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Original contribution

Distinction of intrahepatic metastasis from multicentric carcinogenesis in multifocal hepatocellular carcinoma using molecular alterations☆,☆☆

Peter Chianchiano MS[a,1], Maryam Kherad Pezhouh MD, MSc[a,1], Amy Kim MD[b], Claudio Luchini MD[c], Andrew Cameron MD, PhD[d], Matthew J. Weiss MD[d], Jin He MD, PhD[d], Lysandra Voltaggio MD[a], Kiyoko Oshima MD[a], Robert A. Anders MD, PhD[a], Laura D. Wood MD, PhD[a,e,*]

[a]*Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA*
[b]*Division of Gastroenterology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA*
[c]*Department of Diagnostics and Public Health, Section of Pathology, University of Verona, Verona 37134, Italy*
[d]*Department of Surgery, The Johns Hopkins University School of Medicine, Baltimore, MD 21231*
[e]*Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA*

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Summary Patients with hepatocellular carcinoma (HCC) frequently have multiple anatomically distinct tumors. In these patients, multifocal HCC could represent intrahepatic metastases (IMs) of a single cancer or multicentric carcinogenesis (MC) with multiple independent neoplasms. To determine the frequency and clinical implications of these 2 possibilities, we performed histological and molecular analysis of 70 anatomically distinct HCCs from 24 patients. We assayed mutations in the *TERT* promoter region by Sanger sequencing and used next-generation sequencing to analyze the entire coding regions of 7 well-characterized HCC driver genes—based on shared or discordant mutations in these genes, we classified the HCCs in each patient as IM, MC, or indeterminate. Mutations in the *TERT* promoter were the most common alteration in our cohort, present in 71% of tumors analyzed. Mutations in the remaining genes occurred in less than 20% of analyzed tumors. We were able to determine the relatedness in 58% of the patients analyzed: MC occurred in 41% of patients, with 33% with exclusively MC and 8% with both MC and IM. IM occurred exclusively in 17% of patients, whereas the remainder were indeterminate. This study highlights the utility of molecular analyses to determine relatedness in multifocal HCC; however, targeted sequencing can only resolve this distinction in approximately 60% of patients with multifocal HCC.

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* Corresponding author at: CRB2 Room 345, 1550 Orleans St, Baltimore, MD 21231.
E-mail address: ldwood@jhmi.edu (L. D. Wood).

[1] These authors contributed equally to this work.

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1. Introduction

Liver cancer is the second leading cause of cancer-related deaths worldwide and, as such, is a great cause of morbidity and mortality [1]. Hepatocellular carcinoma (HCC), the most common malignant primary liver neoplasm, occurs most frequently in the background of a variety of underlying chronic liver diseases, including viral hepatitis, fatty liver disease, and chronic biliary tract disease [1,2]. Patients with HCC frequently have multiple anatomically separate tumors, which may have both clinical and biological implications [3-6].

Two distinct biological processes can lead to multifocal HCC. First, 1 primary HCC can spread to additional locations in the liver, representing intrahepatic metastasis (IM). In addition, because HCC frequently occurs in the background of underlying liver disease, multifocal HCC can also represent multiple independent cancers, also known as multicentric carcinogenesis (MC). Although these 2 possibilities are conceptually quite distinct, they cannot be reliably distinguished based on routine clinical and pathological analyses. Multiple previous studies have attempted to distinguish IM and MC based on molecular alterations, and these studies have reported widely disparate frequencies of these 2 possibilities. Most of these studies have focused on loss of heterozygosity as a marker of relatedness, sometimes with arbitrary thresholds to distinguish between IM and MC [5,7-9]. Such analyses are complicated by widespread chromosomal alterations in HCC, which could lead to mischaracterization of tumors based on copy number alone [10]. Even with this limitation in mind, some of these studies have shown poorer prognosis for patients with IM, highlighting potential clinical implications of this distinction [11]. A few studies have reported relatedness assessment based on point mutations assayed by targeted or whole-exome next-generation sequencing (NGS)—such techniques can more definitively distinguish IM and MC in patients with multifocal HCC [12-14]. However, to date, these techniques have not yet been applied to a large cohort of multifocal HCCs.

