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Distinction of intrahepatic metastasis from multi-centric carcinogenesis in multifocal hepatocellular carcinoma using molecular alterations

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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,

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Supplementary data

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targeted sequencing can only resolve this distinction in approximately 60% of patients with multifocal HCC.

Keywords

Multifocal hepatocellular carcinoma; Multicentric carcinogenesis; Intrahepatic metastasis; Sanger sequencing; Next-generation sequencing

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 μ L of ATL buffer (Qiagen) plus 20 μ L of Proteinase K Solution (Qiagen). Following this, 2 μ L of MaxMag Protease (Applied Biosystems) and 15 μ L 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 μ L of buffer AL (Qiagen) was added along with 50 μ L carrier RNA solution (49 μ L AL+ 1 μ L carrier RNA at 1 μ g/ μ L [Qiagen]) and set to incubate for 5 minutes at room temperature. Contents were then transferred to a QIAamp MiniElute Column (Qiagen), and washing and elution were performed according to the manufacturer's instructions for QIAamp DNA FFPE tissue kit.

2.3. Sanger sequencing

Sanger sequencing was conducted to analyze the sequence of the *TERT* promoter region. The region containing the 2 oncogenic hotspots (chr5: 1 295 228 C>T and 1 295 250 C>T) was amplified by polymerase chain reaction (PCR) using the following primers: 5'-CAG CGC TGC CTG AAA CTC-3' and 5'-GTC CTG CCC CTT CAC CTT-3' [19]. PCR conditions were as follows: 94°C for 2 minutes; 3 cycles of 94°C for 15 seconds, 64°C for 30 seconds, and 70°C for 30 seconds; 3 cycles of 94°C for 15 seconds, 61°C for 30 seconds, and 70°C for 30 seconds; 3 cycles at 94°C for 15 seconds, 50°C for 30 seconds, and 70°C for 30 seconds; 30 cycles at 94°C for 15 seconds, 57°C for 30 seconds and 70°C for 30 seconds; and 70°C for 5 minutes [21]. Representative PCR products were then run on a 2% agarose gel to confirm the presence of the 163-base pair product. Sanger DNA sequencing was performed by The Synthesis and Sequencing Facility at Johns Hopkins School of Medicine. Three microliters of DNA template was mixed with 3 μ L of primer, 5 μ L of BigDye Mix (Thermo Fisher), and 1 μ L of glycerol and DMSO mix (2:1 of 50% glycerol to DMSO). Sample was loaded onto the Applied Biosystems 3730xl DNA Analyzer and held at 95°C for 7 minutes followed by 25 cycles at 95°C for 15 seconds, 50°C for 15 seconds, and 60°C for 4 minutes. Samples were run in both the forward and reverse direction for confirmation of result. Mutation status of the *TERT* promoter hotspot was determined by visual inspection of Sanger sequencing data using FinchTV v.1.4.0.

2.4. Next-generation sequencing

NGS was performed using a custom Ion Ampliseq targeted sequencing panel designed using the Ion Ampliseq Designer (version 5.6; Life Technologies, Carlsbad, CA, USA). The panel (314 amplicons in 2 primer pools) targeted the entire coding regions of 7 previously well-characterized HCC driver genes (*ARID1A*, *ARID2*, *AXIN1*, *CTNNB1*, *TERT*, *TP53*, and *UBE3C*), each of which has been reported to be mutated in >10% of HCCs in at least 1 previous large-scale sequencing study (Supplementary Table 2). Genomic DNA was quantified using the Quantifiler Human DNA Quantification kit (Applied Biosystems) according to the manufacturer's instructions before performing library preparation. Library preparation was conducted using the Ion Ampliseq Library Kit 2.0 (Life Technologies) and subsequently loaded into 318v2 chips and sequenced using an Ion Torrent Personal Genome Machine (Life Technologies) using the manufacturer's suggested protocol (Supplementary Table 3). Following sequencing, data were aligned to the hg19 human reference genome, and variants were called and visually verified using the NextGENe software (v2.4; SoftGenetics, Chicago, IL) and NextGENe Viewer (v2.4; Softgenetics), respectively. For any sample with a unique somatic mutation (ie, a mutation that was not identified in any other samples from that patient), we repeated library preparation and sequencing as described above. The mutation was excluded as a technical artifact if it was not identified in the replicate.

