Clonal Origin of Multifocal Hepatocellular Carcinoma

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BACKGROUND: Hepatocellular carcinoma is the most common primary tumor of the liver. Patients frequently have multiple histologically similar, but anatomically separate tumors. The clonal origin of multiple hepatocellular carcinomas is uncertain. METHODS: The authors analyzed 31 tumors from 12 different patients (11 women, 1 man), who had multiple hepatocellular carcinomas involving 1 or both lobes. Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue using laser capture microdissection. DNA was analyzed for loss of heterozygosity (LOH), X chromosome inactivation status, and TP53 gene mutations. RESULTS: Ten (83%) of the 12 patients showed LOH in at least 1 of the analyzed microsatellite markers. Concordant LOH patterns between separate hepatocellular carcinomas in individual patients were seen in 8 (80%) of 10 cases, whereas discordant patterns were seen in 2 (20%) of 10 cases. Five (50%) of 10 informative female patients showed identical nonrandom X chromosome inactivation patterns in multiple tumors; 1 case showed discordant nonrandom X chromosome inactivation pattern. TP53 mutations were identified in 8 (67%) of 12 patients. Tumors in 7 (88%) of these 8 patients showed different point mutations. Three patients (Cases 4, 5, and 10) had tumors with additional TP53 point mutations, indicating additional genetic abnormalities in these tumors. CONCLUSIONS: The data suggested that the significant proportion of patients with multifocal hepatocellular carcinomas have tumors of common clonal origin.

KEYWORDS: liver, multifocal, clonality, loss of heterozygosity, TP53 mutation.

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and the third leading cause of cancer mortality worldwide. The incidence of HCC in the United States is rising, as individuals who contracted hepatitis C virus (HCV) infection approximately 4 decades ago develop cirrhosis with its attendant risks of HCC.1,2 Because no effective anticancer drugs exist for treatment of HCC, therapeutic options are limited by number, size, and location of tumors, which determine their resectability or access to interventional radiological techniques. It is thus pertinent that a significant number of patients present with multiple tumors and at an advanced stage.

The origin of multifocal hepatocellular carcinoma is uncertain, and cannot be determined solely by histologic features. However, previous studies have shown that clarification of the relationship among multiple tumors may be clinically important.3 Some investigators believe that multifocal hepatocellular carcinomas result from intrahepatic metastasis of a primary tumor, a view that is supported by the unique drainage system of the liver, frequent finding of vascular invasion, and histologically similar tumors. Satellite lesions were frequently observed surrounding the larger tumor lesion in our cases. Other investigators suggest that multifocal tumors in patients with chronic liver disease represent clonally independent tumors arising as a consequence of field effect. It is important to note that neither mechanism is mutually exclusive; both intrahepatic metastasis and field effect may play important roles in the pathogenesis of multifocal hepatocellular carcinoma. However, useful clinical information may be gained through detailed genetic characterization and comparison of clonality between separate tumors.

In this study, we analyzed loss of heterozygosity (LOH), X chromosome inactivation, and TP53 gene mutation status of 31 separate tumors from 12 patients. Our goal was to clarify the clonal relationships of multifocal HCC in these patients.

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MATeRIALS AND METHODS

Patients
This study included 12 patients with HCC who underwent hepatectomy or orthotopic liver transplantation between 1997 and 2003 at the participating institutions. None of the patients had received pretransplant adjuvant therapy. Tumors were diagnosed by light microscopy with criteria established for malignant tumors of the liver according to the World Health Organization classification system.4 Tumors were classified into 4 grades (well, moderate, poor, and undifferentiated/anaplastic) based on a modified Edmondson and Steiner grading system.4,5 All patients had multiple tumors involving 1 or both liver lobes. Tumors separated by at least 2 cm of intervening non-neoplastic tissue were considered to be multifocal. Cases were staged according to the 2002 American Joint Commission on Cancer TNM staging system.6 Cases were evaluated for background disease, tumor number, size, histology, grade, and vascular invasion. Elastin and CD31 stainings were performed to exclude the possibility of vascular involvement in small lesions. All patients were from North America. The Indiana University Institutional Review Board approved this study.

