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Tumor Progression in Hepatocellular Carcinoma May Be Mediated by *p53* Mutation

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ABSTRACT

Human hepatocellular carcinoma (HCC) often contains intratumoral subpopulations of heterogeneous cellular differentiations within each tumor. To analyze the genetic alterations of *p53* in the heterogeneous subpopulations, we examined 68 intratumoral nodular lesions within 34 HCCs composed of two distinct subpopulations. The cellular differentiations were determined histologically by Edmondson's grading system. Nine (26.5%) of 34 HCCs examined were found to have genetic alterations in exons 5 to 8 of the *p53* gene, resulting in amino acid substitutions. Three of these nine HCCs with *p53* mutations showed genetic heterogeneity of the *p53* gene within each tumor; one HCC had a single missense mutation at codon 210 (asparginine to 210-serine) in an intratumoral lesion of Edmondson Grade II and double missense mutations at codons 210 and 217 (asparginine to 210-serine and valine to 217-alanine) in another intratumoral lesion of Edmondson Grade III. The remaining two HCCs had *p53* mutations only in lesions of a higher grade. In total, the *p53* mutations were detected in none of eight Edmondson Grade I lesions, in five of 29 Grade III lesions (17.2%), in eight of 26 Grade III lesions (30.8%), and in three of five Grade IV lesions (60.0%). Thus, our data revealed that the *p53* mutations were closely related to the progression of HCC and that, in certain cases, malignant cells which acquired the *p53* mutations might develop into dedifferentiated subpopulations within individual HCC.

INTRODUCTION

The sequential multistages of tumor progression have been analyzed, and they are often histologically associated with cellular dedifferentiation (1, 2). In the progression of these tumors, a subpopulation of less-differentiated cells may arise, grow within the tumors, and eventually display a heterogeneous appearance (2). The cellular differences within a single tumor are represented by the term "tumor heterogeneity" (3, 4). Recent investigations have suggested that such tumor progression might be related to multistep genetic alterations (5, 6). An analysis of the genetic alterations among the tumor heterogeneity of differentiation, therefore, should be appropriate to study their roles in tumor progression. Human HCC[2] also progresses with dedifferentiation and, interestingly, some HCCs form gross intratumoral lesions within tumors, which are typically characterized by a less-differentiated nodule surrounded by a well-differentiated nodule of HCC (8–10). In some cases of such HCC, intratumoral nodular lesions could be separated from the others within each tumor (10). Thus, HCC could be an appropriate model to study the relationship between some genetic alterations and cellular differentiation.

The *p53* gene is one of the tumor suppressor genes, and its mutations in the conserved regions, which span exons 5 to 8 (11), have been identified in numerous human carcinomas (12). Several reports have suggested that the *p53* mutations are suspected to be a late event in the multistep carcinogenesis of such malignancies as carcinomas of the colon (13), brain (7, 14), thyroid (15), and liver (16). However, there have been few reports on the genetic alterations of *p53* in cellular heterogeneity within a single tumor (7, 17). In this paper, we studied the mutation of the *p53* gene from demarcated intratumoral nodular lesions within HCC that, showed heterogeneous cellular differentiation. These results suggested that the mutation of *p53* might play a role in the progression of HCC to a less-differentiated subpopulation.

MATERIALS AND METHODS

Tissues and DNA Extraction. We examined 34 HCCs, which had formed distinct nodular lesions of identified heterogeneous differentiation within each tumor. All cases had been surgically resected in the Department of Surgery II, Kyushu University Hospital, between 1979 and 1991. The histological grade of tumor differentiation was then assigned according to the Edmondson grading system (18). These tissues were fixed within formalin, embedded in paraffin, and stained with hematoxylin-eosin. After microscopical identification, 102 specimens (68 intratumoral lesions from 34 HCCs and 34 noncancerous liver tissues surrounding HCC) were carefully dissected from paraffin-embedded tissues with a scalpel according to the histological diagnosis (Fig. 1). Then, the paraffin was removed from the samples with 1 ml of xylene, and the samples were washed with 100% ethanol and digested in 100 μl of lysis buffer [50 mM Tris-HCl (pH 7.5) containing proteinase K to a final concentration of 0.5 mg/ml] at 50°C for 72 h (19). After phenol-chloroform extraction, genomic DNA was precipitated with ethanol. All possible precautions were taken to avoid contamination.

