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Novel predictive and prognostic strategies of hepatitis B virus related hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is a common malignancy and an important cause of cancer death worldwide. Chronic hepatitis B virus (HBV) infection is the major cause of HCC. Recent studies of HBV-induced carcinogenesis not only discovered many new biomarkers but also developed a novel theory: Cancer Evolution-Development (*Cancer Evo-Dev*). *Cancer Evo-Dev* provides an evolutionary insight of developing more reasonable predictive and prognostic strategies. Characterizing chronic inflammatory microenvironment of cancer evolution, genetic polymorphisms of inflammatory factors, and HCC-related HBV mutations that negatively selected by host immunity may help greatly in identifying HBV-infected individuals who are more likely to develop HCC or benefit from HCC prophylactic options. Gene expression signatures and somatic mutation profiles reflect the different patterns of signaling pathway networks underlying tumor heterogeneity and can be applied to improve the molecular classification and prognostic stratification of HCC patients. Mutant cells that survive the selection can retro-differentiate into tumor initial cells and aggressive sub-clones. Detection of mutants or their hallmarks in cell-free DNA in peripheral blood potentially improve the early diagnosis, prognosis prediction, and personalized treatment of HBV-caused HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed cancers and an important cause of cancer death worldwide. Annually, there are 782,500 HCC incident cases and 745,500 HCC-caused deaths worldwide.[1] Developing countries in East Asia and Sub-Saharan Africa contribute 80% of new HCC cases and related deaths.[2] Chronic infection of hepatitis B virus (HBV) is the major etiological reason for HCC in these areas, which contributes 80-90% of HCC patients.[3,4] According to a cohort study conducted in Taiwan, the cumulative lifetime (age 30 to 75 years) incidences of HCC for men and women that positive for hepatitis B surface antigen (HBsAg) were 27.38% and 7.99%, far more than those of men and women negative

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for HBsAg and anti-hepatitis C virus (1.55% and 1.30%).[5] Besides, HBV infection is also responsible for the increasing trend of HCC in western countries because of the travel and immigration of HBV infected populations.[6] Most HCC patients are diagnosed at advanced stage and cannot accept resection operation or liver transplantation.[7] Approximately 70% patients that have curative hepatectomy will relapse within 5 years.[8] Both the narrow therapeutic window and the high recurrence rate highlight the importance of developing more rigorous surveillance and more active prevention for chronic HBV infected subjects with high HCC risk, and tailoring more suitable treatment options for HCC patients, which depend on continuously discovering promising biomarkers as well as developing carcinogenesis theory for the specific prophylaxis.

Cancer Evo-Dev is a novel scientific theory describing the mechanism of HBV-induced hepatocarcinogenesis.[9] The central aspects of its framework are as follows. Carcinogenesis is an evolutionary process under the microenvironment of chronic non-resolving inflammation. This microenvironment is characterized by immune imbalance due to the interaction between the genetic predisposition of immune/proinflammatory molecules and HBV infection. Cytidine deaminases and their analogous are persistently activated by proinflammatory factors and subsequently induced mutations both in host and viral genomes. Mutant cells are mostly eliminated by selective pressures. Only a small proportion can survive in the inflammatory microenvironment because the somatic mutations alter signaling pathways. Those surviving clones usually share some characteristics of stem cells and gradually retro-differentiate into cancer initialing cells.

This theory was presented based on recent outcomes of HBV-related carcinogenesis researches, mainly including molecular epidemiological studies, cancer genomic mutation analyses, and signaling transduction researches.[10-20] Those breakthroughs not only improved the understanding of cancer evolution from different aspects but also discovered many novel biomarkers and therapeutic targets. Therefore, this theory can provide an evolutionary insight of predicting HCC risk and developing more reasonable predictive and prognostic biomarkers and therapeutic targets. Here, we summarize the important novel viral, inflammatory, genetic, and protein biomarkers of HCC occurrence and prognosis and evaluate them through the lens of *Evo-Dev* theory.

EVALUATING THE MICROENVIRONMENT OF CANCER EVOLUTION

In the evolution process of HBV-induced hepatocarcinogenesis, inflammatory microenvironment plays an important role via facilitating the generation of viral and host genetic mutation and also providing selective pressure. Therefore, the characteristics of the microenvironment in different evolutionary phases and in different populations can be used to stratify HBV-infected individuals with different risk of developing HCC. Although inflammatory microenvironment is a complex system, it can be elucidated in two aspects: HBV itself and immune imbalance.

