in the diagnosis of HCC. Furthermore, as another condition of difficulty in early diagnosis, it must be emphasized that the sensitivity of ultrasound in detecting small cancer is low in patients with NAFLD^[181,182].

Along with a generally more advanced stage of cancer diagnosis, there are additional reasons why the prognosis of patients with HCC related to NAFLD appears to be worse than patients with HCC of different etiology^[6,177]. In fact, the prognosis is negatively affected by a greater number of comorbidities, especially cardiovascular, to which this subgroup of patients is exposed. The highest rate of co-morbidities such as obesity, the highest mean age of patients with NAFLD and often delayed diagnosis lead to fewer liver transplants for these patients^[6,168,170,177]. Finally, the advanced stage of the disease is often a limitation for the applicability of radical treatments (resection or ablation) in favour of purely palliative interventions (chemoembolization or pharmacological therapy). In fact, liver resection and transplantation are only practiced in 17.8% and 4.4% of cases, respectively^[177]. As a result, the death rate in HCC secondary to NAFLD (61% of patients die within one year of diagnosis) is higher than in HCC secondary to viral hepatitis (50% of deaths within one year of diagnosis), with a shorter average life expectancy of 5 months^[6]. Piscaglia *et al.*^[168] observed an average survival of 25.5 months from diagnosis in patients with NAFLD-HCC, versus an average 33.7 months of patients with HCV-related HCC. However, when the patient is eligible for curative treatment, survival does not appear to vary between HCC related to NAFLD and other etiologies^[181].

The rs738409 polymorphism of phospholipase domain similar to the patatine containing 3 (*PNPLA3*) has been reported to be an independent risk factor of HCC in patients with metabolic cirrhosis (odds ratio 1.40)^[183]. In particular, homozygosity GG was associated with the onset of HCC at a younger age, in patients with a shorter history of cirrhosis. Furthermore, it is associated with a worse prognosis^[181]. The rs738409 polymorphism seems to alter the export of lipoproteins and lipogenic activity, thus causing the hepatic accumulation of fatty acids with consequent increase of lipid β -oxidation, as well as the production of ROS, increasing the risk of progression of fibrosis hepatic and HCC development^[183].

An alteration of the intestinal microbiota is often associated with NAFLD and this condition contributes to exacerbating the inflammatory liver^[184]. In particular, there is the appearance of endotoxemia that interacts with TLR receptors on Kupffer cells and hepatic stellate cells triggering a cascade of inflammatory signaling causing the production of TNF- α , IL-1 β , IL-6 and ROS^[185]. This condition contributes to the progression of liver injury and to carcinogenesis as reported above for ALF.

Pathogenic mechanisms of NAFLD-related HCC

Most of the HCC secondary to NAFLD occurs in the cirrhotic liver following the mechanism shown above for other etiologies. However, the literature shows a consistent set of evidence on how the development of HCC may also occur in patients without evidence of significant hepatic fibrosis^[133,168]. As shown in mouse models^[186], hepatic steatosis secondary to the metabolic syndrome is a pre-malignant condition, long before cirrhosis. In this regard, obesity and type 2 diabetes mellitus can be considered as independent risk factors for the onset of HCC^[78,172,187].

The pathogenic mechanisms involved in the carcinogenesis of liver cancer in NASH, with or without significant fibrosis, could be related to chronic low-grade inflammation induced by obesity and the metabolic syndrome and mediated by the crucial role of insulin resistance. The development of oxidative stress, lipid peroxidation and mitochondrial damage also play a fundamental pathogenic role. Moreover, it has been shown that alterations of the intestinal microbiota, the presence of gene polymorphism and IR induced hyperinsulinemia can be significant co-factors for the development of NASH and HCC. These data support the concept of “multiple hits hypothesis” in which several factors cooperate in the pathogenesis of NAFLD and HCC^[188-190].

The IR, the cornerstone of the metabolic syndrome, is able to induce the onset of HCC by increasing the release of free fatty acids from the adipose tissue and subsequent accumulation in the hepatocytes on one hand and inducing the formation of ROS and subsequent oxidative stress with mitochondrial damage and endoplasmic reticulum dysfunction on the other hand^[167]. Moreover, IR is able to alter the balance between pro-inflammatory cytokine production (IL-6, TNF- α , leptin, resistin) and those anti-inflammatory (adiponectin) with a significant increase in pro-inflammatory cytokine^[191], leading to a chronic hepatic and systemic inflammatory state. The IR-induced TNF- α stimulation leads to the activation of the nuclear factor Kappa B (NF- κ B) and of the N-terminal kinase c-Jun (JNK) on one side, as well as to the overexpression of tumor growth promotion genes^[192] on the other side. The increase in free fatty acids and TNF- α and ROS production are all powerful activators of JNK, overexpressed in more than half of the cases of HCC, which in turn causes the phosphorylation of the substrate-1 of the insulin receptor (IRS-1). The signalling mediated by IRS1 can therefore act as a stimulus for cell survival, promoting the proliferation of hepatocytes through the mitogenated protein kinase and PI3K and inhibiting cell apoptosis by blocking the TGF- β 1^[193]. IR-induced hyperinsulinemia is also able to stimulate the production of growth factors such as IGF-1. All the previously mentioned pathways are able to cause liver inflammation and aberrant stimulation of several genes that are crucial in regulating cell growth and inhibition of apoptosis^[175,194].

The cytokinetic imbalance associated with the release of unsaturated fatty acids also contributes to inhibition of tumor suppression factors (for example, phosphatase and homologue of the tensin, PTEN)^[195] and inhibition of apoptotic cell abilities^[175]. In this regard, IR-induced oxidative stress can increase lipid peroxidation, thus leading to the production of trans-4-hydroxy-2-nonenal, which in turn can interact with DNA and cause mutations in the oncosuppressor gene p53, thus favouring hepatocarcinogenesis and the progression of HCC^[196].

The IR-induced increase in TNF- α and IL-6 also stimulates leptin production^[197]. The latter acts as a growth factor activating the Janus-activated kinase (JAK), which in turn stimulates signal transducers and activators

of transcription 3 (STAT3) and extracellular signal-regulated kinases (ERK)^[169,198,199]. Such leptin-induced pathways represent early events in the promotion of the survival and proliferation of pre-neoplastic cell clones, thus favoring the development of HCC and its invasion and metastasis^[198,199]. Furthermore, higher levels of leptin are closely related to an increased risk of recurrent HCC after curative treatment^[200]. On the other hand, IR inhibits the production of adiponectin, an adipokine with anti-inflammatory functions, as well as anti-atherogenic, anti-proliferative, pro-apoptotic, insulin-sensitizing and anti-angiogenic factors^[169]. In fact, this cytokine is able to stimulate the activation of JNK and induce cell apoptosis^[201]. Reductions in adiponectin levels appear to be closely associated with the risk of carcinogenesis^[202].

Similar to what is observed in ALD, deficiency in the autophagy mechanism is also observable in NAFLD, causing reticuloendothelial damage and cellular oxidative stress and contributing to the formation of an environment suitable for the development of HCC^[203].

The immune system may also participate in the complex multifactorial mechanisms of hepatocarcinogenesis. In fact, the metabolic stress promotes the migration of immune cells in the liver, while the T cells CD8 + and Natural Killer (NK), stimulated by the cell damage caused by NAFLD, interact with the hepatocytes activating the signaling cascades that feed the pre-existing state inflammatory^[204]. In this way, they can establish a further vicious circle that worsens hepatocyte damage, promoting the progression of NAFLD towards the development of HCC.

DECLARATIONS

Authors' contributions

Designed the study: Adinolfi LE

Contributed equally to the search for literature and the writing of the text: Nevola R, Rinaldi L, Giordano M, Marrone A

Reviewed the manuscript: Adinolfi LE, Nevola R

Approved the final version: all authors

Availability of data and materials

Not applicable.

Financial support and sponsorship

There was no financial support or sponsorship for this study.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Adult African Americans undergoing cadaveric liver transplantation for hepatocellular carcinoma within the Milan criteria have the lowest 5-year survival among all the ethnic groups in the United States: analysis of USA national data between January 2002 and June 2013

Michele Molinari¹, Subhashini Ayloo², Allan Tsung¹, Patrick Bou Samra¹, Naudia Jonaissaint³

¹Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, USA.

²Department of Surgery, Rutgers New Jersey Medical School, Newark, NJ 07103, USA.

³Department of Medicine, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, USA.

Correspondence to: Prof. Michele Molinari, Division of Transplantation, University of Pittsburgh Medical Center, 3459 Fifth Avenue, N758, Pittsburgh, PA 15213, USA. E-mail: molinarim@upmc.edu

How to cite this article: Molinari M, Ayloo S, Tsung A, Bou Samra P, Jonaissaint N. Adult African Americans undergoing cadaveric liver transplantation for hepatocellular carcinoma within the Milan criteria have the lowest 5-year survival among all the ethnic groups in the United States: analysis of USA national data between January 2002 and June 2013. *Hepatoma Res* 2018;4:56. <http://dx.doi.org/10.20517/2394-5079.2018.71>

Received: 1 Jun 2018 **First Decision:** 23 Jul 2018 **Revised:** 7 Aug 2018 **Accepted:** 10 Aug 2018 **Published:** 20 Sep 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cai-Hong Wang **Production Editor:** Huan-Liang Wu

Abstract

Aim: To investigate the potential effects of recipient ethnicity on the short and long-term outcomes of patients undergoing liver transplantation (LT) for hepatocellular carcinoma (HCC) in the United States. We performed a retrospective study using the standard transplant analysis and research (STAR) files with the primary aim of assessing short and long-term survival of different ethnic groups undergoing LT for HCC in the United States.

Methods: The study population was represented by adults (age ≥ 18) who received a first-time cadaveric LT for HCC between 1 Jan 2002 and 30 Jun 2013. Recipients of LT for other primary and secondary malignancies were excluded. Other exclusion criteria were: transplants from grafts recovered from living or donors after cardiac death, split grafts, multi-visceral or redo transplants, and LT performed across ABO incompatible blood groups. Survival analysis stratified by recipient ethnicity was performed using the Kaplan-Meier method. Proportional hazard model analysis was used to assess the effect of predictors of survival. Characteristics utilized in the Cox regression model were selected a priori.

Results: The study population was represented by 6048 recipients with an average age of 58 years and 20% being females. The majority of patients were Caucasians (67%), followed by Hispanics (14.2%), African Americans



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(8.8%) and Asians (8.6%). Overall 30-, 60-, 90-day and 1-year mortality was 1.7%, 2.3%, 3.0% and 8.8% respectively with no statistically significant differences among ethnicities. Log-rank comparisons however showed that African American had the lowest 5-year survival with statistically significant differences in comparison to all other ethnic groups ($P \leq 0.001$). At multivariate Cox-regression analysis, African American ethnicity remained an independent predictor for increased mortality (HR = 1.524; 95% CI: 1.283-1.803; $P < 0.001$) after adjusting for the recipient and donor age, recipient sex, recipient history of diabetes and recipient functional status at the time of transplantation.

Conclusion: Short-term outcomes of African Americans undergoing cadaveric LT for HCC are similar to other ethnic groups. However, African American ethnicity is an independent predictor of lower 5-year overall survival when compared to all other ethnic groups.

Keywords: Hepatocellular carcinoma, ethnicity, survival, Cox-regression, liver transplantation, predictor

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world^[1] with over 1,000,000 new patients diagnosed every year and 250,000 cancer-related deaths^[2]. The worldwide incidence of HCC is unequally distributed with South-East Asia and Sub-Saharan Africa having the highest incidence while the lowest is recorded in Western Europe and North America^[3]. Geographical differences of the incidence of HCC reflect variations of the most common risk factors for HCC such as viral hepatitis B (HBV) and C (HCV), aflatoxin, alcohol consumption and genetics^[3,4]. However, over the last decades, the incidence of HCC has steadily increased in Western countries due to a rise in the incidence of HCV and non-alcoholic steatohepatitis (NASH)^[5].

Treatment modalities for HCC depend on patient age and comorbidities, tumor characteristics and degree of liver disease and portal hypertension in addition to other factors such as local expertise and resources^[6]. Liver resection and transplantation provide the best long-term survival^[7-9] followed by ablative therapies, locoregional and systemic chemotherapy^[7,8,10,11]. Despite the survival advantage of hepatic resection and liver transplantation (LT), most patients are unable to undergo surgery because of their advanced tumors or the presence of co-morbidities. Even after radical resections, cirrhosis predisposes to the development of recurrent disease in 50%-80% of patients within 5 years^[12,13]. Consequently, LT remains the best treatment as it addresses both the tumor and cirrhosis^[14,15]. Nevertheless, only 10%-12% of patients with HCC are transplanted due to the limited number of donors^[16-22].

Previous studies have reported that in the United States, LT for HCC is performed less frequently in non-Caucasians than in recipients of other ethnicities^[23-25]. The reasons for these disparities are not completely understood but there is some evidence suggesting that disadvantaged ethnic groups face more barriers to access healthcare and are more frequently diagnosed with advanced diseases^[23,24,26].

To be listed for a LT in the United States and Europe, patients with HCC must fulfill not only strict oncological criteria^[15] but also other requirements such as evidence of adequate social support, financial stability, the absence of active mental disorders, abstinence from substance abuse and adherence to diagnostic and therapeutic recommendations. These requirements, especially the ones linked to financial status, might affect certain demographic or socioeconomic groups more than others^[27], but are necessary to optimize the outcomes of LT recipients.

Since all LT candidates have to satisfy similar inclusion criteria, we hypothesized that there should not be differences in short and long-term outcomes among different ethnic groups, and since studies on ethnicity

and outcomes of patients undergoing LT for HCC in the United States are lacking, the primary aim of this study was to assess if African American had short- and long-term outcomes similar to recipients of other ethnic groups.

METHODS

Study design

The United network for organ sharing (UNOS) standard transplant analysis and research (STAR) files were used to identify a retrospective cohort of patients who underwent LT for HCC in the United States between 1 Jan 2002 and 30 Jun 2013. The study was conducted and reported per recommendations from STROBE statement^[28,29] and did not require approval by the ethics review board of our institution.

Rationale and aims of the study

There has been some controversy regarding the possible reasons why some ethnic groups have inferior survival than Caucasian recipients after LT^[30-32]. Nair *et al.*^[33] have previously reported that being African American or Asian American were risk factors for inferior long-term outcomes after LT. On the other hand, Lee *et al.*^[34] did not find any association between race and post-LT outcomes after adjusting for age, gender, total bilirubin, creatinine and prothrombin time. In more recent years, Wong *et al.*^[30] analyzed the 2002-2012 STAR files and concluded that African Americans had significantly lower survival compared with non-Hispanic whites affected by HCV, alcoholic liver disease, and HCC after adjusting for several demographic and clinical characteristics. To the best of our knowledge this was the only study that assessed the outcomes of LT recipients stratified by their ethnicity after the MELD score was introduced in the USA for the allocation of liver grafts. Although this study had the advantage of including a large number of patients, it was limited by the fact that several predictors of long-term survival were not included in the final Cox-regression analysis, and that the study was not specifically designed for patients with HCC. Because of these limitations, we performed a retrospective analysis of the STAR files with the primary aim of testing the null hypothesis, that there were no significant differences in the overall survival of patients with documented HCC and who belonged to different ethnic groups.

Inclusion and exclusion criteria

All adults (age ≥ 18 years) undergoing LT for HCC were candidates for this study. No restriction of race, citizenship or UNOS region were applied. Recipients of LT for other primary and secondary malignancies (e.g., cholangiocarcinoma, hepatoblastoma, hemangiosarcoma, neuroendocrine metastasis) were excluded. Other exclusion criteria were: transplants from grafts recovered from living or donors after cardiac death, split grafts, multi-visceral or redo transplants, and LT performed across ABO incompatible blood groups. Additional exclusion criteria were lack of records on short and long-term outcomes, the absence of HCC in the explanted liver or the presence of variables with values that were deemed implausible for adult recipients or for deceased donor LTs^[35]. Cutoffs for those values were: recipient height either ≤ 120 cm or ≥ 240 cm, cold ischemia time ≥ 24 h. No imputations of missing data were performed, and recipients who had more than 10% of unreported values were excluded.

Variables and outcomes

Variable collected for LT recipients were age at the time of transplant, sex, donor and recipient body mass index (BMI), ethnicity, presence of renal failure requiring hemodialysis before surgery, history of diabetes (either type I or II), mortality within 30-, 60-, 90-day and 1 year after surgery, main cause of death, date of death or date of last follow up, cold ischemia time (h), UNOS region where patients were transplanted. Additional variables collected for the donors were age, sex, height and weight or BMI.

Recipient overall survival was estimated by the difference between the date of transplantation and the date of death from any cause using the Kaplan-Meier method. Censoring was used for recipients who were still alive

on 30 Jun 2013, or who were alive at the time of the last follow-up or if they underwent re-transplantation (date of redo LT surgery).

Covariates used for Cox regression analysis

The presence of renal failure requiring hemodialysis prior to LT and history of diabetes (type 1 or type 2 diabetes) were used as 2-level categorical variables (absent or present). Ethnicity was categorized into five groups: Caucasian, African American, Hispanic, Asian and Multiracial including other minorities such as Hawaiian or Native American. The time on the wait list was calculated from the day of listing for LT to the date of surgery irrespective of the length of time that the patient spent in an inactive state. The waiting time was then categorized into four periods: less than 3 months, 3.1-6 months, 6.1-12 months and longer than 1 year. Recipient functional status at the time of LT was measured using the UNOS classification based on the validated Karnowski performance status^[36-38]. Recipient functional status was reported in the STAR files in 10% increments with 10% representing a patient who was moribund to 100% who represented a fully active and normal individual without complaints and no evidence of disease. Patient functional status was used as a two-level categorical variable: less than 60% and 60% or higher. Recipient educational level was stratified into six categories: elementary or middle school (grade 1-8), high school (grade 9-12), college or technical school, associate or bachelor degree, post college or graduate degree. BMI was estimated using the World Health Organization (WHO) formula: weight (kg)/height (m²). The WHO definition of overweight and obesity were used to classify recipients and donors in three categories: normal weight (BMI 18.5-24.9 kg/m²), overweight (BMI 25-29.9 kg/m²), obese (BMI ≥ 30 kg/m²). Obesity was further classified as class I (BMI 30-34.9), class II (BMI 35-39.9) and class III (BMI ≥ 40). Data for different BMI classes were not adjusted for the presence of ascites as the quantitative contribution of this to the patients' BMI was not reported in the STAR files.

Statistical analysis

The sample size of patients was fixed due to the retrospective design of this study. Continuous variables were reported by estimates of central tendency (means or median) and spread [standard deviation and interquartile range (IQR)] while frequency and percentages were used for categorical data. Survival analysis was performed using the Kaplan-Meier method^[39] and after assessing that the assumptions of the Cox model were met, proportional hazard model analysis was used to assess the effect of predictors of survival after LT. Pre-transplant characteristics utilized in the Cox regression model were selected a priori. Donor variables used as covariates for proportional hazard model were: age and BMI. Recipient variables used as covariates for Cox regression model were: age, sex, the presence of type I or II diabetes, need for dialysis prior to LT, level of education, BMI, time spent on the wait list and functional status. Survival analysis was also adjusted for cold ischemia time and for the UNOS region where the transplant surgery was performed. The UNOS region 1 was chosen as the reference category and the follow-up time was restricted to 5 years after LT. Since previous studies suggested that African Americans had the lowest post LT survival among all the ethnicities, we compared patients of African descent to patients belonging to other ethnicities.

For the calculation of the hazard ratios (HR), Caucasian ethnicity, female sex, functional status lower than 60%, waiting time equal or less than 3 months, post college or graduate degree were selected as references. Adjusted HR (AHR) were calculated using Caucasian patients undergoing LT as a reference. All statistical analyses were performed using SPSS Statistics for Windows, Version 24 (IBM Corporation, United States). Statistical significance was defined when *P* values were equal or less than 0.05, and 2-tailed tests were used for all statistical analyses.

RESULTS

During the study period, 9723 patients were recorded in the STAR files as recipients of a cadaveric LT with HCC being the primary indication for surgery. Cold ischemia time longer than 24 h was logged in 13

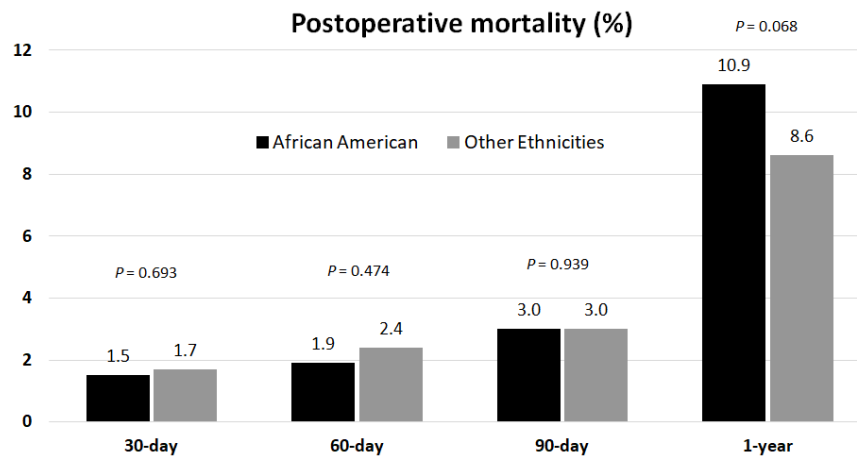


Figure 1. Analysis of the frequency of postoperative mortality observed in African American patients vs. patients of other ethnicities undergoing first-time cadaveric liver transplant for hepatocellular carcinoma at 30-, 60-, 90-day and at 1 year after surgery

recipients and 3019 patients had no HCC in their final surgical pathology report of their explanted livers and were excluded. After the additional removal of 643 recipients who had more than 10% of missing data, we identified a cohort of 6048 LT recipients who represented the study population. The average age of the recipients was 58 years and females represented 20% of the cohort. Most patients were Caucasians (67%), followed by Hispanics (14.2%), African Americans (8.8%) and Asians (8.6%). Detailed demographic and clinical characteristics of the study population are summarized in [Table 1](#).

Clinical and socio-economic characteristics

When compared to all other ethnic groups, the cohort of African American recipients had a higher percentage of women (26.2% vs. 19.7%; $P \leq 0.001$), was younger with an average age of 57 years vs. 58 years ($P = 0.005$), had a greater proportion of patients who required hemodialysis before LT (2.3% vs. 0.7%; $P \leq 0.001$), had fewer patients who had to wait longer than 6 months for LT (34.2% vs. 40.8%; $P = 0.02$), had a lower level of education and received a graft from younger donors (42.6 years vs. 44.2 years; $P = 0.030$). Detailed comparisons between African American patients and the rest of the cohort are reported in [Table 2](#).

Postoperative mortality and survival

The median follow-up of the cohort was 7.6 years (95% CI: 7.5-7.8). During this period, 2079 patients had died (34.3%), 3762 were censored (62.2%), and 207 patients (3.4%) were lost at follow-up. Overall 30-, 60-, 90-day and 1-year mortality was 1.7%, 2.3%, 3.0% and 8.8% respectively with no statistically significant differences between African Americans and other ethnicities [[Figure 1](#)].

[Table 3](#) reports the primary causes of death of patients who died within 5 years after LT. Graft failure was the most frequent cause of death among African Americans (16.6%), followed by multiorgan failure (15.4%) and recurrent malignancy (15.4%). On the other hand, the most frequent known causes of deaths in patients belonging to other ethnic groups were recurrent malignancy (31.1%), graft failure (11.4%) and infections (9.0%) ($P < 0.001$).

Kaplan-Meier survival function showed that the 5-year probability of survival for all patients who underwent LT for HCC was 69% [[Figure 2](#)]. Comparisons of survival functions by ethnicity showed that African American had the lowest 5-year survival with statistically significant differences between African Americans and all the other ethnic groups ($P \leq 0.001$) [[Figure 3](#)].

Table 1. Demographic and clinical characteristics of the study population (6048 liver transplant recipients)

Characteristics	Value
Age, years, mean, (SD)	57.9 (6.9)
Sex, <i>n</i> (%)	
Female	1,224 (20.2)
Ethnicity, <i>n</i> (%)	
Caucasian	4,054 (67.0)
African American	531 (8.8)
Hispanic	859 (14.2)
Asian	522 (8.6)
Multiracial or others	82 (1.4)
Recipient BMI, mean, (SD)	28.5 (5.0)
Recipient BMI, category, <i>n</i> (%)	
Underweight	3 (0)
Normal weight	1,535 (25.4)
Overweight	2,420 (41.0)
Obesity class I	1,342 (22.2)
Obesity class II	545 (9.0)
Obesity class III	143 (2.4)
Donor BMI, mean, (SD)	27.6 (5.4)
Presence of renal insufficiency requiring dialysis, <i>n</i> (%)	50 (0.8)
Presence of diabetes, <i>n</i> (%)	
No	4,254 (70.3)
Type 1 or type 2	1,742 (28.8)
Unknown	52 (0.9)
MELD score, mean, (SD)	12.1 (4.5)
Hospital stay (day), mean, (SD)	10.6 (13.0)
Months spent on the waiting list, <i>n</i> (%)	
0-3	2,370 (39.2)
3.1-6	1,245 (20.6)
6.1-12	1242 (20.5)
Longer than 12 months	1,191 (19.7)
Functional status at the time of transplantation, <i>n</i> (%)	
Less than 60%	958 (15.8)
60% or more	4,795 (79.2)
Unknown	295 (4.8)
Education, <i>n</i> (%)	
Elementary or middle school (grade 1-8)	326 (5.4)
High school (grade 9-12)	2,333 (38.6)
College or technical school	1,376 (22.8)
Associate or bachelor degree	805 (13.3)
Post-college or graduate degree	338 (5.6)
Unknown	870 (14.4)
UNOS region, <i>n</i> (%)	
Region 1	274 (4.5)
Region 2	605 (10.0)
Region 3	888 (14.7)
Region 4	734 (12.1)
Region 5	968 (16.0)
Region 6	301 (5.0)
Region 7	549 (9.1)
Region 8	483 (8.0)
Region 9	422 (7.0)
Region 10	512 (8.5)
Region 11	312 (5.2)
Donors' age, years, mean, (SD)	41.1 (15.9)
Cold ischemia time, hours, mean, (SD)	6.7 (2.5)

BMI: body mass index

Table 2. Demographic and clinical characteristics of the study population stratified by recipient ethnicity: African Americans (8.8%) vs. other ethnicities (91.2%)

Characteristics	Other Ethnicities (no. 5,517)	African American (no. 531)	P value
Age, years, mean, (SD)	58.0 (6.9)	57.1 (6.9)	0.005
Sex, <i>n</i> (%)			
Female	1,085 (19.7)	139 (26.2)	≤ 0.001
Body mass index, mean, (SD)	28.5 (5.0)	28.3 (5.2)	0.593
Recipient BMI, Category, <i>n</i> (%)			
Underweight	3 (0.1)	0 (0.0)	
Normal weight	1,398 (25.3)	137 (25.8)	
Overweight	2,253 (40.8)	227 (42.7)	
Obesity class I	1,230 (22.3)	112 (21.1)	0.273
Obesity class II	508 (9.2)	37 (7.0)	
Obesity class III	125 (2.3)	18 (3.4)	
Donor BMI, mean, (SD)	27.6 (5.4)	27.3 (5.6)	0.260
Presence of renal insufficiency requiring dialysis, <i>n</i> (%)	38 (0.7)	12 (2.3)	≤ 0.001
Presence of diabetes, <i>n</i> (%)			
No	3,867 (70.1)	387 (72.9)	
Type 1 or type 2	1,600 (29.0)	142 (26.7)	0.226
Unknown	50 (0.9)	2 (0.4)	
MELD score, mean, (SD)	12.1 (4.4)	12.1 (5.0)	0.794
Hospital Stay (day), mean, (SD)	10.5 (13.1)	11.5 (12.1)	0.110
Months spent on the waiting list, <i>n</i> (%)			
0-3	2,141 (38.8)	229 (43.1)	
3.1-6	1,125 (20.4)	120 (22.6)	
6.1-12	1,142 (20.7)	100 (18.8)	0.022
Longer than 12 months	1,109 (20.1)	82 (15.4)	
Functional status at the time of transplantation, <i>n</i> (%)			
Less than 60%	881 (16)	77 (14.5)	
60% or more	4,372 (79.2)	423 (79.7)	0.413
Unknown	264 (4.8)	31 (5.8)	
Education, <i>n</i> (%)			
Elementary of middle school (grade 1-8)	310 (5.6)	16 (3.0)	
High school (grade 9-12)	2,110 (38.3)	223 (42.0)	
College or technical school	1,251 (22.7)	125 (23.5)	
Associate or bachelor degree	750 (13.6)	55 (10.4)	0.010
Post college or graduate degree	314 (5.7)	24 (4.5)	
Unknown	781 (14.2)	88 (16.6)	
UNOS region, <i>n</i> (%)			
Region 1	256 (93.4)	18 (6.6)	
Region 2	485 (80.2)	120 (19.8)	
Region 3	804 (90.5)	84 (9.5)	
Region 4	685 (93.3)	49 (6.7)	
Region 5	927 (95.8)	41 (4.2)	
Region 6	295 (98.0)	6 (2.0)	≤ 0.001
Region 7	514 (93.6)	35 (9.5)	
Region 8	439 (90.9)	44 (9.1)	
Region 9	382 (90.5)	40 (9.5)	
Region 10	454 (88.7)	58 (11.3)	
Region 11	276 (88.5)	36 (11.5)	
Donors' age, years, mean, (SD)	44.2 (15.9)	42.6 (16.0)	0.030
Cold ischemia time, hours, mean, (SD)	6.7 (2.5)	6.6 (2.5)	0.393

BMI: body mass index

At univariate Cox regression analysis, ethnicity, age, history of diabetes and functional status at the time of transplantation were independent predictors of survival after LT. At multivariate analysis, African American ethnicity remained the strongest independent predictor for increased mortality in comparison to Caucasian

Table 3. Primary cause of death after cadaveric liver transplantation for hepatocellular carcinoma by recipient ethnicity

The primary cause of death, <i>n</i> (%)	Other ethnicities, <i>n</i> (%)	African American, <i>n</i> (%)	<i>P</i> value
Cardiovascular	101 (7.7)	19 (11.2)	0.185
Graft failure	150 (11.4)	28 (16.6)	1.115
Cerebrovascular complications	16 (1.2)	3 (1.8)	0.622
Pulmonary complications	46 (3.5)	7 (4.1)	0.766
Renal insufficiency	11 (0.8)	2 (1.2)	0.664
Multiorgan failure	95 (7.2)	26 (15.4)	0.001
Infections	119 (9.0)	15 (8.9)	0.810
Hemorrhagic complications	31 (2.3)	2 (1.2)	0.305
Malignancy	408 (31.1)	26 (15.4)	0.001
Unknown	333 (25.4)	41 (24.3)	0.554
Total number (%)	1310 (100)	169 (100)	-

recipients (reference group) (HR = 1.524; 95% CI: 1.283-1.803; $P < 0.001$) after adjusting for the recipient and donor age, recipient sex, recipient history of diabetes and recipient functional status at the time of transplantation [Table 4].

DISCUSSION

Over the past decades, there has been an increasing awareness that cancers have unique mutations in signaling pathways^[40] and that patient socio-economic factors and ethnicity might play a significant role in short and long-term outcomes^[41]. Contrary to the new genomic techniques that have shown biological differences among cancers of similar type^[42], causes responsible for of health disparities among patients of different socio-economic status or ethnicities remain unclear.

Socio-economic conditions are difficult to define and may fluctuate over time^[43]. Several studies have shown that vulnerable socio-economic groups are less likely to undergo screening or surveillance programs for HCC and are less likely to be treated^[24,32,44-46] but possible ethnic differences in the long-term survival after LT for HCC remains poorly studied^[47].

In a retrospective analysis of 754 patients with HCC eligible for LT at Mount Sinai Hospital in New York between 2003 and 2013, Sarpel *et al.*^[27] found that the odds of being transplanted were significantly lower for African Americans than Caucasians (OR = 0.55, 95% CI: 0.33-0.91). They also analyzed all the steps necessary for the evaluation and listing of these patients in the hope of finding barriers that could be removed in the future, but they were unable to identify any specific one. Similarly, Siegel *et al.*^[23] investigated the Surveillance, Epidemiology, and End Results (SEER) database with the main focus of assessing if there were racial disparities in utilization of LT in patients with HCC. They found that during the period between 1998 and 2002, African Americans and Asians were less likely to receive a LT than other ethnic groups. Because of the lack of granular data on many socio-economic factors, the authors were unable to identify the main reasons for those differences, but they hypothesized that access to transplant centers, referral bias, comorbidity and severity of underlying liver disease might have been the main causes why African Americans and Asian Americans had lower rates of LT. Similar findings were reported by other investigators^[30,48].

More recently, Moylan *et al.*^[49] have found that African American were less likely to receive a LT (OR 0.75; 95% CI: 0.59-0.97) during the pre-MELD era and were more likely to die or become too sick for transplant compared to Caucasians (OR 1.51; 95% CI: 1.15-1.98). However, after changes in the allocation of liver grafts that occurred with the introduction of the MELD score, ethnicity was no longer associated with waitlist death or lower rate of LT.

Table 4. Univariate and Multivariate Cox proportional hazard model of mortality of patients undergoing cadaveric liver transplantation for hepatocellular carcinoma. The adjusted Hazard Ratio was calculated by including both clinical and socio-demographic characteristics

Characteristics	Unadjusted HR	95% CI	P value for unadjusted HR	Adjusted HR (*)	95% CI	P value for adjusted HR
Recipient ethnicity			< 0.001			< 0.001
Caucasian (reference)	1			1		
African American	1.484	1.262-1.746	< 0.001	1.524	1.283-1.803	< 0.001
Hispanic	0.799	0.679-0.939	0.007	0.785	0.656-0.940	0.008
Asian	0.602	0.483-0.751	< 0.001	0.618	0.485-0.787	< 0.001
Multiracial or Other Ethnicities	0.610	0.360-1.033	0.066	0.733	0.431-1.246	0.251
Donor age (year)	1.010	1.007-1.014	< 0.001	1.010	1.007-1.014	< 0.001
Recipient age (year)	1.020	1.012-1.028	< 0.001	1.019	1.010-1.027	< 0.001
Recipient sex (female as reference)	1			1		
Male	0.935	0.822-1.063	0.306	0.988	0.861-1.134	0.697
Donor BMI	1.006	0.997-1.016	0.193	1.002	0.991-1.012	0.766
Recipient BMI	1.002	0.992-1.013	0.714	0.996	0.985-1.008	0.788
Cold ischemia time (hour)	1.018	0.998-1.038	0.082	1.013	0.992-1.034	0.233
Presence of diabetes (Type 1 or 2)	1.182	1.029-1.270	0.013	1.065	0.567-2.000	0.844
Dialysis prior to transplant	1.263	0.731-2.181	0.420	1.109	0.612-2.009	0.734
MELD score	1.005	0.994-1.017	0.370	1.002	0.989-1.014	0.812
Functional status at the time of transplantation			< 0.001			< 0.001
Functional status < 60% (reference)	1			1		
Functional status ≥ 60%	0.752	0.617-0.918	0.005	0.696	0.602-0.806	0.044
UNOS region			0.165			0.149
Region 1 (reference)	1		1	1		
Region 2	1.27	0.955-1.690	0.101	1.216	0.897-1.649	0.207
Region 3	1.181	0.900-1.551	0.230	1.216	0.903-1.637	0.198
Region 4	0.904	0.679-1.203	0.489	1.010	0.742-1.374	0.951
Region 5	0.824	0.623-1.089	0.174	0.906	0.669-1.225	0.521
Region 6	0.913	0.661-1.262	0.582	1.097	0.778-1.546	0.598
Region 7	0.978	0.728-1.315	0.883	0.989	0.723-1.354	0.947
Region 8	0.882	0.647-1.204	0.430	0.983	0.708-1.365	0.918
Region 9	1.264	0.942-1.695	0.118	1.171	0.854-1.604	0.326
Region 10	1.139	0.852-1.522	0.381	1.217	0.887-1.668	0.223
Region 11	1.279	0.930-1.758	0.130	1.286	0.909-1.820	0.155
Waiting time (month)			0.390			0.430
0-3 months (reference)	1			1		
3.1-6 months	1.050	0.914-1.206	0.489	1.110	0.955-1.291	0.172
6.1-12 months	0.908	0.782-1.054	0.206	0.995	0.842-1.177	0.956
> 12 months	0.966	0.835-1.118	0.643	1.085	0.922-1.278	0.324
Education			0.064			0.173
Elementary or middle school (grade 0-8)	0.964	0.750-1.240	0.512	1.146	0.877-1.497	0.317
High school (grade 9-12)	0.915	0.790-1.060	0.182	0.969	0.827-1.135	0.697
College or technical school	0.819	0.693-0.967	0.013	0.882	0.738-1.053	0.164
Associate or bachelor degree	0.831	0.686-1.007	0.014	0.891	0.728-1.091	0.082
Post college or graduate degree (reference)	1			1		

The adjusted HR (*) was calculated including clinical and sociodemographic variables. Clinical characteristics used for the adjustment were: donor and recipient age, recipient sex, recipient body mass index (BMI), MELD score, history of diabetes and dialysis, functional status. Social characteristics used for the adjustment were the highest level of education obtained by the recipient. The surgical characteristic used for the adjustment was the cold ischemia time. Other characteristics used for the adjustment of the HR were the UNOS region where the transplant occurred and the length of waiting time

Despite these positive changes, other investigators continued to report that African Americans have the lowest survival rate among all LT recipients for benign conditions^[31-33,50,51]. These findings were confirmed by Wong *et al.*^[30] who analyzed the STAR files from 2002 to 2012 and found that African American with HCC

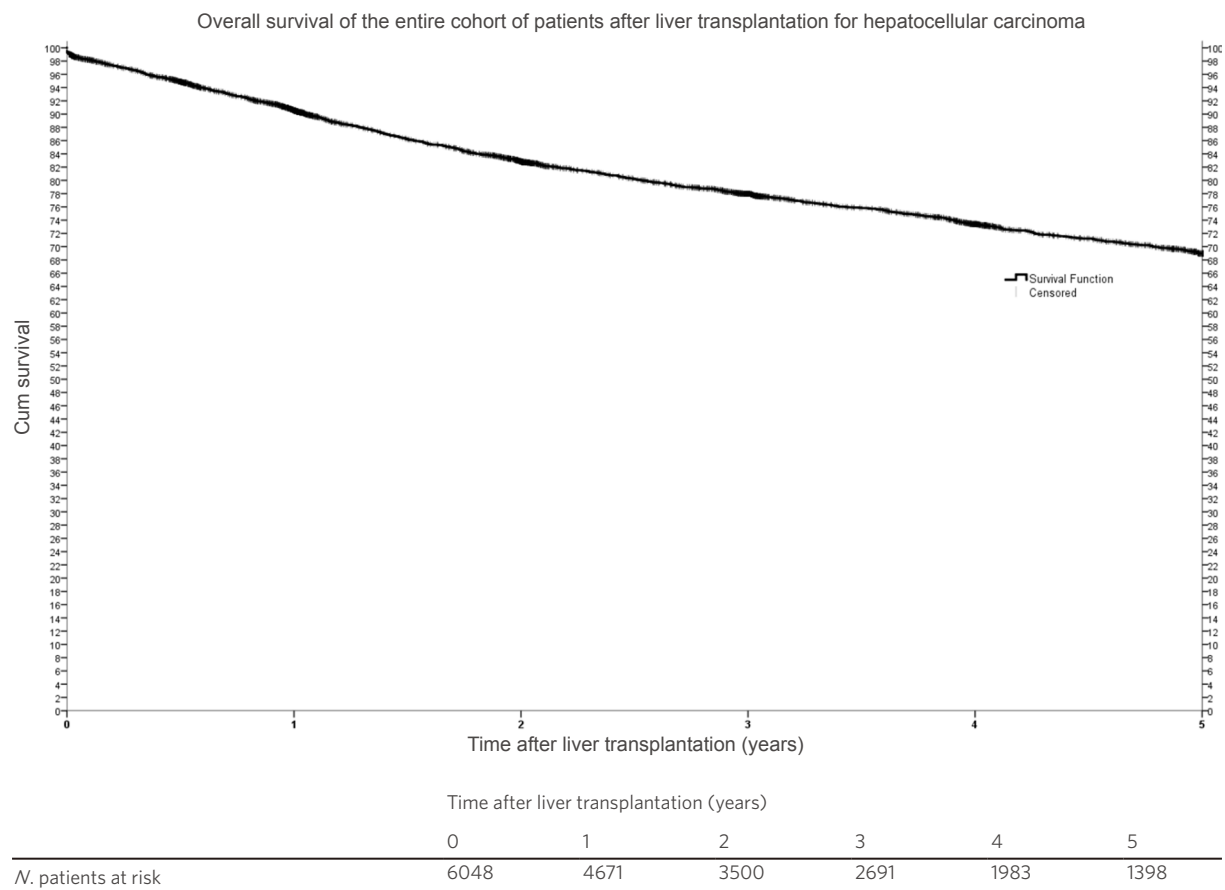
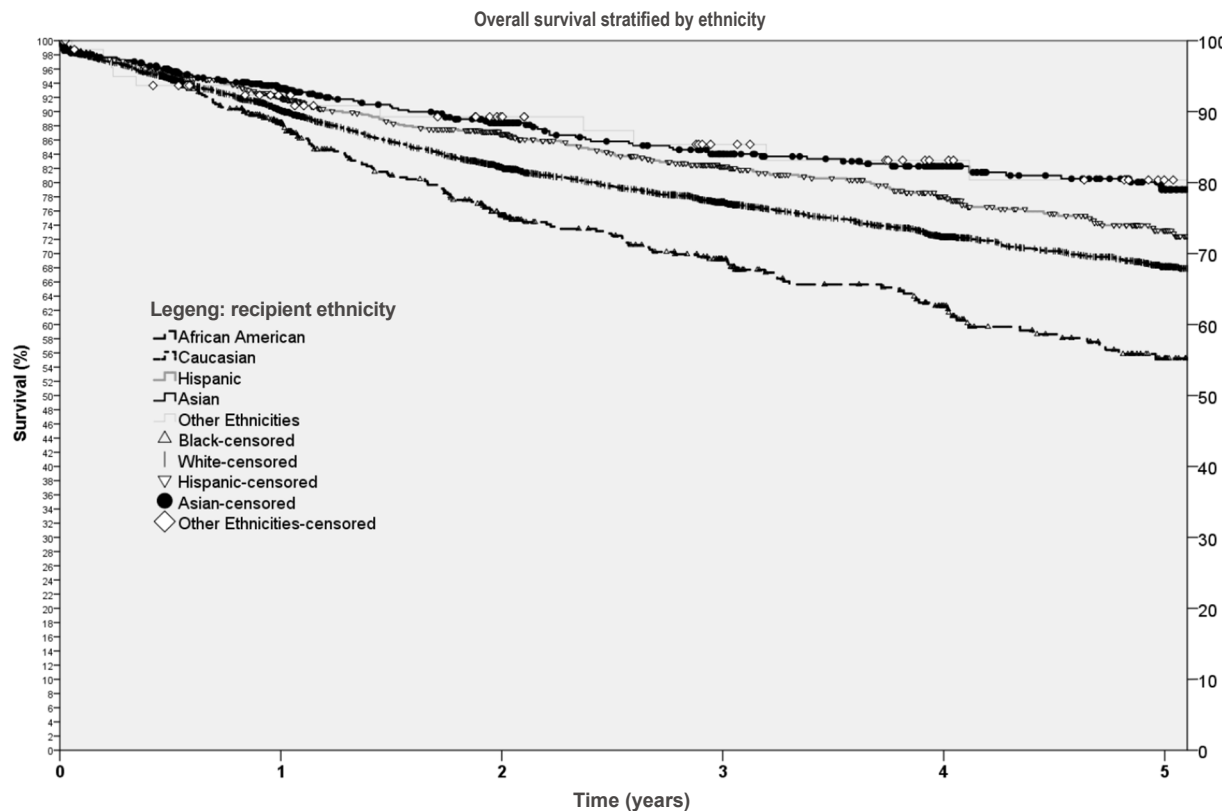


Figure 2. Kaplan Meier survival function representing the 5-year overall survival of all patients undergoing liver transplantation in the United States from 1 Jan, 2002 to 30 Jun, 2013

(HR, 1.49; 95% CI: 1.25-1.79), HCV (HR 1.30; 95% CI: 1.19-1.41) and alcoholic liver disease (HR 1.52; 95% CI: 1.19-1.94) had inferior survival compared to other ethnic groups. One of the limitations of previous studies was the fact that they did not adjust survival analyses for known risk factors such as donor characteristics, cold ischemia time, recipient comorbidities and did not exclude patients whose explanted liver did not have HCC.

Therefore, we analyzed only LT recipients with confirmed HCC with the main intent of testing the null hypothesis that after adjusting for clinical and socio-economic factors, African Americans should have short and long-term outcomes comparable to other ethnic groups. When compared to other ethnicities, we found that African Americans had lower education level, were more frequently affected by renal dysfunction requiring dialysis (2.3% vs. 0.7%) and had a shorter period on the waiting list. Although 30-, 60-, 90-day postoperative mortality was similar between African American and other ethnic groups, their 1-year mortality was higher and their survival started to diverge from all the other ethnicities.

Only 56% of African Americans were alive after 5 years vs. 68% of Caucasians ($P \leq 0.001$), 73% of Hispanics ($P \leq 0.001$) and 79%-81% of Asians and other minorities ($P \leq 0.001$). Multivariate analysis confirmed that African American ethnicity remained the strongest independent predictor of lower survival (HR 1.5; 95% CI: 1.2-1.8) after adjusting for donor and recipient age, sex, BMI, cold ischemia, diabetes and renal insufficiency, MELD score, functional status, waiting time, level of education and UNOS region. These findings rejected our original hypothesis that the outcomes of LT recipients with HCC should be similar among different ethnic groups.



	Time after liver transplantation (years)					
N. patients at risk	0	1	2	3	4	5
African Americans	531	395	275	202	142	89
Caucasians	4,054	3,124	2,329	1,790	1,295	922
Hispanics	859	669	509	382	295	196
Asians	522	416	333	274	215	161
Multiracial or other minorities	82	66	52	40	32	25

Figure 3. Kaplan-Meier survival functions of patients undergoing liver transplantation for hepatocellular carcinoma in the United States stratified by ethnicity. The probability of 5-year survival was 81% for patients belonging to multiracial or other minorities, 79% for Asians, 73% for Hispanics, 68% for Caucasians and 56% for African American ($P \leq 0.001$)

Patients who undergo LT are only a fraction of the number of patients who are referred but fail selection due to insufficient social support, inability to travel to transplant centers or lack of resources including health-care insurance. And, since most of the transplant centers in the United States use comparable criteria for screening patients with inadequate socio-economic resources, and use the Milan criteria for staging HCC irrespective of patient ethnicity, we advanced the hypothesis that unless there were biological reasons, there should not be significant ethnic differences in outcomes after LT.

Overall the results of this study are not novel, yet there are several methodological differences that distinguish our study from others. First of all, we included only patients who had documented HCC in their explanted livers. Confirmation that all recipients in this study had HCC is important because up to 11% of patients who are diagnosed with HCC by imaging tests without biopsy prior to LT end up having no pathological evidence of neoplastic lesions in their explanted livers^[52]. Second, before we analyzed the long-term outcomes, we confirmed that there were no significant differences in perioperative mortality between African Americans and other ethnic groups. Proving that the risk of death at 30-, 60-, and 90-day after LT was similar between the two groups supported the concept that there were no fundamental differences in

pre-existing conditions among different ethnic groups. Third, we performed a multivariable analysis to assess if ethnicity was an independent predictor of patient survival after adjusting for many clinical and demographic factors selected a priori. Among these factors, we included patient characteristics as well as donor and intraoperative variables shown to be associated with long-term outcomes such as cold ischemia time, donor age as proxy for the quality of the liver grafts, history of diabetes, presence of renal failure requiring dialysis prior to transplantation and recipient functional status^[53].

To the best of our knowledge, our study is also the very first to explore if the causes of death after LT were different between African Americans and other ethnic groups. We found that the primary causes of death were similar between African Americans and other ethnic groups except that African Americans had a two-fold risk dying of multiorgan failure (15.4% vs. 7.2%) and half the risk of developing recurrent HCC or new onset of other malignancies (15.4% vs. 31.1%). Although these findings are provocative and would suggest the presence of biological differences among ethnic groups, further investigations are needed as these results might be due to reporting bias, misdiagnosis or erroneous data entry.

Besides the retrospective design of this study, there are several other limitations that are worth mentioning. Although the STAR files have the advantage of containing data on a very large number of transplant recipients, it does not provide enough granularity on the type of insurance, socio-economic status and other personal information that might be important when trying to analyze the impact of socio-economic factors on recipients outcomes and it is subject to data entry errors and miscoding. It is well known that the introduction of random errors reduces the reliability of studies making significant findings less likely^[54]. Therefore, although we recognize the existence of some degree of inaccuracy in the dataset, we suspect that miscoding had occurred randomly with no differences in the frequency of events among ethnic groups. Another limitation is the fact that, there is lack of clear definitions of ethnicities^[55]. Therefore, stratifications of outcomes in this and all other previous studies were performed using self-reported ethnicity. This process has been the norm for health researchers, but self-reporting is a moderate to weak substitution for ancestral genotyping^[56]. Consequently, our results have to be interpreted with some caution since overlapping between ethnic groups is expected. In addition, while our survival analyses were adjusted for many important variables, certain factors that may affect post LT survival such as adherence, HCV status or differences in the pharmacodynamic of immunosuppression medications were not available.

The effect of ethnicity on the pharmacokinetic of commonly used immunosuppressive agents is often underestimated. In a study on immunocompetence between African Americans and Caucasians, Nagashima *et al.*^[57] found that, among patients receiving a tacrolimus-based regimen, African Americans had reduced immunosuppressive effects in comparison to Caucasians with an increased risk of acute cellular and chronic graft rejection^[58,59]. Regarding HCV status, Velidedeoglu *et al.*^[60] found that recipient ethnicity was an independent predictor of survival only in recipients affected by HCV. These findings suggested that the lower survival observed in African Americans may be related to the presence of hepatitis C rather than socio-economic conditions. Unfortunately, due to many missing data on the HCV status of patients with HCC, we were unable to adjust for this important factor. Since the introduction of new antiviral medications that provide sustained virological response in African Americans similar to other ethnic groups, we suspect that HCV positive status will play a very small role in the overall survival of patients undergoing LT in the future.

In conclusion, the findings of our study are several. The first is that the short-term outcomes of African American recipients of cadaveric LTs for HCC are similar to patients belonging to other ethnicities. Second, we confirm that African Americans have the lowest 5-year survival rate among all the ethnic groups after adjusting for several clinical and socio-demographic characteristics. Third, that African American ethnicity and poor functional status at the time of LT are the two strongest predictors of inferior survival.

Previous investigators have suggested that differences in the socioeconomic status might be responsible for the lowest survival observed among African Americans. We recognize that there are many factors that were not accounted in our analysis such as type of health care insurance, household income, serum alpha-feto-protein, number and size of the largest tumor, cellular differentiation and vascular invasion. However, due to similar oncological and socio-economic criteria equally applied across all ethnicities during the evaluation and selection of LT recipients, there might be biological reasons, rather than socio-economic factors responsible for the survival differences observed among ethnic groups undergoing LT for HCC.

DECLARATIONS

Authors' contributions

Michele Molinari designed the study, performed the statistical analysis and wrote the manuscript, Allan Tsung reviewed the statistical analysis and the manuscript, Subhashini Ayloo designed the study, reviewed the manuscript and the statistical analysis, Patrick Bou Samra revised the manuscript and performed the review of the literature, Naudia Jonassaint designed the study, reviewed the manuscript and the statistical analysis.

Availability of data and materials

Data and materials are available from the corresponding author on reasonable request.

Financial support and sponsorship

None.

Conflicts of interest

The authors declare that there are no conflicts of interest related to this study.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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New insights on hepatocellular carcinoma: epidemiology and clinical aspects

Claudio Puoti

Liver Unit, INI Research Institute and Clinics Grottaferrata, Rome 00046, Italy.

Correspondence to: Dr. Claudio Puoti, Liver Unit, INI Research Institute and Clinics Grottaferrata, Rome, Italy.
Email address: puoti@epatologia.org

How to cite this article: Puoti C. New insights on hepatocellular carcinoma: epidemiology and clinical aspects. *Hepatoma Res* 2018;4:57.
<http://dx.doi.org/10.20517/2394-5079.2018.67>

Received: 25 May 2018 **First Decision:** 17 Jul 2018 **Revised:** 29 Jul 2018 **Accepted:** 1 Aug 2018 **Published:** 21 Sep 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cai-Hong Wang **Production Editor:** Zhong-Yu Guo

Abstract

Primary liver cancer represents the 4th most common tumor in males (4% of all cancers) and the 7th most common tumor in females (2.3% of all cancers), with a prevalence of 53/100,000 in males and 22/100,000 in females (male-to-female ratio = 2:1). In the majority of the cases, hepatocellular carcinoma (HCC) develops in patients with cirrhosis and thus the risk factors for HCC and chronic liver disease are overlapping. Viral infections (hepatitis B virus, hepatitis C virus), alcohol and fat (nonalcoholic fatty liver disease/non-alcoholic steatohepatitis) represent the main risk factors for development of HCC on cirrhotic liver. Several prospective studies reported that at present HCC does represent the first cause of death of cirrhotic patients, while in the past morbidity and mortality in cirrhosis were mainly determined by other non-neoplastic complications of the disease. From a clinical point of view, staging systems in HCC should define outcome prediction and treatment assignment. Due to the nature of HCC, the main prognostic variables are the tumor stage, liver function and performance status. The most accepted clinical classification of HCC has been proposed by the Barcelona Clinic Liver Cancer. The BCLC staging system has come to be widely accepted in clinical practice and is also being used for many clinical trials of new drugs to treat HCC. Therefore, it has become the *de facto* staging system that is used.

Keywords: Alcohol, cirrhosis, hepatitis B virus, hepatocellular carcinoma, hepatitis C virus, non-alcoholic steatohepatitis

INTRODUCTION

Hepatocellular carcinoma (HCC) represents one of the most common human neoplasm, being one of the leading mortality worldwide^[1,2]. The main feature of HCC consists in that it affects mostly patients with liver



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cirrhosis, often of viral [hepatitis B virus (HBV), hepatitis C virus (HCV)] or dysmetabolic origin; it means that subjects with HCC suffer from three distinct diseases: the cancer, the cirrhosis and the virus, making more difficult the clinical management of these people.

Indeed, it is known that the mechanisms of hepatocarcinogenesis result from the combination of several causes, such as genetic, immunological, virus-related, environmental and host factors. Host-related factors include male gender, age of at least 50 years, family predisposition, obesity, advanced liver fibrosis or cirrhosis and co-infection with other hepatotropic viruses and human immunodeficiency virus. Environmental factors include heavy alcohol abuse, cigarette smoking, and exposure to aflatoxin^[3].

From a clinical point of view, it should be considered that these people, often critically ill individuals, often elderly, may suffer from clinically relevant abnormalities of haemostasis, renal function and electrolyte balance, and finally often suffer from systemic diseases (heart, lung).

At present HCC does represent the first cause of death of cirrhotic patients^[2], while in the past morbidity and mortality in cirrhosis were mainly determined by other complications of the disease, such as hepatic encephalopathy, upper digestive bleeding from esophageal varices, spontaneous bacterial peritonitis and hepatorenal syndrome. This is mainly due to both early diagnosis and optimized treatment of non-oncologic complications, that increasing life expectancy might be in parallel with the increase of the HCC incidence.

This review is aimed at analyzing available data on the epidemiology and on the clinical aspects of HCC, focusing on the current knowledge about the management of the disease.

EPIDEMIOLOGY

Available international epidemiological data show that primary liver cancer represents the 7th most common tumor in males (4% of all cancers) and the 13th most common tumor in females (2.3% of all cancers), with a prevalence of 53/100,000 in males and 22/100,000 in females (male-to-female ratio = 2:1)^[1,2]. The lifetime (up to 74 years of age) risk of diagnosis of HCC is 17‰ in men (1/59) and 5‰ in women (1/199). Primary liver cancer is the 5th cause of mortality in men (3rd in subjects 50-69 years old) and the 7th in women (4.5% of malignancy-related mortality)^[4,5].

Anyway, relevant geographical differences exist. In Chinese and in African populations, the mean age of patients with the tumor is appreciably younger. This is in sharp contrast to Japan, where the incidence of HCC is the highest in the cohort of men aged 70-79 years. The pattern of HCC occurrence has a clear geographical distribution, with the highest incidence rates in East Asia, sub-Saharan Africa, and Melanesia, where around 85% of cases occur. In developed regions, the incidence is low with the exception of Southern Europe where the incidence in men is significantly higher than in other developed regions^[2].

There is a growing incidence of HCC worldwide. Overall, the incidence and mortality rates were of 65,000 and 60,240 cases in Europe and 21,000 and 18,400 cases in the United States in 2008, respectively. It is estimated that by 2020 the number of cases will reach 78,000 and 27,000, respectively^[2].

Several factors are known to be associated with a higher incidence of HCC: (1) male gender; (2) increasing age; (3) environmental and geographic factors; (4) metabolic and genetic factors (e.g., non-alcoholic steatohepatitis (NASH), genetic hemochromatosis); (5) viral infection; (6) alcohol intake; (7) oncogenic factors (e.g., aflatoxin); and (8) histological stage.

In the majority of the cases, HCC develops in patients with cirrhosis and thus the risk factors for HCC and

chronic liver disease are overlapping^[1]. From a clinical point of view, it means that most of these patients do suffer from three different diseases at the same time, the cirrhosis and the cancer.

In the past morbidity and mortality in cirrhosis were mainly determined by other complications of the disease, such as hepatic encephalopathy, upper digestive bleeding from esophageal varices, spontaneous bacterial peritonitis, hepatorenal syndrome. This is mainly due to both early diagnosis and optimized treatment of non-oncologic complications, that increasing life expectancy might be in parallel with the increasing incidence of HCC^[6]. A significant cohort effect exists, as highest age-specific rates occur among persons aged 75 or older^[7].

The most common causes of HCC in the Western countries are nonalcoholic fatty liver disease (NAFLD)/ NASH, HCV infection, followed by alcohol abuse, mixed viral hepatitis plus alcohol abuse, and HBV infection^[1,7-9]. Indeed, previous epidemiological studies have clearly shown that the prevalence and incidence of HCC significantly differ in developing countries vs. developed countries, as In Eastern Asia and Middle Africa the age-adjusted incidence rate ranges from 20 to 28 cases per 100,000 in men, while this is less than five per 100,000 in Northern Europe, Australia and America^[10]. More in detail, among risk factors in western countries (Europe, America), HCV infection accounts for 60%-70%, HBV for 10%-15%, alcohol for 20% and other risk factors (NASH, hemochromatosis, *etc.*) for the remaining 10%. By contrast, in several areas of Asia and Africa, HBV infection is the higher risk factor (up to 70%), while HCV, alcohol and NASH represent less than 10%-20%.

The annual incidence of HCC in HBV cirrhotics exceeds 2%, while in chronic carriers without cirrhosis the incidence varies between 0.4% and 0.6%, according to gender, age, viral load, geographic area^[8,11,12]. In patients with hepatitis C virus (HCV) infection the increased risk do coincide with the development of cirrhosis, when the yearly incidence varies between 3% and 8%^[13-15]. In patients with genetic hemochromatosis the annual incidence of HCC following establishment of cirrhosis has been calculated to be up to 5%^[3,16]. From a clinical point of view, it has been clearly shown that once the cause of liver damage has been removed, the incidence of HCC decreases, although it is not fully eliminated^[8].

HBV AND HCC

Although rather uncommon, HCC may develop in subjects with chronic HBV infection even in the absence of cirrhosis. In patients with HBV-related cirrhosis, the risk of HCC directly correlates with the degree of serum viral load (serum HBV DNA levels), with the adjusted hazard ratio higher in persons with HBV DNA > 10⁵ cp/mL than in those with HBV DNA levels < 10⁴ cp/mL. It has been clearly shown that the eradication or suppression of the HBV replication by interferon or analogues nucleos(t)ides, significantly reduces, although not eliminate, the risk of HCC in patients with cirrhosis. Antiviral treatment also decreases the risk of hepatic events, liver-related and all-cause mortality over a 5-year observation period, particularly among those with maintained viral suppression^[17-19].

Several factors seem to increase HCC risk among HBV carriers: demographic (male gender, older age, ethnicity, family history of HCC), viral (high viral load, genotype, longer duration of infection, co-infection with HCV, HIV or HDV), clinical (cirrhosis) and environmental (exposure to aflatoxin, heavy alcohol abuse or cigarette smoking). It has been widely reported that chronically infected males have a higher risk of developing HCC if compared to females (2:1 to 3:1)^[20,21] while in developed countries, HCC is rare in patients under 40 years^[20].

One could conclude that: (1) the natural history of chronic hepatitis B is dramatically improved by antiviral treatment; (2) prevention of HCC is not achieved in the absence of stable viral suppression; (3) patients with stable viral suppression show lower rates of hepatic decompensation as well as liver-related mortality and

HCC incidence; and (4) maintained HBV DNA suppression does not fully eliminate the risk of HCC in patients with pre-existing cirrhosis^[19].

Several prediction models of hepatocellular carcinoma development in chronic HBV patients have been proposed. The PAGE-B score has been suggested for assessing HCC risk in HBV afflicted patients^[20].

HCV AND HCC

For whom it concerns the natural history of HCV-related cirrhosis, it has been clearly showed that the progression of chronic HCV hepatitis to cirrhosis is greatly influenced by the age of the patients: 5% of patients under 40 years and 20% of those over 40 years progress to cirrhosis in less than 20 years^[20,22]. HCC risk in chronic HCV patients depends on the severity of fibrosis stage and the rate of progression is approximately 2%-6% per year. It has been established that HCV infected patients have a 15-20 fold risk of developing HCC compared with HCV negative patients^[22].

Previous papers reported that HCV patients achieving sustained virological response (SVR) have a significant reduction of life-threatening complications, such as liver failure and HCC. Cardoso *et al.*^[23] reported the cumulative incidence of HCC and of liver-related complications stratified according to the response to interferon (IFN) treatment, thus confirming that patients with SVR had a paramount reduction of the incidence of HCC with respect to those without SVR. These data were confirmed by Singal *et al.*^[24].

The recent development and widespread availability of the new DAAs of II generation have increased the rate of SVR up to 90%-95%, rapidly decreasing the prevalence of HCV infection. Due to the lack of significant side effects, on the contrary of previous treatment with IFN plus ribavirin, also HCV patients with advanced liver disease or contraindications to IFN might receive this therapy. Although no adequate long-term follow-ups are to date available, it is possible to predict that the incidence of HCC in HCV cirrhotic patients with stable viral eradication will greatly decrease in the next future^[25].

Development of HCV-related HCC in subjects with normal liver has been rarely reported^[26,27].

In conclusion, it is possible to affirm that: (1) in patients with chronic C hepatitis and cirrhosis no correlation exists between serum HCV RNA levels and the severity of the disease, in contrast with HBV-related disease^[28]; (2) in patients with HCV cirrhosis, HCC development is significantly reduced in SVR, while no differences are seen between non responders and untreated people^[23]; (3) prevention of HCC is not achieved in the absence of SVR^[29-31]; (4) due to the paramount virological efficacy of the new DAAs it is possible to predict that in the next years the incidence in HCV cirrhosis will be dramatically reduced^[32].

LIVER STEATOSIS AND HCC

Fatty liver (NAFLD/NASH) and obesity at present represent the leading cause of HCC, at least in developed countries, probably becoming in the next future the main cause for developing HCC^[1-4,33]. In comparison with the lot of papers on the prevalence of HCC in patients with HBV/HCV chronic infections, epidemiological data regarding the prevalence and incidence of HCC in patients with fatty liver are relatively scarce. A systematic review^[34] reported a prevalence of 0%-3% on a follow-up period between 5.6 and 21 years in the whole population of NAFLD/NASH people^[6]. When only patients with steato-cirrhosis were considered, the incidence raise to 2.4% within a follow-up period of 7.2 years and 12.8% with a 3.2-year follow-up^[34].

In a study of HCC management in a realworld setting, including 18,031 patients with HCC in 14 countries (2005-2012), NAFLD accounted for 10%-12% of underlying liver diseases in Europe and North America^[35].

According to data from the Surveillance, Epidemiology and End Results (SEER)-Medicare linked database between 2004 and 2009, NAFLD represented the third most common cause of HCC, after hepatitis C and alcohol-related disease, diagnosed in 14.1% of patients with HCC^[36]. During the six-year study period, an average annual increase of 9% was reported in patients with NAFLD, compared with a 13% increase in patients with hepatitis C.

In a United States population-based study, NAFLD was classified as the most common risk factor for the development of HCC (59%) with a cumulative incidence of 0.3% over a 6-year follow-up^[37]. In another prospective community-based study which evaluated the outcomes of patients with NASH and cirrhosis, 11.3% of patients developed HCC after a mean follow-up of 7.6 years^[38].

As to the prevalence of NAFLD in patients with HCC, several studies showed that steatosis at present does surpass HCV and HBV as the first cause of HCC, ranging from 25% to 35% of all cases.

Moreover, other studies reported development of HCC in non cirrhotic NASH liver^[39,40]. Thus, it is not surprising that NAFLD is the most rapidly increasing indication for liver transplantation (LT) due to HCC.

Beyond advanced liver disease itself^[41], several other factors might interact to increase the risk of HCC in patients with NAFLD, as follows: (1) type 2 diabetes; (2) obesity; (3) genetic background; and (4) co-factors (HBV, HCV, alcohol abuse).

ALCOHOL AND HCC

The relationship between alcohol-related cirrhosis and HCC is now well defined. Alcohol abuse is not only one relevant cause of chronic liver disease and cirrhosis, but strongly interacts with other causes of liver damage, such as HBV and HCV, worsening the progression of the disease and the development of HCC.

The risk of alcohol-related HCC depends upon several factors, as age, gender (more pronounced among females), duration and quantity of alcohol consumption. The 10-year cumulative risk of HCC in patients with alcoholic cirrhosis ranges from 7% to 30%^[42].

Although quantity and duration of alcohol consumption have been associated with ALD progression^[43] and an increased risk for developing HCC^[44], not all patients who chronically overconsume alcohol develop alcoholic cirrhosis and/or HCC. Instead, progression to ALD is influenced by the interaction between consumption and a constellation of host factors, leading to the development of cirrhosis and HCC in only a subset of patients. In other words, although the threshold for development of alcohol-related chronic liver disease is well established (> 30 alcohol units for men and > 20 alcohol units for women), it has not been yet defined the duration/quantity threshold above which the risk for HCC is strengthened^[42].

Probably, other factors might accelerate the progression toward the HCC: genetic factors, ethnicity, first-pass metabolism, volume of distribution, and gastric alcohol dehydrogenase kinetics.

RISK OF HCC AND SURVEILLANCE

According to International Guidelines^[1,2,4,5,8], several groups of patients are considered to have a higher risk of developing HCC, and in these people a strict 6-mo surveillance is mandatory, as follows: (1) cirrhotic patients of any etiology, regardless of Child-Pugh class; (2) non cirrhotic patients with chronic hepatitis B; (3) inactive hepatitis B carriers with viraemia > 2000 UI/mL (evidence 3b, strength B for Western patients; evidence 1b, strength A for Asian patients); (4) non cirrhotic patients with chronic hepatitis C and liver fibrosis \geq F3 Metavir, or \geq 10 kpa at transient elastography (evidence 5, strength D for Western patients;

Table 1. Comparison of demographic, clinical and US risk factors for HCC development

	Lower risk	Higher risk
Gender	Female	Male
Age	< 50 year	> 60 year*
Etiology	Single	Multiple
Co-factors	No	Yes
Child	A	B/C
US pattern	Fine	Coarse nodular
Macronodules	No	Yes
AFP	Normal	Increased

*Asian, African (> 20-40 years). HCC: hepatocellular carcinoma

evidence 3b, strength B for Asian patients); and (5) successfully treated patients with chronic hepatitis B and C (undetectable viraemia), but belonging to any of the previous at risk categories prior to starting antiviral treatment.

The recently published American guidelines^[8]: (1) recommend surveillance of adults with cirrhosis because it improves overall survival (quality/certainty of evidence: moderate - strength of recommendation: strong); (2) suggest surveillance using ultra-sound (US), with or without alpha-fetoprotein (AFP), every 6 months (quality/certainty of evidence: low strength of recommendation: conditional); and (3) suggest not performing surveillance of patients with Child-Pugh class C cirrhosis unless they are on the transplant waiting list, given the low anticipated survival for these patients. (quality/certainty of the evidence: low strength of recommendation: conditional).

In conclusion, the lower risk for developing HCC on cirrhosis is seen in: (1) female gender; (2) age < 50 years; (3) fine US eco-pattern, no macronodules; (4) Child A class; and (5) normal alfa fetoprotein levels.

By contrast, patients at the higher risk of HCC show the following features: (1) male gender; (2) age > 60 years, long history of disease; (3) Asian, African (> 20-40 years); (4) Child B/C classes; (5) persistently high AFP; and (6) US-pattern “coarse nodular” [Table 1].

CLINICAL ASPECTS

The knowledge of the natural history of the disease and prognostic predictors is crucial to estimate the outcome of a given individual and the potential impact of conventional or investigational treatments, as well as to design prospective trials. Survival has improved because of the advancement of the time of diagnosis (lead-time bias) and the increase in the therapeutic efficacy^[45].

When managing HCC in cirrhotic patients, it should be considered that these people: (1) often are critically ill individuals; (2) often elderly; (3) may suffer from clinically relevant abnormalities of haemostasis, renal function and electrolyte balance; and (4) often suffer from systemic diseases (heart, lung).

Several clinical factors might increase mortality in these patients, thus worsening the natural history of the disease and hampering the possibility of effective treatments, such as extra hepatic diseases (lung, heart, kidney diseases), diabetes, obesity, *etc.*

It should be considered that cirrhotic patients often are elderly people, with a long history of disease. Furthermore, the relative risk of liver cancer (95% confidence interval) in obese elderly persons with BMI > 35 kg/m² has been calculated at 4.52 vs. 1.68 of all other cancer^[46]. In these subjects, surveillance for HCC improves the survival of elderly cirrhotic patients by expanding the percentage of cancers amenable to

effective treatments^[47]. Several studies have shown that elderly patients with HCC have a worse prognosis compared to non-elderly ones, but such difference is not due to higher age, but rather seems to be the consequence of undertreatment^[48]. In elderly patients undergoing treatment, survival was unaffected by age^[49].

From a clinical point of view, staging systems in HCC should define outcome prediction and treatment assignment. Due to the nature of HCC, the main prognostic variables are tumor stage, liver function and performance status^[8].

The most accepted clinical classification of HCC has been proposed by the Barcelona Clinic Liver Cancer^[3]. The BCLC staging system has come to be widely accepted in clinical practice and is also being used for many clinical trials of new drugs to treat HCC. Therefore, it has become the de facto staging system that is used, and it was first endorsed by the EASL^[8], and thereafter by the AASLD guidelines for the management of HCC^[3].

This clinical classification does stratify patients with HCC into 5 different stages (stage 0 and stages A to D), according to the ECOG Performance Status (PST) and the Child Pugh Classification. Each stage is further subdivided according to four pre-established prognostic clinical and biochemical parameters (size of the nodule, number of nodules, portal pressure, bilirubin levels).

Beyond its clinical utility, the BCLC staging allows to allocate stage-specific treatment strategies and predicts expected survival.

In summary: (1) The main established parameters for the definition of the stage of HCC are: 1) tumor status; 2) number and size of nodules; 3) presence/absence of macrovascular invasion; 4) presence/absence of extrahepatic spread; 5) liver function; 6) Child-Pugh class; 7) serum bilirubin; 8) albumin levels; 9) presence/absence of portal hypertension; 10) physical status; 11) ECOG classification; and 12) presence of symptoms; (2) prognosis prediction is defined by variables related to tumor status (size, number, vascular invasion, N1, M1), liver function (Child-Pugh's) and health status (Eastern Cooperative Oncology Group, ECOG); and (3) treatment allocation incorporates treatment dependant variables, which have been shown to influence therapeutic outcome, such as bilirubin, portal hypertension or presence of symptoms-ECOG.

The 5-stage classification^[1,4-6] categorizes patients into very early HCC (stage 0), early HCC (stage A), intermediate HCC (stage B), advanced HCC (stage C) and end-stage HCC (stage D).

Stage 0 - patients in the BCLC stage 0 are well-preserved liver function, belonging to the Child Pugh class A and with a performance status 0. In this "very early" status there is a single nodule with size < 2 cm (or carcinoma in situ) without vascular invasion/satellites; portal pressure and bilirubin may be normal or increased. In the first case, patients are suitable for curative treatment as resection; on the contrary, if portal pressure and/or bilirubin levels are increased or extra-hepatic associated disease are present, resection might be contraindicated, and patients should undergo other curative treatments, such as liver transplantation, or local ablation with percutaneous ethanol injection (PEI) or radiofrequency ablation (RFA).

Stage A - patients in the BCLC stage A (early stage) show the following features: (1) single HCC nodule > 2 cm but < 5 cm, or three nodules < 3 cm; (2) ECOG 0; (3) Child Pugh Class A or B; and (4) absence/presence of associated extra-hepatic diseases.

In the absence of associated diseases, the patients might be candidates to liver transplantation; otherwise, local ablation with PEI or RFA should be considered. Single tumors beyond 5 cm are still considered for

surgical resection as first option, because if modern MRI is applied in pre-operative staging, the fact that solitary large tumors remain single and with no macrovascular involvement - which might be common in HBV-related HCC - reflects a more benign biological behaviour^[8].

Variables related to liver function are relevant for candidates to resection. Absence of clinically relevant portal hypertension and normal bilirubin are key predictors of survival in patients with single tumors undergoing resection^[50,51]. Similarly, Child-Pugh class A is the strongest prognostic variable in patients undergoing local ablation, along with tumor size and response to treatment^[50]. Since liver transplantation may potentially cure both the tumor and the underlying liver disease, variables mostly related with HCC have been clearly established as prognostic factors (single tumors < 5 cm or 3 nodules < 3 cm), defining the so-called Milan criteria.

Stage B - patients in the intermediate stage B show multinodular asymptomatic HCC without an invasive pattern. Liver function may be preserved (Child A), or early decompensation might be seen (Child B). Performance Status is = 0. These patients might receive a survival benefit from transarterial chemoembolization, while other treatments such PEI or RFA should be avoided.

Stage C - these subjects suffer from advanced HCC (N1, M1), that consists of macroscopic vascular invasion (portal vein invasion), extrahepatic spread (lymph nodes and metastasis) or cancer-related symptoms (performance status 1-2). They cannot receive treatments other than first line therapy with sorafenib.

Stage D - patients with terminal stage (stage D) have decompensated cirrhosis (Child C) and PST > 2. Only supportive, symptomatic treatment can be offered.

Prognosis and survival

Due to the high clinical variability among the different stages of the BCLC classification, a significant difference in terms of survival exist.

Patients presenting with very early (stage 0) and early-stage diseases (stage A) represent 20%-30% of patients with HCC. This group, suitable for curative treatments such as resection, liver transplantation, or local ablation with PEI or RFA, have a 5-year survival of 50%-70%.

By contrast, patients in intermediate stage B and more advanced stage C stages, who account for 50%-60% of patients, have a poorer prognosis, presenting a 3-year overall survival of 10%-40%. Finally, symptomatic subjects with end-stage disease (stage D; 10%-20%) have a survival < 3 months.

Several new tools will be available to identify cirrhotic patients at higher risk to develop HCC, such as DNA-fusion genes, genetic mutations and epigenetic changes, messenger RNA (mRNA), non-coding RNA-including microRNAs (miRNA), long non-coding RNAs (lncRNA) and other species, proteins and post-translational protein modifications (e.g., phosphorylation), metabolites and antibodies, AFP L3, des-gamma carboxy prothombin^[8,52,53]. In the next future, these tools would be possible biomarkers for prognosis, diagnosis and as therapeutic targets for hepatocellular carcinoma.

OPEN ISSUES AND CONCLUSIONS

Despite substantial advancements in the knowledge and the management of patients with cirrhosis and HCC, several controversies and open issues exist, regarding the timing of surveillance, the optimal diagnostic tools, the increase of HCC after treatment with new DAAs, etc.^[8].

There is considerable debate regarding this latter issue. Indeed, two years ago, two papers from Spain and

Italy suggested an unexpected high rate of early HCC recurrence in patients with HCV-related HCC treated with new DAAs^[54,55]. There are several hypotheses as to why DAA treatment may lead to higher recurrence rates in HCC, one of which is that the activation of regeneration mechanisms through cure of inflammation could lead to growth of precancerous lesions. Another hypothesis involves the liver-specific microRNA 122, which reduces tumorigenesis, angiogenesis and intrahepatic metastasis, and is downregulated by DAA therapy^[56].

Further papers do not confirm this suggestions, showing that DAA treatment is not associated with HCC recurrence after viral clearance in patients with HCV-related cirrhosis and previous history of HCC^[57,58].

Another issue to be further evaluated regards the diagnostic evaluation of suspected HCC with multiphasic CT or multiphasic MRI^[8]. The AASLD recommends diagnostic evaluation for HCC with either multiphasic CT or multiphasic MRI because of similar diagnostic performance characteristics. The selection of the optimal modality and contrast agent for a particular patient depends on multiple factors beyond diagnostic accuracy. These include modality availability, scan time, through-put, scheduling backlog, institutional technical capability, examination costs and charges, radiologist expertise, patient preference, and safety considerations^[8].

In conclusion, in the 21st century, HCC in patients with cirrhosis should be rather regarded as a preventable and treatable disease with current available treatments and not as the beginning of the end, leading inevitably to death. Diagnosis of HCC at present no more implies a “Chronicle of a death foretold”.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Staging of hepatocellular carcinoma

Sedat Karademir

Department of General Surgery, Section of HPB Surgery and Organ Transplantation, Ankara Guven Hospital, Ankara 06540, Turkey.

Correspondence to: Prof. Sedat Karademir, Department of General Surgery, Section of HPB Surgery and Organ Transplantation, Ankara Guven Hospital, Ankara 06540, Turkey. E-mail: sedatkarademir@gmail.com

How to cite this article: Karademir S. Staging of hepatocellular carcinoma. *Hepatoma Res* 2018;4:58.
<http://dx.doi.org/10.20517/2394-5079.2018.40>

Received: 16 Apr 2018 **First Decision:** 29 Jun 2018 **Revised:** 13 Aug 2018 **Accepted:** 22 Aug 2018 **Published:** 26 Sep 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Yuan-Li Wang **Production Editor:** Zhong-Yu Guo

Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths in the world. In contrast to other cancers, survival of patients with HCC is determined by the extent of the tumor in addition to underlying liver disease and its functional reserve. From risk factors to management, HCC reveals a considerable geographic and institutional variation throughout the world. Although many staging and/or scoring systems have been proposed, each prognostic system has several benefits and limitations on its own. Therefore, there is currently no globally accepted system for HCC due to the extreme heterogeneity of the disease. In this review, currently available staging systems for assessing the prognosis of HCC, their uses, limitations, and future prospects are revisited.

Keywords: Hepatocellular cancer, staging and scoring systems, risk factors, survival

INTRODUCTION

Cancer staging systems are important for identification of appropriate therapies and prediction of prognosis for individual patients. Staging in cancer also helps to create a common language in clinical investigations and research^[1]. Since last 2 decades, several staging systems have been proposed for hepatocellular carcinoma (HCC). However, no single system has been universally accepted. Each staging system reflects the features of its own patient population that has been studied on. From risk factors to the treatment given for HCC, considerable geographic and institutional variations exist worldwide. In addition to heterogeneity of the tumor, in each country, availability of surveillance programs, quality of medical technology and accessibility to treatment may influence the prognosis of HCC patients. A staging system which is found to be useful in Western countries may not be similarly suitable for Eastern population. However, external validation of a proposed system for different patient groups worldwide is crucial to reach a common guideline for the management of HCC. So, the



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Table 1. Components of HCC scores and staging systems published in the recent years

Staging systems	Liver functional reserve	Performance status, symptoms	AFP	Tumor status				Other
				Number	Size	Vascular invasion	Metastasis	
Okuda (1985) ^[6]	Ascites, albumin, bilirubin				Yes			
CLIP (1998) ^[7]	Child-Pugh score		Yes	Yes	Yes	Yes		
French (1999) ^[8]	Bilirubin	Karnofsky scale	Yes			Yes		Alk-P, PVT
BCLC (1999) ^[3]	Child-Pugh score	EGOS PST		Yes	Yes	Yes	Yes	
AJCC TNM-7 (2010) ^[5]				Yes	Yes	Yes	Yes	
CUPI (2002) ^[9]	Ascites, bilirubin	Symptoms	Yes	Yes	Yes	Yes	Yes	Alk-P
JIS (2003) ^[10]	Child-Pugh score			Yes	Yes	Yes	Yes	
m-JIS (2006) ^[57]	+ICG-R15 (-encephalopathy)			Yes	Yes	Yes	Yes	
bm-JIS (2008) ^[58]	Child-Pugh score		Yes	Yes	Yes	Yes	Yes	AFP-L3, DCP
Tokyo (2005) ^[11]	Albumin, bilirubin			Yes	Yes			
BALAD (2006) ^[60]	Albumin, bilirubin		Yes					AFP-L3, DCP
ALPCS (2008) ^[48]	Child-Pugh score	Symptoms	Yes		Yes		Yes	Alk-P, Urea, PVT
TIS (2010) ^[63]	Child-Pugh score		Yes	Total tumor volume				
MESIAH (2012) ^[12]	MELD, albumin	Age	Yes	Yes	Yes	Yes	Yes	
HKLC (2014) ^[4]	Child-Pugh score	EGOS PST	No	Yes	Yes	Yes	Yes	
ITA.LI.CA (2016) ^[68]	Child-Pugh score	EGOS PST	Yes	Yes	Yes	Yes	Yes	

HCC: hepatocellular carcinoma; CLIP: Cancer of the Liver Italian Program; BCLC: Barcelona Clinic Liver Cancer; AJCC: American Joint Committee of Cancer; TNM: tumor-node-metastasis; CUPI: Chinese University Prognostic Index; JIS: Japan Integrated Staging; m-JIS: modified JIS; bm-JIS: biomarker combined JIS; BALAD: bilirubin-albumin-AFPL3-AFP-DCP; ALPCS: advanced liver cancer prognostic system; TIS: Taipei Integrated Scoring System; MESIAH: model to estimate survival in ambulatory HCC; HKLC: Hong Kong Liver Cancer; AFP: alpha-fetoprotein; AFP-L3: AFP-Lens culinaris agglutinin-reactive; DCP: des-gamma-carboxy prothrombin; EGOS PST: Eastern Cooperative Oncology Group performance status; ICG-R15: indocyanine green clearance; PVT: portal vein thrombosis

search for a simple, reliable, reproducible and comprehensive staging system continues.

Most HCCs develop upon chronic diseases of the liver, mainly B or C viral hepatitis. Due to the underlying liver disease, prognosis of HCC depends not only on extend of the tumor but also on functional reserve of liver, overall health status of the patient and the treatment given for HCC^[2]. For an accurate prognostication of HCC, parameters which look at all these aspects of prognosis must be included in staging process. In addressing interrelationship of prognostic factors in HCC, several staging systems have been developed but only a few have been widely used and validated.

To date, various parameters have been studied to be of prognostic usefulness in patients with HCC. Parameters based on systematic reviews of the literature and/or expert opinions^[3-5] as well as variables that were significant in multivariable Cox survival analyses^[6-12] were incorporated in these staging/scoring systems. Besides the simple patient related demographic data such as age and gender, many other specific biochemical and clinical variables of liver function, tumor burden and biology as well as age-related clinical consequences and comorbidities have been included in regression analysis of the different studied populations worldwide^[1] [Table 1]. Several biomarkers have also been studied for their prognostic significance in patients with HCC^[13].

The treatment options for patients with HCC are expanding. Depending on the stage of the disease, surgical resection, percutaneous ablation, transarterial chemoembolization and transplantation are being performed either singly or as combination of various modalities. For patients with advanced disease, sorafenib, a multikinase inhibitor is also available. The choice of therapy is influenced by several factors including stage of tumor and severity of underlying liver dysfunction as well as availability of resources and of expertise. Thus, to reach a single staging system and treatment algorithm applicable to all patients with HCC seems to continue to be challenging.

Table 2. Okuda scoring system^[6]

Parameters of advance disease	
Tumor involving > 50% of the liver	
Ascites	
Albumin < 3 g/dL	
Bilirubin > 3 mg/dL	
Stage I	No positive parameter
Stage II	1 or 2 positive parameter(s)
Stage III	3 or 4 positive parameters

CURRENTLY AVAILABLE STAGING SYSTEMS FOR HCC

Okuda score

Okuda staging system was proposed in 1985 based on a study of 850 HCC patients^[6]. This system is the first to combine tumor size (\leq or $>$ 50% of the entire liver) with the variables of liver function such as ascites (presence and absence), serum albumin (\leq or $>$ 3.0 g/dL) and bilirubin levels (\leq or $>$ 3.0 mg/dL). Based on these variables, patients are classified into three stages (I: not advanced; II: moderately advanced; III: very advanced) with different outcomes [Table 2]. Okuda staging system was accepted and widely used as an improved classification system for HCC. However, at the time of its introduction, most HCC cases were diagnosed in the advanced stage (18.5% had surgery). It hardly differentiates the less advanced patients. Therefore, Okuda system is not suitable for the majority of current HCC patients, who are often diagnosed at an early, asymptomatic stage of the disease with possible indication for today's therapeutic modalities. Also, there are major concerns about this system. Considering recent advances in imaging techniques, the only tumor related variable, tumor size (\leq or $>$ 50% of the entire liver) is defined somewhat arbitrarily. It does not include vascular invasion, multicentricity or extrahepatic spread of tumor which definitely affect patient outcomes^[14]. Instead of differentiating early from advance stages, it was found to be useful mainly to identify end-stage patients (stage III), that should be excluded from therapeutic trials due to their poor prognosis. When compared with modern staging systems, it has been shown to have lower predictive capacity^[15-19]. Despite these shortcomings, the Okuda staging system has remained a widely accepted and simple classification system for HCC.

Cancer of the Liver Italian Program score

The Cancer of the Liver Italian Program (CLIP) score was proposed by an Italian group in 1998 based on a retrospective analysis of 435 HCC patients treated at 16 Italian institutions^[7]. Of these, only 12 (2.8%) had surgery and 247 (56.8%) underwent locoregional therapy. CLIP was designed to overcome the deficiencies of the tumor-node-metastasis (TNM) system. It takes into account the Child-Pugh status of the patient with tumor characteristics including tumor morphology and extension, the portal vein thrombosis and levels of alfa-fetoprotein (AFP) assign a score (0, 1, 2) to each variable [Table 3]. Patients are classified into seven groups according to the sum of these scores (0-6). CLIP is easy to calculate, well correlated with survival. CLIP-0 patient has a better prognosis in comparison to one with CLIP-6 (42.5 mo vs. 1.0 mo of median survival). However, in this system, information regarding underlying liver diseases, performance status and extrahepatic metastasis which affect the outcomes were lacking. Additionally, it does not offer any appropriate therapy for HCC patients.

This scoring system was validated prospectively in 196 HCC patients and showed greater predictive power than Okuda staging system^[20]. Although, the CLIP score was developed using an appropriate method and has been externally validated in several (Canadian, Italian and Japanese) cohorts^[18-21], this score has some limitations when applied to patients with the early stage of HCC. In countries like Japan, where many smaller tumors are detected based on the established screening system for HCC, the CLIP score cannot effectively identify early-stage patients who can benefit from radical treatment.

Table 3. CLIP scoring system^[7]

	Scores
Child-Pugh stage	
A	0
B	1
C	2
Tumor morphology	
Uninodular and extension ≤ 50%	0
Multinodular and extension ≤ 50%	1
Massive or extension > 50%	2
Alpha-fetoprotein (ng/dL)	
< 400	0
≥ 400	1
Portal vein thrombosis	
No	0
Yes	1

CLIP: Cancer of the Liver Italian Program

Table 4. French scoring system^[8]

	Scores			
	0	1	2	3
Karnofsky index (%)	≥ 80			< 80
Serum bilirubin (μmol/L)	< 50			≥ 50
Serum alkaline phosphatase (ULN)	< 2		≥ 2	
Serum alpha-fetoprotein (μg/L)	< 35		> 35	
Portal obstruction (sonography)	No	Yes		

ULN: upper limit normal

In different studies, nearly 80% of the patient population is classified as having a CLIP score of 0-2 which shows its poor stratification ability^[18,22,23]. One possible reason may be the definition of tumor extension (less or more than 50% of total liver volume) which is somewhat subjective and may compromise the reliability of CLIP in predicting patient outcomes^[24-27]. Still, CLIP is recently ranked first for its ability to predict survival^[22].

GRETCH Score

GRoupe d'Etude et de Traitement du Carcinoma Hépatocellulaire (GRETCH) system was proposed by the French group Goupe d'Etude et de in 1999^[8]. This system was constructed with the analysis of 761 HCC patients treated at 24 centers. The group has created a score quite similar to the CLIP aiming at a simple classification that would predict survival. Unlike CLIP, GRETCH further includes performance status but lacks tumor morphology information. GRETCH staging divides the patients into three risk groups (A, B, C) on the basis of performance status, serum bilirubin, serum alkaline phosphatase (ALP), serum AFP and portal vein obstruction on ultrasound [Table 4]. The overall survival (OS) differs markedly for the three groups, with a one-year survival rate in group A (low risk to death) of 72%, compared to 34% in group B (intermediate risk of death) and 7% in group C (high risk of death). The strength of this system is that it is based on baseline characteristics that are routinely available at diagnosis and the scores allocated to the respective predictive factors are based on the estimated Cox regression coefficient. However, in this study, 53% of HCC patients did not receive any specific therapy, while only 7.4% underwent surgical resection. Therefore, this score may not be suitable for predicting the survival of HCC patients who undergo surgical resection. In addition, this cohort mostly included patients at advanced stages. A recent comparison with other staging systems has shown that it has limited prognostic capacity in patients with early HCC^[15].