Tissue Samples and Laser Capture Microdissection
Formalin fixed, paraffin-embedded tissue was available in all cases. Serial 5-μm sections were prepared from formalin-fixed, paraffin-embedded tissue and stained with hematoxylin and eosin. Tumors and normal tissues were microdissected using a PixCell II Laser-Capture Microdissection system (Arcturus Engineering, Mountain View, Calif) as described previously (Fig. 1).7-10 Tissues were deparaffinized with xylene, rehydrated through 100%, 95%, and 70% ethyl alcohol, and light stained with hematoxylin and eosin. The microdissected cells were incubated overnight at 37°C in 50 μL of digestion buffer containing 10 nM Tris-HCL, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Tween 20, and 5 mg/mL of proteinase K (pH 8.3). Typical microdissection yields 600 to 1000 cells, and approximately 4 to 6 ng of genomic DNA is recovered.

LOH Analysis
Genomic DNA was amplified according to standard protocol. Primer sequences were obtained from the Genome Database. Approximately 100 to 200 pg of genomic DNA was used as template in the polymerase chain reaction (PCR) system. Four highly polymorphic microsatellite loci, which were previously shown to have LOH in HCCs,11,12 were selected for analysis: 9p21 (D9S161), 11q13 (D11S970), 9p21 (IFNA4, D9S171), and 17p13.1 (TP53). An α-32P-deoxyadenosine triphosphate incorporation PCR was used to ensure the highest sensitivity. Each PCR reaction was run with matching normal control DNA and negative controls. The PCR products were heat denatured for 3 minutes at 95°C and immediately chilled on ice before loading to a 6% polyacrylamide gel. Autoradiography was performed at −70°C, with exposure time as necessary.

Microsatellite banding patterns at each locus in control DNA were classified as homozygous/noninformative (showing only 1 allele) or heterozygous/informative (showing 2 different alleles).10,13 Noninformative cases were excluded from further analysis. Informative patients were divided into 2 groups: Group 1 was negative for LOH, in which the patient’s tumor and control DNA showed identical allelic patterns; Group 2 was positive for LOH, which was determined by comparison of band intensity of corresponding alleles from normal and tumor cells. LOH was defined as ≥75% loss of intensity of an allele band against normal control. Each reaction was repeated separately at least twice, and similar results were obtained.

Tumors from the same patient demonstrating concordant allelic loss patterns were considered to have a common clonal origin, whereas tumors with discordant allelic loss patterns were considered clonally independent.

X Chromosome Inactivation Analysis
X chromosome analyses were performed in the 11 female patients as previously described.9,14 Eight-microliter aliquots of DNA extract were digested with and without 1 Unit of HhaI restriction endonuclease (New England Biolabs, Beverly, Mass) in total volume of 10 μL for 2 hours. Control reactions for each tumor sample were incubated without HhaI. After incubation, 3 μL of digested or nondigested DNA was amplified in a 25-μL polymerase chain reaction volume containing 0.1 μL of α-32P-labeled deoxyadenosine triphosphate (3000 Ci/mmol), 4 mmol/L AR-sense and AR-antisense primers, 4% dimethyl sulfoxide, 2.5 mmol/L MgCl2, 300 mmol/L deoxynucleotide triphosphate, and 0.5 Unit of Taq DNA polymerase (Perkins-Elmer Cetus, Foster City, Calif). Primers used in the reaction were: AR-sense, 5’-TCC AGA ATC TGT TCC AGA GCG TGC-3’; and AR-antisense, 5’-GCT GTG AGT GTT CCT CAT-3’. PCR amplification was performed with an initial denaturation step of 95°C.
for 5 minutes followed by 38 cycles as follows: 95°C for 40 seconds, 63°C for 40 seconds, 72°C for 60 seconds, and the final extension step at 72°C for 7 minutes. The PCR products were then mixed with 4 µL of loading buffer containing 95% deionized formamide, 20 mmol/L EDTA, 0.05% bromophenol, and 0.05% Xylene Cyanole FF (Sigma Chemical Co., St. Louis, Mo). Samples were heated for 5 minutes at 95°C and chilled on ice. Three microliters of the PCR product was loaded onto a 6.5% polyacrylamide denaturing gel. The samples were separated by electrophoresis for 3 hours at 80 W, followed by autoradiography (Kodak Company, Rochester, NY) for 8 to 18 hours as necessary. Normal control samples were considered informative when 2 AR allele bands were