HBV

Despite the high incidence of HCC in HBV-infected population, only small percentages of chronic hepatitis B (CHB) patients develop HCC. HBV variables can serve as clues to identify distinctive outcomes of HBV-infected populations, and to guide the personalized preventive medication accordingly.

HBV replication

The level of HBV replication directly reflects the selective stress from the inflammatory environment, which can influence the evolution of HCC as well. Currently, HBV DNA load is regularly applied in clinic as an indicator of initiating antiviral treatment. It has been demonstrated by various studies that HBV DNA load increases the risk of HCC in CHB patients.[21-23] High level of HBV DNA load either in serum or liver tissue can also predict poor postoperative prognosis in HCC.[24] Hepatitis B e antigen (HBeAg), encoded by HBV precore region, is another marker for active replication of HBV. HBeAg positivity has been proved to be associated with an increased risk of HCC.[25] However, due to HBeAg seroconversion during the natural course of HBV infection, HBeAg expression is not usually high in HCC patients, explaining the reasons that HBeAg positivity is not significantly associated with an increased risk of HCC in some case-control studies.[14] Thus, HBV DNA load should be a more reliable indicator in the prediction of HCC.

HBV genotypes

According to a sequence divergence of no less than 8% in whole viral genome, HBV can be classified into eight genotypes A to H, which can be further classified into sub-genotypes if the sequence divergence is between 4% and 8%.[26] Variant genotypes are distributed unevenly around the world, and the predominant one in mainland China is genotype C (68.3%), followed by genotype B (25.5%).[27] Under selection pressure from inflammatory microenvironment, the fates of different genotypes/sub-genotypes are distinct in a given population. Genotype C HBV infection is an independent risk factor for HCC development.[16,21,28,29] Meanwhile, genotype B HBV infection was associated

with the development of HCC in young patients (< 50 years old).[30] Our study further revealed that genotype B2 HBV infection was related to HCC recurrence, and that HBV genotype C2 HBV was predominant in HCC patients, which was related to its high prevalence.[31] As the HBV genotype is usually identified through a complex procedure that includes extracting HBV DNA, polymerase chain reaction, sequencing, and phylogenetic analysis, the wide application of HBV genotype/subgenotype for preliminary screening in community is limited.

HBV mutations

In the process of HBV-HCC evolution, one of the most prominent molecular events is the generation of HBV mutation, especially mutations in the preS region and basic core promoter (BCP) region of HBV genome. Due to lack of proof reading capacity, HBV genome has a higher mutation rate than other DNA viruses. Moreover, inflammatory factors induced by HBV infection can activate the expression of apolioprotein B mRNA editing enzyme catalytic polypeptides (APOBECs). HBV genome can be degraded and edited by APOBECs.[32] Most HBV mutants are cleared by host immune system, and only those that gained the ability to escape immune eradication survived. The mutant viruses, in return, keep on stimulating the immune system and maintain the inflammatory microenvironment. The HBV mutations reflect, to some extent, the selection pressure of host immune system and serve as risk factors of HCC.

Our recent study of HBV mother-to-child transmission revealed that mutated viruses lost their advantages in infecting infants, whereas the wild-type HBV had advantage of infecting newborn's hepatocytes, interestingly, the HCC-risk HBV mutations was being gradually selected since the establishment of chronic infection.[10] Mutations in HBV the preS region (including A2962G, A2964C, C3116T, C7A, T105C, and preS start codon mutation) and mutations in the BCP region (including C1653T, T1753V, and A1762T/G1764A) were independently associated with an increased risk of HCC.[11,15,21,33] Mutations in combination (combo mutations) can enhance the validity of predicting the occurrence of HCC.[21,33,34] HBV combo mutations of C1653T, T1753V, and A1762T/G1764A increase the validity of HCC prediction compared with single HBV mutation.[21] The HBV mutations can improve the sensitivity and specificity of HCC prediction model based on age, gender, cirrhosis and HBV DNA loads.[21,25,35]

