

**Review**

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# Tumor Heterogeneity in Hepatocellular Carcinoma: Facing the Challenges

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Circulating tumor cell · Circulating tumor DNA · Clonality · Hepatocellular carcinoma · Tumor heterogeneity

**Abstract**

Tumor heterogeneity in hepatocellular carcinoma (HCC), such as that found in second primary tumors after curative treatment, synchronous multifocal tumors of different clonality, or intratumor heterogeneity, poses severe challenges for the development and administration of systemic molecular targeted therapies. Various methodologies, including historical DNA ploidy analysis, integrated hepatitis B virus DNA analysis, DNA fingerprinting, and next-generation sequencing technologies, are used to explore tumor heterogeneity in HCC. It is estimated that 30%–60% of recurrent or metastatic tumors harbor clones different from the primary tumor, 22%–79% of synchronous tumors vary clonally, and 12%–66% of single tumors contain intratumor heterogeneity. Substantial intertumor and intratumor heterogeneity renders biomarker identification, which is critical for the development and administration of molecular targeted therapy, challenging when applied to a single tumor biopsy specimen. The use of circulating tumor cells or circulating tumor DNA to evaluate overall tumor heterogeneity may help resolve this problem. This article reviews previous studies of tumor heterogeneity and discusses the implications and future opportunities regarding tumor heterogeneity in HCC.

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## Introduction

In the era of molecular targeted therapy, identifying predictive biomarkers is crucial for the successful implementation of personalized medicine. However, the tumor heterogeneity demonstrated by recent genomic studies may pose challenges for delivering precision medicine [1, 2]. For example, Gerlinger et al. performed exome sequencing on various regions of primary renal cell carcinomas and associated metastatic sites. A total of 128 non-synonymous mutations were identified, and more than 60% of them were not detected at all sequenced sites [3]. Taking a single tumor biopsy, which is currently common practice, may underestimate the mutational burden in a cancer patient and in a single tumor. For lung adenocarcinoma, a type of malignancy with well-established oncogene addiction loops and effective molecular targeted agents, recent studies have demonstrated substantial intratumor heterogeneity by using multiregion whole exome or genome sequencing [4, 5]. An ongoing prospective study of patients with lung cancer aims to determine the clinical significance of this intratumor heterogeneity and clonal evolution by using multiregion and longitudinal tumor sampling and genetic analysis [6].

Hepatocellular carcinoma (HCC) is a complex malignancy caused by various etiologies, including hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol consumption, and metabolic diseases. Although different etiologies may induce various oncogenic pathways, most HCCs are preceded by a history of chronic hepatitis and liver cirrhosis, which provide a pro-oncogenic microenvironment. Early-stage HCC frequently recurs after curative treatment. The recurrent tumor may arise from the intrahepatic metastasis of the primary tumor or may be a second primary HCC. In addition, it is common to have synchronous and multifocal HCCs at presentation because of the “field cancerization” effect. Furthermore, recent data indicate that intratumor heterogeneity at the phenotypic and genetic levels occurs frequently in HCC.

Tumor heterogeneity is not a trivial concern because it greatly complicates the development of molecular targeted agents for HCC. Despite constant efforts, no targeted agents have been successfully approved for HCC other than sorafenib, a multikinase inhibitor targeting Raf kinase and vascular endothelial growth factor receptor. Tumor tissue-based studies have not yet identified useful biomarkers for predicting sorafenib efficacy in HCC, probably because of the lack of representative HCC tumor tissues. However, if tumor heterogeneity between recurrent and primary tumors, among different synchronous tumors, and among various regions of a single tumor does play a crucial role in HCC, our current practice based on a single biopsy or archived tissues would be seriously inadequate for identifying valid biomarkers for HCC.

Herein, we systematically review the literature on tumor heterogeneity in HCC, focusing on the clonal aspects of primary and recurrent tumors, intertumor heterogeneity, and intratumor heterogeneity. Furthermore, we discuss the opportunities and implications of using new technologies, such as next-generation sequencing and circulating tumor cells (CTCs) or circulating tumor DNA (ctDNA), to address the problems of tumor heterogeneity.

## Clonality of Primary and Recurrent Tumors

Curative resection is the indicated therapy for early localized HCC. However, following curative resection, approximately 70% of patients with localized HCC develop recurrence [7, 8]. The incidence of HCC recurrence generally peaks during the first year after resection, and then declines, but gradually increases again 2 years after curative treatment [9, 10]. Previous studies have attributed early recurrence (within 2 years of curative treatment) to

residual tumor or intrahepatic micrometastasis, whereas late recurrence (more than 2 years after curative treatment) has been attributed to de novo development of a second primary tumor (fig. 1a) [9, 11]. However, these hypotheses have not been completely validated.

