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Review

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New strategy to distinguish clonal origin of RHCC/MHCC between intrahepatic metastasis and multicentric occurrence

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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 resent cancer statistics of China showed that its incidence was in the fourth place,

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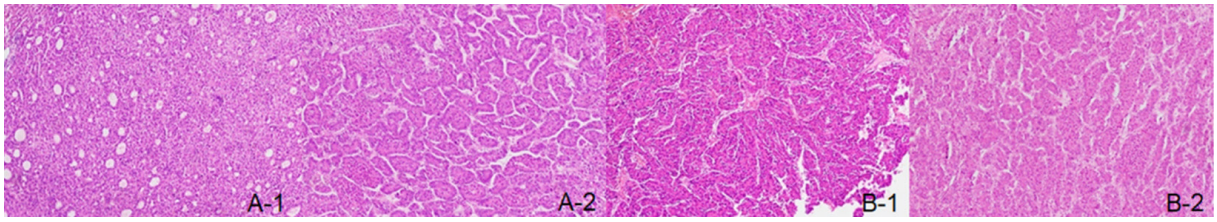

Figure 1. Hepatocellular carcinoma with different histological appearance and similar histological appearance. A-1: pseudoglandular pattern; A-2: thick trabecular pattern; B-1: thick trabecular pattern; B-2: thick trabecular pattern

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 solidary 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].

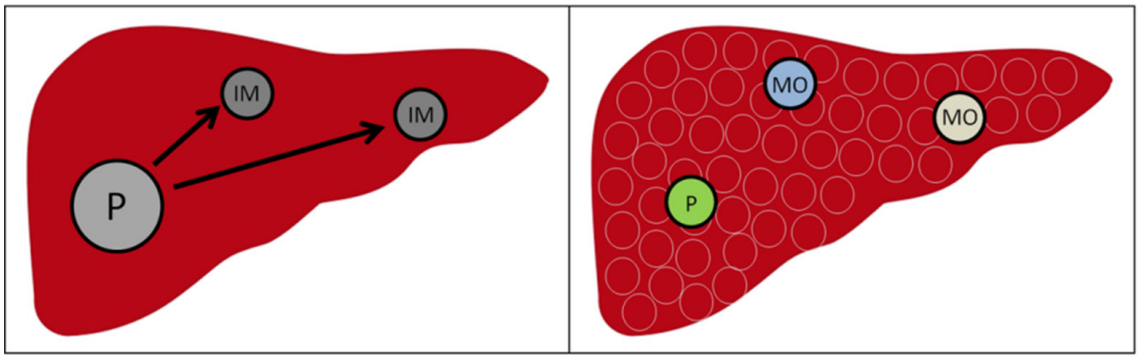

Figure 2. Mechanism of clonal origin with IM type and MO type in recurrent hepatocellular carcinoma/multinodular hepatocellular carcinoma. P: primary hepatocellular carcinoma; IM: intrahepatic metastasis; MO: multicentric occurrence

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 chem otherapy 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.

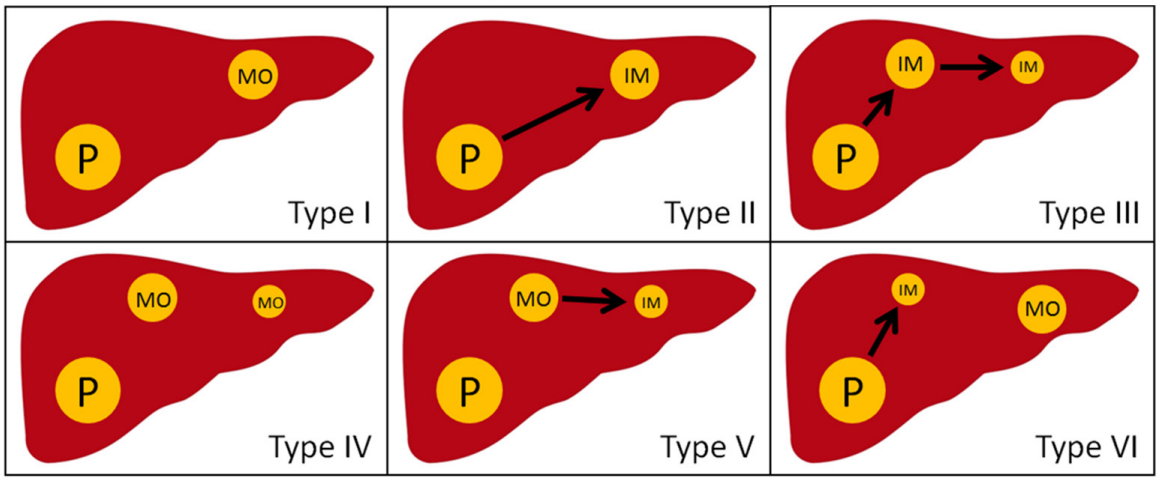

Figure 3. Six subtypes of clonal origin in recurrent hepatocellular carcinoma/multinodular hepatocellular carcinoma. P: primary hepatocellular carcinoma; IM: intrahepatic metastasis; MO: multicentric occurrence

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 microdissecton-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 ≥ 30% difference (number of different LOH loci/number of informative loci × 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, *et al*.	[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

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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