The carcinogenic effects of HBV can be blocked by antiviral treatments. In our prospective hospital-based cohort study, antiviral treatment against HBV using interferon and nucleoside analogues (NAs) significantly reduced HCC occurrence (13.90/1,000 *vs.* 7.70/1,000 person-years, $P = 0.005$).[36] Furthermore, proved by a cohort study and randomized clinical trial, treatment with NAs can also significantly reduce the risk of early recurrence (hazard ratios, 0.41; $P < 0.001$).[13] However, levels of those protective effects are distinct among HBV-infected subjects with different viral mutations. Antiviral treatment with NAs cannot reduce HCC risk in patients without A1762T/G1764A or C1653T and in those with T1753V.[36] The protective function of antiviral treatments for postoperative recurrence cannot be observed in the HCC patients expressing carboxylic acid-terminal truncated HBV X protein (Ct-HBx) in their liver remnants.[13]

Immune imbalance

Immune imbalance is responsible for the maintenance of chronic non-resolving inflammation and subsequently provides a fertile microenvironment for cancer evolution. Immune imbalance can be reflected by the proportion shift of immune cells, abnormal activation of inflammatory pathways, and genetic predisposition of inflammatory molecules, which can serve as biomarkers for HCC prediction and prognosis.

Immune cells

The liver is enriched with innate immune cells such as macrophages and natural killer (NK) cells, as well as adaptive immune cells such as $CD8^+$ cytotoxic T cells, $CD4^+$ T helper cells and B cells, playing an important role not only in host defenses against invading microorganisms and tumor transformation, but also in liver injury and repair. Their presence or enrichment can be seen as predictive or prognostic factors for HCC. $CD8^+$ T in liver tissues, for example, is the protective factor, while the enrichment of M2 macrophages and T helper 17 cells (Th17) as well as the imbalance between $CD8^+$ T cells and regulatory T (Treg) cells or between Th1 and Th2 are the risk factors of HCC.[37] Immune cells that infiltrated into HCC tissues function distinctly on HCC prognosis. Intratumoral natural killer cells and $CD8^+$ T cells indicate good prognosis, while intratumoral Treg cells, neutrophils, and M2 macrophages indicate poor prognosis.[37]

Inflammatory pathways

The abnormal alteration of inflammatory pathways can be reflected by hallmark cytokines. Biomarkers indicating the abnormal activation of inflammatory pathways can also predict the occurrence and recurrence of HCC.[38,39] For example, Wnt/β-catenin signaling pathway plays an important role in inflammation-induced carcinogenesis via regulating the expression of cytokine-induced human inducible nitric

oxide synthase.[40] Activation of Wnt/β-catenin pathway contributes to HCC development. The hallmarks of Wnt/β-catenin pathway, Wnt-1 and Wnt3a, have both predictive and prognostic value.[37,41,42] Likewise, signaling pathways such as phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, and insulin-like growth factor pathway also play an important role in hepatocarcinogenesis.[43]

Genetic polymorphisms of immune/inflammatory molecules

Genetic polymorphisms of immune/inflammatory molecules can also serve as predictive biomarkers for HCC development. For example, genetic polymorphisms of signal transducer and activator of transcription 3 (*STAT3*), class II human leukocyte antigen DP (*HLA-DP*), *HLA-DQ*, miRNA-122-binding site, pre-miR-218, nuclear factor-kappaB (*NF-κB*), and its inhibitor IkappaBalpha are significantly associated with HCC risk.[12,17,18,44-47]

IDENTIFYING SIGNATURES OF SIGNALING PATHWAY ALTERATION

Gene signatures

The alteration of signaling pathways confers stemness characteristics and competitive advantages to cancer cells. These alterations usually affect complex signaling networks that cannot be represented by a signal gene. More than 300 published microarray studies of human HCC samples provide sufficient information regarding tumor gene expression profiles.[48] The accumulation of data regarding differentially expressing genes makes it possible to conduct meta-analysis and subsequently determine gene signatures. Recent gene signature studies are summarized in Table 1.[49-66] Gene signatures developed in those studies were used to separate patients into 2 or more subgroups with different clinical outcomes, phenotypes, and altered signaling pathways. The methods of developing gene signatures fall into two major groups. The first group of gene signatures was generated in case-control studies with the data of training cohort or published gene expression data. Most of the gene signature studies belong to this group.[50,52,53,55,57,59,61-65] The second group of gene signatures concerning defined phenotypes or signaling pathways was derived from the data of cell or animal model studies.[49,51,56,58,60] For examples, Lee *et al*.[49] developed a gene signature of stemness from the gene profiling data of rat fetal liver tissue and Kaposi-Novak *et al*.[51] developed a gene signature of Met signaling pathway using the Met deficient mouse model. The predictive value of novel gene signatures was usually evaluated in cohort studies. High risk patients that were identified through cluster analysis or score model based on gene signatures were prone to have unfavourable clinical outcomes, such as poor overall survival and early recurrence.