Figure 1. Multifocal hepatocellular carcinoma (HCC) (Case 7) is shown. (A) Laser microdissection of multifocal HCC is shown: (A1) the HCC lesion before microdissection and (A2) the lesion after microdissection. (A3) Laser captured cells are shown. (B) *TP53* gene mutation detected by direct sequencing is shown. (B1) Normal tissue is shown. (B2-4) Different foci of HCC demonstrated different mutations in codon 176 and codon 177 at exon 5. The mutated codons were underlined.
present after incubation with HhaI endonuclease. Further analysis was carried out only in informative cases. The nonrandom X chromosome inactivation pattern was defined as ≥75% of the band intensity of 1 allele was lost after HhaI digestion. Tumors from the same patient with identical allelic inactivation patterns were considered monoclonal, whereas independent clonal origin was suggested when tumors showed alternate allelic inactivation patterns.15,16

TP53 Gene Mutation Analysis
Sequencing of exons 5, 7, and 8 of the TP53 gene was performed according to described protocols.17,18 The amplified PCR products were sequenced using the dideoxynucleotide chain termination method on an automated sequencer by PCR-product sequencing kit. Identical TP53 mutations among tumors supported common clonal origin, whereas different TP53 mutations among tumors supported different clonal origin with the context of other genomic changes.

RESULTS
We studied a total of 31 separate tumors from the 12 patients. The clinicopathologic features are shown in Table 1. Eleven patients were women, and 1 was a man. The patients ranged in age from 25 to 68 years, with a mean age of 55 years. All 12 patients had synchronous tumors. Chronic liver disease was present in 10 (83%) of 12 patients, and 6 (60%) of these patients had cirrhosis. HCV infection was the main cause of chronic liver disease. None of the patients was hepatitis B virus positive. Tumors ranged in number from 2 to 4, and from 0.3 to 9.0 cm in diameter. All of the separate tumors from an individual patient had similar histology; 11 cases showed a trabecular pattern, and 1 patient had a fibrolamellar variant. Eleven cases were well or moderately differentiated; tumor was poorly differentiated in Case 6. Ten (83%) of the 12 cases had vascular invasion. Pathologic stages were as follows: pT2 in 2 (16%) cases, pT3 in 5 (42%) cases, and pT4 in 5 (42%) cases.

LOH analysis was performed in the 31 separate tumors. The analyzed microsatellite markers showed an informative rate of 11 (92%) of 12 at D9S161, D11S907, and IFNA and 12 (100%) of 12 at TP53. The frequency of allelic loss at each marker was the following: 45% at D9S161, 18% at D11S907, 55% at IFNA, and 42% at TP53 (Table 2). The number of microsatellites showing allelic loss ranged from 1 to 2 per tumor. Eight (80%) of the 10 cases demonstrated identical allelic loss patterns, suggesting a common clonal origin. The remaining 2 (20%) informative cases had discordant patterns, supporting an independent clonal origin. X chromosome inactivation was performed in the 11 female patients. Ten (91%) of the 11 patients were informative (Table 2). Five (50%) of the 10 informative female patients (Cases 2, 4, 5, 6, and 11) demonstrated a concordant pattern of nonrandom X chromosome inactivation, supporting a common clonal origin. One case (Case 10) showed alternate X chromosome inactivation, indicating an independent clonal origin. The remaining 4 cases (Cases 1, 7, 8, and 12) showed both alleles after methylation-sensitive restriction enzyme digestion (Fig. 2).

Direct DNA sequencing of exons 5, 7, and 8 of the TP53 gene was conducted in the 31 separate tumors. Point mutations were identified in 8 (67%) of 12 patients (Table 2) (Fig. 1). Tumors in 7 (88%) of these 8 patients showed different point mutations. Three patients

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### Table 1. Clinicopathologic Features of 12 Patients With Multifocal Hepatocellular Carcinoma

