Intratumor Heterogeneity in Hepatocellular Carcinoma

Juliane Friemel1,2, Markus Rechsteiner1, Lukas Frick1, Friederike Böhm1, Kirsten Struckmann1, Michèle Egger1, Holger Moch1, Mathias Heikenwalder1,3, and Achim Weber1

Abstract

Purpose: Morphologic intratumor heterogeneity is well known to exist in hepatocellular carcinoma (HCC), but very few systematic analyses of this phenomenon have been performed. The aim of this study was to comprehensively characterize morphologic intratumor heterogeneity in HCC. Also, taken into account were well-known immunohistochemical markers and molecular changes in liver cells that are considered in proposed classifications of liver cell neoplasms or discussed as molecular therapeutic targets.

Experimental Design: In HCC of 23 patients without medical pretreatment, a total of 120 tumor areas were defined. Analyzed were cell and tissue morphology, expression of the liver cell markers cytokeratin (CK)7, CD44, α-fetoprotein (AFP), epithelial cell adhesion molecule (EpCAM), and glutamine synthetase (GS) along with mutations of TP53 and CTNNB1, assayed by both Sanger and next-generation sequencing.

Results: Overall, intratumor heterogeneity was detectable in the majority of HCC cases (20 of 23, 87%). Heterogeneity solely on the level of morphology was found in 6 of 23 cases (26%), morphologic heterogeneity combined with immunohistochemical heterogeneity in 9 of 23 cases (39%), and heterogeneity with respect to morphologic, immunohistochemical, and mutational status of TP53 and CTNNB1 in 5 of 23 cases (22%).

Conclusions: Our findings demonstrate that intratumor heterogeneity represents a challenge for the establishment of a robust HCC classification and may contribute to treatment failure and drug resistance in many cases of HCC. Clin Cancer Res; 21(8); 1951–61. © 2014 AACR. See related commentary by Nault and Villanueva, p. 1786

Introduction

Hepatocellular carcinoma (HCC) generally arises in the context of chronic liver diseases, including chronic viral hepatitis, alcohol-induced liver injury, or other metabolic, dietary, or toxic factors such as fatty liver disease or aflatoxin ingestion (1) and ranks number 3 among the leading causes of cancer mortality worldwide (2). It is worthy of note that dietary-induced liver cancer is one of the fastest growing cancer in the United States (3). For a pathologic stratification, tumor stage and grade are the only valid prognostic factors to date. Attempts to classify HCC by immunohistochemical markers or molecular genetic characteristics added fundamental knowledge to our understanding of hepatocarcinogenesis but so far have hardly been applied in routine surgical pathology and postoperative management (4, 5). This stands in contrast to the widely accepted classification of hepatocellular adenoma based on morphology, immunohistochemistry, and genetics that has been included in the latest edition of the World Health Organization (WHO) classification (6, 7) and has implications for patient management.

It is well-known that HCC frequently display heterogeneous growth patterns and/or cytologic features within one and the same tumor. This might either reflect plasticity of phenotypes or intratumor genetic heterogeneity. Both have been described in several solid tumor entities, including skin, breast, and kidney cancer (8–12). In small HCC, intratumor heterogeneity with respect to histologic differentiation grade and proliferative activity was reported to occur in up to 64% of HCC measuring 3 to 5 cm in diameter and in 25% to 47% of HCC smaller than 2 cm (13, 14). Nonetheless, especially in larger HCC, the true extent of intratumor heterogeneity with respect to morphologic, immunohistochemical, and molecular features has not been systematically assessed. The aim of this study was to systematically investigate intratumor heterogeneity in HCC by a comprehensive analysis of tumor area–specific morphology, correlated with clinically relevant immunohistochemical markers (15–21). Cytokeratin (CK)7 is a marker of biliary as well as progenitor cell differentiation. CK7/19, in conjunction with TP53 mutations, has been described as a marker of putative HCC progenitor cells (15), and its expression has been correlated to early tumor recurrence (22). Likewise, CD44, epithelial cell adhesion molecule (EpCAM), and α-fetoprotein (AFP) have been described to be expressed in hepatic progenitor cells and/or HCC (18–21). In addition, AFP is a serum marker for HCC (23). The enzyme glutamine synthetase (GS), a marker of pericentral non-neoplastic hepatocytes, is an indicator of Wnt signaling, and together with nuclear accumulation of β-catenin, a surrogate marker for CTNNB1 gene...
Translational Relevance