2.5. Determination of relatedness

Sequencing data of adequate quality were obtained from 70 tumors from 24 patients with multifocal HCC. After examining the pattern of somatic mutations in each tumor, we classified the relatedness of the tumors in each patient into 3 categories: MC, IM, or indeterminate

(IND) (Table 1). Because the mutations at the *TERT* promoter occur in an oncogenic hotspot, we considered these mutations separately from those in the other driver genes assessed by NGS. Cases with discordant *TERT* mutations, with or without additional discordant mutations in other driver genes, were classified as MC. Importantly, there were no cases with discordant *TERT* mutations and concordant mutations in other driver genes, highlighting the accuracy of MC designation based on discordant *TERT* mutations. Cases with concordant mutations in the *TERT* promoter as well as at least 1 additional concordant mutation in a driver gene were classified as IM. Cases with only concordant *TERT* promoter mutations or no mutations in either of the assays were classified as IND, as such concordant genetic profiles could represent related tumors (IM) or occur independently in separate neoplasms (MC). Cases with concordant mutations in the *TERT* promoter as well as discordant mutations in other driver genes were classified as MC—the discordant mutations suggest that the *TERT* promoter mutations were independently acquired.

3. Results

Of the patients originally evaluated for inclusion in the study, Sanger sequencing and NGS were successfully performed on all tumors from 24 patients. In all, we analyzed a total of 70 tumors from 24 patients in this study, with 2 to 6 separate tumors evaluated per patient (Table 2). Hepatitis C virus infection was the most common underlying liver disease, present in 15 patients (63%). In addition, 3 patients had hepatitis B virus infection (1 who also had hepatitis C virus), and 1 patient had alcoholic liver disease. Seventeen patients (71%) had cirrhosis. Nine patients were treated with partial hepatectomy, whereas the remaining 15 received liver transplants.

The complete list of somatic mutations identified in our cohort is provided in Supplementary Table 4. Mutation at the oncogenic hotspot in the *TERT* promoter region was detected in 50 (71%) tumors—21 (88%) patients had at least 1 tumor with a *TERT* promoter mutation. Mutations in the other driver genes were less frequent (Table 3). *CTNNB1* mutations were seen in 11 (14%) tumors—8 (33%) patients had at least 1 tumor with a *CTNNB1* mutation. *TP53* mutations were identified in 3 (4%) tumors in 2 (8%) of patients. *AXIN1* mutations were detected in 5 (7%) tumors in 2 (8%) of patients, and *ARID1A* mutations in 3 (4%) of tumors in 2 (8%) of patients. We did not identify any mutations in *ARID2* or *UBE3C* in our cohort.

Based on these somatic mutations, we categorized the relatedness of the tumors into 3 categories: IM, MC, and IND (Table 1, Fig. 1). Eight patients (33%) were characterized as MC, whereas 4 patients (17%) were categorized as IM (Fig. 1). Two patients had more than 2 tumors analyzed and had both MC and IM. In the remaining 10 patients (42%), the results were IND, as the tumors shared no mutations other than those in the *TERT* promoter hotspot, which (because of their high prevalence) could occur independently in unrelated tumors.

Tumors were characterized as MC if they had discordant *TERT* promoter mutations or concordant *TERT* promoter mutations with discordant somatic mutations in other driver genes (Fig. 2). The exemplary patient in Fig. 2 had 3 anatomically separate HCCs. One

tumor (tumor N) was wild type at the *TERT* promoter hotspot and thus was clearly independent from the other 2 tumors in this patient, which had the same classic oncogenic mutation at this position. However, in addition to the *TERT* promoter mutation, we also identified mutations in *CTNNB1* and *ARID1A* in tumor I that were absent in tumor L, indicating that all 3 tumors in this patient arose independently. MC was observed in a total of 8 patients (33%) (Fig. 1).

Tumors were classified as IM when they had concordant somatic mutations in driver genes other than the *TERT* promoter (Fig. 3). Of note, the *TERT* promoter status was also concordant in all such patients. The exemplary patient in Fig. 3, who had concordant mutations in *CTNNB1* and the *TERT* promoter hotspot, also had concordant histologic findings. IM was observed in a total of 4 (17%) patients (Fig. 1).