PCR Primers. To carry out the PCR amplification of DNA from conserved exons 5 to 8 of the human *p53* gene, we used the following primers as essentially described by Hsu *et al.* (20):

exon 5 5′TGTTCACTTGTGCCCTGACT3′
5′CAGCCCTGTCGTCTCTCCAG3′
exon 6 5′TGGTTGCCCAGGGTCCCCAG3′
5′TTAACCCCTCCTCCCAGAGA3′
exon 7 5′AGGGGTCAGCGGCAAGCAGA3′
5′AGGCGCACTGGCCTCATCTT3′
exon 8 5′TTCCTTACTGCCTCTTGCTT3′
5′AGGCATAACTGCACCCTTGG3′

PCR-SSCP. PCR-SSCP was performed as previously described (21–23). Briefly, 1 μg of genomic DNA from paraffin-embedded tissues was amplified by PCR in 10 μl of a solution containing 10 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 1 mmol of each deoxyribonucleoside triphosphate, 0.5 μl of [α-^{32}P]dCTP, and 0.5 units of *Taq* DNA polymerase (Promega Co., Madison, WI), and then 30 cycles of amplification were carried out on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) under the following conditions: 1 min at 94°C (denaturation); 1 min at 58°C (annealing); and 1 min at 72°C (polymerization). For the SSCP analysis, 1 μl of each PCR product was heated at 90°C with 50 μl of the formamide dye mixture (95% formamide:20 mM EDTA:0.05% xylene cyanol:0.05% bromophenol blue), and then 1 μl of the preparation was electrophoresed on a 6% polyacrylamide gel with cooling by fans. The gel was then dried on filter paper and exposed to X-ray film at −80°C for 12 h with an intensifying screen.

Cloning and Sequencing. For the cloning of the *p53* gene, PCR was performed with a 10-fold volume of the PCR solution as described above, without [α-^{32}P]dCTP, using 1 μg of the genomic DNA. The amplified product was purified by electrophoresis using low-melting-point agarose. The purified product was then treated with 1 unit of polynucleotide kinase and 2.5 units of Klenow's fragment of *Escherichia coli* DNA polymerase I in 20 μl of solution and then ligated to the pUC118 vector. The sequence of DNAs from mixed recombinant colonies was determined for both of the strands by the dideoxy ribonucleotide chain termination method, using a Sequenase Version 2.0 kit (United States Biochemicals, Cleveland, OH).

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[2] The abbreviations used are: HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