Several studies have reported comprehensive genetic analysis of HCCs, identifying the most prevalent somatic genetic alterations in this tumor type [10,15-18]. Mutations in the promoter region of the telomerase reverse transcriptase (*TERT*) gene are the most common somatic mutation in HCC with overall prevalence of 40%-60%; the prevalence varies between studies depending at least in part on the underlying liver disease in the analyzed patients [19,20]. Whole-exome and whole-genome sequencing analyses of HCC have demonstrated that *TP53* and *CTNNB1* are also key driver genes in hepatocellular tumorigenesis [10,15-18]. Although the prevalence of mutations in these genes also varies with underlying liver disease etiology, a recent study of almost 250 HCCs reported *TP53* mutations in 26% and *CTNNB1* mutations in 39% [18]. Additional well-characterized driver genes with recurrent somatic mutations in HCC include *AXIN1*, *ARID1A*, and *ARID2*, each of which is mutated in approximately 10% of HCCs [18].

In this study, we determined the relatedness of anatomically separate HCCs by analyzing somatic mutations in the frequently altered driver genes described above to differentiate between IM and MC. By focusing on genes altered by somatic point mutations in at least 10% of HCCs, this study identifies unique somatic mutations that can be used to confidently determine relatedness in the majority of analyzed cases.

2. Materials and methods

2.1. Identification of cases

This study was approved by the Institutional Review Board of The Johns Hopkins Hospital. We searched the pathology archives at The Johns Hopkins Hospital from 2006 to 2015 to identify liver resections and explants with multiple anatomically distinct HCCs. Clinical information and histological slides were reviewed for 40 patients with multifocal HCC. Tumor locations and sizes were recorded from the pathology report in each case. The growth pattern of each tumor was evaluated by a pathologist (M. K. P.) and categorized as pseudoglandular (PS), solid (S), trabecular (T), or clear cell (CC) based on morphology on hematoxylin and eosin (H&E)–stained sections (Supplementary Table 1).

2.2. DNA extraction

DNA was extracted from a single formalin-fixed, paraffin-embedded block of each tumor and matched nonneoplastic tissue. Regions of tumor were identified on an H&E section by a pathologist (M. K. P.), and these regions were cored using a 0.6-mm needle. For matched nonneoplastic DNA samples, absence of tumor was confirmed on an H&E section by a pathologist (M. K. P.), and then tissue was scraped from 5 unstained slides. Genomic DNA was extracted using a combination of QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) and the MagMAX FFPE DNA Isolation Kit (Applied Biosystems, Foster City, CA) as described below.

For tumor cores, tissue was deparaffinized by incubation at 56°C for 5 minutes in deparaffinization solution (Qiagen). For slides of nonneoplastic tissue, tissue was deparaffinized for 5 minutes in xylenes and then scraped with a clean razor blade. Following deparaffinization, tissue was incubated on an agitating thermomixer for 16 hours at 56°C and 800 rpm in 180 μL of ATL buffer (Qiagen) plus 20 μL of Proteinase K Solution (Qiagen). Following this, 2 μL of MaxMag Protease (Applied Biosystems) and 15 μL of MagMax DNA Digestion Additive (Applied Biosystems) were added to each sample, followed by incubation at 60°C at 300 rpm for 60 minutes and then 80°C for 30 minutes without agitation. After cooling to room temperature, 150 μL of buffer AL (Qiagen) was added along with 50 μL carrier RNA solution (49 μL AL+ 1 μL carrier RNA at 1 μg/μL [Qiagen]) and set to incubate for 5 minutes at room temperature. Contents were then transferred