# Variable Intra-Tumor Genomic Heterogeneity of Multiple Lesions in Patients With Hepatocellular Carcinoma

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**BACKGROUND & AIMS:** Many patients with hepatocellular carcinoma (HCC) have multiple lesions (primary tumors, intrahepatic metastases, multiple occurrences, satellite nodules, and tumor thrombi); these have been associated with a poor prognosis and tumor recurrence after surgery. We investigated the clonal relationship among these lesions on the basis of genetic features. **METHODS:** We collected 43 lesions and 10 matched control samples (blood or nontumorous liver) from 10 patients with hepatitis B virus–associated HCC treated at Tianjin Cancer Hospital (China) from January 2013 through May 2014. We performed exome and low-depth, whole-genome sequencing on these samples. Genomic aberrations, including somatic mutations and copy number variations, were identified using germline DNA as control. We compared the genetic features of different lesions from each patient and constructed phylogenetic trees to depict their evolutionary histories. **RESULTS:** In each patient, mutations shared by all the lesions were called *ubiquitous mutations*. The percentage of ubiquitous mutations varied from 8% to 97% among patients, indicating variation in the extent of intratumor heterogeneity. Branched evolution was evident, with somatic mutations, hepatitis B virus integrations, and copy number variations identified on both the trunks and branches of the phylogenetic trees. Intrahepatic metastases and tumor thrombi contained some, but not all, of the mutations detected in their matched primary lesions. By contrast, satellite nodules shared approximately 90% of mutations detected in primary lesions. In a patient with multicentric tumors, 6 lesions were assigned to 2 distinct groups, based on significant differences in genetic features. In another patient with combined hepatocellular and intrahepatic cholangiocarcinoma, the physically separate HCC and cholangiocarcinoma lesions shared 102 mutations. **CONCLUSIONS:** The extent of intratumor heterogeneity varies considerably among patients with HCC. Therefore, sequence analysis of a single lesion cannot completely characterize the genomic features of HCC in some patients. Genomic comparisons of multiple lesions associated with HCCs will provide important information on the genetic changes associated with tumor progression.

*Keywords:* Liver Cancer; Metastasis; Spread; Pathogenesis.

Liver cancer is the second leading cause of cancer-related deaths worldwide.[1] It claims more than 700,000 lives per year and the global death toll continues to increase. Hepatocellular carcinoma (HCC) is the most frequent type of primary liver cancer, accounting for approximately 80% of such incidences. Various risk factors can lead to HCC, and hepatitis B virus (HBV) infection is one of its leading causes.[2,3]

At present, surgical removal is the primary treatment choice for patients with HCC.[4] However, the prognosis remains poor, mainly owing to the high intrahepatic recurrence rate after resection,[5,6] which is associated closely with the fact that HCC patients often harbor multiple lesions (MLs). Genomic sequencing has shown great power in profiling the genomic landscape of HCC,[7–12] and some types of MLs have been investigated.[7,8,12] However, a comprehensive genomic analysis of all major types of MLs associated with HCC has not been performed. In particular, collection of MLs from the same patient is necessary to show their clonal relationship. In addition, multiregion sequencing has shown substantial intratumor heterogeneity (ITH) in many cancer types, reflecting complex tumor clonal architecture and profound implications in tumor characterization, therapy management, and drug resistance.[13–18] Genomic studies of MLs in the same patient provide an ideal system to elucidate ITH in HCC.

MLs can be divided into 2 groups according to their spatial distribution in the liver: lesions in the liver tissue and tumor thrombi (TT) (portal vein TT or bile duct TT) (Figure 1). The former group contains the primary tumor and separate secondary lesions. Based on their clinicopathologic features, secondary lesions can be subdivided into intrahepatic metastasis (IM) and multicentric occurrence (MO;

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***Abbreviations used in this paper:*** **cHCC-ICC, combined hepatocellular and intrahepatic cholangiocarcinoma; CNV, copy number variation; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; IM, intrahepatic metastasis; ITH, intratumor heterogeneity; MCT, multicentric tumor; MG, multiple occurrence group; MLs, multiple lesions; MO, multiple occurrences; P, primary; PG, primary group; SN, satellite nodule; TT, tumor thrombus.**

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0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2015.12.033