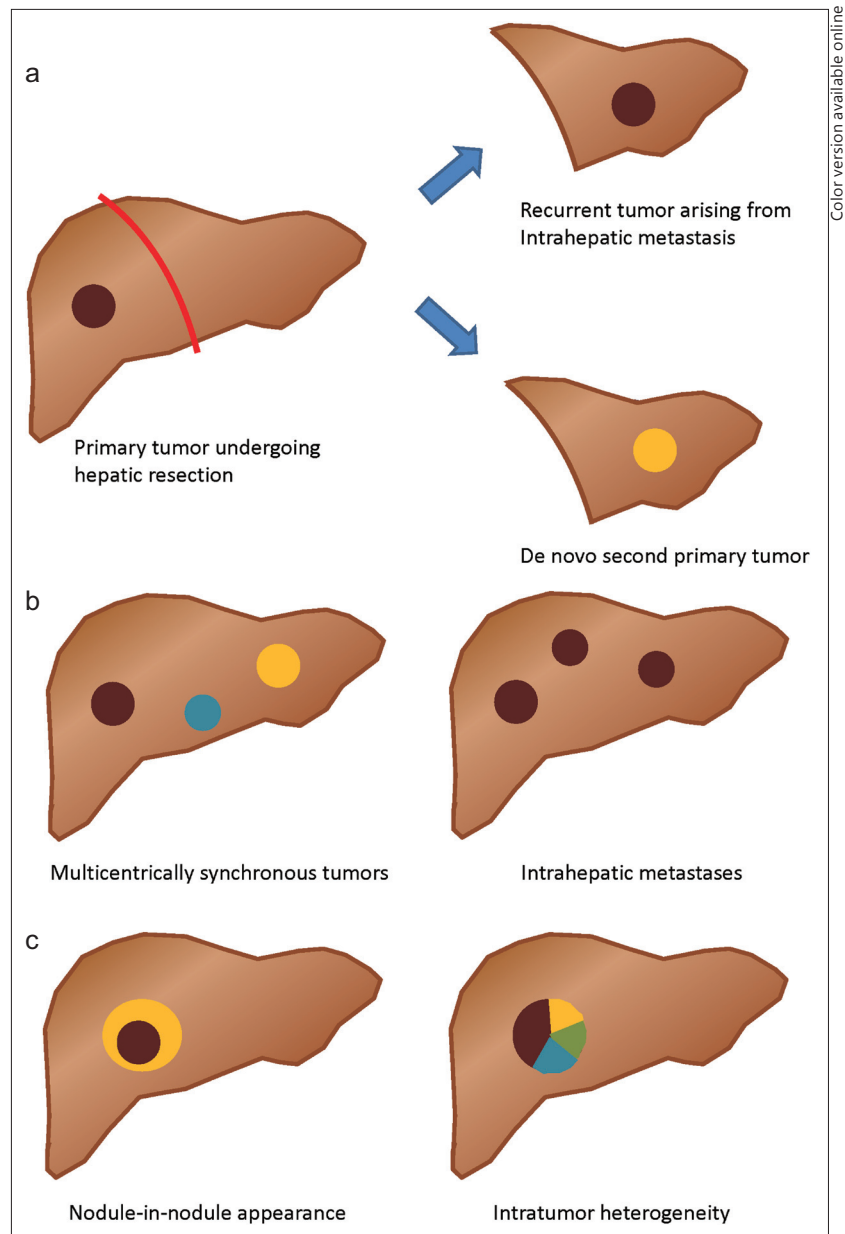
A previous study used integrated HBV DNA as the determinant of clonality for recurrent tumors and found that 60% (3/5) were de novo second primary tumors [12]. Furthermore, by using markers such as DNA ploidy, comparative genomic hybridization (CGH), and the loss of heterozygosity (LOH) of certain microsatellite markers, some studies have identified 42%–53% of recurrent or metastatic tumors with clones different from the primary tumors [13–15]. A recent Chinese study with a large patient cohort of HBV-related HCC determined the clonality of primary and recurrent HCC by analyzing the LOH of 10 microsatellite markers. Thirty percent (48/160) of patients with recurrent HCC were considered to have multicentric occurrence (i.e., tumors of different clonality). The median relapse time was 15.6 months for patients considered to have true recurrence but was approximately 2 years for those with multicentric occurrence ( $p=0.001$ ) [16]. Table 1 summarizes the key findings of studies that assessed the clonality of primary and recurrent HCC.