| Case No. | Age, y/Sex | Background Disease | No. of Tumors | Size of Largest Tumor, cm | Histologic Type | Histologic Grade | Vascular Invasion | Pathologic TNM Stage |
|----------|------------|--------------------|---------------|---------------------------|----------------|----------------|------------------|---------------------|
| 1        | 59/Woman   | CH                 | 2             | 1.6                       | Trabecular      | 2              | –                | II                  |
| 2        | 52/Woman   | C, HCV             | 2             | 2.2                       | Trabecular      | 2              | –                | III                 |
| 3        | 60/Man     | C                  | 2             | 2.8                       | Trabecular      | 2              | –                | III                 |
| 4        | 68/Woman   | H, C               | 2             | 2.8                       | Trabecular      | 2              | –                | III                 |
| 5        | 58/Woman   | CH                 | 2             | 2                         | Trabecular      | 2              | –                | II                  |
| 6        | 64/Woman   | C, HCV             | 2             | 4                         | Trabecular      | 3              | +                | III                 |
| 7        | 56/Woman   | C                  | 3             | 3                         | Trabecular      | 2              | +                | IV                  |
| 8        | 58/Woman   | CH                 | 4             | 9                         | Trabecular      | 2              | +                | IV                  |
| 9        | 60/Woman   | CH                 | 4             | 3                         | Trabecular      | 2              | +                | IV                  |
| 10       | 39/Woman   | N                  | 2             | 5                         | Trabecular      | 2              | +                | III                 |
| 11       | 65/Woman   | C                  | 3             | 3.5                       | Trabecular      | 1              | –                | IV                  |
| 12       | 25/Woman   | N                  | 3             | 8                         | Fibrolamellar   | 1              | –                | IV                  |

CH indicates chronic hepatitis, –, negative; C, cirrhosis; HCV, hepatitis C virus; +, positive; H, hemochromatosis; N, normal.
4, 5, and 10) also had tumors with additional \(TP53\) point mutations, indicating further accumulation of genetic abnormalities. Normal tissue did not harbor any \(TP53\) mutations in these exons.

**DISCUSSION**

In this study, we used a combination of molecular techniques, including LOH, X chromosome inactivation analysis, and \(TP53\) gene mutations by direct sequencing to elucidate the clonal relationship in patients with multifocal HCC. Multifocality is an important prognostic indicator in HCC; however, there is no consensus on whether multiple tumors are monoclonal in origin or arise independently. Several molecular markers have been used to access clonality in HCC. To our knowledge, this is the first study to use a combination of LOH, X chromosome inactivation, and \(TP53\) sequencing to investigate

**Table 2. LOH, X Chromosome Inactivation, and TP53 Mutation Analysis of Multifocal HCC**

| Case No. | Sex | Tumors | LOH Microsatellite Markers | X Chromosome Inactivation | TP53 Mutation Analyses |
|----------|-----|--------|--------------------------|---------------------------|------------------------|
|          |     |        | D9S161 D11S907 IFNA TP53  | Exon 5 | Exon 7 | Exon 8 |
| 1        | Woman | N | | | | |
| T1 | | | | | | |
| T2 | | | | | | |
| 2        | Woman | N | | | | |
| T1 | | | | | | |
| T2 | | | | | | |
| 3        | Man | N | | | | |
| F1 | | | | | | |
| F2 | | | | | | |
| 4        | Woman | N | | | | |
| T1 | | | | | | |
| T2 | | | | | | |
| 5        | Woman | N | | | | |
| T1 | | | | | | |
| T2 | | | | | | |
| 6        | Woman | N | | | | |
| T1 | | | | | | |
| T2 | | | | | | |
| 7        | Woman | N | | | | |
| T1 | | | | | | |
| T2 | | | | | | |
| T3 | | | | | | |
| 8        | Woman | N | | | | |
| T1 | | | | | | |
| T2 | | | | | | |
| T3 | | | | | | |
| T4 | | | | | | |
| 9        | Woman | N | | | | |
| T1 | | | | | | |
| T2 | | | | | | |
| T3 | | | | | | |
| T4 | | | | | | |
| 10       | Woman | N | | | | |
| T1 | | | | | | |
| T2 | | | | | | |
| 11       | Woman | N | | | | |
| T1 | | | | | | |
| T2 | | | | | | |
| T3 | | | | | | |
| 12       | Woman | N | | | | |
| T1 | | | | | | |
| T2 | | | | | | |
| T3 | | | | | | |