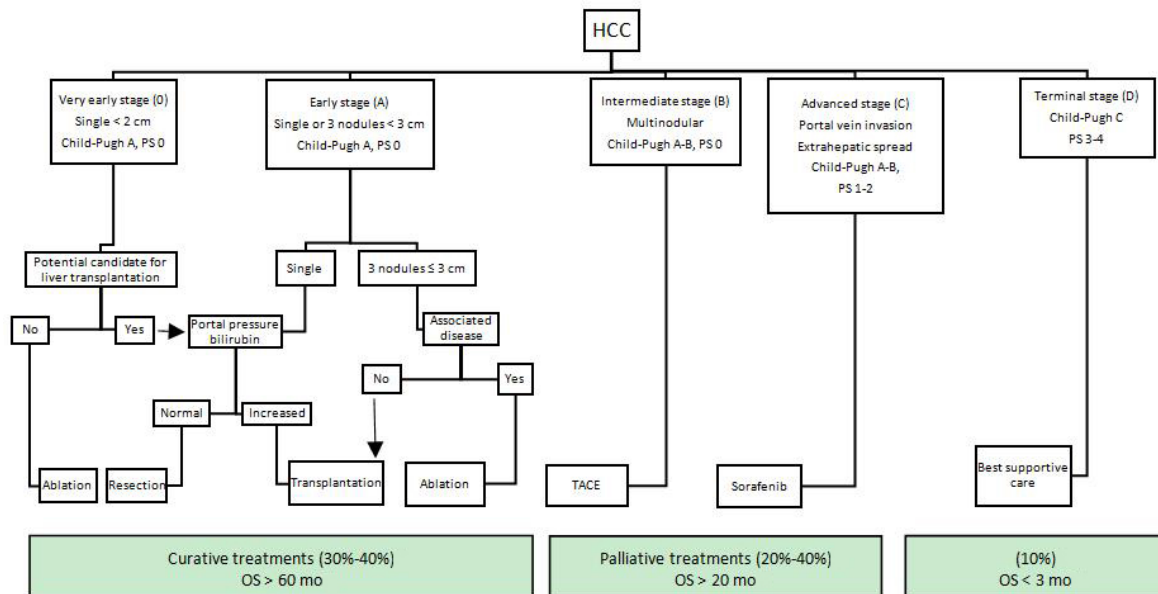


Figure 1. BCLC staging system. PS: performance status; OS: overall survival; BCLC: Barcelona Clinic Liver Cancer

Another issue regarding GRETCH score is use of ultrasound in face of recent advances in imaging. More informative techniques rather than ultrasound may be used to evaluate portal venous anatomy.

Barcelona Clinic Liver Cancer classification

The Barcelona Clinic Liver Cancer (BCLC) classification for HCC was proposed by Llovet *et al.*^[3] in 1999. The BCLC staging system is developed from the results obtained in the setting of several cohort studies and RCTs^[1,3,28]. As it is not based on identification of prognostic factors from a regression analysis, BCLC is not able to predict the mortality in HCC patients^[26]. The notable feature of BCLC is being the first system which recommends evidence-based clinical treatment for each patient at different stages [Figure 1]. Its treatment algorithm has also been recognized as a guideline by renown societies (the American Association for the Study of Liver Diseases, the American Gastroenterological Association and European Association for the Study of the Liver) for management of HCC^[29,30].

BCLC includes predictors of prognosis in HCC patients including tumor extension, liver functional reserve and overall physical status (PS). Tumor extension contains the number of tumors, tumor size and presence of portal vein invasion or extrahepatic metastasis. Child-Pugh grade is replaced for liver functional reserve and the PS is determined by corresponding Eastern Cooperative Oncology Group (ECOG) performance status^[31]. Patients are subsequently assigned to five categories (0, A, B, C and D) based on these variables: stage 0 (very early stage) describes patients with well-preserved liver function (Child-Pugh A), one asymptomatic tumor < 2 cm and no vascular invasion or satellites. Stage A (early stage) covers Child-Pugh A or B patients with one tumor of any size or 2-3 tumors all < 3 cm. Stage B (intermediate-stage) defines patients with Child-Pugh A or B status, multiple tumors without vascular invasion or extrahepatic metastasis. Child-Pugh A or B patients with vascular invasion or extrahepatic metastasis of tumor and in relatively good PS (1-2) are assigned to stage C (advanced stage). Finally, stage D (terminal stage) corresponds to patients with Child-Pugh C status in any tumor stage and poor PS (> 2)^[3,32].

Overall, the BCLC staging system identifies early, intermediate, advanced, and end stage HCC patients who may benefit from curative therapies, palliative treatments or best supportive care. Curative treatment options such as surgical resection, liver transplantation and ablation are recommended for patients with

early stage HCC (stages 0 and A) (median OS > 60 mo). For stage B patients, palliation with transarterial chemoembolization (TACE) is recommended with median OS of 20 months. Sorafenib, multikinase inhibitor, was added to treatment repertoire in 2008 for patients with advanced disease (stage C) (median OS = 11 mo). And, for patients at terminal stage with life expectancy of < 3 months, best supportive care is recommended.

Compared to Okuda and CLIP systems, early stage HCC is defined in more details (number and size of nodules, the associated comorbidities and the portal vein pressure) in BCLC which makes it more suitable to select early stage patients who could benefit from curative therapies^[15,33]. However, the BCLC has shown to have lower prognostic ability than CLIP score regarding advance HCC^[34,35].

BCLC has been externally validated in several Western countries where main etiologies are hepatitis C virus (HCV) infection and alcohol abuse^[2] and are found to have a better ability to predict survival than most other staging systems^[23,36]. Although BCLC has been widely used in Western countries, many Asian experts find its treatment modalities to be too conservative. In contrast to BCLC, Asian guidelines indicate surgical resection and TACE for more advanced tumors. Even then, some studies by Asian groups proved BCLC to be a superior staging system^[37].

Despite its popularity, several studies have shown that BCLC staging system has some limitations. These are mainly related to the heterogeneity of BCLC stages B and C patients in respect to tumor burden and liver function^[38]. For example, patients with multinodular disease without vascular invasion are assigned to intermediate stage (BCLC B) and only a single therapeutic option, TACE is offered. However, resectability of multifocal HCC is closely related to location of tumors. A patient with multiple small tumors confined to the same lobe may still be considered as a good candidate for resection, instead of transarterial chemoembolization. Additionally, tumors with portal invasion (BCLC C) are recommended to be treated only with sorafenib^[29]. For these patients, there are studies which suggest extending the indication for surgery^[39-41] or chemoembolization^[42,43]. Even for a Child-Pugh C patient with HCC within the Milan criteria, the possibility of liver transplantation may be considered. On the other end, in BCLC 0 and A patients with early stage HCC, a single liver tumor is resected only in absence of portal hypertension where it might not affect survival in many resected patients. In current practice, sequential or combined treatments are highly preferred in the multidisciplinary management of HCC (TACE followed by resection or LT, TACE + RF or sorafenib). Under these circumstances, BCLC's one-to-one correspondence treatment recommendations for each stage may not be suitable for use in actual clinical practice^[26,44]. Another critic on BCLC is regarding the controversial prognostic role of variable ECOG PS which is somewhat subjective and may be affected by liver function and cancer symptoms^[45].

The BCLC has shown to have lower prognostic ability than CLIP score regarding advance HCC^[36,46,47]. A new score, advanced liver cancer prognostic system (ALCPS) was proposed by Yau *et al.*^[48] aiming at improving patients selection but found to be too complex for daily clinical practice as it includes eleven variables with different coefficient as in the Chinese University Prognostic Index (CUPI) score^[9].

TNM (AJCC)

The TNM classification was developed by the American Joint Committee on Cancer (AJCC) and International Union for Cancer Control (UICC) and has been updated regularly since the first edition was published in 1977. This system is successfully used by oncologists in several fields. However, the classical staging system based on TNM is not used for HCC. It assesses the extension of the primary tumor, lymph node involvement and extrahepatic metastasis but does not include any measurements of liver function or the health status of the patient. Because of this it has often been used in combination with other criteria such as the Child-Pugh classification or included in other grading systems such as the CLIP score. TNM staging

Table 5. Simplified staging system (Vauthey *et al.*^[51]-TNM 6th edition)

sT1	Single tumor without vascular invasion
sT2	Single tumor with vascular invasion or multiple tumors, none > 5 cm
sT3	Multiple tumors, any > 5 cm or tumor(s) involving major branch of hepatic vein(s)
F0	Grade 0-4 fibrosis (no fibrosis to moderate fibrosis)
F1	Grade 5-6 (severe fibrosis or cirrhosis)
Stage I	sT1 N0 M0
Stage II	sT2 N0 M0
Stage IIIA	sT3 N0 M0
Stage IIIB	Any sT N1 M0
Stage IV	Any sT any N M1

s: simplified; TNM: tumor-node-metastasis

system has been mostly tested in the surgical setting and showed poor prognostic prediction in early HCC patients undergoing either resection^[49] or transplantation^[50].

Vauthey *et al.*^[51] developed a simplified staging system for HCC in 2002 which was adopted as the TNM staging system of AJCC/UICC after minor changes (6th Edition) [Table 5]. It was derived from the finding of a cohort of 557 HCC patients who underwent surgical resection. The authors identified independent predictors of mortality (major vascular invasion, microvascular invasion, severe fibrosis/cirrhosis, multiple tumors and a tumor size greater than 5 cm) using a multivariate analysis. Based on these variables, the AJCC T classification reclassified and a simplified stratification was proposed: sT1: single tumor with no vascular invasion; sT2: single tumor with microvascular invasion or multiple tumors, none more than > 5 cm and sT3: multiple tumors, any > 5 cm or tumor(s) with major vascular invasion. The simplified staging system divides patients into 3 independent prognostic groups (5-year survival rates: stage I 55%, stage II 37% and stage III 16%). The new system may improve the stratification of resected tumors, even though it is controversial whether they will apply to nonsurgical patients. As TNM staging relies on detailed histopathologic examination which requires two fine-needle biopsies, this might be associated with risk of tumor seeding^[52].

The current AJCC/UICC 7th edition is a modification of the simplified staging system and has become widespread since 2010^[5]. The major change between the 6th and the 7th AJCC staging system is that the new system imposes heavier prognostic weight on major vascular invasion as a potential predictive factor for poor prognosis^[53]. The main limitation of this staging system is that it fails to account for liver function whereas it is well known that prognosis of HCC patients also relies on features related to liver cirrhosis^[49,50].

Japan Integrated Staging Score

The Japan Integrated Staging Score (JIS Score) was proposed by Kudo *et al.*^[10] in 2003. It is derived from a cohort of 722 HCC patients treated at two Japanese institutions. The JIS score combines the Child-Pugh grade with the Japanese TNM (Liver Cancer Study Group of Japan- LCSGJ) which is based on three parameters (vascular invasion, single vs. multiple nodules, diameter ≤ vs. > 20 mm) to address the specific deficiency of LCSGJ for not having included liver function evaluation. Patients with a Child-Pugh grades A, B and C status are allocated a score of 0, 1, and 2, respectively. Patients with the TNM stage by LCSGJ of stages I, II, III and IV are allocated to score of 0, 1, 2 and 3, respectively^[54] [Table 6]. Patients are subsequently classified into six groups (0-5) based on the sum of these scores. Statistically significant differences are observed between the survival curves for almost all JIS scores. The cumulative 10-year survival rates of the best prognostic groups in the CLIP staging system (CLIP score 0) and JIS staging system (JIS score 0) were 23% and 65%, respectively. The authors concluded that the JIS score stratifies patients with early diagnosed HCC better than the CLIP score. The same group externally validated the JIS score in 4525 HCC patients treated at five Japanese institutions in 2004^[55]. In a study of 1679 patient, the JIS score has been compared with the BCLC and CLIP and found to be superior in prognostic determination^[56]. Since the JIS score was

Table 6. Japanese Staging System (LCSGJ-TNM)^[55] and Japan Integrated Staging (JIS) score^[10]

LCSGJ-TNM	
T criteria	
Single tumor	
Size < 2 cm	
No vascular involvement	
T1	All 3 features
T2	2 of 3 features
T3	1 of 3 features
T4	None of 3 features
Stage I	T1 N0 M0
Stage II	T2 N0 M0
Stage III	T3 N0 M0
Stage IVA	T4 N0 M0 or any T N1 M0
Stage IVB	Any T N0-1 M1
Japan Integrated Staging (JIS)	
Stage I	0
Stage II	1
Stage III	2
Stage IV	3
Child-Pugh A	0
Child-Pugh B	1
Child-Pugh C	2

LCSGJ: Liver Cancer Study Group of Japan; TNM: tumor-node-metastasis, TNM: tumor-node-metastasis

developed based solely on Japanese HCC patients, prospective validation studies are required in Western population. The other limitations inherent in the LCSGJ staging system such as inaccurate weighting of size and vascular involvement as well as the lack of incorporation of microscopic pathology information remain.

This score was further improved a few years later with the modified-JIS in which the encephalopathy item is replaced by the indocyanine green clearance (ICG-R15), due to an early HCC screening in Japan and a preferred surgical orientation^[57]. The substitution of ICG-R15 for encephalopathy in Child-Pugh grade might have reflected individual differences more accurately among patients who underwent hepatic resection, because none of the patients in the present study had any encephalopathy before operation^[57].

JIS has been recently refined as biomarker combined JIS (bm-JIS) by including AFP, AFP-Lens culinaris agglutinin-reactive (AFP-L3) and des-gamma-carboxy prothrombin (DCP) which allowed better survival predictions^[58]. However, two of those markers are not frequently used in Western countries where HCC is also often being diagnosed at more advanced stages. Thus, this score has not been evaluated on patients from Western countries.

CUPI score

The CUPI for HCC was identified on the basis of a cohort of 926 Chinese patients, most of them with hepatitis B virus (HBV) related cirrhosis^[9]. CUPI combines the conventional TNM system and a number of other factors of liver function and tumor load (serum bilirubin, ascites, ALP, serum AFP and asymptomatic disease on presentation) [Table 7]. Patients are subsequently divided into three groups (low-risk, intermediate-risk and high-risk) according to the sum of the weights of the six prognostic factors. The median survival for the low-risk, intermediate-risk, and high-risk groups were 10.1 months, 3.7 months and 1.4 months, respectively. The authors estimate that this classification has better estimation of survival than CLIP score and Okuda stage, although its discriminatory power in early stages is questionable, as the best 1-year survival was around 50%. In 2011, the group validated the CUPI system in another cohort of 595 HCC patients with predominant HBV infection^[59].

Table 7. Chinese University Prognostic Index^[9]

	Scores
TNM stage	
I and II	-3
IIIa and IIIb	-1
IVa and IVb	0
Asymptomatic disease on presentation	-4
Ascites	3
Alpha-fetoprotein (≥ 500 ng/mL)	2
Total bilirubin ($\mu\text{mol/L}$)	
< 34	0
34-51	3
≥ 52	4
Alkaline phosphatase (≥ 200 units/L)	3

The prognostic factors used in this system are readily available in daily clinical practice and the score is determined based on the estimated Cox regression coefficient. However, CUPI was derived from a cohort of HCC patients primarily with HBV infection (79% of the whole cohort). Thus, this system may not be suitable for application in Western populations with predominant HCV infection or a history of alcohol abuse. Another criticism levelled at CUPI was that only a small proportion of early-stage HCC patients have received surgery (10.4%). Most of the patients in this cohort were in late stage and received only supportive care (58.4%)^[9]. Therefore, this system may not be preferred for assessing patients who undergo curative treatment, such as surgical resection or radiofrequency ablation (RFA). In comparison to other staging systems, the CUPI has not shown a prognostic advantage over other systems and has failed to gain widespread acceptance and usage. Moreover, though it is mainly used in Asian populations with a background of hepatitis B, still there is no evidence that CUPI has universal applicability among liver cancer patients of other races.

Tokyo score

Tokyo score was proposed by Tateishi *et al.*^[11] in 2005 based on a retrospective analysis of 403 HCC patients treated by percutaneous ablation at the University of Tokyo and was validated in 203 HCC patients who underwent surgery resection at the same institution. The main purpose of this study is to develop new prognostic scoring system for patients at early-stage who are candidates for radical therapy, such as percutaneous ablation or surgical resection. They used only serum albumin and bilirubin values as indicators of remnant liver function. This system consists of four factors: tumor size, number of tumor nodules, serum albumin and bilirubin which can be easily obtained from daily laboratory data or images before surgery [Table 8]. Scores are assigned to each of the four variables according to the estimated regression coefficient. Patients have total scores ranging from 0 to 6, Tokyo-0 patient having a better prognosis than those patients with Tokyo-6 (five-year survival rates of 78.7% vs. 14.3%, respectively). In validation study, Tokyo staging system has shown to have a predictive ability equal to CLIP and better than BCLC classification^[11].

Tokyo score is useful in Japanese patients with early stage HCC requiring radical therapy but not suitable for use in patients with advanced stages of disease. Thus, its validation is required in Western population. Performance status and cancer-related symptoms have not been included in Tokyo score because most HCC patients in Japan were diagnosed at an early, asymptomatic stage of the disease due to nationwide screening program for viral hepatitis and surveillance in high-risk groups for HCC.

Bilirubin-albumin-AFPL3-AFP-DCP score

Bilirubin-albumin-AFPL3-AFP-DCP (BALAD) score is proposed by Toyoda *et al.*^[60] in 2006 for the purpose of providing a simple and objective staging system that requires no imaging studies, pathological or clinical

Table 8. Tokyo score^[61]

Parameters	Scores
Albumin (g/dL)	
> 3.5	0
2.8-3.5	1
< 2.8	2
Total bilirubin (mg/dL)	
< 1	0
1-2	1
> 2	2
Tumor size (cm)	
< 2	0
2-5	1
> 5	2
Tumor number	
≤ 3	0
> 3	2

Table 9. BALAD score^[60]

	Bilirubin-albumin score				BALAD score			
	0	1	2		0	1	2	3
Serum bilirubin (mg/dL)	< 1.0	1.0-2.0	> 2.0	Bilirubin-albumin score*	A	B	C	
Serum albumin (g/dL)	> 3.5	2.8-3.5	< 2.8	Number of elevated tumor markers	0	1	2	3

*A: 0-1 points; B: 2-3 points; C: 4 points; BALAD: bilirubin-albumin-AFPL3-AFP-DCP

evaluations. This score is derived from the findings of a cohort of 2600 HCC patients treated at five Japanese institutions. BALAD scoring system is based on 5 serum markers: bilirubin and albumin as indicators of liver functional reserve, lens culinaris agglutinin reactive AFP-L3 > 15%, AFP > 400 ng/dL and DCP > 100 mAU/mL as factors reflecting tumor progression [Table 9]. Based on the sum of the scores assigned to these factors Japanese population of HCC could be stratified into six groups with distinct survivals. The discriminative ability of the BALAD score is comparable to that of the CLIP score and JIS score. The BALAD score has been validated in an independent cohort of Japanese population of HCC, as well as in the Caucasian population of HCC, and the score is consistently able to stratify the outcome of HCC into six group of patients with distinct median OS^[54,61]. It has also been studied in a Chinese patient population of HBV related HCC and shown to have the corresponding median OS of BALAD scores of 1, 2, 3 and 4 as 26.6, 8.3, 2.6, and 1.9 months, respectively^[62]. Although the BALAD score is a simple and objective tool for staging HCC it is not easy to measure the AFP-L3 and DCP values in routine clinical practice worldwide.

ALPCS

The ALPCS (Advanced Liver Cancer Prognostic System) was constructed by Yau *et al.*^[48] in 2008. It is derived from the analysis of a cohort of 1470 advanced HCC patients treated at a single center. To classify advanced HCC patients not indicated for surgical resection or locoregional therapy the authors identified 11 prognostic factors (ascites, abdominal pain, weight loss, Child-Pugh grade, ALP, serum total bilirubin, serum AFP, serum urea, tumor size, portal thrombosis and lung metastasis) using a multivariate Cox model and a point is given for each prognostic factor according to its statistical weight [Table 10]. Patients are subsequently divided into three prognostic groups (good: score ≤ 8, intermediate: 9-15 and poor: ≥ 16) [Table 11]. Survival curves for each prognostic group show clear differences, with a median OS of 7.9, 3.2 and 1.4 months for the good, intermediate and poor prognostic groups, respectively. The authors showed that the discriminatory ability of the ALPCS is significantly better than that of the Okuda system and CLIP score. However, the majority of patients included in this study (73% of the whole cohort) were hepatitis B-related HCC. Therefore, ALPCS needs to be validated in a Western population with predominant HCV infection or alcohol abuse. In

Table 10. Advanced liver cancer prognostic system^[48]

Parameters	0	1	2	3	4	5
Ascites	No		Yes			
Abdominal pain		No		Yes		
Weight loss	No		Yes			
Child-Pugh	A		B			C
ALP (IU/L)	≤ 200			> 200		
Total bilirubin (mg/dL)		≤ 2	2-3		> 3	
Urea (mmol/L)		≤ 8.9		> 8.9		
Portal vein thrombosis		No			Yes	
Tumor size	≤ 5 cm			> 5 cm		Diffuse
Lung metastasis		No			Yes	
Alpha-fetoprotein (ng/mL)	≤ 400				> 400	

ALPCS: advanced liver cancer prognostic system; ALP: alkaline phosphates; IU: international unit

Table 11. Survival for each prognostic group of corresponding ALPCS score

Prognosis	Score	3 month survival
Good (0-8)	0-2	> 0.81
	3-6	0.72-0.8
	7-8	0.66-0.69
Intermediate (9-15)	9	0.63
	10-12	0.51-0.59
	13-14	0.42-0.47
	15	0.38
Poor (16-39)	16	0.33
	17-19	0.21-0.29
	20-22	0.1-0.17
	≥ 23	< 0.1

Table 12. Taipei Integrated System^[63]

	Scores			
	0	1	2	3
Total tumor volume (cm ³)	< 50	50-250	250-500	> 500
Child-Pugh	A	B	C	
Alpha-fetoprotein (ng/mL)	≤ 400	> 400		

addition, considering 11 factors included into the system, calculation of the score is somewhat complicated in daily clinical practice.

Taipei Integrated Score System score

The Taipei Integrated Score System (TIS) was proposed by Hsu *et al.*^[63] in 2010. This system is derived from the study of a cohort of 2030 HCC patients undergoing different treatment modalities at a single institution in Taiwan. The authors included the total tumor volume (TTV) as an indicator of tumor burden and combined it with Child-Pugh grade (A, B and C: 0, 1 and 2 points, respectively) and AFP (<400 vs. > 400 ng/mL: 0 vs. 1 point) [Table 12]. Calculated TTV was categorized into four groups (< 50 cm³, 50-250 cm³, 250-500 cm³ and > 500 cm³: 0, 1, 2 and 3 points, respectively). The score identified six distinct prognostic groups. TIS shows superior prognostic value compared with the four current staging systems (CLIP, BCLC, JIS and Tokyo) for the whole cohort, independently of the treatment modality (curative or palliative). However, in a subgroup of 936 patients treated with curative intent, TIS failed to CLIP probably related to vascular invasion (a factor in the CLIP but not in the TIS) that was observed in 36.7% of the patients. Although the TTV based staging system is a useful and reliable system, it has some limitations. First, all tumors are not spherical. Therefore, TTV value may not be accurate in cases involving tumors that are infiltrative or numberless. Second, TIS

Table 13. MESIAH Score^[12]

MESIAH Score
- 0.232* (age in decades)
+ 0.099* (MELD)
- 0.391* (serum albumin level)
+ 0.290* (tumor size)
+ 0.153* (tumor number)
+ 1.122* (vascular invasion)
+ 1.130* (extrahepatic metastasis)
+ 0.082* (serum alpha-fetoprotein level)
+ 1

MESIAH: model to estimate survival in ambulatory HCC; MELD: model for end-stage liver disease

was constructed based on the results for a cohort of HCC patients with predominant HBV infection (HBV 51%, HCV 27%). Therefore, it needs to be externally validated in Western population.

Model to estimate survival in ambulatory HCC patients score

The model to estimate survival in ambulatory HCC patients score (MESIAH score) was developed by Yang *et al.*^[12] from the Mayo group, in 2012. MESIAH score is derived from a cohort of 477 HCC patients treated at the Mayo Clinic [derivation cohort (DC)] and 904 HCC patients treated at a Korean institution [validation cohort (VC)]. Validation was done using a data set that is racially, geographically, chronologically and diagnostically disparate from the derivation set. The DC differed from VC with regard to the underlying liver disease (DC = HCV 81% *vs.* VC = HBV 75%) and treatment modality (DC = transplantation 31%, resection 17%, TACE 25% *vs.* VC= resection 13%, TACE 57%). The authors identified independent predictors for survival in a multivariate Cox model [age, model for end-stage liver disease (MELD) score, serum albumin level, tumor size, tumor number, vascular invasion and extrahepatic metastasis], thus creating a new risk score [Table 13]. The authors include MELD as an indicator of liver disease severity. MELD has been shown to be a useful measure of hepatic insufficiency since it was adopted as a standard to determine organ allocation priorities among liver transplant candidates in the USA and elsewhere^[64], MELD is consisted of only laboratory variables (bilirubin, INR, creatinine) which are widely available and reproducible. The prognostic value of the MESIAH score was confirmed in the VC. The predictive accuracy of MESIAH is highly stable, irrespective of the underlying liver disease and/or treatment modality. More recently, the same group validated this score in another cohort of 1969 HCC patients with predominant HBV infection (74.6%) treated at a Korean institution^[65]. The discriminatory ability of the MESIAH score is better than that of the BCLC, CLIP, JIS and Tokyo. However, calculating the MESIAH score is somewhat complicated in daily clinical practice. Considering the advantages of superior predictive accuracy and objectivity and reproducibility of the prognostic factors, independent of the underlying liver disease and treatment modality, the MESIAH score is one of the most promising staging systems for evaluating HCC patients.

Hong Kong Liver Cancer classification

The Hong Kong Liver Cancer (HKLC) classification was developed by a Hong Kong group in 2014^[4]. Like the BCLC, HKLC links HCC stages to treatment options. This system is based on four established prognostic factors: ECOG PS, Child-Pugh grade, liver tumor status and presence of extrahepatic vascular invasion or metastasis [Figure 2]. HKLC was derived from the results of a cohort of 3856 HCC patients primarily with HBV infection treated at single institution. Based on these prognostic factors, patients are classified in five main groups and nine subgroups with distinct survival outcomes. In the authors' analysis, HKLC classification exhibits better prognostic value than the BCLC classification. Regarding to problematic issues of BCLC such as heterogeneity of the stages B and C, and rigidity of treatment allocation, HKLC is able to better stratify patients in these stages into distinct groups with better survival outcomes based on more aggressive treatment recommendations than that observed in the BCLC treatment algorithm. Interestingly,

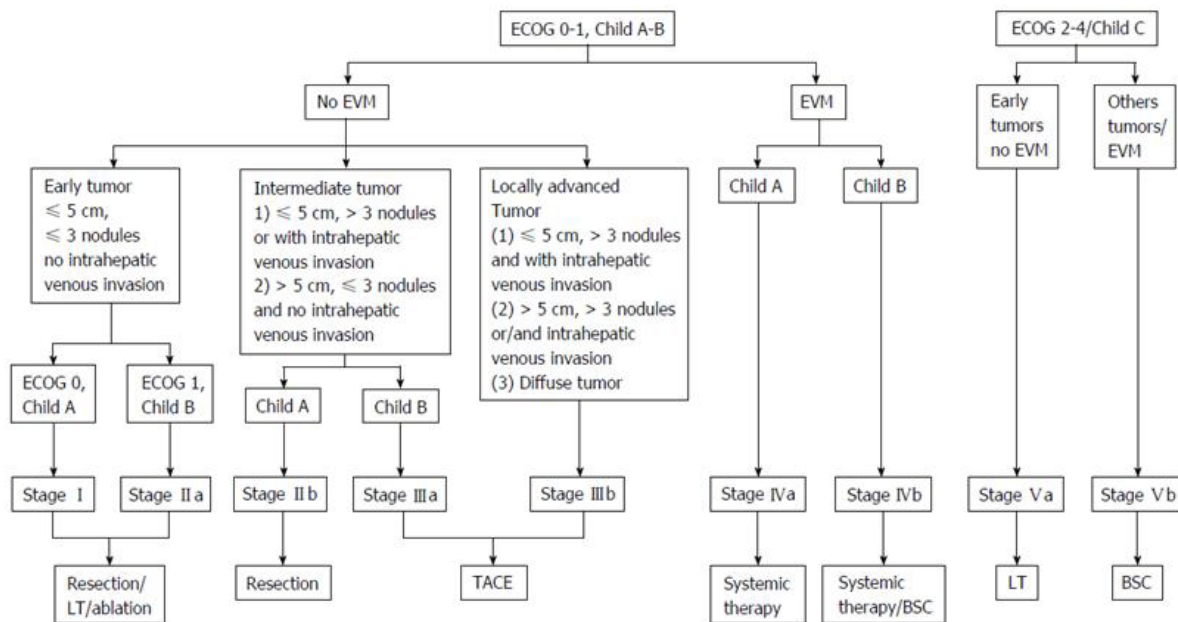


Figure 2. HKLC classification (from Adhoute *et al.*^[66] Usefulness of staging systems and prognostic scores for hepatocellular carcinoma treatments). EVM: extrahepatic vascular invasion/metastasis; BSC: best supportive care; TACE: transarterial chemoembolization; ECOG: Eastern Cooperative Oncology Group

patients staged BCLC B and HKLC II had a survival probability of 52% at 5 years if they underwent surgical resection as first treatment, compared with a survival probability of 18.7% at 5 years if they received first-line TACE. This algorithm expands the scope of surgical resection. It offers expanded treatment recommendations such as surgical resection for BCLC B patients, TACE for BCLC C patients or LT for BCLC D patients which should be verified in a large-scale prospective study.

HKLC classification was derived from a cohort of HCC patients with predominant HBV infection (80% of the whole cohort). Recently, the capability of HKLC in European cohorts have been challenged^[66,67]. In a recent study, based on the pooled data from three European prospective cohort of 1693 patients with HCC, the BCLC staging system found to predict better in OS for European patients than the HKLC staging system^[67]. Twice more patients were eligible for a curative therapy with the HKLC algorithm, but its survival benefit remains to be investigated.

ITA.LI.CA integrated staging system

The ITA.LI.CA staging system was developed by Italian Liver Cancer Study group in 2016 for the purpose of providing a new prognostic system for HCC including both tumor staging to be used in the clinical management and an integrated prognostic score to predict patient survival^[68]. This retrospective study was derived from the analysis of a cohort of over 5000 HCC patients from Italy (mainly HCV). External validation was performed using data from a Taiwanese cohort of over 2600 HCC patients (mainly HBV). Tumor staging (0, A, B1, B2, B3, C) is based on tumor characteristics such as largest tumor diameter, number of nodules, intra- and extrahepatic macroscopic vascular invasion, extrahepatic metastases [Table 14]. Multivariable survival model is then used to calculate the relative prognostic value of ITA.LI.CA tumor stage, ECOG performance status, Child-Pugh score (CPS), and AFP > or ≤ 1,000 µg/L in predicting individual survival. Based on the model results, an ITA.LI.CA integrated prognostic score (from 0 to 13 points) is constructed [Table 15]. In the authors' analysis, the model had better discriminant ability than any of the existing staging systems (BCLC, CLIP, JIS, MESIAH and HKLC stage).

Table 14. The ITA.LI.CA tumor staging system^[68]

Diameter of largest tumor (cm)	Number of tumors	Vascular invasion or metastasis	Stage
≤ 2	1	No	0
≤ 3	2-3	No	A
2-5	1	No	A
3-5	2-3	No	B1
> 5	1	No	B1
> 5	2-3	No	B2
≤ 5	> 3	No	B2
> 5	> 3	No	B3
Any	Any	Intrahepatic	B3
Any	Any	Extrahepatic	C

Table 15. Development of the ITA.LI.CA integrated prognostic score

	Prognostic factor	Points
ITA.LI.CA Tumor staging	0	0
	A	1
	B1	2
	B2	3
	B3	4
	C	5
ITA.LI.CA functional score		
Child-Pugh score	5	0
	6	1
	7	1
	8	2
	9	2
	10-15	3
ECOG PST	0	0
	1	1
	2	1
	3-4	3
AFP (μg/L)	≤ 1,000	0
	> 1,000	2

ECOG PST: Eastern Cooperative Oncology Group performance status; AFP: alpha-fetoprotein

Some aspects of the ITA.LI.CA system are rooted in the BCLC staging system. Different than BCLC, ITA.LI.CA subclassifies BCLC stage B patients into B1, B2, and B3 categories based on degree of intrahepatic tumor burden and presence of intra-extrahepatic metastases. Finally, the serum biomarker AFP was incorporated as a surrogate for occult vascular invasion, distant metastases, or aggressive tumor biology. Although the model demonstrated good prognostic discrimination among study patients, it should be noted that there are significant differences in cancer etiology and treatment choices between European and Asian populations. Additionally, most patients in both cohorts had good performance status, compensated cirrhosis, and early or intermediate stage tumors. Whether ITA.LI.CA staging system would perform as well in cohorts with advanced tumor stage remains to be determined. Furthermore, it needs to be validated in prospective studies with more contemporary cohorts.

Biomarkers

Early diagnosis and treatment of HCC is crucial for achieving long term survival. Detection of tumor biomarkers is one of the main methods in reaching this goal. AFP is widely used as serum biomarker for HCC diagnosis, however, the diagnostic accuracy of HCC with serum AFP exhibits both sensitivity and specificity far below satisfaction, especially with small sizes of HCC^[69]. With the development of new technology and advances in research, a number of new and specific biomarkers of HCC have been

discovered. Besides AFP, Lens culinaris agglutinin-reactive AFP (AFP-L3) and DCP have been incorporated in current staging systems as factors reflecting tumor progression (BALAD and bm-JIS). Although some reports have not been consistent about the significance of both AFP-L3 and DCP in the diagnosis of HCC^[70], they are considered as promising biomarkers especially for the diagnosis of small HCC with low level of AFP^[71]. To improve their diagnostic sensitivity and specificity, combined tests of AFP, AFP-L3 and DCP are often applied in clinical practice^[72].

Vascular endothelial growth factor (VEGF), an endothelial cell mitogen that promotes neovascularization and endothelial cell proliferation, significantly increases in serum of HCC patients compared with control individuals and correlates with venous invasion, advanced tumor stage and poor prognosis^[73,74]. Furthermore, the expression of VEGF in HCC tissues was related to invasiveness and metastasis of HCC^[75]. However, VEGF may also be increased in other cancers and its value for early diagnosis of HCC is also unclear^[76]. Additionally, other biomarkers such as Golgi glycoprotein 73, a transmembrane Golgi glycoprotein, and Glypican 3, a cell-surface heparan proteoglycan have been studied and found to be promising biomarkers which have high sensitivity and specificity for the diagnosis of small HCC with negative AFP^[71]. With the development of genomics and proteomics, more and more new biomarkers will be discovered and used in clinical settings to diagnose different stages of HCC.

CONCLUSION

Over the past 20 years, diagnostic tools and treatment modalities have improved and screening programs have led to earlier diagnosis of HCC. Liver transplantation, hepatic resection, RFA, and transarterial chemoembolization have all been used in these patients to achieve a curative therapy. However, according to the degree of hepatic functional loss and heterogeneous nature of the tumor, optimal management for these patients remains controversial. Therefore, there is an increasing need for a staging system that can reflect the prognosis and permit the better stratification of these patients for clinical trials. To date, several staging systems have been proposed with various combinations of clinical, biochemical and pathological factors. However, search for a comprehensive staging system with appropriate treatment options applicable worldwide to all HCC patients despite geographical, financial and etiologic differences continues.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Role of the contrast-enhanced ultrasound in the diagnosis of HCC in cirrhotic liver

Francesco Loria¹, Antonello Parlato¹, Giuseppe Loria¹, Luciano Frosina², Giuseppe Crea³, Salvatore Basile³, Caterina Alessio¹, Giuseppe Di Leo¹, Adele De Caridi¹, Vittorio Maschio¹, Nicola Zizzi¹, Orazio Trapuzzano¹, Salvatore Giuseppe Galea¹

¹Department of Radiology, Giovanni Paolo II Hospital, Lamezia Terme ZC 89015, Italy.

²Department of Radiology, Policlinico G. Martino University of Messina, Messina ZC 98100, Italy.

³Department of Radiology, Palmi Hospital, ASP RC, Palmi ZC 89015, Italy.

Correspondence to: Dr. Francesco Loria, Department of Radiology, Giovanni Paolo II Hospital Lamezia Terme, Lamezia Terme ZC 89015, Italy. E-mail: francescoloria956@alice.it

How to cite this article: Loria F, Parlato A, Loria G, Frosina L, Crea G, Basile S, Alessio C, Di Leo G, De Caridi A, Maschio V, Zizzi N, Trapuzzano O, Galea SG. Role of the contrast-enhanced ultrasound in the diagnosis of HCC in cirrhotic liver. *Hepatoma Res* 2018;4:59. <http://dx.doi.org/10.20517/2394-5079.2018.75>

Received: 8 Jun 2018 **First Decision:** 1 Aug 2018 **Revised:** 28 Aug 2018 **Accepted:** 29 Aug 2018 **Published:** 28 Sep 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Yuan-Li Wang **Production Editor:** Zhong-Yu Guo

Abstract

The development of second generation ultrasound (US) contrast-medium and specific imaging techniques with dedicated softwares, allows to observe the liver perfusion in real time, becoming an useful and less invasive method to describe precisely the vascularization of hepatic lesions. This significantly increased the ability of US to detect and characterize focal liver lesions. The aim of this review article is to evaluate the role of contrast enhancement US in the diagnosis of hepatocellular carcinoma in cirrhotic liver, with reference to the guidelines of American Association for the Study of Liver Diseases, European Association for the Study of the Liver and European Federation of Societies for Ultrasound in Medicine and Biology.

Keywords: Contrast-enhanced ultrasound, hepato-cellular carcinoma, cirrhotic liver

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common malignancy of the liver. Ultrasound (US) examination and measurement of serum levels of alpha-fetoprotein (AFP) represent the most common screening method for HCC^[1].

However, the conventional grayscale US and Color-Power Doppler US show limited ability in characterizing



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liver tumors^[2-5] and the sensitivity of these biomarkers in the detection of early HCC or small lesions is limited. AFP levels may also be elevated in other malignancies, such as intrahepatic-cholangiocarcinoma (ICC) or colon cancer, as well as during follow-up of chronic viral hepatitis^[6].

The study of vascularization within the nodule in focal liver lesions (FLLs) in a cirrhotic liver is considered to be useful in identification and characterization with various imaging techniques^[7-16].

With the development of a second generation of US contrast-agent and real-time contrast-specific techniques, contrast-enhanced ultrasound (CEUS) has been widely used in clinical studies and has greatly improved the diagnostic ability of US in identification of FLLs^[16-21].

CONTRAST MEDIA

Currently, there are four US contrast agents for liver studies: (1) SonoVue (BraccoSpA, Milan Italy introduced in 2001) that consists of stabilized gaseous microbubbles (sulfur hexafluoride) equal to or smaller than red blood cells, with a diameter of less than 7 μm , stabilized inside a phospholipid shell; (2) Definity (Lantheus Medical, Billerica, MA, USA, introduced in 2001) consists of stabilized microbubbles of perflutren with a lipid shell; (3) Optison (GE Healthcare) consists of stabilized microbubbles of human serum albumin with octofluoropropane; and (4) Sonazoid (Daiichi-Sankyo, GE Tokio, Japan, introduced in 2007) that consists of stabilized gaseous microbubbles (perfluorobutane) with phospholipid shell (hydrogenated egg phosphatidyl serine)^[22].

Definity and Optison have been authorized only in USA and Canada for cardiological imaging; in Canada Definity is used also for other body districts. Sonazoid is used only in Japan and SonoVue in Europe and China. In Europe only Optison is used for cardiological imaging.

In consideration of what previously said, in our article we will exclusively refer to SonoVue, the only US contrast medium authorized in Europe for the study of FLLs.

Basic of CEUS

The contrast media SonoVue consists of microbubbles of stabilized phospholipids containing sulphure-hexafluoride, with the same or inferior dimension of red blood cells (diameter inferior to 7 μm). Due to their small size the microbubbles act as an “blood pool agent” and allow the real time study of the macro- and micro-vascular circulation for several minutes^[23-25].

The interaction between the microbubble blood pool and the incident US beam is the key to understand the mechanism of action of the US contrast agent and its clinical applications. When the microbubbles are hit by the US beam at low mechanical index (MI) ($< 100 \text{ kPa}$ - $\text{MI} < 0.1$), they are exposed to a low-level positive (compression) and negative (dilatation) sound pressure. In this case the microbubbles behave in a linear way as simple reflectors, without breaking. In this way a linear reflection phenomenon is generated which results in a wide reinforcement of the scattering coming from the circulating blood. Increasing the acoustic intensity of the incident beam (MI between 0.1 and 1), the oscillation becomes more intense and asymmetric and the physical behavior of the microbubbles becomes non-linear. Because of non-linear reflection, if the microbubbles are hit by an acoustic beam with this intensity, they generate a reinforcement of the fundamental signal and a harmonic energy.

The non linear behavior of the microbubbles shows itself in a way not dissimilar to stationary tissue. The main advantage that derives from the use of US contrast media is that the amount of the signal coming from the second harmonic, which originates from the microbubbles, is of a length greater than that coming from stationary tissues.

Therefore, thanks to the use of specific software, the linear signals are deleted from the tissues and the images are formed only thanks to the non-linear signals coming from the microbubbles. The use of these more powerful acoustic waves, however, causes the breaking of part of the micro-bubbles. To minimize this phenomenon, we have chosen to work at low mechanical indices. This study technique allows to cancel the signal coming from the tissues and to have pure images coming exclusively from the microbubbles^[25-29].

Although the correct setting of the US scanner and the scanning techniques are important for avoiding artifacts^[30], MI and inadequate gain are the two main causes of error in the visualization of the signals coming from the tissues.

PROTOCOL OF SURVEILLANCE OF HCC

In our institute, we use a HCC surveillance protocol in patients with cirrhosis, based on the six-monthly dosing of alpha-fetus protein serum levels and on the execution of a six-monthly hepatic US examination in patients in the Child Pugh class A and B. In patients in the Child Pugh class C, the US can be also performed every three months.

DIAGNOSIS OF HCC

Baseline us

HCC typically appear as hypoechoic compared to the surrounding hepatic parenchyma. It can also appear as isoechoic, hyperechoic or with mixed echogenicity, with a typical characteristic of nodule in nodule. About 50% of HCC can appear as a nodule with peripheral hypoechoic halo^[22]. Both the conventional Color-Doppler and the Power-Doppler US have a limited ability to describe intralesional vascularization, because they are insensitive to slow and deep blood flows^[31,32]. Generally the Doppler HCC pattern is characterized by an arterial vascularization with a basket pattern due to thin blood vessels that surrounds the nodule^[11,22,33].

CEUS procedures

Before starting the CEUS evaluation, it is mandatory to perform an evaluation in B-mode; in particular it is necessary to analyze the site, the size, dimensions, echogenicity of the lesion and its relationship with the other structures. An evaluation of the vascular pattern of the lesion in Color-Doppler is useful to define the eventual presence of central or peripheral vascular vessels. Once the target lesion has been identified, the specific mode of imaging must be selected for the contrast with a low MI. SonoVue is injected into the antecubital vein with a bolus, followed by a bolus flush of a solution of 10 mL of sodium chloride. To avoid destroying the micro-bubbles during the injection, the calibre of the needle must not be less than 20 gauge^[22]. The target lesion and the surrounding parenchyma are observed for 5-10 min in real time and registered in a video clip. The arterial phase is defined as 0-30 s from the injection, the portal phase 31-75 and the late phase from 75-180 s up to 10 min^[31].

CEUS

The most common appearance in cirrhotic liver of HCC is an hyper-arterial enhancement compared to the surrounding hepatic tissue [Figure 1], which is found in 93.5%-97% of cases^[31,33-38] and generally appear homogeneous and intense. In the nodules that have diameters larger than 2 cm, hyper-enhancement can also be non-homogenous because of the area of necrosis within the lesion [Figure 2]. A slight peripheral enhancement is found in 5 (34.6%) of cases of HCC; it can represent the tumor capsule [Figure 3] or blood vessel around the lesion^[31,33-39]. In the majority of cases HCC shows a precocious enhancement compared to the surrounding tissue, in particular, the rates of detection of the hyper-enhancement in lesions < 1.0 cm, 1.0-2.0 cm and 2.0-3.0 cm are respectively 67%, 83%-88% and 92%-100%^[3,31,36-40] [Table 1]. Furthermore other lesions like dysplastic nodules and hyper-vascularized hemangioma can have the same contrast enhancement pattern^[41].

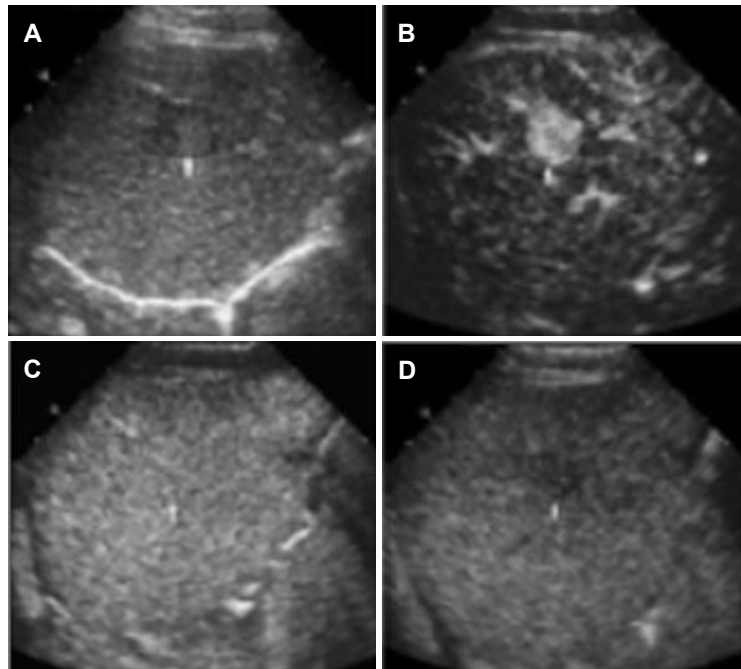


Figure 1. A: US shows a hypoechoic hepatocellular carcinoma (HCC); B: arterial phase (19 s) shows a homogeneous hyper-enhancement of the lesion; C: portal phase image (82 s): the nodule is isoechoic; D: late portal phase (190 s): the HCC is slightly hypoechoic with respect to surrounding liver

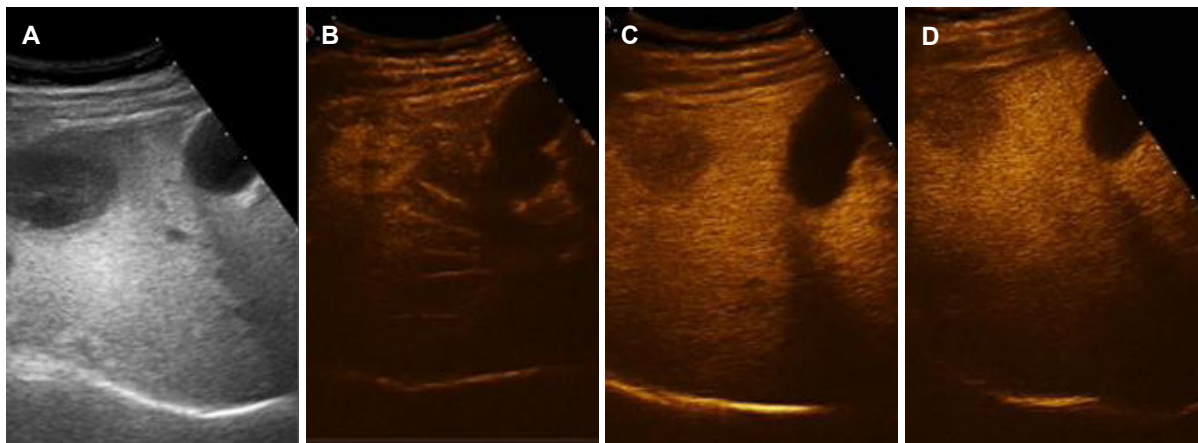


Figure 2. A: US shows a hypoechoic hepatocellular carcinoma (HCC) (> 2 cm); B: arterial phase (26 s) shows an inhomogeneous hyper-enhancement of the lesion; C: portal phase image (70 s) shows wash-out of contrast medium; D: late phase image (95 s): the HCC is hypoechoic with respect to surrounding liver

To increase the specificity of CEUS on the basis of these findings, a demonstration of the washout-phase is decisive and its presence also depends on the size of the nodule: the wash-out is described only in 20%-30% of nodules with diameters of 1-2 cm and in 40%-60% of nodules with diameters of 2-3 cm^[22,38,42-59].

The speed of the wash-out can define the level of differentiation of HCC: poorly differentiated show rapid wash-out, while the well differentiated HCC tends to be iso- or hypo-enhanced compared to parenchyma in the portal or late venous phase^[21,31,60-62] [Figure 4].

Table 1. Typical enhancement of hepatocellular carcinoma in the arterial phase based on the size of lesion

Size lesion (cm) rate of detection of the hyper-enhancement in lesion	
< 1.0 cm	67%
1-2 cm	83%-88%
2-3 cm	92%-100%

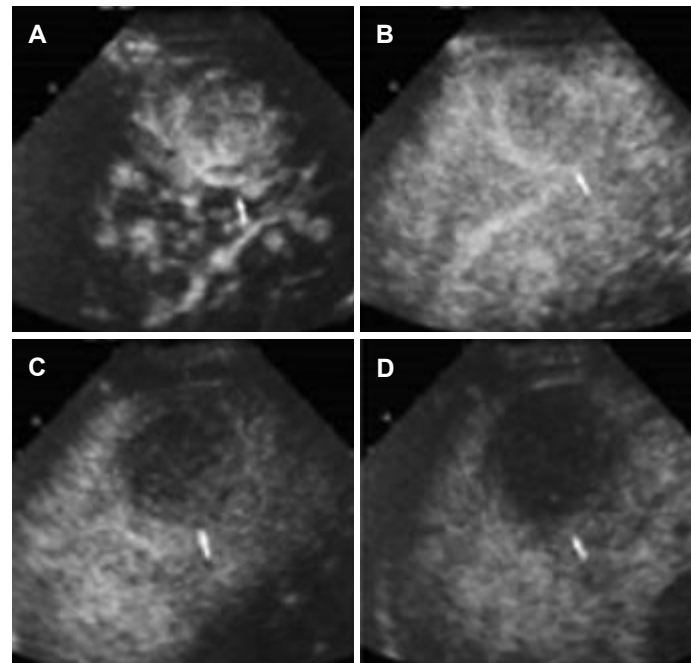


Figure 3. A: Arterial phase (18 s) shows a heterogeneous hyper-enhancement of the lesion; B: portal phase (32 s): the nodule is slightly hypoechoic; C: portal phase (90 s): the nodule is hypoechoic; D: late portal phase (180 s): the nodule is remarkably hypoechoic with respect to the surrounding liver. Capsule of the lesion is well represented (arrows) more evident in A and B

In order to increase the sensitivity of the diagnosis of HCC, in the cirrhotic liver it is useful to observe for more than 4 min, in fact in these cases the wash-out tends to start later, generally not before 60 s after the injection, and in a quarter of cases it appears after only 180 s^[40]. For this reason the presence of precocious wash-out (< 60 s) has been described in HCC poorly differentiated and in cases of ICC^[22,40,61-62].

In conclusion, a hyper-enhancement in the arterial phase, followed by a washout in the late phase is a typical CEUS pattern in HCC in cirrhotic livers^[63]. Usually regenerative/dysplastic nodule doesn't show this kind of pattern contrast enhancement that appears similar to the parenchyma.

DISCUSSION

In 90% of cases the development of hepatocarcinoma occurs through a multi-step path in which the lesion passes from a benign to a malignant lesion following an order summarized in Table 2. During this long process, a reduction in the normal arterial blood supply and the contemporary and progressive increase in newly formed tumor vessels (neo-angiogenesis) were detected. The development of second generation contrast-medium and specific imaging techniques with dedicated softwares, allows to observe the perfusion of the lesion in real time, becoming an useful and less invasive method, in describing precisely blood supply of nodule^[31]. However, in clinical practice, non invasive diagnosis of HCC is relatively recent. Until 2000 the diagnosis of HCC occurred through invasive biopic studies and successive histologic diagnosis^[22].

Table 2. Development of hepatocellular carcinoma (HCC)

Large regenerative nodule
Low dysplastic nodule
High dysplastic nodule
Nodule of HCC
well differentiated - moderately differentiated - poorly differentiated

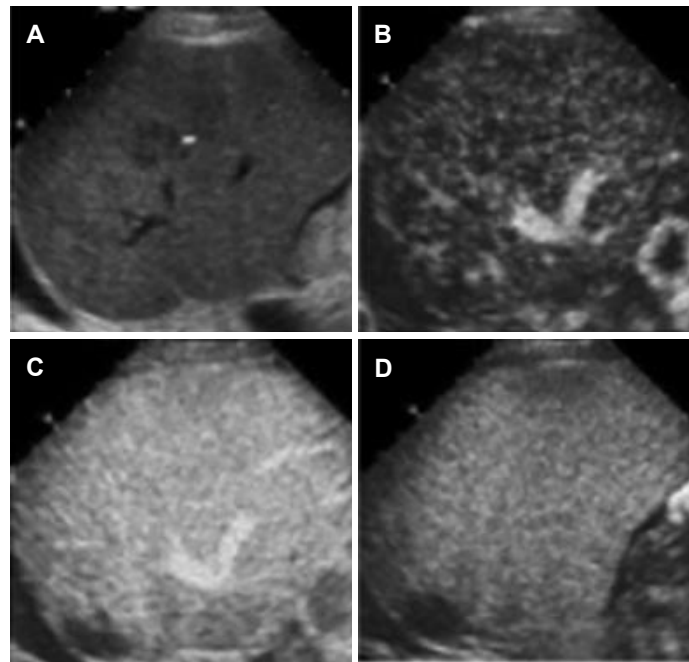


Figure 4. A: US shows a hypoechoic nodule; B: portal phase (32 s): arterial phase (23 s) shows a homogeneous isoenhancement of the lesion; C: portal phase (52 s): the nodule is isoechoic with respect to the surrounding liver; D: late portal phase (280 s): the hepatocellular carcinoma (HCC) is isoechoic with respect to the surrounding liver

In 2001 a group of experts European Association for the Study of the Liver (EASL) on HCC in Barcelona reported, for the first time, the criteria for a non invasive diagnosis^[64]. These criteria required only the presence of a certain dynamic contrast enhancing behavior: the uptake of a contrast medium during the arterial phase documented through CT, angiography magnetic resonance imaging (MRI) or US. Therefore, in a cirrhotic liver, were considered HCC the nodule lesions with a diameter bigger than 2 cm that showed this uptake of contrast medium in 2 different imaging modalities or showed this contrast enhancing impregnation in a single imaging modality but with serum levels of AFP bigger than 400 ng/mL. In all other cases a biopsy was necessary^[22,64].

In 2005 EASL and American Association for the Study of Liver Diseases (AASLD) reached a new radiological signal to further distinguish HCC: wash-out in the venous/late phase^[5,22]. So the non invasive diagnosis of HCC was based both on the presence of uptake of the contrast medium in the arterial phase and on the wash-out in the venous/late phase. For nodules larger than 2 cm these radiological criteria should have been present in just one imaging modality; for nodules of the dimensions of 1-2 cm these radiological signs should have been shown in at least two imaging modalities (CT, MRI and CEUS). The AFP was eliminated from the diagnostic algorithm due to some limitations^[5,22]. Due to the ability to visualize in real time the perfusion of hepatic lesions, CEUS can have a foremost role in the diagnosis of HCC; however it is currently accepted in variable ways in national and international guidelines. At the moment, CEUS is recommended by European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) and is part of the Japanese guide-

Table 3. Recommendations of European Federation of Societies for Ultrasound in Medicine and Biology for the use of contrast-enhanced ultrasound

The characterization of the nodules
To make a rapid diagnosis (however, CT or MR remain necessary, if not contraindicated, for the stadition
When CT and MR are inconclusive especially in nodules that can't be submitted to biopsy
To contribute to selecting a nodule when they are many or have different contrast patterns
To monitor the changes in the nodule
After an inconclusive histology

lines for HCC^[22,23,65,66], but it has been removed from American and EASL guidelines^[48,53]. The main reason for this exclusion lies in the possibility of a mistaken diagnosis between ICC and HCC using only CEUS^[67,68]. Furthermore this exclusion from AASLD guidelines is also related to the fact that, in the United States, contrast enhancing agents are not authorized for the study of the liver and so CEUS is not available. However, in clinical practice, the probability of mistaken diagnosis is minimal when CEUS is carried out by an expert physician^[69], because the ICC shows a rapid wash-out. Apart from this, in recent years a significant variability has been described, that has made the use of CEUS still more controversial^[69]. In 2010 AASLD recommended that, for nodules bigger than 1 cm, the non invasive diagnosis for HCC can be determined with a single means of imaging (CT multidetector or MRI with dynamic contrast)^[53], if the typical contrast enhancement pattern is present; however when typical radiological aspects are not present and the behavior of the nodule is not characteristic, it is necessary to evaluate the nodule through a second imaging technique or with a biopsy^[53]. This change is based on the conclusion of several studies that have demonstrated that the use of a single contrast technique causes a reduction in the positive predictive value that remains higher than 90%^[42,59], they highlight a higher specificity than the typical radiological sign^[41,70]. AASLD guidelines suggest the necessity of adhering closely to imaging protocol and carrying out non invasive diagnosis of HCC in expert centers^[2,53].

Recent EASL guidelines are similar to those of AASLD, suggesting the use of multiphase imaging CT and up to date MRI for non invasive diagnosis of HCC^[48]; in particular for nodules between 1-2 cm, a single imaging technique is advised when carried out exclusively in excellent centers and with high grade radiological equipment or 2 imaging techniques when these criteria are not present and are carried out in inferior contexts. Such prudent recommendations of EASL guidelines are based on evidence of equivocal data concerning non invasive diagnosis of nodules 1-2 cm^[22,48,53]. EFSUMB suggests a very different role for CEUS, describing it separately in two patients subgroups, with and without cirrhosis; this because of the great difference between types of hepatic nodules in cirrhotic and non cirrhotic livers^[22-23]. In cirrhotic livers, among the recommendations of EFSUMB for the use of CEUS^[23] are summarized in Table 3. The multicenter German Society for Ultrasound in Medicine (DEGUM) included 1349 patients with FLLs diagnosed on US; CEUS was compared to the biopsy in 75% of cases and in 25% with contrast enhancement (CE) CT or CE-MRI. The accuracy of CEUS was 90.3%^[71-75].

Another two DEGUM studies evaluated the capacity of CEUS in the characterization of FLL, comparing CEUS in the first study with CE-CT and in the second with CE-MR. In both cases there were no statistically significant differences^[75-77]. In 2012, Goto *et al.*^[78] reported a major sensibility and sensitivity of baseline US in comparison with CEUS, using Sonazoid, in the detection of HCC during the post-vascular phase. In the differential diagnosis between HCC and ICC there is some controversy about the role of washout: in the late phase the wash-out of HCC seems to be less marked than the other liver neoplasms like ICC and metastasis^[23,38,69,79]. Reanalyzing the data of the studies, Guo and Xu^[80], found that the clinical consequences that come from this risk do not seem to justify the complete removal of CEUS as an imaging technique in the characterization of FLL. With regard to this, further positive evidence is being gathered: Li *et al.*^[81] evaluated in the first place the usefulness of CEUS in differentiating ICC from HCC in cirrhotic patients through a detailed analysis of the characteristics of temporal enhancement. Therefore, in a cirrhotic liver if a nodule shows a hyper-enhancement

in the arterial phase followed by a precocious and marked washout in the portal phase, the nodule is highly suspected of ICC; HCC, however, shows a moderate washout in the portal phase and, sometimes, can show iso-enhancing compared to surrounding parenchyma. These results have provided the last evidence to reprove the opinion of AASLD^[80].

The meta-analysis with evidence from 1998 to 2016 of Zhang *et al.*^[82] showed that CEUS was a useful diagnostic instrument for distinguishing HCC from other FLLs and, in conclusion, could also become a front line imaging instrument in the future. Masuzaky *et al.*^[83] and Chan *et al.*^[84] reported that CEUS has an important role in patient candidates to the treatment with radiofrequency ablation (RFA), increasing the detection of HCC that are not seen or poor seen on B-mode US and provides real-time guidance of RFA with good short-term treatment responses. Intrinsic limitations of CEUS vary in relation to patient characteristics (cooperation, obesity, meteorism), characteristics of lesion (site-dimensions-depth) and the CEUS experienced operator.

Another important limitation of CEUS compared to cross sectional image formation is that only one FLL can be evaluated at a time and the repeated administration in bolus of SonoVue is necessary to evaluate other FLLs. However, in clinical practice, only 2 and 3 FLLs situated in the same segment lobe can be simultaneously and easily examined with CEUS^[85]. On CEUS, the evaluation of enhancement is statistically significant in relation to the depth; in particular, at a depth greater than 9 cm from abdominal wall, only 58% of FLL present the same arterial enhancement compared to the corresponding phase in multi-slice CT; this contrasts with 95% of the lesions situated more superficially^[86].

Some studies have demonstrated that a number of lesions, varying 5%-25%, remain untermated after CEUS, because they do not present a characteristic pattern^[86]. Contrast-enhancing agents until today have not demonstrated cardio-, hepatic- or nephro-toxic effects. It is not necessary to carry out laboratory tests to evaluate hepatic or renal function before their administration. There is limited data about use during pregnancy, breast-feeding or in pediatrics. In a retrospective study^[87] of 23,188 investigations with SonoVue the rate of serious adverse events was only 0.0086% (29 cases), including a pseudo- anaphylactic shock and a bronchospasm, but there were no fatalities.

CONCLUSION

CEUS is a non invasive, rapid, economical and accurate method for the diagnosis and management of HCC in cirrhotic patients; moreover it is repeatable, less stressful and less invasive for the patients and doesn't require exposure to radiation. CEUS is not nephro-toxic and is non allergenic. When the nodular lesions are controlled in the cirrhotic liver, CEUS allows a rapid characterization with good precision when carried out by a medical expert.

DECLARATIONS

Authors' contributions

Designed the work, collection and data analysis, critical revision of the article and final approval of the version to be published: Loria F, Parlati A, Loria G

Contributed to the execution of the ultrasound examination and to the interpretation of the results and to the bibliographic research: Loria F, Parlati A, Loria G, Frosina L, Crea G, Basile S, Alessio C, Di Leo G, De Caridi A, Maschio V, Zizzi N, Trapuzzano O, Galea SG

Contributed also to revision of the article: Loria F, Parlati A, Loria G, Frosina L, Crea G, Basile S, Alessio C, Di Leo G, De Caridi A

Contributed also to drafting the article: Loria F, Parlati A, Loria G, Maschio V, Zizzi N, Trapuzzano O, Galea SG

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Liver transplantation for hepatocellular carcinoma - non-cancer factors and implications for improving outcome beyond standard tumor criteria

Arno Kornberg, Martina Schernhammer

Department of Surgery, Klinikum rechts der Isar, Technical University Munich, Munich D-81675, Germany.

Correspondence to: Dr. Arno Kornberg, Department of Surgery, Klinikum rechts der Isar, Technical University Munich, Munich D-81675, Germany. E-mails: ArnoKornberg@aol.com

How to cite this article: Kornberg A, Schernhammer M. Liver transplantation for hepatocellular carcinoma - non-cancer factors and implications for improving outcome beyond standard tumor criteria. *Hepatoma Res* 2018;4:60. <http://dx.doi.org/10.20517/2394-5079.2018.86>

Received: 6 Jul 2018 **First Decision:** 16 Aug 2018 **Revised:** 27 Aug 2018 **Accepted:** 28 Aug 2018 **Published:** 29 Sep 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Yuan-Li Wang **Production Editor:** Zhong-Yu Guo

Abstract

Liver transplantation (LT) is recognized as best treatment option in patients with early hepatocellular cancer (HCC) in underlying liver cirrhosis. Apart from tumor size and number implemented in the Milan criteria, which are current worldwide standards for patient selection, several biological tumor factors have been identified to affect cancer-specific outcome. In particular, grading and vascular tumor invasions were shown to correlate with aggressive biological tumor behavior and poor survival following LT. Identifying tumors with favorable biology is one important approach for expanding the pool of eligible liver recipients beyond the Milan burden limits. Improving the immunological state and condition for appropriate defense against circulating cancer cell attack may be another important prognostic aspect. Therefore, there is increasing interest in non-cancer factors related to the peritransplant period that may influence the oncological outcome by providing negative immunomodulatory actions. Considering and modulation of these non-HCC factors of prognosis might contribute in safely expanding the HCC LT selection criteria.

Keywords: Hepatocellular carcinoma, liver transplantation, tumor biology, non-cancer factors, outcome

INTRODUCTION

In the last 40 years, liver transplantation (LT) has developed as a generally accepted standard procedure in the treatment of a wide range of end-stage liver diseases. Especially liver replacement for hepatocellular carcinoma (HCC) in underlying liver cirrhosis became a phenomenal story of clinical success in oncological surgery^[1].



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Due to cirrhosis-related portal hypertension (PH) and liver dysfunction, these patients are mostly not eligible for hepatic resection, so that only palliative treatment options have frequently been possible in former days^[2]. In particular, the implementation of the so-called Milan criteria (MC) in 1996 for realizing a strict and rigid selection process based on radiographic tumor size and number (one tumor nodule ≤ 5 cm, or up to 3 HCC nodules each ≤ 3 cm, no macrovascular invasion) established LT as best curative treatment option in early stage HCC patients^[3]. The pre-MC era was characterized by high posttransplant tumor recurrence rates and mortality, which was not acceptable in view of donor organ shortage^[4,5]. In contrast, numerous validation trials have clearly shown that Milan-based LT for HCC produces excellent long-term survival rates above 70% at 5 years, which was absolutely comparable to those of other transplant indications^[6-8]. Therefore, the MC have been implemented as standard selection features in large public allocation systems, such as the United Network of Organ Sharing (UNOS) and Eurotransplant^[9,10]. Currently, in times of model for end-stage liver disease (MELD) score based organ allocation, priority is still given to patients with HCC meeting the MC^[11,12].

With increasing experience in rescue LT of marginal donor grafts and living donor liver transplantation (LDLT), who are both independent from MELD-based allocation rules, it became evident in recent years that the MC are too rigid and very often unjustifiably preclude patients with beyond MC tumors from potentially curative treatment^[13-15]. In order to increase the pool of eligible transplant patients, several expanded macromorphologic tumor selection criteria have been proposed, such as the University of California San Francisco (UCSF) and the registry based Up-to-seven (UTS) criteria^[16-18]. However, as shown in the metroticket concept, increasing “distance” from the MC burden limits enhances the oncological risk^[18]. In addition, differences between radiologic and pathologic tumor staging additionally hamper the clinical applicability of tumor size based selection approaches^[19,20]. Poor differentiation and vascular (micro/macro) invasion of the tumor were identified as most important predictors of unfavorable tumor biology in the LT setting^[21-23].

However, both histopathologic features may not adequately be assessed prior to LT by radiographic tools or by using tumor biopsy^[24-26].

The identification of patients with aggressive tumor behavior is one important clinical practice to safely expand the pool of eligible liver transplant recipients beyond the MC^[27-29]. Different surrogate markers of tumor biology were shown to improve the selection process beyond the MC, such as alpha-fetoprotein (AFP)^[30,31], protein induced by Vitamin K absence II (PIVKA-II)^[32,33], serological inflammatory markers [C-reactive protein (CRP); neutrophil lymphocyte ratio (NLR), platelet lymphocyte ratio (PLR)]^[34], 18F-fluorodeoxyglucose (FDG) uptake on positron emission tomography (PET)^[35,36] and tumor downstaging under locoregional treatments^[37,38]. Apart from that, there is increasing evidence that not only tumor-specific characteristics, but also non-cancer factors may decisively influence cancer-specific outcome. Beneficial modulations of these non-HCC related factors might probably be another useful approach to improve post-LT prognosis, since HCC recurrence is the major risk factor for poor overall survival (OS). Therefore, it was the major aim of this manuscript to review the current available clinical data on the prognostic impact of non-tumor factors on post-LT HCC recurrence and tumor-specific survival.

The role of immunology and inflammation

Immunocompetence is a major prognostic factor of outcome in cancer patients. However, a specific characteristic of neoplasia is that it induces a state of inflammation and immunosuppression, which may additionally impair prognosis^[39]. Since the postulation of the link between inflammation and cancer by Virchow in 1863, important molecular mechanisms of cancer-induced pro-inflammatory response reactions have been identified. Malignant cells were shown to release inflammatory and immunosuppressive cytokines to their local environment, promoting tumor invasiveness and growth. In addition, cancer itself may induce

systemic immunosuppression through multiple mechanisms and effector cells, such as T-cell exhaustion, T-regulatory cells, myeloid-derived suppressor cells and M2 macrophages^[40]. In this context, HCC has an exceptional position, since 90% of the cases develop in underlying cirrhosis and fibrosis, which are promoted by chronic liver inflammation. Liver damage and necroinflammation induced by alcoholic disease, non-alcoholic fatty liver disease (NAFLD) and in particular by chronic viral hepatitis comprise a substantial risk of carcinogenesis^[41,42]. Activation of the innate immune system, hepatocyte death with production of damage-associated molecules (DAMPs), T cell exhaustion, and upregulation of pro-inflammatory cytokines [interleukin (IL)-2, IL-7, IL-12, IL-15, IFN- γ] seem to be major molecular mechanisms. Thereby induced local and systemic pro-inflammatory reactions and immunosuppression lead to replication stress, DNA damage and genetic instability, which may result in development of liver cancer and impact cancer treatment^[42,43].

Another important aspect is that liver dysfunction is another important prognostic factor enhancing tumor progression. The liver plays a key role in maintaining immunocompetence. In addition to numerous other mechanisms triggered by its unique blood supply, it has an essential capability to remove gut-derived microbial compounds, and hosts a great variety of innate and adaptive immune cells (sinusoidal cells, hepatic stellate cells, Kupfer cells, dendritic cells), and is able to preserve immunotolerance to non-pathogenic and inflammatory triggers. Decrease of these immunological efficacies result in a persistent up-regulation of inflammatory stimuli which may promote carcinogenesis. For example, increased levels of circulating T regulatory cells were shown to be associated with increased mortality of HCC patients^[42,43].

Currently, 2 major ways of posttransplant HCC recurrence are postulated: (1) growth of pre-LT undetected extrahepatic micrometastases; and (2) engraftment of circulating tumor cells (CTC) that have been released during transplant procedures^[44]. Both ways of metastasis are significantly promoted by immunological dysbalance^[34]. In particular, patients with advanced HCC stages are at an extraordinary oncological risk post-LT, since macromorphologic tumor load correlates with unfavorable tumor features, such as poor grading and vascular invasion, and thereby with numbers of CTC^[45,46]. A prevailing state of immunosuppression and pro-inflammation in the peritransplant period might, therefore, be particularly dangerous for advanced HCC LT patients. Consequently, recipients' factors (cirrhosis, sarcopenia), liver graft quality, surgical procedure and post-LT immunosuppressive treatment as non-cancer features affecting the immunological state have to be considered in order to safely expand the patient selection criteria.

Recipients' factors

Background liver cirrhosis

Progressive liver cirrhosis induces complex pro-inflammatory and immunosuppressive mechanisms referred to as cirrhosis-associated immune dysfunction (CAID) syndromes^[47]. This may impair outcome following non-surgical treatment and hepatic resection^[48,49]. This aspect has not yet been intensively studied in the LT setting so far, which may be due to the fact that most HCC transplant patients present with less severe Child A or B cirrhosis and liver dysfunction are cured by liver replacement, probably implying that CAID has no influence on posttransplant clinical course. However, some interesting recent data have shown that the extent of background native cirrhosis may affect cancer-specific outcome in the LT setting [Table 1]. Already in 2008, Ioannou *et al.*^[50] demonstrated in a large study cohort using the UNOS database, that apart from increased AFP level, laboratory (lab.)MELD score ≥ 20 was the most important predictor of poor post-LT survival. Again by using the UNOS dataset of 3519 liver transplants, Halazun *et al.*^[51] identified pretransplant rising (lab.)MELD score as an independent predictor of microvascular invasion (MVI) on explant pathology, which in turn was the most important factor of poor cancer-specific outcome. Others have recently confirmed the oncological significance of background cirrhosis severity in the liver transplant setting^[52-54]. In a series of 243 transplant candidates with HCC, Faitot *et al.*^[55] demonstrated that clinically evident portal PH was

Table 1. Impact of stage of underlying cirrhosis on post-LT HCC recurrence

Ref.	n	Characterization of cirrhosis severity	Impact on post-LT outcome
Ioannou <i>et al.</i> ^[50]	4453	(lab.) MELD score ≥ 20	Calculated MELD score ≥ 20 was the most important predictor (HR = 1.61; 95%CI 1.3-2.1) of poor post-LT survival, along with AFP level. The risk of post-LT death was almost doubled in patients with either AFP level ≥ 455 ng/mL or MELD score ≥ 20 (HR = 1.97; 95%CI 1.6-2.5)
Halazun <i>et al.</i> ^[51]	3519	Pre-LT rising (lab.) MELD score	Rising pre-LT MELD score proved to be an independent predictor of MVI on explant pathology (OR: 1.46, CI 1.13-1.88; $P=0.004$), which was the most important factor of poor post-LT outcome
Macdonald <i>et al.</i> ^[52]	1074	(lab.) MELD score	Calculated MELD score was identified as an independent predictor of HCC recurrence or death after LT (HR = 1.03; 95%CI 1.01-1.05; $P = 0.005$), along with AFP level and donor risk index
Komorowski <i>et al.</i> ^[53]	142	(lab.) MELD score	Apart from AFP level, pretransplant calculated MELD score turned out to be an independent and significant predictor of RFS (HR = 1.16)
Foerster <i>et al.</i> ^[54]	304	(lab.) MELD score ≥ 15	Calculated MELD score ≥ 15 was an independent promoter of poor OS (HR = 1.028; 95%CI 1.002-1.053; $P = 0.033$), with HCC relapse to be the major reason of mortality
Faitot <i>et al.</i> ^[55]	243	Clinically evident portal hypertension	PH was an independent predictor of drop out from the waiting list due to tumor progression (OR = 2.79; 95%CI 1.02-7.69; $P = 0.04$). In an intent-to-treat analysis, post-LT OS was significantly lower in PH patients when compared to those without PH ($P = 0.044$). However, PH had no significant impact on outcome in the transplanted patients

AFP: alpha fetoprotein; HCC: hepatocellular carcinoma; HR: hazard ratio; LT: liver transplantation; lab: laboratory; MELD: model for end-stage liver disease; MVI: microvascular invasion; OS: overall survival; PH: portal hypertension; RFS: recurrence-free survival

an independent promoter of drop out from the waiting list due to tumor progression. In an intent-to-treat analysis, post-LT OS was significantly lower in PH patients when compared to those without PH. However, PH had no significant impact on outcome in the subgroup of transplanted patients [Table 1].

Sarcopenia

Nowadays, it is undoubtedly that recipients' functional status has a major prognostic impact on liver transplant recipients^[56,57]. In recent years, involuntary loss of muscle mass and strength, referred to "sarcopenia", was shown to be an early predictor of frailty and poor outcome. Sarcopenia is a feared complication in consuming chronic diseases like cancer, sepsis, renal function and liver cirrhosis^[58]. The pathogenesis of sarcopenia in cirrhotics is multifactorial and not fully understood. It seems to be a response to protein-energy malnutrition, metabolic catabolism, and patients' inactivity^[59,60]. Although there is currently no worldwide standard measurement and index of sarcopenia, depletion of skeletal muscle mass and function estimated by cross-sectional abdominal imaging were demonstrated to be a significant risk factor for wait list mortality, prolonged intensive care duration, complicated hospital stay, severe infections, metabolic syndrome and overall poor outcome in liver recipients, independent from underlying indication^[57,61-63].

The pathophysiological mechanisms accounting for such fatal complications are not completely defined. However, it seems to be quite clear that sarcopenia and in particular sarcopenic obesity negatively affect immunocompetence via pro-inflammatory cytokines and adipokines, such as IL-1, IL-6, tumor necrosis factor (TNF)- α and leptin. Apart from that, secretion of the myokine IL-15 is decreased, which has negative effects on growth and differentiation of B and T lymphocytes, natural killer cells, macrophages and monocytes. Thus, a persistent state of immunosuppression and inflammation arises, which is not only enhancing morbidity and mortality, but may also promote cancer development^[64-67]. A large retrospective analysis including 1257 HCC patients following curative and non-curative treatments has recently identified sarcopenia as an independent promoter of mortality and HCC recurrence^[68].

Apart from that, several studies on hepatic resection have shown that risk of HCC recurrence is significantly higher in sarcopenic patients compared to those without muscle waste^[69-72]. These data suggest that, with special regard to high immunosuppressive load early post-LT, sarcopenia-related depression of the

Table 2. Impact of sarcopenia on post-LT HCC recurrence

Ref.	n	Surgical procedure	Impact on overall outcome	Multivariable impact on post-LT HCC relapse
Itoh <i>et al.</i> ^[72]	153	LDLT	Low SVR was associated with poor RFS ($P = 0.01$) and OS ($P = 0.03$.)	Low SVR was identified as an independent promoter of poor post-LDLT outcome
Kim <i>et al.</i> ^[73]	92	LDLT	Cumulative HCC recurrence probability was significantly higher in sarcopenic vs. non-sarcopenic MC Out patients ($P = 0.044$). HCC recurrence rates were 36.1% and 5.0% in sarcopenic and non-sarcopenic patients.	Sarcopenia was identified as an independent predictor of HCC relapse (HR = 2.25; 95%CI 1.18-76.32; $P = 0.034$), along with AFP
Sharma <i>et al.</i> ^[74]	118	DDLT	Overall post-LT survival was significantly lower in patients with low BMD compared to those with high BMD ($P = 0.018$)	Low BMD was identified as an independent predictor of post-LT mortality in HCC LT patients (HR = 0.90; 95%CI 0.83-0.90; $P = 0.03$)

BMD: bone mineral density; CI: confidence interval; DDLT: deceased donor LT; HCC: hepatocellular carcinoma; HR: hazard ratio; LDLT: living donor liver transplantation; MC: Milan criteria; SVR: skeletal muscle-to-visceral fat area ratio

immunocompetence may also increase the oncological risk in LT patients [Table 2]. In a subset of 153 patients following LDLT for HCC, low skeletal muscle-to-visceral fat area ratio (SVR) was shown to predict poor recurrence-free survival (RFS) and OS. In addition, low SVR was identified as an independent and significant prognostic factor for post-LT outcome^[72]. Kim *et al.*^[73] have specifically studied the impact of sarcopenia in series of 92 LDLT patients with Milan Out HCC. Tumor recurrence rate was 36.1% in sarcopenic patients and only 5% in those without muscle depletion. Apart from AFP level and MVI, sarcopenia was identified as an independent and significant promoter of HCC relapse. In series of 118 HCC LT patients, Sharma *et al.*^[74] were able to demonstrate that bone mineral density (BMD), an early predictor of sarcopenia, is an independent predictor of post-LT mortality, with HCC recurrence to be the most common cause of death. A recent meta-analysis by Chang *et al.*^[75] including 13 studies and 3111 HCC patients after curative treatments concluded that sarcopenia is correlated with both, all-cause mortality (HR = 1.95; 95%CI 1.6-2.37) and tumor recurrence (HR = 1.76; 95%CI 1.27-2.45).

Implementing clinical features of sarcopenia in pretransplant decision making, such as the ability to walk, may significantly improve selection process and outcome^[63]. In addition, perioperative interventions like intense physiotherapeutic rehabilitation and nutritional treatment are able to improve posttransplant OS^[76-78]. Whether this may have a beneficial impact on oncological outcome post-LT needs to be further assessed.

Immunological dysbalance associated to malnutrition should be discovered early before sarcopenia has been established. In this context, Nagai *et al.*^[78] have identified peritransplant lymphopenia, which is considered a surrogate marker of immunosuppression and poor nutritional status, as an independent predictor of both, impaired OS and RFS following LT for HCC.

Liver graft injury and marginal liver grafts

Hepatic ischemia reperfusion (I/R) injury

I/R injury to the liver graft is an inevitable process during harvesting, preservation, storage and final implantation of the organ, triggered by consecutive cold and warm ischemia periods. Severe hepatic I/R damage increases the risk of posttransplant early allograft failure and immunological complications^[79]. Currently, there is growing evidence from experimental studies that immune damage and pro-inflammatory response reaction induced by allograft hypoxia promote the oncological risk^[80,81]. Although the precise molecular mechanisms have not yet been identified, it seems to be evident that I/R damage has cancerogenic capabilities via different molecular approaches and levels^[82]. Simply put: (1) hepatic I/R produces a pro-cancer microenvironment via microvascular disturbances, tissue hypoxia and angiogenesis; (2) resulting pro-inflammatory response reactions render HCC cells to be more aggressive by supporting mechanisms of cell adhesion, migration and invasion; and (3) hepatic I/R injury stimulates circulatory progenitor and immune cells to support post-LT HCC relapse.

Table 3. Impact of cold and warm ischemia times on HCC recurrence following LT

Reference	n	Impact on tumor-specific outcome post-LT	Impact on tumor-specific outcome in unfavorable HCC phenotype
Nagai <i>et al.</i> ^[85]	391	Cumulative incidence of HCC recurrence was significantly higher in CIT > vs. < 10 h ($P = 0.015$), and for WIT > vs. < 50 min ($P = 0.036$). CIT (HR = 1.9; 95%CI 1.06-3.04; $P = 0.03$) and WIT (HR = 2.84, 95%CI 1.44-4.85; $P = 0.003$) were both identified as independent predictors of HCC relapse	CIT > 10 h (HR = 2.6; 95%CI 1.23-5.49; $P = 0.01$) and WIT > 50 min (HR = 3.23; 95%CI 1.24-8.38; $P = 0.01$) correlated independently with HCC recurrence in patients with vascular tumor invasion but not in those without
Kornberg <i>et al.</i> ^[84]	103	Apart from PET+ status, AFP > 400 ng/dL and beyond MC HCC, WIT > 50 min was identified as an independent and significant promoter of post-LT HCC relapse (HR = 52.5; 95%CI 6.0-458.1; $P < 0.001$). RFS rates at 1 and 3 years post-LT were 97.2% and 92.8% in WIT ≤ 50 min, and 61.4% and 42.0% in WIT > 50 min, respectively ($P < 0.001$)	In Milan In patients, HCC recurrence rate was 0% in limited but 42.2% in extended WIT ($P = 0.001$). In the Milan Out subset, 10 of 13 patients with WIT > 50 min (76.9%), but only 6 of 27 patients with WIT ≤ 50 min (22.2%) developed HCC relapse ($P = 0.001$). WIT was identified as the only independent and significant risk factor in patients with PET+ tumors (OR 15.5; 95%CI 3.0-101.5; $P < 0.001$)
Grat <i>et al.</i> ^[85]	90	Apart from beyond MC tumors, pre-LT AFP level and male donor sex, CO-LT (HR = 5.88; 95%CI 1.86-18.58; $P = 0.003$) and prolonged total ischemia time (HR = 1.48; 95%CI 1.06-2.07; $P = 0.02$) were identified as independent predictors of tumor recurrence	In MC In patients, RFS rates at 3 years post-LT were 100% and 66.7% following PB-LT CO-LT ($P = 0.003$). Corresponding data in MC Out patients were 77.8% and 48.9% ($P = 0.031$), respectively
Orci <i>et al.</i> ^[86]	9724	Warm ischemia time > 19 min was independently associated with HCC recurrence (HR = 4.26; 95%CI 1.20-15.1; $P = 0.025$)	

AFP: alpha fetoprotein; CI: confidence interval; CIT: cold ischemia time; CO-LT: conventional liver transplantation; HCC: hepatocellular carcinoma; HR: hazard ratio; MC: Milan criteria; OR: odds ratio; PB-LT: piggy back liver transplantation; PET: positron emission tomography; RFS: recurrence-free survival; WIT: warm ischemia time

Transfer of these insights to the clinical transplant setting is still hampered by lack of clear standards of hepatic I/R injury measurement^[83,84]. However, there is convincing evidence that duration of cold (CIT) and warm ischemia times (WIT), which are the major triggers of I/R damage to the liver graft, correlate with risk of HCC recurrence post-LT [Table 3].

In a series of 391 LT patients with HCC, Nagai *et al.*^[85] reported that CIT > 10 h and WIT > 50 min were independent and significant predictors of overall and early post-LT HCC recurrence. In addition, both correlated independently with risk of tumor recurrence in patients with but not in those without vascular tumor infiltration.

Our transplant group was able to confirm the prognostic importance of ischemia time in a subset of 103 LT patients with HCC^[84]. Both CIT (468 vs. 375.5 min; $P = 0.001$) and WIT (58.4 vs. 45.7 min; $P = 0.001$) were significantly longer in patients with compared to those without HCC relapse. Apart from PET+ status, AFP > 400 ng/dL and beyond MC tumors, WIT > 50 min was identified as an independent and significant promoter of post-LT HCC relapse^[84]. RFS rates at 1 and 3 years post-LT were 97.2% and 92.8% in WIT ≤ 50 min, and 61.4% and 42.0% in WIT > 50 min, respectively ($P < 0.001$). In addition, WIT was able to further stratify the oncological risk in unfavorable HCC phenotype, such as PET+ tumors [Table 3].

Another interesting approach by Grat *et al.*^[86] has focused on outcome differences between piggy back (PB) and conventional (Co) LT procedures for HCC. Among others, shorter duration of anhepatic phase and WIT were reported to be major outcome advantages of PB-LT (without clamping and replacement of the inferior caval vein) in comparison to CO-LT (including clamping and replacement of the inferior caval vein). In their series of 90 patients, RFS rates at 1, 2 and 3 years post-LT were 97.0%, 92.2%, and 89.4% for PB-LT, but only 75.6%, 56.0%, and 56.0% for CO-LT, respectively ($P = 0.0006$). Apart from beyond MC tumors, pre-LT AFP level and male donor sex, CO-LT and prolonged total ischemia time were identified as independent predictors of tumor recurrence. In addition, RFS rates were significantly different in MC In and MC Out patients when being stratified according to transplant procedure [Table 3].

Table 4. Impact of donor age on HCC recurrence

Reference	n	Impact on post-LT HCC recurrence
Sharma <i>et al.</i> ^[95]	94	Median donor age was 49 y and 36 y in patients with and without HCC relapse ($P = 0.008$). Along with number and largest diameter of tumor nodules, donor age was identified as the only pre-LT available independent risk factor of tumor recurrence (HR = 1.06; 95%CI 1.02-1.10; $P = 0.002$)
Vagefi <i>et al.</i> ^[96]	5002 (UNOS database)	Cumulative incidence of HCC recurrence at 1-, 2-, 3-, and 4-year post-LT was 3%, 5.1%, 6.4% and 7.3% in donors < 60 y, but 4.5%, 8.3%, 10.4% and 11.8% in donors > 60 y ($P < 0.05$). Apart from non-local organ sharing, donor age ≥ 60 years was reported to be the only independent donor-related predictor of HCC recurrence (HR = 1.42; 95%CI 1.09-1.84; $P = 0.009$)
Orci <i>et al.</i> ^[87]	9724 (SRTR database)	Donor age > 60 y (HR = 1.38; 95%CI 1.10-1.73; $P = 0.006$) was identified as an independent promoter of HCC relapse

CI: confidence interval; HCC: hepatocellular carcinoma; HR: hazard ratio; LT: liver transplantation

In another study including 9724 liver transplant recipients of the Scientific Registry of Transplant Recipients (SRTR) database, WIT ≥ 19 min was associated with increased risk of HCC relapse in uni- and multivariable analysis. However, the authors did not stratify data according to MC^[87].

Marginal liver grafts

The dramatic shortage of appropriate donor livers enhances the risk of patients' drop-out due to tumor progression and/or morbidity or mortality related to cirrhosis progression during waiting times. Therefore, the so-called extended criteria donor grafts (ECD) are increasingly used for decreasing the fatal discrepancy between demand and donor organ availabilities^[88]. In order to avoid penalizing patients with standard criteria HCC or other indications, marginal liver grafts, such as steatotic livers, living donor liver grafts, donor livers after cardiac death (DCD) and older donor grafts are currently accepted for patients with advanced HCC stages, not at least as these patients frequently present with compensated liver function. However, such ECD livers are more susceptible to severe I/R damage, which may impair immunological and oncological outcome^[89].

Steatotic donor livers

In recent years, liver steatosis has become a serious medical issue due to growing rates of diabetes, obesity, metabolic syndrome and alcohol abuse. Consequently, the numbers of explanted, offered and finally accepted steatotic liver grafts has significantly increased in recent years. However, donor graft steatosis is associated with overall poorer outcome post-LT^[90]. Based on histopathologic assessment, we distinguish between mild (< 30%), moderate (30%-60%) and severe (> 60%) liver steatosis, whereby particularly recipients of the latter are subject to an extraordinary risk of hepatic I/R damage with risk of post-LT allograft failure^[91]. In an experimental setting, Orci *et al.*^[92] have shown that I/R injury contributes to more severe intrahepatic and remote HCC recurrence with enhanced liver steatosis. Although statistical significance was lacking, Teng *et al.*^[93] reported on a clear trend of higher HCC recurrence rates in recipients of moderate-to-severe steatotic (50%) compared to non-steatotic grafts (28.7%) and mild steatosis (20.8%). In a large registry trial ($n = 3007$), Orci *et al.*^[87] reported that graft steatosis > 60% was an independent promoter of HCC recurrence post-LT (HR = 1.65; 95%CI 1.03-2.64; $P = 0.037$).

Donor age

The use of elderly donor livers increases the risk of early post-LT graft loss, arterial and biliary complications, and immunological insults. Particularly presence of hepatitis C and prolonged ischemia times are known triggers of the negative impact of older donor grafts^[94]. In recent years, there is growing evidence that donor age may also affect oncological outcome in HCC LT patients [Table 4]. In a retrospective study of 94 liver recipients, Sharma *et al.*^[95] were the first to identify donor age as an independent predictor of HCC recurrence, along with number of tumor lesions and size of the largest tumor diameter. Two large registry studies have subsequently confirmed the oncological importance of donor age. Apart from non-local organ sharing, donor

Table 5. Impact of graft size on outcome in LDLT for HCC

Reference	n	Impact of GRWR on post-LDLT outcome	Impact of GRWR on outcome in advanced HCC
Hu <i>et al.</i> ^[105]	295	OS was significantly better in GRWR $\leq 0.8\%$ vs. $> 0.8\%$ ($P = 0.009$). RFS tended to be better in GRWR > 0.8 ($P = 0.133$). GRWR $> 0.8\%$ was identified as independent predictor of poor OS (HR = 2.166; 95%CI 1.173-4.001; $P = 0.013$), along with vascular invasion	
Li <i>et al.</i> ^[106]	597	RFS rates at 1-, 3- and 5 years were 75.9%; 73.3%, and 71.7% in GRWR $< 0.8\%$, and 86.4%, 80.8% and 77.9% in GRWR $\geq 0.8\%$, respectively ($P = 0.17$). The corresponding OS rates were 87.8%, 80.3% and 78.7% (GRWR $< 0.8\%$), and 93.5%, 87.1%, and 84.1% (GRWR $\geq 0.8\%$; $P = 0.017$)	The 1-, 3- and 5-year RFS rates in MC Out patients were 52.4%, 49.3% and 49.3% in GRWR $< 0.8\%$, and 76.5%, 68.3%, and 64.3% in GRWR $\geq 0.8\%$ ($P = 0.049$). The corresponding OS rates were 77.1%, 65.3%, and 61.5% (GRWR $< 0.8\%$), and 90.2%, 80.1%, and 77.5% (GRWR $> 0.8\%$, $P = 0.047$). No significant effect of GRWR on outcome in Milan In patients was found

CI: confidence interval; GRWR: graft-to-recipient body weight ratio; HR: hazard ratio; OS: overall survival; RFS: recurrence-free survival

age ≥ 60 years was reported to be the only independent donor-related predictor of HCC recurrence in a study of 5002 patients of the UNOS database^[96]. Comparably, Orci *et al.*^[87] reported on an independent prognostic effect of donor age > 60 years (HR = 1.38; 95%CI 1.10-1.73; $P = 0.006$), when analyzing 9742 patients of the SRTR database. Adequate donor-recipient age matching was shown to improve overall long-term outcome in recipients of older donor grafts^[97]. However, no data exists on the oncological impact of such a matching policy.