### Intertumor Heterogeneity

The question whether multiple hepatic tumors in HCC patients develop multicentrically from different clones or arise from a single original tumor via intrahepatic metastasis has been investigated for decades (fig. 1b). Multiple HCCs arising by these two different mechanisms are likely to have different effects on patient prognosis and theoretically should be treated differently. However, the implications of these differences for clinical practice have not been systematically addressed.

Previous studies with small patient cohorts, mostly from Taiwan and Hong Kong, have analyzed DNA ploidy, integrated HBV DNA patterns with or without other markers, or the LOH of specific microsatellite markers to determine the clonality of multiple HCCs, and observed that 22%–61% of patients had synchronous tumors of different clonality [15, 17–22]. A study conducted in the UK used the arbitrarily primed polymerase chain reaction (AP-PCR) technique to compare the DNA fingerprints of 55 HCCs from 13 cirrhotic liver explants. The results unexpectedly showed that no two tumors had identical electrophoretic patterns [23]. A recent Japanese study that evaluated the promoter methylation status of multiple tumor suppressor genes as clonal markers also identified frequent (79%, 15/19 of patients) multicentric and synchronous tumors [24]. Table 2 summarizes studies that explored the clonality of multiple HCCs by using various methods.

High-throughput molecular analyses, such as next-generation sequencing, have been used to reveal the landscape of genetic alterations in numerous malignant diseases, including HCC [25]. One study employed multiomics analyses, including genomics and proteomics data, to investigate the clonality of multiple tumors in two patients with HBV-related HCC. The HBV DNA integration pattern, genomic mutations such as indels and substitutions, copy number variations, and chromosomal structure were similar in different tumors from one patient who was believed to have intrahepatic metastasis and died of recurrent disease shortly after curative resection. By contrast, the multiple tumors of the other patient had distinct HBV DNA integrations and genomic alterations, and the patient showed no recurrence for more than 2 years after the resection of multifocal HCC [26]. This study proved that comprehensive multiomics analyses can be used to characterize multifocal tumors, facilitate clinical decision making, and help implement personalized medicine.



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**Fig. 1.** Tumor heterogeneity of HCC. **a** Recurrent HCC after hepatic resection may arise from intrahepatic metastasis of the primary tumor or may be a second de novo primary tumor. **b** Multiple HCCs may occur multicentrically (intertumor heterogeneity) or may arise from intrahepatic metastasis. **c** Intratumor heterogeneity may arise from a dedifferentiation process (nodule-in-nodule appearance, left) or be clonal evolution within a single HCC tumor (right).

### Intratumor Heterogeneity

Intratumor heterogeneity is a characteristic of many solid tumors [3, 27–29]. Tumor cells undergo evolutionary processes and natural selection that lead to diverse clones in a single tumor. Intratumor heterogeneity is believed to play a crucial role in tumor resistance to cancer therapy, including molecular targeted agents. Intratumor heterogeneity may be

**Table 1.** Studies on the clonality of primary and recurrent HCCs

Study/year	Methods	Ratio of different clonality in recurrent HCC	Etiology
Chen et al., Taiwan/1989 [12]	Integrated HBV DNA (Southern blot analysis)	3/5 (60%)	HBV
Yoshida et al., Japan/1992 [13]	DNA ploidy (microspectrophotometry)	11/25 (44%); lung metastasis	HCV predominant
Chen et al., Taiwan/2000 [14]	CGH + integrated HBV DNA	13/31 (42%)	HBV in 19
Morimoto et al., Japan/2003 [15]	LOH	10/19 (53%)	HCV predominant
Li et al., China/2008 [16]	LOH	48/160 (30%)	HBV