In the era of targeted molecular therapies, intratumor heterogeneity is an emerging challenge to successful cancer therapy, as it may result in evasion of therapy of distinct cell subpopulations or in development of therapy resistance as an adaptive phenomenon to specific inhibiting therapies. Therefore, investigating the frequency of and comprehensively characterizing intratumor heterogeneity is crucial. In hepatocellular carcinoma (HCC), it is known that tumors can display intratumoral heterogeneous morphology and histologic differentiation grades. In our study, we aimed to make a link between morphologic intratumor heterogeneity, immune phenotypes [cytokeratin (CK)7, CD44, α-fetoprotein (AFP), epithelial cell adhesion molecule (EpCAM), glutamine synthetase (GS)], and genetic heterogeneity of the two most important driver mutations in HCC (CTNNB1 and TP53), of which β-catenin (CTNNB1) represents a potential therapeutic target. We used multiregional sequencing, supplementing Sanger sequencing with next-generation sequencing which added a high level of accuracy to our analyses. Our results illustrate that intratumor heterogeneity is a frequent finding in HCC, which has implications not only for targeted therapies but also for establishing a robust HCC classification in daily clinical practice.

Materials and Methods

Patients and tissues
Twenty-three patients with an initial diagnosis of a primary HCC receiving surgical treatment between 2003 and 2009 at the University Hospital Zurich were included in this study. Excluded were cases with any kind of pretreatment (e.g., chemotherapy, TACE) and cases with substantial tumor necrosis (>20%). Necrotic tumor areas, ranging between 3% and 20%, were not included in the analyses. Tumor and adjacent normal liver tissues were distinguished different areas within the same tumor, scanned images of which β-catenin (CTNNB1) represents a potential therapeutic target. We used multiregional sequencing, supplementing Sanger sequencing with next-generation sequencing which added a high level of accuracy to our analyses. Our results illustrate that intratumor heterogeneity is a frequent finding in HCC, which has implications not only for targeted therapies but also for establishing a robust HCC classification in daily clinical practice.

mutations (17, 24). Analyses were supplemented by multiregion-al amplicon sequencing of the 2 genes most frequently mutated in HCC, TP53 and CTNNB1 (β-catenin; refs. 5, 23, 25, 26).

Immunohistochemistry

All slides were stained with antibodies against CK7, CK19, CD34, CD44, EpCAM, AFP, β-catenin, and glutamine synthetase, a downstream target of β-catenin (28, 29), using the following antibodies: CK7: Dako M7018 Cl. Ov-Tl 12/30, dilution 1:100; CK19: Abcam ab 9221, dilution 1:200; CD34: Ventana QBEnd/10 (prediluted); CD44: Becton Dickinson G44-26, dilution 1:100; EpCAM: Dako Ber-EP4, dilution 1:40; AFP: Ventana polyclonal, dilution 1:40; glutamine synthetase: Abcam ab 16802 polyclonal, dilution 1:800 and Biocare Medical Cl. 6, dilution 1:500; β-cate-nin: Transduction Lab Inc. 610154 Cl. 14, dilution 1:50. Glutamine synthetase and CK7 were prepared as single and double stainings (OptiView, Ventana) with individual pretreatments (GS: CC1 mild, CK7: Protease 4). All other stainings (Ventana GX and Ventana Bench Mark Ultra) were performed as single stainings: CD34 (Ventana ultraView, CC1 mild), CD44 (Ventana ultraView, CC1 8), AFP (Ventana OptiView, CC1 mild), CK19 (Ventana ultraView, P1 4), and β-catenin (Ventana ultraView, CC1 mild).