Living donor liver grafts

LDLT has been established as an appropriate alternative approach to fight organ shortage and, thereby, to decrease risk of drop out from the waiting list, especially in Eastern countries where the number of deceased donor liver transplants (DDLT) is significantly restricted. Allocation of these organs is not regulated by public institutions, so that the indication is independent of strict tumor size limitations. Therefore, LDLT is particularly attractive for advanced HCC patients, who may otherwise not be offered a transplant option via HCC exceptional MELD allocation, but rather transferred to palliative treatments^[98,99]. However, apart from the donors' risks related to major hepatectomy, there are important oncological issues that have to be considered.

Liver grafts from living donors are principally small for size and, thus, exposed to an enhanced acute phase attack, which is an established promoter of cancer^[82,100]. Another important oncological aspect is that fast track LDLT without HCC MELD-related waiting time may select more aggressive tumors that otherwise would have been identified and probably rejected^[101]. Based on current mainly retrospective studies of the Eastern and Western transplant regions, the impact of reduced liver graft size compared to full-size donor livers on HCC recurrence remains finally unclear. One meta-analysis including 7 studies and 1310 patients did not find significant outcome differences between both transplant procedures, also when stratified according to MC^[102]. In contrast, a more recent meta-analysis by Grant *et al.*^[103] including 633 LDLT and 1232 DDLT patients provided evidence for reduced RFS following LDLT. Prospective multicenter studies are need, implementing standardized tumor selection criteria, comparable neoadjuvant tumor treatments and intent-to-treat outcome data, which seems to be illusionary with regard to different strategies and mentalities between Eastern and Western countries.

What seems to be equally important is, whether LDLT is principally able to produce acceptable outcome in beyond Milan patients, which by definition may also be lower than those for Milan In patients. Regarding this, it became apparent in recent years that post-LDLT 5-year RFS rates far beyond 50% are possible in MC Out patients when implementing parameters of biological tumor aggressiveness, such as AFP, PIVKA II or PET-status^[98,104]. Apart from that, size of the living related donor graft may be another important prognostic factor that should be considered [Table 5]. In a series of 295 HCC patients following LDLT, Hu *et al.*^[105]

reported on significantly better 1- and 3- year OS rates in graft-to-recipient body weight ratio (GRWR) $\leq 0.8\%$ vs. $> 0.8\%$ ($P = 0.009$), whereas the corresponding RFS rates tended to be different ($P = 0.133$). Besides vascular invasion, GRWR was identified as the only independent and significant prognostic factor for OS. Analyzing 597 consecutive LDLT patients, Lee *et al.*^[106] were able to demonstrate that RFS in Milan Out patients was significantly better in GRWR $< 0.8\%$ ($P = 0.049$) [Table 5].

DCD

In order to cope with dramatic donor organ shortage, donors after cardiac or circulatory death have been increasingly used in recent years. In comparison to LT using donors after brain death (DBD), DCD LT is characterized by repeat and prolonged WIT, higher susceptibility to I/R damage, increased rate of post-LT graft failure, higher rates of re-transplants, and impaired overall outcome^[107,108]. The impact of applying DCD liver grafts on the oncological outcome is currently assessed controversially. Using the SRTR database, Croome *et al.*^[109] demonstrated inferior survival after DCD LT (55.86% at 5-year post-LT) compared to DBD LT (63.77% at 5-years post-LT; $P < 0.001$) in HCC patients, without including data on tumor recurrence. More recently, several large single-center studies did not find a significant difference in cancer-related outcome between both transplant procedures^[110,111]. Using the SRTR database, Oric *et al.*^[87] failed to identify a negative prognostic impact of DCD grafts when being compared to DBD livers. However, WIT exceeding 19 min proved to be an independent predictor of HCC relapse in the subset of DCD liver recipients (HR = 4.26; 95%CI 1.2-15.1, $P = 0.025$).

Improving cancer-specific outcome by mitigating I/R injury

Several approaches to improve tumor-specific outcome by reducing hepatic I/R injury are currently under experimental and clinical consideration.

Orci *et al.*^[112] demonstrated that ischemic preconditioning prior to I/R injury reduced tumor load in an experimental setting of rat liver steatosis to an equal level as in non-steatotic control grafts. The same group recently demonstrated in another experimental study that remote ischemic preconditioning may reduce I/R injury and modulate the gut-liver axis, finally alleviating HCC recurrence^[113].

In a retrospective clinical analysis, our transplant group was able to demonstrate that early post-LT treatment with prostaglandin E1 (PGE1) reduces hepatic I/R damage and provides beneficial immunomodulatory capabilities, finally improving cancer-specific outcome^[114]. In a series of 106 HCC LT patients, RFS rates at 3- and 5-year post LT were significantly better in the PGE1-treatment group (87.9%; 85.7%) compared to the non-PGE1 subset (65.3%; 63.1%; $P = 0.003$). In addition, rate of early HCC relapse within 1 year from LT was significantly higher without PGE1 treatment (34% vs. 5.1%; $P < 0.001$). When stratified according the MC, PGE1-therapy did not exert an independent prognostic impact in Milan In, whereas it was identified as a significant and independent promoter of RFS in patients with MC Out patients (HR = 5.09; 95%CI 1.64-15.76; $P = 0.005$)^[114].

The increasing use of different hypo- or normothermic extracorporeal liver perfusion systems may be another promising approach to expand the pool of transplantable ECD livers. Pre-transplant assessment of organ viability and reducing susceptibility to hepatic I/R are the suggested scope of application. In fact, the safety and feasibility of ex-situ machine preservation have already been demonstrated. First clinical trials suggested reduced morbidity and mortality in recipients of high risk organs that were pretreated with extracorporeal machine perfusion devices^[115-117]. Just recently, He *et al.*^[118] from Guangzhou transplant center presented the first case of “ischemia-free transplantation” of a severely steatotic graft by using normothermic machine perfusion without stopping blood supply, already initiated during donor liver harvesting. So far, there are no

Table 6. Impact of intraoperative blood loss and red blood cell transfusion on post-LT outcome

Reference	n	Overall post-LT outcome	Cancer-specific outcome in unfavorable HCC phenotypes
Teng <i>et al.</i> ^[125]	223	IOBL was identified as an independent predictor of OS when stratified according: Milan: HR = 1.039; 95%CI 1.021-1.057; $P < 0.001$ UCSF: HR = 1.039; 95%CI 1.002-1.057; $P < 0.001$ Fudan: HR = 1.035; 95%CI 1.018-1.052; $P < 0.001$ Hangzhou: HR = 1.020; 95%CI 1.000-1.040; $P = 0.046$	
Liu <i>et al.</i> ^[126]	479	Cumulative 1- and 3-year RFS rates were 30.5% and 42.0% in IOBL ≤ 4 L, and 52.6% and 62.8% in IOBL > 4 L ($P < 0.001$). IOBL > 4 L was identified as an independent promoter of overall HCC recurrence (HR = 2.32; 95%CI 1.60-3.36; $P < 0.001$) and early post-LT (within 1 year) tumor relapse (HR = 2.45; 95%CI 1.64-3.66; $P < 0.001$). Red blood cell transfusion had no prognostic impact	IOBL > 4 L was identified as an independent predictor of tumor recurrence in tumors with vascular invasion (HR = 2.86; 95%CI 1.76-4.64; $P < 0.001$) but not in those without vascular invasion (HR = 1.57; 95%CI 0.87-2.85; $P = 0.138$)
Kornberg <i>et al.</i> ^[127]	111	Post-LT RFS rates at 3 and 5 years' post-LT were 91.9% and 91.9% in IOBL ≤ 1500 mL, but only 43.9% and 37.1% in IOBL > 1500 mL ($P < 0.001$). IOBL was identified as independent predictor of beneficial RFS (HR = 3.91; 95%CI 1.496-10.210; $P = 0.005$) of the entire study group, whereas red blood cell transfusion had no independent prognostic significance	IOBL was identified as an independent prognostic factor for RFS in Milan Out patients (HR = 3.66; 95%CI 1.138-11.766; $P = 0.029$) and PET+ patients (HR = 4.13; 95%CI 1.482-11.524; $P = 0.007$). Application of > 3 red blood cell units proved to be an independent oncological factor in Milan Out (HR = 4.98; 95%CI 1.442-17.185; $P = 0.011$) and PET+ patients (HR = 2.98; 95%CI 1.071-8.280; $P = 0.037$)
Nagai <i>et al.</i> ^[78]	391	Red blood cell transfusion was a strong univariate (HR = 1.03; 95%CI 1.01-1.05; $P = 0.001$) but not an independent (HR = 1.02; 95%CI 0.99-1.05; $P = 0.14$) predictor of post-LT HCC recurrence	
Seehofer <i>et al.</i> ^[133]	336	Apart from microvascular tumor invasion ($P < 0.001$), blood transfusion was identified as the only significant independent predictor of HCC recurrence ($P = 0.033$)	The negative impact of blood transfusions on RFS was more pronounced in patients with ($P = 0.023$) than in those without vascular tumor invasion

CI: confidence interval; HR: hazard ratio; IOBL: intraoperative blood loss; LT: liver transplant; OS: overall survival; PET: positron emission tomography; RFS: recurrence-free survival; UCSF: University of California San Francisco

clinical data on the oncological impact of extracorporeal machine perfusion in HCC patients.

Perioperative complications

In recent years, postoperative complications, such as bleeding, bile leakage, ascites, liver failure, infection and need of reoperation were shown to significantly impair overall and cancer-specific outcome following liver resection for HCC^[119-121]. In the LT setting, surgical complications reduce the overall prognosis in HCC patients. Dai *et al.*^[122] have recently identified complications grade IIIA or more according to Clavien-Dindo classification as only independent predictor of poor overall outcome (HR = 1.108; 95% CI 1.45-34.71; $P = 0.015$) in a series of 99 LT patients with HCC. Just recently, a study from Washington DC demonstrated in a series of 428 patients that re-operation following LT was an independent predictor of graft loss (OR = 5.125; 95%CI 1.35819.552; $P = 0.016$)^[123].

Intraoperative bleeding is still a major determinant of perioperative complications and a need of early reoperation in HCC patients. In times of increasing MELD scores and decreasing liver graft quality, blood loss remains a critical issue in LT, despite significant improvements in surgical techniques and homeostasis management^[124]. There is increasing evidence that the extent of intraoperative blood loss (IOBL) may not only increase early morbidity and mortality, but also promote post-LT HCC recurrence [Table 6].

In a study including 223 HCC LT patients, Teng *et al.*^[125] identified IOBL as an independent prognostic factor for poor OS, independent from the selectin criteria applied. However, the authors did not provide data on oncological outcome. The same group subsequently demonstrated in a series of 479 patients that, apart from recipients age, beyond MC status, AFP > 400 ng/mL and vascular invasion, IOBL > 4 L was an independent predictor of overall HCC recurrence and early post-LT (within 1 year) tumor relapse. In addition, IOBL was independently correlated with tumor recurrence in patients with but not in those without vascular invasion^[126].

We have recently studied the impact of IOBL with a cut-off value of 1500 mL in 111 LT patients with HCC^[127]. Post-LT RFS rates at 3 and 5 years were 91.9% and 91.9% in the low, but only 43.9% and 37.1% in the high IOBL subset ($P < 0.001$). Along with PET-status, tumor grading and AFP level, IOBL was identified as an independent predictor of cancer-specific survival. Furthermore, IOBL correlated independently with cancer relapse in unfavourable tumor phenotypes, such as Milan Out and PET+ tumors, but not in low-risk HCC^[127].

Enhanced spread of occult cancer cells, aggravation of I/R injury to the graft and induction of pro-inflammatory and immunosuppressive mechanisms are currently discussed as underlying cancerogenic mechanisms^[125-129]. Apart from that, IOBL increases the need of red blood cell transfusion, which in turn enhances the oncological risk by induction of pro-inflammatory and immunosuppressive mechanisms^[130,131]. In a meta-analysis including 5635 cases, allogeneic blood transfusion was shown to significantly increase the risk of HCC recurrence at 1, 3, and 5 years following liver resection^[132]. Nagai *et al.*^[78] identified red blood cell transfusion as a strong univariate factor, but it had no independent prognostic significance on post-LT HCC relapse. In a retrospective analysis including 336 LT patients, Seehofer *et al.*^[133] identified red blood cell transfusion as an independent promoter of HCC recurrence, along with vascular tumor invasion. The negative prognostic impact of blood transfusion was particularly evident in patients with vascular invasion. We have recently identified application of > 3 red blood cell units as significant and independent prognostic factor in patients with Milan Out HCC and patients with PET-positive tumors^[127].

Whether the observed oncological risks are related to IOBL or rather to transfusion remains still unclear. In any case, limiting the risk of intraoperative bleeding and, thereby, need of red blood cell transfusion seems to be critical for improving post-LT cancer-specific outcome, particularly in patients with unfavourable tumor stages [Table 6]. As has been shown by several recent studies, intraoperative blood salvage and autologous re-transfusion do not increase the oncological risk and should increasingly be considered, in order to avoid allogeneic transfusion^[134,135].

Post-transplant immunosuppression

Post-transplant immunosuppressive treatment is recognized as a major risk factor for HCC recurrence following LT. In an immunocompetent patient, the innate immune system is able to recognize and destroy CTC. But in the transplant setting, postoperatively high immunosuppressive doses are administered in order to achieve liver graft acceptance, which depresses the natural anti-cancer properties of the immunological defence. Apart from development of de-novo cancers, this may lead to acceleration of metastatic spread, implantation and growth of circulating tumor tissue in HCC patients^[136,137].

Despite a large number of studies on this topic, the most optimal immunosuppressive concept for HCC LT patients has not yet been defined. This may be due to the fact that the vast majority of trials are of retrospective character with significant differences regarding patients' selection criteria, transplant procedure, applied immunosuppressive protocols and post-LT surveillance program. The major conclusions that can be drawn from current available data are the following: (1) early post-LT reduced exposure to calcineurin inhibitor (CNI) is an important factor of improved tumor-specific outcome post-LT [Table 7]. The CNIs cyclosporine and tacrolimus are still the main immunosuppressants used in the setting of LT. Apart from immunoregulatory properties, CNIs are also able to render oncogenes to promote tumor cell aggressiveness and invasiveness, growth and metastasis^[138,139]. As shown by an Italian group, early post-LT dose reduction of CNIs has a favourable effect on cancer-specific outcome^[140,141]. In a large 2 European center study including 219 HCC patients, Rodríguez-Perálvarez *et al.*^[141] reported that higher exposure to CNI (mean tacrolimus trough level > 10 ng/dL or cyclosporine trough concentrations > 300 ng/dL) within the first months post-LT enhanced the risk of HCC relapse (27.7% vs. 14.7% at 5 years; $P = 0.007$). Early post-LT reduced CNI exposure was identified as an

Table 7. Immunosuppressive approaches to reduce the oncological risk after LT

Reference	n	Immunosuppressive approach	Impact on tumor-specific outcome
Vivarelli <i>et al.</i> ^[140]	70	Reduced CsA exposure (≤ 189.6 ng/mL)	Mean CsA exposure was 278.3 ± 86.4 ng/mL in patients with, and 169.9 ± 33.3 ng/mL in those without HCC recurrence. Reduced CsA exposure was identified as the only independent predictor of HCC recurrence ($P < 0.001$)
Vivarelli <i>et al.</i> ^[141]	130	Reduced CNI exposure (CsA ≤ 220 ng/mL; Tac ≤ 10 ng/mL)	Apart from tumor grading, MVI and AFP level, exposure to CNI was identified as the only independent predictor of HCC relapse (HR = 4.01; 95%CI 1.33-12.09; $P = 0.014$)
Rodriguez-Peralvarez <i>et al.</i> ^[142]	219	Reduced CNI exposure (CsA ≤ 300 ng/mL; Tac ≤ 10 ng/mL)	Apart from tumor nodule diameter, micro- and macrovascular invasion, exposure to CNI was identified as independent predictor of HCC relapse (HR = 2.82; 95%CI 1.4-5.8; $P = 0.005$). Reduced CNI exposure resulted in a significantly better RFS in MC Out patients ($P = 0.004$), whereas there was a trend of improved tumor-specific outcome in Milan Out patients ($P = 0.09$)
Liang <i>et al.</i> ^[149]	2950	SRL-based IS	SRL-based regimens led to improved overall survival at 1 (OR = 4.53; 95%CI 2.31-8.89), 3 (OR = 1.97; 95%CI 1.29-3.00) and 5 years (OR=2.47; 95%CI 0.21-0.83) post-LT. In addition, HCC recurrence rate was significantly decreased (OR = 0.42; 95%CI 0.21-0.83)
Menon <i>et al.</i> ^[150]	474	SRL-based IS	SRL-based IS resulted in lower recurrence rate (OR = 0.3; 95%CI 0.16-0.55; $P < 0.001$), lower recurrence-related mortality (OR = 0.29; 95%CI 0.20-0.70; $P = 0.005$) and lower overall mortality (OR = 0.35; 95%CI 0.20-0.61; $P < 0.001$) compared to CNI-based IS
Cholongitas <i>et al.</i> ^[151]	3666	mTORi-based IS	HCC recurrence rate was significantly lower in mTORi-based IS (8%) compared to CNI-based protocol (13.8%; $P < 0.001$)
Zhang <i>et al.</i> ^[152]	7695	SRL-based IS	SRL-based IS prolonged 1-year (OR = 2.44; 95%CI 1.66-3.59), 3-year (OR = 1.67; 95% CI 1.08-2.58) and 5-year (OR = 1.68; 95%CI 1.21-2.33) OS compared to the control group. SRL resulted in lower HCC recurrence rates (OR = 1.68; 95%CI 0.37-0.98), lower recurrence-related mortality (OR = 0.58; 95%CI 0.42-0.81) and lower overall mortality (OR = 0.62; 95% CI 0.44-0.89) compared to SRL-free regimens

CI: confidence interval; CNI: calcineurin inhibitor; CsA: cyclosporin A; HCC: hepatocellular carcinoma; IS: immunosuppression; mTORi: mammalian target of rapamycin inhibitor; OR: odds ratio; SRL: sirolimus; Tac: tacrolimus

independent predictor of favourable cancer-specific outcome. Stratified according the pathologic MC, reduced CNI exposure resulted in a significantly better RFS in Milan In patients, whereas there was a clear trend of improved RFS in Milan Out patients ($P = 0.09$), respectively^[142]; and (2) the protective effect of sirolimus (SRL) based immunosuppression is still inconclusive.

The use of mammalian target of rapamycin inhibitors (mTORis), such as rapamycin (SRL) and everolimus (EVL) provide anti-cancer effects by inhibiting the PI3K/Akt/mTOR pathway beyond its immunosuppressive capabilities^[143,144]. Therefore, many hopes had been placed in this immunosuppressant in recent years for reducing the risk of post-LT HCC recurrence without affecting the immunological outcome^[145-148]. Several systematic reviews and meta-analyses in the past suggested a significant benefit of SRL in HCC LT patients^[149-151] [Table 7]. Just recently, Zhang *et al.*^[152] presented data on an updated meta-analysis including the largest number of patients ($n = 7695$) from a total of 11 studies. The authors reported that patients treated with SRL demonstrated lower recurrence rates, lower recurrence-related mortality and lower overall mortality compared to SRL-free regimens. Whether advanced HCC patients were particularly benefiting from SRL was, however, not adequately assessed. The only prospective, randomized, multicenter, open-label study recently finalized, however, did not find a significant improvement of OS and RFS beyond 5 years^[153].

Currently, several approaches to achieve recipient tolerance by IS weaning protocols in order to reduce long-term CNI-induced complications, such as hyperlipidemia, cardiovascular events, renal dysfunction and de-novo carcinoma are under consideration^[154-157]. About 25% of liver transplant patients were reported to be suitable for complete IS withdrawal without increasing the risk of patient and graft loss. Probably, the application of non-invasive biomarkers predicting “operational tolerance” might permit significant reduction

in a higher number of liver recipients^[154]. As suggested in small study samples, this might be a promising IS-based approach to reduce the oncological risk in LT patients with HCC. However, larger prospective studies are needed.

CONCLUSION

As pointed out in this review, there are several important non-HCC related factors of prognosis that have to be considered in LT for HCC. However, comparability of related studies is rather limited by their mostly retrospective character and the use of different outcome variables [Tables 1-7]. Nevertheless, there is growing evidence that these non-oncological features trigger a series of unfavorable immunomodulatory processes related to inflammation and immunosuppression, and thereby promoting the oncological risk following LT. This may be particularly relevant for patients with advanced HCC stages, who are per se exposed to an increased risk of HCC recurrence. Therefore, these non-oncological factors should play an important role in individual decision making. The presented data suggest that adequate patient and graft selection, limitation of ischemia time, reduction of surgical complications and minimizing post-LT immunosuppressive drug load may be essential components for preserving immunobalance and, thereby, for improving cancer-specific survival.

Since all of these features are well-known prognostic factors that are generally affecting outcome of LT patients even without underlying malignancy, it is a particular challenge to determine the individual transplant benefit based on both tumor biology data and non-HCC variables. In this context, there is currently no applicable clinical algorithm which is implementing both aspects for risk assessment. However, what became clear from our review is that such an approach should include concepts of mitigating hepatic I/R damage not only to improve early posttransplant patient and graft survival, but to reduce the potency of metastatic tumor cell implantation and growth. Thus, the HCC patients' selection criteria might be safely expanded beyond current macromorphologic tumor burden limits.

DECLARATIONS

Authors' contributions

Conceived the study, analyzed data and wrote the manuscript: Kornberg A
Analyzed data and revised the manuscript: Schernhammer M

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Novel diagnosis and therapy for hepatoma targeting HBV-related carcinogenesis through alternative splicing of FIR (PUF60)/FIR Δ exon2

Kazuyuki Matsushita¹, Tyuji Hoshino²

¹Department of Laboratory Medicine & Division of Clinical Genetics and Proteomics, Chiba University Hospital, Chiba, 260-8677, Japan.

²Department of Physical Chemistry, Chiba University, Chiba 260-8670, Japan.

Correspondence to: Dr. Kazuyuki Matsushita, Department of Laboratory Medicine & Division of Clinical Genetics and Proteomics, Chiba University Hospital, Chiba, 260-8677, Japan. E-mail: kmatsu@faculty.chiba-u.jp

How to cite this article: Matsushita K, Hoshino T. Novel diagnosis and therapy for hepatoma targeting HBV-related carcinogenesis through alternative splicing of FIR (PUF60)/FIR Δ exon2. *Hepatoma Res* 2018;4:61. <http://dx.doi.org/10.20517/2394-5079.2018.81>

Received: 18 Jun 2018 **First Decision:** 24 Jul 2018 **Revised:** 29 Jul 2018 **Accepted:** 1 Aug 2018 **Published:** 29 Sep 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cui Yu **Production Editor:** Zhong-Yu Guo

Abstract

Aim: Disturbed alternative splicing of far upstream element-binding protein-interacting repressor (FIR) was found to be unable to repress c-Myc transcription and so it might be important for suppressing tumor development. FIR is a splicing variant of poly (U)-binding-splicing factor (PUF60), and forms complex with other splicing factors. FIR/PUF60 is a splicing factor of U2 small nuclear ribonucleoprotein auxiliary factor family, Thus FIR/PUF60 is a multifunctional protein. The expression of exon2-lacking splicing variant of FIR, FIR Δ exon2, is elevated in many cancer tissues and promotes tumor development by disabling FIR-repression to sustain c-Myc activation. FIR Δ exon2, as a dominant negative of FIR, opposed apoptosis in cancer cells. FIR/FIR Δ exon2 interacts with degron pocket of F-box and W (Typ) D (Asp) repeat domain-containing 7 and inhibits proteolysis of substrates proteins. Recently, FIR/PUF60 was identified as a versatile regulator of transcriptional and post-transcriptional steps in expression of hepatitis B virus (HBV) pregenomic RNA (pgRNA) expression.

Methods: Small molecular chemical compounds against FIR and FIR Δ exon2 were screened among 2,3275 chemicals by natural product depository array (RIKEN, Wako, Saitama, Japan).

Results: Nine chemicals against FIR and four chemicals against FIR Δ exon2 were identified as candidates of interacting chemicals. Interestingly, BK697 contains WD -like structure. Among them, BK697 against FIR Δ exon2 inhibited hepatoma cell growth.

Conclusion: Therefore, FIR (PUF60)/FIR Δ exon2 is multifunctional and applicable for clinical use for HBV suppression and hepatoma treatment. Together, one clue to the development of hepatoma diagnosis and therapies directed against FIR/FIR Δ exon2/PUF60 with small molecular weight chemicals that inhibit HBV cccDNA replication.



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Keywords: Hepatocellular carcinoma, hepatitis B virus, covalently closed circular DNA, far upstream element-binding protein-interacting repressor, poly (U)-binding-splicing factor, F-box and W (Typ) D (Asp) repeat domain-containing 7, natural product depository array, U2AF homology motif, U2AF homology motif ligand motif

INTRODUCTION

C-Myc is overexpressed in the majority of colorectal cancers and is required for tumor maintenance^[1,2]. The far upstream element (FUSE) is a sequence required for proper expression of the human *c-Myc* gene. The FUSE is located 1.5 kb upstream of *c-Myc* promoter P1, and binds the FUSE binding protein1 (FUBP1), a transcription factor stimulating *c-Myc* expression in a FUSE dependent manner^[3,4]. FUBP1 is overexpressed and regulates proliferation and migration of hepatoma cells^[5-7]. Yeast two-hybrid analysis revealed that FUBP1 binds to a protein that has transcriptional inhibitory activity termed the FUBP1-interacting repressor (FIR), and FIR was found to engage the transcriptional factor IIH [TFIIH/p89/xeroderma pigmentosum type B (XPB)] helicase and repress *c-Myc* transcription^[8]. FIR induces apoptosis *via* c-Myc suppression, and is thus a suitable cancer therapy^[9,10]. Adenovirus-FIR or Sendai virus-FIR vectors gene therapy for nasopharyngeal cancer were reported^[11-14]. Up to 60% of all human genes present at least one alternative splice variant^[15]. Disturbed alternative splicing (AS) in cancer cells or hepatitis B virus (HBV) virus affect host's immune response^[16,17]. AS has been documented to play a significant role in human disease and DNA repair in cancers^[18-21]. A splicing variant of FIR that lacks exon2, FIR Δ exon2, failed to repress c-Myc and inhibited FIR-induced apoptosis suggesting FIR Δ exon2 is a dominant negative of FIR in human cancers^[22]. On the other hand, FIR is a splicing variant form of poly(U)-binding-splicing factor (PUF60)^[23,24]. Anti-PUF60 autoantibodies are reported to be detected in the sera of autoimmune diseases such as dermatomyositis, Sjogren's syndrome or idiopathic inflammatory myopathy^[25,26]. Further, the combination of anti-FIRs antibodies with other clinically available tumor markers such as anti-p53 antibodies, CEA, and CA19-9 further improved the specificity and accuracy of diagnosis^[27,28]. Besides, haploinsufficiency of FIR mouse model promoted p53-dependent T-cell acute lymphoblastic leukemia progression^[29]. SAP155, a subunit of the essential splicing factor 3B (SF3B) subcomplex in the spliceosome, is required for proper P27Kip1 pre-mRNA splicing, and P27Kip1 arrests cells at G1^[30,31]. Moreover, spliceostatin A (SSA) or pladienolide, a natural SF3B inhibitor, markedly inhibited P27 expression by disrupting its pre-mRNA splicing with striking cell killing effects^[32,33]. Further, FIR/PUF60 is required for transcriptional and post-transcriptional regulation of HBV pgRNA expression^[34]. To develop novel diagnosis and therapy for hepatoma targeting FIR (PUF60)/FIR Δ exon2, small molecular chemical compounds against FIR Δ exon2 were screened among 2,3275 chemicals by natural product depository (NPDepo) array at RIKEN (Japan) to develop anti-cancer drugs^[35-37]. Finally, small inhibitory chemicals against FIR/FIR Δ exon2 for hepatoma therapy will be discussed.

METHODS

Cancer cell lines

Human cervical SCCs (HeLa cells), gastric cancer cells (NUGC4), HLE cells and HLF cells were purchased from the American Type Culture Collection (<https://www.atcc.org/>). These cells were treated as described previously^[18]. All cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS; Invitrogen, Tokyo, Japan) and 1% penicillin-streptomycin, and they were cultured at 37 °C in a humidified atmosphere containing 5% CO₂.

Protein extraction, western blotting and antibodies

Culture medium was removed, and the cells were washed twice with cold (4 °C) phosphate buffered saline (PBS), lysed with 1:20 β -mercaptoethanol and 2x sample buffer, and incubated at 100 °C for 5-min. Whole-cell lysates were assayed for protein content (Bio-Rad, Hercules, CA, USA), and 10 μ g of proteins were separated

by sodium dodecyl sulfate -poly- acrylamide gel electrophoresis on 7.5% or 10%-20% XV PANTERA gels and transferred onto polyvinylidene fluoride membranes using a tank transfer apparatus. The membranes were blocked with 0.5% skim milk in PBS overnight at 4 °C. Antigens on the membranes were detected with enhanced chemiluminescence detection reagents (GE Healthcare UK Ltd., Buckinghamshire, UK). Membranes were incubated with primary antibodies [Supplementary Table 1] for 1 h at room temperature, followed by three 10-min washes with 1xPBS/0.01% Tween 20. Membranes were then incubated with commercial secondary antibodies [Supplementary Table 1], followed by three 15-min washes with 1xPBS/0.01% Tween 20. The primary mouse monoclonal antibody against FIR's C-terminus (6B4) was described previously^[22].

Small molecular chemical compounds screening against FIRΔexon2

Small molecular chemical compounds against His-tagged FIR (His-FIR) and His-tagged FIRΔexon2 were screened among 2,3275 chemicals of NPDepo at RIKEN as described previously^[35-37]. Briefly, His-FIR (645 μg/mL) and FIRΔexon2 (652 μg/mL) proteins were applied to NPDepo array that contains 2,3275 natural chemical compounds. FIRΔexon2 inhibitor BK697 was diluted in dimethyl sulfoxide (DMSO) at the concentration of 10 mM, stored in room temperature, treated into HeLa and NUGC4 cell lines with different concentrations at different time intervals (see details in figure legends). Briefly, on day one, NUGC4 cells or HeLa cells were prepared in Iscove's modified Dulbecco's medium supplemented with 10% FBS. On day two, candidate chemicals that inhibit FIRΔexon2 protein were diluted in DMSO at the concentration of 10 mM and added as 10 L or 20 L/well/2 mL in the medium (final concentration in medium was 50 mol/L and 100 mol/L respectively) or added as 20 L or 60 L/well/2 mL medium (final concentration in medium was 100 mol/L and 300 mol/L respectively). 100 mol/L or 300 mol/L of BK697 was treated to NUGC4 cells for 24 h, 50 mol/L or 100 mol/L of BK697 was treated to NUGC4 cells or HeLa cells for 6 h, 24 h and 48 h at 37 °C in a CO₂ incubator.

Screening procedures of natural small molecular weight chemical compounds that potentially bind to FIR/FIRΔexon2

Small molecular weight chemical compounds potentially bound to FIRΔexon2 were previously identified [Figure 1] from the NPDepo at RIKEN, which were a collection of the isolates from natural products, build by Dr Hiroyuki Osada (RIKEN, Japan) and his coworkers^[35-37].

Procedure of in silico screening

In the process for searching potent compounds, in silico screening was performed from the commercial chemical database. First, 1000 compounds were selected from the Namiki database that contains 5 million chemical entries, from the viewpoint of structural similarity to natural product that was identified to be bound to FIR in our previous work. Second, 125 compounds were extracted from the selected 1000 chemicals in terms of the electrostatic potential caused by the distribution of positive and negative charges. Finally, 5 compounds were purchased from a supplier for experimental assay. Namiki database (Namiki Shoji Co., Ltd., Tokyo, Japan, <https://www.namiki-s.co.jp/english/>) was a collection of commercially available screening-candidate chemicals.

Display of three-dimensional structure of F-box and WD repeat domain-containing 7 (FBW7)

To examine the possibility of molecular interaction between FBW7 and FIRs from structural viewpoint, two crystal structures were downloaded from protein data bank (PDB, <https://www.rcsb.org/>). One is a complex structure of FBW7 (PDB entry code: 2OVR). The other is the structure of an U2AF homology motif (UHM) domain in complex with UHM-ligand motif (ULM) of SAP155 (PDB entry code: 2PEH). Both crystal structures were visualized by PyMOL (DeLano, W. L.; The PyMOL Molecular Graphics System, Schrödinger, LLC).

MTS assay (Cell proliferation assay)

One day before the chemical treatment, cells were cultured in 100 μL medium in flat-bottomed 96-well plates

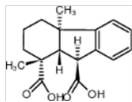
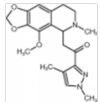
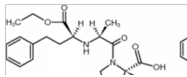
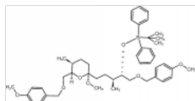
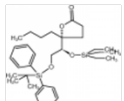
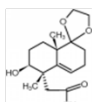
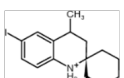
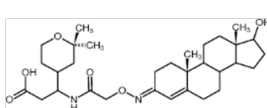
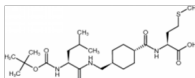
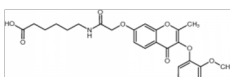
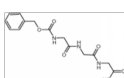
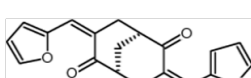
IUPAC name	Structure	His-FIR	His-FIR Δ exon2
1,4a-Dimethyl-2,3,4,4a,9,9a-hexahydro-1H-fluorene-1,9-dicarboxylic acid			3+
1-(1,4-Dimethyl-1H-pyrazol-3-yl)-2-(4-methoxy-6-methyl-5,6,7,8-tetrahydro-[1,3]dioxolo[4,5-g]isoquinolin-5-yl)-ethanone		2+	
1-[2-(1-Ethoxycarbonyl-3-phenyl-propylamino)-propionyl]-pyrrolidine-2-carboxylic acid (compound with but-2-enedioic acid)			3+
tert-Butyl-[1-(4-methoxy-benzyloxymethyl)-4-[2-methoxy-6-(4-methoxy-benzyloxymethyl)-5-methyl-tetrahydro-pyran-2-yl]-2-methyl-butoxy]-diphenyl-silane		3+	
5-Butyl-5-[2-(tert-butyl-diphenyl-silanyloxy)-1-triethylsilanyloxy-ethyl]-dihydro-furan-2-one		3+	3+
3',5',6',7',8',8'a-hexahydro-6'-hydroxy-5',8'a-dimethyl-, (5'S,6'S,8'aS)-Spiro[1,3-dioxolane-2,1'(2'H)-naphthalene]-5'-acetamide			2+
6-iodo-4-methylspiro[3,4-dihydro-1H-quinolin-1-ium-2,1'-cyclohexane]		2+	
3-(2,2-Dimethyl-tetrahydro-pyran-4-yl)-3-[2-(17-hydroxy-10,13-dimethyl-1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-cyclopenta[a]phenanthren-3-ylideneaminoxy)-acetylamino]-propionic acid		3+	
2-({4-[(2-tert-Butoxycarbonylamino-4-methyl-pentanoylamino)-methyl]-cyclohexanecarbonyl}-amino)-4-methylsulfanyl-butyric acid		1+	
6-{2-[3-(2-Methoxy-phenoxy)-2-methyl-4-oxo-4H-chromen-7-yloxy]-acetylamino}-hexanoic acid		3+	
[2-(2-Benzyloxycarbonylamino-acetylamino)-acetylamino]-acetic acid		3+	
3,7-Bis-furan-2-ylmethylene-bicyclo[3.3.1]nonane-2,6-dione		1+	

Figure1. Structures of small molecular weight chemicals that were interacted with His-tagged far upstream element-binding protein-interacting repressor (His-FIR) or His-FIR Δ exon2 screened by natural product depository (NPDepo) (RIKEN, JPN). 3+: strong interaction; 2+: moderate interaction; 1+: weak interaction; IUPAC: International Union of Pure and Applied Chemistry

so that the cells will reach 40%-80% confluent at the time of chemical treatment. After 24 h incubation at 37 °C/5% CO₂, cells were treated with chemicals. After 24 h incubation at 37 °C, CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI, USA) was added to each well according to the manufacturer's instructions. Briefly, CellTiter 96® AQueous One Solution Reagent was warmed up and added to each well (20 L/well), incubated for 1 h at 37 °C. Then, 10% SDS solution was added to each well (25 L/well). Cell viability was determined by measuring the absorbance at 490 nm using a 550 Bio-Rad plate reader. All samples are tested in duplicate, absorbencies were tested 3 times. Same volume of DMSO was used as negative control. Same volume of 3% H₂O₂ was used as positive control.

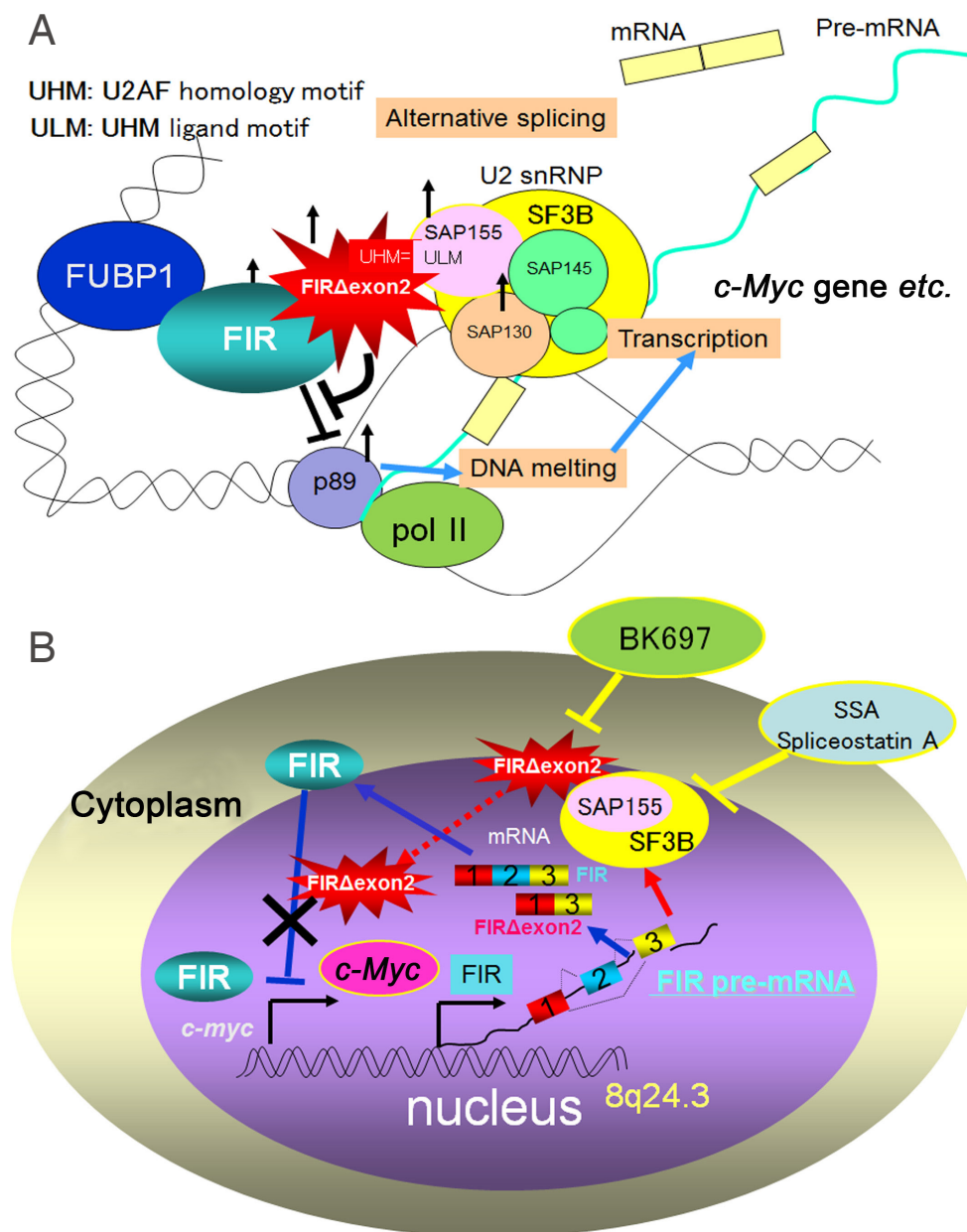


Figure 2. (A) Elevated expression of *c-Myc* has been detected in a broad range of human cancers, indicating a key role for this oncogene in tumor development. Far upstream element-binding protein-interacting repressor (FIR) gene and *c-Myc* gene locates at 8q24.3. An interaction between FIR (FBP interacting repressor) and transcriptional factor IIH helicase was found to repress *c-Myc* transcription and so might be important for suppressing tumor formation. FIR is alternatively spliced in colorectal cancer lacking the transcriptional repression domain within exon 2 (FIRΔexon2) that inhibit FIR as a dominant negative form of FIR. FIRΔexon2 potentially forms a heterodimer with FIR and thus FIRΔexon2 interferes with FIR to bind to far upstream element of *c-Myc* promoter where FIR binds. FIR and FIRΔexon2 form a homo- or hetero-dimer, which makes a complex with SAP155. SAP155 is a subunit of the essential splicing factor 3B (SF3B) subcomplex in the spliceosome. The interaction between SAP155 and FIR/FIRΔexon2 potentially integrated cell cycle progression and *c-Myc* transcription through P89 suppression; (B) FIR/FIRΔexon2/SAP155 interaction is pivotal for cancer development and differentiation and is thus a potent target for cancer screening and treatment. These results strongly suggest that FIRΔexon2 antagonized FIR in *c-Myc* transcriptional suppression and simultaneously interferes with SF3B in splicing during tumor progression. Importantly, Spliceostatin A that is a strong chemical inhibitor of SF3B resulted in *c-Myc* overexpression probably due to the FIR downregulation. FIR: FUBP1-interacting repressor

RESULTS

Mechanism in carcinogenesis of FIR/FIRΔexon2/PUF60 as a target for cancer diagnosis and therapy

Previous studies revealed that FUBP1, FIR (PUF60)/FIRΔexon2, SAP155, and SAP130 were over expressed in hepatocellular carcinoma (HCC) tissue^[18]. Additionally, FIR/FIRΔexon2 mRNA levels were increased in HCC^[38]. Recent studies have been revealed regarding direct protein interactions between UHM family and ULM family [Supplementary Figure 1]^[39-41]. SAP155/SAP145/SAP130 subunits consist of SF3B complex and UHM of FIR/PUF60 directly binds to ULM of SAP155 (SF3B1) [Supplementary Figure 1A-C]^[32,41,42]. Further, FUBP1, FIR (PUF60)/FIRΔexon2, SAP155, and SAP130 were over expressed in hepatitis C virus (HCV)-related HCC tissue and FIR (PUF60)/FIRΔexon2 reflects DNA damage [Supplementary Figure 1D]^[18]. Bleomycin-induced DNA damage decreased SAP155 and significantly increased FIR/FIRΔexon2 mRNA expression as well as the FIRΔexon2: FIR ratio in hepatoblastoma (HLE and HLF) cells^[18]. Therefore, FUBP1/FIR (PUF60)/FIRΔexon2 proteins, mRNAs and/or autoantibodies against these peptides are highly possible biomarker candidates for hepatoma diagnosis. Anti-FIR/FIRΔexon2 autoantibodies were detected in several gastrointestinal cancers^[27,28]. Anti-FIR/FIRΔexon2 autoantibodies in the sera of HCC patients are now under investigation. Given FIRΔexon2 is a dominant negative regulator of FIR/PUF60, FIRΔexon2 inhibition is an advantageous target for cell growth suppression [Figure 2A]. Inhibition of SF3B (SAP155) by siRNA or SSA resulted in *c-Myc* overexpression possibly due to the FIR downregulation [Figure 2B]^[1]. Knockdown of SAP155 or FIR was used to investigate their reciprocal influence on each other and on *c-Myc* transcription, pre-mRNA splicing, and protein expression^[31]. FIR and FIRΔexon2 were co-immunoprecipitated with SAP155^[31]. UHM of FIR/PUF60 at carboxyl-terminus directly binds to W (Tyr) D (Asp)-domain of SF3B1 (SAP155) as ULM^[42,43]. The tight FIR/FIRΔexon2-SAP155 interaction disables established FIR and SAP155 functions disturbing the synthesis of normally spliced FIR mRNA. FIRΔexon2 potently forms a heterodimer with FIR and thus FIRΔexon2 interferes with FIR to bind to FUSE [Supplementary Figure 1D]. These results strongly suggest that FIRΔexon2 antagonized FIR in *c-Myc* transcriptional suppression and simultaneously interferes with SF3B in splicing during tumor progression. Therefore, both common and discriminating recognition elements in the UHM-ULM binding interface provide a rationale for a structural basis for specific UHM-ULM interactions and a platform of intermolecular interactions governing disease-related AS in eukaryotic cells^[40]. For instance, SF3B1 (SAP155)/FIR/PUF60 complex is a target of cancer therapy. In these scenarios, low molecular weight artificial chemical, BK697, was synthesized by in silico screening that targets FIRΔexon2 in this study [Figure 2B]. Small molecular chemical compounds against FIRΔexon2 were screened among 23,275 chemicals of NPDepo by Dr Hiroyuki Osada and his colleagues (RIKEN, Wako, Saitama, Japan) to develop cancer therapy [Figure 1]. Nine small molecular chemicals were identified by NPDepo screening against FIR and four chemicals against FIRΔexon2 as candidates of interacting chemicals [Figure 1].

The interaction of FIRΔexon2 and WD-like domain of FBW7 and in silico screening of small molecular chemical compounds against FIR/FIRΔexon2 for cancer therapy

FBW7 frequently is mutated in hematopoietic tumors^[44]. FBW7 is a member of the Skp1-Cull-F-box type ubiquitin ligase complex and is involved in degradation of various growth-related proteins, Notch1, *c-Myc*, *c-Jun*, and cyclin E *via* the proteasome system^[44], indicating FBW7 is a tumor suppressor in cancer development and progression^[45]. Remarkably, three-dimensional structure analysis revealed the hypothetical inhibitory mechanism of FBW7 function by FIR/FIRΔexon2 [Figure 3]. The binding structure between SAP155 (SF3B1) and one of the splicing factors containing UHM, SPF45, was already clarified by X-ray crystal analysis (PDB code: #2PEH) [Figure 3A and B]. In the 2PEH structure, the crystal unit cell contains two SPF45 recombinant proteins (a.a. 301-401) and two SAP155 partial peptides (a.a. 333-342). SPF45 has an amino sequence of LNGRYFGGRVKA [Figure 3A] and similar sequences are commonly seen at the C-terminal domains of FIR and U2AF65 [Figure 3B]. According to the crystal structure, 2PEH, SPF45 makes a strong interaction with a WD part of SAP155 at the domain of the above-mentioned conserved sequence.

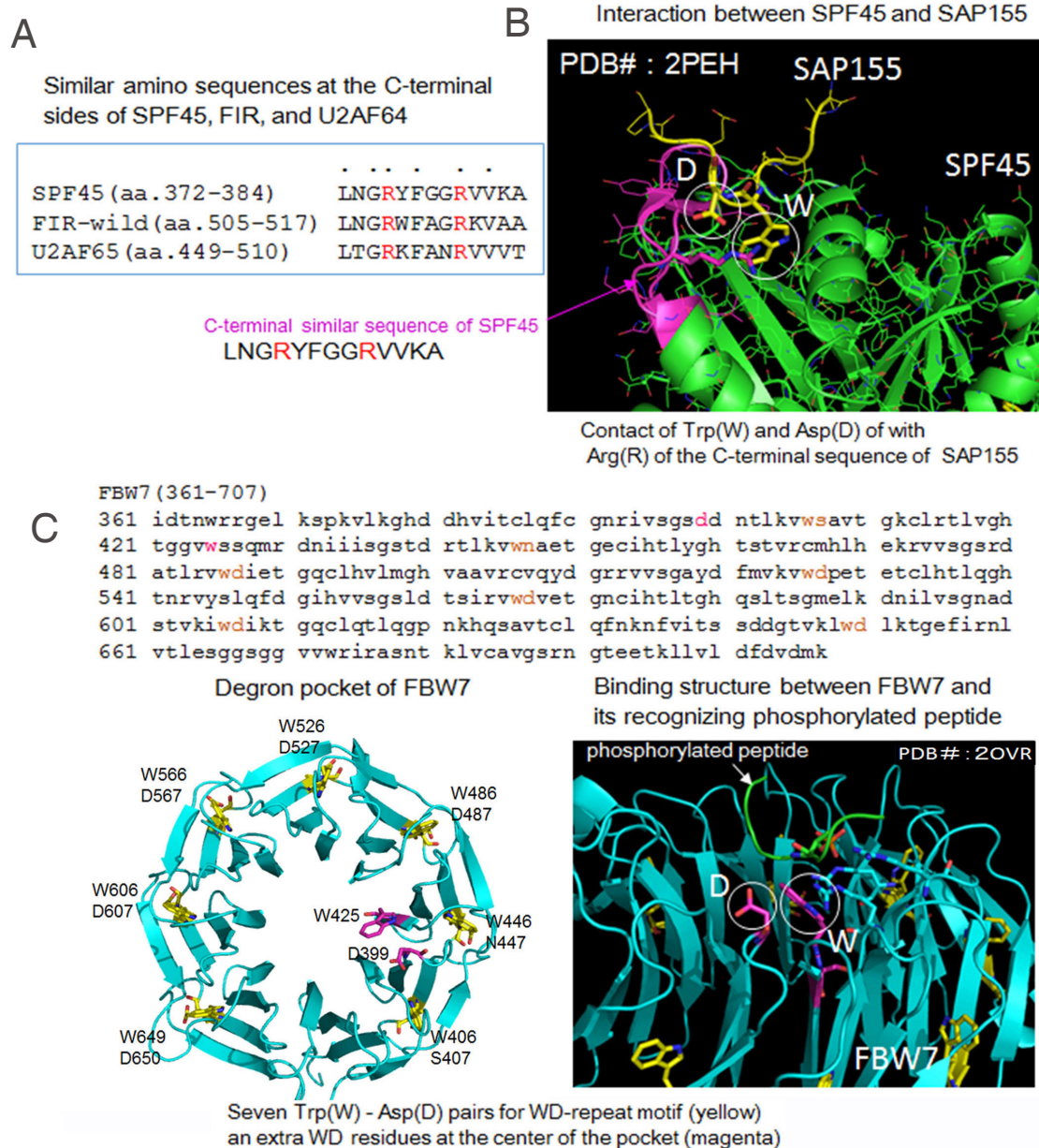


Figure 3. (A) The binding structure between splicing factor 3B (SF3B) and one of the splicing factors containing U2AF homology motif, SPF45, was already clarified by X-ray crystal analysis (protein data base code: #2PEH). In the 2PEH structure, the crystal unit cell contains two SPF45 recombinant proteins (a.a. 301-401) and two SF3B partial peptides (a.a. 333-342); (B) SPF45 has an amino sequence of LNGRYFGGRVVKA and similar sequences are commonly seen at the C-terminal domains of far upstream element-binding protein-interacting repressor (FIR) and U2AF65. According to the crystal structure, 2PEH, SPF45 makes a strong interaction with a WD part of SF3B at the domain of the above-mentioned conserved sequence. FIR and U2AF65 are also expected to interact with SF3B through the domains with the similar amino sequences; (C) F-box and WD repeat domain-containing 7 (FBW7) has many WD motifs and most of the motifs are involved in the conformational stabilization of the WD-repeated domain. Although those WD motifs are not related to the ligand recognition of FBW7, there is an extra pair of W425 and D399 at the center of the WD-repeated domain. Most of the ligands of FBW7 are the amino peptides that include phosphorylated Thr or Ser, because three Arg residues are located at the center of the WD-repeated domain and hold the negatively charged peptides by phosphorylation. The extra pair of W and D at the WD-repeated domain will not be involved in the ligand recognition of the phosphorylated peptides, but the WD pair can interact with the peptide with the above-mentioned conserved sequence from the structural viewpoint. Hence, FIR may be bound to the WD-repeated domain and block the function of FBW7. FIR: FUBP1-interacting repressor; WD: W (Tyr) D (Asp)

FIR and U2AF65 are also expected to interact with SAP155 through the domains with the similar amino sequences. FBW7 has many WD motifs and most of the motifs are involved in the conformational stabilization of the WD-repeated domain. Although those WD motifs are not related to the ligand recognition of FBW7, there is an extra pair of W425 and D399 at the center of the WD-repeated domain. Most of the ligands of FBW7 are the amino peptides that include phosphorylated Thr or Ser, because three Arg residues are located at the center of the WD-repeated domain and hold the negatively charged peptides by phosphorylation [Figure 3C]. The extra pair of W and D at the WD-repeated domain will not be involved in the ligand recognition of the phosphorylated peptides, but the WD pair can interact with the peptide with the above-mentioned conserved sequence from the structural viewpoint. Hence, FIR may be bound to the WD-repeated domain and block the function of FBW7 [Figure 3C]. Together, latent disturbance of FBW7 by FIR/FIR Δ exon2/PUF60 in cancers inhibit degradation of substrate proteins.

FBW7 expression was decreased significantly in esophageal squamous cell carcinoma (ESCC)^[46]. Conversely, FIR and FIR Δ exon2 were overexpressed in ESCC. Especially, the knockdown of SAP155 (SF3B1), a splicing factor required for proper AS of FIR pre-mRNA, decreased cyclin E^[46]. Therefore, disturbed AS of FIR generated FIR/FIR Δ exon2 with cyclin E overexpression in esophageal cancers, indicating that SAP155 siRNA potentially rescued FBW7 function by reducing expression of FIR and/or FIR Δ exon2^[46]. A novel low molecular weight chemical, BK697, with WD-like domain structure that inhibits FIR/FIR Δ exon2 [Figures 2B and 3]^[46], indicating simultaneous downregulation of FBW7 and E-cadherin accompanied with disturbed splicing of FIR is required for migration [or epithelial-mesenchymal transition (EMT)] in cancers.

Cell growth inhibition by in silico-screened compounds against FIR Δ exon2 protein

A small molecular weight chemical that has WD-like motif was identified by NPDepo screening [Figure 1-top, Figure 4-(A), (C)]. From computer screening to search synthesized chemicals that mimicking the structure of the identified compound using Namiki database (Namiki Shoji Co., Ltd., Tokyo, Japan) that was composed of commercially available chemicals [Figure 4-(B), (B')]. Recently, FIR Δ exon2 was suggested to be potentially bound to the substrate-binding degron pocket of FBW7. Since the substrate-binding degron pocket of FBW7 contains a unique structure of Trp (W) and Asp (D) combination (WD motif) and the WD motif is expected to interact with FIR Δ exon2 [Figure 3]. Actually, chemical skeleton of the two synthesized compounds were regarded as a WD mimicking form [Figure 4 (A)-(D)]^[46]. All of the compounds bear a chemical skeleton of aromatic ring connected to carboxyl group with a short linker. Hence, these compounds are analogues of WD motif of FBW7 [Figure 4]. From these chemical structural findings of WD mimicking form, several compounds were selected from the chemicals that have been synthesized in our previous studies [Figure 4]^[46-49]. Synthesized compounds were intended to inhibit FIR Δ exon2 protein function.

Low molecular weight artificial chemical, BK697, that inhibits FIR Δ exon2 protein function suppressed tumor cell growth

Affiliated small molecular weight chemicals that have WD-like motif screened by NPDepo [Figure 5A, square]. Based on the computer screening, lots of similar chemicals were designed and seven compounds were selected for treating with HLE and HLF cells to examine cell growth inhibition [Figure 5A, arrows]. Expectedly, BK697 effectively suppressed hepatoblastoma cells, HLE and HLF cells [Figure 5B]. BK697 suppressed FIR/FIR Δ exon2 expression on dose-dependent manner in NUGC4 cells [Figure 5C, left] and HeLa cells [Figure 5C, right]. Particularly, FIR/PUF60 is required for HBV cccDNA replication^[34], BK697 is a promising candidate for hepatoma treatment by suppressing HBV cccDNA. Previously, FIR has been revealed to contribute to the splicing of PKM1 to PKM2 in mice thymic lymphoma using six-plex tandem mass tag quantitative proteomic analysis in mice model [Table 1]^[50,51]. SAP155 (SF3B1) and FIR/PUF60 are required for E-cadherin expression through engaging in its mRNA editing that is pivotal for cell-cell adhesion or EMT^[52]. Together, BK697 suppressed cell growth through interfering FIR Δ exon2 with binding to analogues of WD-like motif of FBW7

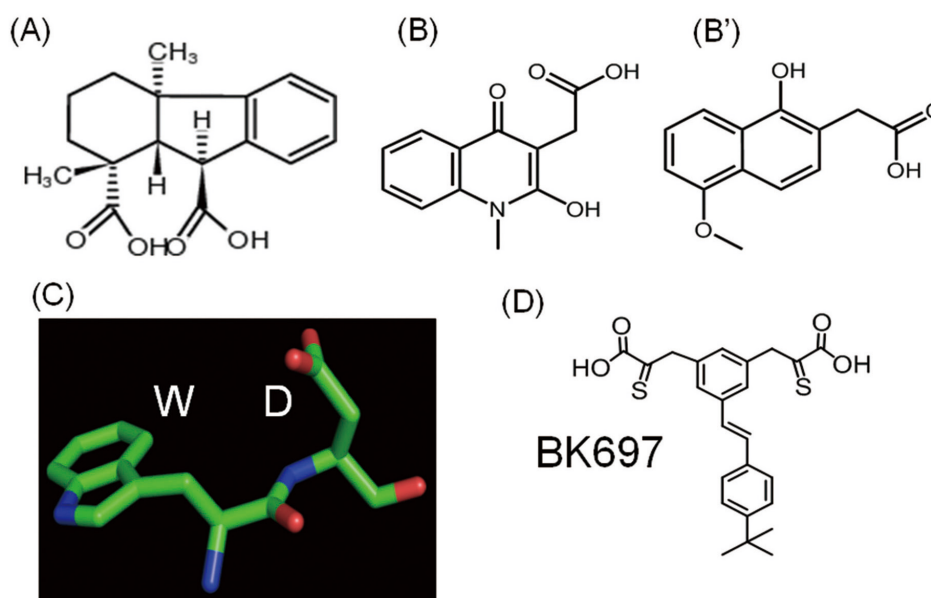
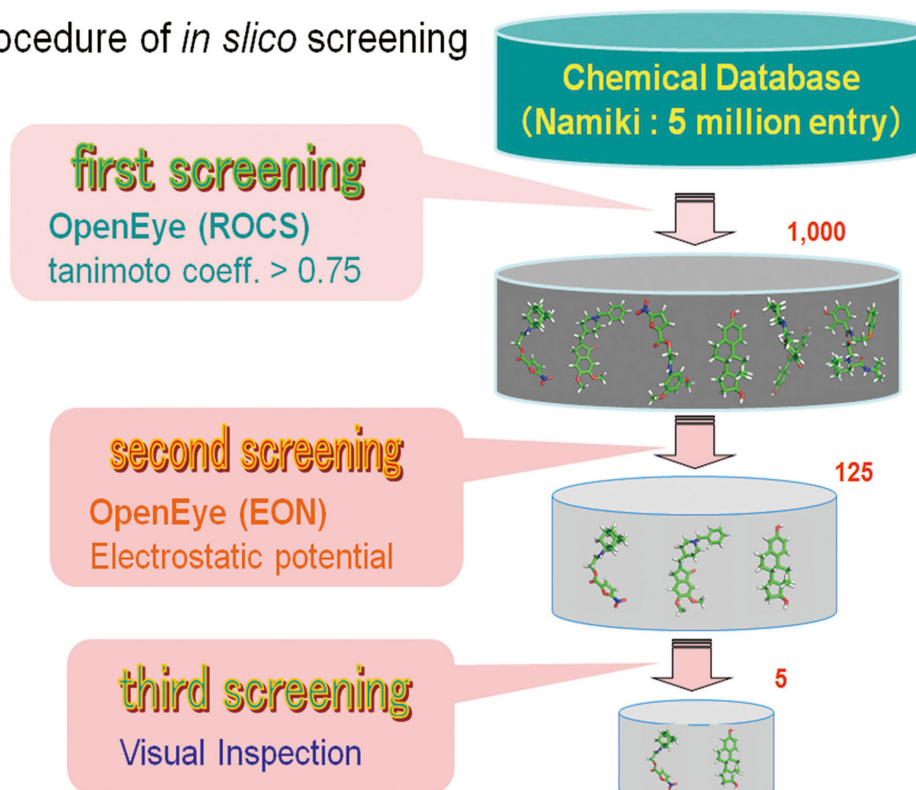
Procedure of *in silico* screening

Figure 4. (A) A small chemical against far upstream element-binding protein-interacting repressor Δ exon2 screened by natural product depository array at RIKEN (Wako city, Saitama, Japan). Two compounds showed inhibitory activity [(B) and (B')] in the cell-based assay. The conformation of the two inhibitory compounds was found to resemble the WD motif (C); Hence, from the similarity to the chemical structure of WD-like motif, we tested several compounds that had been already synthesized in our previous studies targeting viral proteins. Based on the tests with the synthesized compounds, we modified the chemical structure and finally identified BK697 (D). WD: W (Tyr) D (Asp)

Table 1. Summaries of studies in far-upstream element-binding protein (FUBP1)/far upstream element-binding protein-interacting repressor (FIR)/FIRΔexon2/poly (U)-binding-splicing factor (PUF60) system related to human diseases

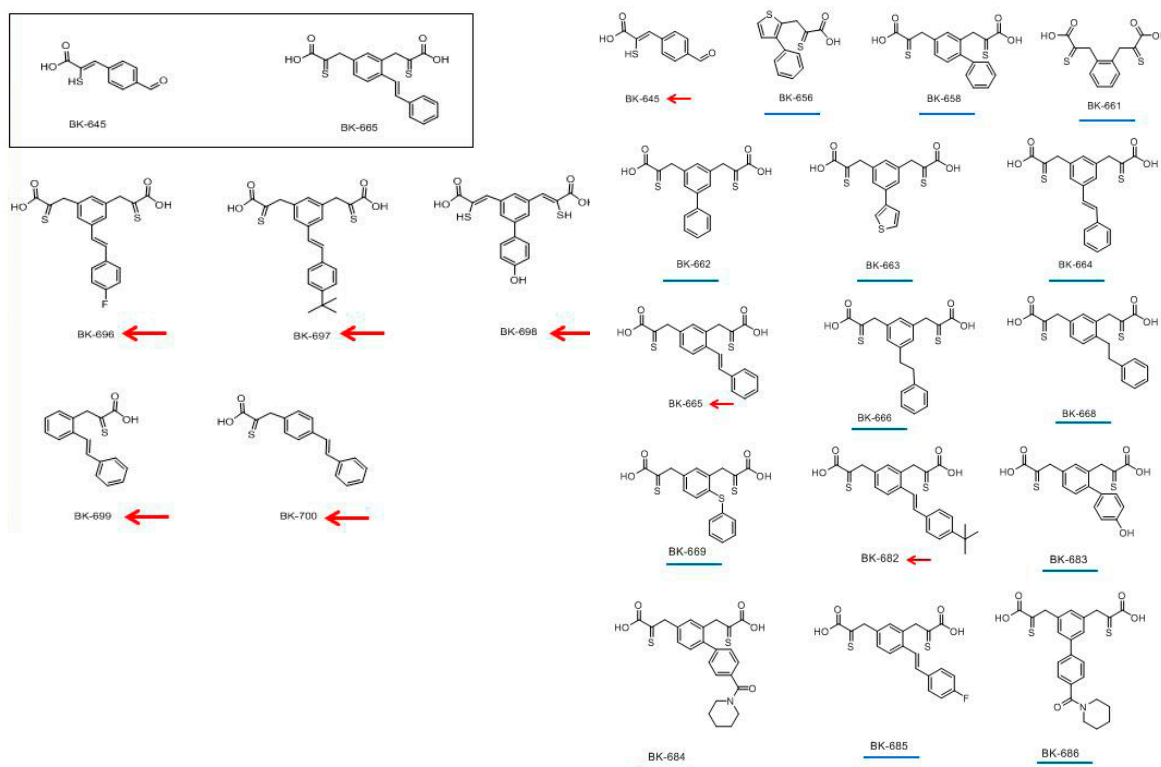
Targets	Functions	References
1	<i>c-Myc</i> gene transcriptional activator	[3,4,7] (far-upstream element (FUSE)/FUBP1)
	<i>c-Myc</i> gene transcriptional repressor	[8] (FIR)
	Apoptosis induction	[4,10-14] (FIR)
	Dominant negative of FIR splicing variant	[16,22,30,38] (FIRΔexon2)
	PKM2/Cancer metabolism	[50]
	DNA damage/cell cycle	[18-20,29-31,46]
	T-cell type acute lymphoblastic leukemia	[43,50]
	SAP155(SF3B1)-FIR(PUF60) interaction in alternative splicing of mRNAs	[23,29,31,40-42]
	F-box and WD repeat domain-containing 7 (FBW7)/proteasome	[44,45]
	autoantibodies/immune reaction	[21,24,25,27,28]
2	Cancers in general	[17-19,21-24,33,34]
	E-cadherin/invasion/metastasis, Epithelial mesenchymal transition (EMT)	[52]
	carcinogenesis	[5,6,56,57] (FUBP1),[9,38,43] (FIRΔexon2)
	tolerance for hypoxia	[53]
3	Hepatoma	[6,18,38,51,56,57]
	proliferation, migration, cancer metabolism, signal transduction, covalently closed circular DNA (cccDNA of HCV)	[34]
4	Hepatitis B virus (HBV)/ hepatitis C virus (HCV)	[34]
	ENI/ENII enhancer region	[34]
	HBV core promotor	[34]
	spliced RNA (HBV RNA)	[17]
	HCV	[55]
	CHARGE syndrome	[59]
	Phenotypic variability of genetic diseases	[59]
4	Rare disease	[59,60]
	Verheji syndrome	[59,60]
	developmental delay, intellectual disability, microcephaly, craniofacial, renal and cardiac defects	[58,59]
	Eye coloboma and complex cardiac malformations	[59,61]
	atrioventricular septal defect and hypoplastic aortic arch, facial dysmorphism, microretrognathia, dysmorphic ears, clinodactyly of the 5th digit on both hands, mild rocker bottom feet and abnormal third sacral vertebra	[61,62]
	microcephaly, short stature, intellectual disability, and heart defects with a de novo c.505C > T variant leading to a p.His169Tyr change in PUF60. (PUF60 deficiency)	[63-65]

in the degron pocket (W425 D399 in the 3D-structure) [Figure 3]. Together, simultaneous downregulation of FBW7 and E-cadherin is potentially pivotal for invasion or metastasis of cancers through EMT and may also contribute to therapeutic target for cancers. Clinically, BK697 and its derivatives are potential candidate anticancer drugs for cancers targeting FBW7 and E-cadherin suppression.

DISCUSSION

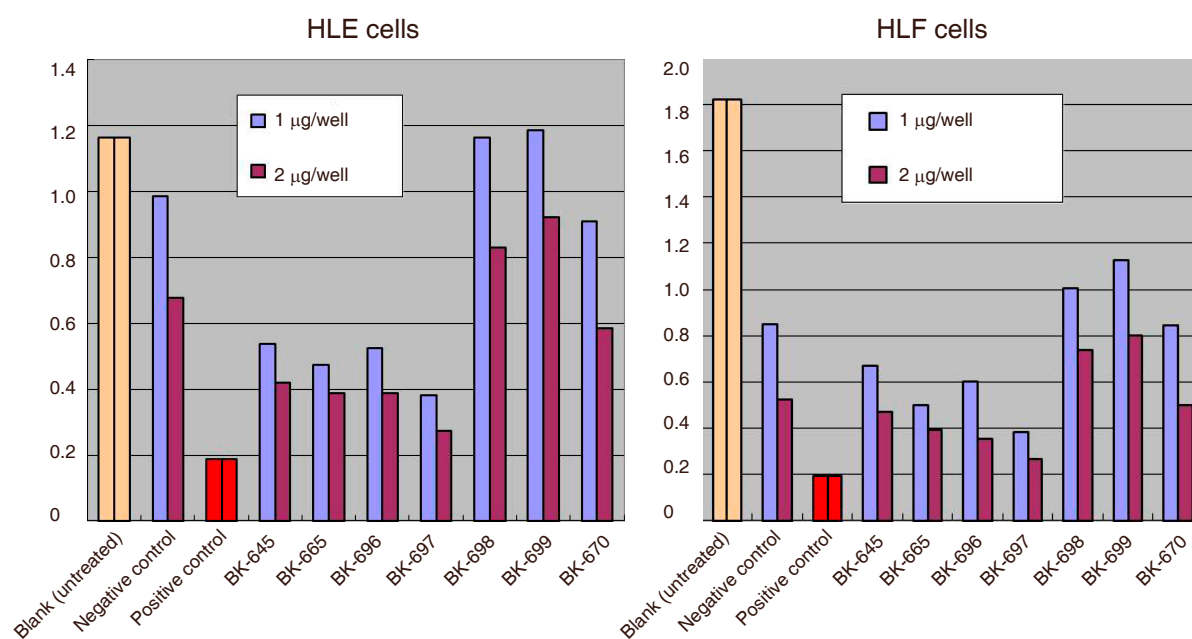
This study demonstrated that FIR strongly repressed endogenous *c-Myc* transcription and induced apoptosis. Most importantly, a splicing variant of FIR, FIRΔexon2, found frequently in human primary colorectal cancer tissue, not only lacked the *c-Myc*-suppressing and apoptosis-inducing action of FIR, but prevented normal FIR from performing these activities. Thus FIRΔexon2 may contribute to tumor progression by enabling higher levels of *c-Myc* expression and greater resistance to apoptosis in tumors than in normal cell [Figure 2A]. The value of FIR and/or FIRΔexon2 detection for cancer diagnosis is under investigation. Recently, PUF60, another FIR splicing variant having exon 5, directly binds to splicing factor SF3B1 with UHM^[39] and inhibition of SF3B (SAP155 is a subunit of SF3B) by natural chemicals demonstrated strong antitumor effect [Figure 2B]^[32,33]. Hypoxia leads to AS of FIR/PUF60 and in PC3 prostate cancer cells^[53]. Given the central role of *c-Myc* in the development of many cancers, and inhibition of splicing function of PUF60 (or FIR itself) with SF3B indicates strong antitumor activity, one route to the development of

A



B

MTS Assay (Cell confluency was 80% at time of drug treatment)



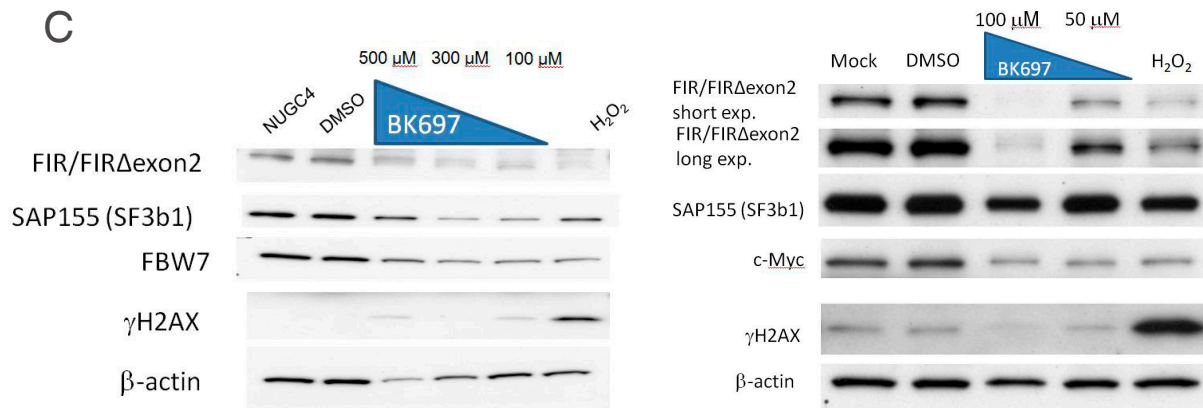


Figure 5. BK697 inhibit far upstream element-binding protein-interacting repressor Δexon2 (FIRΔexon2) that is considered as a dominant negative of FIR. (A) From computer screening to search synthesized chemicals that mimicking the structure of the identified compound using Namiki database (Namiki Shoji Co., Ltd., Tokyo, Japan) that was composed of commercially available chemicals after natural product depository array at RIKEN (Japan). Affiliated chemicals were screened and indicated in the square (square). Based on the computer screening, lots of similar chemicals were designed and seven compounds were selected for treating with HLE and HLF cells to examine cell growth inhibition (arrows); (B) BK697 effectively suppressed hepatoblastoma cells, HLE and HLF cells. BK697 effectively suppressed hepatoblastoma cells, HLE and HLF cells by MTS assay (see materials and methods). Small molecular weight indicated arrows (A) were examined the cell growth suppression in HLE and HLF cells. All samples are tested in duplicate, absorbencies were tested 3 times. Same volume of DMSO was used as negative control. Same volume of 3% H₂O₂ was used as positive control; (C) BK697 suppressed FIR/FIRΔexon2 expression on dose-dependent manner in gastric cancer cells (left) and HeLa cells (right). Note SAP155 (SF3B1) was also suppressed by BK697 along with FIR/FIRΔexon2 expression. H2AX is a marker of DNA damage. FIR: FUBP1-interacting repressor; HLE: hepatoblastoma cell line; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; DMSO: dimethyl sulfoxide

cancer therapies directed against c-Myc and splicing of SF3B inhibition may go through FIR and its splicing variants. In this study, BK697 has been screened to target SAP155-binding FIRΔexon2 for cancer therapy [Figure 2B]. According to recent cancer gene therapy, adenovirus-mediated (Ad) TP53 gene transfer is frequently used, together with cis-dichloro-diammineplatinum administration or ionizing radiation^[1,10]. As for Ad-FIR or Sendai virus-FIR vector, the transduction efficiency showed that the efficacy in preclinical trials and combination treatment with standard chemoradiation and Ad-FIR/Sendai-FIR gene therapy may be an attractive modality in the future^[10-14].

HBV has a small (3.2 kb), partially-double stranded, relaxed-circular DNA genome that encodes four overlapping open reading frames (ORFs)^[54]. The genomic transcripts from these overlapping four ORFs act mRNAs for precore, core and polymerase. The genomic transcript that encodes both core and polymerase is multifunctional and referred to as pgRNA^[54]. The core protein binds to HBV covalently closed circular DNA (cccDNA). The cccDNA forms a minichromosome in the nucleus of the hepatocyte^[54]. Recent nucleoside analogues and interferons treatment for HBV-positive patients do not achieve complete clearance of viral genome cccDNA in the nucleus [Figure 6]. To our interest, PUF60 was identified as a versatile regulator of transcriptional and post-transcriptional steps in expression of HBV 3.5 kb, precore plus pgRNA^[34]. This is the first to identify a host cell factor (protein) involved in not only positively regulating viral gene expression but also negative regulation of the same viral life cycle^[34]. Therefore, FIR/PUF60 is also a novel promising target to inhibit HBV cccDNA transcription as well as interfering FBW7 function [Figure 6]. Given the FIR/PUF60 is required for HBV cccDNA replication^[34] and novel small molecular weight chemicals including BK697 that suppresses FIR/PUF60 expression [Figure 5C], those chemicals have advantage to eliminate HBV cccDNA than other strategies as recent nucleoside analogues and interferons treatment. Further, the amino terminus of FIR was necessary to repress transcription from the c-Myc promoter by suppressing FUBP1, FUBP [Figures 2 and 6]^[22]. FUBP1/FIR(PUF60)/TFIIH system FIR suppresses endogenous c-Myc

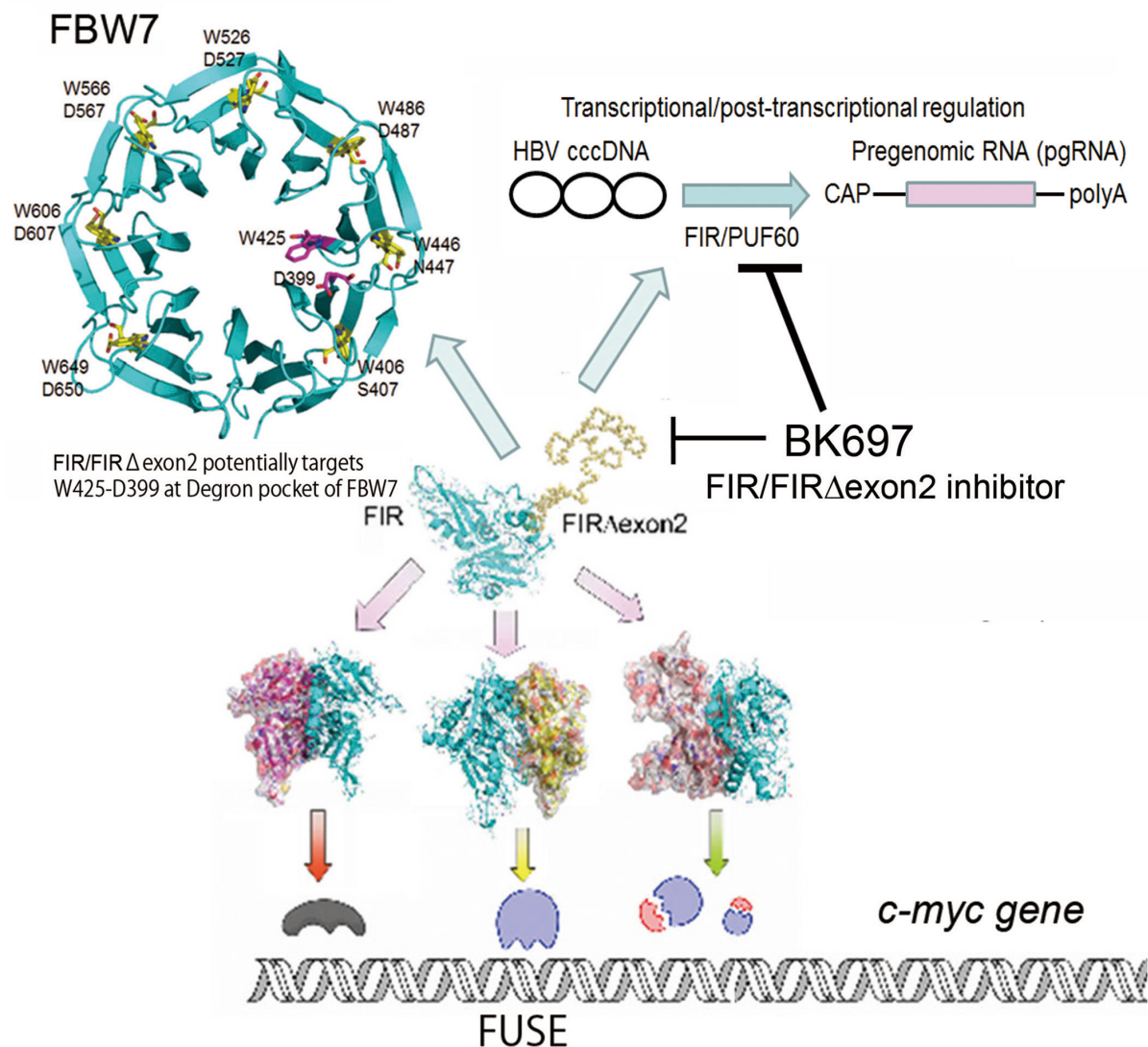


Figure 6. Far upstream element-binding protein-interacting repressor Δ exon2 (FIR Δ exon2) inhibitor BK697 inhibited the growth of the HeLa cells. BK697 is a candidate anticancer drug for inhibiting hepatitis B virus (HBV) replication for hepatoma therapy. FIR/poly (U)-binding-splicing factor (PUF60) has three different functions. (1) A *c-Myc* gene transcriptional repressor; (2) disturbance of substrate proteins degradation through competing with the access to degron pocket of F-box and WD repeat domain-containing 7 (FBW7); and (3) transcriptional/posttranscriptional regulation of HBV covalently closed circular DNA (cccDNA). Targeting FIR/PUF60 is a promising strategy for cancer therapy. FIR: FUBP1-interacting repressor; WD: W (Typ) D (Asp)

at transcriptional level by its amino terminal domain [Figure 2A]. Further, FUBP1 facilitates persistence HCV replication in HCC cells^[55]. FUBP1 as well as FIR (PUF60) is required for tumor growth in HCC^[38,56,57]. Additionally, FUBP1/FIR (PUF60)/TFIIH complex potentially support the growth of hepatoma by *c-Myc* gene transcriptional activation and HCV replication [Table 1]. Further, FUBP1/FIR/FIR Δ exon2/PUF60 are expressed in HCC tissue and less expressed in the normal tissue in developed cells^[18,38]. Therefore, small molecular weight chemicals targeting FUBP1/FIR/FIR Δ exon2/PUF60 system are expected to be harmless.

Recently, FIR/PUF60 and human rare disease are reported [Table 1]. CHARGE syndrome shows an autosomal-dominant, multiple congenital anomaly symptom characterized by vision and hearing loss, congenital heart disease, and malformations of craniofacial and others^[58]. Pathogenic variants in CHD7 of CHARGE

syndrome patients were present in 15 of 28 individuals (53.6%), whereas 4 (14.3%) individuals had other pathogenic variants such as RERE, KMT2D, EP300, or FIR/PUF60^[59]. A two base pair deletion was identified in the *PUF60* gene, which is one of three genes in the critical region of the 8q24.3 microdeletion syndrome (Verheij syndrome) that shows intellectual disability^[60]. In 2013, patients with microdeletions of chromosome 8q24.3 including FIR/PUF60 were found to have developmental delay, microcephaly, craniofacial, renal and cardiac defects were found in six patients with variants in FIR/PUF60^[61]. Eye coloboma and complex cardiac malformations belong to the clinical spectrum of PUF60 variants^[62,63]. The fetus presented atrioventricular septal defect and hypoplastic aortic arch, facial dysmorphism, microretrognathia, dysmorphic ears, clinodactyly of the 5th digit on both hands, mild rocker bottom feet and abnormal third sacral vertebra^[64]. An individual was reported with microcephaly, short stature, intellectual disability, and heart defects with a de novo c.505C > T variant leading to a p.His169Tyr change in PUF60^[65]. The publications that show the direct interaction between FIR/PUF60 deficiency and human disease have been accumulating [Table 1]. FIR/PUF60 deficiency-associated amino-acid substitutions, even within a single RNA recognition motif, altered selection of competing 3' splice sites (3'ss) and branch points of a FIR/PUF60-dependent exon and the 3'ss choice was also influenced by AS of FIR/PUF60^[65]. FIR/FIRΔexon2/PUF60 is a promising target to the development of cancer diagnosis and therapies directed HBV, HCV, FBW7 as well as c-Myc. Together, FIR/PUF60/FIRΔexon2 are multifunctional through AS and applicable for clinical use for HBV suppression especially for hepatoma treatment.

DECLARATIONS

Acknowledgements

The authors appreciate Dr Hiroyuki Osada and his coworkers (RIKEN, Wako, Saitama, Japan) for screening compounds bound to FIR/FIRΔexon2 from a chemical library which was a collection of the isolates from natural products.

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Matsushita K

Performed data acquisition, as well as provided administrative, technical, and material support: Hoshino T

Availability of data and materials

Materials used in this study are generally available for readers as described in the materials and methods.

Financial support and sponsorship

This study was supported in part by Grant-in-Aid 26460667 for priority areas in cancer research from “the Ministry of Education, Science, Sports and Culture of Japan” (KAKENHI), AMED (Japan Agency for Medical Research and Development), “Chiba Foundation for Health Promotion & Disease Prevention” and was partly supported by Extramural Collaborative Research Grant of Cancer Research Institute, Kanazawa University to K.M.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Quantitative hepatitis B surface antigen in predicting recurrence of hepatitis B-related hepatocellular carcinoma after liver transplantation

James Fung^{1,2,3}, Danny Ka-Ho Wong^{2,3}, Yasuhito Tanaka⁴, Regina Lo^{3,5}, Tiffany Wong^{1,6}, Kenneth Siu-Ho Chok^{1,6}, Albert Chi-Yan Chan^{1,6}, Tan-To Cheung^{1,6}, Wing-Chiu Dai^{1,6}, Kelvin Ng^{1,6}, Kevin Ng⁶, Man Kwan^{3,6}, Irene Ng^{3,5}, Wai-Kay Seto^{2,3}, Ching-Lung Lai^{2,3}, Man-Fung Yuen^{2,3}, Chung-Mau Lo^{1,3,6}

¹The Liver Transplant Center, Queen Mary Hospital, Hong Kong, China.

²Department of Medicine, the University of Hong Kong, Hong Kong, China.

³State Key Laboratory for Liver Research, the University of Hong Kong, Hong Kong, China.

⁴Department of Virology and Liver Unit, Graduate School of Medical sciences, Nagoya City University, Nagoya 467-8601, Japan.

⁵Department of Pathology, the University of Hong Kong, Hong Kong, China.

⁶Department of Surgery, the University of Hong Kong, Hong Kong, China.

Correspondence to: Dr. James Fung, the Liver Transplant Center, Queen Mary Hospital, Hong Kong, China. E-mail: jfung@gastro.hk

How to cite this article: Fung J, Wong DKH, Tanaka Y, Lo R, Wong T, Chok KSH, Chan ACY, Cheung TT, Dai WC, Ng K, Ng K, Kwan M, Ng I, Seto WK, Lai CL, Yuen MF, Lo CM. Quantitative hepatitis B surface antigen in predicting recurrence of hepatitis B-related hepatocellular carcinoma after liver transplantation. *Hepatoma Res* 2018;4:62. <http://dx.doi.org/10.20517/2394-5079.2018.92>

Received: 26 Jul 2018 **First Decision:** 9 Aug 2018 **Revised:** 30 Aug 2018 **Accepted:** 30 Aug 2018 **Published:** 29 Sep 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Yuan-Li Wang **Production Editor:** Zhong-Yu Guo

Abstract

Aim: Recurrence of hepatocellular carcinoma (HCC) after liver transplantation (LT) for chronic hepatitis B (CHB) can be associated with reappearance of hepatitis B surface antigen (HBsAg). The current study determined the significance of HBsAg qualitatively and quantitatively using a highly sensitive assay in recurrent HCC after transplantation.

Methods: Consecutive patients with HBV-related HCC with LT were included. Oral nucleos(t)ide analogues without hepatitis B immune globulin were used as hepatitis B virus (HBV) prophylaxis. Quantitative HBsAg levels were performed at time of transplant, at 1 month, 3 and 6 months post transplant using a highly sensitive (hs)-HBsAg assay.

Results: One hundred and fourteen patients were included, with a median follow-up of 80 months, with 24 cases of HCC recurrence, and a cumulative rate of 20.7% at 5 years. There was significant correlation between time of tumor recurrence and time of HBsAg reappearance ($r = 0.551$, $P = 0.027$). Early HCC recurrence was associated with higher median level of hs-HBsAg at the time of transplant (72.85 vs. 69.70 IU/mL, $P = 0.018$). Using a hs-HBsAg cut-off level of 0.0005 IU/mL, patients with levels above this threshold at 3 and 6 months were



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associated with higher rate of early HCC recurrence (28.6% *vs.* 3.0% and 26.9% *vs.* 2.9% respectively, both $P = 0.0006$). There was no significant difference in HCC recurrence between positive and negative HBsAg using the conventional qualitative HBsAg assay.

Conclusion: Serum hs-HBsAg levels of ≥ 0.0005 IU/mL at 3 to 6 months after LT is associated with higher rates of early HCC recurrence, and may be useful as an early tumor marker.

Keywords: Hepatitis B, hepatocellular carcinoma, transplantation, hepatitis B surface antigen, recurrence

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide, and the third most common cause of cancer death^[1]. In the majority of cases, HCC develops on the background of chronic liver disease and established cirrhosis. The prevalence of HCC is the highest in the Asia-Pacific region, where chronic hepatitis B (CHB) infection is the dominant cause^[2,3]. For many patients, liver transplantation (LT) remains the only curative option. By removing the diseased liver, transplantation potentially cures both the tumour and the underlying cirrhosis. However, not all patients with HCC are eligible for transplantation. The most widely adopted criteria to determine eligibility for transplantation include the Milan and the University of California, San Francisco (UCSF) criteria^[4,5]. Despite adhering to the selection criteria, there is still a risk of tumour recurrence of approximately 20% after transplantation^[6].

Previous studies have shown different risk factors associated with HCC recurrence after LT, including higher degree of immunosuppression, higher number of tumour nodules, size of the largest lesion, older donor age, presence of vascular invasion, higher pre-operative alpha-fetoprotein (AFP), and higher neutrophil to lymphocyte ratio^[7-12]. For patients transplanted for hepatitis B virus (HBV)-related HCC, a high pre-operative viral load and inflammatory activity have been shown to be associated with HCC recurrence^[13]. Other studies have shown an association between HCC and HBV recurrence after LT^[14]. A recent study on CHB patients treated with lamivudine monophylaxis after LT showed a higher HCC recurrence rate in those who were hepatitis B surface antigen (HBsAg) positive after transplantation^[15]. Another study demonstrated the detection of HBV DNA and covalently closed circular DNA (cccDNA) in tumour cells, suggesting that HBV replication in tumour cells may contribute to the recurrence of HBV^[16].

The fact that the rate of HCC recurrence in CHB is similar to HCC secondary to other causes after LT suggests that the underlying liver pathology is not a significant predictive factor^[17]. The association of positive viral markers with HCC recurrence, rather than posing as risk factors per se, may signify these as potential tumour markers in predicting HCC recurrence. The aim of the current study was to determine the significance of HBsAg qualitatively and quantitatively in the recurrence of HCC after LT in patients with CHB.

METHODS

All patients with HBV-related HCC with LT performed from June 2003 to December 2010 at Queen Mary Hospital (Hong Kong) were included. The selection of patients eligible for LT was carried out using the UCSF criteria (solitary tumour not exceeding 6.5 cm, or a maximum of 3 tumour nodules totaling up to 8 cm with each nodule not exceeding 4.5 cm)^[5]. Initial tumour evaluation was performed with triphasic computer tomography (CT) scan of the abdomen and thorax, and with radionuclide bone scan to exclude skeletal metastasis. Dual tracer C11-acetate and 18F-fluorodeoxyglucose positron emission tomography was used in some patients in place of bone scan. Imaging was performed at 3-6 monthly intervals to confirm that the patients remain within the criteria. Bridging loco-regional therapy using transarterial chemoembolization, radiofrequency ablation or high intensity focused ultrasound was offered to patients with a prolonged waiting time. None of the patients received systemic chemotherapy post-transplant. None of the patients had evidence

of extrahepatic spread or major vascular invasion at the time of transplantation.