**Table 2.** Studies exploring the clonality of multiple HCCs

Study/year	Methods	Ratio of different clonality in patients with multiple HCC	Etiology
Kuo et al., Taiwan/1987 [17]	DNA ploidy (Feulgen method)	4/14 (29%)	HBV predominant
Hsu et al., Taiwan/1991 [18]	Integrated HBV DNA (Southern blot analysis)	16/28 (57%)	HBV
Sheu et al., Taiwan/1993 [19]	DNA fingerprinting + integrated HBV DNA	11/18 (61%)	HBV in 9
Sirivatanauskorn et al., UK/1999 [23]	DNA fingerprinting (AP-PCR)	13/13 (100%)	Alcohol predominant
Cheung et al., Hong Kong/2002 [20]	cDNA microarray + p53 status + integrated HBV DNA	2/6 (33%)	HBV
Ng et al., Hong Kong/2003 [21]	LOH + CGH + integrated HBV DNA	4/11 (36%)	HBV in 10
Morimoto et al., Japan/2003 [15]	LOH	2/9 (22%)	HCV predominant
Lin et al., Taiwan/2005 [22]	LOH	8/16 (50%)	HBV in 11
Nomoto et al., Japan/2007 [24]	Promoter hypermethylation	15/19 (79%)	HCV in 13

more pronounced in HCC because the “nodule-in-nodule” appearance of HCC is commonly detected using imaging studies [30–33]. This characteristic growth pattern reflects a dedifferentiation process in a differentiated HCC and further contributes to the heterogeneity within a single tumor (fig. 1c).

Previous studies with small sample sizes have analyzed DNA ploidy, LOH of microsatellite markers, and other DNA fingerprints to evaluate the clonality within specific HCC tumors and identified that 12%–66% had intratumor heterogeneity [15, 17, 34–37]. A Japanese

**Table 3.** Studies evaluating the intratumor heterogeneity of HCC

Study/year	Methods	Ratio of HCC tumors with intratumor heterogeneity	Etiology
Kuo et al., Taiwan/1987 [17]	DNA ploidy (Feulgen method)	2/17 (12%)	HBV predominant
Okada et al., Japan/1995 [34]	DNA ploidy (flow cytometry)	7/28 (25%)	HCV predominant
Oriyama et al., Japan/1998 [35]	DNA ploidy (flow cytometry)	9/20 (45%)	HCV in 12
Sirivatanauksorn et al., UK/1999 [36]	DNA fingerprinting (AP-PCR)	13/31 (42%)	Alcohol predominant
Saeki et al., Japan/2000 [37]	Restriction landmark genomic scanning	4/6 (66%)	HCV in 5
An et al., Japan/2001 [38]	Histology	14/41 (34%)	HCV predominant
Morimoto et al., Japan/2003 [15]	LOH	1/7 (14%)	HCV predominant
Friemel et al., Switzerland/ 2014 [39]	Histology + IHC + <i>TP53/CTNNB1</i> mutations	5/23 (22%)	Mixed

IHC=immunohistochemistry.

study analyzed the histological differentiation of 41 small HCCs (less than 3 cm in diameter) and observed that 14 of them (34%) showed heterogeneous histological differentiation [38]. Another recently published study combined histology, immunohistochemical staining ( $\beta$ -catenin, glutamine synthetase, CK7, CD44, AFP, and EpCAM), and mutations of *TP53* and *CTNNB1* to determine the intratumor heterogeneity of HCC. The majority of patients (20/23, 87%) showed intratumor heterogeneity based on at least one of the aforementioned histological, immunophenotypic, or genetic factors. Among the 23 patients, 5 (22%) showed intratumor heterogeneity with regard to all the tested factors [39]. These findings challenge the previous knowledge and classifications of HCC based on phenotypes and molecular changes [40, 41]. Table 3 summarizes the findings of studies on the intratumor heterogeneity of HCC.

Recently, Tao et al. reported mutation profiles from multiple regions of a primary HCC and recurrent tumors by using whole genome and exome sequencing in a single patient. The study dissected the tumor progression patterns by identifying different clones of the primary tumor and additional mutations (foreground mutations) that led to intrahepatic metastasis [42]. The findings confirmed that tumor heterogeneity and evolution can be analyzed with high resolution at the nucleotide level. Additional studies on large HCC patient cohorts are warranted.

### Exploiting CTCs or DNA to Evaluate Tumor Heterogeneity in HCC

Various methods using cell density gradients, cell size differences, and specific surface markers have been developed to isolate CTCs in patients with solid tumors. Two studies have evaluated circulating EpCAM-positive cells as CTCs in patients with HCC and demonstrated that the presence of such cells in the blood stream was associated with poor prognosis

[43, 44]. However, during the epithelial–mesenchymal transition, a process that is required for invasion and metastasis, epithelial markers such as EpCAM could be lost. Using EpCAM-based CTC-isolation methods may result in a substantial loss of CTCs. Recently, an asialoglycoprotein receptor–ligand-based separation method was developed to identify CTCs in HCC patients, but this method requires further validation [45, 46].