Although the tumor gene signatures were identified by different studies with various comparison strategies, they shared some genes conferring cancer stemness. For instance, a group of genes related to proliferation and epithelial cell adhesion molecule (EpCAM)-positive phenotype were included in 8 gene signatures summarized in different studies and all associated with poor prognosis.[48] Gene signatures from adjacent non-tumor tissues were also reported to be significantly associated with HCC recurrence, indicating that the histological “normal” adjacent tissue may be at the early stage of cancer evolution. That highlights the need of biopsy-based gene signature detection for specific individuals, like HBV-infected patients. However, signatures from adjacent tissues obtained in different studies are lack of genes in common. Cross validations are needed to consolidate the criteria. Altered expression patterns of the genes in HCC are usually caused by epigenetic modifications in their regulatory elements and somatic mutations of their repressors.

Somatic mutation profiles

Somatic mutations are genetic basis of carcinogenesis. The values of somatic mutations depend on their impacts on related signaling pathways. By changing patterns of signaling transduction, somatic mutations on a small proportion of genes can promote cancer evolution, which are categorized as “driver mutations”.[19] As a matter of fact, some outstanding somatic mutations in HBV-HCC occur in the genes responsible for epigenetic modifications-chromatin remodeling including *ARID1A* and *ARID2* and methylation such *MLL4*.[67,68] Due to survival competition and the positive selection of inflammatory microenvironment, driver mutations accumulate sufficiently to promote malignant transformation of hepatocytes.

The distribution, combination, and dynamic patterns of driver mutations reflex the pressure of microenvironmental selection and growth advantage of hepatocyte subsets. The high frequent mutations can have clinical values as biomarkers for targeted therapy, classification, and prognostic prediction.[67-71] For instance, homozygous deletions were detected in 40% of HCC patients and were significantly associated with poor survival ($P < 0.0001$).[68]