A 2-point score was applied for glutamine synthetase (negative vs. positive) and for β-catenin (nuclear vs. non-nuclear staining). For CK7, CK19, CD34, CD44, and AFP staining, the percentage of immunoreactive cells with respect to each tumor area was semi-quantitatively evaluated and grouped according to previously described scoring systems (15): >50% positive cells, 5%-50% positive cells, <5% positive cells (also referred to as scattered cells) or negative. Intratumor expression was considered heterogeneous if expression intensities ranged from “negative” to “positive” (for glutamine synthetase), from “nuclear” to “non-nuclear” (for β-catenin), or from “negative” to ≥5% positive cells (CK7, CK19, AFP, CD34, CD44). All staining intensities were compared with peritumoral liver tissue. Morphologic and immunohistochemical results were evaluated by at least 2 different pathologists (J. Friemel and L. Frick or A. Weber).

DNA extraction and Sanger sequencing

DNA was extracted from the microdissected tumor areas using a DNA extraction and purification kit adapted for FFPE samples (NucleoSpin, FFPE DNA, Machery-Nagel and GFX PCR purification, GE healthcare). For amplification of exons 5 to 8 of the TP53
gene, oligonucleotide primer pairs were used as described (30). PCR was performed in a total volume of 30 μL with 100 to 150 ng purified tumor DNA, 0.2 mMol/L dNTPs, 2.5 mMol/L MgCl2, 400 mMol/L primer mix (forward and reverse), and 1 unit Taq polymerase (AmpliTaq Gold, Applied Biosystems). The S1000 Bio-Rad thermocycler (Bio-Rad Laboratories) was used for amplification at 95°C initial denaturation for 5 minutes; 40 cycles of 58°C annealing for TP53 (45 seconds)/57°C for CTNNB1 (1 minute); elongation at 72°C (45 seconds for TP53)/1 minute for CTNNB1); final elongation step at 72°C for 7 minutes (TP53)/10 minutes (CTNNB1). A BigDye Terminator kit version 3.1 was used for sequencing reactions. For sequencing CTNNB1, we used an inner forward primer (5'-TAAAGTGAATTCAATGACAAATC-3'). Capillary electrophoresis was performed on an ABI 3130 xL DNA analyzer (Applied Biosystems). Sequences were analyzed using Bioedit software and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Mutations were identified by comparison with reference sequences (CTNNB1, NM_19041; TP53, NM_001120612.2) and described according to international conventions (31).

HCC that showed intratumor heterogeneity on the molecular level by Sanger sequencing were additionally resequenced by deep sequencing on a 454 Junior Sequencer platform (Roche).

DNA quantification and amplicon library preparation for deep sequencing

Amplicon library preparation started with the target-specific amplification using bidirectional fusion primers as described earlier (30). CTNNB1 fusion primers were designed using NetPrimer software (www.premierbiosoft.com; see Supplementary Table S1). Primers carried adaptor sequences and individual multiplex identifiers (MID). A single amplification step was applied for TP53 exons 5 to 8 in 50 μL reactions containing 50 ng of tumor DNA from FFPE samples, 0.2 mMol/L dNTPs, 0.5 mMol/L MgCl2, 200 mMol/L fusion primer, and 1 unit iProof Polymerase (Bio-Rad). After 2 minutes of initial denaturation at 98°C, each amplification cycle was performed as follows: 15-second denaturation at 98°C; 30-second annealing with 58°C annealing temperature for exons 5 and 6, 56°C for exon 7, and 62°C for exon 8 of TP53; and 30-second elongation at 72°C. Final elongation was performed at 72°C for 5 minutes after 40 cycles. A 2-step amplification protocol for exon 3 of CTNNB1 was used. The first step included a preamplification with the outer forward primer that was used in Sanger sequencing (forward1) and reverse primer (Supplementary Table S1). Reactions were carried out in 20 μL volume containing 25 ng of DNA, 0.2 mMol/L dNTPs, 0.5 mMol/L MgCl2, 200 mMol/L mix primer, 0.5 unit iProof polymerase (Bio-Rad) and 18 amplification cycles at 53°C annealing temperature. For the second amplification step, the PCR products of β-catenin were diluted 1:50 and admixed to 0.2 mMol/L dNTPs, 0.5 mMol/L MgCl2, 400 mMol/L fusion primer mix (forward 2 and reverse in Supplementary Table S1), 1 unit iProof polymerase (Bio-Rad). Amplification program consisted of 2 minute initial denaturation at 98°C; 35 cycles of 15 second denaturation at 98°C, 30 second annealing at 56°C, 30 second elongation at 72°C; final elongation at 72°C for 5 minutes. Amplicon purification and quantification were performed with an AugenCourt Ampure kit (Beckmann Coulter) and a QuantIT PicoGreen dsDNA assay kit (Invitrogen Life Technologies), respectively. The diluted library with pooled equimolar amounts of 1 × 10^6 molecules/μL served as input for emPCR (Lib-A by Roche) reactions (30 μL of 1 × 10^5 molecules for A-beads and 30 μL of 1 × 10^5 molecules for B-beads resulting in a molecule per bead ratio of 0.6:1).