Intriguingly, some cases had evidence of both IM and MC, in which at least 2 tumors had concordant alterations in other driver genes, whereas at least 1 other tumor was discordant for all mutations analyzed (Fig. 4). In the exemplary patient in Fig. 4, tumors I and J shared mutations in *ARID1A* and were wild type at the *TERT* promoter, indicative of IM. However, tumor B lacked *ARID1A* mutation, demonstrating cooccurring MC. This pattern of co-occurring MC and IM was observed in 2 (8%) patients (Fig. 1).

Pathological features of the tumors, including tumor size and histologic growth pattern, are reported in Supplementary Table 1. Although no cases of IM had discordant histological patterns, concordant histology was present in both IM and MC. In addition, a pattern in tumor size of 1 large lesion with additional much smaller lesions (suggestive of satellite lesions) was not reliably associated with IM. Finally, there was no relationship between IM/MC categorization and underlying liver disease, and although the prevalence of IM was higher in noncirrhotic livers, these results were not statistically significant.

The cumulative risk of recurrence for multifocal HCC patients with IM versus MC is presented in Fig. 5. In the Kaplan-Meier analysis, there is a trend of a higher cumulative risk of recurrence for patients with IM compared with those with MC, but the results do not reach statistical significance (log-rank test: 0.43, hazard ratio [HR]: 1.76, 95% confidence interval [CI]: 0.43–7.28). A multivariate analysis (considering age, sex, and type of surgery) also failed to demonstrate a statistically significant difference in risk of recurrence in patients with IM versus MC (HR: 1.13, 95% CI: 0.15–8.50, $P = 0.90$).

4. Discussion

Our study demonstrates the utility of small somatic mutations to determine relatedness of multifocal HCC—we were able to distinguish IM from MC in almost 60% of the patients in our cohort. In 8 patients (33%), a single Sanger sequencing reaction was sufficient to categorize the tumors as MC due to the discovery of discordant *TERT* promoter mutations. Of these 8 patients, 6 were further confirmed to have additional discordant mutations in our NGS, and none of these patients had concordant mutations in the other driver genes, highlighting the accuracy of the initial MC categorization based on the *TERT* mutation. However, concordant *TERT* promoter mutations are more difficult to interpret, as concordant

mutations could occur in related tumors or could occur independently in unrelated tumors. Of the 16 cases with concordant *TERT* promoter mutations, 2 had discordant somatic mutations in the subsequent NGS experiment, demonstrating the potential to overcall IM based on *TERT* promoter mutation alone. Thus, for cases with concordant *TERT* promoter status, additional somatic mutations are required to accurately resolve the relatedness, highlighting the utility of NGS in these cases.

The results of our study have biological implications for hepatic tumorigenesis, as well as possible future clinical implications. We demonstrate that at least 25% of multifocal HCC patients have IM and at least 41% have MC, highlighting that each process accounts for a sizeable proportion of multifocal HCC patients. In a small percentage of patients with multifocal HCC, both processes occur. The distinction between MC and IM is not currently used to stage or otherwise prognosticate HCC. However, our clinical analysis demonstrated a nonsignificant trend of increased cumulative risk of recurrence in patients with IM; it is unclear whether the lack of statistical significance is due to our sample size or a true lack of influence on risk of recurrence. Further studies in larger cohorts are needed to further explore this question and verify the potential significance of the trend in our cohort. Thus, although not currently used in the clinical care of HCC patients, the distinction between MC and IM may be clinically relevant in the future if larger studies confirm the trend seen in our study. In our cohort, there were no clinical or pathological features that were predictive of MC or IM. Surprisingly, neither the pattern of tumor size nor the similarity in tumor morphology was associated with HCC relatedness. Although all cases of IM shared similar histological features, many cases of MC also demonstrated similar morphology, indicating that this histological similarity is not a specific feature of IM. Similarly, distinct morphologies were observed between different nodules in some cases categorized as MC, but this was not a sensitive marker for MC in our cohort. Thus, these features should not be used to infer relatedness of multifocal HCCs. Our results underscore the role of molecular analyses if the distinction between IM and MC will influence the clinical care of the patient.