Immunosuppression

The primary immunosuppressive agent used was tacrolimus, with a target therapeutic level of 8-10 ng/mL in the first 3 months, and a lower range of 5-8 ng/mL beyond 3 months. For patients who were intolerant of tacrolimus, cyclosporine was used. For patients who were intolerant of calcineurin inhibitors or requiring additional immunosuppression, mycophenolate mofetil, sirolimus, and corticosteroids were used. Intravenous hydrocortisone and basiliximab were administered peri-operatively.

Prophylaxis for hepatitis B

Oral nucleos(t)ide analogues were given as prophylaxis after transplantation according to protocol for prevention of recurrent graft hepatitis. Prior to November 2007, lamivudine was used, with additional rescue therapy for those with evidence of lamivudine resistance. For those with pre-existing lamivudine resistance, lamivudine together with adefovir (and later tenofovir) was given. From November 2007 onwards, entecavir replaced lamivudine as the primary HBV prophylactic agent. As the center adopted an all-oral antiviral regimen, hepatitis B immune globulin (HBIG) was not used in the peri-operative or post-transplant period.

Surveillance for hepatitis B

Patients were followed up routinely at 3-monthly intervals once stable, or at shorter intervals depending on the clinical need. HBV serology was performed at routine follow up visits, including HBsAg, anti-HBs, and HBV DNA. The qualitative HBsAg tests were performed using the Architect HBsAg assay (Abbott Diagnostics, Abbott Park, IL, USA), with a lower limit of detection (LLOD) of 0.05 IU/mL. Anti-HBs were measured using the Architect anti-HBs assay (Abbott Diagnostics, Abbott Park, IL, USA), with a LLOD of 10 mIU/mL. HBV DNA was measured initially using the Cobas Amplicor assay (Roche Diagnostics, Branchburg, NJ) with a LLOD of 300 copies/mL, and later with the COBAS Taqman assay (Roche Molecular Systems, Branchburg, NJ) with a LLOD of 20 IU/mL.

To determine the predictive value of HBsAg in early HCC recurrence, quantitative HBsAg levels were performed at the time of transplant, at 1 month, 3 and 6 months post transplant on available stored sera kept at -20 °C using a highly sensitive semi-automated immune complex transfer chemiluminescence enzyme immunoassay (ICT-CLEIA) in a HISCL-2000 chemiluminescence immunoassay analyzer (Sysmex, Kobe, Japan)^[18]. Briefly, the samples were incubated with anti-HBs together with magnetic microparticles coated with anti- 2,4-dinitrophenol monoclonal antibodies. The samples were then washed and further incubated. The reaction was then performed using the 12GC PLUS Magstration System (Precision System Science, Matsudo, Japan), and the results calculated using an in-house standard curve. The positive results were then confirmed with anti-HBs neutralizing antibodies. This highly sensitive HBsAg (hs-HBsAg) assay had a LLOD of 0.0005 IU/mL.

Surveillance for HCC

Regular surveillance for HCC was performed after LT with AFP and contrast CT or magnetic resonance imaging (MRI) scans of the abdomen and thorax at 3-6 monthly intervals during the initial 5 years after transplantation and at 6-12 monthly intervals thereafter.

Immunohistochemistry

Staining for HBsAg was performed on recurrent HCC specimens. The primary antibody HBsAg (clone: S1-210, diluted 1:100, Signet) was applied to 4-µm-thick, 10% formalin-fixed, paraffin-embedded tissue sections. The sections were first deparaffinized, rehydrated, and washed with xylene, graded alcohol and distilled water. The slides were applied on Leica Bond-III autostainer. No antigen retrieval was required. The specific antibody was located by a linking post primary antibody conjugated to a peroxidase-labeled polymer (biotin-free) that

Table 1. Patient characteristics

Parameter	Value
Total	112
Age (years)	55 (30-67)
Gender	
Males	97 (86.6%)
Females	15 (13.2%)
Follow-up length (months)	80.5 (2-145)
Type of transplant	
Living donor	80 (71.4%)
Deceased donor	32 (28.6%)
Tumour characteristics	
Tumour size (cm)	3 (1-8)
Tumour number	1 (1-15)
Alpha-fetoprotein (ng/mL)	18 (1-33858)
Viral parameters	
Hepatitis B e-antigen positive	35 (31.3%)
HBV DNA (log IU/mL)	2.52 (1.54-9.75)
HBV DNA undetectability	44 (39.3%)
Hepatitis B surface antigen (IU/mL)	70.88 (0.0009-75.91)
Antiviral therapy at transplantation	
Lamivudine	51 (45.5%)
Entecavir	46 (41.1%)
Lamivudine + adefovir	10 (8.9%)
Lamivudine + tenofovir	4 (3.6%)
Entecavir + adefovir	1 (0.9%)

Continuous variables expressed as median values (range)

recognized mouse and rabbit immunoglobulins. Hydrogen peroxidase was applied to remove endogenous peroxidase activity. The polymer complex was then visualized with an appropriate diaminobenzidine (DAB) chromogen. The sections were counterstained with hematoxylin. Appropriate positive and negative controls were used.

Informed consent was obtained from all patients for collection and storage of clinical specimens for use in the current project, and approved by the Ethics Committee Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong Western Cluster (UW 05-359 T/1022).

Statistical analysis

All statistical analyses were performed using the SPSS version 17.0 (SPSS Inc, Chicago, IL). Categorical variables were analyzed using the Chi-squared test, and Fisher's exact test when appropriate. Mann-Whitney test was used to analyze continuous variables with skewed distribution, and Kruskal-Wallis test used for continuous variables with more than 2 categories. Bivariate correlation for continuous variables was performed using the Pearson test. The cumulative incidences of HCC recurrences and survivals were analyzed using the Kaplan-Meier method, with log-rank testing for comparison. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

A total of 114 consecutive patients were transplanted for HBV-related HCC from June 2003 to December 2010. Of the 114 patients, 2 patients did not have HBsAg determined after transplantation due to early mortality from cardiac arrest, leaving 112 in the final analysis. The median follow-up was 80.5 months (range, 2 to 145), with a median age of 55 years (range 30-67) and a male predominance (86.6%). Of the 112 patients, 80 (71.4%) and 32 (28.6%) underwent living-related and deceased-donor LT respectively. The patient characteristics, tumour characteristics, and the types of antiviral regimen used at the time of transplantation are summarized in Table 1. The tumour size and number were based on explant histology. At the time of transplant, 31.3% were Hepatitis Be Antigen (HBeAg) positive and the median HBV DNA and HBsAg level was 2.52 log IU/mL and 70.88 IU/mL respectively. There was no correlation between pre-transplant HBsAg levels and HBV DNA

($P = 0.451$) or AFP ($P = 0.402$). There was no difference in pre-transplant HBsAg levels between the numbers of HCC nodules (1-3; $P = 0.620$), and HCC differentiation (well, moderate, poor; $P = 0.740$).

Post transplant hepatitis B status

The majority of patients underwent loss of HBsAg shortly after transplantation, with a cumulative rate of HBsAg seroclearance of 90.7% at 6 months after transplantation [Figure 1A]. Only 5 patients remained persistently positive for HBsAg after transplantation without evidence of seroclearance. A total of 27 patients had re-appearance of HBsAg after initial HBsAg seroclearance after transplantation. The cumulative rate of HBsAg re-appearance was 8.5%, 18.0%, 21.9%, and 26.4% at 1, 2, 3, and 5 years respectively, with no further increase thereafter. There was no difference between living-related and deceased-donor LT with respect to re-appearance of HBsAg ($P = 0.945$).

Despite the absence of HBIG administration, 64 (57.1%) of the patients had detectable anti-HBs titer after transplantation [Figure 1B]. The majority occurred within the early post transplant period, with a median time to antibody development of 2 months (range, 0-137), and a median peak antibody level of 133 mIU/mL (range, 11 to > 1000). The detectable antibody titres represented a transient phenomenon as 56 (87.5%) had subsequent disappearance of antibodies with a median time of 7 months from the time of antibody appearance (range, 0 to 131).

All patients achieved undetectable HBV DNA post transplant, with a cumulative rate of undetectable HBV DNA of 97.3% at 6 months. Virological rebound was defined as a 1 log increase from nadir. The cumulative rate of virological rebound was 20.5% and 31.0% at 5 and 10 years after transplantation respectively. A total of 28 patients had evidence of virological rebound, of which 5 had no evidence of rtM204 mutation, and 6 with pre-existing rtM204 mutation. The remaining 17 patients had newly detected rtM204 mutation, and all were treated with additional nucleos(t)ide analog therapy.

Overall recurrence of HCC

There were 24 cases of HCC recurrence during the follow-up period. The overall cumulative rate of HCC recurrence was 9.0%, 14.4%, 20.7%, and 24.3% at 1, 3, 5, and 10 years respectively [Figure 1C]. The AFP at the time of LT was higher for those with early HCC recurrence compared to those without (38 vs. 14 ng/mL respectively, $P = 0.027$). The sensitivity of an elevated AFP (> 20 ng/mL) at 1, 3, 6, and 12 months in diagnosing early HCC recurrence, as defined by recurrence within 3 years after transplantation, was 8%, 7%, 21%, and 20% respectively. There was no difference in tumour size between those with and without early recurrence ($P = 0.835$). The median number of HCCs on explant was higher for those with early recurrence (3 vs. 1, $P < 0.001$).

HBV DNA levels with HCC recurrence

There was no difference in the HBV DNA levels at the time of transplantation between those with and without early HCC recurrence (both groups had median HBV DNA levels at the LLOD, $P = 0.231$). There was no significant differences in the rate of early HCC recurrence between those with and without undetectable HBV DNA levels at 1, 3, 6, and 12 months after transplantation ($P = 0.448$, $P = 0.579$, $P = 0.308$, and $P = 0.608$ respectively).

HBsAg status and HCC recurrence

There was significantly lower HCC recurrence rates for those with persistent loss of HBsAg compared to those that remained HBsAg positive (7% vs. 40.0% respectively at 5 years post transplant, $P = 0.012$). Twenty-seven patients had re-appearance of HBsAg after initial HBsAg seroclearance. For those with evidence of HBsAg seroclearance, re-appearance of HBsAg was associated with a significantly higher rate of HCC recurrence compared to those who remained HBsAg negative (56.1% vs. 7% respectively at 5 years post transplant, $P < 0.001$) [Figure 2A].

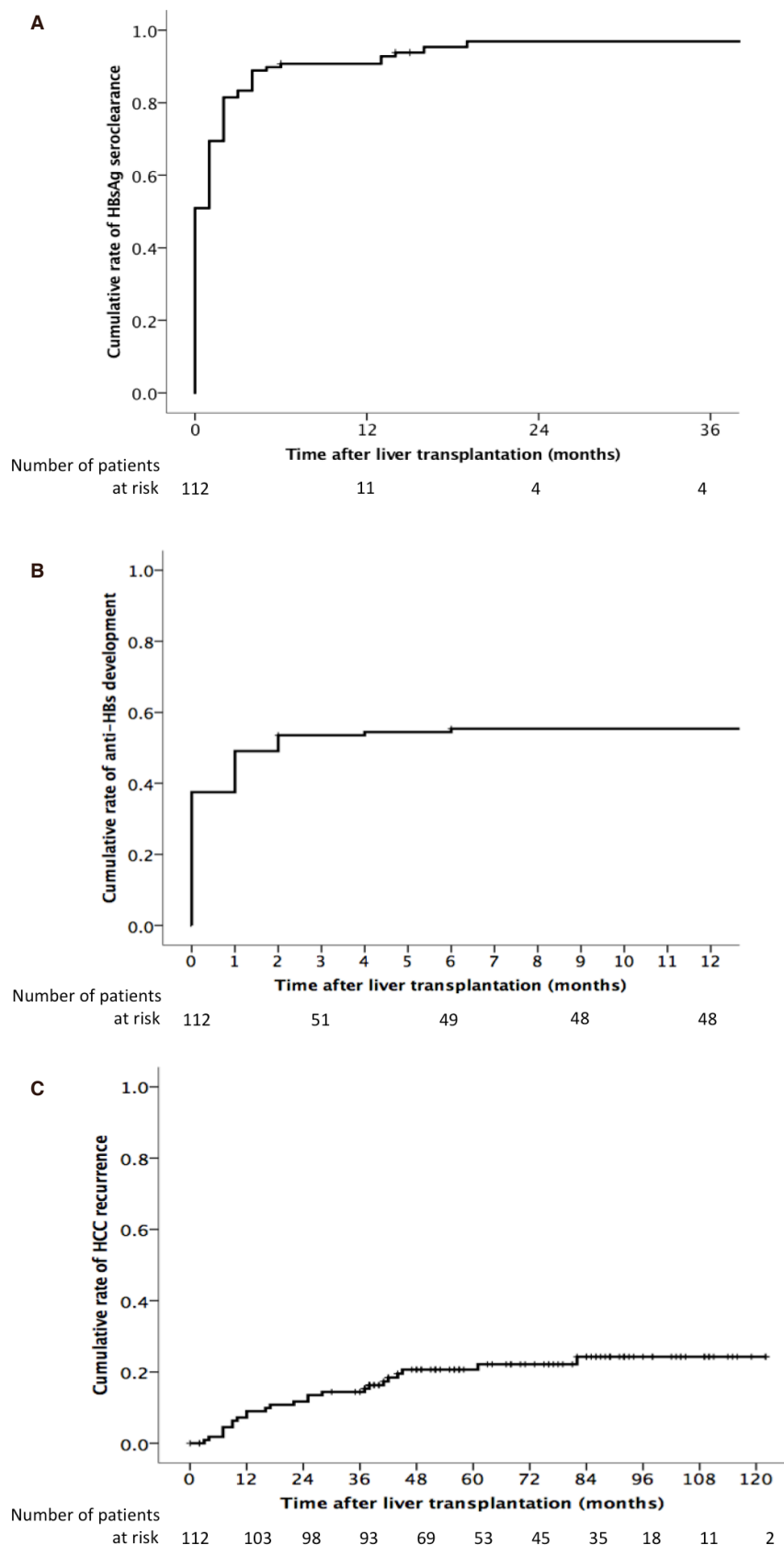


Figure 1. A: Cumulative incidence of HBsAg seroclearance after liver transplantation; B: cumulative incidence of the development of detectable anti-HBs after liver transplantation; C: cumulative incidence of recurrent hepatocellular carcinoma after transplantation

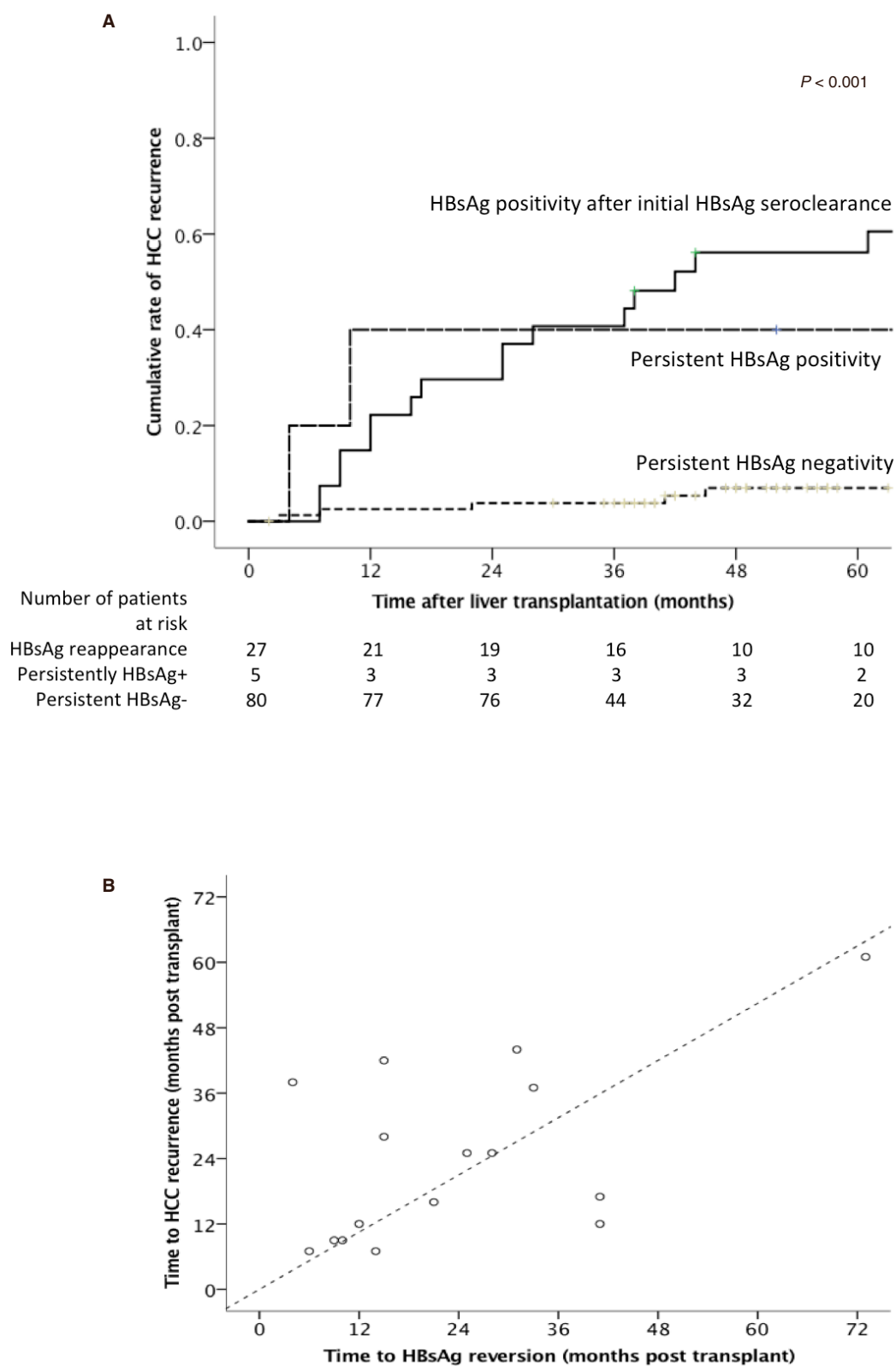


Figure 2. A: Cumulative incidence of recurrent hepatocellular carcinoma according to HBsAg status after liver transplantation; B: correlation between time of hepatocellular carcinoma recurrence and HBsAg seroreversion after liver transplantation

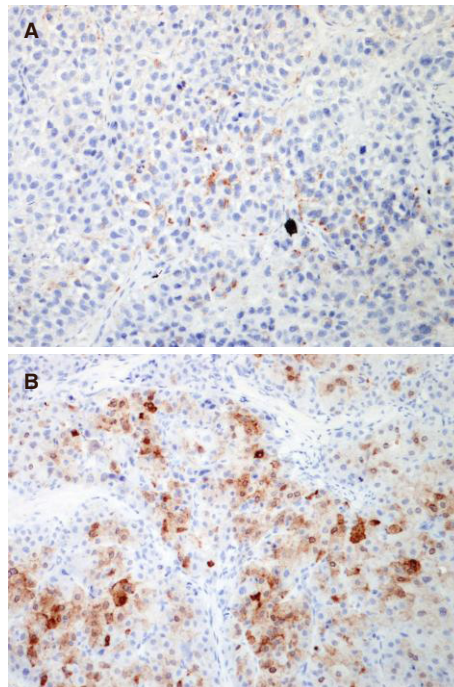


Figure 3. A and B: Immunohistochemical study for HBsAg showing positive cytoplasmic staining in metastatic tumour cells in lungs

A total of 16 patients lost HBsAg after transplantation, with subsequent re-appearance of HBsAg and recurrence of HCC. There was a significant correlation between the time of tumour recurrence and the time of HBsAg re-appearance ($r = 0.551$, $P = 0.027$), as shown in [Figure 2B](#). There was no significant difference in the median time of recurrence of HCC vs. re-appearance of HBsAg after LT (21 months vs. 18 months respectively, $P = 0.809$). Of these 16 patients, 2 had recurrence limited to the liver, and the remaining 14 patients had extra-hepatic metastatic lesions to the lungs, bones, lymph nodes, and adrenal glands. Histology from the site of recurrence was available for 12 of 16 patients, with specimens from 2 patients staining positive for HBsAg (both from metastatic lung tissues) [[Figure 3](#)]. One had recurrence at 9 months after transplantation, with HBsAg re-appearance at 10 months. The other patient had recurrence at 7 months, with HBsAg re-appearance at 14 months.

Quantitative HBsAg levels with HCC recurrence

There was a significant higher median level of hs-HBsAg at the time of transplant for those with early HCC recurrence compared to those without (72.85 vs. 69.70 IU/mL respectively, $P = 0.018$). After transplant, the median hs-HBsAg levels at month 1, 3, 6, and 12 was 0.0008 (range, 0-50.6855), 0 (range, 0-1.0827), 0 (range, 0-0.1642), and 0 (range, 0-0.1310) IU/mL respectively. Using a hs-HBsAg cut-off level of 0.0005 IU/mL, patients with levels ≥ 0.0005 IU/mL was associated with a significantly higher rate of early HCC recurrence compared to those with lower levels at 3 months post transplant (28.6% vs. 3.0% respectively at 3 years post transplant, $P = 0.006$) [[Figure 4A](#)], and at 6 months post transplant (26.9% vs. 2.9% respectively, $P = 0.006$) [[Figure 4B](#)]. In contrast, using the conventional qualitative HBsAg assay, there was no significant difference in HCC recurrence observed between positive and negative HBsAg status at 3 and 6 months post transplant ($P = 0.845$ and $P = 0.449$, respectively). No significant difference in early HCC recurrence rate was observed at 1 month post transplant using this cut-off ($P = 0.162$) [[Figure 4C](#)].

DISCUSSION

In CHB patients who achieve HBsAg seroclearance by conventional assays, a substantial proportion of

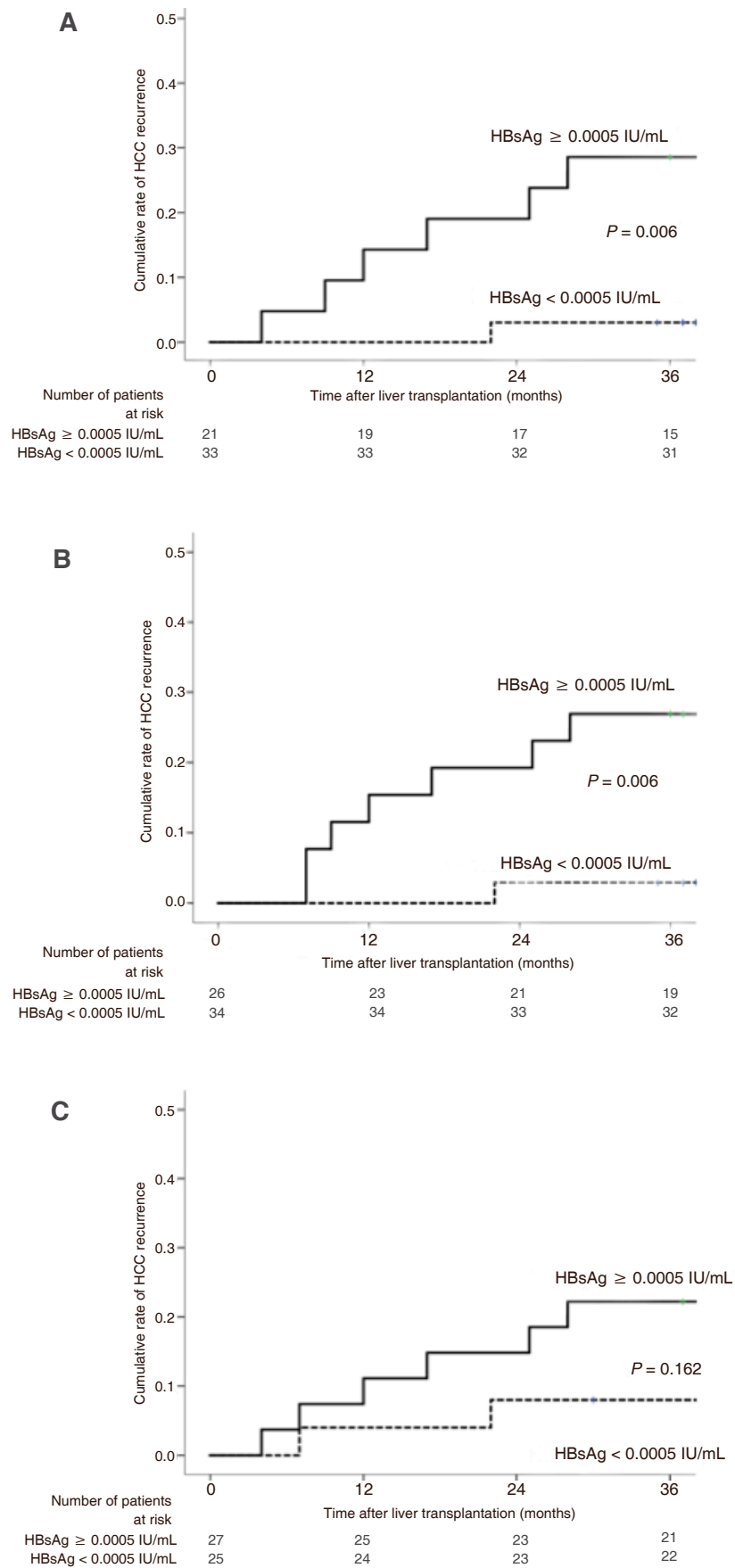


Figure 4. A: Cumulative incidence of hepatocellular carcinoma recurrence according to hs-HBsAg level at 3 months after liver transplantation; B: cumulative incidence of hepatocellular carcinoma recurrence according to hs-HBsAg level at 6 months after liver transplantation; C: cumulative incidence of hepatocellular carcinoma recurrence according to hs-HBsAg level at 1 month after liver transplantation

patients may show detectable HBsAg using the hs-HBsAg assay, especially those who were negative for anti-HBs^[19]. This would suggest that HBsAg seroclearance only implies undetectability below the standard quantitative assay. For the first time, the current study demonstrated that quantitative HBsAg levels using a highly sensitive assay could be used to predict higher chance of HCC recurrence after LT for HBV-related HCC. By using a completely HBIG-free regimen for hepatitis B prophylaxis, the study population presented a unique opportunity to study the characteristics of HBsAg both qualitatively and quantitatively after transplantation, and its association with early HCC recurrence. The administration of HBIG will preclude any useful determination of HBsAg, as these will largely become undetectable through binding to anti-HBs, which is consistently kept at a high level through the use of regular injections. Oral nucleos(t)ide analogues, although effective in suppressive HBV DNA to undetectable levels, has significantly less effect on the HBsAg levels. Previous studies have demonstrated that even potent oral antiviral therapy may not reduce HBsAg levels despite prolonged HBV DNA suppression to undetectable levels^[20].

The current study showed a significantly higher rate of HCC recurrence in those who failed to achieve HBsAg seroclearance after transplantation (50.0% vs. 23.4% respectively at 8 years post transplant, $P = 0.024$). Furthermore, for patients who remained HBsAg positive after transplant, the recurrence of HCC was early (within the first year of transplant). It is possible that micrometastasis present at the time of transplant may be responsible for the persistence of HBsAg. In addition, a significantly higher HCC recurrence rate was observed in those who had HBsAg seroreversion compared to those who remained negative for HBsAg (60.5% vs. 9.4% respectively at 10 years post transplant, $P < 0.001$). The association of recurrence HCC and HBV have also been described in a smaller cohort of patients previously^[21]. Since extra-hepatic tumour tissue stained positive for HBsAg, it is likely that malignant tumour cells are able to support HBsAg production. Moreover, there was correlation between the timing of HBsAg re-appearance and HCC recurrence ($r = 0.551$, $P = 0.027$), raising the possibility that HBsAg may be useful as a marker of HCC recurrence. The temporal relationship suggests that the HBsAg is unlikely to have a role in the pathogenesis of HCC recurrence, but rather HCC may be responsible for the HBsAg reversion.

To date, the only tumour marker currently readily available for post transplant HCC recurrence is serum AFP, which in the current study, showed low sensitivity (7% to 21%) as an early predictor after transplantation. In addition, a significant proportion of HCC did not secrete AFP. The finding that hs-HBsAg levels above the LLOD (≥ 0.0005 IU/mL) at 3 and 6 months can be associated with higher rates of early HCC recurrence suggests that this may be used as an early tumour marker. In contrast, the less sensitive conventional HBsAg measurement did not show a significant difference at 3 or 6 months post transplant with respect to a positive or negative HBsAg status and subsequent HCC recurrence. This may be due to the fact that during the early post transplant period, tumour load is likely to be extremely low for those with recurrence, and therefore the HBV DNA may not be detectable and HBsAg may not be quantifiable by conventional assays.

One of the consistent observations with HBIG-free prophylaxis after transplantation is the development of detectable transient levels of anti-HBs titers shortly after transplantation, despite the absence of HBIG administration (including the current study)^[22-24]. This is most likely due to passive transfer of antibodies from the donor, and is largely non-sustainable. Importantly, this phenomenon may preclude the use of quantitative HBsAg as a useful tool for predicting HCC recurrence in the very early phases (within 3 months) after transplantation, and provide explanation as to why the HBsAg levels were not predictive at 1-month post transplant period in the current study. Other intermediate replication markers such as the hepatitis B core-related antigen may be more useful, and deserves further study.

There are several limitations to the current study. Firstly, quantitative hs-HBsAg was performed in available stored samples only. A previous study looking at the change in HBsAg levels with the conventional assay in stored sera found no significant changes over time^[25]. Secondly, this cohort consists of patients largely in the

lamivudine era, and hence accounts for the high rate of virological rebound due to the development of rtM204 mutations. The virological rebound observed would likely have impact on the predictive value of quantitative HBsAg in predicting HCC recurrence. Thirdly, there was a lower proportion with deceased-donor LT with a lower recurrence rate, thus precluding any comparison with those undergoing living-donor LT.

To conclude, serum hs-HBsAg levels of ≥ 0.0005 IU/mL at 3-6 months after LT have been shown to be associated with higher rates of early HCC recurrence, and may be a useful tool as an early tumour marker in the post transplant setting.

DECLARATIONS

Acknowledgements

Dr. Shintaro Oagawa exams the hs-HBsAg assay.

Authors' contributions

Planning and conduction of study, collecting and interpreting data, drafting manuscript: Fung J

Conduction of study, laboratory supervision and work, critical appraisal: Wong DKH

Laboratory work, critical appraisal: Tanaka Y, Ng K

Laboratory work, collecting and interpreting data, critical appraisal: Lo R

Conduction of study, collecting data, critical appraisal: Wong T, Chok KSH, Chan ACY, Cheung TT, Dai WC, Ng K

Laboratory supervision and work, critical appraisal: Kwan M, Ng I

Conduction of study, critical appraisal: Seto WK

Design of study and critical appraisal: Lai CL

Planning & design of study, critical appraisal, and study supervision: Yuen MF

Critical appraisal and study supervision: Lo CM

Availability of data and materials

On request.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

This study has been performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients for collection and storage of clinical specimens for use in the current project, and approved by the Ethics Committee Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong Western Cluster (UW 05-359 T/1022).

Consent for publication

Not applicable.

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Review

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Alpha-fetoprotein as a predictor of hepatocellular carcinoma recurrence following liver transplantation

Evangelia M. Fatourou, Abid R. Suddle, Michael A. Heneghan

Institute of Liver Studies, King's College Hospital, Denmark Hill, London SE5 9RS, UK.

Correspondence to: Prof. Michael A Heneghan, Institute of Liver Studies, King's College Hospital, Denmark Hill, London SE5 9RS, UK.
E-mail: michael.heneghan@nhs.net

How to cite this article: Fatourou EM, Suddle AR, Heneghan MA. Alpha-fetoprotein as a predictor of hepatocellular carcinoma recurrence following liver transplantation. *Hepatoma Res* 2018;4:63. <http://dx.doi.org/10.20517/2394-5079.2018.62>

Received: 17 May 2018 **First Decision:** 3 Jul 2018 **Revised:** 18 Jul 2018 **Accepted:** 30 Jul 2018 **Published:** 10 Oct 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Jun-Yao Li **Production Editor:** Zhong-Yu Guo

Abstract

Alpha-fetoprotein (AFP) has been increasingly recognised as a valuable marker in predicting HCC recurrence post-liver transplantation. Moreover, its secretion has been associated with poor histological tumour characteristics as it reflects an aggressive tumour biological behaviour. This review aims to summarise the emerging evidence on the use of AFP either as an independent marker, or as a variable incorporated into prognostic models. For this purpose, an electronic PubMed literature search was performed. Due to the heterogeneity of the reported studies, drawing clear conclusions about the optimum AFP cut-off level to predict recurrence is difficult. Models that include AFP at different cut-offs have been shown to be superior to Milan criteria in predicting disease recurrence, but need to be prospectively validated in order to confirm their prognostic value. Until more refined methods for selecting patients become available, existing evidence supports the use of AFP in decision models for liver transplantation.

Keywords: Alpha-fetoprotein, hepatocellular carcinoma, liver transplantation, recurrence, survival

INTRODUCTION

Hepatocellular carcinoma (HCC) is expected to become a leading cause for liver transplantation (LT) following curative treatment for hepatitis C and after more widespread acceptance of the practice of tumour down-staging for patients originally considered beyond LT criteria^[1]. It has been estimated that patients with HCC currently represent 30%-35% of the waiting list population in Europe and in an era of organ shortage, selecting the best candidates for LT with the lowest risk of post-transplant recurrence poses a clinical challenge^[2].

It is evident that even after applying the most restrictive tumour burden selection criteria, 10%-15% of patients



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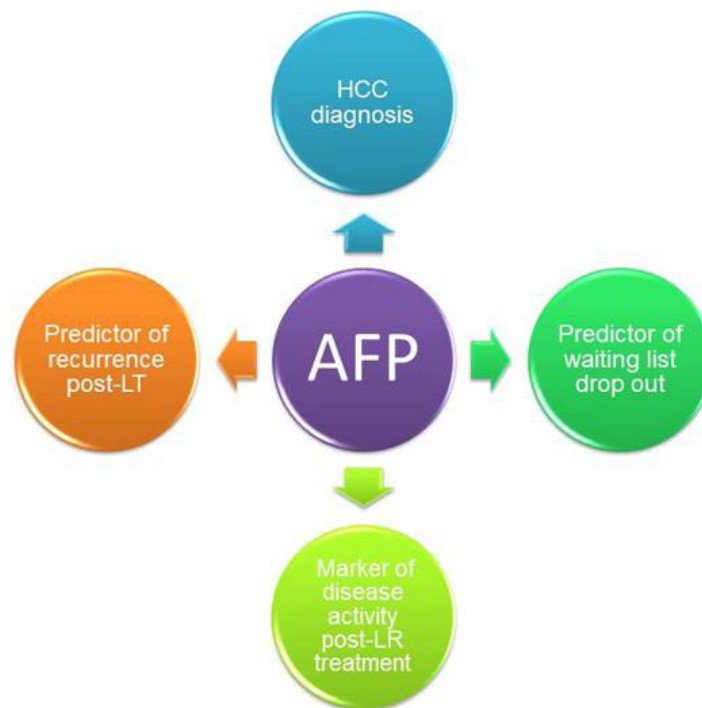


Figure 1. The predictive role of AFP pre- and post-LT. AFP: alpha-fetoprotein; HCC: hepatocellular carcinoma; LT: liver transplantation

with HCC still develop HCC recurrence post-LT^[3]. It is also apparent that factors beyond tumour size and number are associated with more aggressive tumour biology and are accountable for increasing the risk of HCC recurrence^[4]. Among these, alpha-fetoprotein (AFP) has been increasingly recognised as a valuable marker in predicting HCC recurrence [Figure 1]^[5]. Although an association between high AFP values and poor outcomes post LT has been established^[6], it has still not been incorporated in the most widely used listing criteria. Several scoring systems encompassing pre-LT serum AFP levels and tumour size criteria have been recently proposed and have shown to have better predictability of recurrence compared to traditional Milan criteria^[7-10].

The aim of this review is to summarise the emerging evidence on the role of AFP as a predictor of HCC recurrence following LT, either as an independent value or after being combined with tumour size criteria into prognostic models. For this purpose, an electronic PubMed literature search was performed from January 2004 to April 2018 by using the following keywords: “alpha-fetoprotein”, “hepatocellular carcinoma”, “liver transplantation”, “recurrence” and “survival”. In this review, we have included all the original studies that evaluated the role of alpha-fetoprotein (AFP) or AFP including models as a predictive marker of prognosis following LT.

AFP AS AN INDEPENDENT PREDICTOR OF RECURRENCE AND SURVIVAL

Several studies have been published with regard to an AFP absolute cut-off value or AFP change on the waiting list, as an independent predictor of prognosis^[5,9,11-13]. In a meta-analysis that included 13 studies^[14], the upper cut-off AFP value varied widely between 20 ng/mL and 1000 ng/mL. Due to this variation between different studies, this meta-analysis was unable to suggest a single cut-off level which could be universally approved across centres. Despite that, a significant correlation between AFP and both post-transplant recurrence and prognosis was established in the majority of the reported studies^[14].

The critical question as to whether downstaging HCC patients with high AFP is feasible and is associated with

similar prognostic outcomes was addressed in a study by Merani *et al.*^[6]. This retrospective analysis included 6817 patients that were listed with a diagnosis of HCC in the Scientific Registry of Transplant Recipients (SRTR) in the United States and showed that downstaging to an AFP level ≤ 400 ng/mL was associated with good survival rates and prognosis, regardless of the initial AFP level. More specifically, patients who were successfully downstaged to AFP ≤ 400 ng/mL had a similar dropout rate (10% in both groups) and post-transplant survival rates (89% vs. 78% at 3 years, $P = 0.11$) to patients with AFP levels persistently ≤ 400 ng/mL.

A strong dose-response relationship between AFP level and post-transplant outcomes was demonstrated in a study that utilised data from the UNOS registry, that included patients ($n = 45,267$) who were transplanted in the US between 2002 and 2011^[15]. Although patients with an AFP < 15 ng/mL prior to transplantation had similar survival rates to patients without HCC, there was a significant decrease in survival as AFP increased; 16-65 ng/mL [adjusted hazard ratio (AHR) = 1.38, 95% CI : 1.23-1.54], 66-320 ng/mL (AHR = 1.65, 95% CI : 1.45-1.88), and greater than 320 ng/mL (AHR = 2.37, 95% CI : 2.06-2.73). In addition, patients with tumours beyond the Milan criteria at listing had excellent post-transplant survival if serum AFP level was ≤ 15 ng/mL (AHR = 0.97, 95% CI : 0.66-1.43). This study also showed that downstaging of AFP following locoregional treatment was associated with improved post-transplant survival and prognosis^[15].

In a prospective study by Lai *et al.*^[12], mRESIST (modified Response Evaluation criteria in Solid Tumours) progression following locoregional treatment and AFP slope > 15 ng/mL/month, as defined by the difference between the initial and the last pre-LT AFP value divided by the time span between the two values, were independent risk factors of recurrence and survival. Patients within and beyond radiological Milan criteria had similar recurrence-free and overall survival rates if they had stable disease post locoregional treatment and/or an AFP slope < 15 ng/mL/month. Similarly, patients within the Milan criteria but with either progressive disease or AFP slope > 15 ng/mL/month were shown to have increased recurrence rates compared to patients within or beyond Milan criteria with no risk factors^[12]. Another retrospective study from the same group, has shown that AFP > 400 ng/mL can result in an 8-fold increase in the risk of recurrence and a combination with a total tumour diameter < 8 cm can result comparable 5 year survival and recurrence rates^[16].

The predictive value of AFP slope, rather than an AFP single value alone, was also examined in a study by Vibert *et al.*^[17], which included 252 patients transplanted between 1985 and 2005, in a single centre. AFP progression, as defined by an increase greater than 15 ng/mL monthly, was significantly associated with reduced 5 year recurrence-free (47% vs. 74%, $P = 0.01$) and overall survival (54% vs. 77%, $P = 0.02$). In the multivariate analysis, progression of AFP was independently associated with recurrence-free and overall survival. Interestingly, all examined static values of AFP prior to transplantation were not correlated with overall or recurrence-free survival. AFP progression was also significantly associated with the presence of vascular invasion and poor histological differentiation, which suggests that it can be a valuable surrogate pre-operative marker of unfavourable histological findings^[17].

Another study by Hameed *et al.*^[11] has shown that setting a cut-off value of 1000 ng/mL as an exclusion criteria for transplantation, would have resulted in a 20% reduction in the rate of HCC recurrence at the cost of excluding only 4.7% of patients listed. Following this observation, our own national (United Kingdom) Transplant guidelines have applied this cut-off level as an exclusion criterion for LT. This study has also demonstrated a strong correlation between AFP and micro-vascular invasion, especially in patients with AFP values varying between 300-1000 ng/mL^[11].

A large retrospective European multicentre study intended to identify variables for selecting patients that would have the best benefit from transplantation^[18]. AFP ≥ 1000 ng/mL, MELD ≤ 13 , mRESIST progressive or complete response and within Milan criteria were associated with a poor intention to treat (ITT) benefit from LT. Based on these risk factors, four benefit groups were identified. Patients that met three out of four

risk factors were shown to have no benefit from LT, and that accounted for 19.2% of the study population. This study has introduced the concept of ITT benefit for transplantation that can stratify patients towards a more effective allocation system.

In a separate study that included patients registered at the SRTR database ($n = 6478$), a total tumour volume (TTV) $\geq 115 \text{ cm}^3$ and AFP > 400 were independent predictors of survival^[19]. After combining the two variables, with patients being beyond criteria if they had either TTV $\geq 115 \text{ cm}^3$ or an AFP > 400 , the composite score efficiently predicted overall survival (HR 2, 95% CI: 1.7-2.4, $P < 0.001$).

A prospective validation of the proposed criteria was subsequently performed on 233 patients transplanted in 3 different centres. Patients with an AFP $> 400 \text{ ng/mL}$ and TTV $> 115 \text{ cm}^3$ were excluded from LT. Although the risk of drop out was higher in patients within TTV/AFP but beyond Milan criteria, a similar recurrence (9.4 vs. 4.4, $P > 0.05$) and survival rate (74.6% vs. 78.7%, $P > 0.05$) was demonstrated between the two groups. To account for the higher drop out and worse ITT survival in the TTV/AFP groups, the proposed listing criteria are recommended to centres with waiting time of 8 months or more^[9].

A retrospective single centre study that included 137 recipients with more than 50% of the total patient number being beyond Milan or University of California San Francisco criteria at pre-transplant imaging, showed that tumour number > 3 based on explant findings, AFP level $\geq 400 \text{ ng/mL}$, microvascular invasion and rejection needing anti-lymphocytic antibodies were independent predictors of recurrence^[20].

Another study from China has also confirmed that AFP $\geq 400 \text{ ng/mL}$ is independently associated with adverse outcomes. Based on prognostic stratification, the Hangzhou criteria were proposed, based on which, patients with total tumour diameter less than or equal to 8 cm, or total tumour diameter more than 8 cm, with histopathologic grade I or II and preoperative AFP level less than or equal to 400 ng/mL simultaneously, were shown to have favourable post-transplant outcomes^[21].

Several other studies have reported on different AFP values as predictors of recurrence in the recent literature. This includes a study that included 101 patients from a single centre in the U.S. showed that AFP $> 100 \text{ ng/mL}$ (OR = 5.0, 95% CI : 1.23-29.71, $P = 0.006$) and tumour size on explant (OR = 4.1, 95% CI : 1.2-13.5, $P = 0.013$) were associated with microvascular invasion and post-LT recurrence^[22]. Another single centre study including 140 HCC patients confirmed the validity of AFP $> 100 \text{ ng/mL}$ as cut-off value in predicting the risk of post-LT recurrence, in patients meeting the San Francisco or up-to-seven criteria (Warsaw criteria)^[23]. The authors have shown that the expanded proposed criteria increased the transplant eligibility rate by 20.3% without compromising post-transplant outcomes.

A multi-centre Korean study that included 688 patients with advanced HCC (beyond Milan criteria on explant) or far advanced HCC (defined as maximum tumour diameter $\geq 10 \text{ cm}$, 10 or more nodules, or accompanying macro-vascular invasion) has shown that both AFP and the biomarker prothrombin induced by vitamin K absence or antagonist-II (PIVKA-II) were significant risk factors for recurrence^[24]. In particular, a sum of AFP plus PIVKA-II < 300 was a better predictor than either marker alone and can provide valuable information on tumour biology and behaviour in advanced HCCs.

Finally, a single centre study of 250 Korean patients has shown that patients with AFP $> 400 \text{ ng/mL}$ had significantly worse disease-free and overall survival^[25]. On discriminative analysis; a cut off value of 54 ng/mL was significantly associated with disease recurrence, whereas cut-off value of 105 ng/mL was a better discriminator of overall survival^[25].

Table 1 summarises the studies that have evaluated different cut-off values of AFP as significant predictors of recurrence and survival following LT.

Table 1. AFP values to predict HCC recurrence in recently published studies

Reference	Year of publication	Number of patients	Country	Study design	AFP Cut-off value	Prognostic endpoint
Duvoux <i>et al.</i> ^[10]	2012	537 TC 435 VC	France	Retrospective Prospective	100 ng/mL, 1000 ng/mL	5-year RFS and OS
Mazzaferro <i>et al.</i> ^[7]	2017	1018 TC 341 VC	Italy China	Retrospective Retrospective	200 ng/mL, 400 ng/mL 1000 ng/mL	5-year OS
Mehta <i>et al.</i> ^[8]	2017	721 TC 340 VC	US Canada	Retrospective Retrospective	100 ng/mL, 1000 ng/mL	5-year RFS and OS
Merani <i>et al.</i> ^[6]	2011	6817	US	Retrospective	400 ng/mL	3-year ITT survival and OS
Berry <i>et al.</i> ^[15]	2013	45,267	US	Retrospective	15 ng/mL, 16-65 ng/mL 66-320 ng/mL, < 320 ng/mL	6-year OS
Lai <i>et al.</i> ^[12]	2013	422	Europe	Prospective	AFP slope > 15 ng/mL/month	5-year RFS and OS
Lai <i>et al.</i> ^[16]	2012	158	Italy	Retrospective	AFP > 400 ng/mL	5-year RFS and OS
Vibert <i>et al.</i> ^[17]	2010	252	France	Retrospective	AFP slope > 15 ng/mL/month	5-year RFS and OS
Hameed <i>et al.</i> ^[11]	2014	211	US	Retrospective	AFP > 1000 ng/mL	1-, 5-year RFS and OS
Lai <i>et al.</i> ^[18]	2017	2013	Europe	Retrospective	AFP > 1000 ng/mL	ITT survival
Toso <i>et al.</i> ^[19]	2009	6478	US	Retrospective	AFP > 400 ng/mL	5-year OS
Toso <i>et al.</i> ^[9]	2015	233	Switzerland Canada	Prospective	AFP > 400 ng/mL	4-year RFS and OS, ITT survival
Ciccarelli <i>et al.</i> ^[20]	2012	137	Belgium	Retrospective	AFP > 400 ng/mL	5-year RFS
Zheng <i>et al.</i> ^[21]	2008	195	China	Retrospective	AFP > 400 ng/mL	1-, 3-, 5- year RFS and OS
McHugh <i>et al.</i> ^[22]	2010	101	US	Retrospective	AFP > 100 ng/mL	1-, 3-, 5- year RFS and OS
Grat <i>et al.</i> ^[23]	2017	140	Poland	Retrospective	AFP > 100 ng/mL	5-year RFS and OS
Lee <i>et al.</i> ^[24]	2018	688	Korea	Retrospective	AFP + PIVKA > 300	5-year RFS and OS
She <i>et al.</i> ^[25]	2018	250	Korea	Retrospective	54 ng/mL 105 ng/mL	5-year RFS 5-year OS

AFP: alpha-fetoprotein; HCC: hepatocellular carcinoma; TC: training cohort; VC: validation cohort; RFS: recurrence-free survival; OS: overall survival; ITT: intention-to-treat; PIVKA: protein induced by vitamin K absence or antagonist

AFP PROGNOSTIC SCORES FOR THE SELECTION OF PATIENTS FOR LT

In the recent literature, several models that combine tumour burden characteristics with pre-operative AFP at different cut-off levels have been proposed. These have been shown to be superior to Milan criteria in predicting tumour recurrence.

AFP score

A prognostic model which includes AFP at two different cut-off levels (100 ng/mL and 1000 ng/mL) and tumour radiological characteristics at listing was developed in a cohort of 597 French patients transplanted for HCC across 16 different centres, and prospectively validated in a cohort of 434 patients registered for LT in France^[10]. The AFP score defined three groups of patients with low risk of HCC recurrence; (1) patients with 1-3 nodules, maximum diameter of the largest tumour of less than 3 cm and AFP ≤ 1000 ng/mL, (2) patients with 1-3 nodules, maximum diameter of the largest tumour of 3-6 cm, and AFP ≤ 100 ng/mL and (3) patients with more than 4 nodules, maximum diameter of the largest tumour of less than 3 cm, and AFP ≤ 100 ng/mL.

A simplified user-friendly version of the model was developed and the score was calculated by adding the individual points from each variable [Table 2]. A cut-off value more than two (2) points discriminated between patients with low and high risk of recurrence. Five-year recurrence rate was 8.8% ± 1.7% vs. 50.6% ± 10.2% ($P < 0.001$) in patients with AFP score ≤ 2 and ≥ 2 and 5-year survival rate was 67.8% ± 3.4% and 47.5% ± 8.1% ($P < 0.002$) respectively^[10].

The AFP score was subsequently validated in a cohort of 574 patients with a high prevalence of viral hepatitis as an aetiological factor for chronic liver disease, who were transplanted for HCC in 4 Italian centres^[26]. An AFP score ≤ 2 again identified a group of patients with low risk of recurrence, even if they were beyond the Milan criteria at listing. Additionally, in a subgroup of patients who underwent a downstaging procedure prior to

Table 2. The AFP score for the prediction of HCC recurrence

AFP model*	
Variables	Points
Largest tumour diameter (cm)	
≤ 3	0
3-6	1
> 6	4
Number of nodules	
1-3	0
≥ 4	2
AFP level (ng/mL)	
≤ 100	0
100-1000	2
> 1000	3

*The score is calculated by adding the individual points for each variable. A cut-off value of 2 discriminates between patients with low and high risk of recurrence. AFP: alpha-fetoprotein; HCC: hepatocellular carcinoma

Table 3. The proposed AFP-UTS criteria for the prediction of HCC recurrence

AFP-UTS criteria
HCC at pre-transplantation radiology within up to 7 criteria*, if AFP < 200 ng/mL
HCC at pre-transplantation radiology within up to 5 criteria*, if AFP 200-400 ng/mL
HCC at pre-transplantation radiology within up to 4 criteria*, if AFP 400-1000 ng/mL

*Considering as up to 7, 5, or 4 the maximum allowed sum of size (in cm) and number of tumours on the last radiology assessment prior to transplantation. AFP: alpha-fetoprotein; HCC: hepatocellular carcinoma

listing, an AFP score ≤ 2 was associated with an excellent 5 year survival and reduced risk of recurrence^[26]. Another validation study which included 327 patients from Latin America has also demonstrated the superiority of the AFP score compared to Milan criteria in predicting post LT recurrence, even in patients who were downstaged in order to fulfil the listing criteria^[27].

In a study that aimed to examine the survival benefit and cost-utility in order to better allocate medical resources, the AFP score was proven to be a useful tool for cost-effectiveness^[28]. LT was a cost-effective treatment in patients with AFP score ≤ 3 and was proven to be cost-ineffective in patients with AFP score > 7 . Although cost-effectiveness should not directly determine eligibility for transplantation, it should be taken into consideration in order to improve organ allocation. Finally, as previously mentioned, in 2013 the AFP model was adopted by the French Organisation for Organ Sharing as the official national listing criteria.

Metroticket 2.0

In a study by Mazzaferro *et al.*^[7] which included in the training set 1018 patients who underwent LT in 3 different centres in Italy, and in the validation set, 341 patients transplanted for HCC in China, a model that consists of the sum of tumour size and number preoperatively and log₁₀AFP, has shown better predictability of recurrence and survival compared to Milan criteria. By using three different cut-off AFP values the authors defined the AFP-adjusted-to-HCC size (AFP-UTS) criteria as shown in Table 3. Patients within compared to beyond the AFP-UTS criteria showed a 5-year overall, HCC-specific and recurrence-free survival of 79.7% vs. 51.2% ($P < 0.0001$), 93.5% vs. 55.6% ($P < 0.0001$), and 89.6% vs. 46.8% ($P < 0.0001$), respectively. An online calculator was also developed (www.hcc-olt-metroticket.org) which provides a 5-year post-transplantation prediction of HCC specific survival based on the pre-operative radiological tumour assessment and the last AFP value. The prediction value can also be refined based on the presence or not of HCV infection, as this can have a negative impact on overall post-transplant survival.

Table 4. The RETREAT score for the prediction of HCC recurrence

RETREAT score	
Predictor	Retreat points
AFP at LT, ng/mL	
0-20	0
21-99	1
100-999	2
≥ 1000	3
Microvascular invasion	2
Largest viable tumour diameter (cm) plus No of viable tumours*	
0	0
1.1-4.9	1
5-9.9	2
≥ 10	3

*The score is calculated by adding the individual points for each variable. The score is zero (0) if no viable tumour identified. AFP: alpha-fetoprotein; HCC: hepatocellular carcinoma; LT: liver transplantation

RETREAT score

The Risk Estimation of Tumour Recurrence after Transplant (RETREAT) score which consists of the sum of the largest viable tumour diameter and number of viable tumours on explant, microvascular invasion and AFP at the time of LT, was developed in a cohort of 721 patients across 3 U.S centres and externally validated in a cohort of 340 patients from a single centre in Canada. The RETREAT score is calculated as shown in Table 4. Patients with a RETREAT score of 0 have a predicting 1 and 5 year recurrence risk of 1% (95% CI : 0.0%-2.1%) and 2.9% (95% CI : 0.0%-5.6%) respectively which can increase to 29.3% (95% CI : 25.5%-50.5%) and 75.2% (95% CI : 56.7%-85.8%) in patients with a RETREAT score of 5 or higher. One of the advantages of the RETREAT score over other proposed scoring systems is that it takes into consideration the effect of pre-transplant locoregional treatment by including only viable tumours into the model equation. Although this score can only be calculated post LT, it can be utilised to determine surveillance strategies, as well as influence decisions on immunosuppression regimens and adjuvant therapies post LT.

In a study by Mehta *et al.*^[13], the RETREAT score was validated by using the United Network for Organ Sharing database in 3275 patients transplanted for HCC, between 2012 and 2014. Based on explant findings, the RETREAT score discriminated well between patients with low and high risk and recurrence and higher scores were associated with poor survival outcomes. Specifically, patients with a RETREAT score of 0 had a 3 year recurrence and survival rate of 1.6% and 91% respectively, whereas, for patients with a RETREAT score of 5 or higher, the 3 year recurrence and survival rates were 29% and 58% accordingly. The RETREAT score was also shown to be superior to Milan criteria on explant, in predicting HCC recurrence. Finally, the RETREAT score was associated with a shorter time to HCC recurrence with a median time to recurrence of 10.9 months (IQR 51, -17.9) in patients with a score ≥ 4^[13].

HALT HCC score

The Hazzard associated with Liver Transplantation for Hepatocellular Carcinoma (HALT-HCC) score was developed based on retrospective data from 420 patients transplanted for HCC in a single US centre, and included MELD-sodium (MELD-Na), tumour burden score (tumour maximum diameter plus number of lesions) and AFP as shown in the following equation; $\text{HALT-HCC} = (1.27 \times \text{tumour burden score}) + (1.85 \times \ln \text{AFP}) + (0.26 \times \text{MELD-Na})$ ^[29]. The HALT-HCC score was externally validated in 13,717 patients that derived from the SRTR and was significantly associated with overall survival (HR 1.06%, 95% CI : 1.05-1.07). Patients were shown to have similar risk of death when stratified by the HALT HCC score, regardless of being within or beyond the Milan criteria prior to transplantation. The advantage of the HALT-HCC score over the other published scores is that it takes into consideration not only the tumour burden and the biological behaviour, but also the underlying liver function at the time of LT^[29].

TRAIN score

In a study by Lai *et al.*^[30], a prognostic score that included radiological response criteria (mRESIST), AFP slope, neutrophil to lymphocyte ratio (NLR) prior to transplantation and waiting time (WT) on the transplant list was developed following retrospective analysis of a single cohort ($n = 179$). The Time-radiological response-AFP-Inflammation (TRAIN) score is calculated as shown in the equation; 0.988 (if mRESIST-progressive disease) $+ 0.838$ (if AFP slope ≥ 15 ng/mL/month) $+ 0.452$ (if NLR ≥ 5.0) $- 0.03 \times \text{WT}^9$ (x month). A Score ≥ 1 has shown an excellent ability to stratify patient in terms of intention to treat survival and recurrence. The TRAIN score allowed a potential 8.9% increase in the patients eligible for transplantation without increasing the recurrence risk that would have otherwise been excluded based on the Milan criteria. One of the main disadvantages of this proposed score is that it can only be applied in patients who have undergone pre-transplant locoregional treatment.

THE PROGNOSTIC ROLE OF AFP IN THE CONTEXT OF LT AS REPORTED IN NATIONAL AND INTERNATIONAL GUIDELINES

Despite the emerging robust evidence on the ability of pre-operative AFP to predict pre-transplant recurrence, only few International transplant societies have implemented this as part of their listing criteria.

The recently revised EASL Clinical Practice guidelines recommend that the conservative Milan criteria are the benchmark for selection of patients with HCC for LT^[2]. Despite this, it is suggested that composite criteria that consider surrogates of tumour biology, (among which AFP is the most relevant), in combination with tumour size and number of nodules is likely to replace conventional criteria for defining eligibility for LT. Although, different cut-off levels have been proposed (100 ng/mL, 200 ng/mL, 400 ng/mL, 1000 ng/mL) no consensus has been reached as to the optimal cut-off level that would best predict HCC recurrence.

In the UK, patients are eligible for LT if they have a single tumour ≤ 5 cm diameter or up to 5 tumours all ≤ 3 cm or a single tumour > 5 cm and ≤ 7 cm diameter, where there has been no evidence of tumour progression (volume increase by $< 20\%$), no extra-hepatic spread and no new nodule formation over a 6-month period. Since 2012, a cut-off AFP level more than 1000 i.u/mL has been used as an exclusion criterion for listing. Tumour rupture, extra-hepatic spread and macrovascular invasion are also considered as absolute contraindications for transplantation^[31].

In 2013, the French Organization for Organ Sharing officially implemented the AFP model^[10] as the national listing criteria for transplantation. This includes AFP at different cut off levels in combination with tumour size and number^[10]. A simplified version of the original model has been proposed [Table 2] and has shown to be superior to Milan criteria in predicting HCC recurrence. The AFP model has been externally validated^[26] and has been shown to discriminate well between patients with low and high risk of recurrence, both in patients within or beyond Milan criteria.

In 2008, the Canadian Society for Transplantation recommended that programs use a combination of total tumour volume (TTV), and AFP as listing criteria^[9]. Patients are eligible for transplantation if they have $\text{TTV} \leq 115 \text{ cm}^3$ and $\text{AFP} \leq 400 \text{ ng/mL}$. Unlike the Milan criteria, the current score allows the eligibility of patients with more than three tumours but with low tumour volume, which has been associated with favourable outcomes^[9]. The TTV is calculated as the sum of the volume of each tumour $[(4/3)\pi r^3]$ based on the maximum radius of each tumour.

Finally, the international consensus conference on LT for HCC which was held in Zurich in 2010 and established internationally accepted statements and guidelines for the conduct of LT, suggested that AFP should be utilised for waiting-list monitoring and following bridging therapy to discriminate between patients with low and high risk for drop out^[32].

CONCLUSION

Successful eradication of hepatitis C virus, following the widespread use and efficacy of the direct acting antiviral (DAA) treatment, has already reduced the number of patients with HCV related cirrhosis requiring LT. As a result, HCC and non-alcoholic fatty liver disease are becoming the leading causes for LT in the USA and in Europe. Transplantation for HCC is challenging, as one has to ensure that disease-free survival remains similar to that of patients transplanted due to benign disease. Similarly, it is equally important to ensure that HCC patients are not disadvantaged and erroneously excluded from liver transplantation, based on tumour volume characteristics alone. It is likely therefore that with the reduction of the HCV burden, transplant programs will become less restrictive. Moreover, with alternative biomarkers and the use of liquid biopsies as prognostic tools in HCC, a more “biological” rather than “morphological” approach to HCC treatment is anticipated.

Until these more refined methods for selecting patients become available, existing evidence supports the use of AFP in decision models for LT. Whether an AFP slope can be more informative compared to a static single value remains unclear. Of the currently available models the “AFP model” is currently the most extensively utilised and validated. The Metroticket 2.0 calculator allows an individualised accurate prediction of post LT recurrence and can be used by different transplant programs, which can be more permissive or restrictive based on the recurrence rates they are willing to accept. Finally, models that incorporate AFP at different cut-offs have shown to be superior to Milan criteria in predicting recurrence, but require to be prospectively validated in order to confirm their prognostic value.

DECLARATIONS

Authors' contributions

Drafted the manuscript: Fatourou EM

Reviewed the manuscript and made the appropriate corrections: Suddle AR, Heneghan MA

Availability of data and materials

Not applicable.

Financial support and sponsorship

Heneghan MA is funded by research support from the King's College Hospital Charity (Orpin Bequest and Kelly Group), and the European Association for the Study of Liver.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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HCV elimination: breaking down the barriers to prison based care

Timothy Papaluca^{1,2}, Alexander Thompson^{1,2}

¹Department of Gastroenterology, St Vincent's Hospital, Melbourne 3065, Australia.

²School of Medicine, Dentistry and Health Sciences, University of Melbourne, Melbourne 3052, Australia.

Correspondence to: Dr. Alexander Thompson, Department of Gastroenterology, St Vincent's Hospital, Melbourne 3065, Australia.
E-mail: alexander.THOMPSON@svha.org.au

How to cite this article: Papaluca T, Thompson A. HCV elimination: breaking down the barriers to prison based care. *Hepatoma Res* 2018;4:64. <http://dx.doi.org/10.20517/2394-5079.2018.53>

Received: 6 May 2018 **First Decision:** 9 Jul 2018 **Revised:** 16 Jul 2018 **Accepted:** 25 Jul 2018 **Published:** 17 Oct 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cui Yu **Production Editor:** Zhong-Yu Guo

Abstract

Hepatitis C virus (HCV) remains a major public health threat worldwide, responsible for 500,000 deaths annually; hepatocellular carcinoma (HCC) remains one of the major causes of HCV-related mortality. The global prevalence of HCV is approximately 1.0%, and in developed countries, injecting drug use continues to be the primary risk factor in incident cases. Targeted treatment of people who inject drugs (PWID) is important for achieving the WHO goals of eliminating viral hepatitis, which will have a significant impact on reducing HCC rates. Due to the close relationship between injecting drug use, incarceration and chronic HCV, the prevalence of HCV is up to 40 times greater within correctional facilities compared with the community. However, very few prisoners are treated for HCV while incarcerated. This is a result of financial, logistical and prisoner barriers to HCV care within correctional facilities. In the era of direct acting antiviral (DAA) therapy which is highly efficacious, time-efficient and safe, modelling studies have identified the benefit of increasing HCV treatment uptake amongst PWIDs to reduce community prevalence via treatment-as-prevention. Despite this, there are few real-world data evaluating DAA therapy within prison settings. In this article, we review the barriers to HCV care within prison systems, the outcomes of traditional HCV treatment programs within prisons and emerging data regarding the benefit of DAA therapy within correctional facilities. We present the mathematical modelling regarding the impact of treatment as prevention amongst PWIDs to eliminate HCV as a public health threat and how the prison fits into this paradigm.

Keywords: Hepatitis C virus, people who inject drugs, prisoner, elimination, direct acting antiviral

INTRODUCTION

Hepatitis C virus (HCV) is a prominent public health issue worldwide. It is estimated that there are over



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70 million people chronically infected with HCV, with a global prevalence of 1.0%^[1]. Over time, HCV mediated inflammation leads to the development of liver fibrosis, and can subsequently cause decompensated cirrhosis and hepatocellular carcinoma (HCC)^[2,3]. HCV accounts for approximately 500,000 deaths per year globally and remains the leading indication for orthotopic liver transplantation in the western world^[4,5]. Viral hepatitis, including HCV, is responsible for > 60% of incident HCCs and was the 7th leading cause of death globally^[6,7].

The advent of direct acting antiviral (DAA) therapy has revolutionised HCV treatment. HCV DAA therapy is highly efficacious, yet simple, safe and short in duration in relation to pegylated interferon- α and ribavirin (PEG RBV) which had significant toxicity and poor efficacy^[8]. The introduction of DAA therapy led the World Health Organisation (WHO) to propose targets for the reduction of HCV incidence and mortality of 80% and 65% respectively by 2030^[9]. In developed countries, injecting drug use continues to be the primary risk factor for acquiring HCV, accounting for the majority of incident cases^[10,11]. As such, treatments scale up amongst people who inject drugs (PWID) are key to achieving these elimination targets, which are supported by multiple HCV modelling studies^[12]. PWIDs however are often marginalised and historically it's been difficult for them to be engaged in care.

There are an estimated 10 million prisoners worldwide, with over 2 million incarcerated in the USA alone^[13,14]. This population has an extremely high prevalence of chronic HCV infection. Conservative estimates suggest there are approximately 1.5 million prisoners living with HCV at any given time^[15]. This reflects the close association between PWID, incarceration and HCV due to the criminalisation of injecting drug use. Incarceration itself is an independent risk factor for HCV infection^[16] and over 50% of PWIDs will spend some time in prison^[17]. Recent modelling has demonstrated that to achieve the proposed WHO elimination targets, HCV treatments must be significantly scaled up amongst PWIDs to influence reductions in HCV incidence and prevalence^[12]. The prison system is an ideal setting for such public health initiatives given these characteristics and can play a key role in elimination efforts. However, to date there has been limited prison based HCV treatment in the setting of prisoner, organisational and funding/policy barriers. In 2015, a survey conducted in 49 of the 50 State Department of Corrections in the US, responsible for the care of 1,348,716 prisoners, estimated that < 1% of prisoners with chronic HCV infection were receiving treatment^[18].

This article explores the prevalence of HCV within correctional facilities, presents the existing literature describing the efficacy of HCV treatment, and discusses the barriers to implementation and upscaling of HCV treatment in prisons. Finally, the opportunity for correctional HCV treatment programs to support HCV eliminations goals in the era of DAA therapy is discussed.

HCV PREVALENCE GLOBALLY, IN PWIDS AND CORRECTIONAL FACILITIES WORLDWIDE

Recent systematic reviews have estimated that HCV prevalence is approximately 1.0% worldwide, with 71 million people affected^[1]. Countries with high HCV viraemia prevalence include those found in Northern Africa, the Middle East and Central Asia, where prevalence may exceed 3%. A systematic review investigating HCV prevalence amongst PWIDs estimated that worldwide, 67% are affected by chronic HCV. Thirty eight of 79 countries, where data regarding HCV prevalence are available, have a HCV prevalence amongst their PWIDs of greater than 60% - these included China (67%), Russia (72.5%) and the USA (73.4%).

Due to the close association between injecting drug use and incarceration, the high prevalence of HCV within the PWID community has created the epidemic in correctional facilities worldwide. Studies demonstrate that 56%-90% of PWID will spend time in prison during their lifetime and HCV prevalence is up to 40 times higher amongst incarcerated populations when compared with the community^[19]. A recent systemic review assessed HCV prevalence amongst incarcerated populations^[15]. The midpoint HCV prevalence was 15.1%, equating to 1,546,500 of prisoners globally being affected by HCV^[13], and prevalence exceeded 10% in six of nine regions worldwide. A second review estimated anti-HCV prevalence at 26% amongst prisoners, and 64% amongst those

Table 1. Barriers to prison-based HCV treatment and potential solutions

Barriers	Solutions
System/prison factors	System/prison factors
Low HCV screening rates	HCV testing for all detainees on incarceration
Short prison sentences	Increase number of DAA prescribers to facilitate local treatment
Low prioritisation of a chronic disease	Promote jurisdiction-wide care to manage frequent prisoner transfer
Frequent interprison transfers interrupting treatment	Increase access to harm reduction strategies
Limited harm reduction strategies	
High rates of dropout in HCV care cascade relating to missed opportunity in:	
Confirming HCV diagnosis	
Referring for assessment	
Commencing therapy	
Prisoner factors	Prisoner factors
Prisoner attitudes and knowledge regarding:	Promote prisoner group education to manage deficiencies in HCV-related knowledge
HCV screening - including fear of diagnosis, difficulty with venepuncture	
HCV therapies - side effects, tolerability, efficacy	
Perceived stigma of HCV treatment	
Motivation	
Economic factors	Economic factors
High list price of HCV DAA therapies	Validation of simplified methods of fibrosis determination (ie APRI) to minimize the need for FibroScan
Limited prison healthcare resources	
Treatment factors	Treatment factors
Toxicity of historical PEG RBV therapy	Utilization of short duration, all oral DAA therapy for HCV
Duration of treatment	Implementation of facilities including telehealth to address limited access to specialist care
Specialist access	Education programs for prison healthcare staff regarding HCV diagnosis and treatment
Knowledge gap among prison medical, nursing and security staff regarding current HCV cascade of care	

DAA: direct acting antivirals; HCV: hepatitis C virus; PEG RBV: pegylated interferon & ribavirin; APRI: aspartate aminotransferase to platelet ratio index

prisoners who were identified as a PWID^[20]. Significant heterogeneity between different regions was observed [Australasia (35%), Central Asia (38%) and Latin America (4.7%)]^[15,20].

HISTORIC HCV TREATMENT AND BARRIERS

Less than one percent of eligible prisoners living with HCV are currently treated while incarcerated^[21,22]. HCV management within the prison relies on screening, clinical and laboratory assessment, specialist assessment, treatment access and confirmation of cure. At each step, there are organisational and financial barriers which have traditionally limited the number of prison based treatment [Table 1].

Screening and assessment

Despite WHO recommendations that all prisoners should be screened for HCV^[23], practice varies greatly worldwide. Only 34% (10/29) of European countries and 20% of the United States jurisdictions report established HCV screening protocols^[24,25]. Furthermore, where HCV screening is available, access to screening may be restricted to prisoners with a risk factor for HCV, such as PWID status or deranged liver biochemistry^[26], despite the fact that incarceration itself is an independent risk factor for HCV infection^[16]. Uptake of screening may be variable. A Canadian study identified that only 30% of prisoners were tested while incarcerated although universal opt-in screening being policy, and HCV screening across 21 English prisons reached less than 3% of prisoners^[27,28]. The cause of this is likely multi-factorial, including the cost of HCV diagnostics, the prioritisation of preventative health care within a prison budget, prisoner movement within prison systems limiting health centre access, and the stigma that can be associated with HCV testing^[29,30]. Screening uptake may also be impeded by prisoner factors including lack of knowledge about HCV or fear of diagnosis^[31]. PWIDs can have very difficult venous access resulting in fear of venepuncture - one prison based study utilised dried blood spot testing for HCV screening and noted a 12.2% increase in uptake^[32]. Barriers to HCV screening may be best addressed by implementing universal opt-out practices in all correction facilities worldwide to increase diagnosis rates and treatment throughput^[29].

Seropositive prisoners require further diagnostic testing to confirm chronic infection and stage liver fibrosis.

In one study only 62% ($n = 1490/2413$) of anti-HCV positive prisoners had a HCV PCR test performed during their incarceration^[28]. Liver imaging and/or elastography can be challenging. More than 85% of English prisons currently transfer prisoners to an external health centre for ultrasound or liver stiffness measurements (FibroScan)^[33].

Treatment

Even when screening is protocolised^[22], linkage to HCV treatment is often low. In Wisconsin, USA, only 18% of 3126 prisoners affected by chronic HCV were assessed for treatment whilst incarcerated^[34]. The need for specialist review can cause considerable delay due to inadequate medical and nursing resourcing on site^[30,35], a delay which may be exaggerated by the need for transfer to public hospital outpatient departments^[33]. Geographical isolation also contributes to delayed assessments. These delays are at odds with the short average prison sentence and frequent prisoner turnover. Prior to the recent introduction of DAAs, the treatment consisted of PEG RBV which was associated with significant side effects and was poorly tolerated. As such many prisoners were ineligible, intolerant or unwilling to undertake HCV therapy^[35,36]. Psychiatric comorbidity was a particular issue, affecting more than 50% of prison populations, and affecting eligibility for treatment^[37,38]. Finally, interprison transfers, frequent in correctional facility networks, can cause disruption and cessation of treatment where treatment programs are siloed to one facility^[31,35]. In the era of well-tolerated and highly efficacious treatments, jurisdiction-wide, coordinated efforts are necessary to develop models of care to overcome these barriers.

The high list price of HCV DAA therapy may also be prohibitive for correctional services due to limitations in fixed healthcare budgets. DAA HCV therapy is expensive. The wholesale price of therapy ranges from \$54,600 to \$147,000 depending on the regimen selected^[39]. Despite this high cost however, due to the much less resource intensive on-treatment monitoring, and the improved efficacy compared with PEG RBV, the price per sustained virological response (SVR) achieved by DAA therapy is cheaper than for PEG RBV^[39].

TREATMENT AS PREVENTION MODELLING, COST EFFECTIVENESS OF PWID TREATMENTS

PWIDs present unique challenges regarding HCV care in the community. A large proportion of PWIDs remain undiagnosed due to limitations in access or availability of appropriate diagnostic services^[40]. Diagnosis rates in the general population in Western Countries vary between 15%-86% in the literature, and the diagnosis rate in active PWID is low^[41]. Once diagnosed, PWIDs are less likely to access medical care compared with their non PWID counterparts, preventing them from accessing effective treatments^[42]. Even in the case of successful linkage to physician care, in the pre-DAA era, less than 20% were commenced on treatment due to multiple patient and physician factors^[40].

The PWID population therefore experiences difficulties progressing through the HCV care cascade. Irrespective of these challenges, to achieve the WHO elimination targets^[9], there needs to be a concerted effort to increase treatments uptake amongst this key population^[43]. Multiple mathematical modelling studies have now clearly demonstrated a benefit at a population level of scaling up HCV treatment amongst PWIDs who contribute most heavily to incidence and prevalence. This notion, referred to as “treatment-as-prevention” (TAsP) not only treats the individuals but reduces incident infections via interrupting transmission^[44]. Targeted treatment of PWID is therefore a priority for eliminating HCV as a public health threat.

Modelling studies of treating PWID in community

Australia has a policy of universal DAA access, irrespective of their underlying degree of fibrosis or PWID status, and has been considered in a mathematical modelling study to assess the impact of treatment scale up amongst PWIDs^[12]. The Australian model accounted for transmission, reinfection, treatment associated costs and progression of liver disease. It was determined that both the incidence and mortality targets could be achieved by 2030 by delivering 4725 treatments/year to PWIDs, equating to 59 out of each 1000 current

PWIDs in Australia. This strategy resulted in a spend of \$A3.895 billion over 15 years compared with inaction, but resulted in 132,000 quality adjusted life years (QALYs) gained. Using a willingness to pay (WTP) threshold of \$AUD50,000 per QALY, this was cost effective with an incremental cost-effectiveness ratio of \$A29,614 per QALY gained. The modelling is reproduced in [Figure 1](#) which demonstrates the rapid reduction in incidence and prevalence utilising this method compared treating those with established liver disease where reductions are only modest [[Figure 1](#)].

Iceland also has a policy of universal DAA access^[45]. Iceland was also evaluated in an mathematical model to establish key targets required to achieve the elimination objectives as stipulated by WHO^[9]. Iceland has a population of 332,000, of which 1300 are estimated to be living with HCV. The modelling determined that if DAA therapy was provided at current treatment levels, accompanied by unchanged HCV testing rates amongst the PWID population, incidence would decrease by 72% by 2030, yet still short of the WHO target. Comparatively, elimination could be achieved by 2030, 2025 or 2020, if 55/1000, 75/1000 or 188/1000 of the country's current PWIDs underwent HCV treatment per year. In all scenarios modelled, the elimination targets were achieved only where treatment was scaled up amongst PWIDs. The researchers did acknowledge that to satisfy this model, an increase in HCV diagnosis amongst Icelandic PWIDs or a 20% increase in harm reduction services is required.

A modelling study regarding PWIDs in Montreal, Canada identified the importance of early HCV diagnosis and prompted linkage to care^[46]. Montreal has 4000 PWIDs who are variably involved in HCV care. The researchers established key variables in their mathematical model including the interval between acquisition and diagnosis, time to linkage to care, lost to follow up rates, referral-to-treatment conversion and the efficacy of treatment, defined as likelihood of SVR12. The modelling demonstrated that the greatest incidence and prevalence reductions of 76% and 4.3/100 person years over 10 years respectively were achieved by maximising treatments delivered to PWIDs, irrespective of fibrosis stage, and by improving the cascade of care throughput. Comparatively, where treatments were delivered only to PWID with hepatic fibrosis or cirrhosis, incidence and prevalence estimates were only modestly reduced as TAsP was not achieved. The TAsP principle is supported by an alternate study^[47] which demonstrated that treating 120/1000 PWIDs per year in the USA, in a climate of 60% HCV prevalence amongst PWIDs, would achieve elimination within 10 years.

HCV TAsP is also cost-effective. Martin *et al.*^[48] utilised a mathematic model which evaluated the impact of HCV therapy delivered to either PWID, ex-PWID or never-PWID in a setting of varying HCV prevalences. The model considered the effect on transmission, interrupting liver disease progression and reinfection after SVR12. In a climate of 40% chronic HCV infection amongst PWIDs, it was most cost effective to provide HCV therapy to PWIDs with moderate fibrosis, followed by PWIDs with mild fibrosis. Both scenarios were more cost effective than treating ex- or never-PWIDs due to the prevention of incident infections via transmission. Treating PWIDs with moderate and mild fibrosis conferred the greatest net monetary benefit of £60,640 and £59,258 respectively (where chronic HCV infection was 20% amongst PWIDs) by minimising future medical expenditures and gaining QALYs. The cost effectiveness of this approach is in contrast to policies which limit the use of DAAs to patients with advanced fibrosis or cirrhosis only, including in the USA, and will result in higher ongoing HCV related costs despite the failure to control the HCV epidemic^[49].

Modelling studies of treating PWID in prison

The role of prison-based HCV treatment programs to reduce disease prevalence within the broader community has been demonstrated with Scottish data^[50]. The model determined 27.7% of incident HCV infections in Scotland related to incarceration, particularly related to the heightened risk of transmission immediately post-release. Via treatment scale up that would reach 80% of all HCV infected PWIDs with a sentence duration of > 16 weeks, this intervention alone could reduce Scotland's incidence and prevalence by 45.6% and 45.5% respectively by 2030, highlighting the key role of prison based programs to achieve elimination.

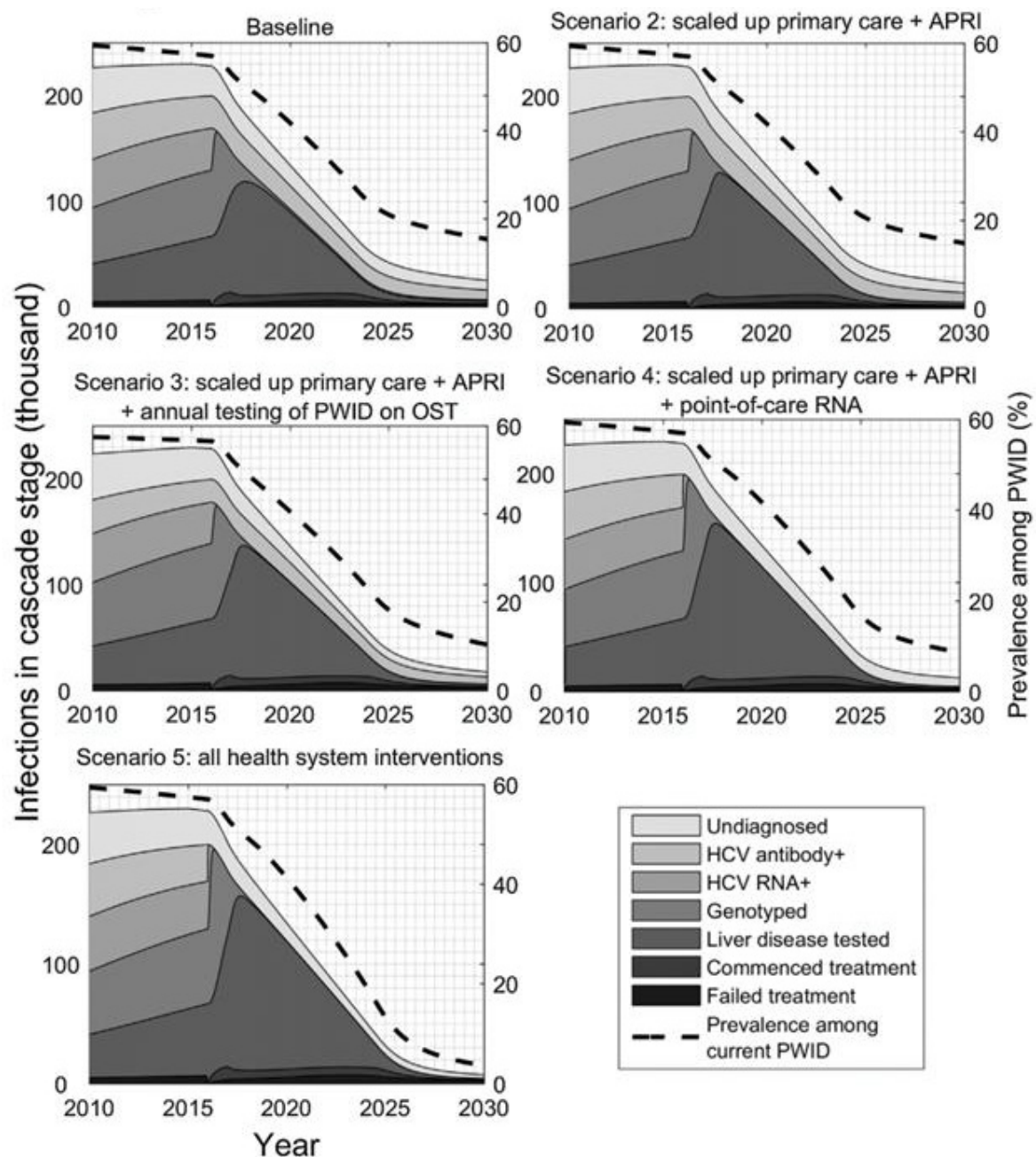


Figure 1. Australian HCV infections in various cascade stages; projected outcomes 2016-2030 under different scenarios, reproduced with permission from Scott *et al.*^[12]. HCV: hepatitis C virus; OST: opiate substitution therapy; PWID: people who inject drugs; APRI: aspartate aminotransferase to platelet ratio index

Modelling specifically pertaining to the prison system in England demonstrated that in the era of HCV DAA regimens of 8-12 weeks duration for all genotypes, by doubling HCV testing rates on prison receptions (currently at 6% with current UK opt in screening practices) and ensuring 10% of prisoners referred with HCV are treated, prison based DAA treatments are likely to be 99% cost effective under a £13,000 WTP per QALY gained^[51].

CURRENT HCV TREATMENT PROGRAMS WITHIN PRISONS

There are limited data describing HCV treatment in the prison setting, particularly in the DAA era [\[Table 2\]](#)^[52-61].

Table 2. Outcomes of prison based HCV treatment programs

	Referred for assessment	Treatment initiated	Treatment	Male (%)	Cirrhosis (%)	PWID (%)	GT 1 (%)	GT 3 (%)	Ceased early	SVR ITT	SVR PP
Farley <i>et al.</i> ^[52]	214	90	IFN RBV	90 (100)	4 (6)	70 (77)	49 (54)	30 (33)	17 (19)	48%	56%
Sabbatani <i>et al.</i> ^[53]	127	39	PEG RBV	38 (97)	1/12 (8)	36 (92)	19 (49)	20 (51)	24 (62)	21%	40%
Chew <i>et al.</i> ^[54]	-	71	PEG RBV	71 (100)	3/59 (5)	61 (86)	46 (65)	9 (13)	38 (54)	28%	59%
Strock <i>et al.</i> ^[55]	211	86	PEG+/-RBV	-	-	75 (87)	-	-	30 (35)	52%	-
Simonovic Babic <i>et al.</i> ^[56]	76	32	PEG RBV	28 (88)	5 (16)	30 (88)	12 (38)	-	4 (13)	53%	63%
Maru <i>et al.</i> ^[57]	138	68	PEG RBV	58 (85)	11 (68)	46 (68)	51 (75)	6 (9)	21 (31)	47%	54%
Aspinall <i>et al.</i> ^[58]	-	291	PEG RBV	261 (90)	8 (3)	180/200 (90)	115 (40) incl GT4	160 (55)	-	61%	83%
Bartlett <i>et al.</i> ^[59]	125	119	DAA	119 (100)	14 (12)	-	44 (37)	72 (61)	-	65%	97%
Blogg <i>et al.</i> ^[60]	-	18	DAA	-	-	-	-	-	-	83%	100%
Sterling <i>et al.</i> ^[61]	220	180	DAA	80 (95)	149 (83)	-	158 (88)	10 (6)	-	52%	94%

HCV: hepatitis C virus; DAA: direct acting antiviral; GT1: genotype 1; GT3: genotype 3; INF RBV: interferon & ribavirin; ITT: Intention-to-treat; PEG RBV: pegylated interferon & ribavirin; PP: per-protocol; SVR: sustained virological response; PWID: people who inject drugs

Treatment for HCV using PEG RBV could be delivered in a manner that was safe and effective, but was resource intensive and only suitable for a small number of prisoners^[25], burdened by high rates of adverse effects and treatment discontinuation. The largest prison based PEG RBV study^[58] specifically demonstrated that treatment outcomes for therapy delivered to a cohort in both the prison and in the community were not significantly different (61% vs. 63%, $P > 0.05$). SVR rates were lower when a prisoner was transferred between prisons while receiving treatment or when released on treatment. Frequent interprison transfer and early parole therefore present challenges for correctional treatment programs. Treatment rates remain low. As recently as 2015, a survey conducted in 49 of the 50 State Department of Corrections in the US, responsible for the care of 1,348,716 prisoners, estimated that < 1% of chronically infected prisoners were receiving treatment at that time^[18].

While modelling demonstrates that HCV DAA treatment is cost effective and efficacious, there are limited real-world data regarding the use of DAAs in correctional facilities worldwide [Table 2]. A recent publication demonstrated HCV micro-elimination within one prison in a jurisdiction of Australia^[59]. HCV DAA therapy was commenced in 119 prisoners. SVR12 data was available for 66 prisoners at the SVR12 time point and on per protocol analysis, SVR12 was achieved in 97% ($n = 64/66$). Where SVR12 data were not available, prisoners had most commonly already been released. No treatment-related serious adverse outcomes were reported. HCV point prevalence decreased over the study period from 12.6% to 1.1%. A survey of almost all the US prison authorities in 2015 indicated that 90% were in contract negotiations to secure HCV DAA therapy for use within their prisons indicating that existing treatment paradigms within correctional facilities are changing and we await further data. Robust data detailing the efficacy of HCV DAA therapy within the prison are needed to demonstrate the contribution that prison treatment programs can make to the elimination agenda.

CONCLUSION

Concerted efforts to increase HCV treatment rates amongst PWIDs are required to eliminate HCV as a public health threat. Correctional facilities provide ready access to large numbers of PWIDs, a population that is challenging to engage and retain in medical care in the community. The nexus between HCV infection, injecting drug use, and drug-related crime manifests a high prevalence of HCV within the prison.

Treatment as prevention for HCV amongst PWIDs is both efficacious and cost-effective. As injecting drug use is the key driver of HCV incidence, the benefit of this approach is the rapid reduction in community HCV prevalence and incidence, achieved by treating a small proportion of current PWIDs. There is clear evidence of these benefits as supported by multiple modelling studies. Traditional approaches have had limited success improving the cascade of care for HCV among PWIDs^[62]. Modelling studies of the impact of TAsP at the

population level support the development and implementation of novel public health platforms, including within the prison system. Such prison-based HCV treatment programs will facilitate the engagement and treatment of PWIDs, a key role in promoting the achievement of the WHO elimination goals by 2030.

Multiple barriers within the prison impede HCV management in prisons. Future studies should evaluate models of care that overcome these barriers. It will be important to evaluate the implementation of enhanced opt-out screening programs in all correctional facilities; the benefit of point of care diagnosis and referral on prison reception for reducing the time for diagnosis to treatment; and the benefit of peer-led prisoner education programs to promote uptake of screening and treatment. Historically HCV therapy for prisoners has been impeded by delays in transfer for specialist assessment, frequent prisoner transfer interrupting treatment and the need for frequent transfer to tertiary centres for monitoring and diagnostics. The development of jurisdiction-wide, comprehensive “in-reach” hepatitis treatment program could overcome these barriers and provide HCV care locally at each prison site, minimising the need for transfers.

In conclusion, prisoner hepatitis treatment programs should be included in HCV elimination paradigms to maximise the TAsP effect. Novel treatment programs are needed to access and scale up treatment in these marginalised populations.

DECLARATIONS

Authors' contributions

Both authors contributed equally to the research and writing of the manuscript.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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New and old biomarkers of hepatocellular carcinoma

Georgios Zacharakis^{1,2}, Ahmad Aleid³, Khaled K Aldossari⁴

¹Endoscopy Unit, Limassol General Hospital, St George's Medical School, University of Nicosia, Nicosia 1700, Republic of Cyprus.

²Endoscopy Unit, Department of Internal Medicine, College of Medicine, Prince Sattam bin Abdulaziz University, University Hospital, Al Kharj 16277, Saudi Arabia.

³Department of Gastroenterology and Hepatology, King Fahad Medical City, Riyadh 11525, Saudi Arabia.

⁴Department of Family Medicine, College of Medicine, Prince Sattam bin Abdulaziz University, University Hospital, Al Kharj 16277, Saudi Arabia.