Deep sequencing workflow and analysis

For the target regions exon 3 of CTNNB1 and exons 5–8 of TP53, deep sequencing was performed on a 454 Junior Sequencer (Roche). Demultiplexing, gene mapping, and variant calling (reference genome GRch37/hg19) were performed with Ampli
con Variant Analyser software (AVA) version 2.7 from Roche with default settings. We included variants that were present in forward and reverse sequences and with at least 50 reads for further analysis (30). For quality control (sensitivity and specificity), we amplified the target regions from independent samples of FFPE extracted tumor DNA and sequenced each in an individual run. This resulted in 8 independently amplified and sequenced replicates. In this resequencing control, we detected the TP53 variant p.E258V in 7 of 8 runs (sensitivity). The background error rate or "noise" (specificity) was calculated from the frequencies of unexpected single-nucleotide changes, insertions, or deletions with very low frequencies and/or strand bias that were detected in only 1 of the 8 replicate analyses (Supplementary Fig. S1). One single-nucleotide change with a strand bias (fw/rev ratio: 2.3) located at a stretch of 4 homopolymers most probably represented a false-positive call. As a result, the calculated error rate of our control was 1.3% over all amplicons. Variants that could only be detected in deep sequencing and not in Sanger sequencing, that is, < 10% frequency, were carefully analyzed for strand biases and coverage: if the minimum coverage of an identified variant was <1,000 or had a strand bias > 1.5, these areas were resequenced.

To determine single-nucleotide variants and the pathogenicity of mutations, every variant was annotated with an algorithm using free access databases. Annotations were taken from ENSEMBL (www.ensembl.org), mutation assessor (www.mutationasses
or.org), Polyphen (http://genetics.bwh.harvard.edu/pph/data/), SIFT (http://sift.jcvi.org), and CHASM (www.chasmsoftware.org).

Statistical analysis was conducted by SPSS 20. Analysis of associations between variables was calculated with the Fisher exact test. Statistical analysis performed on the level of all defined tumor areas was regarded as exploratory, as these areas were not completely independent due to the fact that several of these belonged to the same tumor. Survival analysis was performed by Kaplan–Meier analysis and log-rank tests. P < 0.05 was considered significant. Hierarchical cluster analysis used the squared Euclid
ean distance as distance measure.

Results

Overall frequency of intratumor heterogeneity in HCC

In 23 HCC, a total of 120 tumor areas were demarcated. Per tumor, a mean of 5 areas (5.3; SD, ±2.6; range, 3–10) was analyzed. The size of the tumor areas ranged from 3 to 332 mm² (mean ± SD, 81 ± 80 mm²). Intratumor heterogeneity was found with respect to 3 different features: morphologic, immu

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heterogeneity on the morphologic and immunohistochemical level. Morphologic intratumor heterogeneity disregarding other aspects was seen in 87% of HCC. Most commonly, the tumors were of solid growth pattern, followed by pseudoglandular growth and trabecular growth pattern (53%, 24%, and 23%, respectively, quantification based on tumor areas). Ten HCC displayed one or more cytologic features (clear cell, n = 5; fatty change, n = 5; pleomorphic cells, n = 2; bile plugs, n = 1) in at least one tumor area. Five HCC displayed different histologic grades in the same tumor. However, there was no significant correlation between specific morphologic patterns, histologic differentiation grades, or cytologic features and positivity of certain immunohistochemical markers (Supplementary Table S2). Three tumors were homogeneous, meaning that there was no variation in morphology or immunohistochemical marker expression within the tumor.