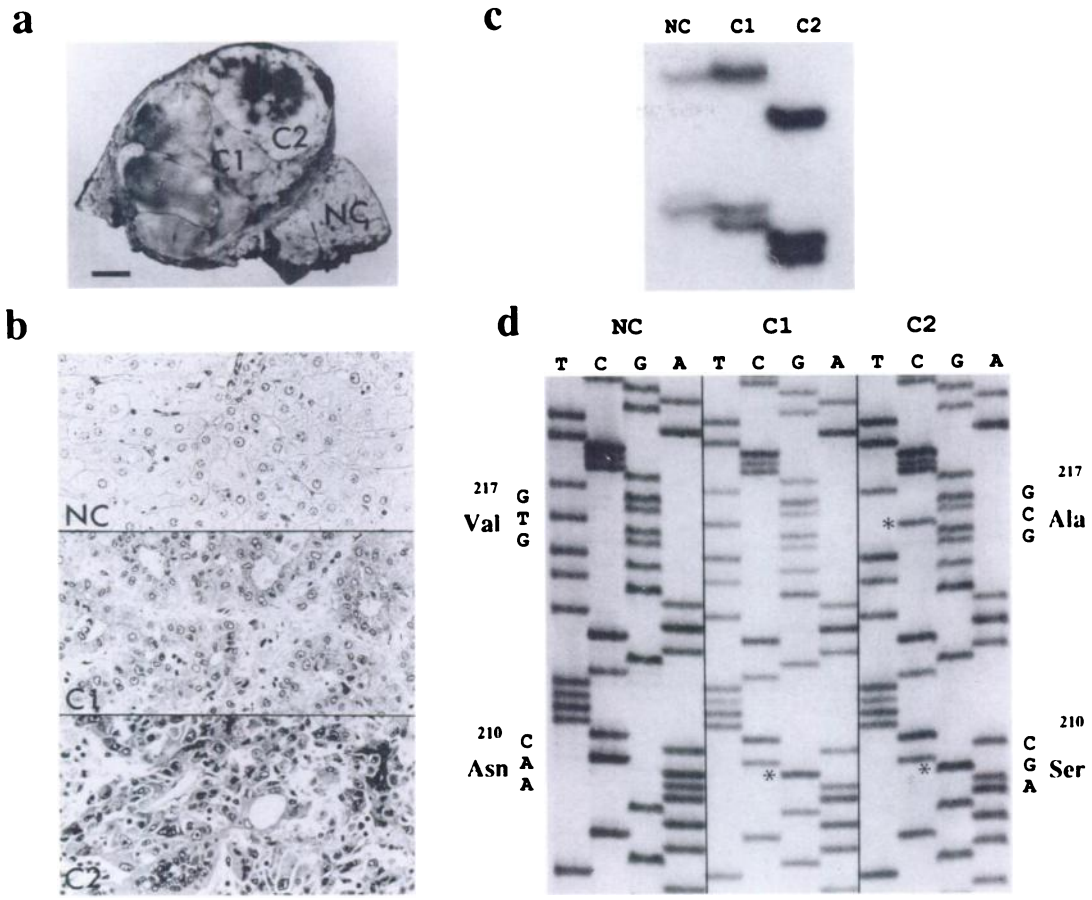

Fig. 1. An example of human HCC containing intratumoral nodular lesions within a single tumor in a partially resected liver (Case 1). *a*, gross view. Distinct intratumoral nodular lesions *C1* and *C2* are observed within a single HCC encapsulated. *NC*, noncancerous liver tissue surrounding HCC. *Bar* = 1.0 cm. *b*, histological view. *NC*, hepatocytes in the liver tissues surrounding HCC showing no cellular abnormalities. *C1*, the lesion composed of malignant cells, Edmonson's Grade II, that are grading in a trabecular and pseudoglandular pattern; *C2*, the lesion composed of malignant cells, Edmondson's Grade III, proliferating in a trabecular pattern. Bizarre giant cells are frequently seen. H & E, × 160. *c*, PCR-SSCP analysis of exon 6 in the *p53* gene from distinct areas. A heterogeneous migration pattern between the intratumoral lesions is shown in SSCP. *d*, DNA sequence analysis of the *p53* gene. The point mutations are indicated by an *asterisk*. While *C1* contained a single mutation at codon 210 (asparginine to 210-serine; *middle lane*), *C2* contained double mutations at codons 210 and 217 (asparginine to 210-serine and valine to 217-alanine; *right lane*), compared to the DNA sequence of *NC* (*left lane*).

RESULTS

PCR-SSCP Analysis. In order to study the mutation of the *p53* gene in HCCs with heterogeneous lesions within each tumor, we first performed a PCR-SSCP analysis on exons 5 to 8 of the gene. The results revealed that 10 of 34 HCCs showed abnormal migration patterns in one of the examined *p53* exons in either or both of the two intratumoral lesions. Three of these ten HCCs demonstrated heterogeneous patterns (Cases 1, 2, and 3; Table 1), and the remaining seven HCCs represented homogeneous patterns between intratumoral lesions (Cases 4, 5, 6, 7, 8, 9, and 10; Table 1). An example of HCC showing heterogeneous SSCP patterns is demonstrated in Fig. 1*c* (Case 1).