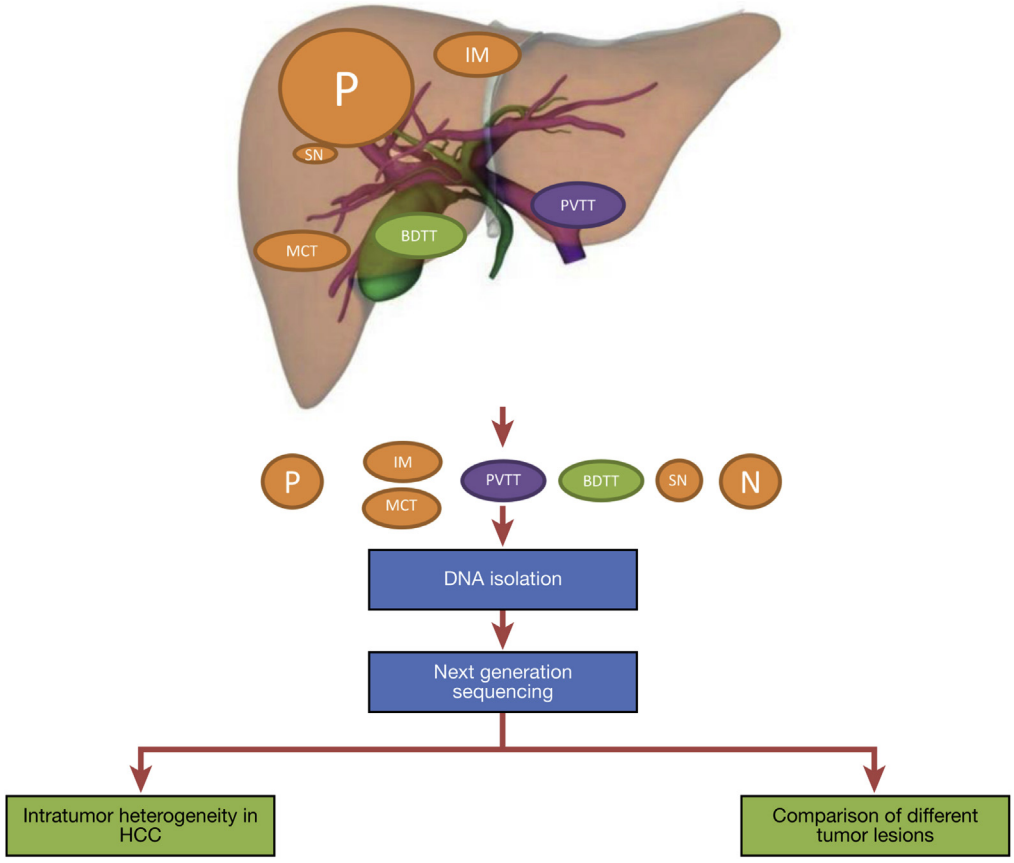


**Figure 1.** Research strategy for genomic comparison of MLs in HCC patients. Schematic illustration of MLs in the liver. The positions of the portal vein (purple) and bile duct (green) are shown. First, surgeons collected the MLs via hepatectomy. DNA was isolated from these lesions and subjected to library preparation and NGS. Finally, genomic information was extracted from the sequence data. BDTT, bile duct tumor thrombus; IM, intrahepatic metastasis; MCT, multicentric tumor; N, adjacent noncancerous tissue or blood; P, primary tumor; PVTT, portal vein tumor thrombus; SN, satellite nodule.

also known as multicentric tumor, MCT). Several clinical studies have reported that IMs and MOs have distinct risk factors and prognosis.[4,19,20] Satellite nodules (SNs), a special form of secondary microscopic lesion in liver tissue, are invisible under conventional imaging modalities such as computed tomography or magnetic resonance imaging. Left unremoved, SNs become potential sources of recurrence in the liver remnant.[21,22] However, whether these SNs are IMs or MOs is difficult to determine using conventional methods.

In the current study, we performed genomic sequencing on MLs from HCC patients (Figure 1 and Supplementary Figure 1). The data show variable extent of ITH among different patients. Comparative analysis of MLs in the same patient was performed to reconstruct the evolutionary history of the tumor. The results presented herein show the genomic complexity of MLs in HCC patients, highlighting the challenges in its diagnosis and treatment strategies.

## Materials and Methods

### *Patient Samples*

MLs and matched blood or adjacent noncancerous liver tissues were collected from HCC patients treated with surgical resection at Tianjin Cancer Hospital from January 2013 to May 2014. Informed consent was obtained from patients enrolled in this study. This research was approved by the institutional review boards of Tianjin Cancer Hospital and Peking University. For further information, see the Supplementary Materials and Methods section.

### *DNA Isolation, Library Preparation, and Next-Generation Sequencing*

DNA (50 ng to 1 $\mu$g) was extracted from each sample and used to prepare exome and whole-genome libraries. Genomic sequencing was performed on Illumina Miseq and Hiseq 2500 platforms (San Diego, CA). For further information, see the Supplementary Materials and Methods section.

### *Whole-Genome and Exome Data Analyses*

Whole genome data were analyzed to plot coverage depth patterns across the genome. Exome data were analyzed for mutations and copy number variations (CNVs) calling. Potential driver mutations, HBV integrations, and recurrent CNVs were determined. For further information, see the "Supplementary Materials and Methods section.

### *Accession Codes*

Sequence data have been deposited in the NCBI Sequence Read Archive under accession number SRP062373.

## Results

### *Genomic Landscape of 10 HBV-Associated HCC Patients*

A total of 53 samples were collected, comprising 43 lesions and 10 matched samples of noncancerous liver tissues or blood (Supplementary Table 1). On average, we obtained 99x exome coverage by uniquely mapping 100-bp reads