**Clinicopathologic findings**

The mean age of the patients was 62 years (SD, ±12.4), and the male:female ratio was 3:8:1. The mean size of the tumors (range, 0.5–18 cm) was 7.4 cm (SD, ±5.4 cm). Large HCC (n = 14) had a mean tumor size of 10 cm (SD, ±4) and small HCC (n = 9) had a mean tumor size of 2 cm (SD, ±0.7).

Table 1 shows the tumor-specific findings and associated clinical background. The tumors were sorted by size and stratified according to TNM (2009) with a distribution of 6/14/3 for the tumor stages pT1/pT2/pT3. Genetic intratumor heterogeneity was more frequently seen in tumors with higher tumor stages (n = 4 of 17 of tumor stage T2 and T3) and larger tumor size of >4 cm (n = 4 of 14). Multinodularity of tumors was frequently associated with morphologic and combined immunohistochemical intratumor heterogeneity (P = 0.023) whereas satellite nodules were generally a rare finding (4 of 23). About 60% of HCC with molecular heterogeneity, 56% of HCC with immunohistochemical heterogeneity, and 33% of HCC with morphologic heterogeneity showed vascular invasion. The median disease-free survival was not significantly different between the 2 groups (26 months in the 5 patients with tumors displaying genetic heterogeneity, compared with 27 months in other patients). Of 4 tested pathologic variables (intratumor heterogeneity, multinodularity, satellite nodules, vascular invasion), only vascular invasion showed significant impact on patient survival (log-rank test: P = 0.04; Supplementary Fig. S2).

Twenty patients with HCC were known to have a chronic liver disease. Eleven patients suffered from chronic hepatitis B or C. Three patients had HCC of unknown etiology. In a total of 5 cases in which tumors displayed all features of intratumor heterogeneity (morphologic, immunohistochemical, and molecular characteristics), there was no predominance of a particular chronic liver disease. Worthy of note is that 2 of 3 patients with HCC of unknown etiology had homogeneous tumors.

**Intratumor heterogeneity of immune phenotypes**

Table 2 provides a heatmap of intratumor heterogeneity of immune phenotypes. Eleven tumors were glutamine synthetase–positive and/or showed β-catenin nuclear positivity in at least one tumor area. The association between these 2 markers was in line with published data (24, 29) and found to be statistically significant (P = 0.006). Consistent glutamine synthetase positivity in all areas of the same tumor was found in only 2 cases, whereas heterogeneous immunoreactivity of glutamine synthetase was seen in 80% of the tumors (n = 8 of 10). Of 15 CK7-positive tumors, 73% exhibited intratumoral heterogeneous CK7 expression (n = 11 of 15). CK7 positivity in all tumor areas of the same tumor was found in 4 HCC. In tumors with intratumoral heterogeneous CK7 expression, a spectrum of different staining patterns was observed: exclusive positivity in glandular structures, widely scattered single-cell staining, or marked foci within single tumor areas (Supplementary Fig. S3). CD44 expression was found in 10 tumors, with 4 showing intratumoral heterogeneous expression. About 57% of AFP-positive tumors showed intratumoral heterogeneous expression (n = 4 of 7). Immunohistochemical AFP expression (heterogeneous and homogeneous) was significantly associated with increased serum AFP (presurgical measurements) >10 μg/L (P = 0.015). One HCC exhibited foci of immunoreactivity for EpCAM in single tumor areas (patient 3). Strong
positivity over all tumor areas was seen in a young patient with a hepatitis B–related HCC (patient 14).

In the analysis of area-specific morphology with respect to immunohistochemistry, no significant associations between morphologic growth pattern and cytologic features were detected (Supplementary Table S2). Remarkably, 71% of areas with clear cell aspect and 48% of pseudoglandular areas showed CK7 positivity.