Using next generation sequencing technology, some

Table 1: Representative gene signature studies of hepatocellular carcinoma

Study	Population	Sample type	Etiology	Gene No.	Different clinical outcomes of subgroups
Lee *et al.*[49]	*n* = 61 (validation 1, Chinese) *n* = 78 (validation 2, European)	Tumor tissue	HBV, HCV	907	Overall survival ($P < 0.001$)
Budhu *et al.*[50]	*n* = 20 (training, Chinese) *n* = 95 (validation, Chinese)	Adjacent liver tissue	HBV,	17	Risk of survival/recurrence HR (95% CI) in validation set: 15.1 (5.0-45.8)/7.9 (2.5-25.0)
Kaposi-Novak *et al.*[51]	*n* = 249 (Caucasian)	Tumor tissue	HBV, alcohol, HCV	24	Overall survival ($P < 0.001$)
Wang *et al.*[52]	*n* = 23 (training, Asian) *n* = 25 (validation, Asian)	Tumor tissue	HBV, HCV	57	Rate of vascular invasion (accuracy: 84%; sensitivity: 86%; specificity 82%)
Boyault *et al.*[53]	*n* = 57 (training, French) *n* = 63 (validation, French)	Tumor tissue	HBV, alcohol, HCV	16	Overall survival ($P < 0.001$)
Woo *et al.*[54]	*n* = 65 (Chinese)	Tumor tissue	HBV	628	Risk of early recurrence (within 2 years after surgery) HR (95% CI): 12.539 (3.59-43.76)
Hoshida *et al.*[55]	*n* = 82 (training, Japanese) *n* = 225 (validation, European)	Adjacent liver tissue	HBV, HCV	132	Risk of late recurrence (more than 2 years after surgery) HR (95% CI) in the validation set: 2.08 (1.03-4.18)
Coulouarn *et al.*[56]	*n* = 139 (Caucasian)	Tumor tissue	HBV, alcohol, HCV	249	Overall survival ($P < 0.001$)
Yoshioka *et al.*[57]	*n* = 42 (training, Japanese) *n* = 97 (validation, Japanese)	Tumor tissue	HBV, HCV	172	Risk of early recurrence (within 2 years after surgery) HR (95% CI) in the validation set: 3.29 (1.83-5.91)
Woo *et al.*[58]	*n* = 61 (validation 1, Chinese) *n* = 78 (validation 2, Caucasian)	Tumor tissue	HBV, HCV	625	Risk of recurrence HR (95% CI) in the Chinese set: 2.84 (1.51-5.34)
Roessler *et al.*[59]	*n* = 247 (validation 1, Chinese) *n* = 139 (validation 2, GEO data)	Tumor tissue	HBV, HCV	161	Risk of early recurrence (within 2 years after surgery) HR (95% CI) in the Chinese set: 2.72 (1.48-4.5)
Villanueva *et al.*[60]	*n* = 287 (Japanese)	Tumor and adjacent liver tissue	HBV, HCV	16 for tumor; 17 for adjacent liver tissue	Risk of recurrence HR (95% CI): 1.75 (1.20-2.53) for tumor signature; 1.92 (1.20-3.06) for adjacent signature
Minguez *et al.*[61]	*n* = 79 (training, Caucasian) *n* = 135 (validation, Caucasian)	Tumor tissues	HCV, HBV, alcohol	35	Risk of vascular invasion HR (95 % CI) in the validation set 3.12 (1.29-7.51)
Weng *et al.*[62]	*n* = 80 (Chinese)	Tumor tissue	HBV	3	Risk of early recurrence (within 1 year after surgery) HR (95% CI): 4.762 (1.764-12.856)
Kim *et al.*[63]	*n* = 139 (training, South Korea) *n* = 292 (validation, South Korea)	Tumor tissue	HBV	65	Risk of poor survival HR (95% CI) in validation the set: 1.36 (1.13-1.64)
Kim *et al.*[64]	*n* = 56 (training, South Korea) *n* = 40 (validation, South Korea)	Tumor and adjacent liver tissue	HBV	127	Overall survival ($P < 0.001$)
Lim *et al.*[65]	*n* = 286 (training, South Korea) *n* = 83 (validation, China)	Tumor tissue	HBV	30	Risk of poor prognosis HR (95% CI) in validation set: 2.048 (1.130-3.712)
Kim *et al.*[66]	*n* = 396 (Chinese)	Tumor tissues	HBV	233 for late recurrence, 65 for early recurrence	Risk of late recurrence HR (95% CI): 2.2 (1.3-3.7) Risk of early recurrence HR (95% CI): 1.7 (1.1-2.6)

HBV: hepatitis B virus; HCV: hepatitis C virus; HR: hazard ratio; CI: confidence interval

basic patterns of HCC somatic mutations have been extensively investigated. The somatic mutations provide a novel genomic insight of molecular classification and prognostic prediction. Some genes including *TP53*, *TERT*, *CTNNB1*, *ARID1A*, and *AXIN1* are proved to be hotspots of genetic alteration [Table 2]. However, specific mutation in a single hot gene is not frequent, ranging from 5% to 20%. Such a low rate

Table 2: Important somatic mutations and related signaling pathways of hepatocellular carcinoma