Correspondence to: Dr. Georgios Zacharakis, Endoscopy Unit, Department of Internal Medicine, Prince Sattam bin Abdulaziz University Hospital, Al Kharj 16277, Saudi Arabia. E-mail: GZacharakis@yahoo.gr

How to cite this article: Zacharakis G, Aleid A, Aldossari KK. New and old biomarkers of hepatocellular carcinoma. *Hepatoma Res* 2018;4:65. <http://dx.doi.org/10.20517/2394-5079.2018.76>

Received: 11 Jun 2018 **First Decision:** 24 Jul 2018 **Revised:** 12 Sep 2018 **Accepted:** 13 Sep 2018 **Published:** 17 Oct 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cui Yu **Production Editor:** Zhong-Yu Guo

Abstract

Hepatocellular carcinoma (HCC) is a significant cause of mortality in patients with chronic liver disease around the world. Development of biomarkers for early HCC detection is a primary public health goal to decrease mortality. The ideal biomarkers should be highly sensitive and specific for surveillance of high-risk populations and early detection of HCC and also be able to predict therapeutic outcome and provide a prognosis on survival. Currently, the new biomarkers do not perform better than the conventional ones such as alpha-fetoprotein in such a way that they could be widely adopted in clinical practice. Another problem is the low sensitivity of these biomarkers in the detection of HCC. Further work on the development of novel biomarkers and on a combination of them is necessary. Advances in identifying unique molecular signatures including genomic, proteomic, metabolomic, and glycomic profiles have improved our understanding of many biological processes involved in HCC. This review focuses on the role of old and new biomarkers in surveillance, diagnosis, prognosis, and prediction of response to therapeutic targets for HCC and provides up-to-date data to health-care providers which would be applied in clinical practice.

Keywords: Hepatocellular carcinoma, biomarkers, diagnosis, surveillance, prognosis, treatment response

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related mortality and morbidity. HCC accounts for about 6% of all newly diagnosed cancer cases worldwide^[1,2]. Risk factors include chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection which contributes up to 85% of HCC cases worldwide^[3].



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Other risk factors include metabolic disorders such as nonalcoholic fatty liver disease (NAFLD) and chronic alcohol consumption^[4]. The frequency of cirrhosis among patients with HCC has been shown to be 85%-95%^[5,6]. The HCC incidence rate among cirrhotic patients has been estimated to be 2%-4% per year^[7]. Cirrhotic patients represent a high-risk group for HCC development and should undergo surveillance for HCC on a regular basis.

Early detection of HCC through surveillance methods have increased patient survival by providing effective initial treatments such as primary curative hepatectomy and locoregional ablative therapy^[8,9]. Surveillance and diagnostic methods for HCC depend on several biomarkers, defined as molecules that can be objectively measured in body fluids.

Although many studies have investigated several biomarkers for the prognosis and the evaluation of HCC, no biomarkers can predict and/or confirm the presence of HCC. There are no validated predictive biomarkers to evaluate the therapeutic response to HCC treatment except for the alpha-fetoprotein (AFP) for the evaluation of ramucirumab treatment efficacy as recently presented by Zhu *et al.*^[10].

The most well-studied HCC biomarkers are (1) the AFP, its isoform lens culinaris agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3); and (2) des- γ -carboxy prothrombin (DCP). However, there are many other molecules that might be taken into account for future studies, including glypican 3 (GPC3), glutamine synthase (GS), heat shock protein 70 (HSP70), cytokeratin 19 (CK19), Golgi protein 73 (GP73), midkine, osteopontin (OPN), squamous cell carcinoma antigen (SCCA), Annexin A2, fibroblast growth factor 3/4 (FGF3/4), micro-RNAs (miRNAs), Long non-coding RNAs (lncRNAs), circulating tumor cells (CTCs), cell-free DNA (cfDNA), and other biomarkers based on proteomic analyses. In addition, genetic signatures might play a role in the prognosis of HCC and therefore, they might be considered among possible disease biomarkers^[9].

Epigenetic modifications are the changes occurring in the gene expression but do not involve changes in the DNA sequence. These modifications include DNA methylation and histone modifications. Interestingly, some enzymes involved in the epigenetic regulation have shown to be involved in HCC pathogenesis. These enzymes include *RASSF1A*, *P16*, *DLC1* RhoA GTPase activating protein, runt related transcription factor 3, and suppressor of cytokine signaling 1^[11,12]. Multiple studies showed that (1) targeting these epigenetic modifiers might be effective in different types of cancers including HCC; and (2) they have the potential to be used as biomarkers for therapeutic response^[13,14]. Histone deacetylase inhibitors such as panalinostat and belinostat have shown therapeutic efficacy in HCC^[15,16].

CURRENTLY USED BIOMARKERS AND LIMITATIONS OF OLD BIOMARKERS

AFP for HCC surveillance in high-risk groups, diagnosis, and prognosis. Is it the ideal biomarker

AFP is the most extensively used old biomarker. AFP is a common biomarker for the diagnosis and surveillance for HCC and it has reached the phase 5 of biomarker development stages (prospective randomized studies aiming to define the clinical utility of biomarkers).

AFP is a large serum glycoprotein that is a part of the serum albumin gene family^[17]. The synthesis of AFP in the liver occurs during the fetal life is repressed during the adulthood^[18]. Therefore, AFP levels often diminish rapidly after birth and remain low throughout the adulthood. However, AFP can be expressed under certain pathological conditions such as chronic liver disease, HCC, germ cell tumors, and gastric cancer^[19].

There have been several investigations concerning the diagnostic utility of AFP suggesting that elevated serum AFP levels (> 20 ng/mL) correlate with an increased risk for HCC development. Although the sensitivity of AFP is excellent, its specificity is low. The use of a higher cutoff value such as of 200 ng/mL drops the sensitivity

to 22% while increases the specificity^[20,21]. Therefore, the use of AFP in clinical practice is limited by the low sensitivity at cutoff values maintaining sufficiently high specificity.

Furthermore, it has not escaped our notice that the heterogeneity of AFP cut off values in the literature could be attributed to several epidemiological factors as the high incidence of HBV infection in Asian HCC patients and the fatty liver in Western countries. In addition, the control group in most of these studies includes subjects without HCC instead of subjects with suspected HCC to ensure prevalence rates comparable to the rates in clinical settings. Finally, several studies suffer from the “verification bias” because the reference standard (CT scan or MRI) had not been performed in all subjects to exclude tumor presence in non-HCC cases.

Although AFP can be used to help define the population at risk of HCC^[22], it has a suboptimal performance as a serological test for surveillance. The following study supports the use of AFP as a single biomarker for surveillance of HCC in particular populations or healthcare environments where ultrasound (US) is not available^[23]. Furthermore, the use of US and AFP levels vs. the use of US alone offered additional detection in 6%-8% of HCC cases. The low specificity of AFP as a biomarker for HCC surveillance could be explained by (1) the transient rise in AFP levels in patients with cirrhosis reflecting an exacerbation of the hepatitis infection or in patients with chronic liver disease; and (2) the flares of underlying liver disease such as HBV, HCV or HCC development^[24].

Furthermore, AFP shows low sensitivity because it is not overexpressed in all HCC patients. It was found that elevated AFP levels were not evident in around 80% of small HCCs^[25]. Only about 10%-20% of HCC tumors at the early stage can present with abnormal AFP serum levels. This observation has been recently found in a molecular subclass of aggressive HCCs (S2 class, *EpCAM* positive)^[26-28]. In addition, AFP levels may be normal in up to 40% of patients diagnosed with early HCC. Based on data from the literature, a summary of the sensitivity and specificity of AFP is shown in Table 1^[21].

Another limitation of AFP includes the suboptimal performance in distinguishing intrahepatic cholangiocarcinoma and HCC. Although AFP (-) was the most sensitive assay for differentiating intrahepatic cholangiocarcinoma (ICC) from HCC (91.1%), its specificity was significantly lower than other markers such as CA242 (+) and carbohydrate antigen (CA) CA19-9 (+)^[29].

This has a critical impact on the outcome of the misdiagnosed patients since surgical resection is generally the preferred therapeutic choice for HCC but not ICC^[30]. As a result, AFP has been excluded in some guidelines on HCC surveillance and diagnosis. In fact, the European Association for the Study of the Liver and the European Organization for Research and Treatment of Cancer (EASL-EORTC) clinical practice guidelines (CPG)^[31] for HCC screening and diagnosis do not include quantitative measurements of serum AFP and recommend surveillance by experienced personnel in the at-risk populations using abdominal US every 6 months. However, the American Association for the Study of Liver Diseases (AASLD) guidelines^[32] recommend surveillance by the US for cirrhotic adults every 6 months with optional use of AFP due to the poor sensitivity and specificity of this biomarker. Shorter follow-up interval (every 3-4 months) is recommended in case of any of these conditions: (1) a nodule of less than one cm has been detected; (2) after liver resection; or (3) after loco-regional therapy. In contrary, the follow-up 3-6 months of serum AFP was included in the diagnostic algorithm of hepatic nodules by the Oriental guidelines for HCC management. Therefore, in the most common guidelines, it is well established and recommended that US should be a part of surveillance and most commonly combined with AFP.

Furthermore, a recent systematic review on HCC surveillance^[33] showed that the most commonly used surveillance tests for HCC in cirrhotic patients were US and AFP. Four studies used only US for HCC surveillance, whereas the rest of studies used the combination of US and AFP at 6-month intervals. The

Table 1. Cut off values for the commonly used biomarkers in the diagnosis of hepatocellular carcinoma; specificity and sensitivity characteristics

Tumor marker	Specificity (%)	Sensitivity (%)
AFP		
> 20 ng/mL*	[80-94]	[41-65]
Elevated serum AFP-L3		
Range: 10%-35%	[83-94]	[37-75]
DCP/PIVKA II		
Range: 60-150 mAU/mL	[70-100]	[41-89]

*Higher cut off values increase specificity up to 100% and decrease sensitivity to less than 20%. AFP: alpha-fetoprotein; AFP-L3: lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; DCP: des- γ -carboxyprothrombin; PIVKA II: prothrombin induced by vitamin K absence

combined surveillance tests had better detection of early-stage HCC compared with no surveillance (OR 2.16, 95% CI 1.80-2.6). On the other hand, the use of US only compared with no surveillance showed better detection of early stage HCC (OR 2.04, 95% CI 1.55-2.68). Also, the use of either the US alone or in combination with AFP showed similar curative rates for the treatment (OR 2.23, 95% CI 1.83-2.71 and 2.19, 95% CI 1.89-2.53, respectively). Unfortunately, no studies have compared US alone vs. US in combination with AFP to detect early-stage HCC or to assess curative therapy. Regarding the improving survival, US plus AFP had a pooled risk ratio of 1.86 (95% CI 1.76-1.97) whereas the US alone had a slightly lower pooled risk ratio of 1.75 (95% CI 1.56-1.98). At present, it is unknown whether the addition of AFP allows for improved survival and which type of surveillance tests, US alone or in combination with AFP has a better-improved survival.

Furthermore, AFP has been incorporated in nomograms or calculators to predict the outcome of hepatic resection^[34] and transplantation^[35]. AFP combined with SCCA might predict the risk of HCC in patients with chronic liver disease^[36,37]. AFP level has been incorporated in many staging systems for HCC patients, such as Cancer of the Liver Italian Program, Chines University Prognostic Index, Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire, and model to estimate survival in ambulatory HCC patients score^[38]. Changes in AFP levels can predict the outcome in patients treated with transarterial chemoembolization^[39] or Sorafenib^[40,41].

Several limitations have been recognized in using AFP levels as a biomarker for HCC. First of all, AFP levels as a prognostic marker cannot help in therapeutic decisions and especially for patients with normal pretreatment AFP levels. Also, there is no consensus on when post-treatment AFP levels should be measured. Finally, the clinical utility of AFP response in patients treated with sorafenib has not yet been validated in prospective studies.

The limitations of AFP use highlight the need to identify novel biomarkers. Given the increasing incidence of HCC, it is necessary to explore whether other new or old serum biomarkers or a combination of them can compete with or complement that of the US and constitute an optimal performance in the diagnosis, prognosis, treatment response, and surveillance of HCC^[9,42].

DCP

Other serum markers such as DCP have also been explored, alone or in combination, in the diagnosis and surveillance of HCC.

DCP is an abnormal prothrombin molecule induced by vitamin K absence (PIVKA II) and posttranslational carboxylation machinery is known as DCP. DCP is an abnormal prothrombin molecule overproduced in HCC patients^[43,44].

Unfortunately, DCP did not offer substantial advantages concerning AFP^[45]. This marker is not used for early detection of HCC. DCP levels have been associated with portal vein invasion and advanced stage of HCC as

with AFP-L3 fraction levels^[46]. Serum DCP-based diagnosis showed suboptimal sensitivity (48%-62%) but satisfactory specificity (81%-98%) in HCC patients^[43,47].

Regarding the role of PIVKA-II as treatment response marker, a recent meta-analysis showed that increased PIVKA-II levels could predict worsening overall survival and recurrence-free survival in patients with HCC who had curative ablation^[48]. More studies are needed to confirm the clinical utility of PIVKA-II for HCC prognosis. At present, none of the above surveillance tests can be recommended to screen patients at high risk for HCC.

Surveillance tests: the combination of AFP + AFP-L3 + DCP markers

To address the problem of the markers being suboptimal due to sensitivity and specificity problems, combined application of DCP- and AFP-based biomarkers has been tested. The combination of AFP-L3, AFP and DCP markers used in 104 patients with HCC, 43% of whom had AFP levels below 10 ng/mL achieved 60.6% sensitivity and 100% specificity^[49]. In another large multicenter case-control study, DCP with AFP immunoassay for HCC detection showed increased sensitivity from 65% to 87%, but specificity dropped from 84% to 69%^[50]. These studies supported the clinical utility of DCP for early-stage HCC diagnosis. However, further studies are needed to validate the effectiveness of DCP alone or as part of a new predictive score for HCC diagnosis. At present, the combination of AFP + AFP-L3 + DCP is included in the diagnostic algorithm of hepatic nodules by Oriental guidelines for HCC management, but not by Western guidelines.

Other biomarkers

In an effort to identify markers with highest sensitivity and specificity for the detection of HCC, many other molecules have been explored, including GPC3, GS, HSP70 (tissue), CK19, GP73, midkine, OPN, SCCA, Annexin A2, FGF3/4, miRNAs, lncRNA, CTCs, and cfDNA. Also, biomarkers obtained by proteomic-based approaches should be taken into account.

Proteomic studies have now identified multiple serum protein fragments with differential expression in HCC such as 70-kDa HSP70 and fructose-1, 6-bisphosphatase 1, the most consistently reported proteins, with upregulation and downregulation, respectively, in HCC^[51]. Therefore, many of these proteins could serve as new biomarkers for HCC diagnosis, surveillance, prognosis, and treatment response. However, there is a limitation of proteomics currently used, the lack of agreement among various studies in reporting changes in protein expression associated with HCC.

Likewise, metabolomic studies investigate changes in lipid- and water-soluble metabolites found in the blood or urine. The scope was to identify a broader array of potential biomarkers for HCC^[52-54]. Also, glycomic studies investigated N-glycosylation patterns that may be associated with cancer development^[55]. N-glycans are complex polysaccharides bound to biomolecules through N-glycosylation and actively involved in several biological processes.

Also, genetic signatures can also be included among oncomarkers with prognostic meaning. Genomic variation between individuals has revealed multiple single nucleotide polymorphisms (SNPs) associated with HCC risk^[56]. However, the high degree of change in gene expression based on patient ethnicity and underlying chronic liver disease makes it difficult to discover gene expression profiles that can reliably predict the risk of HCC.

In conclusion, many molecules have been explored as possible biomarkers for HCC, and some of them are described below.

GPC3, GS, HSP70

GPC3 is a cell-surface proteoglycan of the glypican family. This proteoglycan is overproduced in HCC cells and plays a pivotal role in regulating tumor growth. So, the soluble NH₂-terminal fragment of GPC3 is

used as a serological biomarker due to its ability to accurately distinguish between patients with small, well-differentiated HCC tumors and those with cirrhosis^[57]. This marker is similar to AFP, showed high specificity but low sensitivity^[58]. Even if it is combined with miRNAs, such as miR-21, only a slight improvement in performance was shown compared with AFP^[59].

Other markers used in combination with GPC3 include HSP70 and GS. HSP70 belongs to a class of genes (heat shock proteins) abundantly overexpressed in advanced HCC as compared to early HCC, and in early HCC as compared to precancerous lesions^[60]. Also, a study by Osada *et al.*^[61] showed a stepwise increase in GS immunoreactivity from precancerous lesions to early and advanced HCC suggested that GS has a role in HCC. In CPG jointly published by EASL-EORTC recommend that GPC3 could be used alone or in combination with HSP70 and GS to distinguish well-differentiated HCC (early and grade 1) from dysplastic nodules of cirrhosis.

NOVEL BIOMARKERS FOR HCC

There is an unmet clinical need to discover better biomarkers for HCC that (1) fully correlate with the tumor stage; (2) can be detected in early HCC; and (3) allow for tumor surveillance and evaluation of therapeutic efficacy. Therefore, the research into novel HCC biomarkers continues. In this section, we briefly discuss the novel biomarkers for HCC, all are under investigation in clinical trials and are not currently used in clinical practice.

CK19

CK19 is a novel HCC biomarker associated with poor prognostic factors in HCC patients due to high risk of microvascular invasion and distant metastasis, as well as worse treatment outcome^[62-64].

GP73

GP73 is a transmembrane protein localized in the Golgi complex. Although it is absent in normal hepatocytes, abundantly overexpressed in HCC patients, compared with cirrhotic patients^[65]. GP73 could be used as a marker in early-stage^[66,67].

OPN

OPN is a glycoprotein, an extracellular matrix protein^[68] expressed in HCC cells and other various types of malignancies^[69]. OPN, although, it has a higher sensitivity in the discrimination of early HCC than AFP according to the clinical study of Shang *et al.*^[70]. The low specificity can be explained by its relationship with more than 30 types of cancers^[71]. Therefore a combination with AFP is necessary to optimize its performance^[70].

SCCA

SCCA is a serine protease inhibitor. It is found in squamous epithelium. The use of SCCA as an additional diagnostic marker with AFP for HCC has been well documented^[72]. Also, it might play a role as a biomarker for response to treatment as there is an inverse correlation with the treatment response for HCC^[73]. Finally, the combination of AFP and SCCA should be investigated in future studies to validate the diagnostic role of SCCA as a predictor for the risk of HCC in patients with chronic liver disease.

Annexin A2

Annexin A2 is a calcium-dependent, phospholipid-binding protein. It is present in the cell surface, and it seems to be implicated in the development and metastasis of HCC^[74]. It has been used as a serological biomarker for diagnosis and prognosis of early-stage HCC patients with higher sensitivity and specificity than AFP^[75].

miRNA

miRNAs are small non-coding endogenous RNAs that have been implicated in various biological roles at the cellular level including apoptosis and oncogenesis^[76]. Some types of miRNAs act as controllers of different

genes during HCC pathogenesis^[77]. There are several types of miRNAs being tested as diagnostic and prognostic markers for HCC. To date, most common methods used for detection are the microarray, PCR and gene sequencing^[78]. As a prognostic factor, low level and down-regulation of miRNA-542 and miRNA-139 are associated with poor prognosis as vascular invasion, larger tumor size and metastatic disease^[79,80]. Expression of miRNA profile in the histopathological analysis after HCC resection can predict the risk of HCC recurrence within the Milan criteria^[81], HCC miRNAs expression varies between cirrhotic and non-cirrhotic HCC^[82]. Remarkably, miRNAs like miR503HG suppress metastasis and inhibit malignant cell migration. Therefore, downregulation is associated with a higher risk of metastatic disease. This discovery may act as a template for future pharmacological targeted treatment^[83]. Several studies compared them with the conventional HCC biomarkers such as AFP, DCP, AFP-L3 or used along with these biomarkers and the results demonstrated that a single miRNA or even better combination of different miRNAs were more sensitive than AFP, DCP, and AFP-L3%. However, the miRNA expression profiles in HCC patients could vary significantly according to the tumor stages. Subsequently, it was difficult to distinguish between patients with different tumor stages. This was a limitation of the diagnostic utility of miRNAs as serological biomarkers.

Later, a panel of circulating miRNAs was developed, with the advances in miRNA screening techniques and the development of new bioinformatics tools achieved higher sensitivity and specificity in HCC diagnosis. Indeed, a miRNA panel, with cutoff 20 ng/mL, showed better diagnostic sensitivity than AFP and similar specificity to AFP especially for the detection of small and early-stage tumors. Also, this miRNA panel could be used as a prognostic score to improve the treatment outcome of HCC patients.

In conclusion, miRNAs are the promising biomarkers in the field of HCC diagnosis, prognosis, and potential therapeutic targets. However, they do not yet fit for the routine clinical setting.

lncRNAs

lncRNA are a unique class which are defined as transcripts of more than 200 nucleotides that present in genome-wide analysis of mammalian transcriptome. Accumulating evidence showed that dysregulated lncRNA had been involved in the pathogenesis of HCC^[84,85]. Lately, lncRNA has been recognized as important regulators for carbohydrate and lipid metabolism; this has led to discovering a novel biomarker “lncRNA Ftx” which stimulate HCC progression and glycolysis. Therefore, lncRNA Ftx may act as a prototype for further research in targeted therapy for HCC^[86]. A recent prospective study suggested combining lncRNA and AFP measurement may be a novel useful marker for HCC regarding diagnosis and prognosis^[87]. Expression of RP11-466I1 in the serum and HCC tissue is associated with poor features like tumor capsule invasion^[88].

CTCs

One of the most adverse prognostic features of HCC is the presence of vascular invasion which leads to hematological spread and distant metastasis of malignant cells. Therefore, detection of CTCs has strategic clinical value in predicting HCC recurrence and monitoring treatment response^[89,90]. Detection of CTCs is associated with poor overall survival and relapse-free survival^[91]. In addition to that, CTCs positivity is significantly correlated with serum AFP level, vascular invasion and TNM stage which can reflect the histopathological status of HCC^[92]. According to a recent meta-analysis of more than 20 studies, the CTC is not used as a sole indicator for diagnosis instead associated with poor clinical and pathological features^[92].

cfDNA

Dysregulated levels of cfDNA have a role in diagnosis, monitoring of treatment response, and even outcome prediction for cancer diseases^[93-95]. Furthermore, single-nucleotide polymorphism of cfDNA such as Ser249 p53 mutation which is commonly found in the plasma DNA, unfortunately, is detected in HCC and non-HCC individuals^[96]. However, differential methylation signatures identified in cfDNA, precede the occurrence

of HCC, which are recommended to be used in combination with the conventional HCC biomarker AFP to improve the accuracy of HCC diagnosis because of lack of robustness^[97,98]. Finally, the monitoring of cfDNA in the urine has been recently reported to be a promising tool to predict HCC recurrence^[99].

Proteomic analysis and serum metabolite biomarkers

An array of proteomic studies coupled with bioinformatics analysis identified serum protein fragments with differential expression in HCC, which possibly could serve as potential HCC biomarkers^[100]. Luo *et al.*^[100] investigated the utility of a serum metabolite biomarker panel of phenylalanyl-tryptophan and glycocholate. They found a higher diagnostic performance for the serum metabolite biomarkers compared with the AFP in terms of differentiating HCC from a high-risk population of cirrhosis.

Core-fucosylated

Core-fucosylated (CF) proteins could be candidate biomarkers in the diagnosis of HCC^[101]. CF such as from fibronectin at site 1007 could differentiate HCC from cirrhosis in patients with alcoholic liver diseases. Also, CF cadherin-5 at site 61 could distinguish between HCC on chronic HCV hepatitis liver disease from cirrhosis. Furthermore, four differentially expressed apolipoprotein isoform proteins could differentiate NAFLD without cirrhosis from NAFLD-related cirrhosis or HCC on cirrhotic NAFLD^[102]. Also, another protein, CD5 antigen-like, a soluble scavenger cysteine-rich protein that modulates inflammatory responses, could distinguish between NAFLD-related cirrhosis from NAFLD without cirrhosis but could not have any diagnostic value for HCC.

Finally, an 11-peak algorithm based on analysis of serum proteins was proven to be more accurate than several conventional biomarkers for early-stage HCC^[103]. Overall, more proteomics probably will identify more HCC biomarkers.

DEVELOPMENT OF NEW HCC BIOMARKERS FOR DIAGNOSIS, PROGNOSIS AND TUMOR RESPONSE PREDICTION

So far, none of the new biomarkers outperform the conventional ones in such a way that it has been widely adopted in clinical practice. However, new data are promising.

Biomarkers for HCC risk assessment

Cirrhotic patients undergo justified periodical screenings to detect the early development of HCC. The identification of host factors such as the various biological pathways involved in liver carcinogenesis may help define specific adapted screening policies. Today, numerous candidate-gene studies have reported associations between SNPs and the presence of HCC^[104].

Unfortunately, the several host SNPs identified so far only partly explain the association with HCC in HCV-infected patients and did not enable good prediction on the individual and population levels^[105]. It seems reasonable that various panels of SNPs should be incorporated into complex models of “genomic risk prediction”, which take into account both host and environmental factors that can influence liver carcinogenesis at the near future.

At present, two biomarkers for HCC risk assessment have been developed including SNPs in germ-line epidermal growth factor associated with HCC on HCV-related cirrhosis and a specific 186-gene signature defining the high risk of HCC development in cirrhotic patients^[106].

Biomarkers for HCC diagnosis

Non-invasive diagnosis using EASL/AASLD criteria allows a confidential diagnosis of most HCCs above 2 cm^[107]. However, imaging is less reliable in one-third of small nodules, and a liver biopsy is often indicated. In this

scenario, EASL guidelines recommend testing the combination of 3 immunohistochemical markers (GPC3, HSP70 and GS). Besides, a 3-gene signature including GPC3, lymphatic vessel endothelial hyaluronan receptor 1, and survivin has also been proposed as an accurate molecular tool (sensitivity of 95% and specificity of 94%) to discriminate dysplastic nodules and HCCs smaller than 2 cm in the setting of HCV etiology^[108]. A step forward in the diagnosis of HCC could be provided by the development of a “liquid biopsy”, i.e., the identification in the peripheral circulation of CTCs or circulating tumor DNA that have detached from a primary tumor^[109]. A recent paper reported preliminary data in 8 tumor types, including HCC^[110].

Prognostic biomarkers for HCC

Regarding prognostic signatures for HCC, the phenotypic and molecular diversity of HCC allows us to identify several new biomarkers.

Changes in AFP levels have been used for prognostic stratification at a cut-off of > 500 ng/dL as a predictor of drop-out in the list of transplantation^[31] and as a predictor of the outcome of patients in phase III trials testing systemic therapies such as transarterial therapies or Sorafenib.

Furthermore, an excellent prognostic ability has also been reported for some genetic signatures obtained from tumor specimens in HCC patients treated by liver resection. Indeed, a 5-gene score based on the expression of *TAF9*, *RAN*, *RAMP3*, *KRT19* and *HN1* genes, represents the most reliable predictor of survival identified so far in multiple cohorts^[111]. Also, neoangiogenesis-related genes (a panel of microRNA associated with regulation of angiogenesis) seem to be hallmarks of fast-growing HCCs and worst survival^[112]. Finally, a 186-gene score from adjacent to tumor tissue was shown to have independent prognostic significance to predict overall survival in HCC patients^[113].

The use of biomarkers as predictors of response to therapeutic targets to HCC

The possibility of using novel biomarkers to predict tumor behavior to targeted therapies is appealing. Such biomarkers are the FGF that are essential pathway components of oncogenesis. FGF3/FGF4 amplification was found to predict increased response to the sorafenib in patients with HCC^[114]. Sorafenib is a targeted therapy, classified as a tyrosine kinase inhibitor, has been the standard of care for patients with advanced HCC for the last decade^[115]. Other predictive markers for sorafenib efficacy include high levels of soluble stem-cell factor receptor c-Kit and low levels of hepatocyte growth factor which have shown a non-significant trend for sorafenib efficacy^[116,117]. Furthermore, patients with HCV-related HCC showed a higher benefit from sorafenib (HR: 0.47) compared to non-HCV patients (HR: 0.81)^[118].

Other targeted therapies for HCC include lenvatinib^[119] and regorafenib^[118] as first-line treatments and cabozantinib^[120] and ramucirumab^[121] as second-line treatments. In phase III REACH-2 trial, Zhu *et al.*^[121] demonstrated that ramucirumab as a second-line treatment achieved a significant and meaningful overall survival benefit with a favorable safety profile in HCC patients with baseline AFP greater than or equal to 400 ng/mL, a population associated with poor prognosis; ramucirumab decreased mortality by 29% vs. placebo as a second-line treatment for patients with advanced HCC and that AFP is a predictor of the efficacy of ramucirumab. Although AFP could predict the efficacy of ramucirumab, there is still a need for more biomarkers that show survival benefits for other HCC treatments^[121].

Another targeted drug for HCC, nivolumab, in a recent phase I-II clinical trial of 260 patients with advanced HCC has shown up to 16% of objective responses, some of them of long duration, obtaining a median overall survival of 16 months^[122]. Again, the biomarkers used, the programmed death-1 and its ligand immunostaining status did not predict response to nivolumab^[123].

Recently, a gene signature capturing the immune class of HCC (~30% of patients) is currently under investigation as a treatment response predictor^[124].

HCC biomarkers that might be used as therapeutic targets

As explained previously, GPC3 is a membrane-associated heparan sulfate proteoglycan that can be used as biomarker for HCC. However, recent studies have shown that it plays a role in cancer pathogenesis and proliferation and therefore it can be used as a therapeutic target to stop the progress and proliferation of tumor cells^[125,126].

Another biomarker and possible therapeutic target is the kinesin family member C1 (KIFC1). Recent studies showed that KIFC1 is overexpressed in HCC tumor tissues compared with non-tumor tissues, therefore, it might be used as a predictor for HCC^[127]. Moreover, in vitro KIFC1 knockdown could effectively decrease the viability of HCC tumor cells, and induce apoptosis and cell death. This highlights that KIFC1 might be used as a biomarker and a therapeutic target for HCC^[127].

Serum marker panels for HCC

Aspartate aminotransferase to platelet ratio index

Aspartate aminotransferase to platelet ratio index (APRI) has been used to assess the risk of fibrosis and cirrhosis among hepatitis C patients. APRI has been recently investigated to predict the risk of cirrhosis-dependent and independent HCC in HBV patients^[128]. A recent study showed that APRI can predict response to transarterial chemoembolization treatment before starting the therapy^[129].

Fibrosis-4 index

A Korean study evaluating the role of Fibrosis-4 (FIB-4) index in predicting HCC among HBsAg positive individuals; they found that FIB-4 has a better predictive of HCC incidence, compared to that of ultrasonographic liver cirrhosis (C-index: 0.775 vs. 0.701; $P = 0.040$)^[130]. On the other hand, some reports show that liver fibrosis index (FIB-4) is not reliable for the prediction of HCC^[131].

Forns test

A study of liver fibrosis indices (APRI, FIB-4 index, and Forns index) showed that Forns index performed before HCV antiviral therapy was a predictor to identify patients with low likelihood of developing HCC after achieving a sustained virologic response^[132]. Moreover, Forns index was found to predict the recurrence and death of patients with hepatitis B-related HCC after curative resection^[133].

CONCLUSION

No biomarker combination is reliable enough to diagnose a lesion as HCC without confirmatory histological or radiological features. None of the new tumor markers outperform the conventional ones in such a way that it has been widely adopted in clinical practice. The diagnostic accuracy, particularly for early-stage HCC, can be improved by combining two or more biomarkers to reach an acceptable ($> 80\%$) sensitivity with a modest decrement in specificity^[49]. For this purpose, the accuracy can also be improved by measuring the overtime variability of the marker. However, all these proposals are waiting for prospective and external validations, and there are no recommended recall policies based on biomarker combinations or variability for the surveillance of patients at risk of developing HCC.

In terms of response to HCC treatment, AFP levels can predict response to ramucirumab treatment; an elevated AFP is a poor prognostic factor for ramucirumab survival benefit. However, future research should develop useful biomarkers for monitoring treatment activity, detecting early resistance to treatment and identifying patients who would more likely benefit from treatment.

DECLARATIONS

Authors' contributions

Reviewed the literature and wrote the manuscript: Zacharakis G, Aleid A, Aldossari KK

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Gender differences in hepatocellular cancer: disparities in nonalcoholic fatty liver disease/steatohepatitis and liver transplantation

Eric M. Wu¹, Linda L. Wong¹, Brenda Y. Hernandez³, Jun-Fang Ji⁴, Wei Jia³, Sandi A. Kwee³, Sumodh Kalathil²

¹Department of Surgery, University of Hawaii, John A. Burns School of Medicine, Hawaii, 96813, USA.

²Department of Medicine, University of Hawaii, John A. Burns School of Medicine, Hawaii, 96813, USA.

³Cancer Center, University of Hawaii, Hawaii, 96813, USA.

⁴Life Sciences Institute, Zhejiang University, Hangzhou 310058, China.

Correspondence to: Dr. Linda L. Wong, Department of Surgery, University of Hawaii, John A. Burns School of Medicine, 550 S. Beretania Street, Suite 403 Honolulu, Hawaii, 96813, USA. E-mail: hepatoma@aol.com

How to cite this article: Wu EM, Wong LL, Hernandez BY, Ji JF, Jia W, Kwee SA, Kalathil S. Gender differences in hepatocellular cancer: disparities in nonalcoholic fatty liver disease/steatohepatitis and liver transplantation. *Hepatoma Res* 2018;4:66. <http://dx.doi.org/10.20517/2394-5079.2018.87>

Received: 9 Jul 2018 **First Decision:** 20 Aug 2018 **Revised:** 26 Sep 2018 **Accepted:** 26 Sep 2018 **Published:** 18 Oct 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cui Yu **Production Editor:** Zhong-Yu Guo

Abstract

Aim: Worldwide, hepatocellular cancer (HCC) is the fourth leading cause of cancer death and occurs 3 times more commonly in males than females. Current surveillance practices do not fully address gender differences in HCC.

Methods: Clinical characteristics and survival were compared between males and females using a prospectively collected database of HCC patients.

Results: In a cohort of 1206 patients, 307 (25%) were female who presented with older age, more non-alcoholic fatty liver disease/steatohepatitis (NAFLD/NASH), family history of HCC, and hypertension. Males (75%) were more likely to use alcohol and cigarettes. Females were more likely to undergo HCC surveillance, have smaller tumor size at diagnosis, and less vascular involvement. Males who met Milan criteria were more likely to undergo liver transplant than women who met the criteria. Median/mean survival was similar between the genders. Multivariate analysis showed that NAFLD/NASH was predictive of mortality for both males and females, age and smoking were predictive of mortality for males, and transplant was predictive of survival for males.

Conclusion: Gender differences in HCC appear related to both behavioral risk factors and biologic factors. Older females with HCC have more NAFLD/NASH and may be overlooked by current surveillance guidelines. These gender disparities may lend support to future studies of gender-based HCC screening.



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Keywords: Hepatocellular carcinoma, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, gender, transplant

INTRODUCTION

Hepatocellular cancer (HCC) is the fourth leading cause of cancer death worldwide and approximately 841,000 new cases are diagnosed annually^[1]. In the US, HCC is one of the few cancers that is increasing in both incidence and death^[2]. Viral hepatitis, a major risk factor for HCC, has declined in relative importance as vaccination for hepatitis B has become almost routine and treatments for chronic hepatitis B and C virus (HBV and HCV) have improved. Concomitantly, metabolic conditions and fat-related liver disease have become increasingly prominent risk factors^[3,4]. Although HCC is historically more common in Asian/Pacific Islanders and males, the incidence is increasing in Hispanics, Blacks, and females^[5].

Although current guidelines on HCC surveillance from leading professional organizations focus on high-risk populations, there is no consensus as to the optimal surveillance in those with non-alcoholic fatty liver disease/steatohepatitis (NAFLD/NASH). A large part of the problem is difficulty in identification of the population at risk as many of these patients have undiagnosed NAFLD/NASH. They are typically followed by only primary care physicians for diabetes or hyperlipidemia or perhaps followed by a hematologist for unexplained thrombocytopenia.

HCC predominantly affects males with incidence two to four times more common in males than females^[6]. The reasons for this gender disparity are complex and may stem from differences in behavioral risk factors, metabolic factors, tumor biology, and treatments received. Of note, there are gender differences in metabolic factors and NAFLD/NASH that may be helpful in developing guidelines for HCC surveillance. Obesity is more prevalent in females than males with currently 38% of US females being obese^[7]. Type II diabetes mellitus is more common in males than females but females are more likely to have cardiovascular disease, myocardial infarction and cerebrovascular accidents^[8]. Males are overall more likely than females to have NAFLD/NASH, however, after the age of 60 years, females are much more likely to have NAFLD/NASH^[9,10]. Estrogen is believed to have a protective role in the development of HCC as differences in subtypes of estrogen receptors expressed in males vs. female have been shown to contribute to the progression of HCV related HCC^[11]. As fat related liver diseases increasingly emerge as the most common cause of chronic liver disease, it is crucial that the relationship between fatty liver disease and HCC is fully explored.

The purpose of this study is to comprehensively evaluate gender differences in a large cohort of HCC patients - to better define populations at risk for evaluation in future surveillance studies.

METHODS

Study participants

A retrospective analysis was conducted using de-identified clinical and outcome data from 1206 HCC cases diagnosed between 1993 and 2017 by a group of physicians associated with a medical center having the only liver transplant program in Hawaii, as well as the only referral center for liver disease for the American territories of the Pacific Basin and other Pacific Island Nations, including Samoa, Guam, Saipan, Micronesia and the Marshall Islands. This clinic and the transplant center were initially affiliated with Hawaii Medical Center-East (formerly St. Francis Medical Center) and after 2012, with the Queens Medical Center. About 60%-70% of HCC cases from the State of Hawaii are seen in this center. Other patients in this cohort were foreign nationals from Asian countries, including China, Japan, Korea, and the Philippines, who pursued medical care in the US. This study was approved by the University of Hawaii Institutional Review Board.

The diagnosis of HCC was confirmed histologically (percutaneous biopsy or at surgery) or with a

combination of imaging and alpha-fetoprotein (AFP). Patients diagnosed in the first decade were included if they had a history of chronic liver disease and a liver mass that was least 2 cm in size and seen on two imaging studies (ultrasound, CT scan or MRI) and one of the following: (1) vascular blush seen on CT scan or MRI; (2) AFP > 200 ng/mL; or (3) arteriogram confirming the tumor. More recently, the diagnosis of HCC was verified with only imaging if a contrast-enhanced study (dynamic CT or MRI) showed typical arterial enhancement with “washout” in the venous phase as described by the American Association for the Study of Liver Disease guidelines^[12,13].

Data collection

Information on demographics, medical history, laboratory results, tumor characteristics, treatment, and survival was obtained from medical records. Demographic data included age, sex, birthplace, and the patient's self-reported ethnicity. Ethnicity was then categorized as “White”, “Asian” (including Filipinos), or “Pacific Islander”. Patients who did not fit into one of these categories or were of mixed ethnicity were subsequently classified as “Mixed”. Patients of mixed race with 50% Pacific Islander ethnicity were categorized as “Pacific Islander”. Risk factor information that was collected included: diabetes mellitus, hyperlipidemia, smoking, viral HBV and HCV, alcohol abuse (defined as greater than two alcoholic beverages daily for at least ten years), and other chronic liver diseases. Information was based on available medical records and interviews, without use of a structured questionnaire. Patients who did not report hyperlipidemia but had a lipid-lowering agent on their current medication list were also classified as having hyperlipidemia. Measured height and weight were used to determine body mass index (BMI). Obesity was defined as BMI ≥ 30 . Patients with no viral, alcohol risk factors or other known liver disease were categorized as NAFLD if documented by imaging or liver biopsy showing steatosis. Those with no viral or alcohol risk factors were classified as NASH if imaging or biopsy showed cirrhosis.

Laboratory data collected (within 2 weeks of initial visit) included bilirubin, albumin, prothrombin time, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), platelet count and AFP. Model for end-stage liver disease (MELD) score and fibrosis markers, fibrosis-4 score (FIB4) and AST/platelet ratio index (APRI) were also calculated. The size and number of the tumor(s) were used to determine the tumor node metastases stage according to the American Joint Commission on Cancer staging manual^[14]. Vascular invasion was only reported as macrovascular invasion based on imaging studies as not all patients had sufficient tissue specimen to provide useful analysis of microvascular invasion.

The proportion of patients with HCC detected with surveillance was noted. Although current guidelines recommend surveillance of patients with cirrhosis and chronic HBV or HCV with AFP and liver ultrasound every six months, there was no uniform screening protocol used in the cohort. Referring physicians used a combination of AFP and/or imaging (ultrasound, CT scan or MRI) at variable intervals. HCC was deemed to be found on “screening” if the referring physician stated that screening was done and/or the patient had a previous imaging study from three to twelve months prior. HCC not found on screening was either diagnosed with symptoms (pain, abdominal mass, weight loss, jaundice) or asymptotically with imaging done for unrelated reasons and incidental discovery of a liver mass.

Treatments

Treatments included liver resection, transplantation, loco-regional therapies (including radiofrequency ablation, cryosurgery, transarterial chemoembolization, and percutaneous ethanol injection) and systemic therapies. Liver resection was considered in Child's A patients and early Child's B patients (Childs Turcotte Pugh score of 7, without any evidence of ascites or encephalopathy). Liver transplantations were considered in patients who had unresectable HCC but met Milan criteria (single tumor less than 5 cm or 2-3 tumors, each less than 3 cm). Liver transplantation was also considered in patients who underwent resection but had recurrence of HCC more than six months after surgery, provided the recurrent tumor met Milan criteria and there was no disease progression while awaiting transplant. Since 2007, liver transplantation was considered in patients who met

UCSF criteria (single tumors less than 6.5 cm, 2-4 tumors with total diameters less than 8.5 cm) provided that their HCC had been downstaged to meet Milan criteria with locoregional therapy and AFP was less than 1000 ng/dL. All liver resections and transplantation were performed by members of our surgical group. The majority of patients on the transplant list underwent locoregional therapy as a bridge to transplant.

Patients were followed with imaging every 3 months after surgery or locoregional therapies for the first year and subsequently every 4-6 months. Most of these patients were followed by the physicians involved in the initial treatment, so follow up and survival were carefully monitored. Deaths were confirmed using the Social Security Death Index and local newspaper obituaries.

Statistical analysis

All analyses were performed using Excel and SPSS statistical software. Categorical variables were analyzed using chi-square analysis and Student's *t*-test was used to determine significant differences in numerical values. Univariate and multivariate logistic regression were used to determine factors that were associated with receiving transplantation. Factors included gender, age < 60 years, hypertension, NAFLD/NASH, family history of HCC, alcohol, smoking, whether they had a screenable disease, obesity, education, HBV, HCV and race. Multivariate Cox proportional hazards regression was used to determine factors that were associated with survival in males and females separately.

RESULTS

Overall cohort

In this cohort of 1206 patients, 899 (74.5%) were male and mean age overall was 62.7 years with 41.6% of patients being 65 years or older. Ethnic distribution was as follows: Asian (59.5%), White (20.2%), Pacific Islander (15.4%), Mixed (2.2%), Hispanic (1.8%) and Black (0.9%). HBV surface Ag was positive in 26.2% and another 10.9% were positive for HBV core Ab but negative for surface Ag. The overall incidence of HCV antibody was 40.8%. About 11% of patients in the cohort had no viral or alcohol risk factors and had documented NAFLD or NASH on imaging or biopsy.

Differences between males and females

Demographics and risk factors

Differences in demographics and risk factors are summarized in Table 1. Females developed HCC at a significantly older age (66.0 years vs. 61.6 years, $P < 0.001$) with a larger proportion greater than 65 years old (53.4% vs. 27.6%). Females trended toward having less incidence of HBV surface Ag, core Ab and HCV positivity however this was not statistically significant. A higher proportion of males were coinfecting with both HCV and HBV (7.0% vs. 3.6%). Overall, females were more often screened for HCC (29.3% vs. 22.7%, $P = 0.02$) and had greater rates of NAFLD/NASH (21.5% vs. 7.2%, $P < 0.0001$) and hypertension (67.2% vs. 54.8%, $P = 0.0007$). Elderly females (≥ 65 years) were more likely than elderly males to have a NAFLD/NASH related HCC (28.0% vs. 14.8%, $P = 0.0006$). Furthermore, elderly females were also more likely to have NAFLD/NASH than younger females, as 46 of 164 older women had NAFLD/NASH compared to 20 of 143 younger women who had NAFLD/NASH (28% vs. 14%, $P = 0.003$). Females with a screenable disease (based on existing practice guidelines) were also more likely to undergo HCC screening than men with screenable disease (41.6% vs. 28.7%, $P = 0.0005$). Males were more likely to smoke (68.4% vs. 38%, $P = 0.0001$) and drink alcohol (52.9% vs. 12.1%, $P = 0.0001$). Females were more likely to have a family history of HCC (8.8% vs. 5.3%, $P = 0.04$). There was no significant difference in educational attainment, viral hepatitis rates, obesity, diabetes and hyperlipidemia.

Laboratory data

Table 2 summarizes differences in laboratory studies. Males had a higher rate of normal AFP (40.6% vs. 31.7%, $P = 0.0064$), higher mean bilirubin (1.8 vs. 1.4, $P = 0.03$), creatinine (1.09 vs. 0.95, $P = 0.01$), AST (90.9 vs. 72.4, $P = 0.001$) and ALT (73.3 vs. 52.4, $P < 0.001$). The MELD score was also higher in males (10.8 vs. 10.0, $P = 0.007$).

Table 1. Demographics and risk factors: comparison between females and males

	Females (n = 307)	Males (n = 899)	P-value
Mean age in years (SD)	66.0 (11.3)	61.6 (11.3)	< 0.001
Age ≥ 65 years	164 (53.4%)	338 (27.6%)	< 0.0001
Race			0.002
Asian	213 (69.4%)	505 (56.2%)	
Black	0	9 (1%)	
Hispanic	4 (1.3%)	18 (2.0%)	
Mixed	8 (2.6%)	19 (2.1%)	
Pacific Islander	39 (12.7%)	147 (16.4%)	
White	43 (14%)	201 (22.4%)	
Finished high school	149/191 (78%)	494/606 (87.5%)	0.29
Hepatitis B sAg+	69/304 (22.7%)	248/896 (27.7%)	0.10
Hepatitis B coreAb+	27/304 (8.9%)	104/896 (11.6%)	0.20
HCV+	112/304 (36.9%)	382/895 (42.5%)	0.08
Alcohol use	37/306 (12.1%)	474/896 (52.9%)	0.0001
Screenable disease	209/307 (68.1%)	705/899 (78.4%)	0.0003
HCC found on surveillance*	87/209 (41.6%)	202/705 (28.7%)	0.0005
NAFLD/NASH	66 (21.5%)	65 (7.2%)	< 0.0001
NAFLD/NASH (age ≥ 65)	46/164 (28.0%)	50/338 (14.8%)	0.0006
Mean BMI	26.3 (5.86)	27.0 (5.32)	0.05
Obesity (BMI ≥ 30)	61 (19.9%)	176 (19.6%)	0.93
Smoking history	114/300 (38%)	607/888 (68.4%)	0.0001
Current Smoker	24/300 (8%)	109/888 (12.3%)	0.04
Diabetes	116 (37.8%)	289 (32.9%)	0.21
Hyperlipidemia	72/304 (23.7%)	203/873 (23.3%)	0.88
Hypertension	160/238 (67.2%)	396/726 (54.8%)	0.0007
Family History of HCC	27 (8.8%)	48 (5.3%)	0.04

*Includes only those with a screenable disease. HCV: hepatitis C; HCC: hepatocellular cancer; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; BMI: body mass index

Table 2. Laboratory data: comparison between females and males

	Females (n = 307)	Males (n = 899)	P-value
Normal AFP	97/306 (31.7%)	363/895 (40.6%)	0.0064
Mean AFP (ng/mL)	14,962 (67797)	13,257 (61588)	0.68
Mean bilirubin (mg/dL)	1.4 (1.97)	1.8 (2.74)	0.03
Mean albumin (g/dL)	3.5 (0.66)	3.5 (0.71)	0.44
Platelets (10 ³ /mm ³)	162.6 (99.8)	169.6 (98.4)	0.29
Creatinine (mg/dL)	0.95 (0.88)	1.09 (0.84)	0.01
AST (U/L)	72.4 (61.8)	90.9 (84.6)	0.001
ALT (U/L)	52.4 (43.4)	73.3 (61.7)	< 0.001
Cholesterol (mg/dL)	163.3 (53.5)	163.8 (42.6)	0.94
Triglyceride (mg/dL)	104.7 (43.9)	123.1 (74.8)	0.81
MELD	10.0 (4.36)	10.8 (4.58)	0.007
APRI	1.2 (2.12)	1.1 (1.68)	0.35
FIB4	5.7 (5.09)	5.3 (4.36)	0.21

AFP: alpha-fetoprotein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; MELD: model for end-stage liver disease; APRI: AST/platelet ratio index; FIB4: fibrosis-4 score

There were no significant differences in mean AFP, albumin, platelets, cholesterol, triglycerides, APRI or FIB4 score between males and females.

Tumor characteristics and treatments

Differences in tumor characteristics and treatments are summarized in Table 3. Males had a larger mean tumor size (6.2 vs. 5.3, $P = 0.003$), with more tumors > 5 cm (43.4% vs. 34.5%, $P = 0.007$). Females had more tumors that met Milan criteria (47.9% vs. 40%, $P = 0.05$). HCC in males more often involved major vessels (12% vs. 7.5%, $P = 0.03$). There were no significant differences in the percentage of patients that presented with a single tumor or the receipt of resection or transplant. However, among the patients that met Milan criteria, men were more likely than women to receive transplant (29.6% vs. 10.9%, $P < 0.0001$).

Table 3. Tumor characteristics and treatments: comparison between females and males

	Females (<i>n</i> = 307)	Males (<i>n</i> = 899)	<i>P</i> -value
Mean tumor size in cm (SD)	5.3 (4.02)	6.2 (4.58)	0.003
Tumor > 5 cm	106 (34.5%)	496 (43.4%)	0.007
Single tumor	213 (69.4%)	588 (65.4%)	0.21
Tumors met Milan criteria	147 (47.9%)	260 (40%)	0.05
Tumor rupture	14 (4.5%)	35 (3.9%)	0.62
Major vascular invasion	23 (7.5%)	108 (12%)	0.03
Liver resection	68 (22.1%)	168 (18.7%)	0.30
Liver transplantation	16 (5.2%)	77 (8.6%)	0.06
%Transplant/met Milan criteria	16/147 (10.9%)	77/260 (29.6%)	< 0.0001

Table 4. Odds-ratios of factors associated with transplantation (modeled using logistic regression)

Factor	Univariate odds ratio (95% CI)	Multivariate odds ratio (95% CI)
Sex (males vs. females)	1.71 (0.98-2.97)	1.48 (0.76-2.88)
Age (< 65 vs. ≥ 65)	9.84 (4.052-21.45)	10.21 (3.88-26.99)
Tumor size	0.81 (0.49-1.33)	
Hypertension	0.61 (0.3-0.96)	0.92 (0.55-1.55)
NAFLD/NASH	0.66 (0.30-1.45)	4.14 (1.42-12.05)
Family history of HCC	1.25 (0.56-2.79)	
Alcohol use	0.93 (0.60-1.43)	
Smoking	0.71 (0.47-1.10)	
Presence of screenable disease	9.91 (3.10-31.61)	11.52 (3.03-43.76)
Obesity (BMI 30+)	1.13 (0.68-1.90)	
Education (≤ 13 vs. > 13 years)	0.51 (0.33-0.79)	0.63 (0.38-1.05)
Hepatitis B positive	0.91 (0.59-1.42)	
Hepatitis C positive	2.34 (1.52-3.60)	1.55 (0.88-2.76)
Race (reference = White)		
Asian	0.58 (0.36-0.94)	1.09 (0.88-2.76)
Hispanic	0.77 (0.17-3.46)	0.56 (0.07-4.60)
Mixed	0.96 (0.27-3.40)	0.36 (0.04-2.93)
Pacific Islander	0.39 (0.18-0.85)	0.58 (0.25-1.36)

HCC: hepatocellular cancer; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; BMI: body mass index

Factors associated with transplantation

Table 4 summarizes differences in factors associated with transplantation. Univariate analysis determined that age < 65 years, presence of screenable disease and having HCV were associated with receiving transplant, while hypertension, having high school or less education and being of Asian or Pacific Islander ethnicity relative to Caucasian ethnicity were associated with lower rates of transplant. Multivariate logistic regression analysis determined that age < 60 years, presence of NAFLD/NASH and having a screenable disease were associated with transplantation. Factors not significantly associated with transplantation included sex, hypertension, educational attainment, HCV infection, or race.

Survival

Survival outcomes are displayed in Figure 1. There was no significant difference in survival between males and females by the log-rank test ($P = 0.69$, see Figure 1). Table 5 summarizes the independent predictors of death. Multivariate Cox proportional hazards regression showed that NAFLD/NASH was a predictor of death in both males and females. Smoking and number of tumors were predictors of death while age less than 65 years, a family history of HCC and undergoing liver transplant were predictive of survival in males.

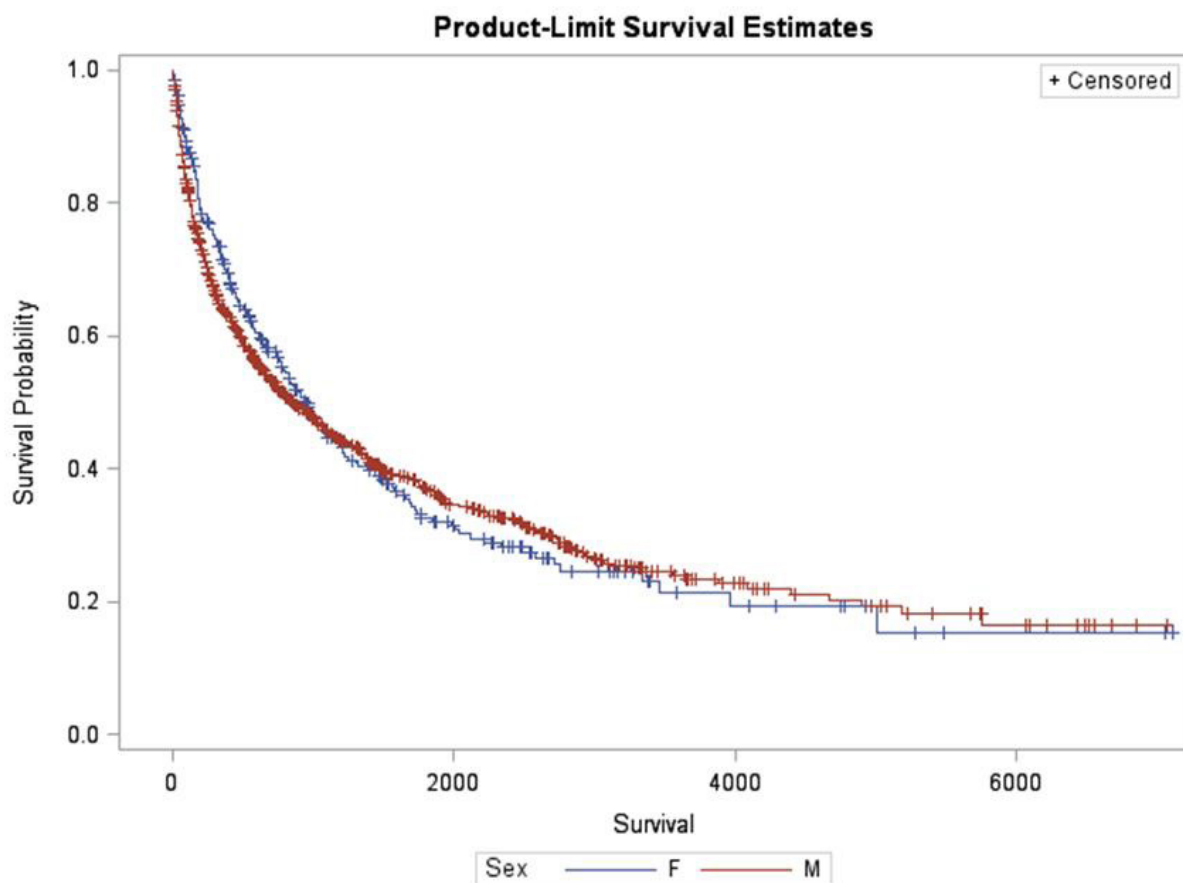
DISCUSSION

Gender differences in HBV and HCV may partially explain the male predominance of HCC, however

Table 5. Factors predictive of death (Cox regression) by gender

Parameter	Hazard ratio (95%CI) males	P-value	Hazard ratio (95% CI) females	P-value
Age (< 65 vs. ≥ 65 years)	0.65 (0.47-0.90)	0.009	0.78 (0.47-1.30)	0.35
Liver transplant	0.47 (0.33-0.68)	< 0.0001	0.66 (0.28-1.53)	0.34
Number of tumors	1.20 (1.06-1.36)	0.003	1.14 (0.78-1.70)	0.48
Hypertension	0.88 (0.67-1.16)	0.38	0.99 (0.58-1.68)	0.97
NAFLD/NASH	2.02 (1.22-3.33)	0.006	2.29 (1.20-4.35)	0.01
Family history of HCC	0.57 (0.34-0.97)	0.038	0.89 (0.38-2.08)	0.78
Alcohol use	0.97 (0.73-1.30)	0.86	1.64 (0.85-3.16)	0.14
Smoking history	1.78 (1.32-2.38)	< 0.0001	1.29 (0.82-2.03)	0.27
HCC found on surveillance	1.22 (0.83-1.79)	0.31	1.31 (0.76-2.23)	0.34

HCC: hepatocellular cancer; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis

**Figure 1.** Kaplan-Meier survival: comparison of males vs. females. Survival is measured in days

geographic variations, hormonal changes, environmental/behavioral risk factors and compliance with antiviral therapies may further influence these differences. Males are more likely to acquire HBV and HCV, develop chronic hepatitis, cirrhosis and HCC. This progression may be related to lower seroconversion after HBV vaccination compared to females, as well as androgen related upregulation of viral production and inflammation^[15,16]. For both HBV and HCV, there is evidence that female gender confers a protective effect against HCC as estrogen decreases IL-6 mediated hepatic inflammation and viral production^[17-19]. While this study cannot make definitive conclusions without knowledge of all HBV and HCV patients at risk, females trended toward having less HBV and HCV although this was not statistically significant. Although a recent meta-analysis reported that co-infection with HBV and HCV did not increase HCC risk^[20], our study did show

that males in the cohort were more likely to be coinfectd.

Behavioral risk factors such as smoking and alcohol are known independent risk factors for HCC^[21]. Alcohol damages the liver through oxidative stress and inflammation that results in a spectrum of fatty changes from reversible damage to cirrhosis. In the US, HCC attributed to alcohol usage is more common in males (27.8%) than females (15.4%)^[22]. In our study, a larger proportion of males had significant alcohol usage compared to females, although we did not exactly quantify the amount of alcohol used nor account for past vs. current alcohol use. Smoking has been shown to increase both the incidence and mortality of HCC, and males in our study were more likely to smoke. Smoking was also an independent predictor of mortality in males in our study, while alcohol did not affect mortality in either gender. Despite the inability to determine dose effects of alcohol and smoking, our data confirms that there are gender differences in behavioral risk factors for HCC.

Gender differences in metabolic risk factors for HCC are important as NAFLD is currently the most common chronic liver disease in western industrialized countries^[23]. Differences in adipocyte metabolism may contribute to the gender disparity in HCC^[24]. Visceral adiposity, more common in males, has been shown to induce a pro-inflammatory state that could increase risk of fibrosis relative to females, who may be protected by estrogen^[25]. This protection may be lost in postmenopausal women, where NAFLD rates have been shown to increase with age relative to men^[26]. The relative increase in visceral adiposity in males may help explain the gender disparity in HCC, as one study showed an association of BMI with HCC risk only in males^[27]. Females in our study had higher rates of NAFLD/NASH than males, with older women having significantly higher rates of NAFLD/NASH than younger women and older men. Our study showed that NASH associated HCC disproportionately affected older women but a longitudinal study of a large population of NASH patients would be necessary to validate this.

Surveillance has been shown to decrease mortality from HCC in multiple retrospective studies^[27]. However, data on gender differences in HCC surveillance have been inconsistent^[28-31] and gender disparities in surveillance rates may impact prognosis^[32]. In this study, females with a screenable disease were more likely to have HCC identified with surveillance, but this did not impact their survival. One possible explanation is that females overall were less likely to have a known screenable disease and more likely to have a fat-related liver disease, an HCC risk factor for which there are no established screening guidelines unless cirrhosis is present. Furthermore, HCC attributed to NAFLD has been shown to frequently develop in non-cirrhotic livers^[33], decreasing the likelihood of early tumor detection. Despite a higher rate of HCC detection through surveillance, a considerable proportion of females at risk for HCC may be overlooked with regards to screening.

Gender disparities in transplantation are well described in the literature, with males tending to undergo transplantation more than females^[33]. Gender disparity may result from the fact that males more commonly present with the leading indications for transplant (alcohol and HCV induced cirrhosis) and are more likely than females to have early-referral to a transplant center^[34,35] while females may have lower MELD scores due to relatively less muscle mass and creatinine^[36,37] and finally, donor-recipient organ size mismatch^[38,39]. In our study, females trended towards meeting Milan criteria and males trended towards having more liver transplants. If only those patients who met Milan criteria were considered as potential transplant candidates, males were significantly more likely to undergo liver transplant. In the multivariate analysis, the significant factors for receiving a liver transplant were age, the presence of NAFLD/NASH, and presence of screenable disease. This may suggest that efforts to improve transplant rates should be directed towards better screening for patients with NAFLD and NASH.

This study did not show a survival difference between the genders but contained a more detailed risk factor analysis than previous studies^[40] which demonstrated that NAFLD/NASH was the only factor associated with

mortality in both genders. Receiving a liver transplant was associated with improved survival in males but not females. While one would expect that liver transplant would improve survival in both genders, perhaps the fewer numbers of females undergoing transplant in our cohort made the overall survival benefit in females less apparent. Females were less likely to receive liver transplants despite being more likely to meet Milan criteria, have NASH/NAFLD and have HCC found with surveillance. Clearly there are other reasons that contribute to getting a liver transplant that could not be delineated in this study which may include insurance issues, substance abuse, comorbidities and potentially cultural issues in a predominantly Asian population. Although we cannot determine causation, our data suggests that NAFLD/NASH may lead to increased mortality due to decreased surveillance in this population and less opportunity for curative therapies. Some of these patients were likely diagnosed with NAFLD but were not followed closely and thus, were allowed to progress to HCC.

A limitation of this study was that it consisted of a single-center retrospective study in a relatively isolated population. Some of the differences in risk factors and treatment by gender might have been affected by ethnicity, as well as cultural and language barriers because more than a third of the patients were born outside the US. It was also difficult to truly separate all of the risk factors to determine causality as many patients had combinations of risk factors and dose/time/severity dependent factors such as alcohol usage, smoking, obesity and diabetes. We also did not collect data on whether a patient was pre or post-menopausal and whether there was any usage of hormone replacement therapy so it was difficult to make conclusions about the contribution of sex steroids on the development of HCC. Finally, we may have underestimated the NAFLD/NASH group, as there were patients with no viral risk factors or alcohol usage, but with metabolic risk factors and not enough information on imaging or biopsy to categorize them as NAFLD/NASH. Despite these limitations, the strengths of our study include a robust sample size, diverse study population, and detailed risk factor data that may not be available in administrative or national cancer databases. Furthermore, because we are Hawaii's only dedicated liver center that sees nearly 70% of Hawaii's HCC cases, we believe that this study gave an accurate view of a state with a high burden of HCC.

We have shown that there are distinct gender differences in behavioral and metabolic risk factors as well as access to liver transplantation that disproportionately affects certain subgroups with regards to HCC. Older women with HCC appear to have higher rates of underlying NAFLD/NASH but this population may be overlooked by current surveillance guidelines, thus losing a valuable opportunity for early tumor detection and treatment. The epidemic of NAFLD/NASH may potentially increase HCC disproportionately in older females but further studies will be needed to validate this. Future efforts should be directed towards better identification of NAFLD/NASH in this population and how to effectively survey these patients for HCC.

DECLARATIONS

Authors' contributions

Conception, data collection: Wong LL

Study design: Wong LL, Hernandez BY

Data analysis: Wong LL, Hernandez BY, Wu EM

Interpretation of results and manuscript writing: Jia W, Kwee SA, Wong LL, Wu EM

Manuscript review: Ji JF, Jia W, Kalathil S, Kwee SA

Availability of data and materials

The dataset used to support this study can only be shared in an IRB-approved collaborative study and with permission from the authors.

Financial support and sponsorship

This work was supported by U.S. National Institutes of Health grant 3P30CA071789-12S6.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

This study was approved by the University of Hawaii Institutional Review Board.

Consent for publication

Not applicable.

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Original Article

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Endolymphatic immunotherapy for advanced hepatocellular carcinoma: an update of our experience

Marialuisa Lugaresi¹, Yuval Katz¹, Riccardo Bertelli¹, Noa Ruhrman¹, Lorenza Puviani¹, Giuseppe Cavallari¹, Caterina De Vinci², Giancarlo Pizza², Bruno Nardo¹

¹Department of Experimental, Diagnostics and Medicine Specialty, S. Orsola-Malpighi Hospital, University of Bologna, Bologna 40138, Italy.

²Module of Immunotherapy, S. Orsola-Malpighi Hospital, University of Bologna, Bologna 40138, Italy.

Correspondence to: Dr. Marialuisa Lugaresi, Department of Experimental, Diagnostics and Medicine Specialty, S. Orsola-Malpighi Hospital, University of Bologna, Bologna 40138, Italy. E-mail: marialuisa.lugaresi2@unibo.it

How to cite this article: Lugaresi M, Katz Y, Bertelli R, Ruhrman N, Puviani L, Cavallari G, De Vinci C, Pizza G, Nardo B. Endolymphatic immunotherapy for advanced hepatocellular carcinoma: an update of our experience. *Hepatoma Res* 2018;4:67. <http://dx.doi.org/10.20517/2394-5079.2018.88>

Received: 9 Jul 2018 **First Decision:** 20 Aug 2018 **Revised:** 25 Sep 2018 **Accepted:** 26 Sep 2018 **Published:** 18 Oct 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cui Yu **Production Editor:** Zhong-Yu Guo

Abstract

Aim: We report an update of our experience on endolymphatic immunotherapy in patients with advanced hepatocellular carcinoma (HCC) not eligible for surgery.

Methods: From 2003 to 2009 we enrolled 39 patients with advanced HCC not suitable for surgery. Patients underwent monthly endolymphatic injections of 1.5×10^6 - 3.0×10^6 IL-2-activated peripheral autologous lymphocytes and 250U of IL-2. Blood biochemistry every 3 months and imaging studies every 6 months were performed. Evaluation of the results was done according to clinical and pathological characters mainly including etiology, Child-Pugh class, size and number of lesions, α -fetoprotein, lymphadenopathy, vascular invasion, Response Evaluation Criteria in Solid Tumours criteria for tumour burden, biochemical parameters and survival rates.

Results: Ten patients completed 12 therapy cycles, 6 received 6 infusions, 10 only 3-4 injection and 13 patients received less than 3 injections and were considered not suitable for evaluation. No clinically significant adverse reactions occurred. Imaging studies showed no significant decrease in tumour mass. Survival of treated patients was significantly higher with respect to control group ($P < 0.0001$). The 1-year survival was 0% in the control group vs. 50% in the treated group. In addition survival of patients who completed 12 therapy cycles appeared higher with respect to patients who underwent less than 6 cycles without reaching statistical significance due to



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the small number of patients. All patients with 12 completed cycles showed an improvement of 9 parameters or more.

Conclusion: Endolymphatic administration of immunotherapy appeared safe, easy to perform and effective in terms of survival. This study should encourage future large scale studies in order to reach a firmer conclusion and define uniform inclusion criteria.

Keywords: Hepatocellular carcinoma, endolymphatic, immunotherapy, survival

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy globally and the third leading cause of malignancy - related mortality worldwide. The incidence of HCC is still higher in some African and Eastern Asian regions. This cancer represents 3%-6% of all solid tumours in the USA and Europe^[1,2]. Hepatitis B virus (HBV)/hepatitis C virus (HCV) infection and alcohol abuse seem to be the main causes of the spread of HCC in Western countries^[3]. Despite the established efficacy of screening programs for at-risk individuals, the diagnosis is usually performed at later stages of disease, wherein the tumour characteristics or liver disease progressions do not allow for curative therapeutic approach^[4,5]. Many criteria have been proposed for the staging of HCC, combining different prognostic factors. The current treatment of HCC is based on the Barcelona Clinic Liver Cancer (BCLC) classification^[6,7] including stages of the disease, macroscopic features of the lesion and liver function parameters as identified by Child-Pugh scoring system. Curative surgical treatment appears suitable in 30%-35% of all diagnosed cases^[8,9], therefore much effort is directed towards new therapeutic agents. Encouraged by the good results obtained from treating metastatic renal cell carcinoma with immunotherapy^[10], we offered the same procedure, with palliative intent, to patients with advanced disease who were not eligible either for hepatic resection or for percutaneous ablation based on BCLC classification obtaining interesting preliminary results^[11] before approval of a new drug for treatment of HCC^[12]. Sorafenib® is the only approved drug for patients with advanced HCC but has shown limited activity^[13]. It acts as a multikinase inhibitor suppressing cell proliferation and angiogenesis. Recently it has been reported that other oncogenic targets may contribute to the anti-proliferative activity of the drug^[14,15]. Herein we report the results of our pilot study in a cohort of patients with HCC in the pre-terminal stage who were not suitable for any curative interventions, before Sorafenib® - period.

METHODS

From January 2003 to March 2009, 39 patients with advanced HCC were enrolled in our study. Among these, 26 underwent at least 3 cycles of immunotherapy, but only 16 who completed at least 6 cycles were able to evaluate the efficacy of the treatment. In 13 patients the treatment was interrupted before the third cycle because of local skin reaction ($n = 1$), early death ($n = 2$) and worsened clinical conditions ($n = 10$). An historical control group is represented of 15 patients with similar characteristics of advanced HCC who underwent standard therapy without immunotherapy. The protocol of the immunotherapy which was already reported by our group^[11] consisted in monthly endolymphatic infusions of 1.5×10^6 - 3.0×10^6 autologous activated lymphocytes (LAK) and 250IU of IL-2. Lymphocytes were obtained through the centrifugation of 30 mL of the patients' peripheral blood on a Ficoll-Hypaque gradient. The lymphocytes were then suspended in Roswell Park Memorial Institute-1640 (Sigma Aldrich, Germany) 2×10^6 /mL and incubated with 20 U/mL of IL-2 at 37 °C for 72 h. After the incubation the cells were washed with saline solution and suspended in 5-10 mL of saline solution containing 250IU of IL-2.

Surgical procedure consisted of three steps. Firstly, the lymphatic vessels on the back of the foot was identified using the standard lymphographic technique (subcutaneous injection of violet patent blue between two finger). Then the main lymphatic was isolated and cannulated with a needle catheter (27G). A syringe containing the

cells suspended in 5 saline mL with 250U of IL-2 was connected to a pump for micro-injections (0.5 mL/min): the infusion lasted 10-20 min. The patients were also i.m. administered with chlorphenamine maleate (GSK, Brantford, UK) and ranitidine (GSK, Brantford, UK) 1 h before the treatment, in order to block H1 and H2 lymphocytes receptors and reduce possible side effects.

For evaluating the impact of the treatment on the tumour mass we adopted Response Evaluation Criteria in Solid Tumours criteria, a well known simple and pragmatic methodology to evaluate the activity and efficacy of therapies towards tumours^[16]. In addition every three months we evaluated 12 biochemical parameters on the peripheral venous blood of the patients, i.e., alanine-amino-transferase (ALT), aspartate-amino-transferase, gamma-glutamyl-transferase (GGT), bilirubin (BIL), alkaline phosphatase (ALP), α -fetoprotein (AFP), platelets, white blood cells, total plasmatic proteins, albumin, prothrombin time, creatinine (Cr). The minimal acceptable response to the therapy was defined as an improvement of at least 7 of these biochemical parameters. Finally we compared the survival rate of the treated patient to that of the non-treated patients (control group).

The present study was conducted in accordance with the ethical standards of the Helsinki Declaration (1964, amended most recently in 2008) of the World Medical Association. The local Institutional Review Board approved the use of the database for this retrospective review of the case files. Each patient provided written consent, and all patient information, including illustrations, were anonymous.

Statistical analysis

Data are represented as mean (range) for continuous variables and as *n* (%) for categorical variables. The χ^2 test or Fisher's test and the Student's *t* test were used to analyse categorical and continuous variables.

Survival analysis was performed using the Kaplan-Meier method and the log-rank test. *P*-values < 0.05 were considered significant. Data were analysed using SPSS (version 15.0) (SPSS Inc., Chicago, IL, USA).

RESULTS

In Table 1 are reported and compared the main clinical and pathological characteristics of treated patients and control group. There were no statistically significant differences between the two groups. Among the 26 patients enrolled in our study, ten patients completed 12 therapy cycles, six received 6 infusions and ten patients underwent only 3 or 4 procedures.

Twelve patients showed a partial response to the therapy that is amelioration of at least 7 out of 12 biochemical parameters considered [Figure 1]. Moreover, all the patients who completed the 12 cycles showed an improvement in 9 or more of the analysed parameters [Figure 1].

All parameters, but ALP and GGT, either improved or remained stable in more than 50% of the cases [Figure 2].

The regression of the neoplastic mass was not evident at the imaging studies in neither group, but in the treated group we observed 34% of patients with stability after 12 cycles and 0% of stability in the other patients treated with ≤ 6 cycles of immunotherapy.

The survival rate was measured from the beginning of the therapy, and analyzed with the Kaplan-Meier curve. The difference between the treated group and the control group was calculated with log-rank test and found to be statistically significant (*P* < 0.0001). The 1-year survival was 0% in the control group vs. 50% in the treated group [Figure 3].

A striking difference (even though not statistically significant due to small numbers of the groups) can be noted between the group of patients who completed the 12 cycles and those with < 6 cycles; 1-year survival was

Table 1. Clinical and pathological characteristics of patients with advanced hepatocellular carcinoma

Parameter	Treated group (n = 26)	Control group (n = 15)	P
Age	Mean 69 years (49-76)	Mean 67 years (52-75)	0.648
> 60 years	20 (77%)	11 (73%)	
< 60 years	6 (23%)	4 (27%)	
Gender			0.693
Male	22 (85%)	12 (80%)	
Female	4 (15%)	3 (20%)	
Etiology			0.972
HCV	5 (19%)	3 (20%)	
HBV	7 (27%)	5 (33%)	
HCV + HBV	4 (15%)	2 (14%)	
Other	10 (39%)	5 (33%)	
Liver			1.000
No cirrhosis	8 (31%)	4 (27%)	
Cirrhosis	18 (69%)	11 (73%)	
Child-Pugh			0.992
A	19 (73%)	11 (73%)	
B	5 (19%)	3 (20%)	
C	2 (8%)	1 (7%)	
Ascites			0.730
Yes	7 (27%)	5 (33%)	
No	19 (73%)	11 (77%)	
Splenomegaly			0.512
Yes	16 (61, 5%)	11 (77%)	
No	10 (38, 5%)	4 (23%)	
α -fetoprotein			1.000
< 200 ng/mL	13 (50%)	8 (53%)	
> 200 ng/mL	13 (50%)	7 (47%)	
N. of HCC lesions			1.000
Single	2 (8%)	1 (7%)	
Multiple	24 (92%)	14 (93%)	
Tumor size			0.644
Single nodule	6.3 cm \times 5.5 cm; 7 cm \times 5.5 cm	7 cm \times 6.5 cm	
Multiple nodules (median, range)	5 cm (2-9 cm)	5 cm (1-8 cm)	
Lymph node positive			1.000
Yes	5 (19%)	3 (20%)	
No	21 (81%)	12 (80%)	
TACE			1.000
Yes	8 (31%)	4 (27%)	
No	18 (69%)	11 (73%)	
Previous liver resection			1.000
Yes	17 (65%)	10 (67%)	
No	9 (35%)	5 (33%)	
Portal vein infiltration e/or thrombi			1.000
Yes	8 (31%)	4 (27%)	
No	18 (69%)	11 (73%)	
Caval thrombi			1.000
Yes	2 (8%)	1 (7%)	
No	24 (92%)	14 (93%)	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; TACE: transarterial chemoembolization

100% in the group that completed 12 cycles vs. 20% in patients with < 6 cycles of therapy [Figure 4].

We compared the characteristics of patients on the basis of the therapy cycles (12 cycles or < 6 cycles) and observed that all ten patients who completed the 12 cycles were Child A and without vascular infiltration of portal vein and seven of them had a value of AFP < 200 ng/mL. However, we have to remark that hepatic reserve and tumor burden of HCC could be affecting the survival of the patients.

Among the remaining 16 patients (group \leq 6 cycles), 11 were Child B and C, 8 showed vascular infiltration, 10 had a value of AFP > 200 ng/mL and 1 patient had bone metastases. These factors (Child B or C, AFP > 200 ng/mL, portal infiltration and the presence of extrahepatic malignancy) may be considered as

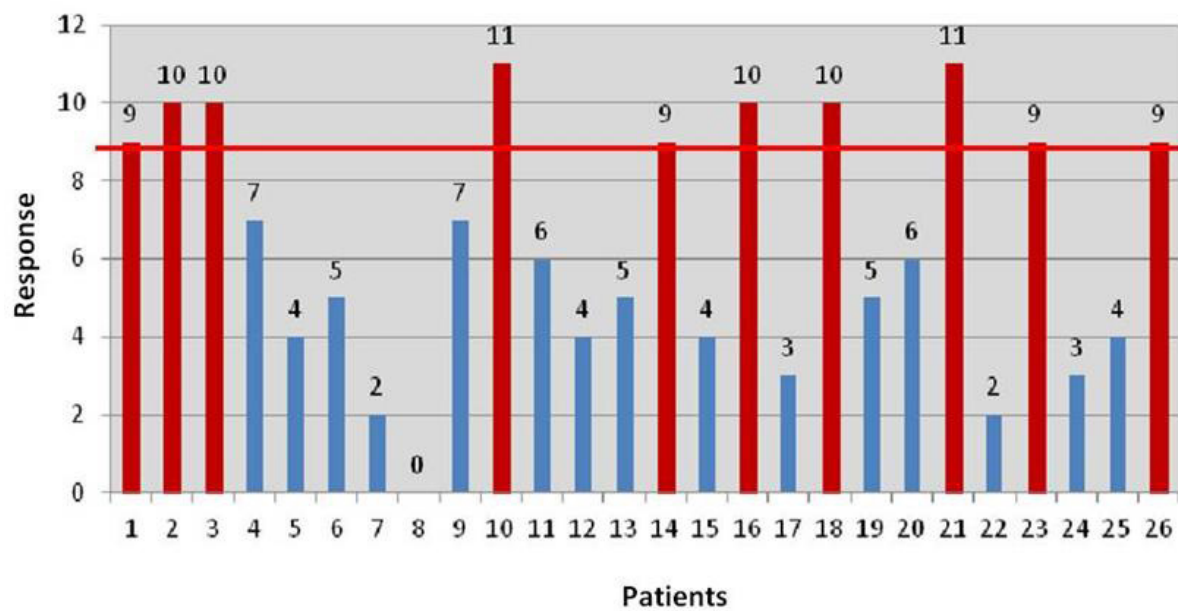


Figure 1. Trend of biochemical parameters in treated patients with endolymphatic immunotherapy according with number of therapy cycles. Patients who completed 12 therapy cycles (red color) vs. patients with less than 6 cycles (blue colour)

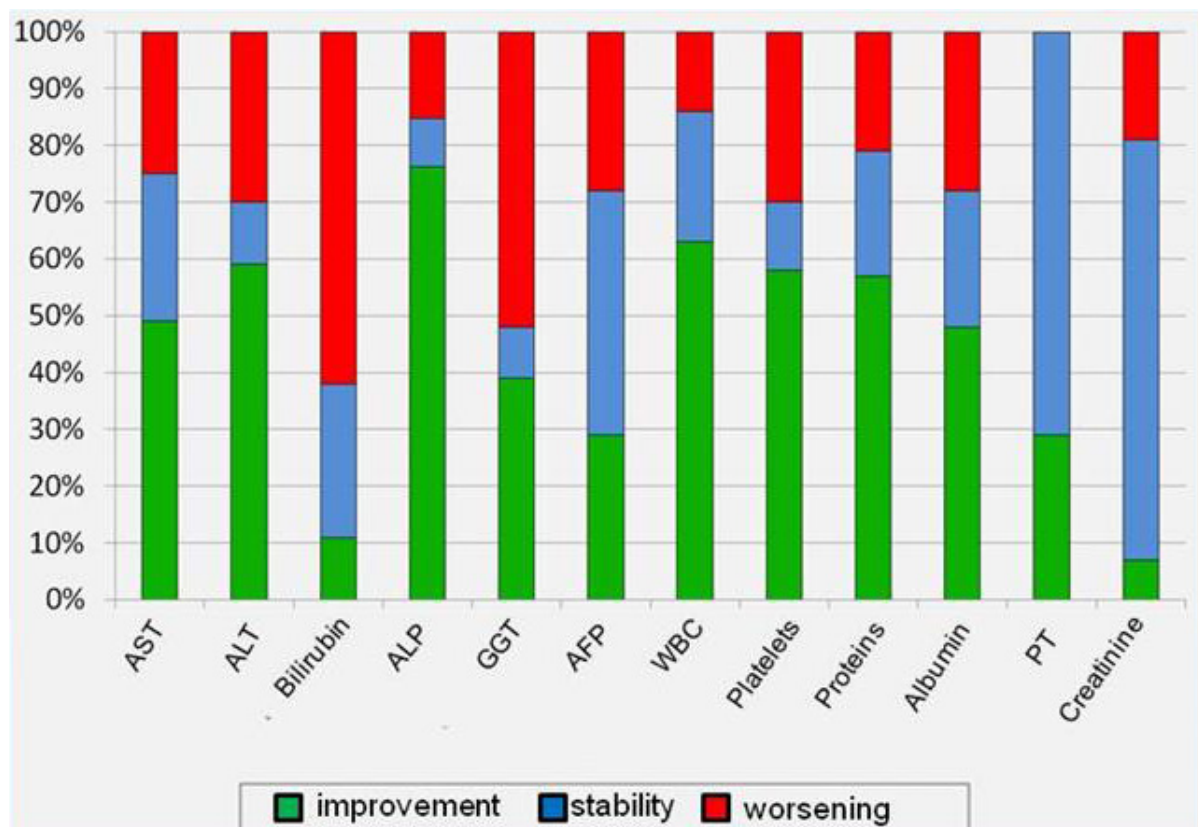


Figure 2. Percentage of improvement (green), stability (blue) or worsening (red) of biochemical parameters. AST: aspartate-amino-transferase; ALT: alanine-amino-transferase; ALP: alkaline phosphatase; GGT: gamma-glutamyl-transferase; AFP: α -fetoprotein; WBC: white blood cells; PT: prothrombin time

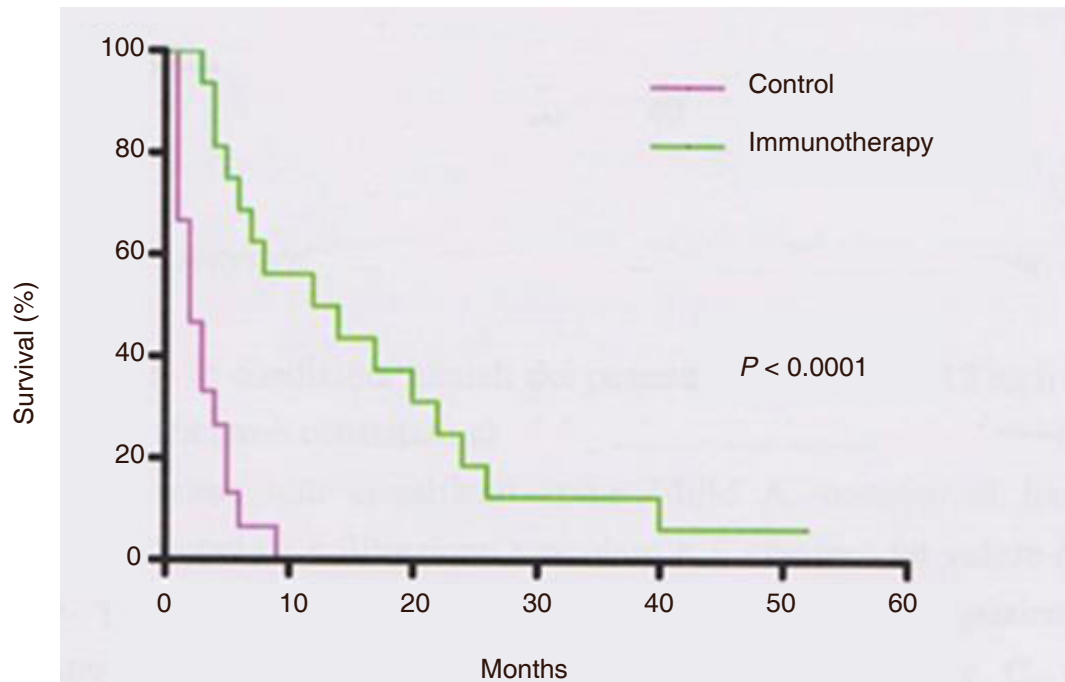


Figure 3. Kaplan-Meier survival analysis of patients with advanced hepatocellular carcinoma (endolymphatic immunotherapy vs. control group) ($P < 0.0001$)

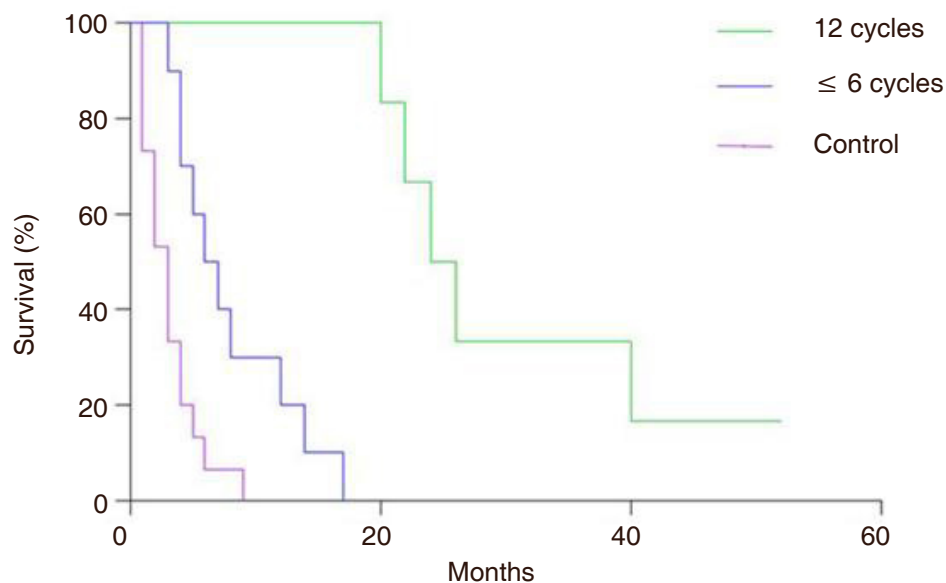


Figure 4. Kaplan-Meier survival analysis of patients with advanced hepatocellular carcinoma according to cycles of immunotherapy therapy and compared to control group ($P = ns$)

poor prognostic factors but are necessary larger studies to define the exclusion criteria of the patients for endolymphatic immunotherapy.

DISCUSSION

HCC is a complex and heterogeneous tumor with multiple genetic aberrations. Several molecular pathways involved in the regulation of proliferation and cell death are implicated in the hepatocarcinogenesis in

addition to major etiological factors, i.e., HBV and HCV virus infections. Continuous oxidative stress also due environmental factors or cellular mitochondrial dysfunction, have recently been associated with hepatocarcinogenesis^[17,18]. At present time Sorafenib®, a multikinase inhibitor, represents the most promising therapeutic agent which has undergone extensive investigation up to phase III clinical trials in patients with advanced HCC. The combination with other target-based agents^[14,15] could potentiate the clinical benefits obtained by Sorafenib®. Recently it has been reported that fasting had synergized with Sorafenib® in hampering HCC cell growth and glucose uptake^[19]. Moreover, fasting could appear to normalize the expression levels of genes which are commonly altered by Sorafenib® in HCC cells. Thus, fasting or fasting-mimicking diet should be evaluated in preclinical studies for potentiating the activity of Sorafenib® in clinical use.

HCC patients are frequently cirrhotic with an associated deficiency of liver function that increases the toxicity of conventional chemotherapy, so immunotherapy could be considered a promising treatment option. Recent papers reporting clinical trials on immunotherapy for patients with advanced HCC mainly outlined the safety and feasibility of such therapeutic approach although the results were inconstant and not comparable^[20,21]. The clinical results obtained by Onishi *et al.*^[20] are very close to our own. Ten patients with HCC, three of whom had pulmonary metastasis, were treated with adoptive immunotherapy using autologous LAK cells plus recombinant IL-2. Patients received 15 µg per day of recombinant IL-2 consecutively (for 14 to 64 days), from day 7 prior to the first leukapheresis, and received 109 to 1010 LAK cells once or twice per week intravenously; the LAK cells had been generated from mononuclear cells obtained through leukapheresis. Previous administration of recombinant IL-2 prior to the first leukapheresis resulted in a remarkable increase of LAK activity in seven of nine cases in whom LAK activity had been poorly inducible even at high concentrations of recombinant IL-2. At the end of the treatment, liver tumor regression (34% and 63%, respectively, of two-dimensional size) was observed in two of two patients with a solitary tumor; no increase of liver tumor size was observed in seven patients with massive or multiple tumors, and no changes in the size or number of pulmonary metastatic tumors in any patients were observed. A decrease of more than 35% in serum α -fetoprotein level was noted in four of nine α -fetoprotein-positive patients. However, child's grades, performance status and LAK activity on entry into the study could not be used as parameters to predict therapy responsiveness. Neither serious side effects, significant changes of serum BIL, ALT nor Cr were noted. Thus, this treatment seems to be well tolerated even in advanced HCC with poor liver function reserve, and tumour regression could be expected in small-burden HCC.

In our study we aimed to demonstrate the efficacy of immunotherapy administered by means of endolymphatic injections while in the literature few studies on advanced HCC treated with different procedures^[20,21] are available. In this first phase of our study we evaluated the safety and efficacy of the endolymphatic infusions of LAK and of IL-2 alone. Despite the small number of patients enrolled, the results obtained seems encouraging in terms of survival rate and improvement of biochemical parameters. We calculated the survival rate of the treated patient compared to historical control group of 15 patients with similar characteristics of advanced HCC who were not treated with endolymphatic immunotherapy (control group). The 1-year survival was 0% in the control group vs. 50% in the treated group.

Moreover concerning the survival a striking but not significant difference was observed between the group of patients who completed the 12 cycles and those who did not; 1-year survival was 100% in the group that completed 12 cycles vs. 20% in patients with that did not complete 12 cycles of therapy (≤ 6 cycles). The immunological basis for the clinical effect on survival, mainly the changes in circulating lymphocytes, was not investigated yet. We observed that patients who underwent 12 cycles had no signs of vascular infiltration, levels of AFP lower than 200 ng/mL, no metastases and a Child-Pugh score of A. Since hepatic reserve and tumour burden of HCC could be the critical factors affecting the survival of the patients, further investigation in a large population of patients is mandatory. However, this analysis may allow us to consider these features as parameters for inclusion in future studies as this category of HCC patients may have the largest benefit from

endolymphatic immunotherapy as a palliative strategy. The regression of the neoplastic mass, however, was not evident at the imaging studies in neither group. The low dosage of IL-2 is responsible for two other important advantages of this treatment: the virtual absence of major side effects and the low costs of the treatment. In conclusion we firmly consider immunotherapy a good prospective for the treatment of HCC both for its efficacy and for the low systemic toxicity in comparison to chemotherapy, which is often unacceptable in patients with a such compromised liver level. On the other hand, the detection of molecular factors predictive of response to anti-cancer agents such as Sorafenib® and the identification of mechanisms of resistance to anti-cancer agents^[22] may probably represent another direction to improve the treatment of HCC.