CK19 positivity was only present in entrapped bile ducts. Similarly, CD34 expression was not found in tumor cells but only in microvessel structures (not shown). Figure 2 illustrates overview and high power of intratumor heterogeneity of all 3 features (patient 13). Tumor areas were defined and schematically illustrated by the H&E slide overview. Microscopic images showed predominantly solid growth pattern with partial glutational synthesis or CK7 positivity. Multiregional sequencing revealed 3 different CTNNB1 mutations: a private S37 and 2 shared mutations (T41 and 15bp deletion at c.352-c.367), whereas an SNP in exon 6 of TP53 (rs1800372) was detected in all tumor areas.

Intratumor diversity of CTNNB1 and TP53 mutational status in HCC

Of 23 HCC, 6 were CTNNB1 mutated and 2 were TP53 mutated. CTNNB1 mutations affected the GSK3β phosphorylation sites, as described (25), and were significantly associated with immunohistochemical positivity for glutamine synthetase ($P = 0.002$) as well as nuclear accumulation of β-catenin ($P = 0.009$).

![Image](image.png)
A total of 120 tumor areas in 23 HCC were sequenced targeting CTNNB1 and TP53. The distribution of wild-type, CTNNB1-, and/or TP53-mutated tumor areas is shown in Table 1 and illustrated for intratumoral heterogeneous cases in Figs. 2 and 3 and Supplementary Fig. S4A. For 8 HCC cases, all tumor areas were validated using deep sequencing to detect mutations with low frequencies and to account for the possibility that wild-type contamination might have led to false-negative results using the Sanger sequencing method. Targeted deep sequencing was performed in 8 individual runs. A total load of about 500,000 enriched beads per run resulted in an average of 79,867 high-quality reads with a mean coverage of 1,442 reads per amplicon. Two additional low-frequency CTNNB1 mutations were discovered by deep sequencing. For example, Sanger sequencing detected a CTNNB1 mutation (I33S) in 5 of 6 tumor areas in patient 1, whereas 6 of 6 areas were mutated at the same location in deep sequencing (Supplementary Fig. S4B). Another additional low-frequency CTNNB1 mutation (S37P) was detected in patient 2 (Fig. 3C). Conversely, 2 CTNNB1 mutations that were detected in tumor areas of patient 2 (S45P, Fig. 3C) and of patient 19 (E54E, not shown) could not be validated in deep sequencing (coverage: 2,297 and 2,111 reads, respectively). Remarkably, 2 HCC with heterogeneous intratumor morphology and immunohistochemistry of 3 markers (CK7, glutamine synthetase, and β-catenin nuclear accumulation) exhibited different CTNNB1 mutations in different tumor areas (Figs. 2C and 3C). Moreover, one tumor (patient 2) carried 3 different CTNNB1 mutations in one single area (Fig. 3C), displaying a rare L31P, a S33A, and a silent mutation P44P (P44P not shown). Analysis of single reads of deep sequencing results revealed that not all nucleotide exchanges were located on the same read (data not shown). Two HCC carried TP53 mutations, one in exon 5 and one in exon 7. Intratumor heterogeneity with wild-type and TP53-mutated tumor areas was seen in one patient with a R175H mutation (patient 6, Fig. 3E). Another TP53 mutation (E258G) was found in all tumor areas in patient 4. Analysis for SNPs of the targeted genes (exon 3 CTNNB1, exons 5–8 TP53) was also taken into account for determining molecular intratumor heterogeneity. Only one SNP (rs1800372) was uniformly detected in the HCC of patient 13 along with intratumoral heterogeneity. Only one SNP (rs1800372) was uniformly detected in the HCC of patient 13 along with intratumoral heterogeneity. However, different tumor samples were not included in the analysis. Multiregional sequencing results were finally displayed as hierarchical cluster analysis (dendrograms of Figs. 2D and 3B, D, F) showing clusters of mutated among wild-type tumor areas.