The clinical applications of CTC or ctDNA isolation may include the early detection of recurrence, the monitoring of treatment efficacy, and predicting prognosis. In the era of molecular targeting therapy, “liquid biopsies” are being actively investigated for surrogate biomarkers of the primary tumor [47]. For example, epidermal growth factor receptor (EGFR) mutations, which are associated with the efficacy of EGFR tyrosine kinase inhibitors, can be detected using various methods involving CTCs or ctDNA in patients with non-small cell lung cancer [48, 49]. Therefore, assessing the molecular heterogeneity of primary and metastatic tumors by using CTCs or ctDNA may be a rational approach, because circulating samples are derived from multiple tumor sites in a patient. Thus, based on the assumption that different clones have a similar tendency to disseminate or shed DNA into the circulation, CTC and ctDNA isolation could potentially reveal a complete picture of the genetic landscape in a longitudinal and dynamic manner. However, this type of study remains relatively unexplored for HCC.

### **Clinical Implications**

Establishing the tumor heterogeneity of HCC may impact clinical decisions and patient management. For patients with early-stage HCC, curative treatments are indicated. If such a patient shows intrahepatic metastasis-related multiple HCC, adjuvant treatment may be beneficial because of the high risk of recurrence. In contrast, for patients with intermediate-stage HCC and multicentric tumors of different clonality, aggressive locoregional therapy may be beneficial. Additional clinical studies are warranted to validate the significance of these hypothetical approaches.

For patients with advanced HCC, several clinical trials of molecular targeted therapy have accepted archived tumor tissues for biomarker testing. In many cases, archived tissues were obtained from primary tumors many years before the development of recurrent and advanced HCC when patients were indicated for systemic therapy. Such “recurrent” tumors could have developed from a clone of the previous primary tumor or could have arisen as a *de novo* second primary tumor. Therefore, using biomarker profiles obtained from archived tissues for predicting the treatment response of a second primary tumor is unjustified. Even if a fresh biopsy is taken for molecular profiling, the inter- and intratumor heterogeneity of HCC may still result in the failure to identify appropriate biomarkers for molecular targeted therapy. With an improved understanding of the significance of tumor heterogeneity in HCC, clinical trials adopting biomarker-enrichment and adaptive-design strategies might constitute a route leading to the successful development of personalized targeted therapy for HCC.

### **Future Directions and Challenges**

Previous studies based on the simple analysis of DNA ploidy, HBV DNA integration, or other DNA fingerprinting method have revealed substantial intertumor and intratumor clonal heterogeneity in HCC. Advances in deep-sequencing and cutting-edge technologies have facilitated understanding of the genetic alterations found in HCC with high resolution, multiple

dimensions, and pathway-driven insights. Future studies using these novel technologies should address the following issues related to the tumor heterogeneity of HCC.

Although HCC is caused by clear etiologies, and some studies have proposed the classification of HCC based on phenotypes, molecular changes, or pathway activation [25, 41], no driver event corresponding to targeted therapy has been identified. The intertumor and intratumor heterogeneity of HCC makes the identification and verification of such driver genetic alterations challenging. Future studies need to evaluate in detail genetic alterations geographically across different regions, within and across different tumors in individual patients, and chronologically along the tumor progression. This information will clarify the evolutionary genetic changes involved in hepatocarcinogenesis and cancer progression, and thus might facilitate the identification of potential key genetic drivers of HCC.

Deep sequencing technologies, however, are still based on “snapshot” tumor biopsies. Although multiregion sequencing reveals higher mutational burdens and possible evolutionary processes, it is unclear how many biopsies are needed to display all subclones of a single tumor, or even multiple tumors; in addition, multiregional sampling is difficult in the clinical setting. Studying CTCs and ctDNA could potentially identify more tumor clones and could potentially build up a complete picture of tumor heterogeneity in a longitudinal and dynamic manner. Further studies are warranted to standardize and validate the methods for collecting CTCs and ctDNA from patients with HCC.

In conclusion, understanding tumor heterogeneity highlights the limitations of current methods for exploring biomarkers in HCC. As high-throughput methods and bioinformatics technologies develop and improve, highly comprehensive genomic studies on large patient cohorts will be facilitated, and such studies will produce new strategies to tackle the challenges of developing novel molecular targeted agents for HCC.

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## Conflicts of Interest

The authors do not have any conflicts of interest to declare.

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