DECLARATIONS

Authors' contributions

Concept and design, data acquisition, data analysis, manuscript preparation: Lugaresi M, Katz Y, Bertelli R, Ruhrman N, Puviani L, Cavallari G, De Vinci C, Pizza G, Nardo B

Critical revision and finalizing of the manuscript: Lugaresi M, Pizza G, Nardo B

Availability of data and materials

The data were strictly obtained from medical records according to the privacy policy and ethics code of our institute.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The local Institutional Review Board approved the use of the database for this retrospective review of the case files.

Consent for publication

Consents from all of the patients were established prior to submission and all records were confidential.

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Review

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Minimally invasive therapies for hepatocellular carcinoma: narrowing the gaps

Kevin M. Sullivan¹, Raymond S. Yeung^{1,2}

¹Department of Surgery, University of Washington, Seattle, WA 98195, USA.

²Center for Advanced Minimally Invasive Liver Oncologic Therapies (CAMILOT), University of Washington, Seattle, WA 98195, USA.

Correspondence to: Dr. Raymond S. Yeung, Department of Surgery, University of Washington, 1959 NE Pacific, Seattle, WA 98195, USA.
E-mail: ryeung@uw.edu

How to cite this article: Sullivan KM, Yeung RS. Minimally invasive therapies for hepatocellular carcinoma: narrowing the gaps. *Hepatoma Res* 2018;4:68. <http://dx.doi.org/10.20517/2394-5079.2018.95>

Received: 21 Aug 2018 **First Decision:** 9 Oct 2018 **Revised:** 18 Oct 2018 **Accepted:** 18 Oct 2018 **Published:** 25 Oct 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cai-Hong Wang **Production Editor:** Zhong-Yu Guo

Abstract

With increasing awareness of the HCC epidemic around the globe, early diagnosis of tumors provides a greater opportunity to benefit patients from liver-directed treatments including surgical resection, ablation, catheter-based therapies and external beam radiation. Development of new approaches and refinement of existing techniques have improved our capabilities to provide efficacious and safe means of local disease control. The choice of treatment for individual patients hinges heavily on factors related to the tumor, underlying hepatic function, and existing co-morbidities. Recent advances in minimally invasive therapies across all disciplines have augmented our ability to eradicate the tumor while preserving liver parenchyma. In this review, we discuss and summarize current minimally invasive options that are available to treat HCCs that are confirmed to the liver, especially in their early stages. Emerging evidence suggest that resection, ablation and radiation can all provide excellent local control, and this opens more options for patients to best suit their needs.

Keywords: Resection, ablation, chemoembolization, radioembolization, Yttrium-90, radiation, laparoscopic, robotic

INTRODUCTION

Hepatocellular carcinoma (HCC) has the sixth highest cancer incidence and is the fourth most common cause of cancer-related mortality worldwide^[1]. In the United States, the average annual percent change in the cancer-related death rate for HCC increased 2.8% from 2003 to 2012, compared to a decrease in the average annual percent change in cancer-related death for the majority of the other top causes of cancer-related death^[2]. Common causes of HCC are cirrhosis due to hepatitis B virus, hepatitis C virus (HCV), or alcoholic hepatitis, with less common etiologies including hereditary diseases such as hemochromatosis or



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liver damage due to toxins like aflatoxin. Chronic liver disease caused by HCV is a significant contributor to the rising trend in Western countries although widespread adoption of effective anti-hepatitis C treatments using direct antiviral agents is beginning to reduce the number of HCV-related HCC cases. Yet, a much larger threat stemming from non-alcoholic steatohepatitis (NASH) will continue to promote the incidence of HCC worldwide as the obesity pandemic reaches all corners of the globe. Unlike those with cirrhosis secondary to viral hepatitis or alcohol abuse, the surveillance for HCC in the setting of non-cirrhotic NASH remains uncertain and without established guidelines. Much effort is focused on finding cost-effective methods such as ultrasound evaluation and serum alpha-fetoprotein (AFP) measurement for early cancer detection in this high-risk group with the understanding that the stage at which HCC is diagnosed strongly influences the outcome of the disease.

As the majority of HCCs remain confined to the liver without distant metastases at the time of diagnosis, liver-directed loco-regional approaches are the mainstay of current treatments. Over the last two decades, the field has witnessed remarkable advances in many areas involving surgery, interventional radiology, radiation oncology, and medical oncology, which are re-shaping the landscape of HCC treatments. In this review, we will highlight progress made in minimally invasive techniques that are currently in use, with the objective of comparing their efficacy based on available evidence. Due to the wide-ranging disciplines and technical demands of individual treatment modalities, we strongly endorse an up-front multi-disciplinary discussion for every case of newly diagnosed HCC. In our Liver Tumor Clinic at the University of Washington, each patient is provided with a consensus recommendation from our multi-disciplinary group consisting of surgeons, radiologists, interventional radiologists, medical oncologists, and radiation oncologists. This approach is continued longitudinally to ensure the most appropriate management given the high risk of recurrent disease. While many patients are considered for liver transplantation, only a limited number undergo such procedure due to organ availability and variable drop-out rates. For those with good liver reserve and limited tumor burden, definitive loco-regional therapies provide excellent disease control. Here, we will summarize recent developments in minimally invasive modalities and their relative efficacy in the treatment of HCC.

ADVANCES IN LIVER-DIRECTED THERAPIES

Minimally invasive techniques for hepatic resection

Surgical resection has remained the gold standard for treatment of localized hepatocellular carcinoma in patients with good liver reserve (i.e., Child's A, B7) and without significant portal hypertension (i.e., hepatic venous pressure gradient < 10 mmHg, platelet count > 100,000/ μ L). Other factors to be considered include the tumor stage [usually Barcelona Clinic Liver Cancer (BCLC) 0, A], tumor biology, and patient's medical comorbidities. The presence of vascular invasion by the tumor and high AFP levels are predictors of poor outcome, and such cases should be thoroughly discussed by a multi-disciplinary tumor board before deciding on surgical resection.

Traditionally, hepatic resection has been performed as an open operation using a variety of abdominal incisions, which are associated with major morbidities. Advances in surgical technique including the application of minimally invasive approaches have significantly reduced morbidities following hepatectomy. Laparoscopic hepatobiliary surgery carries the same advantages of minimally invasive surgery in all other realms, namely decreased length of hospitalization, reduced wound complications, and improved postoperative pain, which translate to faster resumption of normal activities. Another notable benefit of laparoscopic hepatic surgery is the tamponade effect created by the carbon dioxide insufflation to reduce hemorrhage from hepatic venous branches. Placement of patient in reverse Trendelenburg position also aims to minimize blood loss by decreasing venous pressure. Early reports of laparoscopic hepatectomy confirmed that the approach was safe with minimal mortality and produced comparable overall survival (OS) and disease-free survival (DFS) to open hepatectomy^[3-5]. In cirrhotic

livers, there is suggestion of reduced post-operative ascites following laparoscopic resection. Subsequent large systematic reviews of laparoscopic vs. open hepatectomy for malignant disease further demonstrated decreased intraoperative blood loss and transfusion requirements, shorter length of hospitalization, and fewer overall complications^[6-8]. With regards to oncologic outcomes in HCC, compared to open resection, laparoscopic resection showed no difference in 1-, 3-, and 5-year OS and DFS^[9]. The indications for laparoscopic approaches continue to evolve to include both minor and major resections^[10]. Based on the recommendations from the Second International Consensus Conference on laparoscopic liver resection, ‘minor’ hepatectomy (e.g., left lateral sectionectomies, resection of segments 4B, 5, and 6) is increasing adopted as a standard practice although high-level evidence based on randomized clinical trials (RCTs) is still pending^[11]. Techniques for minimally invasive “major” resections are still developing, and no consensus has been adopted, but suffice to say that laparoscopic liver surgery demands a high skill level with advanced experience in both open resection and laparoscopic proficiency. Overall, many high-volume centers perform roughly half of their liver resections minimally invasively.

As the robotic platform expands, experience with robot-assisted liver resection (RALR) has increased dramatically. The robotic approach affords advantages over traditional laparoscopy including optics with increased magnification and the ability to visualize the surgical field with depth perception. In addition, the robotic system allows for greater degrees of freedom in the instruments due to the wrist-like action at joints, facilitating tasks such as suturing for hemorrhage control. For these reasons, it has been suggested that the robotic approach is easier to learn as a method of minimally invasive liver surgery^[11]. In a review by Salloum *et al.*^[12] summarizing the experience of 447 cases of RALR reported in 14 series, the authors concluded that there is no clear advantage of RALR over conventional laparoscopic hepatectomy at this time, but more vigorous study designs are necessary to draw meaningful conclusions between different techniques. Similar to the costs of laparoscopic surgery, increased intraoperative times and equipment costs of RALR compared to open liver resection are often offset by reduced complications and hospital length of stay. Our own experience indicates that it is a viable alternative to open liver resection even when cost is taken into consideration^[13]. Reviews of mostly retrospective data have generally found no difference in postoperative outcomes including mortality, morbidity, length of hospitalization, and margin status between laparoscopic and robotic hepatectomy^[14-16]. Laparoscopic hepatectomy did demonstrate lower blood loss^[16] and reduced operative time as well as cost compared to robotic surgery^[15]. Progress in imaging technology, haptic feedback, vascular control, and artificial intelligence will accelerate the adoption of the robotic platform, and therefore an additional minimally invasive option versus open resection, for hepatobiliary surgery in the future. Once considered a large open operation with significant morbidity, hepatic resection can now be considered a minimally invasive therapy in many instances.

Ablation of hepatic tumors

The ablation of HCC is another option typically utilized in BCLC 0/A-stage tumors that are less than 3 cm in size. Ablation can be performed using several techniques including thermal, chemical, or non-thermal. Thermal ablation typically consists of radiofrequency ablation (RFA), which is the application of an electrical current through the tissue to generate heat and cause coagulation necrosis. RFA has emerged as the most commonly used ablation technique overall, either via a minimally invasive or open surgical approach. The long-term results are satisfactory with reported local recurrence rates at 5 years ranging from 10%-32% and OS has been shown to be 40%-68% at 5 years^[17-24]. Several clinical trials have shown it to be superior to percutaneous ethanol injection^[25-28]. Alternatively, microwave ablation (MWA) uses electromagnetic energy rather than electric current to generate heat, and is less reliant on heat conduction compared to RFA. Both methods report similar local control and complication rates^[29]. In a RCT of RFA vs. MWA, the local recurrence rate for RFA was found to be 10% at 2 years compared to 24% for the MWA group, although this trend was not found to be statistically significant^[30]. But neither RFA nor MWA should be used when the tumor is adjacent to major vascular or biliary structures, and instead, irreversible

electroporation (IRE) may be considered for these lesions. IRE involves the application of an electric field above a threshold that causes irreversible damage to the cell membrane but below the threshold causing thermal damage thus minimizing coagulative necrosis. The non-thermal nature of this technique allows potential application when lesions are near important structures^[31]. Large-scale clinical data remains scarce for this technique, with retrospective studies showing local tumor progression rate within the first year of 20%-34%^[32,33] and progression free survival rate of 70% at 12 months^[32,33]. Overall, the two thermal ablation techniques (RFA and MWA) appear to provide similar outcomes for patients with HCC lesions less than 3 cm located away from major vascular or biliary structures and while more data is required, the IRE technique is promising as an alternative for small lesions located next to major structures.

Current practice advocates a minimally invasive approach to liver tumor ablation such that treatments can usually be performed on an out-patient basis. For tumors lying deep in the liver parenchyma, image-guided percutaneous approach is often feasible. However, for lesions that are near the periphery of the liver where it comes within 1 cm of the visceral structures (e.g., stomach, duodenum, colon, gallbladder, diaphragm), we prefer a laparoscopic approach to safely avoid injuries to such organs. In patients with sub-diaphragmatic lesions (e.g., segment 7, 8) especially in the setting of multiple prior open abdominal surgeries involving the right upper quadrant, we recommend a minimally invasive thorascopic approach. Open ablations are reserved for patients who are undergoing laparotomies for other indications.

Trans-arterial therapies for HCC

For patients with multinodular tumors (> 3) and those larger than 5 cm (i.e., BCLC stage B), catheter-based therapies are recommended if otherwise not a resection candidate^[34]. Options for catheter-based therapies include transarterial bland embolization, chemoembolization (TACE), or radioembolization (TARE) using yttrium-90 (Y90) glass beads. For these patients who have contraindications to undergo resection or ablation, TACE has been demonstrated in RCTs to be superior in terms of survival compared to supportive care^[35,36]. For Y90 radioembolization, the SARAH trial in Europe did not demonstrate a difference in OS with Y90 vs. sorafenib as first-line therapy, but did show better local tumor response and improved quality of life, as indicated by lower total and median numbers of treatment-related adverse events in the Y90 group^[37]. Similarly, SIRveNIB trial in Asia did not demonstrate an OS difference when comparing Y90 radioembolization to sorafenib, but similarly showed increased tolerability to treatment with radioembolization^[38]. Importantly, liver-directed Y90 treatment was not inferior to sorafenib as first-line therapy for patients with advanced HCC confined to the liver, thus providing meaningful options for these patients.

Comparing lobar TACE with TARE, both methods appear to have similar OS^[39-44]. Patients undergoing TARE benefit from longer time to progression^[43] and progression-free survival^[45] compared to TACE with shorter hospitalization stays^[41,42]. In a comparative effectiveness study of various transarterial strategies based on network meta-analysis, chemo- and radio-embolization provide improved tumor objective response over control (supportive care) and bland embolization, but did not show survival benefit over bland embolization alone^[46].

In recent years, there is a trend towards the use of selective, high-dose radioembolization, so-called radiation segmentectomy, for HCCs that receive their arterial supply predominantly from one segmental artery; these lesions tend to be located more peripherally rather than central tumors that often draw blood supply from multiple segmental branches. In the appropriate patients, Y90 segmentectomy is designed to deliver higher radiation dose to the target lesion while sparing more of the non-tumor liver. In a retrospective experience of 178 patients undergoing segmental catheter-based treatments for HCC at our institution, propensity score-matched analysis highlights 92% complete response of the index lesion following Y90 segmentectomy compared with 74% in the TACE group^[45]. Progression-free survival was

significantly longer following TARE, but significant OS benefit was not achieved. Larger multi-center experience will be necessary to better inform us of the clinical value of this approach.

Radiation therapy: photons and protons

Radiation is another modality available in the loco-regional treatment of HCC for patients who are not surgical candidates and in whom catheter-based approaches are not preferred or have failed prior TACE. Bilobar multifocal tumors and proximity to hollow viscus can pose technical challenges to external beam radiotherapy, as with patient with poor liver reserve (e.g., $\geq B9$) or fluctuating ascites. Historically, the use of external beam radiation therapy (EBRT) was limited by radiation induced liver disease (RILD). The advances in modern technique known as stereotactic body radiation therapy (SBRT) allows for the delivery of more precise radiation to the lesion of interest while sparing normal liver and other structures. Several phase I and II studies of photon SBRT have found favorable local control rates of 78%-96% and OS of 58%-94% at 1 year with acceptable toxicity (8%-39% grade 3 or greater, RILD 4%-7%)^[47-52]. While SBRT relies on photons to deliver radiation dose, charged particles such as protons have emerged as an alternative technique to deliver radiation. The advantage of proton beam therapy is the ability to control the energy along its beam path, thus minimizing the exit dose. This allows for precise delivery of the radiation dose to the lesion and sparing greater liver parenchyma. Phase I/II studies using proton therapy found 2 to 3 year OS of 50%-63% with 0%-6% grade 3 or greater toxicities^[53-56]. No RCT has been performed directly comparing photon SBRT and proton beam therapy, but both modalities appear safe and effective in the treatment of HCC. The enormous cost of installing a proton center limits its widespread use. Nonetheless, modern techniques in external beam radiotherapy has emerged as an effective alternative for the local control of HCC in patients who are not suitable to undergo resection or ablation.

COMPARISON OF MODALITIES FOR LOCO-REGIONAL TREATMENT OF HCC

Resection vs. ablation

For patients who are stage BCLC 0 and A, resection and ablation are recommended as treatment modalities. Several prospective RCTs have attempted to evaluate which of the two modalities, if any, is superior. An early study from China investigated percutaneous ablation vs. open surgical resection and found statistically equivalent OS of 68% and 64% respectively, as well as statistically equivalent DFS rates of 46% and 52% respectively^[57]. Greater morbidity and the only death reported in the study occurred in the surgical group. A second RCT from China, in contrast, found that 5-year OS was higher in the open resection group compared to the percutaneous RFA group (75% vs. 55%, respectively) with lower recurrence rates of resection compared to the RFA group (42% and 63%, respectively)^[58]. However, the open resection group had a greater rate of adverse events than the RFA group. A third study again from China comparing percutaneous RFA with open hepatectomy did not find a difference in 3 year OS between RFA and resection (67% vs. 75%, respectively), with no difference in the recurrence rate at 3 years (38% vs. 50% for resection and RFA, respectively) but a higher complication rate in the resection group^[59]. A more recent study from Hong Kong which included long term follow-up to 10 years, showed statistically similar OS of 48% in the open resection group and 42% for the RFA group. Recurrence-free survival was 29% in the resection group and 18% in the RFA group, which did not meet statistical significance^[60]. In this study, the postoperative complication rate did not differ between the two although RFA did have shorter length of stay. Taking all prospective RCTs into account, it appears that the survival and recurrence rates are similar between RFA and resection, especially for smaller tumors (i.e., ≤ 3 cm) with the added benefit of fewer complications with ablation. However, no trial has evaluated the outcome of ablation against those of laparoscopic or robotic hepatectomy, which is expected to have lower morbidity compared to open resection. Other factors include methods of ablation such that higher local recurrence has been reported following percutaneous ablation compared with laparoscopic or open procedure. Collectively, for HCCs ≤ 3 cm, clinical outcomes are comparable between ablation and resection, thus selection between the two modalities lies with providers' experience and patients' preference. Our institutional bias is to

offer a minimally invasive approach for either ablation or resection that will provide optimal local control while preserving liver reserve.

Resection vs. TACE

As trans-catheter based techniques developed in managing HCC, the effectiveness of TACE was evaluated against resection as the standard. To date, one RCT in China has been performed directly comparing the two treatment modalities in patients with multiple resectable HCC lesions that fell outside of the Milan criteria. The 3-year OS was significantly higher in the hepatectomy group at 52%, compared to 18% in the TACE group^[61]. Similar results are reported in several propensity score matched non-randomized clinical trials, all showing an overall statistically significant improved OS with resection (18%-54% at 5 years) compared to TACE (12%-34% at 5 years)^[62-66]. A recent meta-analysis which included an additional 12 non-randomized controlled trials also found improved OS, 1-, 3-, and 5-year OS with resection compared to TACE with equivalent procedure related mortality^[66]. Across all studies, the findings of improved survival after resection compared to TACE were consistent across BCLC stages studied. Therefore, in patients with resectable HCC, hepatectomy is superior to TACE, however, there exists a role of catheter-based approaches in patients with potentially resectable HCC but with limited hepatic reserve.

Ablation vs. TACE

In patients with HCC who are not resection candidates, other treatment options of the loco-regional disease include ablation or catheter-based approaches. While no RCT has been performed comparing the two, they have been compared using propensity-score matching analysis in retrospective studies. A retrospective study from Taiwan found that in patients within the Milan criteria (single tumor less than 5 cm, or 3 or fewer nodules less than 3 cm) with performance status of 0, OS was significantly better in the RFA group compared to the TACE with drug eluting beads group (77% vs. 62% at 3 years, respectively)^[67]. In patients with worse performance status (≥ 1), survival difference was no longer evident. In other retrospective studies from China and Japan, RFA improved survival of BCLC o/A patients compared with patients who were also BCLC o/A but instead received TACE, but this difference was attributable to differences in co-morbidities between the two groups^[68,69]. One of these studies did find that the cumulative recurrence rate was higher following TACE. Currently when HCC is unresectable but ablatable, thermal ablation remains the treatment of choice in BCLC o/A patients. Otherwise, TACE is a viable alternative in providing a survival benefit over supportive care.

Radiation therapy vs. other loco-regional treatments

Radiation therapy has grown in popularity for its potential uses in loco-regional management of HCC. Few retrospective studies have evaluated radiation vs. ablation; a propensity matched analysis based on SEER database (2004-2012) found that ablation was associated with improved survival compared to EBRT in patients with tumors greater than 3 cm, while EBRT and ablation were equivalent in patients with tumors less than 3 cm^[70]. A separate retrospective study of SBRT vs. RFA also showed no significant difference in survival between SBRT and RFA, nor time to progression for tumors less than 2 cm^[71]. However, for larger tumors, it reported the opposite findings with improved time to local progression in the SBRT group vs. the RFA group. One RCT has been performed comparing proton therapy to TACE therapy for HCC meeting transplant criteria. Results of an interim analysis demonstrated no difference in OS at 2 years, but there is a trend towards improved progression-free survival and local tumor control favoring the proton radiation therapy group^[72]. Further prospective evidence is needed in order to draw conclusions about the effectiveness of radiation therapy, but the data thus far indicates it will play a major role in the management of HCC.

SELECTION OF TREATMENT MODALITY

With the expansion of options that are currently employed in loco-regional management of HCC, clinicians are faced with the challenge of selecting the most appropriate treatment for individual patients. In the era of

Table 1. Compilation and comparison of reported data from prospective clinical studies

Treatment modality	Local control	Overall survival at 1 year	Rate of adverse events (grade ≥ 3)
Open surgical resection ^[57-61,78]	96%-99%	93%-98%	16%-55%
Percutaneous or laparoscopic ablation ^[26,30,57-60,78]	87%-96%	87%-98%	4%-9%
TACE or TARE ^[36-38,61,72]	45%-68%	40%-77%	7%-54%
External radiation ^[47,48,72]	78%-96%	58%-94%	0-39%

[†]Based on the Clavien-Dindo classification system; ^{*}predominately radiofrequency ablation rather than MWA; ^{**}these studies use largely non-selective techniques (e.g., lobar treatment); ^{***}both photon and proton radiotherapy included. TACE: transarterial chemoembolization; TARE: transarterial radioembolization; MWA: microwave ablation

personalized medicine, the spectrum of minimally invasive liver-directed therapies outlined above allows for a greater number of patients to potentially benefit from these survival-prolonging treatments. Advances in precise tumor targeting have led to better preservation of hepatic function in patients with underlying liver disease; this is particularly relevant to those who are not transplant candidates. Based on current evidence, the rates of local tumor control following hepatic resection, thermal ablation, and external beam radiation therapy are approaching parity for small HCCs, but there has not been any direct comparison across all modalities to account for confounders, and long-term results are lacking for the newer techniques [Table 1]. Excluding transplantation, which benefits a small fraction of patients, surgical resection offers the best chance of cure while the results of thermal ablation for HCC ≤ 3 cm is on par with that of hepatectomy. At present, both modalities are considered curative with the major difference between the two being the severity of treatment-related morbidity, but through the use of laparoscopic or robotic liver resection, the gap has been minimized. The choice between resection and ablation for small HCCs comes down to provider's preference based on tumor location, liver reserve and co-morbidities. For those who are at higher risk for general anesthesia, radiation, either internal (Y90) or external (SBRT), offers excellent local control. While these options are considered palliative in the past, current evidence using selective Y90 segmentectomy and SBRT/proton radiation yield approximately 90% local control at 2 years. Currently, there are only a handful of studies using radiation segmentectomy reporting such high rates of success, but if confirmed in larger long-term studies, radiation may carry similar efficacy as ablation or resection. Results from on-going trials will better define the role of these modalities, but if they live up to their expectations, clinicians will have the luxury to offer a variety of minimally invasive treatment options that best suit the patient and his/her clinical scenario including factors related to the tumor, liver reserve, performance status, as well as cost and social circumstances. The large socioeconomic impact of new therapies has led to financial toxicity for many patients diagnosed with cancer, which can limit access and treatment adherence leading to adverse outcome^[73]. Greater emphasis on fiscally responsible care is particularly relevant to HCC management given the wide disparity in the cost of surgery, ablation, radiation and systemic therapies. Based on Markov modelling, it has been suggested that RFA is more cost-effective than SBRT as the initial management of unresectable HCC, however, for recurrent disease, SBRT was favored over repeat RFA^[74]. Another study demonstrated that the addition of TACE to sorafenib or non-sorafenib chemotherapy is more cost effective than systemic therapy alone^[75]. As the financial burden rises, some resources may become limiting, and physicians and their patients will need to have open discussions regarding the wise utilization of available options that meet their personal goals.

In summary, loco-regional treatments of HCC are improving across all disciplines. Current and future directions include the investigation of combination strategies. For example, a number of trials have examined the addition of radiation therapy to TACE, which was shown to have improved OS and progression free survival in patients with macroscopic vascular invasion compared to sorafenib^[76]. Combination TACE plus radiation therapy also showed improved rate of complete response and DFS compared to TACE alone^[77]. Further, the combined use of minimally invasive loco-regional therapies and systemic drugs such as kinase inhibitors and immunotherapies is also being examined with the hope of improving the chance of cancer-free survival while preserving quality of living.

DECLARATIONS

Authors' contributions

Contributed to all aspects of the article including topics of coverage, format of discussion, writing and editing of the manuscript: Sullivan KM, Yeung RS

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Stemness features in liver cancer

Margherita Correnti¹, Richell Booiink², Giovanni Di Maira³, Chiara Raggi^{1,3}, Fabio Marra³

¹Center for Autoimmune Liver Diseases, Humanitas Clinical and Research Center, Rozzano 20089, Italy.

²Department of Biomaterial Science and Technology, University of Twente, Enschede 7500AE, The Netherlands.

³Department of Clinical and Experimental Medicine, University of Florence, Florence 50141, Italy.

Correspondence to: Dr. Fabio Marra, Department of Clinical and Experimental Medicine, University of Florence, Florence 50141, Italy. E-mail: fabio.marra@unifi.it; Dr. Chiara Raggi, Department of Clinical and Experimental Medicine, University of Florence, Florence 50141, Italy. E-mail: chiara.raggi@unifi.it

How to cite this article: Correnti M, Booiink R, Di Maira G, Raggi C, Marra F. Stemness features in liver cancer. *Hepatoma Res* 2018;4:69. <http://dx.doi.org/10.20517/2394-5079.2018.96>

Received: 26 Aug 2018 **First Decision:** 26 Sep 2018 **Revised:** 24 Oct 2018 **Accepted:** 27 Oct 2018 **Published:** 5 Nov 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cai-Hong Wang **Production Editor:** Zhong-Yu Guo

Abstract

Heterogeneity is a cardinal hallmark of cancer, including primary liver cancer (PLC), and occurs at different layers including putative cell-of-origin. Current evidence suggests that within cellular subpopulations in PLC there are stem-like cells, the cancer stem cells (CSCs). The CSC concept has been recently proposed as an explanation of such intra-tumor heterogeneity. According to this model, CSCs are responsible for tumor initiation, recurrence, metastasis as well as drug-resistance. However, although the CSC hypothesis is intriguing and supported by a large number of experimental studies, there are still open questions regarding the origin of putative CSCs. Since chemo-resistance and recurrence represent major issues in PLC treatment, the development of new therapeutic strategies is needed, for which a good understanding of tumor behavior and in particular of CSCs biology is an imperative prerequisite. In this review we summarize the regulatory pathways that support CSC features in PLC. Moreover, we highlight the key features of hepatic CSC, in terms of enhanced drug-resistance, increased metastatic potential and metabolic rearrangement. Knowledge of the molecular mechanisms underlying CSC biology may provide novel options for PLC combination therapies.

Keywords: Hepatocellular carcinoma, cholangiocarcinoma, cancer stem cells, tumor heterogeneity, drug-resistance

MULTIPLE CELLS-OF-ORIGIN OF PRIMARY LIVER CANCER

Primary liver cancer (PLC) is one of the most common cancers worldwide and the second leading cause of cancer-related mortality^[1,2]. The major forms of PLC comprise hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA)^[1,3-5]. HCC accounts for approximately 90% of all PLCs^[1,3], while CCA is the



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second most common form and accounts for about 5% of all PLCs^[3-5]. HCC causes over 600,000 deaths worldwide annually, and its incidence and mortality are increasing at a fast rate^[6-10]. On the other hand, CCA is characterized by a very poor prognosis, with a 5-years survival lower than 20%, and its incidence and worldwide mortality are also increasing^[5,11-13]. The high mortality rate of CCA may depend on its non-specific or silent clinical features and the lack of specific markers that make it difficult to diagnose^[14-16].

Many studies carried out in these last years have attempted to define which type of epithelial cell [hepatocytes, cholangiocytes, hepatic progenitor cells (HPCs) or all three] should be considered as the PLC cell of origin^[17]. For a long time, HCC and CCA have been commonly accepted to derive from hepatocytes and cholangiocytes, respectively. Since mature hepatocytes and cholangiocytes have an enormous self-renewal capacity and longevity, they meet the requirements to be targets for oncogenesis^[17-23]. Detailed analyses of a wide range of PLC tumor types have reported that a rare form of combined HCC-CCA (cHCC-CCA) has intermediate characteristics between HCC and intrahepatic CCA (iCCA), suggesting that they could share the same stem/progenitor cell origin^[18-24]. In this regard, since most PLCs arise on the background of chronic liver disease in the presence of an extensive activation of the HPC compartment (the so-called ductular reaction), several studies suggested that PLCs can be derived from HPCs rather than from mature cell types^[25]. HPCs situated in the canal of Hering physiologically act as a reserve cell compartment activated in case of liver damage or when mature hepatocytes and/or cholangiocytes replication is compromised. These cells are bipotential, and may differentiate into either hepatocytes or cholangiocytes^[26-28]. During the differentiation in malignant cells, bipotential HPCs undergo maturation arrest and give rise to a spectrum of tumor phenotypes with both admixed hepatocellular and cholangiocellular features, such as cholangiolocellular carcinoma and cHCC-CCA^[29-31]. Additionally, a new subtype of CCA-like HCC (CLHCC) has been discovered and characterized as HCC expressing CCA-like traits^[32]. CLHCC co-express embryonic stem cell (ESC) traits and hepatoblast-like genomic signatures, suggesting a HPC origin. These lines of evidence provided important insight into the heterogeneous progression of PLCs, which imply a common evolutionary origin from cells at different developmental stages^[31-33]. The hypothesis of a progenitor cell origin has been supported by new advancement in genome wide analysis. Indeed, it has been suggested that iCCA and HCC are closely related at molecular level^[19,29,34,35], since both tumor types share common copy number variations^[11,36].

Such phenotypic variability and presence of progenitor cell features in PLC can be explained in two ways: either the cell of origin is a progenitor cell with acquired genetic alterations or, alternatively, mature tumor cells de-differentiate acquiring progenitor cell features during carcinogenesis (de-differentiation theory^[37-40]). Interestingly, new findings provide direct evidence that any cell in the hepatic lineage can be the cell of origin of PLC^[41]. In this regard, it has been recently suggested the development of iCCA by lineage conversion of malignant hepatocytes, through a co-activation of both Notch and protein kinase B (AKT) signaling, contributes to the acquisition of stem/progenitor cell features^[42,43]. In spite of the marked plasticity in the underlying cells of origin, current evidence suggests that most PLCs are derived from undifferentiated cells with stem-like capabilities^[40].

UNDERSTANDING THE CONCEPT OF CANCER STEM CELL

Extensive clinical and pathobiological heterogeneity at the level of cellular morphologies, genetic fingerprints and responses to therapies is a cardinal hallmark of cancer, including PLC. Such tumor complexity may reflect the presence of different cell subtypes with distinct self-renewal and differentiation potentials^[40,44-46]. The traditional view of cancer development is based on a stochastic model, which states that every malignant cell may undergo genetic and/or epigenetic alterations and clonally expand to initiate tumor growth. Thus, every cell within the tumor may be equally responsible for tumor initiation and progression^[47-51]. Unlike the stochastic model, the hierarchical or cancer stem cell (CSC) model may explain intra-tumor heterogeneity representing tumor as a hierarchically organized tissue with CSCs at the apex in the pyramid and more committed and differentiated tumor cell types progressively down^[47-50].

According to this model, CSCs represent a fraction of cells resident in the tumor endowed with stem-like features like the ability to self-renew and differentiate into heterogeneous tumor cell progeny as well as with the unresponsiveness to treatments^[52,53], and represent the unit of selection within the tumor, while any other bulk tumor cells lead to clonal exhaustion^[50]. More importantly, CSCs are thought to be a unique cellular subset responsible not only for tumor initiation but also for tumor growth maintenance, tumor recurrence and metastasis, showing intrinsic resistance to chemotherapeutic drugs compared to bulk tumor cells^[52,54-56]. In this view, the existence of CSCs represent an entirely distinct dimension of intra-tumoral heterogeneity^[57].

Interestingly, a third model has been recently proposed to explain the intra-tumor heterogeneity, the so-called “CSC plasticity model”. According with this theory, tumor cells represent a very plastic and dynamic population, with the ability to continuously shift between non-CSC and CSC states, in response to intrinsic and extrinsic stimuli. In this view, the stochastic and the CSC model not only are not mutually exclusive, but can be integrated with each other, adding a new level of tumor complexity^[58].

The idea that tumor initiation and progression are driven by stem-like cells is still a subject of debate, since the first time it was proposed^[59] until today. While CSC existence has been confirmed in a growing range of hematologic and solid tumors (e.g., acute myeloid leukemia, pancreatic cancer, breast cancer, lung cancer, hepatocellular carcinoma, head and neck cancer, colon cancer, prostate cancer, melanoma, and glioblastoma), no agreement has yet been reached regarding the origin of putative CSCs^[60]. Some reports have indicated that CSCs can originate from normal resident stem cells, due to their inherent self-renewal capacity and long life span that can allow them to accumulate oncogenic and epigenetic modifications, resulting in malignant transformation. Alternatively, CSCs may originate from more committed progenitor cells^[47], or even from differentiated non-CSCs that re-acquire stem cell properties by de-differentiation or reprogramming processes^[61,62]. Thus, tumor hierarchical organization does not imply that CSCs originated from normal stem cells, and the CSC model does not address the cell-of-origin, that represents the normal cell that acquires the first cancer-promoting mutation(s) and is not necessarily related to the CSC concept^[63,64]. These considerations interconnect with the debate on the true nature of the cell-of-origin of PLC. While it has already been accepted that HCC progression is driven by CSCs^[22,65-69], very few studies have indicated the presence of CSCs in CCA^[70] (reviewed in^[71]).

REGULATORY PATHWAYS INVOLVED IN PLC-ASSOCIATED STEMNESS

Many of the identified CSC regulatory pathways are also known to be involved in normal stem-cell maintenance as well as in self-renewal potential and pluripotency of embryonic stem cells^[72-77]. Here, we will briefly review the key regulatory pathways that support stemness features in the context of PLC [Figure 1].

Wingless-type MMTV integration site family member (Wnt)/ β -catenin pathway

Disruption of Wnt/ β -catenin signaling results from both genetic and epigenetic changes in many tumors, including PLC. Wnt/ β -catenin canonical signaling pathway appears to be involved in stemness maintenance in both embryonic and cancer stem cells^[78,79]. Extracellular Wnt ligand binds to Frizzled cell surface receptors leading to increased cytoplasmic β -catenin levels, with the following induction of Wnt key target genes^[31,55,80]. Notably, β -catenin is expressed in 58% of CCA, mutated in 8% of cases and it is considered an early determinant in CCA-progression^[71]. In up to 90% of HCCs, the Wnt receptor FZD-7 is overexpressed, and 20%-40% of HCCs have unusual cytoplasmic and nuclear accumulation of β -catenin^[81]. Moreover, in 25% of HCCs, β -catenin and Axin1 mutations are observed^[69,81].

Notch signaling pathway

The Notch canonical signaling plays an important role in cell differentiation, proliferation and apoptosis, as well as in stem cell and HPCs maintenance^[31,71,82,83]. Moreover, Notch signaling is implicated in bile duct morphogenesis (reviewed in^[84]), and dysfunction in this pathway may result in reduced detoxification,

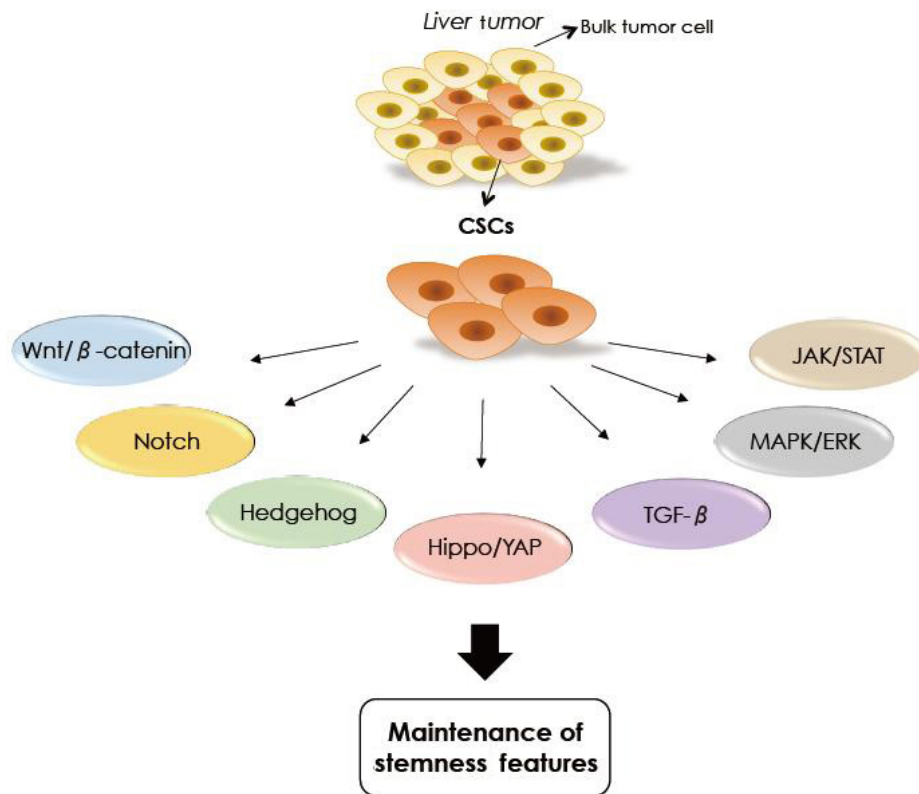


Figure 1. Regulatory pathways of liver cancer stem cells (CSCs). Primary liver cancers are heterogeneously composed by bulk tumor cells and CSCs. Liver CSCs are characterized by the activation of several molecular regulatory pathways that contribute to support the maintenance of CSC stemness features, including Wnt/ β -catenin, Notch, Hedgehog, Hippo/Yes-associated protein (YAP), transforming growth factor- β (TGF- β), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling pathways

ultimately leading to liver damage and iCCA development. Interestingly, the expression of Notch receptors 1 and 3 correlates with CCA progression and poor survival^[71], whereas overexpression of Notch receptors 1 and 4 in HCC exerts tumorigenic effect^[85]. Moreover, in up to 30% of HCCs, nuclear expression of Notch 1 and 3 is associated with the presence of stem cell signatures, supporting the role of Notch in promoting the expansion of the CSC niche^[81,86]. Since Notch signaling can contribute to either CCA or HCC, it has been suggested that this pathway could be deregulated in bipotential HPCs^[82].

HEDGEHOG SIGNALING PATHWAY

The Hedgehog (Hh) pathway regulates embryonic development, cell differentiation, regeneration and stem cell biology. The aberrant activation of the Hh pathway has been reported in different malignancies^[87], and its correlation with prognosis is well known^[88]. In addition to HCC carcinogenesis and HPC proliferation, activation of Hh pathway promotes CCA proliferation^[71,79]. Notably, Sonic Hh (Shh) is the predominant ligand in the liver and is overexpressed in over 60% of HCCs^[31,69,81,89].

Hippo signaling pathway

The Hippo signaling cascade is an evolutionarily conserved pathway involved in organ development^[90-92]. This pathway has been implicated in multiple events during tumor onset. Strong evidence indicates a significant role of Hippo signaling in regulating stem cells, including HPCs^[93-95]. Yes-associated protein 1 (YAP1) is a primary effector of the Hippo cascade and is frequently expressed in HCC and cHCC-CCA mixed tumor types, which retain stemness-related features^[94]. Furthermore, constitutive activation of YAP in bile ducts, in association with AKT, seems to be essential in inducing CCA in a murine biliary injury model^[31,96].

Phosphatidyl inositol 3-kinase/AKT signaling

AKT plays a critical role in many human cancers, including HCC and CCA^[3,97]. AKT signaling can be triggered downstream of tyrosine kinase receptors activation, phosphatidyl inositol 3-kinase (PI3K) constitutive activation or loss of phosphatase and tensin homolog (*PTEN*)^[3]. *PTEN* deletion results in the proliferation of a CD133+ population^[71,98]. PI3K signaling promotes stem-like properties of HCC cells and it is implicated in HCC chemo- and radio-resistance as well as in epithelial-to-mesenchymal transition (EMT) and metastasis^[99-104]. Notably, the co-activation of *AKT* and neuroblastoma rat sarcoma viral oncogene homolog (*N-RAS*) oncogenes leads to development of cHCC-CCA-like liver tumors, through the expansion of HPCs or malignant conversion of hepatocyte into progenitor-like cells^[42].

Mitogen-activated protein kinase/extracellular signal-regulated kinases signaling pathway

The mitogen-activated protein kinase (MAPK) cascade regulates many important cell function, such as proliferation, invasion and survival and is critical for HPCs proliferation^[71]. Gain-of-function mutations of *KRAS* are some of the most frequent mutations observed in iCCA, defining a class of patients characterized by poor outcome and enriched in CCA stem like-cells and tumor recurrence predicting signatures. Moreover, these mutations are also detected in patients with primary sclerosing cholangitis, suggesting that this could be an early event that contributes to the malignant transformation of cholangiocytes^[36]. It is known that the MAPK pathway is directly associated with HCC cell growth and tumor-initiating capability^[105-107]. Moreover, the long non-coding RNA H19 is highly expressed in HCC cells, where it activates the MAPK/extracellular signal-regulated kinases signaling pathway, regulating oxidative stress and chemotherapy resistance of CD133+ HCC CSC^[108].

Transforming growth factor- β signaling

The transforming growth factor- β (TGF- β) pathway plays a key role in self-renewal and maintenance of an undifferentiated stem cell state. Its disruption is implicated in CCA development through impairment of stem cell differentiation and deregulated proliferation of HPCs^[98]. Nonetheless, the role of TGF- β in PLC development is still controversial. Indeed, TGF- β acts as a tumor suppressor early in tumor initiation, whereas at late stages it promotes tumor growth, metastasis and EMT. It has been demonstrated that TGF- β 1/Snail activation induces EMT in CCA both *in vitro* and *in vivo*, and this is associated with a higher CCA aggressiveness^[109]. Moreover, TGF- β is upregulated in 40% of HCCs^[69,81,89], and it may promote HCC progression via regulatory T cells recruitment and subsequent creation of a tumor suitable microenvironment^[110,111].

Janus kinase/signal transducers and activators of transcription signaling

Several lines of evidences highlight the central role of interleukin (IL)-6/signal transducers and activators of transcription 3 (STAT3) signaling in CCA. Binding of IL-6 to the gp130 receptor leads to Janus kinases (JAKs) (JAK1, JAK2 and TYK2) and STAT3 activation, inducing the transcription of target genes essential for cell growth, differentiation and proliferation (reviewed in^[34,112]). STAT3 signaling is also involved in maintenance of CSC population^[113-115] and EMT-triggering in diverse tumors, including PLC^[116,117]. Increased IL-6 expression has been reported to drive CSCs expansion through STAT3 activation in HCC^[118]. Moreover, a recent study has demonstrated that EMT+ metastatic CSCs can be generated in a β 2SP^{+/-} mouse model of HCC, mainly due to overexpression of IL-6 in addition to the partial disruption of TGF- β signaling^[119].

KEY FEATURES OF LIVER CSCS

Drug-resistance

A fundamental aspect contributing to poor PLC survival rate is the unresponsiveness to conventional therapies^[11,12]. Currently, effective treatment is limited to surgical resection for both HCC and CCA, as well as liver transplantation for HCC. Unfortunately, 80% of HCC patients are diagnosed at an advanced tumor stage, which is not amenable to curative treatment^[8-10]. Although other treatment procedures (e.g.,

cryosurgery, radiofrequency ablation and embolization) are also available, they are mostly palliative approaches and the treatment regime is shifting towards systemic chemotherapy^[9]. Moreover, more than 70% of patients with early-stage HCC develop post-surgery recurrence^[9,110]. Likewise, CCAs are generally asymptomatic in early stages and are usually diagnosed at an advanced unresectable stage. Moreover, although chemotherapy improves the patients' quality of life, it still remains only a palliative treatment^[5,13,120]. Therefore, the majority of patients with unresectable CCA undergoes a rapid decline in clinical conditions and dies within 12 months of the onset of symptoms. Thus, PLC still remains a fatal disease, mainly due to frequent tumor recurrence and chemoresistance.

CSCs represent a peculiar sub-compartment of tumor cell population crucially involved in recurrence, metastasis as well as drug resistance^[86,121] [Figure 2]. CSCs can escape drug-induced cell death through different intrinsic and external mechanisms. The intrinsic mechanisms consist of enhancement of DNA damage repair pathways, self-renewal ability of CSCs, high expression of drug efflux-related proteins and over activation of growth- and other stem-related pathway. The external mechanisms refer to the role of the tumor microenvironment (TME) on CSC drug resistance. This includes TME-derived EMT signals, hypoxia stimulation and angiogenesis trigger^[122]. Consistently, increasing evidence suggests that sorafenib resistance in HCC correlates with the activation of EMT and enrichment of CSC traits^[123-125].

Several CSC markers seem to be implicated in drug resistance, such as CD13, that protects PLC CSCs from apoptosis and ROS-dependent DNA damage induced by different chemotherapeutic drugs (e.g., 5-FU)^[86]. The HCC epithelial cell adhesion molecule (EpCAM)+ CSCs also show chemo-resistance against genotoxic agents like 5-FU^[9]. Next, CD133+ HCC CSCs exhibited chemo-resistance to fluorouracil and doxorubicin through AKT and Bcl-2 pathway activation. Furthermore, CD133+ CSC and CSC spheres isolated from HCC cell lines display enhanced resistance to a panel of chemotherapeutic drugs (e.g., paclitaxel, methotrexate, vinblastine, cisplatin, carboplatin, docetaxel, irinotecan, etc.)^[126,127]. According to these data, we have recently demonstrated that CCA CSCs isolated by tumor sphere assay possess higher resistance to common chemotherapeutic agents^[70]. Additionally, laminin-332 expression is fundamental for maintaining self-renewal abilities of hepatic CSCs and for inducing mTOR-associated resistance to doxorubicin and sorafenib. Laminin-332 not only protects hepatic cancer cells against chemotherapy but also stimulates simultaneously cell proliferation upon sorafenib exposure, and it has been hypothesized that while laminin-332 may induce quiescence in PLC in "normal" circumstances, under cellular stress (e.g., sorafenib treatment) it could stimulate PLC cells to react by enhancing their proliferation^[17,86].

Metastatic activity

The spread of circulating tumor cells (CTCs) in the blood plays a major role in tumor recurrence and metastasis initiation. Nevertheless, only a subset of CTCs can survive in the bloodstream, migrate to distant sites and establish secondary tumors. Consistent with CSC-hypothesis, stem-like CTCs might represent a potential source for cancer relapse and metastasis^[121,128] [Figure 2]. In fact, mature tumor cells have only a short blood circulation time and mostly die through natural apoptosis. CSCs, however, have shown to have significantly higher viability, enhanced homing ability into the bloodstream as well as higher distant metastasis initiation capability compared to other tumor cells^[121,122,128]. According to the CSC-hypothesis, circulating CSCs (cCSCs) are particularly difficult to eradicate, with a consequent permanence of minimal residual disease and tumor recurrence^[121,128].

Some putative markers has been proposed for identification of liver cCSCs. It has been demonstrated that CD90+ cCSCs express key stem-like genes (e.g., *BMI1*, *CD44*, *OCT4*, *WNT3A*, *STAT3* and *HIF-1α*) at very high levels, also when compared to tissue CD90+ CSCs^[121,129,130]. Moreover, CD90+ CXCR4+ cCSCs are able to initiate tumor metastasis formation in transplanted mice, enhancing the metastasis initiating ability of CSCs^[121,131]. Considering that intercellular adhesion molecule 1 (ICAM1) inhibition by shRNA results in reduced metastasis in mice, ICAM1 has been proposed as another cCSC marker in PLC patients^[121,132]. An

explanation for the different metastatic activity observed between CSCs and other tumor cells might be the EMT status of CSCs, which enables them to have a prominent role in the metastasis and invasion^[122]. Malignant cells undergo molecular changes typical of EMT, which represents a key stage of the metastatic multistep process, and eventually undergo a mesenchymal-to-epithelial transition (MET) to generate secondary tumors in target organs. Hence, CSCs mediate tumor metastasis by maintaining plasticity to transition between epithelial or mesenchymal states, and the EMT process represents the potential link between CSCs and circulating metastasis-initiating cells^[121,133]. For example, in the CCA cell line TFK-1, TGF- β 1 is able to induce not only EMT, but also CSC generation with a consequent decreased sensitivity to the chemotherapeutic agent 5-FU. Furthermore, the EMT-related overexpression of hepatic transmembrane 4L six family member 5 (TM4SF5) has a potential role in generating HCC cCSCs with metastatic properties through interaction with CD44^[121,134]. In addition, HCC CSCs isolated by sphere assay are associated with an enhanced expression of the variant isoforms of CD44, which are related to CSC chemo-resistance, as well as with an increased frequency of intrahepatic metastasis when injected in the spleen of NOD-Rag1^{null} IL2r γ ^{null} double mutant mice (NRG mice). Also in this case, enhancement of the EMT correlates to the metastatic potential and CSC state^[135]. Another study has revealed that CD44 is associated with a mesenchymal phenotype in HCC cell lines, and knockdown of CD44 reverses EMT and inhibits lung metastasis of HCC cells in a murine model^[136]. Another gene expression analysis of microarray data from 238 HCC cases has revealed an enriched EMT signature in CD90+ stem-like cells^[137]. Finally, a recent study has found that CD44 protein levels are enhanced after TGF- β 1 treatment and that interaction between CD44 and TGF- β 1 induces EMT and CSC phenotypes through β -catenin signaling in HCC^[138]. All these findings strengthen the hypothesis of an existing link between EMT and CSC cellular states in relation with the metastatic process.

Metabolic reprogramming

Starting from the pioneering work of Otto Warburg, several observations have indicated that tumor genetic alterations imply also cell metabolism reorganization^[139,140]. In particular, it has been shown that tumor cells produce ATP via glycolysis and accumulate extracellular lactate even under normoxic conditions^[140,141], and often present a limited or absent mitochondrial oxidative phosphorylation (OXPHOS)^[140]. Although metabolic reprogramming is currently considered a hallmark of cancer, no consensus has been reached on the metabolic features of CSCs, which are very plastic and capable of either reside in a dormant state, or rapidly proliferate to replenish the tumor mass. A number of studies suggest that CSCs more strongly favor the glycolytic pathway compared to bulk tumor cells, while other studies report that mitochondrial oxidative metabolism is the prevalent source of energy for CSC (reviewed in^[141]) [Figure 2]. However, even if investigation of PLC metabolism is still at its very beginning in comparison with other tumor systems, recent evidence has revealed the importance of the metabolic rearrangement in PLC CSCs. CD44+ CCA CSCs adapt their redox status regulation according to their needs and contribute to reactive oxygen species (ROS) defense promoting glutathione synthesis by way of xCT (a cysteine-glutamate transporter), resulting in evasion of cell death^[142]. Moreover, CD133+ HCC CSCs are characterized by high glycolytic metabolism with concomitant overexpression of glycolytic genes and enhanced extracellular acidification rate, demonstrating that CD133+ cells are more glycolytic compared to CD133- cells. Further, CD133+ cells stemness features are significantly reduced when glycolysis is inhibited^[143]. Extensive transcriptome and metabolome analysis of CD133+ HCC cells revealed the key role of MYC in the regulation of glycolytic metabolism in HCC CSCs^[144].

There is also an increasing interest in lipid metabolism and specifically in alterations in lipid and cholesterol-associated pathways. It is well known that proliferating tumor cells require lipids and cholesterol, and they may increase the uptake of exogenous lipids and lipoproteins or hyper-activate metabolic pathways deputed to produce lipids and cholesterol. When specifically looking at the stem cell compartment, it has been demonstrated that stem-like cells rely on fatty acid oxidation (FAO) for the generation of ATP and NADH^[145] [Figure 2]. Metabolism analysis has revealed that NAD⁺ concentrations

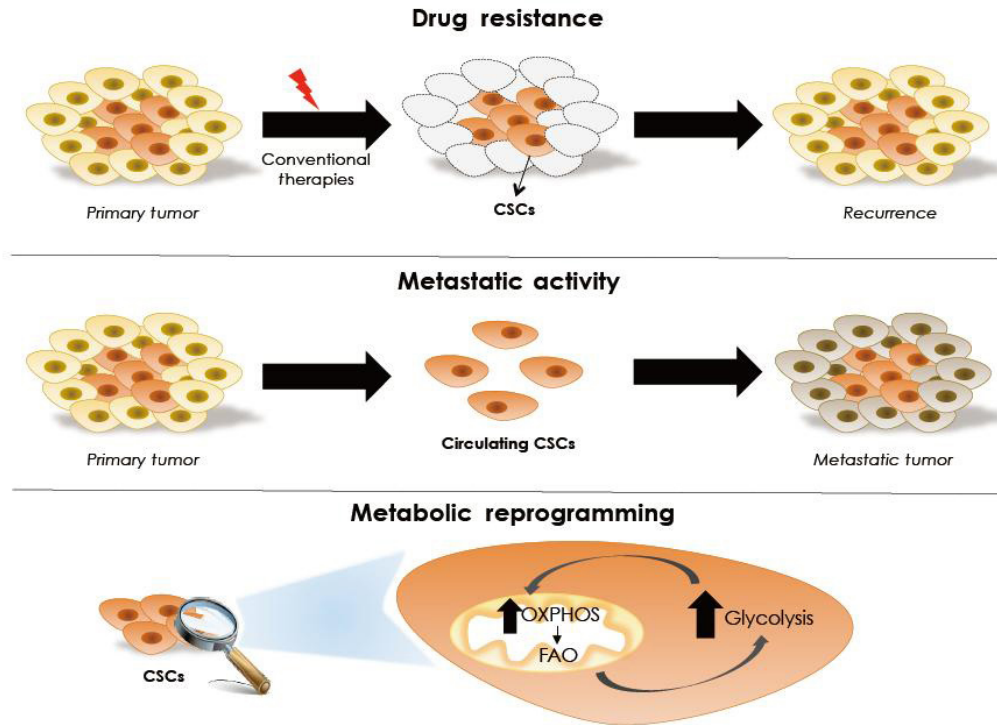


Figure 2. Key features of liver cancer stem cells (CSCs). Liver CSCs present some common key functional features. CSCs represent a peculiar sub-compartment of tumor cells crucially involved in drug resistance. Current therapeutic strategies for the treatment of hepatic cancer mostly focus on the inhibition of tumor growth, resulting in the death of only bulk tumor cells. CSCs are able to survive thanks to intrinsic and extrinsic mechanisms of chemo-resistance and subsequently they can give rise to primary liver cancer recurrence. Moreover, CSCs are the only cells endowed with metastatic potential. While mature tumor cells mostly die through natural apoptosis into blood circulation, CSCs, however, have a significantly higher viability, enhanced homing ability into the bloodstream as circulating CSCs as well as higher distant metastasis initiation capability. Next, CSCs are characterized by metabolic changes, but, no consensus has been reached on the metabolic features of CSCs, which are very plastic and so able to modify their metabolic features according to specific needs. Then, CSCs can exhibit enhanced glycolytic activity as well as increased mitochondrial oxidative phosphorylation (OXPHOS) with subsequent increased fatty acid oxidation, depending on tumor context. All these features make CSCs particularly hard to eradicate. FAO: fatty acid oxidation

are increased in CD133+ cells, and this is directly correlated with SIRT1-dependent enhanced FAO^[144]. In HCC, genome-wide transcriptional profiling and Ingenuity pathway analysis have suggested NANOG to be the connecting point between FAO and stem-like features, because of its simultaneous OXPHOS repression and FAO activation actions^[145]. Moreover, it has been observed that stearoyl-CoA desaturase 1 (SCD1), a central enzyme involved in the conversion of saturated fatty acids into monounsaturated fatty acids (MUFAs), regulates liver CSCs^[146]. In addition, enhanced activation of SCD1 and the consequent production of MUFAs appear to be a potential hallmark of CSCs^[141].

All these findings prompt metabolic plasticity as a central force that enables CSCs to modify their replicative capabilities according to specific needs [Figure 2]. Further, emerging evidence suggests that CSCs may adopt specific metabolic phenotypes based on their location within tumor mass^[147].

CONCLUSIONS AND CLINICAL IMPLICATIONS

Unresponsiveness to current conventional therapies remains one of the major challenges in PLC. Current therapeutic strategies for the treatment of hepatic cancer mostly focus on the inhibition of tumor growth, with unsatisfactory results. Future treatments are likely to target CSCs and their specialized niche. In this view, it is imperative to decipher the molecular mechanism behind chemoresistance of PLC cells and especially of CSCs, with the objective to develop novel therapeutic strategies targeting features, markers or signaling pathways essentials for CSC biology.

Since CSCs are characterized by metabolic changes, drugs that inhibit OXPHOS have been studied as potential anticancer agents. Metformin, which interfere with OXPHOS by inhibiting NADH-coenzyme Q oxidoreductase (complex I), is a key example and has been shown to be particularly cytotoxic for CSCs, as well as for cells with mutations in OXPHOS complex I^[148,149]. Despite metabolic studies in the field of liver CSC are still at an early stage, the dual inhibition of glycolytic and mitochondrial energy pathways may represent a promising superior therapeutic approach to effectively eradicate heterogeneous liver CSCs and to overcome therapeutic resistance.

Moreover, since EMT pathway and CSC features seem to be intimately linked, improving our understanding of these cellular states may help to develop novel therapies. The plasticity of CSCs further suggests that simultaneously targeting CSCs existing in both epithelial and mesenchymal states rather than either state alone is needed to achieve complete tumor eradication^[150]. Hence, future investigations in this direction are imperative.

It is important to underline that the development of CSC-specific therapeutic strategies imply the presence of a common recognized method for isolation and subsequent characterization of liver CSCs. During the last decade a large number of studies have aimed to identify liver CSCs and several attempts have been made to enrich liver CSCs. Common strategies for PLC CSC enrichment, varied from the widely used classical antigenic approach that relies on surface CSC markers detection (e.g., CD133^[46,56,151-154], CD44^[71,153-155], OV6^[156], CD90^[129,130,157], EpCAM^[22,68,71,158], CD13^[159], CD24^[153,154,160], CD47^[161]) to functional techniques including side population (SP) analysis^[65,162-165], Aldefluor assay^[166-169] and tumor-sphere formation^[65,67,70,170,171]. In all different published studies, enriched PLC CSC subsets have been then tested in immune-deficient mice for the *in vivo* tumorigenic potential^[22,56,65,67,68,129,130,151,152,155,156,159-162,166,170].

One important challenge in developing new therapeutic strategies is the dynamic and plastic behavior of tumor cells, especially of CSC. As it's well known, a central role in the regulation of cancer cell plasticity is played not only by genetic alterations, but also by epigenetic changes, including DNA methylation, histone modifications and non-coding RNA (ncRNA) activity^[58]. By acting at transcriptional, post-transcriptional and translational level, ncRNAs represent key regulators of CSCs by modulating several biological processes including asymmetric division, unresponsiveness to treatments and EMT, thus affecting tumor progression and recurrence^[58]. In addition, recent studies also suggest that similar to normal stem cells, CSCs seem to reside in specialized microenvironment ("CSC-niche")^[46,50,70,172], whose signals can support self-renewal and drug-resistance features and, thereby, may influence the plasticity of CSCs^[173-177]. Therefore, targeting only CSCs may not be enough, and continued development of therapies targeting CSCs and their microenvironment in combination with chemotherapy may be essential to improve the outcomes of PLC patients.

DECLARATIONS

Authors' contributions

Analysis of publications and drafting of the manuscript: Correnti M, Booiink R, Di Maira G

Critically revised the manuscript: Raggi C, Marra F

Read and approved the final manuscript: All authors

Availability of data and materials

Not applicable.

Financial support and sponsorship

Raggi C and Correnti M were supported by the Italian Foundation of Cancer Research (AIRC). Work on liver cancer in Dr. Marra's laboratory is supported by AIRC, Istituto Toscano Tumori (ITT), Fondazione Umberto Veronesi, University of Florence.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Direct-acting antivirals and hepatocellular carcinoma occurrence and recurrence in hepatitis C virus-related liver cirrhosis: fact or fiction

Alberto Zanetto[#], Sarah Shalaby[#], Alberto Ferrarese, Chiara Becchetti, Salvatore Sciarrone, Giacomo Germani, Marco Senzolo, Martina Gambato, Francesco Paolo Russo, Patrizia Burra

Multivisceral Transplant Unit, Department of Surgery, Oncology and Gastroenterology, Padua University Hospital, Padua 35128, Italy.

[#]Authors contributed equally.

Correspondence to: Dr. Patrizia Burra, Multivisceral Transplant Unit, Department of Surgery, Oncology and Gastroenterology, Padua University Hospital, Via Giustiniani 2, Padua 35128, Italy. E-mail: burra@unipd.it

How to cite this article: Zanetto A, Shalaby S, Ferrarese A, Becchetti C, Sciarrone S, Germani G, Senzolo M, Gambato M, Russo FP, Burra P. Direct-acting antivirals and hepatocellular carcinoma occurrence and recurrence in hepatitis C virus-related liver cirrhosis: fact or fiction. *Hepatoma Res* 2018;4:70. <http://dx.doi.org/10.20517/2394-5079.2018.102>

Received: 25 Sep 2018 **First Decision:** 16 Oct 2018 **Revised:** 5 Nov 2018 **Accepted:** 6 Nov 2018 **Published:** 21 Nov 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cui Yu **Production Editor:** Huan-Liang Wu

Abstract

Since the widespread adoption of new direct-acting antiviral agents (DAAs), the approach to hepatitis C virus (HCV) infection has changed profoundly as almost all patients can be cured regardless of the stage of their liver disease. On the other hand, there are a few conflicting reports on the risk of hepatocellular carcinoma (HCC) occurring and recurring in patients given DAA-based therapy. The present review focuses on the latest and most relevant literature providing evidence on the occurrence and recurrence of HCC after HCV antiviral treatment with the new DAAs. Retaining the distinction between HCC occurrence and recurrence, we also discuss its patterns of presentation and speculate on the possible pathogenic mechanisms. We offer our personal viewpoints on this important issue, which has kept clinicians second-guessing in real-world clinical practice, when dealing with HCV eradication in the setting of advanced liver disease in this interferon-free era.

Keywords: Hepatitis C virus, direct-acting antiviral agent, occurrence, recurrence, hepatocellular carcinoma and liver cirrhosis

INTRODUCTION

The development of safe and effective treatments for hepatitis C virus (HCV) infection has been a major concern for hepatologists in the last few decades. The era of interferon (IFN)-based treatment regimens was plagued with frequent, severe adverse events necessitating a strict follow-up and prompt management of



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complications, while sustained virological response (SVR) rates were often not very high. The prescription of IFN was also constrained by disease stage and a patient's comorbidities, making any attempt to eradicate HCV unfeasible in the case of end-stage disease.

The last few years have seen a major step forward in the treatment of HCV with the introduction of all-oral therapies. Direct-acting antiviral agents (DAAs) have since revolutionized the management of HCV patients, achieving high eradication rates with an excellent safety profile^[1]. The restrictions that IFN treatments imposed have been consigned to history and SVR rates have consistently exceeded 90%, regardless of the chosen antiviral schedule^[2-5]. These unexpected, striking results led to the assumption that HCV could be virtually eradicated and on a global level, interrupting the natural history of the disease without incurring any severe side effects. In fact, it has been demonstrated that liver function can improve after the virus has been eradicated, with a significant reduction in the hepatic venous pressure gradient in most patients^[6-8]. This improvement has prompted the delisting of some patients with advanced disease^[9,10], but the greatest benefit is seen in cases of well-compensated cirrhosis with no clinically significant portal hypertension^[11].

Bearing in mind that chronic HCV infection is one of the main risk factors for the onset of hepatocellular carcinoma (HCC), lower rates of the latter's occurrence/recurrence were expected in the HCV-eradicated population, whatever the stage of their liver disease was. Accordingly as previously seen for patients successfully treated with IFN^[12], the incidence rate of HCC dropped from 7.2/1000 person-years among patients with no SVR to 1.1/1000 person-years among those who achieved a SVR, as recently reported by Janjua *et al.*^[13]. The widespread use of DAAs is clinically and economically cost-effective for society in the short and long term, justifying the provision of this treatment for patients with all stages of liver disease^[14-17]. In fact, current treatment guidelines issued by the European and American Associations for the study of the liver recommend a timely treatment for all infected patients in order to prevent not only hepatic but also extra-hepatic HCV-related complications^[18-20], and thereby contain tumor-unrelated mortality^[21].

Solid data on the long-term outcome of cirrhotic patients treated with these new regimens are still unavailable, however, and DAA registration trials did not distinguish between patients with and without a history of HCC^[3,22-34]. Patients with HCC were treated with DAAs regardless of any presence of concomitant or prior HCC, or any other cancer. Great concern was raised, however, by the unexpected report from Reig *et al.*^[35] of a presumable time-related association between DAA treatment and recurrent HCC with an aggressive pattern. This prompted several groups to analyze their results in an effort to establish whether this red flag raised on DAAs was justifiable.

To date, controversial data have emerged on HCC occurrence/recurrence after HCV eradication with IFN-free treatment regimens. Most reports come from single-center, often retrospective, observational studies, with differences in patients' characteristics and length of follow-up [Tables 1 and 2]. The picture consequently remains unclear for now, with a marked heterogeneity, even in terms of the control groups considered: some authors compared DAA-treated patients with those treated with IFN-based regimens; others compared them with untreated patients; and retrospective cohorts belonging to different eras were often involved. This might be partly attributable to several factors. For a start, HCC onset was not an endpoint in the initial DAA registration studies. Secondly, trials included patients with both chronic hepatitis C (CHC) and cirrhosis, with or without a history of HCC, and usually with a follow-up after treatment too short for the purpose of assessing HCC onset or recurrence. The incidence of HCC is also difficult to compare between patients given DAAs as opposed to IFN-based regimens because the former group also includes patients with decompensated cirrhosis, who are at higher risk of developing HCC^[36]. The present review summarizes all the latest and most relevant literature regarding this issue, providing evidence on the occurrence and recurrence of HCC after HCV antiviral treatment with the new DAAs. Retaining the distinction between HCC occurrence and recurrence, we also provide a distinction between those studies comparing DAAs and no treatment, and

Table 1. Hepatocellular carcinoma occurrence after direct-acting antiviral agents treatment

Authors, year	Type of study	Patients	Cirrhotic patients (%)	Child-Pugh	%	Control group	Fol-low-up (me-dian)	HCC occur-rence rates	Time between DAA treatment and HCC occur-rence
Conti <i>et al.</i> ^[37] , 2016	Retrospective	285	100	CTPA CTPB	88.7 11.3	-	6 mo	3.16%	NA
Cheung <i>et al.</i> ^[40] , 2016	Prospective	377	100	CTPA CTPB CTPC	17.2 72.7 10.1	Untreated	15 mo	4%	NA
Foster <i>et al.</i> ^[2] , 2016	Prospective	467	87.5	CTPA CTPB CTPC	17.4 72.6 10	Untreated	6 mo	5.4%	NA
Zeng <i>et al.</i> ^[96] , 2016	NA (letter)	31	100	NA			15 mo	0%	NA
Kozbial <i>et al.</i> ^[50] , 2016	NA (letter)	195	100	NA	NA		12 mo	6.6%	NA
Kobayashi <i>et al.</i> ^[45] , 2017	Retrospective	77	NA	NA		IFN-based	48 mo	3- and 5-year cumulative 1.3% and 3%, vs. 1% and 2.2% in controls ($P = NS$)	NA
Romano <i>et al.</i> ^[42] , 2018	Prospective	2279	85.7	CTPA CTPB	91 9		7.4 mo	1-year cumulative 2.1	NA
Cardoso <i>et al.</i> ^[51] , 2016	Retrospective (letter)	54	100	-	-	-	12 mo	7.4%	7.6 mo after HCV-RNA undetectability
Affronti <i>et al.</i> ^[47] , 2016	Retrospective (abstract)	105	100	CTP > 7	80%	Relapse after IFN-free	15 mo	1-year cumulative 4.4% ($P < 0.002$ vs. controls)	NA
Muir <i>et al.</i> ^[41] , 2016	Prospective	859	100%	NA	-	-	12 mo	1%	NA
Buonfiglioli <i>et al.</i> ^[39] , 2016	Prospective (abs)	285	100	NA	-		6 mo	3.2%	NA
Carrat ^[97] , 2016	Prospective (abs)	2156	63	NA	-	-	18 mo	4.3%	NA
Ji <i>et al.</i> ^[44] , 2017	Prospective (Abs)	165	NA	NA	-	IFN-RBV	14 mo	Not different between groups	NA
Innes <i>et al.</i> ^[98] , 2017	Retrospective (Abs)	570	100	NA	-	IFN-based	22 mo	7% (not different between groups)	NA
Calvaruso <i>et al.</i> ^[99] , 2017	Retrospective (Abs)	3447	77.8	CTPA CTPB	68 9.2	-	8.5 mo	1.44% overall 1.69% in CTPA 4.37% in CTPB	NA
Issachar <i>et al.</i> ^[100] , 2017	Retrospective (Abs)	273	NA		-	-	15 mo	2.1%	NA
Bielen <i>et al.</i> ^[48] , 2017	Retrospective (Abs)	332	NA	CHC + CTPA	100	-	-	1.5%	NA
Waziry <i>et al.</i> ^[61] , 2017	Metanalysis	9 studies	100	NA	-	IFN-based studies	-	RR 0.68, 95% CI 0.18-2.55, $P = 0.5$	NA
Ioannou <i>et al.</i> ^[49] , 2017	Retrospective	26483	16.8	-	-	IFN based cohort	72 mo	3.8% in IFN-FREE + IFN 2% in IFN-FREE only	NA
Nagaoki <i>et al.</i> ^[43] , 2017	Retrospective	154	NA	NA		IFN based cohort	23 mo	Cumulative 1 and 5-year: 0.6% and 9% ($P = ns$ vs. control group)	22 mo after end of treatment
Nagata <i>et al.</i> ^[46] , 2017	Retrospective	669	NA	NA	-	IFN-based cohort	20 mo	Cumulative 3-year: 1.4% ($P = 0.49$ vs. controls)	NA

Kanwal <i>et al.</i> ^[101] , 2017	Retrospective	22500	39	NA	-	Relapse after SVR		Cumulative 1-year - Overall 1.18% - SVR 0.9% - Relapse 3.4%	5.2 mo in SVR patients vs. 6.1 mo in non NA SVR, after end of treatment
Ogata <i>et al.</i> ^[102] , 2017	Retrospective	1170	NA	NA	-	-	1.3 years	1.9%	0.5 years after end of treatment
Deterding <i>et al.</i> ^[103] , 2017	Retrospective	863	100	CTPA CTPB CTPC	69.9 13.7 1.7	-	-	1.4%	NA
Finkelmeier <i>et al.</i> ^[104] , 2018	Retrospective	819	32.8	CTPA CTPB CTPC	78 19 3	IFN-based cohort	8.8 mo	3.1% vs. 5.4 in controls ($P = NS$)	312 days after end of treatment
Li <i>et al.</i> ^[53] , 2018	Retrospective	5834	19.9%	NA	-	IFN-based cohort	-	0.86% (22.8 per 1000 person year; $P = NS$ vs. IFN)	NA
Romano <i>et al.</i> ^[42] , 2018	Prospective	3917	75.5	CTPA CTPB	80.7 11.9	Untreated	17.4 mo	1.4% overall - 0.42% in F3 patients - 1.88% in cirrhotics	31.8 w after treatment start

HCC: hepatocellular carcinoma; DAA: direct-acting antiviral agent; NA: not available; NS: not significant; IFN: interferon; CTP: Child-Turcotte-Pugh; SVR: sustained virological response; CHC: chronic viral hepatitis; RBV: ribavirin

those ones including a control group of patients treated with IFN. Finally, we discuss HCC patterns of presentation, speculating on the possible pathogenic mechanisms.

HCC OCCURRENCE

DAAs vs. no treatment

Conti *et al.*^[37] retrospectively analyzed the occurrence of HCC in compensated patients with cirrhosis with no history of liver cancer, who achieved a SVR after IFN-free treatment regimens. They found an HCC occurrence rate of 3.1% within 6 months after treatment, which was higher than what was previously observed in the natural history of untreated HCV-related cirrhosis^[38]. These preliminary findings were confirmed in a subsequent publication by the same authors^[39]. The occurrence of HCC after IFN-free treatment was again similar to the rate seen in untreated patients in another prospective English study by Cheung *et al.*^[40] in patients with decompensated disease. It is worth noting that most of these new cancers were diagnosed within the first 3 months of therapy, which might mean that the cancer was already there when the antiviral treatment was started. These two studies were underpowered due to a short follow-up, and might well have underestimated the true incidence of HCC, but data coming from studies with a longer follow-up substantially confirmed these results^[41]. In particular, our experience comes from a large sample of patients treated at several centers in northern Italy with a median follow-up of 17.4 months^[42]. During this period, the HCC occurrence rate in the sub cohort of cirrhotic patients was much the same as (or even lower than) expected without antiviral therapy. The incidence of HCC significantly dropped after the first year in both Child-Turcotte-Pugh (CTP)-A and CTP-B patients (Mantel-Cox test, $P = 0.00008$). The reason for this is unclear, but it might relate to a greater reduction in intrahepatic inflammation in the longer term after stopping the antiviral therapy. Foster *et al.*^[2] prospectively compared the outcome of 467 patients treated with DAAs in the UK in 2014 with a group of untreated cirrhotic patients finding no difference in HCC occurrence rates within 6 months. Even though the incidence found in the English cohort was almost twice as high as in the Italian study by Conti *et al.*^[37], we have to consider that the patients were much more severely decompensated.

It seems that the SVR obtained with DAAs does not substantially change the natural incidence of HCC in cirrhotic patients, in the short to medium term at least. Patients already in the advanced fibrotic stage before

Table 2. Hepatocellular carcinoma recurrence after direct-acting antiviral agents treatment

Authors, year	Type of study	Patients	Cirrhotic patients (%)	Child-Pugh	%	Control group	Follow-up (median)	HCC recurrence rates	Time between last HCC treatment and DAA start	Time between DAA treatment and HCC recurrence
Reig <i>et al.</i> ^[35] , 2016	Retro-spective	58	94.8	CTPA CTPB CTPC	91 5.4 3.6	-	5.7 mo	28%	11.2 mo (8.7 mo in patients with recurrence, 15 mo in those without)	3.5 mo after DAA start
Conti <i>et al.</i> ^[37] , 2016	Retro-spective	59	100	CTPA CTPB	88.7 11.3	-	6 mo	28.8%	376 days (446 days in patients with recurrence, 360 days in those without)	NA
ANRS collaborative study group on hepatocellular carcinoma ^[56] , 2016	Retro-spective HEP-ATHER cohort	189	85	NA	-	Untreated	20 mo	0.73/100 person-month ($P = 0.87$ vs. controls)	NA	NA
ANRS collaborative study group on hepatocellular carcinoma ^[56] , 2016	Retro-spective CirVir cohort	13	100	CTPA	100	Untreated	59 mo	1.1/100 person-month ($P = 0.75$ vs. controls)	Included patients were considered to be in remission at least 3 mo following implementation of at least one curative procedure	37.1 mo (one patient)
Cheung <i>et al.</i> ^[40] , 2016	Prospective	29	100	CTPA CTPB CTPC	17.2 72.7 10.1	-	15 mo	6.9%	NA	20 w and 26 w after treatment start (two patients)
Zavaglia <i>et al.</i> ^[57] , 2017	Retro-spective (letter)	31	100	CTPA CTPB	81 19	-	8 mo	3.2%	19.3 mo (1.7 mo since last assessment)	8 mo (one patient)
Petta <i>et al.</i> ^[59] , 2017	Retro-spective	58	94.8	CTPA CTPB CTPC	91 5 4	IFN based cohort	18 mo	Cumulative 1 and 5-year: 12.9% and 39.1% (P NS vs. controls)	NA	NA
Cabibbo <i>et al.</i> ^[58] , 2017	Prospective	143	100	CTPA CTPB	86 14	Untreated	8.7 mo	6-,12-,18-month recurrence: 12%, 26.6%, 29.1%. No differences in terms of time to recurrence with untreated patients	NA	NA
Ikeda <i>et al.</i> ^[105] , 2017	Restro-pective	177	NA	NA	-	Untreated	20.7 mo	Recurrence rates at 1st and 2nd year were 18.1 and 25.0% in pts with DAA therapy and 21.8 and 46.5% in those without DAAs, ($P = 0.003$)	10.7 mo	NA

HCC: hepatocellular carcinoma; DAA: direct-acting antiviral agent; NA: not available; NS: not significant; IFN: interferon; CTP: Child-Turcotte-Pugh; SVR: sustained virological response; CHC: chronic viral hepatitis; RBV: ribavirin

Table 3. Supposed immunological derangements induced by direct-acting antiviral agents viral eradication

Reduced homing of leucocytes towards the liver (HCC-specific and non-specific CD8+ T cells)
Normalization of the NK-cell compartment
Decreased TRAIL-expression
Enhanced proliferation of few isolated malignant cells already present at treatment starting
Lack of continuous IFN-stimulation in the liver
Changes in miR-122 levels
Increase in serum vascular endothelial growth factor with increased liver cancer angiogenesis
More aggressive immunologic pattern already present, before the immune changes due to DAAs occur
Partial reversion of histone modifications induced by chronic HCV infection

HCC: hepatocellular carcinoma; NK: natural killer; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand; IFN: interferon; DAA: direct-acting antiviral agent; HCV: hepatitis C virus

HCV eradication carried the same neoplastic risk afterwards. The benefits of eradicating the virus, as opposed to leaving cirrhotic patients untreated, would probably emerge more clearly in the much longer term, when the effects of having put a stop to the continuous virus-related damage would become more evident.

DAAs vs. IFN-based regimens

Some authors compared DAA-treated patients with those given IFN to see whether the novel, rapid virus-eradicating mechanism was able to modulate HCC occurrence as much as previous treatments had done. But the two populations being compared not only belonged to different historical periods, but also happened to differ considerably because of the greater accessibility of today's new drugs. The comparison was also further undermined by the excessively broad inclusion criteria used in most of such studies (in which both patient groups had CHC or cirrhosis, for instance).

Nagaoki *et al.*^[43] investigated HCC occurrence after daclatasvir/asunaprevir treatment in 154 patients with CHC or cirrhosis. After appropriate propensity score matching analysis with a historical cohort of 244 patients treated with IFN-based regimens, the cumulative HCC incidence at 1, 3 and 5 years was respectively 0.6%, 9% and 9% for the DAA group, and 0.4%, 3% and 5% for the IFN group ($P = 0.053$).

The above findings were confirmed in another two Asian cohorts from China^[44] and Japan^[45]. On multivariate analysis, the risk of HCC onset was significantly associated with alcohol abuse as a cofactor, but not with IFN-based as opposed to IFN-free antiviral treatment, after adjusting for age, gender and baseline cirrhotic status.

Nagata *et al.*^[46] retrospectively compared large cohorts of patients treated with IFN-based vs. DAA regimens in Japan who had CHC or cirrhosis with no history of HCC, but no sub analysis of cirrhotics alone was available. When the authors used propensity score analysis to reduce the bias of different follow-ups after achieving a SVR (6.8 vs. 1.8 years, respectively), they found the 3-year cumulative occurrence rate of HCC was similar in the two groups (3.3% vs. 1.4%, $P = 0.49$). Notably, the cumulative incidence of HCC was significantly lower for patients achieving a SVR in both groups so that SVR after IFN-free regimens was likewise associated with a lower rate of HCC development as in the previously-mentioned study by Cheung *et al.*^[40] and even in the cohort described by Affronti *et al.*^[47] which had a great percentage of decompensated patients. In a retrospective multicenter analysis involving 15 centers in Belgium, Bielen *et al.*^[48] found that early HCC occurrence rates were similar in patients with CHC or compensated cirrhosis treated with DAAs, with or without IFN (DAAs plus IFN vs. DAAs alone: 3.6% vs. 1.1%). Lastly, Ioannou *et al.*^[49] found SVR associated with a significant reduction in HCC developing in HCV-related cirrhosis compared with situations when the treatment failed, whatever the type of treatment (IFN, DAA, or combinations of both), after the mean 6.1 years of follow-up.

Conversely, Kozbial *et al.*^[50] and Cardoso *et al.*^[51] reported their experiences, from Austria and Portugal respectively, of an unexpectedly high incidence of HCC, which however remained isolated reports. Additionally, the whole cohorts' baseline characteristics were unavailable and patients with F3 fibrosis were included, making it impossible to compare these results with other studies.

After the initial rush to report on small populations with short follow-ups, some longer-term studies on larger cohorts have begun to emerge. One recent study using real-world data found DAA-based HCV treatment unassociated with any increased risk of incident liver cancer. This risk even seemed to be lower than in either untreated or IFN-treated patients, suggesting that the benefits of DAA treatment will become more apparent with time. In the study by Singer *et al.*^[52], after adjusting for gender, age and disease stage, DAA treatment was associated with a significantly lower risk of liver cancer by comparison with no treatment (adjusted hazard ratio (HR) = 0.84, 95% CI: 0.73-0.96), or IFN-based treatment in the pre-DAA era (HR = 0.69, 95% CI: 0.59-0.81). Using the Electronically Retrieved Cohort of HCV-Infected Veterans database, Li *et al.*^[53] found that, among the cirrhotics with a SVR, neither the HCC incidence rate nor HCC-free survival differed significantly between the DAA and IFN groups ($P = 0.78$; and log-rank, $P = 0.17$). Both treated groups had a significantly lower probability of developing HCC than the untreated group (log-rank, $P = 0.0004$).

In short, though extremely heterogeneous, the above-mentioned studies seem to suggest (with the exception of a few isolated cases) that, regardless of how it is achieved, a SVR lowers the likelihood of HCC, albeit to a different degree depending on the stage of liver disease. Once a SVR has been achieved, it seems that comorbidities and lifestyle begin to have a major role, and should therefore be taken into account.

Taking another perspective, Cucchetti *et al.*^[21] estimated the influence of DAA regimens on patient mortality using a Markov model. They found DAA-based antiviral treatment associated to a drastic reduction in mortality unrelated to cancer before any onset of HCC, with only a slight increment in the HCC occurrence rate. The 20-year mortality due to causes other than HCC dropped by 21.9% in patients without varices, and by 27.5% in those with varices. Thus, assuming the cancer risk remains unchanged, the larger number of survivors generated a longer lifetime risk of developing HCC.

HCC RECURRENCE

DAAs vs. no treatment

The first warning came from a Spanish multicenter study^[35] showing a high rate of HCC recurrence in compensated cirrhotic patients considered to be in oncological remission after undergoing resection (34.5%), ablation (55.2%), and trans arterial chemoembolization (10.3%), and receiving DAA treatment, with a median follow-up of 5.7 months. This finding was supported by the same authors' comparisons of HCC recurrence rates, after surgical resection in one prospective study (in which HCC recurrence rates at 4 months were 13.5% in high-risk patients, and 3.8% in low-risk cases), and after ablation in another prospective series (unpublished data) (2.45% at 4 months and 27.6% at 12 months), as well as in the double-blind, placebo-controlled STORM trial^[54]. Interestingly, the highest rate of recurrence (41.17%) was seen in patients with a short interval (< 4 months) between HCC treatment and latest imaging assessment of complete response. In this regard, Cammà *et al.*^[55] analyzed the data in the Reig study, and showed that the probability of HCC recurrence during the first 6 months after starting DAAs was twice as high for patients with a shorter interval between their HCC treatment and their latest assessment of complete response compared with patients with longer intervals (< 15%). This may mean that the high early tumor recurrence rate described by Reig and co-workers was driven largely by individual cases, including those initially observed, initiated on DAAs shortly after being treated for HCC.

The paper by Reig *et al.*^[35] was not the only one to suggest that particular attention should be paid to managing patients with HCC receiving DAA treatment. Conti *et al.*^[37] confirmed that DAA-induced HCV eradica-

tion does not reduce HCC recurrence in the short term. The design of this latter study was very similar to the Spanish one. The crude HCC recurrence rate was almost identical to the one reported by Reig *et al.*^[35], but the follow-up after starting DAA was longer, thus leading to a lower time-based incidence.

Here again, however, the study design did not allow for the HCC recurrence rate to be defined as higher, lower or the same as expected in the natural history of the disease.

Other studies analyzing different populations did not confirm such a high risk of HCC recurrence following DAA-based therapy. The largest study concerned a French multicenter cohort^[56] in which two different groups of pre-transplant patients were prospectively followed up: the HCC recurrence rates were similar in DAA-treated and untreated patients.

A rather similar low rate of HCC recurrence in cirrhotic patients treated with DAAs was also reported by Zavaglia *et al.*^[57] along with Cheung *et al.*^[40] reporting respectively on compensated and decompensated cirrhotic patients.

Similarly, Cabibbo *et al.*^[58] found 6-, 12- and 18-month recurrence rates comparable with the figures reported in the literature for untreated patients, and the time to recurrence was much the same too. A history of HCC recurrence and tumor size emerged as two independent risk factors, and the authors suggested they be used to stratify patients by risk of early HCC recurrence.

Besides the few initial alarming studies, in which probably the HCC recurrence just happened to coincide with their antiviral treatment follow-up, it is becoming clear that DAAs do not substantially change the risk of recurrence in patients with advanced liver disease and previous history of HCC. Indeed, in these patients, in which the neoplastic process did already take place, the risk of recurrence remains high, appearing not be influenced by viral eradication. It is possible in fact that, contrariwise to patients without any previous tumoral history, the process, once triggered by active viral replication on a cirrhotic ground, becomes independent from the replication status so that recurrence rates and timings remain unmodified after treatment being modulated by HCC characteristics instead.

DAAs vs. IFN-based regimens

The experience of the Italian Liver Cancer Group, recently reported by Petta *et al.*^[59], demonstrated that both IFN-based and IFN-free HCV clearance result in longer times to tumor recurrence in patients with HCC radically treated with either resection or ablation, with no significant difference between the two virus eradication treatments.

In a European multicenter study by Kolly *et al.*^[60], the time elapsing between HCC treatment and DAA initiation emerged as a predictor of recurrence, in line with the analysis by Cammà *et al.*^[55].

Obtaining a SVR with DAAs (as opposed to IFN-based treatment or no treatment) does not seem to enhance the risk of HCC recurrence, which appears to be better predicted, again by other tumor- and patient-related variables. That said, studies on cancer recurrence should always report the tumors' baseline characteristics, the type of treatment administered, and the time elapsing before starting DAA to enable results to be interpreted correctly. In a meta-analysis, Waziry *et al.*^[61] recently confirmed the uncertainty of correlating HCC occurrence or recurrence with DAAs: they found no evidence for a correlation between IFN-free regimens and HCC development, and confounders such as a shorter mean follow-up or older age emerged as potential biases influencing the studies that sounded the alarm. The authors confirmed that HCV eradication reduces the risk of HCC in patients who achieve a SVR, while older age, advanced cirrhosis, and worse baseline patient features were independent predictors of HCC onset in the DAA-treated population, which helps to

explain their apparently higher risk (3.1 vs. 1.1/100 per years). In another meta-analysis conducted on 24 studies^[62], the factors associated with recurrent HCC included a history of HCC recurrence, and a shorter interval between HCC complete response and DAA initiation. This led the authors to recommend delaying DAA treatment for at least 6 months after HCC treatment, thus enabling a longer immune surveillance of existing microscopic HCC clones. Delaying DAA treatment could also allow more time to assess HCC treatment response, thereby minimizing the chances of misclassification bias. Such a delay was merely a precautionary (not evidence-based) suggestion, said the authors, that might be adopted in clinical practice while we wait for this HCC-DAA issue to be solved. Even though we still need more long-term evidences to disconfirm the possible role of DAA-mediated viral eradication in enhancing HCC recurrence, which is supported also by the lack of those immune-modulating properties held by IFN, current available evidences are not supporting this hypothesis. To help further evidences clarify this issue, clinicians should always document correct assessment of response after HCC treatments, possibly shortly before DAA start, and estimate recurrence risk on tumors' features and patients' related risk factors. Additionally, when comparing DAAs-treated patients with those treated with IFN, adjustments for disease stages should always be conducted as baseline risks have different reference ranges.

HCC PATTERN

Occurrence

Nakao *et al.*^[63] investigated the pattern of *de novo* HCC, reporting 6 cases of pathologically-confirmed HCC in patients with a SVR after treatment with DAAs. All these patients' tumors were single nodules, moderately differentiated and growing rapidly: these unconventional features (when compared with previous series) might overlap with the unexpected early tumor recurrence as described by Reig *et al.*^[35]. In our own experience, with the northern Italian cohort^[42], we found what seemed to be a more aggressive pattern of HCC presentation: among 16 patients developing HCC (29.1% of the sample), 8 (14.5%) presented with multiple nodules of various size, 8 (14.5%) with an infiltrative diffuse HCC, 6 (10.9%) with portal thrombosis, and 4 (7.2%) with extrahepatic metastases. Given the clinical importance of these findings, Renzulli *et al.*^[64] aimed specifically to examine the radiological features of microvascular invasion (MVI) in a retrospective analysis of 344 consecutive patients with HCV-related cirrhosis treated with DAAs and followed up for 48-74 weeks. After DAA treatment, HCC developed in 29 patients (11/29, 38% multi-nodular); forty-one HCC nodules were detected (27 of them recurrent), with imaging suggestive of MVI in 29/41 (70.7%) nodules, even in 17/29 (58.6%) nodules 10-20 mm in diameter. On the other hand, MVI was only present in 17/51 (33.3%) of the HCC nodules developing before any DAA treatment ($P = 0.0007$). These surprising data come from different cohorts and cannot be attributed simply to a lack of surveillance, because patients were strictly followed up. That said, it is important to remember that all these alarming findings came from small cohorts, and often from retrospective single-center experiences. The picture they paint contrasts with the report on the large historical French cohort^[65], in which cancer presented as a single nodule in 69.6% of cases, as 2 or 3 nodules in 19.8%, and was infiltrative or with more than 3 nodules in only 10.8%.