Area-based analysis of mutational status and the expression of immunohistochemical markers and morphology revealed a positive association between CTNNB1 mutations, nuclear β-catenin (24), and glutamine synthetase positivity (29). CTNNB1 mutations and AFP positivity were inversely associated as has been observed before (32). Furthermore, a pseudoglandular growth pattern was frequently seen in CTNNB1-mutated tumor areas (45%). CD44 positivity was present in 70% of TP53-mutated areas (33). The association between TP53 mutation and β-catenin immunohistochemistry found in our cohort was due to a single TP53-mutated HCC (patient 4) with nuclear β-catenin staining in all tumor areas (Table 3).

Discussion

In this study, we systematically characterized intratumor heterogeneity in HCC on the level of morphology, immune phenotype, or mutational status of CTNNB1 and TP53, respectively. With this approach, we found tumor heterogeneity of at least one feature in the majority of HCC (20 of 23; 87%). Although based on a relatively small number of cases, our findings have implications for HCC biology, HCC classification, and HCC-targeted therapy in the era of personalized medicine.

Morphologic intratumor heterogeneity in HCC has been reported earlier by Kenmochi and colleagues (13), who found 2 or more histologic differentiation grades in more than half of the cases they had studied. An and colleagues (14) also analyzed heterogeneity with respect to the histologic grade and described different tumor grades in 11 of 41 surgically resected small HCC (<3 cm in diameter). After studying tumor heterogeneity on 3 different levels, we now confirm and further define this phenomenon, having detected heterogeneity in different combinations revealing a strict hierarchy: (i) only on the level of morphology, (ii) morphologic heterogeneity combined with immunohistochemical heterogeneity, or (iii) a 3-feature intratumor heterogeneity with respect to morphologic, immunohistochemical, and the mutational status of TP53 and CTNNB1. Morphologic and immunophenotypic heterogeneity within a tumor might either reflect variable generic aberrations or tumor cell plasticity without underlying genetic differences. Our data suggest that both apply to HCC. Notably, about 50% of HCC reveal heterogeneous expression of 2 key immunohistochemical markers in liver pathology, that is, CK7 and glutamine synthetase, respectively, which also are used for HCC classification and prognostication (15). Not unexpectedly, we observed that the frequency of morphologic intratumor heterogeneity was associated with larger tumor size and higher tumor stage, although it did not reach statistical significance, most likely due to a small sample size.

Studying TP53 and CTNNB1, we found a heterogeneous intratumor mutational status in 22% of HCC. Different types of CTNNB1 mutations in one tumor sample were also reported before by Van Nieuh and colleagues (34) and Huang and colleagues (35). Park and colleagues analyzed HCC as well as precursor lesions (36). These findings show that CTNNB1 mutations, generally considered as...
Implications for Tumor Classifications and Targeted Therapies

Table 3. Associations of CTNNB1 mutations and TP53 mutations with morphology and immunohistochemistry

<table>
<thead>
<tr>
<th>Areas with CTNNB1 mutations (n = 22)</th>
<th>Areas with TP53 mutations (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of areas</td>
<td>OR</td>
</tr>
<tr>
<td>Morphology</td>
<td></td>
</tr>
<tr>
<td>Solid</td>
<td>8 (36%)</td>
</tr>
<tr>
<td>Trabecular</td>
<td>4 (18%)</td>
</tr>
<tr>
<td>Pseudoglandular</td>
<td>10 (45%)</td>
</tr>
<tr>
<td>Clear cell aspect</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Fatty change</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td></td>
</tr>
<tr>
<td>β-catenin nucl. +</td>
<td>14 (64%)</td>
</tr>
<tr>
<td>GS+</td>
<td>20 (91%)</td>
</tr>
<tr>
<td>CK7+</td>
<td>13 (59%)</td>
</tr>
<tr>
<td>AFP+</td>
<td>3 (14%)</td>
</tr>
<tr>
<td>CD44+</td>
<td>8 (36%)</td>
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<tr>
<td>EpCam+</td>
<td>0</td>
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</tbody>
</table>

NOTE: p values based on the Fisher exact test. Bold numbers indicate significant associations of mutational status with morphology or immune phenotypes, percentages refer to the total number of CTNNB1/TP53-mutated tumor areas; the predominant growth pattern of each tumor area was used for the analysis, cytologic features (if present) were additionally recorded.