One unexpected result in our study was the low prevalence of mutations in *TP53* (occurring in only 4% of tumors and 8% of patients); *TP53* mutations have previously been reported in >20% of HCCs [18]. There are at least 2 possible explanations for this result. First, several previous studies have reported variation in driver gene mutation prevalence according to the etiology of underlying liver disease. It is possible that the etio-logic makeup of our cohort is different than that of previously reported studies, contributing to the discrepancy in *TP53* mutation prevalence. In addition, our cohort of multifocal HCCs is clinically unique from those cohorts of unifocal HCCs typically analyzed in large sequencing studies. It is possible that *TP53* mutation is less common in patients with multifocal HCC. Our cohort is not sufficiently powered to robustly address this question, but another small cohort of multifocal HCC patients also showed a lower *TP53* mutation prevalence than previously reported [22].

Our study does have limitations. Although we included all cases of multifocal HCC at a tertiary care center over a 10-year period, our cohort size is still limited. This is in part due to the difficulty in obtaining high-quality NGS data from formalin-fixed, paraffin-embedded tissue—of the 40 cases that we attempted to analyze, only 24 (63%) provided data of sufficient quality to be included in our study. In addition, a sizeable proportion (42%) of our

patients remained IND after both Sanger and NGS analyses, further limiting the size of the cohort available for survival analysis. This problem is inherent in the genomic landscape of HCC; because there are few frequently altered driver genes in HCC, targeted NGS analyses will not identify nonhotspot alterations in some tumors. Resolving these IND cases would require more molecular analysis, such as whole-exome sequencing, to identify additional somatic mutations to serve as markers of relatedness.

In a subset of our patients, alternative explanations for the mutation patterns should be considered. In patients classified as MC based on discordant mutations in driver genes other than the *TERT* promoter, it is possible that these mutations were acquired in the subclone of the tumor that underwent IM and may not be indicative of genetic independence. However, a recent study analyzing intratumoral genetic heterogeneity in large HCCs suggests that such heterogeneity is not a significant confounding factor in our analysis [22]. In this study, approximately 89% of driver gene mutations were shared between multiple regions of the same HCC tumor nodule, suggesting that genetic heterogeneity with respect to driver genes is not common in HCC and thus unlikely to influence our categorization of relatedness in the majority of cases in our cohort. In the same study, the authors also analyzed a cohort of multifocal HCCs in which the relatedness was conclusively determined with more extensive genetic analysis [22]. In this cohort, 82% of cases of IM shared all driver gene mutations between the tumor nodules, again suggesting that genetic heterogeneity in driver genes is not a significant confounding factor in our study.

Another subset of patients that should be considered is those with concordant mutations in the *TERT* promoter as well as another driver gene. In our study, these patients were classified as IM, but it is theoretically possible that both shared driver gene mutations could have arisen independently by chance. However, aside from the *TERT* promoter hotspot, all the other mutations identified in our study have a low prevalence in HCC—the next most frequently occurring mutation (p.S45P in *CTNNB1*) was present in only 2.9% of HCCs in a recent large sequencing study from The Cancer Genome Atlas [23]. Thus, the probability of 2 tumors independently acquiring the same p.S45P mutation is <0.1%. All other mutations identified in our cohort occurred at lower prevalence in The Cancer Genome Atlas data set and thus would have an even lower probability of being shared in unrelated tumors by chance. Taken together, these results suggest that independent acquisition of any mutation other than those at the *TERT* promoter hotspot is very unlikely, supporting the robustness of our IM categorizations. A final subset of patients deserving separate consideration is those with more than 2 tumors in which there was strong evidence for MC in 1 tumor, but additional tumors remained IND because of only a shared *TERT* promoter mutation. These patients may represent additional cases of cooccurring IM and MC that cannot be resolved by our targeted sequencing assay.

Although several previous studies have attempted to address the question of the origin of multifocal hepatocellular carcinoma, our study adds to the field in multiple important ways. Previous studies have assessed relatedness in multiple ways, although copy number alteration/loss of heterozygosity analysis has been commonly used [5,7–9]. The definition of relatedness based on such alterations is not clear cut, both because the prevalence of such alterations is quite high in HCC and because there is significant heterogeneity with respect