LOH indicates loss of heterozygosity; HCC, hepatocellular carcinoma; N, normal control; |, both alleles present; -, no mutation detected; T, different tumor focus; \(\uparrow\), loss of upper allele; \(\downarrow\), loss of lower allele; NI, noninformative.
Overall, 5 of 12 cases of multifocal HCCs (Cases 3, 5, 6, 9, and 11) showed unequivocal evidence of monoclonal origin. Abnormalities in the TP53 gene are common in a wide variety of malignancies, and recent studies suggest that prognosis in patients with HCC correlates to mutations of TP53. In the current study, point mutations in exons 5, 7, and 8 of the TP53 gene were identified in 8 (67%) of 12 patients. Seven (88%) of these 8 patients had different point mutations, and 3 patients (Cases 4, 5, and 10) had tumors with additional TP53 point mutations. One case (Case 10) showed alternate X chromosome inactivation and different TP53 mutations between tumors in both exon 5 and exon 8, further supporting independent clonal origin. In Case 8, there was no TP53 mutation detected in the tumors, although 1 tumor showed LOH with discordant allele loss patterns. This seemed to support the notion that compared with X chromosome inactivation and LOH, TP53 point mutations are a later event, reflecting genomic instability and a dynamic process of differentiation. This is helpful in assessing the clonality of multiple tumors. There appears to be an association between TP53 mutations and microvascular invasion in HCC. Our results supported this association, as vascular invasion was identified in all but 1 of our cases with TP53 mutations. Mutations of TP53 appeared to be a late event in hepatocarcinogenesis.

The combination of our results confirms that the origin of multifocal HCC is heterogeneous. Some patients with multifocal HCCs had independent clonal origin. Chen et al used hepatitis B virus DNA integration assay as a marker of clonality and found that approximately...
50% of multifocal HCCs represent independent (de novo) tumors. Nomoto et al used the methylation status of multiple tumor suppressor genes as clonal markers in 19 patients with multicentric and recurrent HCC. Those investigators determined that 74% of recurrent HCCs were independent lesions. The varied incidence of tumors with independent clonal origin may be related to variant population groups or geographical differences, because the major risk factors in Asia are different from those in North America.

Recently, Nakata et al analyzed gene expression profiles of multifocal HCC by microarray analysis, and found different gene expression patterns in some multcentric HCCs, suggesting independent origin. Field effect has been proposed in the pathogenesis of a wide variety of multifocal tumors, including clear cell renal cell carcinoma and neuroendocrine tumors of the enteropancreatic axis, urinary tract, and head and neck. Field carcinogenesis is associated with cancer stem cells in the affected field, which maintain themselves through self-renewal and propagate through differentiation; each cancer stem cell and its lineage possess a unique set of biological features genetically, epigenetically, and phenotypically. Clonal cancers are thought to represent clonal expansion of 1 or few cancer stem cells. Multifocal HCCs may represent the lineage of 1 cancer stem cell migrating within the affected field, giving rise to clonally identical tumors, or separate cancer stem cells distributed within the shared field, giving rise to clonally dissimilar tumors. This model may eventually lead to new understanding of tumor multifocality, for it expands the concept of carcinogenesis from a point to a field.

Clinically, determination of clonal origin may be important in staging patients with multiple HCCs. The current TNM staging system (2010 revision) assigns patients with a solitary tumor with vascular invasion or multiple tumors, none >5 cm, as pT2 stage. Patients with multiple tumors >5 cm or tumor involving a major branch of the portal or hepatic vein are considered pT3a or pT3b, respectively. Our findings may have important clinical implications, because independent tumors may behave less aggressively than tumors arising from biologically more aggressive tumors, which have acquired metastatic capacity. It is important to distinguish multiple tumors that arise independently from multiple tumors derived from intrahepatic metastasis (common clonal origin). Recent molecular studies of LOH suggest that fractional allelic imbalance may also provide useful information in addition to TNM stage.

Several caveats should be considered in the interpretation of our results. A discordant X chromosome inactivation and LOH pattern supports an independent clonal origin. Detection of discordant TP53 gene mutations in multiple tumors may also support independent clonal origin (for example, Case 7). In Case 12, there were no LOH, X chromosome inactivation, or TP53 mutations detected; therefore, this case was not informative of clonal status. Several other limitations to our study must also be considered. The numbers of cases available was relatively small. The panel of clonal markers we used may not be optimal in all patients. Specifically, X chromosome analysis only provides useful information in females, whereas HCCs have a male preponderance. We also recognize that molecular techniques such as DNA microarray analysis may provide further insights into this process. Nevertheless, we believe that the 3 different methods we used for determination of clonality (LOH, X chromosome inactivation, and TP53 gene sequencing) were adequate to study this important clinical question, and the data are supportive.

In summary, our findings suggest that a high percentage of patients with multifocal HCCs have common clonal origin. A better understanding of the genetic relationships between multiple tumors may be clinically important in assessing prognosis, and selecting therapeutic options in these patients.

CONFLICT OF INTEREST DISCLOSURES
The authors made no disclosures.

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