Study	Population and sequencing method	Etiology	Mutation frequency of important genes	Global gene mutation frequency of signaling pathways
Guichard *et al*.[67]	*n* = 24 (training), whole exome equencing; *n* = 125 (validation), Sanger sequencing	Alcohol, HBV, HCV, NASH	*CTNNB1* (32.8%), *TP53* (20.8%), *ARID1A* (16.8%), *PIK3CA* (1.6%)	Wnt/β-catenin pathway (49.6%), p53/cell cycle pathway (32.8%), chromatin remodeling (22.4%), PI3K/Ras pathway (12.8%)
Kan *et al*.[68]	*n* = 88, whole genome sequencing	HBV	*CTNNB1* (16.0%), *IL6R* (26.0%), *TP53* (35.2%), *AXIN1* (5.0%)	Wnt/β-catenin pathway (62.5%), JAK/STAT pathway (45.5%), p53 pathway (43.2%), Apoptosis (45.5%)
Ahn *et al*.[69]	*n* = 231, whole exome sequencing	HBV, HCV	*CTNNB1* (16%), *TP53* (32%), *CCND1* (5%), *RPS6KA3* (5%), *ARID1A* (7%)	Wnt/β-catenin pathway (31%), p53 pathway (37%), cell cycle pathway (23%), PI3K/Ras pathway (12%), chromatin remodeling (34%)
Totoki *et al*.[70]	*n* = 608, whole exome sequencing	HBV, HCV	*CTNNB1* (31%), *TP53* (31%), *ARID2* (10%), *NF1* (4%), *TERT* (54%), *NFE2L2* (5%)	Wnt/β-catenin pathway (66%), p53 signaling (72%), chromatin remodeling (67%), PI3k/mTOR signaling (45%), telomere maintenance (68%), Nrf2/Keap1 pathway (19%)
Schulz *et al*.[71]	*n* = 235, whole exome sequencing	Alcohol, HBV, HCV, NASH	*CTNNB1* (37%), *TP53* (24%), *TERT* (60%), *ARID1A* (13%), *ALB* (13%), *AXIN1* (11%), *CDKN2A* (9%)	Wnt/β-catenin pathway (54%), p53 pathway (49%), telomere maintenance (60%), PI3k/mTOR pathway (51%), MAP kinase pathway (43%), hepatic differentiation (34%), epigenetic regulation (32%), chromatin remodeling (28%)

HBV: hepatitis B virus; HCV: hepatitis C virus; NASH: nonalcoholic steatohepatitis; *CTNNB1*: catenin beta 1; *TP53*: tumor suppressor p53; *ARID1A*: AT rich interactive domain 1A; *PIK3CA*: phosphoinositide-3-kinase catalytic alpha polypeptide; *IL6R*: interleukin 6 receptor; *CCND1*: cyclin D1; *RPS6KA3*: ribosomal protein S6 kinase polypeptide 3; *ARID2*: AT rich interactive domain 2; *NF1*: neurofibromin 1; *TERT*: telomerase reverse transcriptase; *NFE2L2*: nuclear factor (erythroid-derived 2)-like 2; *CDKN2A*: cyclin-dependent kinase inhibitor 2A; JAK: Janus kinase; STAT: signal transducer and activator of transcription; MAP: methionine aminopeptidas

limits the application of a single mutation. For example, *RB1* somatic mutation can serve as an independent predictor for poor cancer-specific survival (HR 2.5, 95% CI: 1.05-5.93, P = 0.038) and early recurrence (OR 3.93, 95% CI: 1.29-11.90, P = 0.015). But the frequencies of *RB1* somatic mutation were only 3.4% and 7% among different studies.[68,69] Similarly, somatic mutations of *CDKN2A* and *FGF-CCND1* were proved to be significantly associated with overall survival (P = 3.0 × 10^{-4} and P = 7.4 × 10^{-6} respectively) and their frequencies were both less than 5%.[70]

Although the spectrums and frequencies of altered genes vary greatly among individuals, they are clustered to pathways or function groups that are closely related with stemness and embryonic characteristics. In this regard, global mutation rates of functionally related genes are added together to define the mutation rate of a given signaling pathway. Mutation rates of Wnt/β-catenin, p53/cell cycle control, JAK/STAT, PI3k/mTOR, and MAP kinas signaling pathways range from 12% to 72%. Similar outstanding outcomes are also observed in function gene groups of chromatin remodeling and telomere maintenance. Ahn *et al*.[69] developed a somatic mutation signature of cell cycle pathway which comprised 4 genes including *RB1*, *MYC*, *CCND1*, and *RBL2*. The total mutation rate of those 4 genes were 23% and the signature was significantly associated with poor cancer-specific and recurrence-free survival (P = 0.002 and P = 0.007, respectively). Therefore, it is promising to use combo somatic mutations as predictive and prognostic biomarkers.