to such alterations within single tumor nodules [8]. These issues lead to wide variations in the estimates of prevalence of IM and MC in these studies, ranging from 80% IM to 80% MC. By using somatic single-nucleotide variants, which are much more specific markers of tumor relatedness, our study provides a more robust estimate of the prevalence of these 2 pheno-types. For example, 1 study used loss of heterozygosity of microsatellite markers, the prevalence of which ranged from 15% to 48% in their cohort—such alterations are much more likely to be shared by chance than point mutations (outside of the TERT promoter hotspot), as the highest prevalence of those identified in our cohort was 3% [8]. Some previous studies have used single-nucleotide variants from whole-exome and whole-genome sequencing in 1–2 patients to clarify relatedness [12–14], but our study is the first to use these alterations in a sizeable cohort of multifocal HCC patients. In these small studies, the large number of identified alterations resulted in clearer definitions of relatedness based on concordance or discordance of tens or even hundreds of mutations, but the driver genes in our assay would have correctly resolved relatedness in the 2 cases in which gene-specific data were available [13,14]. Although we acknowledge that more comprehensive sequencing could resolve the relatedness in a greater proportion of our cohort, our use of targeted NGS ensures the clinical applicability of our results, as this is the approach applied to clinical tumor samples in the majority of molecular pathology laboratories.

Through targeted Sanger sequencing and NGS, our study has demonstrated the minimum frequencies of IM and MC in our cohort of multifocal HCCs, indicating that each process occurs in a significant proportion of multifocal HCC patients. However, we also show the limits of targeted sequencing in resolving the relatedness of the tumors in these patients; whole-exome sequencing will be required to confidently classify all cases. Still, our targeted sequencing assay can categorize the majority of patients, highlighting the utility of this approach to determine relatedness in patients with multifocal HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Relatedness of multifocal HCCs. The graph indicates the percentage of patients with each pattern of relatedness (as determined by molecular alterations).

Fig. 2. Exemplary patients with MC. For each anatomically separate tumor, morphology is shown in the upper panel, with mutations in *TERT* and other driver genes listed below the corresponding histological image. In this patient (MFHC1), discordant mutations in *TERT*, *ARID1A*, and *CTNNB1* suggest 3 independent primary HCCs, indicating MC. H&E stain, original magnification $\times 20$.

Fig. 3. Exemplary patient with IM. For each anatomically separate tumor, morphology is shown in the upper panel, with mutations in *TERT* and other driver genes listed below the corresponding histological image. In this patient (MFHC21), shared mutations in *TERT* and *CTNNB1* indicate that these tumors are genetically related, representing IM. H&E stain, ×20.

Fig. 4. Exemplary patient with both MC and IM. For each anatomically separate tumor, morphology is shown in the upper panel, with mutations in *TERT* and other driver genes listed below the corresponding histological image. In this patient (MFHC11), 2 tumors share the same mutation in *ARID1A*, indicating IM. The third tumor is wild type for all mutations assayed, indicating that there is also an independent primary HCC in MC. H&E stain, ×20.

Fig. 5.

Kaplan-Meier curve for risk of recurrence in patients with IM versus MC. Patients with IM (green line) had a trend toward increased risk of recurrence compared with patients with only MC (blue line) that did not reach statistical significance (log-rank test: 0.43, HR: 1.76, 95% CI:0.43–7.28). Patients with both IM and MC were analyzed in the IM group.

Table 1

Determination of relatedness of multifocal HCCs using molecular alterations

		Other driver genes		
		Concordant	Discordant	No mutations
<i>TERT</i>	Concordant	IM	MC	IND
promoter	Discordant	N/A	MC	MC

Abbreviation: N/A, not applicable (no cases had this pattern).

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Table 2

Clinical and pathological features of multifocal HCC cohort

Feature	n (%)
Total no. of patients	24
Total no. of tumors	70
Hepatitis C viral infection	13 (54%)
Cirrhosis	17 (71%)
Age, mean $\pm$ standard deviation	63.2 $\pm$ 8.2
Male	19 (79%)
Minimum tumors per patient	2
Maximum tumors per patient	6
Liver resection	9 (38%)
Liver transplant	15 (62%)

Table 3

Somatic mutations identified in multifocal HCCs

Gene	Tumors, n (%)	Patients, n (%)
<i>TERT</i> promoter	50 (71%)	21 (88%)
<i>TP53</i>	3 (4%)	3 (13%)
<i>AXIN1</i>	5 (7%)	2 (8%)
<i>CTNNB1</i>	11 (16%)	8 (33%)
<i>ARID1A</i>	3 (4%)	2 (8%)
<i>ARID2</i>	0 (0%)	0 (0%)
<i>UBE3C</i>	0 (0%)	0 (0%)