Abbreviations: AFP, α-fetoprotein; EpCAM, epithelial cell adhesion molecule; GS, glutamine synthetase; nucl., nuclear positivity.

driver mutations in HCC, are not uniformly present in all tumor regions within the same tumor and reflect a rather late event in hepatocarcinogenesis. TP53 and CTNNB1 are both used as molecular classifiers for HCC (26, 37). For instance, the transcriptome classification proposed by Boyault and colleagues depicts 6 subgroups of HCC; 2 groups are associated with TP53 and 2 other groups with CTNNB1 alterations (37). The intratumor heterogeneity we found on the molecular and morphologic levels indicates a high degree of genetic instability in HCC. This stands in contrast to hepatocellular adenoma, for which a solid body of literature exists without evidence for relevant histologic heterogeneity (38, 39). Furthermore, on the basis of the few data available on genetic heterogeneity in hepatocellular adenoma (HCA) when progression to HCC was studied (40), it can be assumed, that adenoma are more homogeneous. The classification of HCA based on the clinical setting, morphology, and genetics is well accepted (6) and has an impact on the clinical management of patients with HCA. For HCC, classification proposals based on genetic changes (26), or gene expression patterns (41, 42), have not proven robust enough so far to be integrated into clinical practice. According to our data, intratumor heterogeneity obviously is an obstacle to the establishment of a comprehensive classification of HCC comparable with the HCA classification (6, 7). Distinct parts of one and the same tumor differ with respect to biomarker expression and mutational status, which illustrates the coexistence of different tumor cell populations in the same tumor. Presumably, the rate of genetic diversity would be even higher if the genetic analysis of individual tumor areas was expanded to analyze more genes.

Molecular analysis, based on testing a small piece of tumor, might underestimate the complexity of tumor genomics. According to our data which are based on analysis of multiple tumor regions, single tumor biopsies might be an insufficient approach to characterize individual HCC. It is conceivable that a significant proportion of HCC already harbors the same genetic complexity and molecular heterogeneity within one and the same tumor that is characteristic of HCC as a tumor entity (1, 43). This obviously impedes the development of a comprehensive and clinically meaningful HCC classification.

The level of intratumor heterogeneity we have demonstrated may result in evasion of therapy when targeting single molecules of hepatocarcinogenesis. In particular, the Wnt/β-catenin pathway is discussed as a potential therapeutic target in HCC (44). Because the efficacy of a molecular therapy is determined by the mutational status of the target, for example, EGF receptor (EGFR) in colorectal and lung cancer treatment or c-KIT in the case of gastrointestinal stromal tumors (GIST), an HCC harboring tumor subpopulations with variable CTNNB1 mutational status (notably before therapy) is a major challenge. Currently, treatment with the multitargeted tyrosine kinase inhibitor sorafenib is the only proposed molecular targeted therapy for HCC, although only a modest survival benefit was demonstrated in a multicenter European HCC trial (45) and even markedly lower benefit in a trial with Asian patients with HCC (46). The intratumor molecular diversity we have shown for the TP53 and CTNNB1 mutational status in a significant proportion of HCC suggests that the development of a molecular targeted therapy for HCC might be more challenging than for tumor entities with more uniform targetable genetic changes.

In summary, in a comprehensive analysis on the level of morphologic, immunohistochemical, and mutational status of TP53 and CTNNB genes, we show that intratumor heterogeneity is a frequent finding in HCC. This finding might be the reason for some of the challenges in developing a robust classification of HCC as well as a molecular targeted therapy for this tumor entity.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Heikenwalder, A. Weber
Development of methodology: J. Friemel, M. Rechsteiner, I. Frick, K. Struckmann, A. Weber
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Friemel, M. Rechsteiner, I. Frick, F. Böhm, M. Egger, H. Mocci, A. Weber
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Friemel, M. Rechsteiner, H. Mocci, M. Heikenwalder, A. Weber
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Rechsteiner, M. Egger, H. Mocci, A. Weber
Study supervision: A. Weber
Other (acquired funding for this study): A. Weber

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Juliane Friemel, Markus Rechsteiner, Lukas Frick, et al.


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