DETECTING CELLS WITH MALIGNANCY POTENTIAL AND THEIR HALLMARKS IN PERIPHERAL BLOOD

Circulating tumor cells

Release of cancer cells into the circulation is common in HCC patients. The appearance of circulating tumor cells (CTC) in the blood stream characterizes the intermediate stage of tumor metastasis process.[72] CTC test can be applied to monitor early metastasis, assess the effectiveness of therapeutic options, and predict the prognosis.[73] A study examining blood samples of 123 HCC patients one month before and after tumor resection indicated that $EpCAM^+$ CTCs were presented in 66.67% of patients and that CTCs count in 7.5 mL blood (CTC7.5) is an independent prognostic factor

of tumor recurrence.[74] Therefore, EpCAM$^+$ CTCs may be used as a real-time parameter for monitoring treatment response. In addition, EpCAM$^+$ CTCs are positive in HCC patients with different BCLC stages and the positive rates of EpCAM$^+$ CTCs in patients of BCLA stage A, B, and C are 11.1%, 19.4%, and 57.9%, respectively.[75] Thus, EpCAM$^+$ CTC is prognostic and predictive in HCC.

Cell-free DNA

Biopsy of HCC may be restricted by the special position of tumors or the poor condition of patients, resulting in the limitation of HCC gene analysis for prognostic and predictive purposes.[76] The necrosis and apoptosis of tumor cells usually release cell-free DNA (cfDNA) into circulation. Based on sequencing technology, genetic and epigenetic information can be obtained from these cfDNA. Detecting cfDNA is a microinvasive method to find early HCC, termed as "liquid biopsy".[77] The abnormities including methylation changes and point mutations in cfDNA can be detected in peripheral blood even before the solid tumor nidus can be detected.

Hypermethylated *RASSF1A* within cfDNA sequence is present in the sera of 93% HCC patients. When combining *RASSF1A* methylation and AFP to diagnose HCC, the sensitivity and specificity increase from 65% and 87% using AFP alone to 77% and 89%, respectively. Serum methylated *RASSF1A* is also prognostic and also reflects the tumor load in HCC patients.[78] A study with a cohort of 151 HCC patients indicated that 4 hypermethylation genes (*RGS10*, *ST8SIA6*, *RUNX2*, and *VIM*) in sera have weak correlation with each other but the combination of the 4 genes as a classifier successfully identified HCC patients from HBV-induced cirrhosis population, with the sensitivity of 85% and the specificity of 96%.[79]

TP53 R249S mutation in cfDNA was proved to have a remarkable ecological correlation with HCC exposure in China and Africa.[80] In a retrospective study using short oligonucleotide mass analysis to exam *R249S* in the plasma ahead of cancer diagnosis, 9 (64%) of 14 patients who developed HCC during the follow-up were positive for *R249S*.[81] Genetic mutation in serum is related to the mutation in tumor tissue. Another study examining the mutations of *CTNNB1*, a gene encoding β-catenin, in HCC patients' sera indicated that *CTNNB1* mutation was not present both in serum and corresponding tumor tissues, although the average mutation rate of *CTNNB1* was about 25% in previous researches.[82] This suggests that clinical application of cfDNA mutations should be mutation signatures rather than single gene mutation.

CONCLUSION

HBV-induced HCC is a common malignancy characterized by high mortality, high recurrence rate, and significant heterogeneity. *Cancer Evo-Dev*, a novel scientific theory of HBV-induced carcinogenesis, provides an evolutionary insight of HCC occurrence/recurrence prediction. From this point of view, recent development of HCC predictive and prognostic strategies can be categorized as three main directions: evaluating the inflammatory microenvironment of cancer evolution via investigating HBV variables and characteristics of immune imbalance, identifying alteration patterns of signaling transformation through signatures of gene expression and somatic mutation, and detecting cells with malignancy potential and their hallmarks in peripheral blood. To validate predictive or prognostic biomarkers, 4 steps should be taken: (1) exploratory research, to discover promising biomarkers; (2) case-control study, to evaluate statistical association between the occurrence/recurrence and biomarkers; (3) cohort study, to validate the sensitivity and specificity of biomarkers; (4) randomized clinical control trail, to determine if the screening and related prophylaxis/treatment can reduce the occurrence/recurrence. Currently, most novel biomarkers were just validated in phase 2 or 3. Further validation and reasonable combination of novel biomarkers should be conducted under the direction of *Cancer Evo-Dev* theory.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethics approval

This review is waived for ethical approval.

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