Recurrence

Reig *et al.*^[35] reported not only on a higher incidence of HCC recurrence, but also on a possibly more aggressive neoplastic pattern in recurrences after DAA treatment: 25% of the recurrences in the original Spanish cohort were multi-nodular, and 20% of them had an infiltrative pattern, despite the fact that the majority of the HCCs included in this analysis were at low risk of recurrence (judging from nodule size, Barcelona Clinic Liver Cancer stage, and histopathology of the resected tumor in patients who had surgery). In the previously-mentioned study by Cabibbo *et al.*^[58], the pattern of recurrence varied: 28 patients developed intrahepatic growths, and 24 of them had a nodular profile, while 5 (one with MVI) developed infiltrative HCC. None of the patients developed extrahepatic metastases.

Very little information is available regarding the characteristics of recurrent tumors, however, so that it is almost impossible to draw any conclusions.

On the other hand, albeit in a very different setting, we were able to compare the histopathological features of HCC on livers explanted from a small cohort of patients transplanted at our center who were treated with DAAs while listed for a transplant with active HCC, and having HCC bridging treatments at the same time. We found no histopathological differences in median number and total volume of HCC nodules, tumor differentiation or MVI^[66] vis-à-vis a contemporary untreated cohort involving patients and tumors with comparable baseline characteristics.

POSSIBLE PATHOGENIC MECHANISMS

Another particular issue that came to light with the first “warning report” concerns the possibility of HCV clearance from the liver, and the consequent impairment of the local immunological microenvironment, having an impact on HCC biology [Table 3]. Chronic stimulation of antigens against HCV infection contributes to virus-specific CD8+ T-cell exhaustion, continuative activation of host-mediated liver inflammation (driven partly by endogenous IFNs), and altered innate immune cell populations^[67,68]. IFN can modulate both innate and adaptive immune system. Different cells and pathways can be involved, including but not limited to inhibition of CD4+ and CD8+ T-cell proliferation, direct activation of natural killer (NK) cells, and suppression of IL-12 production by monocytes^[63]. The immune “restoration” that follows IFN-based antiviral therapy, together with eradication of the virus, was considered to be the pathophysiological explanation for the decrease incidence of HCC in patients achieving SVR with IFN-based antiviral therapies. There has been speculation that the mechanism by which patients with cirrhosis on IFN-free treatment might experience a higher HCC rate could relate to a reduced tumor-specific immune surveillance, particularly as concerns the HCC-specific CD8+ T-cells. In fact, DAA-induced HCV eradication could lead to a rapid decline in HCV-specific and non-specific T-cells from the liver, with a reduced homing of leukocytes towards the liver. This weaker infiltration by lymphocytes has been shown to correlate with a higher risk of HCC recurrence^[69-73]. Debes *et al.*^[74] recently speculated on other potential mechanisms of immune derangement during DAA therapy, involving NK-cell activity and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression. These immune mediators have consistently been found increased during HCV infection, potentially blocking HCC cell proliferation while attempting to contain the virus^[75,76]. The demonstrated DAA-induced normalization of the NK-cell compartment, together with the decrease in TRAIL receptor 2 expression, could therefore be responsible for a less efficient immune surveillance, and thus favor HCC recurrence. Whether or not the high HCC recurrence rate could be due to radiologically-undetectable tumors growing rapidly remains to be seen, however. Cheung *et al.*^[40] suggested that the rapid recurrence of HCC noted in DAA-treated patients might be prompted by the enhanced proliferation of a few isolated malignant cells already present when the treatment started giving rise to a rapid tumor growth, rather than by any *de novo* clone development. Indeed, the regeneration mechanisms activated by the rapid cure of inflammation, and differences in the immunological environment compared with IFN-based treatments could be responsible for the unrestricted growth of precancerous lesions or small malignant cell clones^[50]. The lack of any continuous IFN stimulation in the liver after eradication of the virus probably has a significant impact on intrahepatic immune responses too, giving rise to a less efficient neoplastic surveillance and enhancing neoplastic cell proliferation after an SVR has been achieved^[77,78]. The peculiarity of the warning in the Spanish report lies in the timing of HCC recurrences, which peaked during antiviral treatment and soon afterwards: this prompted several groups to investigate molecular changes occurring during this particular time frame. For instance, miR-122 concentrations were found to correlate with virus-induced liver inflammation and HCV-RNA levels, and serum levels decreased in patients with a SVR treated with a 12-week course of paritaprevir/ritonavir + dasabuvir or ombitasvir^[79]. MiR-122 has a central role in suppressing viral replication and it reduces tumorigenesis, angiogenesis and intrahepatic metastasis^[80], serving as a marker of disease status and response to therapy^[81-83]. During the first two weeks of the DAA treatment, changes in miR-122 levels were similar across genotypes, and comparable with or without ribavirin. Interestingly, miR-122 remained below the baseline levels throughout post-treatment week-12 in patients who subsequently achieved

a SVR, whereas they began to return to baseline levels after the second week of treatment in patients who did not^[79]. Villani *et al.*^[84] also observed an early rise in serum levels of vascular endothelial growth factor (VEGF) and a change in the inflammatory pattern 4 weeks after initiating DAA treatment, suggesting an increased liver cancer angiogenesis and tumor growth during this time. These changes returned to normal after the end of treatment, however. In this regard, Faillaci *et al.*^[85] recently reported the result of a prospective study that confirmed the role of VEGF pathways in HCC occurrence and recurrence. In a cohort of 183 patients with cirrhosis, 14/28 (50.0%) with previous HCC recurred while 21/155 (13.5%) developed *de novo* HCC. DAA therapy was associated with a significant increase of VEGF expression and this was significantly correlated with an increased rate of HCC occurrence/recurrence in “high-risk” patients. These patients were characterized by a baseline elevated and abnormal activation in liver tissues of neo-angiogenetic pathways, as shown by increased level of angiopoietin-2. From a clinical perspective, they presented a greater severity of baseline liver disease, as shown by higher portal collateralization and liver fibrosis scores. VEGF increased during DAA therapy, remaining elevated during follow-up, and significantly correlated with serum angiopoietin-2. Furthermore, angiopoietin-2 expression in the primary HCC or in cirrhotic tissue before DAAs was independently related with risk of HCC recurrence [odds ratio (OR), 1.137; 95% CI, 1.044-1.137; $P = 0.003$] or occurrence (OR, 1.604; 95% CI, 1.080-2.382; $P = 0.019$).

Debes *et al.*^[86] found 12 different soluble tumor markers (out of 22 tested, including markers of apoptosis, cytokines and growth factors) that were significantly higher before DAA treatment in patients who developed *de novo* HCC than in matched controls who did not. This raises the possibility of patients who eventually develop HCC already having a more aggressive immunological pattern, even before any immune changes due to DAAs occur, suggesting that the immune profile modulating HCC growth is attributed more to patients' prior individual characteristics than to changes induced by DAA-mediated HCV clearance. Epigenetic effects could be affected by DAA-mediated HCV eradication too. It was recently found that the marked changes in histone methylation induced by chronic HCV infection were only partially reversed by eradicating the virus with DAAs^[87]. An abnormal transcription could contribute to driving HCC tumorigenesis, alongside the other mechanisms already discussed. What is clear is that immune system plays a crucial role in terms of neoplastic surveillance^[88]. Trying to translate this concept into clinical practice, different prognostic scores that include also immunological variables have been conceived and successfully implemented in different types of tumor, including HCC. In this regard, systemic immune-inflammation index, neutrophil to lymphocyte ratio, platelet to lymphocyte ratio, aspartate aminotransferase-lymphocyte ratio index (ALRI), and albumin/bilirubin score (ALBI) have been shown to predict both survival and recurrence risk in patients with HCC^[89-91]. Recently, Casadei Gardini *et al.*^[92] tested the applicability of these scores in the specific setting of HCC recurrence after DAA, showing that ALBI and ALRI scores are promising practical tools able to stratify the risk of HCC development/recurrence in DAA treated patients. More particularly, at multivariate analysis, increase in ALBI grade ($P = 0.038$, HR = 2.35, 95% CI: 1.05-5.25) and ALRI ($P = 0.008$, HR = 1.05, 95% CI: 1.01-1.09) were independently associated with HCC development and recurrence, respectively. Importantly, risk of recurrence was adjusted for the time from HCC treatment.

CONCLUSION

In summary, the available data are not consistent enough to judge the risk of HCC occurrence/recurrence after IFN-free HCV eradication treatment. To better understand the reportedly diverse rates of liver cancer development, several factors should be taken into account, such as the achievement of a SVR, and the severity of liver dysfunction (the higher the Child-Pugh score, the higher the risk of HCC). We should consider patients' comorbidities and lifestyles too, particularly factors that might have an additional procarcinogenic potential, such as diabetes, smoking, alcohol abuse, and so on. Another important issue in assessing HCC recurrence risk is the time frame between tumor eradication and starting DAA treatment: this is not always taken into account, but it could well help to explain the contrasting results between different studies. Large cohort studies and meta-analyses suggest that there is no direct correlation between these two events. Addi-

tionally, more recent studies are starting to exclude early occurrences/recurrences from risk analyses as they could be an expression of undetected clones rather than induced by DAAs-mediated eradication, overestimating the real post-treatment incidences. Besides, the rates of new-onset HCC appear more homogeneous than the recurrence rates across the various studies, indicating that DAAs do not modify the incidence of HCC in the short term after HCV eradication. The populations investigated differed considerably in many aspects, however, and baseline characteristics were not always available, making data comparisons difficult. The very discordant HCC recurrence rates between different studies mean that, for the time being, it is virtually impossible to draw any useful conclusions.

Given the uncertainty surrounding the HCC occurrence and recurrence rates, despite their having been widely investigated in many different settings, specific pathogenic studies are still needed to demonstrate the link, if any, between DAA-mediated virus eradication and liver carcinogenesis, especially if an aggressive pattern is confirmed in HCCs occurring or recurring afterwards. Sudden changes prompted by DAAs in a chronically-inflamed liver might disrupt its anti-tumor response, but we still have too little evidence to attempt to see the whole picture. Hopefully, further translational studies will shed light on who are those patients that should be considered at high-risk of developing HCC recurrence, giving clinicians new biomarker or clinical scores that can help them in the clinical practice.

Our strategy for now is to eradicate HCV in early-stage disease, to rule out any HCC before starting antiviral treatment, and then to strictly follow up cirrhotic patients after they have achieved a SVR (based on the current EASL-EORTC clinical practice guidelines for HCC surveillance)^[93]. A strict follow-up is especially necessary in certain settings, such as patients on the waiting list for liver transplantation. Until further studies prove otherwise, we prefer not to delay antiviral treatment in well-compensated cirrhotic patients in order to avoid further liver deterioration and extrahepatic complications of HCV.

Dedicated, long-term prospective randomized interventional studies with proper controls are much needed to clarify this important issue, but the numerous variables involved in HCC occurrences and recurrences, and differences in screening protocols (often involving operator-dependent procedures, different timings, and no proper control groups) will make this a challenge. Significant changes in HCV epidemiology are also to be expected in the near future, as the virus will be virtually eradicated in the early stages of the infection. This will lead to a drastic reduction in cases of HCV-related end-stage liver disease, and correlated HCC^[94], meaning that the question of whether or not to treat patients' HCV because of any associated risk of HCC will become largely irrelevant.

Randomizing patients for such intervention would not be ethical, given the clearly-demonstrated benefits of DAA therapy in patients with cirrhosis. This includes those with decompensated disease, who are at highest risk of HCC, who also gain from the additional chance of being delisted for transplantation, and thus allowing organs to be allocated to others^[9,95].

DECLARATIONS

Authors' contributions

Bibliographical search, drafting of the manuscript, approval of the final version: Zanetto A, Shalaby S, Ferrarese A

Bibliographical research: Ferrarese A, Becchetti C, Sciarrone S, Germani G, Senzolo M, Gambato M, Russo FP

Critical revision of the manuscript and final approval: Burra P

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Efficacy and safety of generic daclatasvir + sofosbuvir \pm ribavirin in treatment of genotype 3 infected hepatitis C patients - a real life experience from Pakistan

Muhammad Umar¹, Tayyab Saeed Akhter¹, Junaid Sadiq², Samar Saleem¹, Shoaib Khokhar¹

¹Centre for Liver and Digestive Diseases, Holy Family Hospital, Rawalpindi Medical College and Allied Hospitals, Rawalpindi 46300, Pakistan.

²Tehsil Headquarter Hospital, Murree 47150, Pakistan.

Correspondence to: Dr. Muhammad Umar, Centre for Liver and Digestive Diseases, Holy Family Hospital, Rawalpindi Medical College and Allied Hospitals, Rawalpindi 46300, Pakistan. E-mail: drumark@yahoo.com

How to cite this article: Umar M, Akhter TS, Sadiq J, Saleem S, Khokhar S. Efficacy and safety of generic daclatasvir + sofosbuvir \pm ribavirin in treatment of genotype 3 infected hepatitis C patients - a real life experience from Pakistan. *Hepatoma Res* 2018;4:71. <http://dx.doi.org/10.20517/2394-5079.2018.31>

Received: 27 Mar 2018 **First Decision:** 19 Apr 2018 **Revised:** 6 Jun 2018 **Accepted:** 18 Oct 2018 **Published:** 30 Nov 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cai-Hong Wang **Production Editor:** Zhong-Yu Guo

Abstract

Aim: Genotype 3 is the most prevalent genotype in Pakistan. Despite a revolution in the treatment of Hepatitis C, genotype 3 is still thought to be difficult to treat genotype. The price of patent direct acting antivirals was thought to a great limiting factor especially for low income countries. In Pakistan low cost generics of daclatasvir and sofosbuvir are easily available for treatment. The aim of our study is to provide real life local data to determine their efficacy and safety.

Methods: This open-label, non-randomized, uncontrolled study was carried out at Center for Liver and Digestive Diseases, Holyfamily Hospital, Rawalpindi. We enrolled patients from March 2016 through March 2018 who were 18 years or older having chronic hepatitis C infection with detectable polymerase chain reaction (PCR), regardless of whether they were treatment naïve or have experienced Interferon in the past. The patients were offered generic sofosbuvir 400 mg and daclatasvir 60 mg once daily with or without ribavirin for a period of 12 to 24 weeks. Follow-up PCRs were performed at 4th week of treatment, end of treatment and 12 weeks post treatment. All those patients were included in the study that had at least one follow-up PCR during or after the course of treatment.

Results: A total of 102 patients were enrolled in the study with a mean age of 48.11 ± 12.70 including 63% males and 37% females. All patients were genotype 3. On 4th week follow up, 31/36 (86.11%) patients had quantitative PCR negative. Out of 102 patients 78 patients had follow up PCR at the completion of therapy with an end of treatment response of about 96.1%. Thirty patients had a follow up at 12 weeks post treatment with a SVR12 of



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83.33% (25/30) amongst which treatment Naïve had a response rate of 84% (21/25), treatment experience 80% (4/5), non-cirrhotics 85.71% (12/14), cirrhotics 81.25% (13/16) and decompensated chronic liver disease patients have a SVR12 of about 83.33% (10/12) respectively. The combination was well tolerated with few side effects, 18.6% patients had itching, 10.8% had insomnia, 8.8% had oral ulcers and 6.9% had fatigue.

Conclusion: Generic sofosbuvir and daclatasvir are cheap, safe and efficacious with a SVR12 of about 83.33% amongst genotype 3 patients. These generics will act as a pivot in the eradication of hepatitis C infection from developing world.

Keywords: Daclatasvir, sofosbuvir, genotype 3, hepatitis C

INTRODUCTION

Eighty million people are suffering from hepatitis C globally having six known genotypes with different distribution throughout the world. Amongst the countries with highest hepatitis prevalence, Pakistan ranks number 2, with the prevalence rate of 6.7% and the most commonly detected genotype is 3^[1]. Before the availability of direct acting antivirals (DAAs), patients were treated with interferon and ribavirin with sustained virologic response (SVR) of as low as 50% using conventional interferon and 57.6% using pegylated interferon in Pakistani population^[2]. The advent of DAAs undoubtedly revolutionized the treatment both in terms of safety and efficacy however genotype 3 is still thought to be difficult to treat genotype^[3].

After approval from FDA in 2013, sofosbuvir a NS5B inhibitor was the leading DAA followed by daclatasvir which is a NS5A inhibitor. The patent price for sofosbuvir is US\$84,000 and daclatasvir is US\$63,000, for a 12 week course^[4]. According to ALLY 3+ trial the combination of sofosbuvir and daclatasvir in genotype 3 patients is safe and efficacious with a SVR12 of 92% in treatment naïve and 89% in treatment experienced patients respectively^[5]. The combination has minimal drug-drug interactions and has safely been tried in patients with liver transplant, renal transplant and HIV co-infected patients as well^[6].

With Pakistan being a developing nation with a population of 29.5% living below poverty line^[7], the price of DAAs is a major issue. With the availability of generics, a combination of sofosbuvir and daclatasvir costs as low as US\$75 for a 12 week course that makes it affordable for the majority of patients in the country. Scarce data are available to determine the safety and efficacy of these low price generic drugs. Our study is one such effort to establish the efficacy and safety of these generics in Pakistani population.

METHODS

This open-label, non-randomized, uncontrolled study was carried out at Centre for Liver and Digestive Diseases, Holyfamily Hospital, Rawalpindi. Holyfamily Hospital is one of the largest tertiary care hospitals that drain not only local population but also patients from northern Punjab, Azad Kashmir and Khyber Pakhtunkhwa provinces. Our Centre is one of the largest gastroenterology centres of the country with well-established liver clinics. Formal approval was conducted from the ethical review board of Rawalpindi Medical University.

Eligible patients from March 2016 through March 2018 who were 18 years or older having chronic hepatitis C infection with detectable polymerase chain reaction (PCR), regardless of whether they were treatment naïve or have experienced Interferon in the past were enrolled in the study. Their cirrhosis status was determined using non-invasive measures like Fibroscan, ultrasound and child class before starting therapy. A high viral load was considered if the pretreatment PCR was $\geq 8 \times 10^5$ IU whereas a low viral load was considered if PCR was $< 8 \times 10^5$ IU. Patients who were of genotype other than 3, pregnant, breast feeding mothers or having active renal disease with GRF < 30 were excluded from the study.

Since the patients were included through non-probability consecutive sampling technique and in addition it was a single group study, lacking any control group based on ethical grounds, hence it was a quasi-experiment study. Keeping the expected proportion of patients with attainment of SVR in Genotype 3 patients as 99% according to recent ALLY 3+ study, the absolute precision as 5% and the level of confidence as 95%, the minimally required sample size was estimated to be 16. This sample size was calculated on OpenEpi, Version 3 sample size calculator. One patient discontinued the treatment due to non-hepatic cause whereas one patient did not comply with the treatment fully due to intolerance.

Treatment advised as per national consensus practice guidelines of Pakistan^[2]. Treatment Naïve or Interferon experienced non-cirrhotic patients were offered generic sofosbuvir 400 mg and daclatasvir 60 mg once daily for 12 weeks. Ribavirin 1000 mg (in patients < 75 kg) or 1,200 mg (in patients > 75 kg) was added to the regimen and the treatment extended for 24 weeks for cirrhotic patients and/or sofosbuvir experienced patients. Patients with decompensated cirrhosis if ribavirin eligible were offered a 12 week course and if ribavirin ineligible a 24 week course respectively.

Patients were followed on regular intervals with PCR at 4 weeks after the start of treatment, at the end of treatment and 12 weeks after completion of treatment. All PCRs performed on Real Time PCR by TagMan Probe and sequence specific primers using Scacae Biotechnology Sa Cyclor-96 instrument with a minimal threshold of 50 IU/mL used for reporting negatives. Hepatitis C virus (HCV) PCR below the threshold of quantification at 4th week of treatment is defined as a rapid virologic response (RVR), at the end of treatment as end of treatment response (ETR) and 12 weeks post treatment as SVR12. Adverse events were documented on each follow-up and patients were asked regarding fatigue, headache, nausea, insomnia, itching, anemia, weakness, rash, loss of appetite, oral ulcers, diarrhea or any other side effects. Follow-up PCRs were performed at 4th week of treatment, end of treatment and 12 weeks post treatment. All those patients were included in the study that had at least one follow-up PCR during or after the course of treatment.

Before the actual data collection were written and verbal informed consent was sought from all the respondents after explaining to them the nature and purpose of study, data were collected by a standardized performa. All the data were entered and analyzed in SPSS v.22. Descriptive analytic component included frequencies and percentages of various categorical variables.

RESULTS

A total of 102 patients were included in the study having HCV genotype 3 amongst which 63% were males and 37% were females. The mean age of participants was 48.11 years (\pm 12.70 years). The mean PCR HCV RNA quantitative levels were 3.5×10^6 IU/mL. The 52 (51%) patients had cirrhosis amongst which 37 (36.3%) were having decompensated liver disease. Study participants who were naïve to any previous Interferon treatment were 84 (82.4%) while amongst remaining 18 (17.6%) patients who had HCV treatment experienced previously, 3 (17%) were non-responders while 15 (83%) were relapsers.

Among participants 36 had a follow up PCR at 4th week of treatment with a RVR of 86.11% (31/36). Out of 102 patients 78 patients had follow up PCR at the completion of therapy with an ETR of about 96.1%. ETR in treatment naïve was 96.92% (63/65), treatment experienced was 92.30% (12/13), cirrhotics was 95.1% (39/41) whereas in decompensated cirrhosis patients was 93.10%(27/29) respectively. Thirty patients had a follow up of 12 weeks post treatment with a SVR12 of 83.33% (25/30) amongst which treatment Naïve had a response rate of 84% (21/25), treatment experience 80% (4/5), cirrhotics 81.25% (13/16) and patients with decompensated cirrhosis had a SVR12 of 83.33% (10/12) respectively. The distribution of virological responses in study participants is displayed in [Figure 1](#). For categorical variables we applied chi-square test to explore association between the treatment status (Naïve, interferon experienced or sofosbuvir experienced) and virological response (RVR, ETR or SVR) but no statistically significant difference was observed in patients

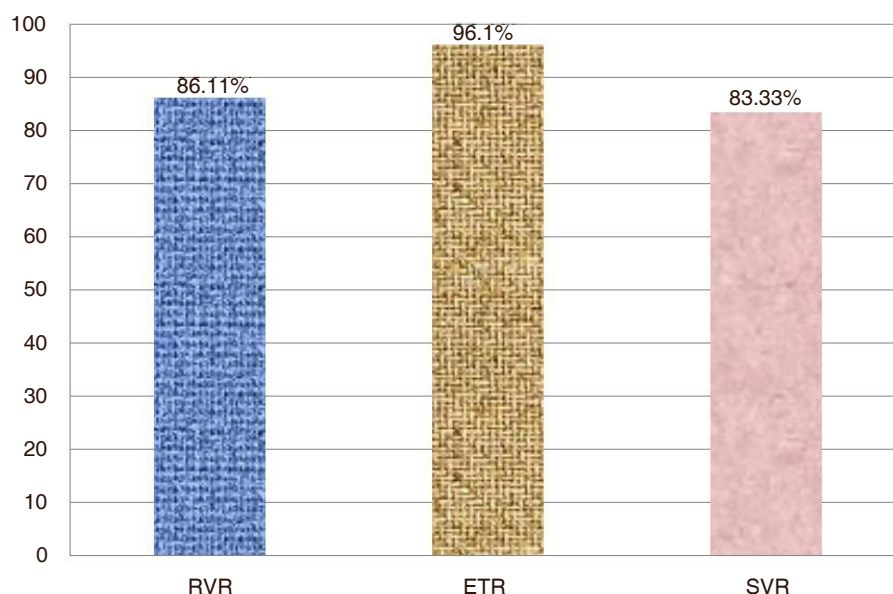


Figure 1. Genotype 3 hepatitis C virus chronic liver disease patients attaining rapid virologic response (RVR), end of treatment response (ETR) and sustained virologic response (SVR) using generic sofosbuvir and daclatasvir

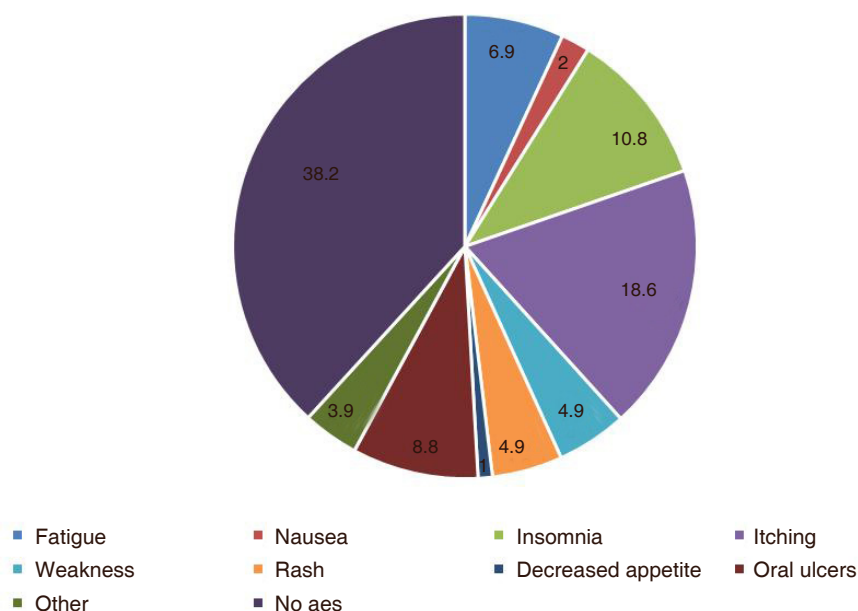


Figure 2. Percentage age of adverse events in patients using sofosbuvir (SOF) and daclatasvir (DAC) with or without ribavirin

whether attained RVR, ETR or SVR 12 or not, based on previous treatment status with all P values > 0.05 . Similarly no statistically significant association was observed between the baseline viral load (high $\geq 8 \times 10^5$ IU or low $< 8 \times 10^5$ IU) and virological response (RVR, ETR or SVR) with all P values > 0.05 .

The combination was well tolerated as only 1 patient was unable to complete the treatment due to side effects. The 18.6% patients had itching, 10.8% had insomnia, 8.8% had oral ulcers, and 6.9% had fatigue whereas 4.9% had weakness and rash, 3.9% had myalgias and 1 patient complained loss of appetite. The percentage distribution of different adverse events is displayed in Figure 2.

DISCUSSION

The availability of direct acting antivirals (DAAs) has really revolutionized the HCV treatment but their high prices has always been an element of criticism and efforts were being made to provide patients, especially in low income countries to get the drugs at cheap rates^[8]. Allowing generics in about 101 developing countries is one such strategy that has led to drastic decline in the prices of DAAs^[9]. But the efficacy and safety of these generics is a big concern that needs scientific evaluation.

The findings of our study are reasonably good and quite comparable with the international data. ALLY 3+ one of the leading study evaluating the sofosbuvir and daclatasvir in genotype 3 patients showed a SVR12 of 90% (45/50) whereas in our study it is 83.33%. Similarly SVR12 in cirrhotics is 86% and treatment experienced is 87% which in comparison with our study are 81.25% and 80% respectively^[5]. Another study using the same combination in genotype 3 patients in a real world cohort exhibit an overall SVR12 of 88%, in treatment naïve patients 92%, treatment experienced 84%, and cirrhotics 89% respectively^[10].

A study from Iran also evaluated the results of generic sofosbuvir and daclatasvir in genotype 3 patients but their results are far superior with SVR12 of 98% (40/41). Furthermore they only include cirrhotic patients in their study and the price of generic drug is about \$1,890 for a 12 week course^[11]. Our results for cirrhotic patients are 81.25% but the cost is only \$75. Several factors can be responsible for this difference in results including the bioequivalence of generics as compared to the branded drugs, compliance, study population and possible underlying drug resistance. For the generics it is mandatory to prove their bioequivalence to meet WHO prequalification standards^[11]. About five different generics from Egypt and India when compared with their originator drug (sofosbuvir or daclatasvir) proved to have similar pharmacokinetics^[12]. However this difference is due to drug quality, underlying resistance or is purely epidemiological, needs further probing.

The safety profile of these generic drugs is also comparable with the international data. In ALLY 3+ study fatigue and insomnia were the major side effects. In our study apart from these major side effects patient also complained of itching and oral ulcers. A study from Egypt using generic sofosbuvir and daclatasvir has described itching as side effect in up to 9.8% of the patients^[12]. Oral ulcers are not a common side effect of new DAAs. However in one of our old study regarding sofosbuvir they were present in 0.7% of the patients^[13], but with this combination rate of oral ulcers was 8.8%. One patient developed intractable ulcers that didn't respond to any supportive therapy and improved only after completion of treatment.

Only one patient was unable to tolerate the treatment and left it after 2-3 days because of worsening decompensation. Patient was already a child class B patient with minimal ascites. After treatment patient's ascites worsened and patient developed encephalopathy. The complications were managed medically but the treatment discontinued. This acute response can be due to some drug related liver injury (DILI) and a few case reports are available in literature describing DILI in patients using sofosbuvir^[14].

In total 6 patients were ribavirin eligible, and amongst them 2 patients developed ribavirin associated hemolysis due to which they were shifted to sofosbuvir and daclatasvir regimen and duration extended to 6 months. Both these patients successfully eradicated the virus.

Out of 30 patients whose SVR were checked 5 patients were unable to eradicate the virus [Table 1]. Four out of these 5 patients were males; a finding consistent with one of our previous study based on sofosbuvir and Rabavirin^[13]. Similarly 4 out of 5 patients were treatment naïve and 1 was Interferon relapser. Our study lacks evaluation to determine the risk factors for poor outcome. Further studies should be carried out to determine the underlined genetic mutations for drug resistance as well as other factors like obesity and diabetes. Our study has less number of patients with follow-up PCR at 12 weeks post treatment to check SVR as compared to the total number of patients enrolled. This lack of follow-up is mainly due

Table 1. Characteristics of patients not achieving SVR12

No.	Age	Gender	PCR (IU/mL)	Treatment status	Child class
1	66	M	4.2×10^6	Naïve	C
2	45	M	5×10^6	Naïve	A
3	50	M	7.4×10^5	Experienced	B
4	55	F	1×10^6	Naïve	A
5	35	M	2.2×10^6	Naïve	A

SVR: sustained virologic response

to non-compliance and affordability issues. Yet still the data are informative enough to establish the importance of generic drugs in the treatment of hepatitis C.

Overall the generic drugs are safe and efficacious. These drugs are not only cost effective but also cost saving and in the long run will help in preventing HCV related decompensated liver diseases, hepatocellular carcinomas and liver related deaths^[15]. Even for patients who are unable to respond to these drugs, new DAAs and their generics will be available in the near future. The generics for Velpatasvir are now available in Pakistan as well. The availability of new and new generic drugs will be the most effective method in eliminating Hepatitis C from the globe by 2030^[12].

In conclusions, Generic sofosbuvir and daclatasvir are cheap, safe and efficacious with a SVR12 of about 83.33% amongst genotype 3 patients. These generics will act as a pivot in the eradication of hepatitis C infection from the developing world.

DECLARATIONS

Authors' contributions

Conceived the research question, performed literature review, formulated discussion: Umar M
 Performed literature review, formulated introduction and discussion, data analysis: Akhter TS
 Formulated research methodology and results, data analysis: Sadiq J
 Data collection: Saleem S
 Data entry: Khokhar S

Availability of data and materials

Departmental data, Center for Liver and Digestive diseases, Holyfamily Hospital, Rawalpindi 46300. Data sharing can be considered on personal request to the primary author.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Surveillance for hepatocellular carcinoma - current status and advances

Kaina Chen¹, Pik-Eu Chang^{1,2}, George Boon-Bee Goh^{1,2}, Chee-Kiat Tan^{1,2}

¹Department of Gastroenterology and Hepatology, Singapore General Hospital, Singapore 169608, Singapore.

²Department of Medicine, Duke-NUS Medical School, Singapore 169857, Singapore.

Correspondence to: Dr. Chee-Kiat Tan, Department of Gastroenterology and Hepatology, Singapore General Hospital, Singapore 169608, Singapore. E-mail: tan.chee.kiat@singhealth.com.sg

How to cite this article: Chen K, Chang PE, Goh GBB, Tan CK. Surveillance for hepatocellular carcinoma - current status and advances. *Hepatoma Res* 2018;4:72. <http://dx.doi.org/10.20517/2394-5079.2018.103>

Received: 5 Oct 2018 **First Decision:** 24 Oct 2018 **Revised:** 2 Nov 2018 **Accepted:** 6 Nov 2018 **Published:** 12 Dec 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cai-Hong Wang **Production Editor:** Huang-Liang Wu

Abstract

Aim: Hepatocellular carcinoma (HCC) is a common cancer worldwide, especially in Asia, with high mortality. Curative options are only available for early-stage HCC, which are usually asymptomatic and best diagnosed through surveillance. Risk factors associated with HCC include liver cirrhosis due to alcohol, chronic viral hepatitis infections and nonalcoholic steatohepatitis. We review the evidence supporting the benefits and drawbacks of HCC surveillance as well as new surveillance modalities.

Methods: A MEDLINE and Cochrane Database search with defined search phrases was performed. Studies published from Jan 2000 to Jul 2018 were reviewed and publications focusing on the benefits and harms of HCC surveillance were qualitatively synthesized. Modalities of HCC surveillance were also reviewed.

Results: A total of 5 randomized controlled trials (RCTs) and 24 cohort studies with sample size of more than 100 each were selected. Significant mortality reduction was demonstrated in 1 RCT. Cohort studies showed overall improved outcomes in the surveillance group with 61.3%-88% of HCC being detected in an early-stage and with up to 80% eligible for curative treatments. A quarter (27.5%) of the surveillance patients experienced additional scans or procedures due to false-positive results. Combination of ultrasound with alpha-fetoprotein increases HCC detection rate. Novel serum markers and liquid biopsy are attractive tools for surveillance as they are non-invasive and convenient.

Conclusion: The current evidence supports HCC surveillance as it detects earlier stage of tumor, allows more curative treatment and improves survival. Further research on hepatocarcinogenesis and novel surveillance modalities will continue to refine surveillance guidelines to reduce HCC-related mortality.

Keywords: Hepatocellular carcinoma, surveillance, surveillance modalities, screening, biomarkers



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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 80% of all primary liver malignancies. Worldwide, it is the fifth most common cancer in males, ninth in females, and over half a million of new cases are diagnosed annually. Asia-Pacific region, East Asia and Sub-Saharan Africa accounts for 82% of all liver cancer cases in the world^[1]. HCC is the second most common cause of cancer-related deaths in 2012, 1% of all deaths in the world can be attributed to HCC every year. The overall survival of HCC was 3%-5%^[2], and mortality to incidence ratio is 0.95^[3], suggesting its poor prognosis attributable to the late stage of diagnosis in most of these cases. An early-stage HCC, on the contrary, is amenable to several curative therapeutic options, and a five-year survival of 70%-75% can be achieved^[4]. Liver cirrhosis may be due to several risk factors including alcohol but chronic hepatitis B or C infections are the most common risk factors of HCC contributing to 70%-90% of the cases, and nonalcoholic steatohepatitis (NASH) is rapidly gaining prominence^[5,6].

Several professional societies, including American Association for the Study of Liver Diseases (AASLD), European Association for the Study of the Liver (EASL), Japanese Society of Hepatology and Asian Pacific Association for the Study of the Liver, have recommended regular surveillance of HCC in at-risk populations^[7-10]. The goal is to identify HCC at an early stage when it is amenable to curative treatment, therefore reducing mortality. Increasing usage of surveillance to detect early HCC is associated with improvement in outcomes^[6]. The strongest evidence for surveillance is seen in patients with chronic hepatitis B infection^[11]. However, whether surveillance for HCC is truly effective and beneficial is still a topic of debate, owing to the concern of the quality and paucity of existing evidence. We conducted a systematic review of the literature to better understand the benefits and disadvantages of HCC surveillance, and the current surveillance modalities.

METHODS

Data sources and searches

A search on the MEDLINE database and Cochrane Database of Systematic Reviews was performed on 19 Jul 2018. Search phrases used were “hepatocellular carcinoma” OR “HCC” OR “Carcinoma, Hepatocellular” OR “liver cancer” OR “Liver Neoplasms” AND “surveillance” OR “screening” OR “Early Detection of Cancer”. We filtered the literature published from January 1 2000 to July 2018 and each literature was manually screened and selected based on our inclusion and exclusion criteria.

Study selection

All primary studies on HCC surveillance published in English, comprising randomized controlled trials, cohort studies, case studies and systematic reviews were included. We defined the term “surveillance” as “repeated use of a test at regular interval over time to detect a previously undiagnosed lesion”. The analysis was focused on the effect of surveillance on survival and/or mortality of HCC patients, with or without adjustment for bias. Particular attention was paid to any lead-time bias analysis for survival reporting. Modalities of HCC surveillance and stages of disease on diagnosis are also included. Exclusion criteria include studies published in foreign languages, studies on patients with recurrent or metastatic HCC, studies irrelevant to primary liver cancer, animal or *in vitro* studies, studies with no mortality/survival data directly comparing surveillance and non-surveillance group, or cohort studies with a sample size of less than 100 in either group.

Data synthesis and analysis

The data were qualitatively synthesized and summarized on the survival and mortality benefit of HCC surveillance.

RESULTS

The literature search yield 4,557 results in PubMed and 273 in Cochrane Library. We manually screened the literature from the title and study aims, and full-text articles of all eligible studies were reviewed. All the

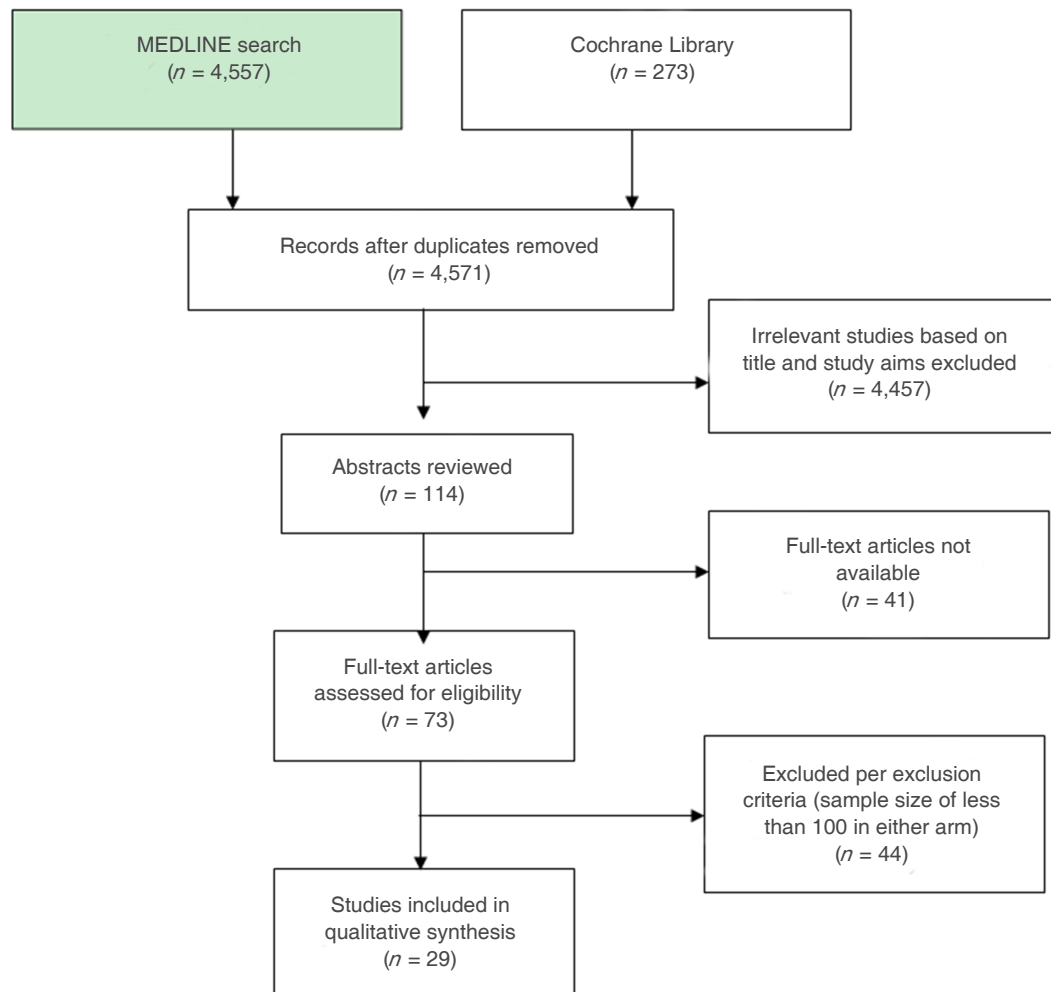


Figure 1. Study selection flowsheet^[64]

randomized controlled trials and cohort studies with more than 100 subjects in the surveillance and non-surveillance groups were included for qualitative analysis [Figure 1]^[12].

Randomised trials

To date, there were only two randomised trials, both done in China, directly comparing patients with surveillance to no surveillance. In both trials, the study population was exclusively patients with chronic hepatitis B infection (positive serum hepatitis B surface antigen). The first study by Chen *et al* in 2003 conducted surveillance with six-monthly serum alpha-fetoprotein (AFP), followed by ultrasound for patients with high AFP levels^[12]. No difference in mortality was found in the two groups. Zhang *et al.*^[11] subsequently conducted surveillance with AFP with US 6-monthly in two randomized groups of hepatitis B patients, and a significant mortality difference was found with a mortality rate ratio of 0.63 (95% CI: 0.41-0.98). These two trials were heavily criticized due to the poor compliance rate in surveillance group, as well as the limited information on study design and a high risk of bias [Table 1].

Other randomized controlled trials (RCT) done in Europe and Taiwan addressed the impact of ultrasound surveillance intervals. Trinchet *et al.*^[13] conducted a multicenter RCT comparing 3-monthly to 6-monthly ultrasound surveillance on HCC patients in France and Belgium. Study population was histology-proven cirrhosis and the main etiologies were alcohol and viral hepatitis. Three-monthly ultrasound detects more

Table 1. Randomised controlled trials on hepatocellular carcinoma surveillance

Author, year	Study period	Sample size (S vs. NS)	Continent	Surveillance modality	Etiology (%)	Stage at diagnosis (%)	Mortality	Survival (%)	Treatment (%)
Chen <i>et al.</i> ^[12] , 2003	1989-1995	3712 vs. 1869	Asia (China)	AFP 6-mthly vs. none	HBV [#] Cirrhosis: NA	I ^{a,*} : 29.6 vs. 6 II: 50.6 vs. 53 III: 19.8 vs. 41 (<i>P</i> = 0.86)	HCC mortality per 100,000: 1,138 vs. 1,114 (<i>P</i> = 0.86)	1-year: 23.7 vs. 9.7 3-year: 7 vs. 4 5-year: 4 vs. 4.1	NA
Zhang <i>et al.</i> ^[11] , 2004	1993-1995	9373 vs. 9443	Asia (China)	US + AFP vs. none	HBV Cirrhosis: NA	I ^b : 60.5 vs. 0 II: 13.9 vs. 37.3 III: 25.6 vs. 62.7 (<i>P</i> < 0.010)	HCC mortality per 100,000: 83.2 vs. 131.5 RR 0.63 (95% CI: 0.41 to 0.98); (<i>P</i> < 0.010)	1-year: 65.9 vs. 31.2 3-year: 52.6 vs. 7.2 5-year: 46.4 vs. 0	Resection: 46.5 vs. 7.5 TACE or PEI: 32.6 vs. 41.8 Conservative treatment: 20.9 vs. 50.7
Trinchet <i>et al.</i> ^[13] , 2011	2000-2006	640 (3 months) vs. 638 (6 months)	Europe (France, Belgium)	US 3 monthly vs. 6-monthly	Histo-proven cirrhosis: all Alcohol: 39.4 vs. 39 HCV: 44.7 vs. 43.6 HBV: 12.8 vs. 12.2 Hemochromatosis: 0.8 vs. 2.3 Others: 2.3 vs. 2.6	Within Milan criteria ^b : 79.2 vs. 71.4 (<i>P</i> = 0.4)	Overall mortality (%): 11.3 vs. 12.1 (<i>P</i> = 0.38)	2-year: 95.8 vs. 93.5 5-year: 84.9 vs. 85.8	LTx: 18.9 vs. 4.3 Resection: 5.7 vs. 9.7 Ablation: 37.7 vs. 44.3 Supportive care 9.4 vs. 17.1 (<i>P</i> = 0.1)
Wang <i>et al.</i> ^[14] , 2013	2006-2010	387 (4 months) vs. 357 (12 months)	Asia (Taiwan, China)	US 4-monthly vs. 12-monthly	HepB: 30 vs. 25.2 HepC: 63 vs. 67.2 Cirrhosis: 87.5 vs. 100 (<i>P</i> = 0.27)	BCLC stage ^c : NA 0: 37.5 vs. 6.7 A: 54.2 vs. 66.6 Others: 8.3 vs. 26.7 (<i>P</i> = 0.017)		1-year: 95.8 vs. 80 2-year: 78.8 vs. 64 5-year: 57.4 vs. 56 (<i>P</i> = 0.399)	Curative Rx: 13 vs. 3 Others: 45.8 vs. 80 (<i>P</i> = 0.049)
Taylor <i>et al.</i> ^[16] , 2017	Markov model	1000 vs. 1000	NA	6-monthly US vs. none	Cirrhosis: all (simulated)	NA	HCC mortality 69 vs. 82 (NNS 77) Harm (additional imaging/biopsy) 150 (NNH 7)	NA	NA

[#]HBV: patients with positive serum Hepatitis B surface antigen; ^{*}including cases diagnosed with HCC within the first two months of enrolment; ^aclinical classification of the China Liver Cancer Study group; stage I (early stage, subclinical disease) included patients with no symptoms (and a tumour usually < 5 cm in diameter) at first diagnosis. Stage III (advanced stage), included patients with severe liver dysfunction. The remaining cases between stage I and III were classed as stage II (middle stage); ^bMilan criteria: one tumor ≤ 50 mm in diameter, or 2-3 tumors ≤ 30 mm in diameter without vascular extension or metastasis (based on computed tomography scan); ^cBCLC staging - stage 0: tumor < 2 cm, performance status (PS) 0 and the Child-Pugh A; stage A: single tumor < 5 cm, or up to 3 tumors all < 3 cm, PS 0 and Child-Pugh A or B; stage B: multinodular HCC, PS 0 and Child-Pugh A or B; stage C: portal, lymph node or organ invasion, or PS 1 or 2, Child-Pugh A or B; stage D: PS > 2 or Child-Pugh C. AFP: alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer staging; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; TACE: transarterial chemoembolization; PEI: percutaneous ethanol injection; NA: not available; NNH: number needed to harm; NNS: number needed to screen; LTx: liver transplant; OR: odds ratio; S: surveillance group; NS: no surveillance group; Tx: treatment; US: ultrasound

small focal lesions, however no survival difference was observed between the 2 randomized groups. A community-based study in Taiwan compared 4-monthly to 12-monthly ultrasound surveillance for viral hepatitis B/C patients with platelet level more than 150,000/mL. More frequent surveillance detected smaller HCCs that were amenable for curative treatment modalities. However there was no significant difference in overall survival^[14] [Table 1].

Poustchi *et al.*^[15] attempted to conduct a RCT on HCC surveillance for cirrhotic patients. After risk and benefits of surveillance were discussed, 99.5% of the patients declined randomization, demonstrating the dif-

Table 2. Cohort studies on hepatocellular carcinoma surveillance

Author, year	Study design	Surveillance modality	Study period	Sample size (S vs. NS)	Continent	Etiology (%)	Stage at diagnosis (%)	Mortality	Survival (%)	Treatment received (%)
Chiang <i>et al.</i> ^[65] , 2017	Retro-spective	≥ 3 vs. < 3 US within 2 years of HCC dx	1997-2010	1,472 vs. 3,149	Asia (Taiwan, China)	Cirrhosis: 80.4 vs. 65 HBV: 42.1 vs. 35.5 HCV: 42.1 vs. 21	NA	NA	5-year: 14.4 vs. 7.7 ($P < 0.001$)	Resection: 15 vs. 10.9 RFA: 6.9 vs. 2.3 PEI: 6.4 vs. 16.6 LTx: 0.1 vs. 0.4 TACE: 18.5 vs. 30.8 Chemo 37.1 vs. 46 RT: 20.5 vs. 22.7
Chaiteerakij <i>et al.</i> ^[63] , 2017	Retro-spective	≥ 1 US within 1 year of HCC dx	2007-2013	103 vs. 343	Asia (Thailand)	Cirrhosis: 96.1 vs. 93.6 HBV: 61.2 vs. 52.2 HCV: 25.2 vs. 17.8 Alcohol: 6.8 vs. 12.2 NASH: 3.9 vs. 11.1	BCLC: A: 80.6 vs. 33.8 B: 12.6 vs. 39.1 C: 4.9 vs. 26.2 ($P < 0.001$)	Adju HR: 0.63 (0.45-0.87) ($P = 0.005$)	Median survival (months): 15.9 ($P < 0.0001$)	Curative Tx (resection, RFA, LTx, PEI): 73.8 vs. 44.9 ($P < 0.001$)
Singal <i>et al.</i> ^[21] , 2017	Retro-spective	Imaging (US, CEUS, CT, MRI), within 6 months of HCC dx	2012-2013	157 vs. 217	United States	Cirrhosis: all HCV: 67.5 vs. 49.8 HBV: 5.1 vs. 6.5 Alcohol: 12.7 vs. 16.1 NAFLD: 12.1 vs. 16.1 Others: 2.6 vs. 11.5	BCLC: A: 63.1 vs. 36.4 B: 15.3 vs. 12.4 C: 6.4 vs. 29 D: 15.3 vs. 22.1 ($P < 0.001$)	1-year mortality 22.3 vs. 39.6 ($P < 0.001$)	Median survival (months): 14.6 vs. 6; Survival: 1-year: 75.3 vs. 53.4 3-year: 68.7 vs. 35.5	Curative: 30.6 vs. 13
Mittal <i>et al.</i> ^[66] , 2016	Retro-spective	≥ 2 Imaging (US, CT, MRI) +/- AFP within 2 years of HCC dx	2005-2010	412 vs. 475	United States	Cirrhosis: all HBV: 4.6 vs. 4.6 HCV: 86.9 vs. 70.1 Alcohol: 90.3 vs. 86.7 NAFLD: 6 vs. 21	BCLC: O/A: 27.2 vs. 11.6 B: 22.8 vs. 22.1 C: 26.5 vs. 35.4 D: 24.2 vs. 15 Adj for: HCC stage, Tx	Adj HR: 0.80 (0.69-0.94)	Median survival (months): 16.8 vs. 9.9	Curative: 20.9 vs. 11.6 Palliative: 59.2 vs. 45.5
Oeda <i>et al.</i> ^[20] , 2016	Retro-spective	US + AFP/DCP/AFP-L3 +/- imaging (CT/MRI)	2004-2012	226 vs. 107	Asia (Japan)	Cirrhosis: all HBV: 10.6 vs. 26.2 HCV: 89.4 vs. 73.8 ($P < 0.001$)	I ^a : 31.4 vs. 9.3 II: 37.6 vs. 23.4 III: 26.5 vs. 42.1 IV: 4.4 vs. 25.2 ($P < 0.001$)	NA	Median survival (months, corrected for lead-time bias): 56.5 vs. 31.4 ($P = 0.011$) 1-year: 81.8 vs. 48.9 3-year: 67.9 vs. 58.1 5-year: 36.6 vs. 34.7 ($P < 0.001$)	Resection: 27.9 vs. 27.1 RFA 49.1 vs. 14 TACE 21.2 vs. 42.1 Others: 1.8 vs. 16.8 Curative: OR 3.213 (1.615-6.319, $P = 0.001$)

van Meer <i>et al.</i> ^[62] , 2015	Retro-spective	AFP +/- imaging +/-	2005-2012	295 vs. 779	Europe (Netherlands)	Cirrhosis: 97 vs. 60 ($P < 0.001$) HBV: 20 vs. 14 HCV: 38 vs. 12 Alcohol: 24 vs. 30 NAFLD: 7 vs. 20	BCLC O: 15 vs. 3 A: 46 vs. 18 B: 21 vs. 14 C: 12 vs. 30	> 9 months surveillance: unadjusted HR 0.55 (0.42-0.73) ($P < 0.001$)	1-year: 68 vs. 55 3-year: 47 vs. 29 5-year: 39 vs. 22	Surgical therapy: 34 vs. 25 RFA: 23 vs. 7
Thein <i>et al.</i> ^[22] , 2015	Retro-spective	US	2000-2010	943 vs. 540	Canada	Cirrhosis: 52.4 vs. 42 Viral hepatitis: all	NA	Lead-time corrected HR ^b : 0.76 (0.64-0.91) vs. 0.86 (0.75-0.98)	Median survival (days, lead-time corrected) ^c : 779 vs. 610 vs. 478 3-year: 42.6 vs. 35.7 vs. 29.9 5-year: 31.9 vs. 22.4 vs. 20.7	Curative (S vs. NS): 59.3 vs. 41.3 ($P < 0.001$)
Nusbaum <i>et al.</i> ^[19] , 2015	Retro-spective	AFP +/- imaging	2007-2012	126 vs. 162	US	Cirrhosis (majority HCV, HBV), no detailed data	Early-stage (I&II): 92% vs. 62% ($P < 0.001$)	Adj HR 0.62 (0.41-0.94)	Overall survival: 63 vs. 49 ($P = 0.006$)	LTx: 53 vs. 23 Surgical (LTx + resection): 61 vs. 33 ($P < 0.01$)
Wu <i>et al.</i> ^[23] , 2015	Retro-spective	US	2002-2007	31704 vs. 21119	Asia (Taiwan, China)	Cirrhosis: 62.5 vs. 38.6 HBV: 28 vs. 27 HCV: 30.8 vs. 12 Alcohol: 11.1 vs. 5	NA	5-year mortality ^d : 69.9 vs. 71.1 vs. 77.2 vs. 81	Median survival (lead-time corrected, year) ^d : 2 vs. 1.54 vs. 0.94 vs. 0.73 vs. 0.54	Curative therapy ^d : 24.3 vs. 26.9 vs. 22.9 vs. 21.3 vs. 18.3
Cucchetti <i>et al.</i> ^[67] , 2014	Retro-spective	US +/- AFP	1987-2012	1084 vs. 296	Europe (Italy)	Cirrhosis: all HBV: 10.2 vs. 12.8 HCV: 61.6 vs. 34.5 Alcohol 8.9 vs. 23 Others 6.8 vs. 13.2	Milan criteria: 78.5 vs. 29.7	NA	3-year: 54.4 vs. 24.2 5-year: 31.1 vs. 12.2	LTx: 3 vs. 0.7 Resection: 14.8 vs. 13.9 RFA/PEI: 41.9 vs. 12.5
EL-Serag <i>et al.</i> ^[68] , 2011	Retro-spective	AFP + US	1998-2007	580 vs. 332	US	HCV: all Cirrhosis: NA	NA	HR: 0.71 (0.62-0.82)	3-year: 22 vs. 13	NA
Stroffolini <i>et al.</i> ^[69] , 2011	Pro-spective	AFP + US	2008-2009	257 vs. 154	Europe (Italy)	Cirrhosis: 97.5 vs. 90.1 ($P = 0.003$) HBV: 14 vs. 15.1 HCV: 61.6 vs. 46 ($P = 0.01$) HBV + HCV: 1.3 vs. 2.2	Single tumor: 65.6 vs. 47.1 ($P < 0.0001$) Multinodular: 30.8 vs. 35.3 Diffuse: 3.6 vs. 17.6 Vascular invasion: 9.6 vs. 26.4 Metastasis: 2.2 vs. 5.6	NA	NA	NA
Yang <i>et al.</i> ^[70] , 2011	Retro-spective	Imaging (US/CT/MRI)	2007-2009	136 vs. 307	US	Cirrhosis: 98 vs. 77	Milan criteria: 63 vs. 20	NA	10 months: 52.9 vs. 33.9 20 months: 25% vs. 13.7 30 months: 9.6 vs. 5.2	Curative: 64 vs. 31

Kuo <i>et al.</i> ^[77] , 2010	Retro-spective	AFP + US, 1 year	2002-2004	318 vs. 1118	Asia (Taiwan, China)	Cirrhosis: all HBV: 48.7 vs. 47.1 HCV: 38.1 vs. 33.4	BCLC: O: 8.2 vs. 3.7 A: 60.4 vs. 23.1 B: 21.7 vs. 35.2 C: 6.9 vs. 30.9 ($P < 0.001$)	NA	3-year: 59.1 vs. 29.3	Curative: 45.6 vs. 22.7 TACE: 47.2 vs. 38.2 Other: 7.2 vs. 39.1
Noda <i>et al.</i> ^[61] , 2010	Retro-spective	Imaging (US/CT/MRI)	2001-2007	124 vs. 116	Asia (Japan)	HCV: all Cirrhosis: 73 vs. 64.7	Milan criteria: 88 vs. 44 ($P < 0.001$)	NA	1-year: 90 vs. 50 3-year: 73 vs. 34 5-year: 54 vs. 9	Curative: 80 vs. 45
Pascual <i>et al.</i> ^[72] , 2008	Retro-spective	US + AFP every 6 months	1996-2005	117 vs. 173	Europe (Spain)	Cirrhosis: all Alcohol: 21 vs. 35 HCV: 61 vs. 35 HBV: 3 vs. 6 Others: 10 vs. 13	Tumor size: < 5 cm: 60 vs. 24 > 5 cm: 9 vs. 28 Multifocal: 14 vs. 32	NA	Mean survival (months): 27 vs. 6	LTx: 15 vs. 3 PEI/RF: 31.6 vs. 12.1 TACE: 39 vs. 20
Tanaka <i>et al.</i> ^[73] , 2006	Retro-spective	US + AFP, 6 months	1991-2003	182 vs. 202	Asia (Japan)	HCV: all Cirrhosis: 84 vs. 76	Milan: 86 vs. 50	NA	Median survival (year): 4.7 vs. 3.1 ($P < 0.001$) 3-year: 67 vs. 51 5-year: 46 vs. 32	Resection: 6 vs. 12 PEI/RFA: 60 vs. 34 TACE: 20 vs. 42 Chemo: 3 vs. 9 ($P < 0.001$)
Toyoda <i>et al.</i> ^[74] , 2006	Retro-spective	AFP/DCP +/- imaging	1968-2004	1050 vs. 591	Asia (Japan)	NA	Stage I: 24 vs. 3.6 Stage II: 33.6 vs. 16 Stage III: 24 vs. 15.7 Stage IV: 18.2 vs. 64.6	NA	3-year: 51.4 vs. 27.1 5-year: 35.9 vs. 18.6	LTx: 21.7 vs. 5.1 Resection: 22.6 vs. 9 TACE: 34.1 vs. 27.2 Others: 7 vs. 19.8
Ando <i>et al.</i> ^[75] , 2006	Retro-spective	AFP and imaging	1995-2000	392 vs. 182	Asia (Japan)	Cirrhosis: NA HCV: 87 vs. 74 HBV: 8.7 vs. 17	Early HCC: 73 vs. 26	NA	3-year: 62 vs. 38	Curative: 56.9 vs. 26 Supportive: 0 vs. 7
Trevisani <i>et al.</i> ^[24] , 2004	Retro-spective	US + AFP every 6-12 months	1998-2001	158 vs. 205	Europe (Italy)	Cirrhosis: all HBV: 9.5 vs. 8.3 HCV: 67.1 vs. 60.5 Alcohol: 5.7 vs. 11.7	Tumor ≤ 3 cm ^a : 68.7 vs. 49.3 vs. 6.7 Multifocal: 11.1 vs. 15.9 vs. 22.4 Advanced: 29.7 vs. 60.9 vs. 74.6	NA	Median survival (months, lead-time corrected) ^a : 24 vs. 21 vs. 7	Resection ^a : 8.4 vs. 2.9 vs. 0 TACE: 28.6 vs. 17.6 vs. 20 Others: 27.3 vs. 42.6 vs. 69.2
Yu <i>et al.</i> ^[18] , 2004	Retro-spective	US	1996-1997	164 vs. 516	Asia (Taiwan, China)	Cirrhosis: 91.9 vs. 68.2 HBV: 67.7 vs. 53.6 HCV: 43.9 vs. 31.3	TNM I: 66.2 vs. 19.3 II: 27.2 vs. 37.2 III: 3.7 vs. 28.0 IV: 2.9 vs. 14.6	NA	Unadj OR of survival at 1-year: 3.57 (5.26 - 2.38) 2-year: 3.7 (5.26 - 2.56) 3-year: 3.57 (5.26 - 2.44)	Resection: 53.5 vs. 34 ($P < 0.0001$) TACE: 35.1 vs. 29.9

Trevisani <i>et al.</i> ^[25] , 2002	Retro-spective	US + AFP every 6-12 months	1988-1998	370 vs. 451	Europe (Italy)	Cirrhosis: all 6 months vs. 12 months vs. NS HBV: 13.6 vs. 20.4 vs. 20.5 HCV: 66.6 vs. 62.5 vs. 55.9 Alcohol: 8.5 vs. 7.2 vs. 13.8	6 months vs. 12 months vs. NS Non-advanced: 68.7 vs. 60.4 vs. 31 Advanced: 31.3 vs. 39.6 vs. 69	NA	Median survival (months, lead-time corrected): 30 vs. 14 3-year: 48 vs. 23	Curative 41 vs. 27 ($P < 0.001$)
Chen <i>et al.</i> ^[76] , 2002	Retro-spective	Clinical markers ¹ + US	1991-1998	4385 vs. 458	Asia (Taiwan)	Cirrhosis: 7 vs. unknown HBV: 65.9 vs. 67.0 HCV: 18.2 vs. 14.9	NA	HR: 0.76 (0.38-1.52)	NA	NA
Yuen <i>et al.</i> ^[60] , 2000	Retro-spective	AFP +/- US	1995-1997	142 vs. 164	Asia (HK)	Cirrhosis: 85.2 vs. 68.9 ($P = 0.0013$) Multifocal: 32.4 vs. 50 PV invasion: 9.2 vs. 38.4 ($P < 0.001$)	Tumor < 3 cm: 40.1 vs. 4.9 Tumor < 5 cm: 61.3 vs. 11.6	NA	Median survival (months): 22 vs. 5	Curative resection: 26.8 vs. 7.9 ($P < 0.001$) TACE: 45.1 vs. 32.3 ($P = 0.03$)

^aTumor stages per Liver cancer study group of Japan guidelines, based on: (1) tumor diameter ≤ 20 mm; (2) single tumor; (3) no vascular invasion; tumors that met three, two, one or none of the conditions were classified as stage I, II, III, or IV respectively; ^bHazard ratio in routine surveillance (≥ 1 US surveillance annually) vs. inconsistent surveillance compared to no surveillance; ^ccomparison groups: routine surveillance vs. inconsistent surveillance vs. no surveillance; ^dcomparison groups: surveillance 1-6 months vs. 7-12 months vs. 13-24 months vs. 25-36 months vs. never screened; ^ecomparison groups: HCC diagnosed from surveillance 6-12 months vs. incidental diagnosis vs. symptomatic diagnosis; ^f6 markers: (1) positive hepatitis B surface antigen (HbsAg); (2) positive antibody for hepatitis C (anti-HCV); (3) alpha-fetoprotein (AFP) ≥ 20 ng/mL; (4) aspartate transaminase (AST) ≥ 40 IU/L; (5) alanine transaminase (ALT) ≥ 45 IU/L; and (6) family history of HCC. Adj HR: adjusted hazard ratio; BCLC: Barcelona Clinic Liver Cancer staging; CEUS: contrast-enhanced ultrasound; CT: computed tomography; DCP: des-gamma-carboxyprothrombin; dx: diagnosis; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HK: Hong Kong; HR: hazard ratio; NA: not available; LTx: liver transplant; OR: odds ratio; RFA: radiofrequency ablation; PEI: percutaneous ethanol injection; S: surveillance group; NS: no surveillance group; TACE: transarterial chemoembolization; TNM: tumor, node, metastasis staging system of the American Joint Committee on Cancer; Tx: treatment; Unadj: unadjusted; US: ultrasound

difficulty of conducting RCTs on HCC surveillance among cirrhotic patients. Hence, Taylor *et al.*^[16] used the Markov model to simulate a HCC surveillance program on cirrhotic patients, and to study the benefit and harm of surveillance. A small absolute mortality benefit was found in the HCC surveillance group, with a number needed to screen of 77. After a focal lesion was identified, further investigations were carried out based on EASL-EORTC (European Association for the Study of the Liver and the European Organization for Research and Treatment of Cancer) recall policy^[17]. However, many more patients experienced additional unnecessary imaging or biopsy due to false positive results, with a number needed to harm of 7 only [Table 1].

Cohort studies

There are a large number of cohort studies on the efficacy of HCC surveillance in our literature search over the past 20 years. We included twenty-four retrospective cohort studies that compared survival and/or mortality of surveillance-detected HCC to incidentally diagnosed HCC [Table 2]. In general, the patients in the surveillance group have chronic viral hepatitis [hepatitis B virus (HBV) and hepatitis C virus (HCV)] infection or cirrhosis of any etiology [Table 2].

Patients in the surveillance group had earlier stages of HCC at diagnosis: 22.8%-80.3% of surveillance group patients had Barcelona Clinic Liver Cancer staging (BCLC) stage 0/A disease. Not surprisingly, more patients in the surveillance compared to non-surveillance group underwent curative HCC treatment [surgical resec-

tion, radiofrequency ablation (RFA)/percutaneous ethanol injection, liver transplant]. Among the reported studies, up to 53.5% of patients in the surveillance group underwent surgical resection^[18], 53% received liver transplant^[19], 49.1% received RFA^[20]. The reported median survival in the surveillance group differs among the studies. Singal *et al.*^[21] reported 14.6 months median survival in patients whose HCC was detected from surveillance imaging [computed tomography (CT)/magnetic resonance imaging (MRI)/contrast-enhanced ultrasound/ultrasound (US)] within 6 months of HCC diagnosis; while Oeda *et al.*^[20] reported 56.5 months of median survival (corrected with lead-time) in the Japanese population, where high-risk cirrhosis patients were screened every 3-4 months with US and serum biomarkers [AFP/AFP-L3/des-gamma-carboxyprothrombin (DCP)] based on Japanese society of Hepatology practice guidelines. Most of these cohort studies carry selection bias (specialist centre referrals), lead-time and length-time bias inherent to the study design. Several studies attempted to correct for the lead-time bias in survival time reporting, based on HCC doubling time (90-120 days)^[21,23-26]. Overall, the data from cohort studies demonstrated that HCC surveillance was associated with early-stage tumor detection and curative treatments. Improved overall survival was evidenced in the surveillance group as well. Thus, the benefits of surveillance included early diagnosis, more treatment options, and prolonged survival compared to no surveillance [Table 2].

Several prospective cohort studies were conducted to investigate the benefit of HCC surveillance in at-risk populations. Two studies examined surveillance in chronic hepatitis B patients. McMahon *et al.*^[26] conducted a population-based prospective study for 16 years on Alaska natives with chronic hepatitis B patients. Surveillance modality was 6-monthly AFP. Surveillance detected more early resectable HCC and accorded significantly longer survival. A study in Thailand by Ungtrakul *et al.*^[27] recruited 2,293 chronic hepatitis B patients and surveillance was carried out with 6-monthly AFP and ultrasound. A high 3-year survival of 90% was observed as most patients were able to receive curative treatments. A Taiwanese group evaluated a community-based HCC surveillance program with abdominal ultrasound. Subjects were selected from a risk score. Mortality in the surveillance group was reduced compared to the control group and the general population^[28]. Overall, evidence supports HCC surveillance in at-risk populations because it detects smaller tumors that are amenable to curative treatment [Table 2].

Harm of surveillance

The study by Taylor *et al.*^[16] simulated HCC surveillance in cirrhotic patients based on EASL-EORTC recall policy [Table 1]. It showed more patients experienced unnecessary biopsy or imaging due to false positive screening results, and the calculated number needed to harm was only 7 compared to a small mortality benefit. Few cohort studies mentioned the harm of HCC surveillance. One retrospective cohort study by Atiq *et al.*^[29] aimed to characterize the correlation of harm and benefits in cirrhosis patients undergoing HCC surveillance. Surveillance-related harm was defined as additional scans, biopsies, or procedures performed for false-positive or indeterminate results. Around one quarter (27.5%) of the patients experienced harm, and it was more often related to ultrasound than AFP. This was associated with hepatology subspecialty care, elevated ALT, and portal hypertension with thrombocytopenia. However, psychological harm and financial harm were not evaluated in this study.

Surveillance modalities

Cancer surveillance tools should be accurate and cost-effective, and able to detect tumor at a stage that cure is possible. HCC usually develops in populations with defined risk factors. Cirrhosis is the major risk factor of HCC development, with an annual incidence of 1.5%, which makes HCC a good target for surveillance^[30,31]. At present, ultrasound and serum AFP are widely accepted as the primary surveillance tools for HCC. Here we reviewed the current evidence of HCC surveillance tools.

Imaging

The recommended surveillance modality differs slightly in different parts of the world, but the majority recommends ultrasound imaging with or without serum AFP^[32].

A shortcoming of ultrasound in HCC surveillance is its relatively low sensitivity and specificity^[33]. A recent retrospective cohort study by Samoylova *et al.*^[34] investigated the predictors for ultrasound failure of HCC detection. It was found that the sensitivity of ultrasound to detect HCC for subjects with BMI ≥ 30 was significantly lower (0.76) compared to those with BMI < 30 group (0.87). Patients with NASH had a ultrasound sensitivity of only 0.59 compared to 0.84 in other etiologies, suggesting 41% of HCC would be missed in this population. Thus we currently lack an ideal first-line imaging modality for surveillance of HCC in patients with NASH despite the latter becoming an increasingly prevalent liver disease worldwide.

A recent systemic review and meta-analysis studied the use of surveillance imaging, with or without AFP, for early detection of HCC in patients with cirrhosis. Thirty-two studies were reviewed and ultrasound was found to have a good sensitivity for detecting any stage HCC. However it performs poorly in detecting early-stage HCC with only 47% sensitivity. The combination of ultrasound with AFP increased the sensitivity (65%) but also lowered the specificity for HCC detection^[33].

Pocha *et al.*^[35] conducted a randomized trial comparing biannual ultrasound vs. annual CT in HCC surveillance of cirrhotic patients. CT has a comparable sensitivity (62.5%) to ultrasound-based surveillance. However, due to its high cost and repeated radiation exposure, no evidence so far supports the use of CT as surveillance modality. Studies comparing MRI and ultrasound showed that MRI has a significantly higher sensitivity than ultrasound (83.7% vs. 25.6%) for HCC detection in cirrhotic patients^[36]. However, the high cost, limited availability of scanners and long scanning time make MRI not ideal as a surveillance tool.

Serum biomarkers

Serum biomarkers are cancer-related molecules or substances that are measurable in the peripheral blood, enabling early cancer detection. They are attractive tools in cancer surveillance and diagnosis as they are noninvasive with the convenience of repeated sample collections.

The most commonly used serum marker in HCC is AFP, which by itself has limited sensitivity and specificity, and serves as an adjunct to imaging in HCC diagnosis. AFP-L3 measures the AFP isoform that is reactive to lens culinaris agglutinin. It is widely used for HCC surveillance in Japan. A recent study on AFP-L3 by Kumada *et al.*^[37] involving 2,830 patients in a HCC surveillance program found that 34.3% of the patients had elevated AFP-L3 1 year prior to the diagnosis of HCC, suggesting that it can be an earlier predictor of HCC development.

DCP, also known as prothrombin-induced by vitamin K absence-II, is an abnormal prothrombin formed in the presence of vitamin K antagonism. The performance of DCP varies among different studies^[38,39]. One study by Ji *et al.*^[40] studied DCP vs. AFP in HBV-related HCC, and concluded that DCP is complementary to AFP in detecting AFP-negative HCC, and excluding HCC in cirrhotic patients with false positive AFP, suggesting its complementary role in HCC surveillance. Similar conclusion was drawn in HCV cohorts by the Italian group^[41].

Other biomarkers studied were GPC3 (plasma membrane bound protein), Golgi protein 73, interleukin-6, and squamous cell carcinoma antigen. These biomarkers have been studied for many years, but had inconsistent performance in different patient populations, precluding its wide use in HCC surveillance.

Liquid biopsy

Recent advances in genomics sequencing technologies allow identification and quantification of cancer genetic material in the circulating blood. This has enabled the discovery of novel biomarkers and increased our understanding of HCC cancer genomics.

Liquid biopsy refers to the sampling of bodily fluid instead of solid tissue for the genetic material of cancer. The most common sampling markers are cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and cell-free RNAs (e.g., miRNA), which are the byproducts of tumor cells. In contrast to solid tumor biopsy, liquid biopsy is less invasive and allows repeated sampling for dynamic evaluation of disease status and prediction of clinical outcomes. Solid tumour biopsy is infrequently done now as it is painful, carries risks of bleeding and iatrogenic tumor seeding. Liquid biopsy has been shown to have higher sensitivity in the early tumor detection and prognostication. The application of liquid biopsy in HCC is still under evaluation.

Liao *et al.*^[42] conducted a meta-analysis to evaluate the use of cfDNA in HCC diagnosis. By quantitatively and qualitatively analysing the concentrations of circulating cfDNA, as well as single-gene methylation alterations, they found that the combination of AFP and cfDNA can attain an optimal sensitivity of 81% and specificity of 96% in the diagnosis of HCC in at-risk patients.

CTCs are mainly studied for its role in cancer recurrence, prognosis, and response to treatments. It has not been investigated in the context of HCC surveillance.

Data on the role of ctDNA in HCC are limited. Zhou *et al.*^[43] studied the size profiles of plasma DNA in 90 HCC patients, and found aberrantly short DNA molecules in HCC patients as well as elevated amounts of mitochondrial DNA. Their presence raises the suspicion of early HCC during surveillance process. The detection of ctDNA can also predict metastasis in 86% of the HCC patients.

Several studies were done on quantification of circulating miRNA to facilitate the diagnosis of HCC in chronic hepatitis^[44-46] and hepatitis C patients^[47]. Li *et al.*^[44] studied the serum miRNA levels of control, HBV and HBV-positive HCC patients. They found that miR-375 alone has a high diagnostic accuracy of HCC compared to control patients, and miRNA expression profiles can differentiate HBV patients from control, and HBV-positive HCC patients from HBV patients. The study suggested that serum miRNAs can be used as noninvasive biomarkers for the diagnosis of HBV infection and HBV-positive HCC. Hung *et al.*^[45] demonstrated that serum circulating miRNAs, miR-122 and miR-let-7b, can differentiate dysplastic nodules from early HCC in chronic hepatitis B patients. A recently published paper by the Vietnamese group collected all the published data on miRNAs in HCC, and established a miRNA panel for HCC diagnosis. Three miRNAs, miR-21, 122, and 192, together with AFP can be combined to diagnose early HCC in hepatitis B patients^[46].

In the current clinical setting, liquid biopsy has limited applications in HCC surveillance owing to the lack of standardized methodology and the high cost of genetic sequencing, which needs to be improved with more studies and standardization of assays. The high cost of genetic sequencing also precludes its use as a surveillance modality for HCC. However, liquid biopsy offers a noninvasive method of characterizing HCC tumor cells' genomic mutations and molecular pathways, hence offers opportunities for further studies on the therapeutic targets in HCC. It is promising as a non-invasive, accurate and convenient surveillance tool for HCC in the future.

DISCUSSION

This study reviewed the current status of the literature on the efficacy, benefit and harm of HCC surveillance, as well as new developments in surveillance modalities. The benefit of HCC surveillance was demonstrated in one RCT and supported by a significant number of cohort studies. Although significant bias may be present, it is not feasible to conduct further randomized trials due to ethical concerns^[15]. Cohort studies demonstrated earlier tumor detection and longer survival in HCC patients diagnosed from surveillance. However, the proportion of patients diagnosed at early-stage and length of survival differs significantly in

different cohorts, suggesting that the benefit is not homogeneous in all HCC patients. Different ethnic origin and HCC etiologies likely contributed to this heterogeneity. Patients with NASH and alcoholic liver disease are more common in the United States than Asian and European population, and has lower risk of developing HCC^[48].

Non-alcoholic fatty liver disease (NAFLD) and NASH are emerging causes of liver cirrhosis and HCC. It has been reported that the yearly HCC incidence among NASH-cirrhotic patients was 2.6%^[49], and a significant number of patients with NAFLD-related HCC did not have cirrhosis^[50,51]. NASH-related HCC patients received significantly less HCC surveillance compared to HCV or alcohol-related HCC patients, and received less HCC-related treatment. However, the one-year survival rate was similar^[51]. At present, AASLD and EASL guidelines do not recommend routine HCC surveillance for non-cirrhotic NASH patients. More studies are needed to develop a cost-effective surveillance program in this population.

Chronic hepatitis C infection had been a major risk factor for liver cirrhosis and HCC in the world. The incidence of HCC in patients with chronic hepatitis C infection was reported to be 1%-4%, higher in patients with cirrhosis^[52-54]. Treatment with direct-acting antivirals (DAAs) has impressive efficacy in Hepatitis C eradication. However, its effect on the long-term clinical outcome was lacking^[55]. Conti *et al.*^[56] found that unfortunately HCC occurrence was not reduced in successfully treated cirrhotic patients. Recently, a systemic review and meta-analysis was performed by Singh *et al.*^[57] on oral DAAs use and risk of HCC development. A total of 8 controlled studies and 36 uncontrolled studies were reviewed, and the estimated incidence of HCC was 3.3% and 1.5% (1 in 67 DAA users) in controlled and uncontrolled studies respectively, not significantly different from the previously reported incidence in chronic hepatitis C patients. Moreover, the HCC recurrence rate was as high as 16.7%-20.1% with DAAs treatment. Hence, continuing HCC surveillance is still important in patients treated with DAA for hepatitis C, even after achievement of sustained virological response.

Two earlier reviews on HCC surveillance were published in 2014. A meta-analysis done by Singal *et al.*^[58] aimed to determine the effect of surveillance on cirrhotic patients. Studies published from 1990 to 2014 were reviewed and pooled odds ratio was calculated on 47 selected studies with a total of 15,158 HCC patients. Surveillance was associated with early-stage cancer detection (OR 2.08 CI 1.8-2.37), curative treatment rates (OR 2.24 CI 1.99-2.52), and prolonged survival (OR 1.9 CI 1.67-2.17), supporting HCC surveillance in cirrhotic patients. On the other hand, Kansagara *et al.*^[59] did a systemic review to study the strength of evidence supporting HCC surveillance. A total of 22 studies were selected and the overall strength of evidence on the effect of screening was very low owing to limited randomized trials and significant confounders in cohort studies. Screening identified early-stage HCC. However, its effect on mortality and survival in chronic liver disease patients is not clear. The conflicted evidence may have contributed to the underutilization of HCC surveillance in some regions.

The harm of HCC surveillance is an important issue but there were few studies published. One retrospective cohort study demonstrated that one fourth of the patients who underwent HCC surveillance required additional tests due to false positive or indeterminate results. This calls for development of new surveillance modalities that minimize false positive results without compromising the diagnostic accuracy for HCC. Although imaging modalities such as contrast-enhanced CT and MRI have high sensitivity and specificity, they are not recommended for surveillance due to the high cost and limited availability. Other than AFP, serum biomarkers are not widely accepted as surveillance tools except in a few countries, such as Japan. More studies are needed to evaluate the clinical utility of novel serum biomarkers and their role in HCC surveillance. Liquid biopsy is the latest tool in cancer diagnosis and prognosis. Emerging evidence indicates that liquid biopsy can be used in HCC surveillance as it is noninvasive and provides a dynamic profile of disease progression.

In conclusion, studies have shown that current surveillance strategies can detect significantly more early stage HCCs: 61.3%-88% were within Milan criteria and 61%-91.7% were BCLC stage 0/A compared to 11.6%-44% and 21%-73.3% respectively for subjects who were not on HCC surveillance^[14,60-62]. Up to 73.8%-80% of the HCC patients in the surveillance group received curative management with a median survival as high as 4.7 years and a 3-year survival of up to 73% compared to only 45% of subjects not on surveillance being amenable to curative therapy with a median survival of only up to 2.6 years^[20,61,63]. Hence, HCC surveillance in at-risk patients is beneficial and improves patient outcome.

Further research on hepatocarcinogenesis and novel surveillance tools will continue to help refine the surveillance guidelines. In particular, further understanding of the hepatocarcinogenesis pathway in non-alcoholic fatty liver disease-related HCC is needed to evolve a surveillance strategy for this huge group of patients. The aim is always to detect more curable HCC in patients with chronic liver disease and hence reduce HCC-related mortality in the near future.

DECLARATIONS

Acknowledgements

We like to thank and acknowledgement our colleague Dr. Yu-Tien Wang for his insightful comments on the article.

Author's contributions

Design of the work: Chen K, Tan CK

Acquisition, analysis of data: Chen K

First draft of the manuscript: Chen K

Writing and editing of the manuscript: Chen K, Chang PE, Goh GBB, Tan CK

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Simple screening method for the diagnosis of nonB-nonC hepatocellular carcinoma

Kazuhiro Nouso^{1,2}, Yoshie Furubayashi¹, Shohei Shiota¹, Akiko Wakuta¹, Ayano Oonishi¹, Kazuya Kariyama¹, Yasuto Takeuchi², Nozomu Wada², Hideki Onishi², Takuya Adachi², Atsushi Oyama², Chihiro Dohi², Tetsuya Yasunaka², Yuki Yasunaka², Fusao Ikeda², Hidenori Shiraha², Akinobu Takaki², Hiroyuki Okada²

¹Department of Gastroenterology, Okayama City Hospital, Okayama 700-8557, Japan.

²Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan.

Correspondence to: Dr. Kazuhiro Nouso, Department of Gastroenterology, Okayama City Hospital, 3-20-1, Kitanagase-omotemachi, Kita-ku, Okayama City, Okayama 700-8557, Japan. E-mail: kazunouso@gmail.com

How to cite this article: Nouso K, Furubayashi Y, Shiota S, Wakuta A, Oonishi A, Kariyama K, Takeuchi Y, Wada N, Onishi H, Adachi T, Oyama A, Dohi C, Yasunaka T, Yasunaka Y, Ikeda F, Shiraha H, Takaki A, Okada H. Simple screening method for the diagnosis of nonB-nonC hepatocellular carcinoma. *Hepatoma Res* 2018;4:73. <http://dx.doi.org/10.20517/2394-5079.2018.93>

Received: 8 Aug 2018 **First Decision:** 31 Aug 2018 **Revised:** 8 Nov 2018 **Accepted:** 9 Nov 2018 **Published:** 12 Dec 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cui Yu **Production Editor:** Huang-Liang Wu

Abstract

Aim: The incidence of non-virus-related nonB-nonC hepatocellular carcinoma (NBNC-HCC) is on the rise. However, screening at-risk individuals using imaging methods is complicated by the large size of the at-risk patient pool. The aim of this study is to develop an effective simple screening method, using blood tests.

Methods: The diagnostic value of aspartate aminotransferase (AST), alpha-fetoprotein (AFP), and des-gamma-carboxy prothrombin (DCP) was analyzed using sera from 203 NBNC-HCC patients and 106 diabetes mellitus patients.

Results: Areas under receiver operating characteristic curves for AST, AFP, and DCP were 0.844, 0.901, and 0.914, respectively. The optimal cut-offs for diagnosing NBNC-HCC based on Youden indices were 30 IU/L, 3.6 ng/mL, and 25 mAU/mL, respectively. On selecting patients who were positive at least one parameter (AST, AFP, or DCP), the sensitivity was 97.5%. This high sensitivity was preserved (98.0%) even in cases of non-advanced HCC (≤ 3 cm, ≤ 3 nodules). Specificity was 72.6%.

Conclusion: This simple triple screen for AST, AFP, and DCP appears to have diagnostic value in NBNC-HCC and could be used to select candidates for further testing using imaging.

Keywords: Hepatocellular carcinoma, screening, diagnosis, alpha-fetoprotein, aspartate aminotransferase, desgamma-carboxy-prothrombin



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INTRODUCTION

Hepatocellular carcinoma (HCC), a frequent complication of chronic hepatitis virus infection, is the second-leading cause of cancer death worldwide^[1]. Due to the development of a HCC screening system with ultrasonography in patients with viral hepatitis, early diagnosis of HCC has been improved in Japan^[2,3]. Moreover, the spread of direct acting antivirals for hepatitis C virus and nucleoside analogues for hepatitis B virus, has curtailed the incidence of hepatitis virus-related HCC^[4-6].

In contrast, the incidence of non-virus-related nonB-nonC HCC (NBNC-HCC) is on the rise^[7]. Aging, excessive alcohol consumption, diabetes mellitus (DM), and non-alcoholic steatohepatitis (NASH) are considered major risk factors for NBNC-HCC^[8,9]. However, periodic screening of patients at risk using imaging modalities, which is the recommended practice by the Japan society of hepatology^[9], is not realistic due to the large number of patients with these risk factors^[10]. Even if screening were limited to only the patients with DM, the estimated patient number in Japan would be over 7 million^[11]. In order to implement a comprehensive screening for NBNC-HCC, the development of effective non-imaging screening methods is necessary.

There are several reports indicating that an elevation in the serum transaminase levels is a risk factor for HCC^[12,13]. In addition, serum levels of alpha-fetoprotein (AFP) were found to reliably predict the development of HCC in patients with chronic hepatitis C virus infection who had achieved a sustained virological response^[14-16]. A third potential biomarker, des-gamma-carboxy prothrombin (DCP) should also be considered as it was reported to be a better marker for HCC than AFP in NBNC-HCC^[17].

In this study, we examined the diagnostic utility of aspartate aminotransferase (AST), AFP, and DCP as non-imaging screening markers for NBNC-HCC.

METHODS

Patients

Between January 2001 and December 2016, 1,285 consecutive patients were initially diagnosed with HCC and treated at the Okayama University Hospital. Among these, 203 patients who were negative for both the hepatitis B virus surface antigen and hepatitis C virus antibody were diagnosed with NBNC-HCC and enrolled to the test group. Additionally, 106 patients with DM treated at the outpatient clinic of Okayama City Hospital were enrolled to the control group. For validation, 86 NBNC-HCC patients treated at Okayama City Hospital were also enrolled.

Diagnosis

HCC was diagnosed using imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), or angiography. Diagnostic criteria for HCC were based on previous reports of hyperattenuation at the arterial phase or hypoattenuation at the portal phase, determined with dynamic CT or MRI, and tumor staining in angiography^[18].

There was no history of cancers, including HCC, in the control group, and abdominal ultrasonography was used to rule out HCC in the 6 months prior to enrollment. None of the patients were on warfarin or vitamin K.

Determination of diagnostic accuracy

Serum levels of AST, AFP, and DCP were compared between the NBNC-HCC and control groups. Sensitivity and specificity for the three markers used in the diagnosis of HCC were analyzed at different cut-offs. In addition, optimal cut-offs were determined using the receiver operating characteristic (ROC) curve and by calculation of Youden index. The rate of patients whose serum levels for any of the three markers were higher than the optimal cut-offs, was also analyzed.

Table 1. Patient characteristics

Variables	NBNC-HCC (n = 203)	DM (n = 106)	P-value
Age (years)	69 (24-90)	65 (25-92.0)	0.024
Sex (male)	158 (77.8%)	64 (60.4%)	0.001
Total bilirubin (mg/dL)	0.8 (0.1-4.8)	0.6 (0.2-1.2)	< 0.001
Albumin (g/dL)	3.8 (2.0-5.1)	4.1 (3.1-5.0)	< 0.001
Platelet ($\times 10^4/\text{mm}^3$)	13.7 (2.2-65.3)	23.5 (2.6-40.6)	< 0.001
AST (IU/L)	42 (14-611)	22 (13-75)	< 0.001
ALT (IU/L)	33 (2-377)	20 (9-247)	< 0.001
Tumor size (mm)	28 (8-200)	NA	NA
Tumor number (> 3)	53 (26.4%)	NA	NA
AFP (ng/mL)	8.5 (0.6-376210)	1.9 (0.5-10.9)	< 0.001
DCP (mAU/mL)	98 (11-1323600)	16 (8-48)	< 0.001

Values are the median (range), unless otherwise indicated; NBNC-HCC: nonB-nonC hepatocellular carcinoma; DM: diabetes mellitus; AST: aspartate aminotransferase; ALT: alanine aminotransferase; AFP: alpha-fetoprotein; DCP: des-gamma-carboxy prothrombin; NA: not applicable

Statistical analysis

The baseline characteristics were summarized as medians and ranges. Differences in the continuous variables were compared using the Wilcoxon rank-sum test, while categorical variables were analyzed using the chi-square test. All significance tests were two-sided, and $P < 0.05$ was considered significant. Analyses were performed using the JMP software program (ver. 13.0, SAS Institute Japan Ltd., Tokyo, Japan).

RESULTS

Patient characteristics

The patient characteristics are shown in Table 1. Patients in the NBNC-HCC group compared with the control group, respectively, were slightly older (median age, 69 vs. 65 years; $P = 0.024$), with a higher percentage of males (77.8% vs. 60.4%; $P = 0.001$). The median tumor size was 28 mm and in 48.8% of cases, the tumor was under 3 cm in diameter with less than or equal to three nodules, and thus treatable by surgical resection or local ablation therapies. AST, AFP, and DCP were significantly elevated in the NBNC-HCC group compared to the control group.

Evaluation of diagnostic accuracy of AST, AFP, and DCP

Area under ROC (AUROC) curve values for AST, AFP, and DCP were 0.844 (95% confidence interval; 0.793-0.884), 0.901 (95% confidence interval; 0.861-0.929), and 0.914 (95% confidence interval; 0.878-0.940), respectively. The optimal cut-off values, as calculated with Youden indexes, were 30 IU/L, 3.6 ng/mL, and 25 mAU/mL, respectively. Positivity rates for the different parameters in the two groups at different cut-offs are shown in Table 2. The combination of the three factors with the optimal cut-offs achieved a high positive rate (97.5%) in the NBNC-HCC group [Figure 1A], which was maintained (98.0%) in a subgroup of patients with non-advanced HCC (≤ 3 cm, ≤ 3 nodules). Using the same cut-offs, the positive rate in the control group was 27.4% [Figure 1B].

Because the control group consisted of patients with DM, we also analyzed the positive rate in NBNC-HCC patients with DM. The rates were 98.5% (66/77) in all HCC patients with DM and 97.0% (32/33) in patients with DM with non-advanced HCC (≤ 3 cm, ≤ 3 nodules). The test positivity rates in NBNC-HCC patients without DM were 97.1% (132/136; all cases) and 98.5% (64/65; non-advanced HCC). No statistical difference in the positive rate was observed when comparing NBNC-HCC cases with and without DM ($P = 0.531$).

We checked the positivity in the validation set and similar result was obtained (81/86, 94.2%). The rate was maintained (61/57, 93.4%) in non-advanced HCC (≤ 3 cm, ≤ 3 nodules).

Table 2. Diagnostic accuracy using different cut-offs

Variables (cut-off)		Positive rate		
		NBNC-HCC	≤ 3 cm, ≤ 3 nodules	DM
AFP	(3.6 ng/mL)*	161 (79.3%)	74 (75.5%)	10 (9.4%)
	(5 ng/mL)	138 (68.0%)	56 (57.1%)	5 (4.7%)
	(10 ng/mL)	96 (47.3%)	29 (29.6%)	1 (0.94%)
DCP	(25 mAU/mL)*	165 (81.3%)	71 (72.5%)	9 (8.5%)
	(40 mAU/mL)	144 (70.9%)	53 (54.1%)	1 (0.94%)
	(100 mAU/mL)	101 (49.8%)	25 (25.5%)	0
AST	(30 IU/L)*	148 (72.9%)	66 (67.4%)	16 (15.1%)
	(40 IU/L)	107 (52.7%)	45 (45.1%)	10 (9.4%)
	(80 IU/L)	24 (11.8%)	6 (6.1%)	0
Combination**		198/203 (97.5%)	96/98 (98.0%)	29/106 (27.4%)
HCC with diabetes		66/67 (98.5%)	32/33 (97.0%)	-
HCC without diabetes		132/136 (97.1%)	64/65 (98.5%)	-

*Optimum value for detecting HCC; **combination of AST, AFP, and DCP; NBNC-HCC: nonB-nonC hepatocellular carcinoma; DM: diabetes mellitus; AFP: alpha-fetoprotein; DCP: des-gamma-carboxy prothrombin; AST: aspartate aminotransferase; HCC: hepatocellular carcinoma

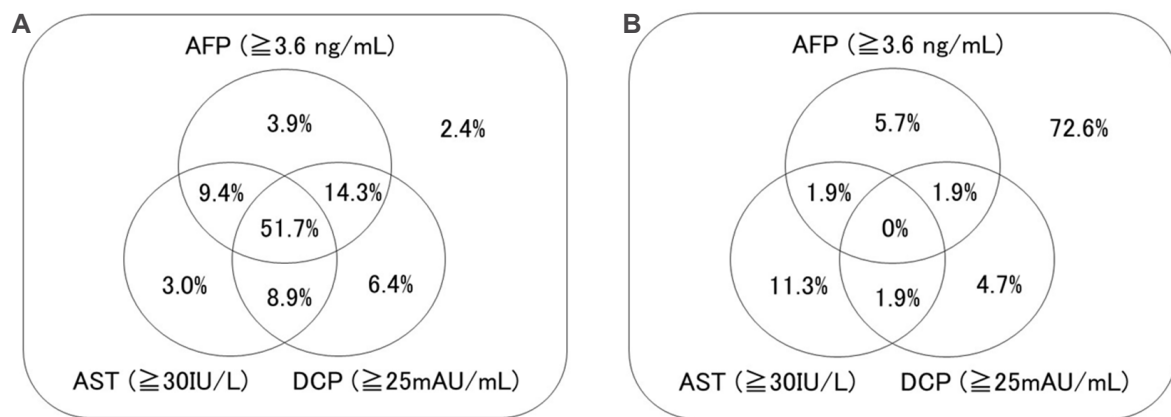


Figure 1. Distribution of marker-positive patients. Distribution of the patients positive for aspartate aminotransferase (AST), alpha-fetoprotein (AFP), and des-gamma-carboxy prothrombin (DCP) in patients with nonB-nonC hepatocellular carcinoma (NBNC-HCC). The majority (97.6%) of patients with NBNC-HCC showed elevations in AST, AFP and/or DCP over the selected cut-offs (A); distribution of the three markers in patients with diabetes mellitus (B). One-fourth (27.4%) of the patients showed elevations in AST, AFP, and/or DCP over the selected cut-offs

DISCUSSION

This study shows, for the first time, that a triple screen for serum AST, AFP, and DCP can be used to identify patients at high risk for NBNC-HCC. Over 95% of patients with NBNC-HCC in this study showed elevations in AST (≥ 30 IU/L), AFP (≥ 3.6 ng/mL) and/or DCP (≥ 25 mAU/mL), regardless of the diabetic status. In contrast, only one-fourth (27.4%) of DM patients without HCC showed elevations in AST, AFP, and/or DCP. These patients can be considered to be at risk for developing HCC, meaning that this triple screening method could be used to identify patients who need further testing using imaging.

AFP is an oncofetal protein originally recognized as an HCC tumor marker^[19]. There are many studies that set the cut-offs at high level because most of the control subjects were active chronic viral hepatitis. As the results, many of them showed low diagnostic abilities. Its sensitivity in HCC diagnosis increased to range between 49% and 71% when using a lower cut-off of 20 ng/mL^[9]. However, AFP has not been considered a tumor marker of choice in NBNC-HCC, as its elevation in this condition is less pronounced than that in hepatitis virus-related HCC^[17]. Recently, AFP has also been identified as a marker of carcinogenic potential of the liver. By using low cut-off (5 ng/mL), it can reliably predict development of HCC in chronic hepatitis C

patients who have achieved a sustained virological response^[14-16]. The positive rate in DM patients was only 4.7%, using the same cut-off level. The rate was still low (9.4%) even when the cut-off was lowered to 3.6 ng/mL, which is the cut-off adopted in this study. Approximately 80% of patients with NBNC-HCC showed an AFP elevation over 3.6 ng/mL. These results indicate that AFP is a good marker for NBNC-HCC with the low cut-off, in agreement with the results obtained in HCV patients with a sustained virological response. It should be noted that the origin of serum AFP, whether from highly carcinogenic liver parenchymal cells or from HCC, remains unclear.

In contrast to AFP, DCP is considered a reliable marker for NBNC-HCC, and was hence included in this combination screen^[17]. The sensitivity and specificity of DCP at a commonly used cut-off (40 mAU/mL) were 70.9% and 99.1%, respectively. Even on lowering the cut-off to 25 mAU/mL to maximize the detection rate, we still observed a high specificity (91.5%). Although DCP testing is not meaningful in patients using drugs affecting vitamin K levels (warfarin, menatetrenone, *etc.*), our results show that it is a good marker for NBNC-HCC in the majority of cases.

Several reports indicate that an elevation in serum transaminase levels increases the risk for HCC^[12,13]. A large cohort study examining over 0.4 million people conducted by a private health screening firm in Taiwan revealed that abnormal AST levels were associated with a 3.3-10.9-fold increased risk for HCC, compared with normal AST levels (< 25 IU/L)^[13]. In contrast, hazard ratio of the patients with abnormal alanine aminotransferase (ALT) levels was not high (1.29). In preliminary analysis with our cohort, the AUROC for ALT (0.68) was significantly lower than that of AST (0.84) ($P < 0.001$). Based on these findings, we included AST in the triple screen.

In this study, we show that the use of a triple screening method increased the sensitivity for HCC to 97.5%. Although the specificity was decreased to 72.6%, this index is useful because it reduced the number of candidates who required further screening to one-fourth of the original population size, which is a more manageable number for screening with imaging. Notably, this screening strategy produced high sensitivity even in patients in the early stages of HCC (tumor size ≤ 3 cm, ≤ 3 nodules), indicating that a triple screen for AST, ALT, and DCP may be useful for early detection of HCC.

We also examined the effect of occult hepatitis B virus infection that might correlate with the development of NBNC-HCC. Among 203 NBNC-HCC, 68 (33.5%) patients were positive for hepatitis B core antibody (HBc-Ab). However, no difference of the positivity of AFP, DCP and AST was observed between the patients with and without HBc-Ab in this cohort (data not shown).

This study has several limitations. First, we could not effectively select the patients with NASH who need further examination with imaging. The patients with NASH often showed deterioration in liver function, which resulted in high AST and/or AFP levels in about 50% of cases. Second, the cut-offs adopted for AST, ALT, and DCP are optimized for each individual marker, but when used in combination, may not optimally delineate the high-risk populations. While the usage of the formula obtained by logistic regression analysis produced a high AUROC (0.971), the calculation was too complex for use in the clinical setting. Furthermore, no data of healthy control was presented although it might strength the conclusion of this study. Given that this is a retrospective case study, another limitation is that use of the triple screen in this population cannot predict the actual risk for developing HCC, but merely has diagnostic ability. Prospective periodic measurement of these markers is required for early detection of HCC.

In conclusion, the screening method developed in this study is easy to use because it is a blood test consisted of AST, AFP and DCP. This study clearly showed that being aware of the new low-cut offs of the markers when we conduct blood tests with any reasons was important in achieving early diagnosis of NBNC-HCC. Although it is necessary to use imaging as a confirmatory test for NBNC-HCC, the use of this triple screen

would reduce the number of patients requiring screening by imaging to one-fourth the original number. Further analysis in a large cohort is required to validate this screening method.

DECLARATIONS

Acknowledgements

We thank Wako Pure Chemical Industries for measuring tumor markers in patients with diabetes mellitus.

Authors' contributions

Design of the work, data analysis, interpretation and preparation of this manuscript: Nouso K

Data collection, data update and acquisition of serum samples: Furubayashi Y

Data collection, database construction: Shiota S, Wakuta A, Oonishi A, Kariyama K, Takeuchi Y, Wada N, Onishi H, Adachi T, Oyama A, Dohi C, Yasunaka T, Yasunaka Y, Ikeda F, Shiraha H, Takaki A

Gave advise for conducting the whole work: Okada H

Availability of data and materials

The data will not be shared because the patients did not give their consents for the data to be analyzed by the third party.

Financial support and sponsorship

Wako Pure Chemical Industries measured tumor markers in patients with diabetes mellitus.

Conflict of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All HCC patients provided written informed consent to use their clinical records for this study. In addition, written informed consent was obtained from the control group patients for tumor marker (AFP and DCP) measurement and use of clinical data. The study protocol conformed to the tenets of the Declaration of Helsinki and was approved by the institute ethics committees.

Consent for publication

Not applicable.

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Review

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Direct-acting antivirals and chronic hepatitis C: towards elimination

Ricardo A. Franco¹, James W. Galbraith², Edgar T. Overton¹, Michael S. Saag¹

¹Department of Medicine, Division of Infectious Diseases, University of Alabama at Birmingham, Birmingham, AL 35294, USA.

²Department of Emergency Medicine, University of Mississippi Medical Center, Jackson, MS 39216, USA.

Correspondence to: Dr. Ricardo A. Franco, Department of Medicine, Division of Infectious Diseases, University of Alabama at Birmingham, 908 20th Street South, CCB 330B, Birmingham, AL 35294, USA. E-mail: rfranco@uabmc.edu

How to cite this article: Franco RA, Galbraith JW, Overton ET, Saag MS. Direct-acting antivirals and chronic hepatitis C: towards elimination. *Hepatoma Res* 2018;4:74. <http://dx.doi.org/10.20517/2394-5079.2018.94>

Received: 13 Aug 2018 **First Decision:** 7 Sep 2018 **Revised:** 17 Nov 2018 **Accepted:** 18 Nov 2018 **Published:** 14 Dec 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cui Yu **Production Editor:** Huang-Liang Wu

Abstract

Hepatitis C virus (HCV) is a major cause of liver morbidity and mortality worldwide with increasing disease burden projected for the next several decades. The timely advent of direct-acting antivirals (DAAs) sparked significant public health responses aimed at HCV elimination by 2030. This review will focus on the implications of the DAAs in terms of medical progress, barriers to HCV elimination as a public health threat, and current gaps that will require further innovation. We utilized PubMed searches with the relevant keywords for articles published in the last 5 years, as well as personal collections of relevant publications. DAAs have proven to be safe and effective. DAAs are well suited for nearly all infected patients, and many countries worldwide have taken on initial treatment scale-up strategies. These unprecedented efforts, albeit significant, face extraordinary challenges related to the high infection burden, stigma, and financial constraints. Currently, few countries are progressing towards HCV elimination, as this attainable public health goal requires explicit, adequately resourced, and coordinated public health prioritization at all levels.

Keywords: Direct-acting antivirals, hepatitis surveillance, hepatitis C elimination

INTRODUCTION

Chronic hepatitis C virus (HCV) is a blood-born viral infection that affects over 71 million people worldwide, representing a major cause of liver morbidity and mortality^[1-3]. HCV chronically infects hosts as a complex mixture of related variants or “quasispecies”, able to genetically evolve and escape host immune responses^[4]. Paradoxically, HCV-specific cytotoxic T-cell immune responses lead to hepatocyte injury, liver fibrosis progression and complications [cirrhosis and hepatocellular carcinoma (HCC)]^[5]. Although no effec-



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tive vaccine is currently available, HCV infection is amenable to cure if potent antivirals fully and quickly suppress virus replication. Sustained virologic response (SVR), i.e., cure, is achieved following therapy completion in > 95% of treated individuals^[6,7]. Direct-acting antivirals (DAAs) have shown superior safety and efficacy compared to interferon-based regimens (> 95% vs. 40% cure rates, respectively), and revolutionized HCV treatment paradigms towards broader access to cure^[8]. The 69th World Health Assembly endorsed the global health sector strategy to eliminate HCV infection by 2030, which can become a reality with expanded use of DAAs^[9]. Here, we describe the current prospects of HCV eradication in the DAA era and ongoing challenges to achieve elimination goals.

CLINICAL IMPACT OF DIRECT-ACTING ANTIVIRALS

In 2012, Lok *et al.*^[10] reported successful treatment of patients who were null responders to peg-interferon and ribavirin, infected with genotype 1a and 1b HCV, who received a 24-week course of asunaprevir, a protease inhibitor, and daclatasvir, a non-structural protein 5A inhibitor. This preliminary, proof of concept study demonstrated that SVR (virologic cure) could be achieved by the combination of two DAAs in patients who did not respond to the standard of care at the time. It also signified the culmination of a sequence of major breakthrough discoveries that followed the cloning of HCV for the first time in 1989^[11]. Such progress in basic science allowed, over the ensuing years, for elucidation of key functions of the HCV genome and the virus life cycle; engineering of “sub-genomic” replicons; and development of functional cell-based *in vitro* systems suitable to screen compound candidates for effective treatment^[12,13]. Lok’s study led the way of an impressive wave of clinical studies, that applied several combinations of DAAs at an extraordinarily fast pace^[14]. From these clinical studies, we learned that DAAs proved to be safe and effective in addressing unmet needs of key subpopulations, traditionally unreached by interferon-based therapies. State-of-art treatment options were made available for patients with human immunodeficiency virus (HIV) co-infection^[15-22], decompensated cirrhosis^[23-28], post-liver transplantation^[29-33], chronic kidney disease^[34,35], renal transplant patients^[36-38], and children^[39,40]. These clinical studies also defined best practices in overcoming HCV resistance. Highly efficacious retreatment strategies could still be utilized for the few patients experiencing DAA-failure and emergence of resistance associated substitutions^[41-46].

The field quickly evolved towards the recognition that HCV can be eradicated from most, if not all, infected individuals, expanding the benefits of virus clearance^[47]. Virologic cure has been shown to universally decrease liver inflammation, reflected by improved aminotransferase levels and reduced rates of liver fibrosis progression. In some patients, achieving SVR also leads to cirrhosis regression and improvement in clinical signs of portal hypertension and end-stage liver disease^[48]. Numerous studies have demonstrated strong associations between SVR and significant reductions in the risk of HCC, liver-related mortality and liver transplantation^[49-51]. In addition to these major clinical benefits, cure of HCV infection ameliorates or facilitates management of extra-hepatic manifestations such as cryoglobulinemia, non-Hodgkin’s lymphoma, diabetes and porphyria cutanea tarda^[52-54]. The lower complexity of DAA therapy has made investigators and clinicians challenge the preconceived notion that expeditious HCV treatment would only benefit highly selected patients who exhibited liver fibrosis METAVIR stage 2 at a minimum^[55]. Well-designed cohort and modeling studies have suggested that early therapy in patients with no significant liver fibrosis have tremendous clinical benefits with SVR^[56-58]. Similarly, patient reported outcome assessments from pivotal DAA trials have shown improvements in overall health-related quality of life and work productivity following successful HCV therapy^[59-61]. These findings build on previous studies reporting reductions in fatigue after HCV cure with interferon and ribavirin^[62]. Taken together, this body of work highlights the extraordinary clinical benefit potential of expanding use of DAAs [Table 1].

In addition, the medical field has clarified the lack of accurate data regarding HCC risk following DAA therapy^[63,64]. Initially, concerns were raised that abrupt HCV viral load suppression using DAAs could hypothetically abolish the immune system surveillance or “brake” defenses to tumor progression. Further meta-

Table 1. Benefits of hepatitis C cure by scaling-up direct-acting antivirals

Primary prevention	Cirrhosis among patients with chronic infection
Clinical	Decreased liver inflammation Reduced rates of liver fibrosis progression Cirrhosis regression Potential improvement in portal hypertension and ESLD Improved management of extra-hepatic manifestations Reductions in insulin resistance Improved energy, cognition and quality of life measures
Secondary prevention	HCC among cirrhotic patients Liver-related mortality and liver transplantation AI disorders, PCT, B-cell non-Hodgkin's lymphoma All-cause mortality
Public health	Expanded cure to special patient populations with unmet needs Cure access to hard-to-reach populations (PWIDs, homeless) Scale-up programs with disease eradication goals
Societal	Cost-effectiveness potential Direct and indirect economic impact Awareness of patients, families, providers and health systems Reductions in stigma Integration and expansion of harm reduction programs

ESLD: end-stage liver disease; HCC: hepatocellular carcinoma; AI: auto-immune; PCT: porphyria cutanea tarda; PWIDs: people who inject drugs

analysis studies, however, demonstrated that (1) HCV cure following DAA therapy in patients with cirrhosis reduces HCC risk to a similar extent as interferon (IFN)-based cure (estimated at 63%-77% reduction); and (2) the beneficial impact on HCC incidence should be markedly higher in the DAA era, given the greater extent that cirrhosis populations are treated with DAAs, and the higher cure rates among these high risk patients^[65]. Expanded use of DAAs will solidify the evidence in favor of decreased HCC risk following DAA-based virologic cure, as exemplified by the study findings of Backus *et al.*^[66], which showed a 83.5% reduction in HCC diagnosis following DAA therapy.

Applying interferon-based HCV therapy among people who inject drugs (PWID) was extremely challenging due to patient, provider, health system, structural, and societal barriers^[67-69]. The availability of DAA therapies with cure rates > 95% have overcome many of these barriers for PWID as they have fewer psychiatric side effects, are simpler (oral, once-daily vs. weekly injections), and shorter in duration. In earlier years, interferon-based therapy had been proved to be safe and effective among PWID^[70], and results of several recent studies have provided substantial insight about DAA use among several PWID subgroups. Among people receiving opioid substitution therapy (OST) with no recent illicit drug use, post-hoc analyses of phase 2 and 3 trials of DAA therapy have demonstrated that the SVR is similar in those receiving and not receiving OST^[71-75]. In the first phase 3 trial to evaluate DAA therapy in people receiving OST, including those with ongoing drug use, treatment completion was 96%, 97% demonstrated > 95% adherence, and the overall SVR was 91%^[71]. Real-world results, among people with a history of injecting drug use (with and without recent drug use), indicated overall treatment completion rates of 93%-100% and SVR rates of 80%-96%^[76-79]. In studies focused on people with recent injecting drug use, 95%-96% of participants completed therapy with SVR rates of 93%-94%^[80,81]. Mathematical modelling adds further support to this strong body of evidence. According to these models, modest scale-up of DAA treatment to 8 per 100 PWID years could lead to substantial reductions in HCV prevalence within these populations, thereby preventing transmission and lowering HCV incidence^[82,83]. Guidelines from the World Health Organization (WHO), the American Association for the Study of Liver Disease/Infectious Diseases Society of America, the European Association for the Study of the Liver, and the International Network for Hepatitis in Substance Users now recommend DAA treatment for PWID^[84-87].

While associations between virologic cure and decreased the risk of liver disease-related death have been established during the interferon-era, all-cause mortality is still the most definite clinical end point with clear interpretation, and an important parameter in considering efforts for DAA treatment scale-up^[50]. Van

der Meer *et al.*^[50] were able to detect all-cause mortality benefit among patients with chronic HCV infection and advanced hepatic fibrosis who achieved SVR to interferon-based treatment. However, the retrospective nature of the study could have led to selection of a relatively healthy cirrhotic HCV population, because interferon therapy is contraindicated in patients with moderate to severe cirrhosis^[50]. This selection bias is minimized by DAA therapies due to improved safety and efficacy profiles, even among patients with higher Model for End-Stage Liver Disease (MELD) scores. There is much anticipation to observe data regarding both all-cause and liver-related survival benefits, as the experience with DAA therapy accumulates. At the latest European Association for the Study of the Liver conference, Calvaruso *et al.*^[88] reported results from a large real-world setting cohort with patients using a variety of DAA regimens. According to the authors, achieving SVR significantly reduced mortality from both liver disease-related and unrelated causes at all stages of liver fibrosis. In another report from the same conference, the European Liver Transplant Registry reported that, while the total number of liver transplants performed in Europe remained stable over the last decade, the percentage of transplants related to HCV fell significantly from 23% in the interferon era to 11% in the DAA era^[89].

BARRIERS TO HCV ELIMINATION

The global burden of viral hepatitis is increasing since 1990, reaching 1.46 million deaths in 2013, exceeding that of HIV (1.3 million), tuberculosis (1.2 million) and malaria (0.5 million deaths). HCV is responsible for approximately 30% of the overall viral hepatitis mortality^[90]. The advent of DAA therapy and its extraordinary clinical impact hold promise that HCV elimination as a public health threat is a reachable goal by 2030. According to the global health sector strategy on viral hepatitis 2016-2021, HCV elimination can be achieved by diagnosing 90% of people infected and treating 80% of the people diagnosed. Such a strategy is predicted to reduce new infections by 90% and mortality by 65%^[9]. This report also established a baseline for tracking progress of this global strategy, where only 20% (14 million) of 71 million people living with chronic HCV knew their diagnosis and a disappointing 7.4% of those diagnosed (1.1 million) started HCV treatment in 2015.

DAAs can only benefit patients who are screened, diagnosed, linked to care, engaged in care and treated^[91]. The HCV care cascade concept, adapted from public health efforts in HIV, identifies multiple missed opportunities to address the HCV burden at local, national and global levels^[92,93]. In order for each HCV infected individual to move down the cascade from diagnosis to HCV treatment, a myriad of variables interact with each other in multifaceted ways. Adapted health care utilization frameworks, such as the Gelberg-Andersen model, are useful tools to examine and understand factors influencing the impact of specific care actions (such HCV screening, linkage to care, engagement, treatment initiation) among vulnerable, high-risk populations^[94]. Health care utilization is in general influenced by traditional predisposing (ethnicity, age, education, gender), enabling factors (source of care, health insurance, income) as well as need (perceived health, medical conditions, awareness of HCV-positive status). For instance, progressive movement of HCV-positive homeless individuals down the cascade would also be influenced by additional, more specific predisposing (histories of child abuse, jail/prison, drug and alcohol use, mental illness, and risky sexual behavior), and enabling factors (barriers to care, competing needs, lack of housing, food security, and case management). It is known that the many of the highest HCV prevalent populations (i.e., PWID, homeless and socioeconomically disadvantaged) often lack access to HCV testing and continuity of care^[94]. Case management and regular sources of care attenuates social vulnerability, and robust support systems are needed in response to these complex and challenging demands^[95-97].

Several determinants of health care utilization among vulnerable individuals, including illicit drug use, often introduce stigma to the care cascade equation, furthering the hardships of those in need of HCV care and cure^[98]. Perceived stigma associated with HCV infection leads to anxiety, fear of transmission to others, reduced intimacy in relationships, denial (reluctance to seek medical care for addiction and/or HCV

treatment) and social isolation^[99]. People living with HCV are frequently blamed for the disease, putting themselves at risk to acquire HIV infection, and viewed as irresponsible, not accountable, “unworthy”^[100,101]. Perceived and real stigma towards HCV, within families and workplaces, affect self-esteem and quality of life, causes delay or impediment to timely diagnosis and treatment, and leads to continuing risk of disease transmission^[102]. The response to stigma requires broad-based, societal educational efforts in order to increase the understanding of this disease, still connected to several pejorative stereotypes^[103,104]. These efforts are expected to bring greater compassion, patient-centered healthcare, and improved coping skills to people living with HCV^[105].

Among the 71 million people infected globally, there is a large burden of HCV infection among PWID, with a 50% prevalence of chronic infection, representing an estimated 5.6 million individuals - 8% of all infections globally^[106]. There is also a large and unquantified number of chronic infections among PWID who have ceased injecting, and HCV morbidity and mortality continues to rise among recent and former PWID^[107]. In 2015, there were 1.7 million new HCV infections globally - this is a greater number than patients who were started on treatment in the same year - with 23% of these new infections attributable to current injecting drug use in many settings^[9,108-111]. Along with unsafe healthcare practices and injections, intravenous drug use is a leading contributor to HCV incidence, especially in the European and Eastern Mediterranean Regions^[9]. Even in areas of the world where the incidence was low in 2015, an increase in transmission may occur at any time, due to epidemic spread associated with injection drug use. Despite years of HCV decline in the US, the incidence of HCV infection doubled between 2010 and 2014, due to an intensifying opioid epidemic and rise in injecting drug use behavior^[112]. The number of reported cases of acute HCV among persons reporting injection drug use has increased, particularly in rural areas^[113,114]. In the US, injection drug use among PWID has resulted in rapid dissemination of HIV and HCV, as well as some transmission of hepatitis B virus (HBV)^[115,116]. There have been few studies evaluating the HCV cascade of care among PWID, and contemporary studies from Australia and Kentucky has similarly shown high prevalence of antibody positivity, poor rates of viral load confirmation and minimal rates of treatment uptake, both during the interferon era and in the first few years of the DAA era^[117,118]. In the Netherlands, access and reimbursement for DAA therapy occurred earlier (since 2014) than many other countries, and cohorts of PWID have been well-characterized. Despite rates of viral load testing as high as 95% among seropositive individuals, DAA uptake has remained low, largely limited by fibrosis staging restrictions that were in effect until October 2015 and subsequently lifted^[119].

Transmission of HCV among men who have sex with men (MSM) infected with HIV has also been reported in Europe, Australia and the US as well as reinfection among HIV-infected MSM who were successfully cured with treatment for hepatitis^[120,121]. No estimates are available to quantify how much this emerging issue contributes to the overall transmission of HCV^[122,123]. The observed risk of reinfection in HIV-infected MSM during the interferon era ranged from 5.3 to 13.2/100 persons years^[121,124,125], including subgroups with multiple HCV reinfections and at risk of transmission of HCV virus with resistant variants^[121,126]. These reinfection rates are higher than the rates observed in retrospective and prospective studies of PWID treated for chronic HCV infection, ranging from 1.21 to 4.9/100 persons years^[127-130]. The role of HIV infection in increasing the risk of HCV reinfection is likely associated with an approximately threefold reduction in rates of spontaneous clearance following acute HCV infection, as well as high-risk sexual practices among predominantly male cohorts representing HIV-infected MSM^[131,132]. Traditionally, individuals at risk of reinfection have been grouped as either HIV-infected MSM or PWID; however, there is clearly a subset of HIV-infected men who both use injection drugs and have sex with men. As such, interventions targeted at both safer sexual practices and safer drug use practices are indicated among HIV-infected MSM.

HCV is highly prevalent among incarcerated populations, with global prevalence over 10%, and considerably higher among incarcerated PWID^[133-135]. Globally, more than 10 million people are incarcerated on a daily basis, with many more annually, making prisons a key setting for implementation of HCV elimination strat-

egies^[136]. The close relationship between injecting drug use, incarceration, and prevalence of blood-borne viruses makes correctional centers a crucial setting for enhanced DAA therapy access and broad prevention strategies^[134]. The United Nations Basic Principles for the Treatment of Prisoners state that prisoners “shall have access to the health services available in the country without discrimination on the grounds of their legal situation”^[137]. Unfortunately, this principle has been infrequently applied in real life and in most countries prisoners have a lesser possibility of assistance and care than other citizens^[138]. Once in prison, overcrowding, violence, separation from family and emotional problems are additional reasons that may induce inmates to start or continue unsafe habits, fueling high incidence rates that exceeds 30 per 100 persons per year^[139-141]. Proper treatment of chronic hepatitis C in prison is rare due to social and educational reasons and, not least, because most inmates with HCV infection remain unaware of their status, and several other barriers (drug abuse, stress, fear, lack of confidence, stigma, difficulty to relate to the health personnel) adds up to the lack of liver disease specialists in prison^[142-145]. Although many prisoners are incarcerated for long periods, the average length of stay can be shorten to weeks or months in several cases, which makes it difficult to complete the clinical itinerary from screening to post-treatment follow-up^[146,147].

Compared to interferon, DAA therapies are easier to roll out in community and outreach settings, but in reality there is a significant lack of experience and engagement in routine HCV screening and treatment in primary care, and misconceptions about whom to screen, risk of progression of liver disease or therapy itself in this setting^[148-150]. Even specialists in liver disease may have limited experience treating HCV, or be selective about which patients they consider as good candidates for therapy and fail to recommend treatment because of concerns about nonadherence, drug use or risk of re-infection^[151,152]. Furthermore, there are insufficient numbers of providers who can and are willing to treat HCV, and insufficient resources for case managers, navigators and social workers in suitable capacity to attend a growing demand of patients in need of treatment^[153].

All-oral treatments are very expensive, with initial wholesale acquisition cost (WAC) of 90,000 US dollars per 3 months treatment course (or \$1000/pill). While the prices of DAAs have decreased rapidly in some countries, they remain variably expensive and remain unaffordable in others^[154]. In the US for example, DAA pricing is influenced by a chain of multiple organizations, including pharmaceutical companies (who determine the WAC), Pharmacy Benefit Managers (PBMs) (intermediaries between the former and health insurance companies), insurance companies (who determine the preferred choice of regimens and out-of-pocket expenses for patients), and specialty pharmacies (who receive dispensing fees and may contract with insurance companies, PBMs, or pharmaceutical companies to provide adherence support, management of adverse effects, and outcome measurements). In this system chain, negotiated drug prices are held as confidential business contracts, with no transparency regarding the actual prices paid for hepatitis C drugs. Nevertheless, the recently observed increases in WAC discounts or rebates have implied a reduction in drug costs to payers^[85,155,156]. In other countries, pharmaceutical companies negotiate pricing directly with the payers (usually a nationalized system), where licensing agreements may allow for production of generic formulations and transparency in negotiated cost of drugs to payers^[155,157]. Increasing generic competition has lowered DAA price, but those remain high (tens of thousands of dollars per treatment course) in developed countries, in those middle-income countries that do not have access to generic formulations, and in those countries who fall outside of license agreements. This creates a heavy financial burden on many health systems and leads to treatment rationing^[154]. Comparatively, generic versions of new HCV medicines have been available for under 500 US dollars per patient in some countries, and the production cost of two DAAs could be as low as 200 US dollars per patient. Hence, further price reductions could be achieved and will be needed to increase the number of patients treated^[157].

In addition to drug cost, the cost of diagnosis and disease evaluation also represent an important financial burden, especially in low to middle income countries (LMICs), which has brought uncertainty as to the opti-

mal testing approaches and who to prioritize for testing in this setting^[158]. Diagnostic testing involves laboratory-based immunoassays required to meet minimum safety, quality and performance standards, and rapid diagnostic tests (RDT) with important role in settings where there is limited access to laboratory infrastructure and/or in populations where access to rapid testing would facilitate linkage to care and treatment^[158]. Directly following a reactive HCV antibody serological test result, the use of quantitative or qualitative nucleic acid testing (NAT) for detection of HCV RNA is recommended as the preferred strategy to diagnose viraemic infection and monitor treatment response. An assay to detect HCV core (p22) antigen, which has comparable clinical sensitivity to NAT, is an alternative to NAT to diagnose viraemic infection^[159]. According to recent WHO guidelines, focused serologic testing with HCV antibody (anti-HCV) should be offered with linkage to prevention, care and services to high-risk populations; general population testing should be approached in settings of high prevalence in the general population (2%-5% infection prevalence); and birth cohort testing should be applied to specific identified birth cohorts of older persons at higher risk of infection and morbidity within populations that have an overall lower general prevalence^[158,159]. Such testing strategies, although incurring in significant cost if applied to massive testing scale-up, should still hold reasonable cost-effectiveness tailored to broad variations in gross domestic product worldwide, although there is lack of evidence among LMICs^[158]. Interestingly, studies have shown that the cost-effectiveness of testing for HCV seems most sensitive to variations in prevalence, treatment efficacy, progression rates from chronic HCV to cirrhosis, and levels of linkage to care and treatment, and relatively insensitive to costs of screening and treatment^[158,160-162]. Another barrier to HCV testing and evaluation scale-up is the cost involved in HCV genotype ascertainment. This is required for a number of DAA regimens available, and certainly makes the use pan-genotypic regimens an attractive cost-effective option, especially in countries with high prevalence of non-GT1 HCV, that could potentially bypass genotype confirmation^[163]. Simplifying testing algorithms and lowering the cost of monitoring can dramatically cut costs of treatment for HCV in the future. For instance, the cost of the current step-wise evaluation algorithms (screening for exposure using serology or RDT; quantitative NAT testing for viremia confirmation, monitoring, efficacy assessment; and genotyping) can be as high as 220-1100 USD; whereas the cost of potential future scenarios (screening for exposure using serology, RDT, oral fluids or dried blood spots; qualitative NAT for viremia confirmation without genotyping, minimal viral load monitoring and efficacy assessment) could be as low as 15-75 USD^[164].

PROGRESS IN PUBLIC HEALTH RESPONSE

Public health strategies addressing the remarkable challenges of HCV elimination has leveraged sound epidemiological data, detailed expert opinion input and mathematical modelling. In order to inform treatment and prevention strategies, as well as public health policy, efforts have focused on gathering country-specific data^[165]. Collectively, evidence estimates suggest that the HCV infection burden is highly variable worldwide. For instance, the population prevalence of HCV viremia seems to range widely, from 0.3% in Austria, England, Germany and France to 7.3% in Egypt. The latter country is clearly unique, even when compared to Portugal, Brazil and the US with viremia prevalence nearing 1.0%-1.2%^[166,167]. Within the estimated viremic population, there are also significant variations in the estimated rates of individuals newly diagnosed in each country (3%-14% per year) and treated (1%-11% per year)^[167,168]. Liver fibrosis burden is also estimated to be greater in countries with more generalized, older epidemics such as Egypt and Brazil, in opposition to younger epidemics with large contributions of PWIDs (Australia, Czech Republic and Australia)^[166]. While the overall number of new HCV infections is expected to decline worldwide, the number of cases with advanced liver disease is expected to increase^[169]. This dichotomy and epidemiological contrasts between countries is fueled by high cumulative prevalence, reason why the global strategy calls for significant reductions of both the number of new infections and HCV-related mortality.

Modeling-based evidence, calibrated by country-specific epidemiological data, shows that sizable reductions in incidence, morbidity and mortality can only occur if high-efficacy therapies are combined with increased diagnosis and treatment access. Yearly treatment rates in the order of 10% are likely to position most coun-

tries on track to achieve HCV elimination targets. However, this is estimated to require a 3 to 5-fold increase in diagnosis and/or treatment rates from baseline; and robust, highly inclusive public health programs, focused on hard-to-reach populations and PWIDs^[167,83]. Much progress is needed to make HCV elimination an explicit and adequately resourced public health priority, using appropriate means at all levels through collaborations between individual citizens, civil society organizations, researchers, healthcare professionals, the private sector, local and national governmental bodies^[170]. Countries have been challenged to disseminate models of enhanced screening and DAA delivery in and outside tertiary care settings, such as community primary care^[171], nurse-led models of care^[172] and prisons^[173]. Studies have demonstrated the utility of nurse-physician partnerships and training programs to improve engagement in HCV care, translated into high proportions of patients receiving counselling, education, and successful treatment with cure rates comparable to contemporary clinical trials, during the interferon and early DAA eras^[171,174-176]. The results of the ASCEND trial suggested that DAAs can be independently administered by primary care physicians and nurse practitioners to challenging sub-populations, setting the foundation to HCV micro-elimination interventions such as the one carried out within the Cherokee Nation Health Services system^[177,178]. HCV elimination should not be an impossible task if taken as a “think global, act local” approach, in which clinics are structured to support vulnerable populations, also in connection with harm reduction venues in the form of needle and syringe services programs (NSP) and co-location of treatment to OST clinics^[179]. For example, Iceland’s geographical isolation and relatively small population- comparable in size to many cities globally - makes it an important case study. In general, Iceland provide favorable conditions for geographically-targeted policies to reduce transmission among PWID (setting up testing and treatment programs, NSPs and OST in consultation with local healthcare and community service providers) without the unpredictable bias of population mobility to and from areas with varying program coverages or HCV epidemiology within the same country^[180]. It is estimated that DAA scale-up to levels already being experienced, coupled with reasonable efforts to diagnose and treat PWIDs, could turn Iceland one the first countries to eliminate HCV as early as 2020^[181].

The European Union (EU) rely on advanced health-care infrastructure, and is uniquely poised to eliminate HCV^[182]. Estimates indicate that over one million people had been identified with positive viremic status by 2015 (36% of total viremic pool) and 133,000 were cured in 2015 alone (4% of the total infected population or 9% of the diagnosed population). The number of cures in that year was higher than the estimated number of new infections (~58,000) added to the number of HCV-infected immigrants (~30,000) believed to have entered the EU. Austria, France, Germany, Netherlands and Spain have led the way with at least 8% of infected individuals cured in 2015. But many other countries (Bulgaria, Croatia, Czech Republic, Finland, Hungary, Latvia, Lithuania, Poland, Romania, Slovakia) have seen greater estimated numbers of new infections than the number of people cured. In order for the EU to be on track with WHO targets by 2025, unrestricted treatment still needs to increase by 25% until then, and annual new diagnosis rates by 2-fold compared to 2015 baseline^[182].

In Australia, an active HCV screening program has led to 82% of HCV-infected population being diagnosed, placing the country on-track to achieve WHO elimination targets. The Australian unrestricted DAA program, launched in March 2016, adopts a fixed priced approach where the country pays a single fee for ad lib access to as much DAA therapy as it can use over a fixed period of time. This approach eliminates the “fee for service” model and instead uses a public health model that incentivizes patients and providers to employ universal screening and treat all who test positive. This has resulted in an estimated 58,500 individuals (26% of total HCV-infected population) initiating treatment through 2017. Treatment uptake has been high among sub-populations at greater HCV transmission risk (22% of PWIDs and > 60% of those with HIV/HCV coinfection initiated DAA treatment in 2016) and the country has enhanced surveillance efforts to track the program’s future results. It is estimated that Australia could eliminate HCV from the continent by 2020^[183].

In the United States, an estimated 260,000 people have received HCV treatment in 2015. This significant treatment volume was mostly due to large uptake of patients with advanced liver fibrosis who had been waiting for DAAs to become available^[184]. Progress estimates towards elimination in the US are greatly impacted by significant increases in HCV incidence experienced from 2011 (16,000 new cases) to 2014 (31,000 new cases), largely driven by the opioid epidemic^[185]. Assuming that the rates of new infection remain the same in the next 14 years, the US can only achieve WHO targets by 2030 if it expands screening to diagnose 80% of individuals infected (50% of infected individuals are diagnosed at baseline), provides unrestricted treatment for all, and maintains the number of treated patients at least 150,000 per year^[184,186]. The Veterans Affairs Health System has taken on robust efforts to increase funding, negotiate reduced costs per cure, screen the majority of patients at risk, expand treatment capacity by utilizing primary care and pharmacy services and have offered unrestricted treatment to 75% their patients in need^[187]. In coordination with the Center for Disease Control and Prevention and the Viral Hepatitis National Plan, multiple ongoing federal and non-federal initiatives take on similar efforts to make a dent in local HCV epidemics across the US^[188].

In 2016, roughly 40,000 Egyptians died of the disease, and nearly 4.5-5 million are currently infected - the highest burden in the world for Egypt's population size^[189]. Following successful negotiations between government and drug makers in 2014, DAAs have become widely available at markedly reduced prices. Since then, more than a million Egyptians have been treated^[190]. In addition to lowering the cost of drugs, Egypt has succeeded in opening new treatment centers, creating electronic portals to enroll patients, and expanding its domestic pharmaceutical industry to ensure a steady pipeline of affordable medications^[191].

Georgia, another country with high HCV prevalence, initiated in April 2015 the world's first program to eliminate hepatitis. With technical assistance from Centers for Disease Control and Prevention (CDC) and key partnership with drug industry to provide DAAs free of charge, the ambitious goal was defined as a 90% reduction in HCV prevalence by 2020^[192,193]. From April 2015 through December 2016, a total of 27,595 persons initiated treatment for HCV infection, among whom 19,778 (71.7%) completed treatment. The number of persons initiating treatment peaked in September 2016 at 4,595 and declined during October-December. Broader implementation of interventions that increase access to HCV testing, care, and treatment for persons living with HCV are needed for Georgia to reach national targets for the elimination of HCV^[194]. Brazil, with an estimated burden of 657,000 people infected, and enhanced DAA access through public health system able to negotiate 90% cost reduction in drug prices, hosted the World Viral Hepatitis Summit in 2017, and presented care cascade estimates that places the country on track of disease elimination by 2030, along with Australia, Egypt, Georgia, Germany, Iceland, Japan, the Netherlands and Qatar^[195]. Taken together, these examples suggest that the largest hurdle to eliminating HCV is the cost of medications, impeding access to therapy in locations where the cost of drugs remains prohibitively expensive.

SURVEILLANCE, ADVOCACY AND POLICY GAPS

As mentioned above, political will to optimize DAA treatment access and reduce costs per cure has been a main driver for the witnessed public health progress. However, much more needs to be accomplished to ensure that the hepatitis treatment goals are reached on a global level. Currently, treatment priorities aim to improve outcomes for individuals with more advanced disease progression. This treatment prioritization aimed at the individual-level misses the opportunity to reduce incident infections at the population level through treatment as prevention aimed at individuals largely driving new infections (i.e., PWID). Treatment prioritization for those with severe liver disease is supported by cost-effectiveness analyses that exclusively accounts for individual health benefits of HCV treatment. These analyses show that treatment of moderate to severe disease is cost-effective but, at high HCV treatment costs, treatment of mild disease should be delayed^[196]. However, due to the relatively long duration of HCV disease progression compared with durations of risk behavior (such as injecting drug use), treatment of those with advanced liver disease is unlikely to have prevention benefit^[197]. On the other hand, models of HCV transmission that incorporate both indi-

vidual and population prevention benefit show that treating PWID could avert secondary infections (treatment as prevention) and be more cost-effective in many settings (where chronic HCV is at 40% prevalence or less among PWID) than treating other patient groups^[198]. Therefore, the public response we have seen thus far creates many opportunities to reach liver mortality reduction targets by 2030, but also many challenges in reaching incidence reduction targets in the same time span. This needs to include unrestricted access to DAAs on a global scale and, in particular, enhanced HCV screening to identify the large proportion of hard-to-reach and undiagnosed individuals. Without a change in public policy that focus on reducing incident HCV infections, eradicating HCV as a pathogen will be near impossible^[199]. A survey of patient advocacy groups from 25 different European Countries, has recently highlighted several specific policies and program gaps in support of elimination efforts. Although fewer countries (8 out of 25) were reported to refuse treatment to people who are currently injecting drugs in 2017, nearly half of these nations were reported to lack a national HCV strategy, and the majority of them lack key components of comprehensive strategies such as disease registries, syringe exchange programs available in all parts of the country, DAA treatment availability in non-hospital settings, and unrestricted access to DAAs^[200].

Much of what is known about public health responses in HCV is based on modeling studies that are limited in their essence to be useful approximations of reality^[201]. Accurate program evaluations towards disease elimination will require robust surveillance systems. Case reporting, based on regular notification by clinicians and laboratories, serological surveys and cancer and death registries are important for measuring the impact of hepatitis infections and evaluating the efficacy of interventions^[202]. However, viral hepatitis surveillance shortcomings have resulted in many WHO Member States (MS) having insufficient data available to guide decision-making^[203]. Among MS in the WHO European Region, key surveillance components currently exist with more than 90% of MS conducting surveillance for acute HBV and HCV infections; however, substantial systemic shortcomings were reported as well, especially in regions where the surveillance of chronic HBV and HCV infections was less common^[204]. Viral hepatitis surveillance systems historically have focused on collecting data on acute infections, primarily for the purpose of identifying outbreaks, suggesting that surveillance systems may not be evolving rapidly enough to keep pace with recent developments in viral hepatitis prevention and treatment^[205]. Besides, the accurate classification of viral hepatitis infection as acute versus chronic is a widely recognized challenge, especially for hepatitis C, and only a minority of MS have no hepatitis cases reported as “undifferentiated” or “unclassified”^[206]. In the US, HCV surveillance has been ongoing since 1982, but the program for chronic disease surveillance is underfunded as only seven jurisdictions receive support from the CDC. Additionally, local health departments are responsible for reporting to the CDC, and the data aggregation across health departments from different governmental levels is not always accurate^[207]. A greater focus on chronic disease surveillance would contribute to better understand the disease burden, assess the impact of prevention and treatment efforts, and maximize the impact of resources^[208].

CONCLUSION

The advent of the DAA era sparked serious efforts towards elimination of HCV infection by 2030, which can become a reality with expanded use of DAAs. All-oral regimens have proven safe and effective in treating key HCV-infected subpopulations, including PWIDs, and allow for cure opportunities to nearly all infected patients with hepatitis C. Programmatic prospects of disease elimination will face many challenges, including: an extraordinary and ever increasing disease burden, the stigmatizing nature of the infection which challenges diagnosis, multiple societal and individual barriers to access care, and, perhaps most importantly, the high treatment costs. Unprecedented progress towards HCV elimination has been experienced in recent years, but only few countries are currently considered to be on track for disease elimination by 2030. Worldwide, countries are investigating strategies to scale-up efficient HCV screening and treatment to the levels necessary to reduce both HCV mortality and incidence. Fundamental changes in societal views, political will, surveillance and adoption of a public health treatment mindset, with sharp reduction in the cost of DAA therapy, will be required for HCV elimination worldwide.

DECLARATIONS

Authors' contributions

Design, literature research, manuscript writing: Franco RA
 Manuscript editing: Galbraith JW, Overton ET
 Manuscript revision: Saag MS

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Franco RA is has served as a consultant to Gilead and Bristol Myers-Squibb and is an investigator on grants paid to his institution from Gilead, Merck and Janssen; Galbraith JW has served on Gilead and AbbVie advisory boards and received grants from Gilead Sciences paid to his institution; Overton ET receives research support for the National Institute of Health, Gilead, Merck and Abbvie paid to his institution and has served as a consultant to Gilead and ViiV; Saag MS is a scientific advisor to Gilead, Merck, and ViiV, and principal investigator on grants paid to his institution from Gilead, Merck, and ViiV.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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The prediction of microvascular invasion of hepatocellular carcinoma using multiple imaging modalities

Hideyuki Tamai

Department of Hepatology, Wakayama Rosai Hospital, Wakayama 640-8505, Japan.

Correspondence to: Dr. Hideyuki Tamai, Department of Hepatology, Wakayama Rosai Hospital, 93-1 Kinomoto, Wakayama-shi, Wakayama 640-8505, Japan. E-mail: hdy-tamai@wakayamah.johas.go.jp

How to cite this article: Tamai H. The prediction of microvascular invasion of hepatocellular carcinoma using multiple imaging modalities. *Hepatoma Res* 2018;4:75. <http://dx.doi.org/10.20517/2394-5079.2018.98>

Received: 10 Sep 2018 **First Decision:** 12 Oct 2018 **Revised:** 20 Nov 2018 **Accepted:** 27 Nov 2018 **Published:** 18 Dec 2018

Science Editor: Guang-Wen Cao **Copy Editor:** Cui Yu **Production Editor:** Huan-Liang Wu

Abstract

To implement an adequate treatment strategy for solitary hepatocellular carcinoma (HCC), the prediction of microvascular invasion (MVI) is crucial. Metastatic recurrences after curative treatments can result from occult metastasis derived from invisible MVI. For predicting MVI, poorly differentiated or non-singular nodular HCC with a high risk of MVI should be evaluated by common imaging modalities such as ultrasound, contrast enhanced computed tomography (CECT), or magnetic resonance imaging (MRI). Summarizing these predictabilities in previous reports, the accuracies for predicting MVI were 78% in contrast enhanced ultrasonography (CEUS), 76%-89% in CECT, and 62%-77% in MRI. Those for predicting poor differentiation were 69%-92% in CEUS, 52%-90% in CECT, and 71%-75% in MRI. Those for predicting non-singular nodular type were 92%-95% in CEUS, 81%-89% in MRI, and 91%-93% in the combination of MRI and CECT. Among common imaging modalities, MRI can provide tissue characterization of the HCC using signal intensity. Gadolinium-ethoxybenzyl diethylenetriamine penta-acetic acid-enhanced MRI including diffusion imaging is the most informative imaging modality to predict MVI. Combination of MRI with other imaging modalities or tumor markers may provide a more accurate predicting for MVI. HCC with a high risk of MVI should be treated as advanced HCC even after curative treatment.

Keywords: Hepatocellular carcinoma, microvascular invasion, histologic differentiation, ultrasound, computed tomography, magnetic resonance imaging

INTRODUCTION

There have been rapid advances in the development of imaging modalities as diagnostic tools in recent years. In hepatocellular carcinoma (HCC), imaging plays a greater part than biopsy in its diagnosis. In addition,



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imaging is used not just for its diagnosis, but also for disease surveillance, determination of tumor stage, evaluation of treatment efficacy, and navigation of local treatments. Importantly, imaging has the ability to predict histologic differentiations that reflect the malignant potential of HCC.

As the HCC tumor grows larger, it has a stronger tendency to invade the adjacent portal vein or hepatic vein. HCC with intrahepatic or extrahepatic metastasis derived from vascular invasion is an advanced cancer that is difficult to treat radically^[1]. However, even if the tumor is solitary on imaging, metastatic recurrences after curative treatments such as surgical resection or local ablation is not uncommon. These metastatic recurrences may result from occult metastasis derived from invisible microvascular invasion (MVI) at the time of diagnostic imaging before treatment was initiated. Therefore, HCC with MVI can be considered as an advanced cancer with occult metastasis. However, there is a limitation in the diagnostic imaging of MVI or occult metastasis. To make an adequate treatment strategy for solitary HCC, the prediction of MVI is crucial. In this review, the present status of the prediction of MVI of HCC using common imaging modalities such as ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI) is presented.

FACTORS RELATED TO MVI IN HCC

HCC develops in a multistep fashion^[2]. Therefore, most HCCs consist of heterogeneously differentiated components. For example, nodule in nodule or mosaic pattern on US imaging represents multistep carcinogenesis^[2]. As histologic differentiation advances from well differentiated to poorly differentiated, the prevalence of MVI becomes higher^[3]. Poor histologic differentiation is a strong predictor of MVI^[4]. A large proportion of poorly differentiated HCCs has MVI and intrahepatic metastasis even when the tumor is small^[3]. As the tumor size increases, a fibrous capsule forms such that a typical HCC is visualized as a nodule within a fibrous capsule. Cancer cell infiltration into the fibrous capsule demonstrates a morphologically invasive feature, and HCCs with infiltrations to the fibrous capsule tend to be poorly differentiated and to have MVI^[5]. Small nodular HCCs can be macroscopically classified into three types such as single nodular (SN), single nodular with extra-nodular growth (SNEG), and contiguous multinodular (CMN)^[6]. Both SNEG and CMN types have a stronger invasive potential, and tend to be more poorly differentiated than the SN type. The prevalence of MVI or microscopic intrahepatic metastasis is also higher in the SNEG and CMN types than in the SN type^[7-9]. Since MVI is strongly associated with histologic differentiation and the macroscopic type of HCC, the accurate prediction of MVI by imaging will require accurate evaluation of these two parameters.

PREDICTION OF MVI USING US

US has the highest spatial resolution among common imaging modalities, therefore it can potentially provide an accurate assessment of the macroscopic morphology of HCC. Moribata *et al.*^[10] reported the correlation between B mode ultrasonogram and histologic differentiation of small HCC. They revealed that most poorly differentiated small HCCs were visualized as hypoechoic tumor with an irregular or unclear margin on B mode US. However, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of diagnosis for poorly differentiated HCC on the basis of their data are 89%, 67%, 19%, 99%, and 69% respectively.

There have been several reports on the prediction of poorly differentiated HCC or MVI using CEUS, based on the evaluation of intra-tumoral angioarchitecture. Sugimoto *et al.*^[11] showed the correlation between the angioarchitecture and histologic differentiation using microflow imaging (MFI) by CEUS. The deadwood pattern of tumoral blood vessels was visualized clearly, but they gradually tapered off and were interrupted suddenly. When HCCs with deadwood pattern were assessed as poorly differentiated, the sensitivity, specificity, positive PPV, NPV, and accuracy of diagnosis for poorly differentiated HCC on the basis of their data are 80%, 96%, 86%, 94%, and 92%, respectively. Tanaka *et al.*^[12] implemented the malignant grading system based on the combined assessment of Kupffer imaging and the maximum intensity projection imaging

made by the accumulation for each MFI sequence using CEUS with perflubutane microbubbles (Sonazoid®; Daiichi-Sankyo, Tokyo, Japan). They classified HCCs into four grades: grade 1 (iso-fine/vascular), grade 2 (hypo-fine), grade 3 (hypovascular), and grade 4 (hypo-irregular). When HCC was assessed as grade 4, sensitivity, specificity, positive PPV, NPV, and accuracy of the diagnosis for poorly differentiated HCC are 100%, 91%, 50%, 100%, and 92% respectively, and those of the diagnosis for MVI are 40%, 92%, 67%, 80%, and 78% respectively.

The correlation between histologic grading and tumor enhancement washout time on CEUS has also been reported^[13-17]. For example, Xu *et al.*^[14] analyzed the enhancement pattern of HCC using time-intensity curve. They observed that the time to peak, contrast-enhanced time, and wash-out time of well differentiated HCCs were longer than those of the moderately to poorly differentiated HCCs, whereas the enhancement slope and clearance slope of the well differentiated lesions were lower than those of the moderately to poorly differentiated lesions. However, Pei *et al.*^[15] reported that washout time is the only significant factor (among all the time-intensity curve parameters) correlated to histologic grading. Washout time in the well-, moderately-, and poorly differentiated HCCs was 36.66 ± 9.61 , 19.37 ± 2.83 , and 11.61 ± 2.78 s, respectively. Feng *et al.*^[17] reported using the washout rate to predict HCC differentiation. They demonstrated that when the cutoff point was set at washout before 40 s from contrast injection, the ability to distinguish poorly differentiated from moderately- and well differentiated HCCs could be performed with a sensitivity, specificity, and area under the curve (AUC) of 24%, 97%, and 0.68, respectively.

Additionally, several reports have demonstrated the correlation between macroscopic HCC type and Kupffer imaging (post vascular phase) using Sonazoid CEUS. Hatanaka *et al.*^[18] reported that the sensitivity, specificity, PPV, NPV, and accuracy of CEUS for predicting for the non-SN type were 80%, 96%, 92%, 89% and 90%, respectively. Tada *et al.*^[19] also reported that those in small HCCs (3 cm or less) were 87%, 93%, 91%, 84% and 94%, respectively. Furthermore, Hatanaka *et al.*^[20] demonstrated that CEUS was more accurate at distinguishing macroscopic type than contrast CT. This could be explained by the difficulty in evaluating the shapes of nodules on contrast CT because of the partial volume effect. Nuta *et al.*^[21] also indicated that HCCs with an irregular defect visualized during Kupffer-phase of CEUS were characterized by more frequent MVI and intrahepatic metastasis. They demonstrated that Kupffer-phase images were more accurate at predicting the macroscopic pathologic type with high grade malignancy (SNEG or CMN type) than conventional B mode (CEUS AUC 0.89 vs. B-mode US AUC 0.78), and diagnostic accuracy was also significantly higher with Kupffer-phase imaging (92%) than with conventional B-mode imaging (74%). The sensitivity, specificity, PPV, NPV, and accuracy of US studies cited in this review were summarized in Table 1 except for not available reports.

PREDICTION OF MVI USING CONTRAST CT

Contrast multi-detector low CT is commonly used for the definite diagnostic imaging of HCC. The diagnostic information obtained by contrast CT is tumor vascularity and morphology. There are some reports about the correlation between histologic differentiation and tumor vascularity. For example, Asayama *et al.*^[22] indicated that the arterial blood supply decreases significantly in poorly differentiated HCCs compared to moderately differentiated HCCs. Sanada *et al.*^[23] demonstrated that small HCCs intermingled with hypovascular areas and hypervascular areas in the arterial phase of contrast CT included poorly differentiated HCC components. Kawamura *et al.*^[24] also reported that heterogeneous enhancement with irregular ring-like structures in the arterial phase was a significant independent predictor of poorly differentiated HCC. On the other hand, fast tumor enhancement washout is also associated with poorly differentiated HCC. Nishie *et al.*^[25] indicated that poorly differentiated HCCs show faster tumor enhancement washout on contrast CT than non-poorly differentiated HCCs. However, Nakachi *et al.*^[26] demonstrated that the diagnostic accuracy for poorly differentiated HCC using tumor enhancement washout in the venous phase was low compared with heterogeneous tumor enhancement in the arterial phase. They showed that sensitivity, specificity, PPV, NPV, and accuracy for predicting poor differentiation in small HCCs (up to 3 cm in diameter) by heterogeneous

Table 1. Studies with ultrasound for predicting poorly differentiation, non-single nodular type, or microvascular invasion

Ref.	Modalities	Findings	Prediction	Sensitivity	Specificity	PPV	NPV	Accuracy
Moribata <i>et al.</i> ^[10]	B-mode US	Irregular or unclear margin	Poorly diff.	89%	67%	19%	99%	69%
Nuta <i>et al.</i> ^[21]	B-mode US	Irregular or unclear margin	Non-SN type	72%	85%	96%	39%	74%
Sugimoto <i>et al.</i> ^[11]	CEUS	Dead wood pattern	Poorly diff.	80%	96%	86%	94%	92%
Tanaka <i>et al.</i> ^[12]	CEUS	Grade 4 (hypo-irregular)	Poorly diff.	100%	91%	50%	100%	92%
Tanaka <i>et al.</i> ^[12]	CEUS	Grade 4 (hypo-irregular)	MVI	40%	92%	67%	80%	78%
Feng <i>et al.</i> ^[17]	CEUS	Washout time < 40 s	Poorly diff.	24%	97%	65%	61%	69%
Hatanaka <i>et al.</i> ^[18]	CEUS	Irregular defect on Kupffer phase	Non-SN type	80%	96%	92%	89%	90%
Tada <i>et al.</i> ^[19]	CEUS	Irregular defect on Kupffer phase	Non-SN type	87%	93%	91%	84%	95%
Nuta <i>et al.</i> ^[21]	CEUS	Irregular defect on Kupffer phase	Non-SN type	93%	85%	97%	73%	92%

PPV: positive predictive value; NPV: negative predictive value; US: ultrasound; SN: single nodular; CEUS: contrast enhanced ultrasonography; MVI: microvascular invasion

tumor enhancement were 75%, 90%, 48%, 97% and 88%, respectively^[26]. Accordingly, heterogenous tumor enhancement even in small HCC is an important observation for predicting HCC with poorly differentiated components.

Some reports show that irregular tumor margin in the venous phase of contrast CT is an important finding for predicting MVI or tumor differentiation. Lee *et al.*^[27] demonstrated that the presence of intra-tumoral vessels and aneurysms, tumor necrosis, attenuation of pre-contrast, the relative timing of washout, intra-tumoral attenuation heterogeneity, tumor margin, and tumor size were correlated with the pathological differentiation of HCC. In particular, the presence of intra-tumoral aneurysm was a highly specific finding for poorly differentiated HCC. Chou *et al.*^[28] showed that the sensitivity, specificity, PPV, NPV, and accuracy of the irregular tumor margin in predicting MVI in their retrospective study were 66%, 86.5%, 82.5%, 72.6% and 76.5%, respectively, and those in their prospective study were 81.7%, 88.1%, 90.7%, 77.1% and 84.3%, respectively^[29]. Reginelli *et al.*^[30] indicated that irregularity in tumor margins, as well as defects of peritumoral capsule are the most significant characteristics predicting MVI in HCC. Wu *et al.*^[31] reported that irregular tumor margin was alone independent predictive factor for MVI among previously proposed predicting factors such as fluorine-18 fluorodeoxyglucose-positron emission tomography (FDG-PET) results and serum tumor markers. Hu *et al.*^[32] demonstrated in a meta-analysis that CT is superior to MRI in evaluating an irregular tumor margin for MVI assessment. Banerjee *et al.*^[33] showed new features of contrast CT that can also accurately predict histological MVI in HCC surgical candidates. These features include: the positivity of radiogenic venous invasion consisting of three separate imaging features; the persistence of discrete arterial tumor enhancement in the venous phase; partial or complete absence of hypodense halo; and absence of tumor-liver difference in the absence of a halo. Zhao *et al.*^[34] demonstrated that the predictive scoring model based on intra-tumoral arteries, non-nodular type of HCC, and absence of the radiological tumor capsule on preoperative CECT is of great value in the prediction of MVI regardless of tumor size. The sensitivity, specificity, PPV, NPV, and accuracy of CT studies cited in this review were summarized in Table 2 except for not available reports.

PREDICTION OF MVI USING MRI

The signal intensity of MRI can also be used to distinguish well differentiated HCC from moderately/poorly differentiated HCC^[35-37]. For example, typical moderately/poorly differentiated HCC show hypointensity on T1-weighted imaging and hyperintensity on T2-weighted imaging. Enomoto *et al.*^[38] reported that hypointensity of tumor on T1-weighted imaging and tumor stain washout during the portal phase of dynamic MRI reflected poorer histological differentiation of HCCs, and the sensitivity, specificity, and the accuracy for diagnosis of poorly differentiated HCC using combined findings of hypointensity on T1-weighted imaging and tumor enhancement washout during the portal phase were 88%, 67% and 71%, respectively. On the contrary, most of well-differentiated HCCs (83%) showed non-hypointensity on T1-weighted image^[38]. Min *et al.*^[39]

Table 2. Studies with contrast enhanced computed tomography for predicting poorly differentiation, non-single nodular type, or microvascular invasion

Ref.	Modalities	Findings	Prediction	Sensitivity	Specificity	PPV	NPV	Accuracy
Nishie <i>et al.</i> ^[25]	CECT	Washout on portal-venous phase	Poorly diff.	63%	72%	38%	88%	70%
Nakachi <i>et al.</i> ^[26]	CECT	Enhancement with non-enhanced area	Poorly diff.	75%	90%	48%	97%	88%
Nakachi <i>et al.</i> ^[26]	CECT	Washout on portal-venous phase	Poorly diff.	100%	55%	22%	100%	60%
Nakachi <i>et al.</i> ^[26]	CECT	Above combination	Poorly diff.	75%	92%	55%	97%	90%
Lee <i>et al.</i> ^[27]	CECT	Intra-tumoral aneurysm	Poorly diff.	18%	99%	93%	77%	78%
Lee <i>et al.</i> ^[27]	CECT	Irregular tumor margin	Poorly diff.	74%	44%	32%	82%	52%
Chou <i>et al.</i> ^[28]	CECT	Irregular tumor margin (retrospective)	MVI	66%	87%	83%	73%	77%
Chou <i>et al.</i> ^[28]	CECT	Irregular tumor margin (prospective)	MVI	82%	87%	91%	77%	84%
Wu <i>et al.</i> ^[31]	CECT	Irregular tumor margin	MVI	87%	73%	43%	96%	76%
Reginelli <i>et al.</i> ^[30]	CECT	Irregular tumor margin	MVI	66%	94%	84%	86%	85%
Reginelli <i>et al.</i> ^[30]	CECT	Incomplete peritumoral capsule	MVI	81%	90%	76%	91%	89%
Banerjee <i>et al.</i> ^[33]	CECT	Positivity of radiogenic venous invasion	MVI	76%	94%	83%	91%	89%
Zhao <i>et al.</i> ^[34]	CECT	Score model (validation cohort)	MVI	82%	83%	74%	88%	N/A

PPV: positive predictive value; NPV: negative predictive value; CECT: contrast enhanced computed tomography; MVI: microvascular invasion; N/A: not available

reported that intra-tumoral fat detected by chemical-shift of T1-weighted image indicates lower risk for MVI of HCC.

The recent advances in MRI instrumentation has allowed high quality diffusion weighted images (DWI) to be obtained. The correlation between the apparent diffusion coefficient (ADC) values on DWI and histologic differentiation have been reported^[40-44], suggesting that low ADC values can be a useful predictor of MVI^[45-47]. However, there was no notable threshold of ADC value for predicting poorly differentiated HCC on meta-analysis^[48]. Park *et al.*^[49] showed that hypervascular HCCs with low ADC value could be interpreted as poorly differentiated HCCs, while it was difficult to differentiate between well- and poorly differentiated HCCs that are hypovascular. Among all ADC parameters, Moriya *et al.*^[50] demonstrated that the minimum ADC value was the most useful in distinguishing poorly differentiated HCC in 3D analysis of ADC histograms. On the other hand, Ogihara *et al.*^[51] indicated that contrast-to-noise ratio (CNR) between the lesion and the liver parenchyma on DWI might be more useful than the ADC values for predicting poorly differentiated HCCs. Iwasa *et al.*^[52] also indicated that DWI CNR and the lesion-to-liver relative contrast ratio (RCR) on DWI are superior in predicting histologic differentiation than the ADC values, T2-weighted RCR, and ethoxybenzyl-hepatobiliary RCR. Mori *et al.*^[53] showed the usefulness of ADC mapping in predicting pre-operative malignant potential of HCC. On the basis of their data, the sensitivity, specificity, PPV, NPV, and accuracy for predicting poorly differentiated HCC is 93%, 68%, 54%, 96% and 75%, respectively, and those for predicting MVI is 89%, 58%, 31%, 96% and 63%, respectively. They suggested that hypointense HCC on ADC mapping are characterized by poor histological differentiation and more frequent microscopic portal invasion^[53]. Zhao *et al.*^[54] showed the usefulness of the combination of the true diffusion coefficient value and an irregular shape on hepatobiliary phase for predicting MVI, and the sensitivity and specificity were improved to 94.4% and 63.6% respectively. Wang *et al.*^[55] reported that other diffusion parameters, such as mean kurtosis value on diffusion kurtosis imaging, and irregular circumferential enhancement on dynamic MRI were independent risk factors for MVI of HCC. The combination of higher mean kurtosis values and irregular shape are potential predictive biomarkers for MVI^[55].

Gadolinium-ethoxybenzyl diethylenetriamine penta-acetic acid-enhanced magnetic resonance imaging (EOB-MRI) is now commonly used for the diagnosis of HCC. With its use, there have been increasing reports of predicting MVI using dynamic MRI including hepatobiliary phase of EOB-MRI. Chang *et al.*^[56] indicated that relatively low arterial enhancement on arterial phase of EOB-MRI and low ADC value were predictive of worse histological grades of HCC. Kim *et al.*^[57] suggested focusing on the peritumoral hypointensity on hepatobiliary phase of EOB-MRI for predicting MVI. The sensitivity, specificity, PPV and NPV

Table 3. Studies with magnetic resonance imaging for predicting poorly differentiation, non-single nodular type, or microvascular invasion

Ref.	Modalities	Findings	Prediction	Sensitivity	Specificity	PPV	NPV	Accuracy
Enomoto <i>et al.</i> ^[38]	Plain + dynamic MRI	Hypointensity on T1-weighted imaging and washout on portal-venous phase	Poorly diff.	88%	67%	N/A	N/A	71%
Mori <i>et al.</i> ^[53]	Plain MRI	Hypointensity on ADC map	Poorly diff.	93%	68%	54%	96%	75%
Mori <i>et al.</i> ^[53]	Plain MRI	Hypointensity on ADC map	MVI	89%	58%	31%	96%	63%
Wang <i>et al.</i> ^[55]	Plain MRI	Mean kurtosis values > 0.917	MVI	70%	77%	70%	77%	74%
Kim <i>et al.</i> ^[57]	EOB-MRI	Peritumoral hypointensity on HBP	MVI	38%	93%	89%	53%	62%
Zhao <i>et al.</i> ^[54]	EOB-MRI	Irregular tumor margin	MVI	50%	88%	69%	76%	75%
Lee <i>et al.</i> ^[58]	EOB-MRI	Arterial peritumoral enhancement	MVI	54%	88%	68%	80%	77%
Lee <i>et al.</i> ^[58]	EOB-MRI	Irregular tumor margin	MVI	70%	69%	51%	83%	69%
Lee <i>et al.</i> ^[58]	EOB-MRI	Peritumoral hypointensity on HBP	MVI	32%	92%	65%	74%	73%
Tada <i>et al.</i> ^[60]	EOB-MRI	Irregular tumor margin	Non-SN type	97%	72%	74%	97%	83%
Chen <i>et al.</i> ^[61]	EOB-MRI	Irregular tumor margin	Non-SN type	96%	79%	87%	94%	89%
Kobayashi <i>et al.</i> ^[62]	EOB-MRI	Irregular tumor margin	Non-SN type	64%	96%	93%	77%	81%
Chen <i>et al.</i> ^[61]	EOB-MRI + CECT	Irregular tumor margin	Non-SN type	98%	84%	90%	94%	93%
Kobayashi <i>et al.</i> ^[62]	EOB-MRI + CEUS	Irregular tumor margin	Non-SN type	85%	95%	94%	88%	91%

PPV: positive predictive value; NPV: negative predictive value; MRI: magnetic resonance imaging; N/A: not available; ADC: apparent diffusion coefficient; MVI: microvascular invasion; EOB-MRI: Gadolinium-ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging; HBP: hepatobiliary phase; SN: single nodular; CECT: contrast enhanced computed tomography; CEUS: contrast enhanced ultrasonography

were 38.3%, 93.2%, 88.5%, 52.6% and 62% respectively. Lee *et al.*^[58] also demonstrated that a combination of two or more of the following; arterial peritumoral enhancement, irregular tumor margin, and peritumoral hypointensity on hepatobiliary phase, can be used as a preoperative imaging biomarker for predicting MVI, with specificity > 90%. Hu *et al.*^[59] also reported in a systemic review and meta-analysis that peritumoral enhancement and peritumoral hypointensity on hepatobiliary phase were highly specific (90%-94%) but low sensitive findings (29%-40%) for predicting MVI.

On distinguishing between the SN type and non-SN type using EOB-MRI, Tada *et al.*^[60] demonstrated that the sensitivity, specificity, and accuracy of EOB-MRI for identifying non-SN were equal to or higher than that using angiography-assisted CT. Chen *et al.*^[61] also compared the diagnostic ability of EOB-MRI and contrast CT. The sensitivities, specificities, and accuracies for the diagnosis of non-SN type were 71.4%, 81.6%, and 75.5% in contrast CT, 96.4%, 78.9%, and 89.3% in EOB-MRI, and 98.2%, 84.2%, and 92.5% in combination, respectively. They concluded that contrast CT combined with EOB-MRI offers a more accurate imaging evaluation for HCC macroscopic classification than either modality alone^[61]. Kobayashi *et al.*^[62] compared the ability of EOB-MRI and CEUS to predict macroscopic type, and found that the sensitivity, specificity, PPV, NPV, and accuracy for the diagnosis of non-SN type were 64.1%, 95.7%, 92.6%, 76.9% and 81.2% in EOB-MRI, 56.4%, 97.8%, 95.7%, 72.6% and 78.8% in CEUS, and 84.6%, 95.7%, 94.3%, 88% and 90.6% in combination, respectively. The combined diagnosis of EOB-MRI and CEUS provides highest diagnostic ability^[62]. Iwamoto *et al.*^[63] also showed that the diagnostic ability for macroscopic classification of nodular HCC of the post-vascular phase of CEUS with Sonazoid was comparable with that of hepatobiliary phase of EOB-MRI, and the combination of the two modalities provided a more accurate diagnostic performance. The sensitivity, specificity, PPV, NPV, and accuracy of MRI studies cited in this review were summarized in Table 3 except for not available reports.

DISCUSSION

This article reviews the current status of predicting MVI using common imaging modalities for the diagnosis of HCC. MVI is strongly associated with histologic differentiation and macroscopic type. Poorly differentiated HCCs are characterized by hypovascular components and faster tumor enhancement washout on dynamic imaging. Non-SN type HCCs are characterized by irregular shape image. The possible mechanism

which could interpret the correlation between imaging features and probability of MVI is that HCC has a strong tendency of invasive growth along with de-differentiation from well to poorly differentiated HCC. Therefore, the accurate diagnosis of histologic differentiation and macroscopic type is essential for accurate prediction of MVI.

US including CEUS is the most non-invasive among imaging modalities. Although most of the previous reports cited in “US” section were about the diagnosis of HCC with poor differentiation or non-SN type, which suggested that direct connection between US and diagnosis of MVI was a few, useful US parameters for predicting poorly differentiated HCC or MVI are irregular intra-tumoral artery, fast washout of tumor enhancement, and irregular tumor margin. MFI should be used to assess the intra-tumoral angioarchitecture, with the deadwood pattern being a highly specific finding for predicting MVI. Although the optimal cut off time is yet unknown, shorter washout time of tumor enhancement is also a specific finding. Based on previous reports on assessing tumor shape by imaging, post-vascular phase (Kupffer phase) of CEUS is considered more accurate than B-mode US or contrast CT, and the diagnostic ability of CEUS for predicting HCC macroscopic type would be equal to that of the hepatobiliary phase of EOB-MRI. However, US has several disadvantages, such as poor visualization due to dead space, artifacts, and deep lesion, and difficulty of whole scan in larger tumors. Therefore, when the whole tumor cannot be scanned by US, another imaging assessment using EOB-MRI or contrast CT is necessary.

Factors to consider in predicting poorly differentiated HCC or MVI on contrast CT are heterogenous enhancement including hypovascular components, fast washout of tumor enhancement, complete or partial absence of peritumoral capsule (halo), the presence of intra-tumoral vessels and aneurysms on venous phase, and irregular tumor margin. Although complete or partial absence of peritumoral capsule, the presence of intra-tumoral vessels and aneurysms, and irregular tumor margin can be easily evaluated in large HCC, these parameters are difficult to assess in small HCCs. The heterogenous enhancement and fast tumor enhancement washout are useful findings in predicting poor histologic differentiation of small HCCs.

Tumor tissue characterization such as water, fat, and metal content, or diffusion of water molecules can be easily obtained by plain MRI. As non-hypointense HCC on T1-weighted image reflects well differentiation of HCC, the MVI risk would be low. Since intra-tumoral fat is also often seen in well differentiated HCCs, HCCs containing fat components would be at low risk of MVI. Accordingly, T1-weighted image including chemical shift is important in predicting low risk of MVI. Previous reports also demonstrated that low ADC value reflects poor differentiation. However, there is no adequate cut-off value to distinguish poorly and non-poorly differentiated HCC on meta-analysis. This may be because the absolute ADC value depends on the MRI equipment coil systems, imagers, vendors, and field strengths^[64]. Since the contrast between tumor and adjacent liver tissue, measured by CNR, and the RCR on DWI, are superior in predicting poor differentiation compared to the ADC values, the assessment of tumor contrast to adjacent liver tissue on DWI or ADC map should be more appropriate and universal in clinical practice than quantification of ADC values. As such, care should be taken when evaluating a very high intense HCC on DWI or low intense HCC on ADC map. When directly predicting MVI using EOB-MRI, the important parameters include irregular margin, arterial peritumoral enhancement (relative hypovascularity), and peritumoral hypointensity on hepatobiliary phase. Although the specificities of these findings for predicting MVI were very high, the sensitivities were low. Therefore, attention should be paid for the false negativity of these findings. To improve the sensitivity for predicting MVI, the combined evaluation with plain MRI including DWI and EOB-MRI may be useful, because the evaluation of diffusion parameters is highly sensitive for predicting poor differentiation or MVI. Among common imaging modalities, the most information for predicting MVI can be obtained from MRI. Furthermore, there are some reports that the combination of EOB-MRI with CECT or CEUS improved the accuracy in predicting non-SN type. As mentioned above, combination of MRI with other imaging modalities may provide a more accurate assessment of malignant potential of HCC.

As other tumor factors related to MVI, tumor markers^[65,66] and FDG-PET uptake^[67] have been reported. As FDG-PET is not used as common imaging modality for the diagnosis of HCC, the papers about the predictability of FDG-PET for MVI were omitted from this review. Although tumor markers such as alpha-fetoprotein and des- γ -carboxy prothrombin (DCP) are closely related to the presence of MVI, they are also highly expressed in patients with benign diseases such as chronic hepatitis and liver cirrhosis^[68]. Furthermore, as several cut-off values according to studies were suggested, the best cut-off value has not been unknown. However, Shirabe *et al.*^[69] reported that a scoring system for predicting MVI using tumor size, serum DCP levels, and FDG-PET uptake can provide a precise prediction of MVI, and the sensitivity and specificity were 100% and 90.9%, respectively. Probably, tumor marker levels would be helpful for predicting MVI.

There are some limitations in this research field. Firstly, although some highly specific or sensitive imaging findings for predicting poorly differentiated HCC or MVI have been reported, there are no highly accurate diagnostic findings. This may be due to limited accuracy of identifying histologic differentiation or MVI from resected specimens. MVI would often be missed if thorough microscopic examination is not performed, and histologic differentiation would be judged as non-poorly differentiated HCC if poorly differentiated components are not dominant. It is difficult to search for MVI throughout the whole tumor using a microscope, and MVI detection depends on the serial slice width of the tumor specimen. The thinner the specimen, the more accurate the MVI detection. Secondly, since most of previous reports are small-cohort, single-center, and retrospective, and diagnostic ability also depends on the performance of imaging equipment, their conclusions might be unreliable and biased. To validate their results, large scale prospective studies are needed. Lastly, adequate treatment strategy based on MVI prediction or histologic differentiation has not been established. At this time, if a HCC patient is predicted to have high risk of MVI as assessed by imaging, it should be treated as advanced HCC even after resection or local ablation.

In conclusion, HCCs with high grade malignant potential can be diagnosed with commonly used imaging modalities. For accurate prediction of MVI in HCC, the diagnosis of poor histologic differentiation or non-SN type is needed, and the combination of MRI with other imaging modalities should be used.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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AUTHOR INSTRUCTIONS

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The journal adopts Gold Open Access publishing model since its establishment and has been distributing contents under Attribution 4.0 International License since October 2017, whereas Attribution-NonCommercial-ShareAlike 3.0 Unported had been adopted by then. Please make sure that you are well aware of these policies.

1.3 Publication Fees

Authors are required to pay Article Processing Charges of 360 US Dollars after the manuscript is officially accepted. For more details, please refer to Article Processing Charges.

1.4 Language Editing

All submissions are required to be presented clearly and cohesively in good English. Authors whose first language is not English are advised to have their manuscripts checked or edited by a native English speaker before submission to ensure the high quality of expression. A well-organized manuscript in good English would make the peer review even the whole editorial handling more smooth and efficient.

If needed, authors are recommended to consider the language editing services provided by Charlesworth to ensure that the manuscript is written in correct scientific English before submission. Authors who publish with OAE journals enjoy a special discount for the services of Charlesworth via the following two ways.

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1.5 Work Funded by the National Institutes of Health

If an accepted manuscript was funded by National Institutes of Health (NIH), the author may inform editors of the NIH funding number. The editors are able to deposit the paper to the NIH Manuscript Submission System on behalf of the author.

2. Submission Preparation

2.1 Cover Letter

A cover letter is required to be submitted accompanying each manuscript. It should be concise and explain why the study is significant, why it fits the scope of the journal, and why it would be attractive to readers, *etc.*

Here is a guideline of a cover letter for authors' consideration:

In the first paragraph: include the title and type (e.g., Original Article, Review, Case Report, *etc.*) of the manuscript, a brief on the background of the study, the question the author sought out to answer and why;

In the second paragraph: concisely explain what was done, the main findings and why they are significant;

In the third paragraph: indicate why the manuscript fits the Aims and Scope of the journal, and why it would be attractive to readers;

In the fourth paragraph: confirm that the manuscript has not been published elsewhere and not under consideration of any other journal. All authors have approved the manuscript and agreed on its submission to the journal. Journal's specific requirements have been met if any.

If the manuscript is contributed to a special issue, please also mention it in the cover letter.

If the manuscript was presented partly or entirely in a conference, the author should clearly state the background information of the event, including the conference name, time and place in the cover letter.

2.2 Types of Manuscripts

There is no restriction on the length of manuscripts, number of figures, tables and references, provided that the manuscript is concise and comprehensive. The journal publishes Original Article, Review, Meta-Analysis, Case Report, Commentary, *etc.* For more details about paper type, please refer to the following table.

Manuscript Type	Definition	Abstract	Keywords	Main Text Structure
Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author includes an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
Case Report	A Case Report details symptoms, signs, diagnosis, treatment, and follows up an individual patient. The goal of a Case Report is to make other researchers aware of the possibility that a specific phenomenon might occur.	Unstructured abstract. No more than 150 words.	3-8 keywords	The main text consists of three sections with fixed section titles: Introduction, Case Report, and Discussion.
Meta-Analysis	A Meta-Analysis is a statistical analysis combining the results of multiple scientific studies. It is often an overview of clinical trials.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Systematic Review	A Systematic Review collects and critically analyzes multiple research studies, using methods selected before one or more research questions are formulated, and then finding and analyzing related studies and answering those questions in a structured methodology.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Technical Note	A Technical Note is a short article giving a brief description of a specific development, technique or procedure, or it may describe a modification of an existing technique, procedure or device applied in research.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Commentary	A Commentary is to provide comments on a newly published article or an alternative viewpoint on a certain topic.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Editorial	An Editorial is a short article describing news about the journal or opinions of senior editors or the publisher.	None required	None required	/
Letter to Editor	A Letter to Editor is usually an open post-publication review of a paper from its readers, often critical of some aspect of a published paper. Controversial papers often attract numerous Letters to Editor	Unstructured abstract (optional). No more than 250 words.	3-8 keywords (optional)	/
Opinion	An Opinion usually presents personal thoughts, beliefs, or feelings on a topic.	Unstructured abstract (optional). No more than 250 words.	3-8 keywords	/
Perspective	A Perspective provides personal points of view on the state-of-the-art of a specific area of knowledge and its future prospects. Links to areas of intense current research focus can also be made. The emphasis should be on a personal assessment rather than a comprehensive, critical review. However, comments should be put into the context of existing literature. Perspectives are usually invited by the Editors.	Unstructured abstract. No more than 150 words.	3-8 keywords	/

2.3 Manuscript Structure

2.3.1 Front Matter

2.3.1.1 Title

The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

2.3.1.2 Authors and Affiliations

Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

2.3.1.3 Abstract

The abstract should be a single paragraph with word limitation and specific structure requirements (for more details please refer to Types of Manuscripts). It usually describes the main objective(s) of the study, explains how the study was done, including any model organisms used, without methodological detail, and summarizes the most important results and their significance. The abstract must be an objective representation of the study: it is not allowed to contain results which are not presented and substantiated in the manuscript, or exaggerate the main conclusions. Citations should not be included in the abstract.

2.3.1.4 Keywords

Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

2.3.2 Main Text

Manuscripts of different types are structured with different sections of content. Please refer to Types of Manuscripts to make sure which sections should be included in the manuscripts.

2.3.2.1 Introduction

The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

2.3.2.2 Methods

Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

2.3.2.3 Results

This section contains the findings of the study. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

2.3.2.4 Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

2.3.2.5 Conclusion

It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

2.3.3 Back Matter

2.3.3.1 Acknowledgments

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

2.3.3.2 Authors' Contributions

Each author is expected to have made substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data, or the creation of new software used in the work, or have drafted the work or substantively revised it.

Please use Surname and Initial of Forename to refer to an author's contribution. For example: made substantial contributions to conception and design of the study and performed data analysis and interpretation: Salas H, Castaneda WV; performed data acquisition, as well as provided administrative, technical, and material support: Castillo N, Young V.

If an article is single-authored, please include "The author contributed solely to the article." in this section.

2.3.3.3 Availability of Data and Materials

In order to maintain the integrity, transparency and reproducibility of research records, authors should include this section in their manuscripts, detailing where the data supporting their findings can be found. Data can be deposited into data repositories or published as supplementary information in the journal. Authors who cannot share their data should state that the data will not be shared and explain it. If a manuscript does not involve such issue, please state "Not applicable." in this section.

2.3.3.4 Financial Support and Sponsorship

All sources of funding for the study reported should be declared. The role of the funding body in the experiment design, collection, analysis and interpretation of data, and writing of the manuscript should be declared. Any relevant grant numbers and the link of funder's website should be provided if any. If the study is not involved with this issue, state "None." in this section.

2.3.3.5 Conflicts of Interest

Authors must declare any potential conflicts of interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there are no conflicts of interest, please state "All authors declared that there are no conflicts of interest." in this section. Some authors may be bound by confidentiality agreements. In such cases, in place of itemized disclosures, we will require authors to state "All authors declare that they are bound by confidentiality agreements that prevent them from disclosing their conflicts of interest in this work." If authors are unsure whether conflicts of interest exist, please refer to the "Conflicts of Interest" of OAE Editorial Policies for a full explanation.

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Research involving human subjects, human material or human data must be performed in accordance with the Declaration of Helsinki and approved by an appropriate ethics committee. An informed consent to participate in the study should also be obtained from participants, or their parents or legal guardians for children under 16. A statement detailing the name of the ethics committee (including the reference number where appropriate) and the informed consent obtained must appear in the manuscripts reporting such research.

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If the manuscript does not involve such issue, please state "Not applicable." in this section.

2.3.3.7 Consent for Publication

Manuscripts containing individual details, images or videos, must obtain consent for publication from that person, or in the case of children, their parents or legal guardians. If the person has died, consent for publication must be obtained from the next of kin of the participant. Manuscripts must include a statement that a written informed consent for publication was obtained. Authors do not have to submit such content accompanying the manuscript. However, these documents must be available if requested. If the manuscript does not involve this issue, state "Not applicable." in this section.

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2.3.3.9 References

References should be numbered in order of appearance at the end of manuscripts. In the text, reference numbers should be placed in square brackets and the corresponding references are cited thereafter. Only the first five authors' names are required to be listed in the references, other authors' names should be omitted and replaced with "et al.". Abbreviations of the journals should be provided on the basis of Index Medicus. Information from manuscripts accepted but not published should be cited in the text as "Unpublished material" with written permission from the source.

References should be described as follows, depending on the types of works:

Types	Examples
Journal articles by individual authors	Weaver DL, Ashikaga T, Krag DN, Skelly JM, Anderson SJ, et al. Effect of occult metastases on survival in node-negative breast cancer. <i>N Engl J Med</i> 2011;364:412-21. [PMID: 21247310 DOI: 10.1056/NEJMoal008108]
Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. <i>Hypertension</i> 2002;40:679-86. [PMID: 12411462]
Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. <i>J Urol</i> 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. <i>J Appl Clin Pediatr</i> 2012;27:1903-7. (in Chinese)
Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. <i>BJOG</i> 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. <i>The genetic basis of human cancer</i> . New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm . [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. <i>Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming</i> ; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. <i>Proc Natl Acad Sci U S A</i> . Forthcoming 2002.

For other types of references, please refer to U.S. National Library of Medicine.

The journal also recommends that authors prepare references with a bibliography software package, such as EndNote to avoid typing mistakes and duplicated references.

2.3.3.10 Supplementary Materials

Additional data and information can be uploaded as Supplementary Material to accompany the manuscripts. The supplementary materials will also be available to the referees as part of the peer-review process. Any file format is acceptable, such as data sheet (word, excel, csv, cdx, fasta, pdf or zip files), presentation (powerpoint, pdf or zip files), image (cdx, eps, jpeg, pdf, png or tiff), table (word, excel, csv or pdf), audio (mp3, wav or wma) or video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv). All information should be clearly presented. Supplementary materials should be cited in the main text in numeric order (e.g., Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2, *etc.*). The style of supplementary figures or tables complies with the same requirements on figures or tables in main text. Videos and audios should be prepared in English, and limited to a size of 500 MB or a duration of 3 minutes.

2.4 Manuscript Format

2.4.1 File Format

Manuscript files can be in DOC and DOCX formats and should not be locked or protected.

2.4.2 Length

There are no restrictions on paper length, number of figures, or amount of supporting documents. Authors are encouraged to present and discuss their findings concisely.

2.4.3 Language

Manuscripts must be written in English.

2.4.4 Multimedia Files

The journal supports manuscripts with multimedia files. The requirements are listed as follows:

Videos or audio files are only acceptable in English. The presentation and introduction should be easy to understand. The frames should be clear, and the speech speed should be moderate.

A brief overview of the video or audio files should be given in the manuscript text.

The video or audio files should be limited to a duration of 3 min and a size of up to 500 MB.

Please use professional software to produce high-quality video files, to facilitate acceptance and publication along with the submitted article. Upload the videos in mp4, wmv, or rm format (preferably mp4) and audio files in mp3 or wav format.

2.4.5 Figures

Figures should be cited in numeric order (e.g., Figure 1, Figure 2) and placed after the paragraph where it is first cited;

Figures can be submitted in format of tiff, psd, AI or jpeg, with resolution of 300-600 dpi;

Figure caption is placed under the Figure;

Diagrams with describing words (including, flow chart, coordinate diagram, bar chart, line chart, and scatter diagram, *etc.*) should be editable in word, excel or powerpoint format. Non-English information should be avoided;

Labels, numbers, letters, arrows, and symbols in figure should be clear, of uniform size, and contrast with the background; Symbols, arrows, numbers, or letters used to identify parts of the illustrations must be identified and explained in the legend;

Internal scale (magnification) should be explained and the staining method in photomicrographs should be identified;

All non-standard abbreviations should be explained in the legend;

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2.4.6 Tables

Tables should be cited in numeric order and placed after the paragraph where it is first cited;

The table caption should be placed above the table and labeled sequentially (e.g., Table 1, Table 2);

Tables should be provided in editable form like DOC or DOCX format (picture is not allowed);

Abbreviations and symbols used in table should be explained in footnote;

Explanatory matter should also be placed in footnotes;

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2.4.7 Abbreviations

Abbreviations should be defined upon first appearance in the abstract, main text, and in figure or table captions and used consistently thereafter. Non-standard abbreviations are not allowed unless they appear at least three times in the text. Commonly-used abbreviations, such as DNA, RNA, ATP, *etc.*, can be used directly without definition. Abbreviations in titles and keywords should be avoided, except for the ones which are widely used.

2.4.8 Italics

General italic words like *vs.*, *et al.*, *etc.*, *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

2.4.9 Units

SI Units should be used. Imperial, US customary and other units should be converted to SI units whenever possible. There is a space between the number and the unit (i.e., 23 mL). Hour, minute, second should be written as h, min, s.

2.4.10 Numbers

Numbers appearing at the beginning of sentences should be expressed in English. When there are two or more numbers in a paragraph, they should be expressed as Arabic numerals; when there is only one number in a paragraph, number < 10 should be expressed in English and number > 10 should be expressed as Arabic numerals. 12345678 should be written as 12,345,678.

2.4.11 Equations

Equations should be editable and not appear in a picture format. Authors are advised to use either the Microsoft Equation Editor or the MathType for display and inline equations.

2.5 Submission Link

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