DNA Sequence Analysis. We determined the DNA sequences of the amplified products of the *p53* gene that showed aberrant migration patterns in SSCP analysis. The results were summarized in Table 1. All of the 10 cases with aberrant SSCP migration patterns contained point mutation. All but one (Case 5) of these point mutations caused amino acid substitutions by missenses. Case 5 had a silent mutation at codon 189 in exon 6, GCC to GCT (alanine). Thus, the *p53* mutations that caused the amino acid changes were found in 9 (26.5%) of the 34 HCCs examined. Three of these nine HCCs showed genetic heterogeneity of *p53* within each tumor (Cases 1, 2, and 3); Case 1 contained a single mutation at codon 210 (asparginine to 210-serine) in one intratumoral lesion of Edmondson's Grade II and double mutations in the other intratumoral lesion of Edmondson's Grade III, *i.e.*, the same mutation at codon 210 (asparginine to 210-serine) and an additional mutation at codon 217 (valine to 217-alanine), as shown in Fig. 1*d*. Case 2 contained a mutation at codon 272 (valine to 272-alanine), and Case 3 contained a mutation at codon 286 (glutamic acid to 286-aspartic acid) in only intratumoral lesions of higher histological grade but no mutations in the other intratumoral lesions of lower histological grade within each tumor. The data suggested that subpopulations which acquired *p53* mutations were able to progress with intratumoral dedifferentiation. The loss of the wild-type *p53* gene in the remaining 6 HCCs represented the same mutations between the intratumoral lesions. Interestingly, an AGG to AGT mutation at codon 249 resulting in arginine to serine substitution was recognized in three HCCs (Cases 7, 8, and 9). This mutation, arginine to 249-serine, was reported to be related to the exposure of aflatoxin B_1, one of the risk factors of HCC (20, 24, 25). Other amino acid alterations were recognized as arginine to serine at codon 175 (exon 5) in Case 4, methionine to isoleucine at codon 246 (exon 7) in Case 5, and aspartic acid to asparginine at codon 281 (exon 8) in Case 10. In these 6 cases which showed the same mutations in both of the lesions, the loss of the wild-type *p53* gene was observed in all HCC lesions (Table 1). There were no detectable abnormalities of the *p53* gene in the surrounding noncancerous liver tissues in any of the examined cases, compared with the published DNA sequence (26).

Table 1 *p53 gene mutations in hepatocellular carcinomas*

Case	Grade[a]	Loss of wild type[b]	Codon	Nucleotide change	Amino acid substitution
1	II	+	210	AAC to AGC	Asparginine to serine
	III	+	210	AAC to AGC	Asparginine to serine
			217	GTG to GCG	Valine to alanine
2	II	-	Wild type		
	III	+	272	GTG to GCG	Valine to alanine
3	I	-	Wild type		
	II	-	286	GAA to GAC	Glutamic acid to aspartic acid
4	III	+	175	CGC to AGC	Arginine to serine
	IV	+	175	CGC to AGC	Arginine to serine
5	I	-	189	GCC to GCT	Silent
	II	-	189	GCC to GCT	Silent
6	III	+	246	ATG to ATC	Methionine to isoleucine
	IV	+	246	ATG to ATC	Methionine to isoleucine
7	II	+	249	AGG to AGT	Arginine to serine
	III	+	249	AGG to AGT	Arginine to serine
8	II	+	249	AGG to AGT	Arginine to serine
	III	+	249	AGG to AGT	Arginine to serine
9	III	+	249	AGG to AGT	Arginine to serine
	IV	+	249	AGG to AGT	Arginine to serine
10	II	+	281	GAC to AAC	Aspartic acid to asparginine
	III	+	281	GAC to AAC	Aspartic acid to asparginine

[a] The histological grade of tumor differentiation was assigned according to the grading system of Edmondson and Steiner (18).
[b] The loss of wild-type allele at *p53* was determined by the PCR-SSCP analysis and the sequencing of the mixed DNAs from pooled PCR clones, as described in the text.

Relationship between *p53* Mutations and Histological Grades of HCC. We summarize the relationship between the *p53* mutations and the differentiation grade of the 68 nodular lesions from 34 HCCs in Table 2. The *p53* mutations were detected in none of 8 lesions classified as Edmondson's Grade I, in 5 of 29 lesions of Edmondson's Grade II (17.2%), in 8 of 26 lesions of Edmondson's Grade III (30.8%), and in 3 of 5 lesions of Edmondson's Grade IV (60.0%). Thus, the frequency of *p53* mutations in HCC lesions increased in close relation to the grade of histological differentiation.

DISCUSSION

Tumor cells are known to be less stable genetically than normal cells, and this instability leads to the development of more mutations in tumor cell genes which are associated with possible regulators of the cell cycle (4). The accumulation of acquired genetic mutations in the cells populating a tumor causes its progression to the more malignant state (2). Our study demonstrated that the frequency of mutations in the *p53* tumor suppressor gene of HCC nodular lesions increased with the grading of cellular dedifferentiation. Several histological studies have confirmed that the tumor progression of HCC, including recurrent tumor, is attributable to cellular dedifferentiation (8–10, 27, 28). Our data, therefore, suggested that the increase of *p53* mutations is associated with the tumor progression of HCC.

The increased genetic instability could produce new subpopulations within the progressing tumor to form clonal heterogeneity. Tumor heterogeneity has been recognized by various characteristics, such as cellular morphology, tumor histology, growth rate, cell products, receptors, enzymes, immunological characteristics, metastatic ability, and sensitivity to therapeutic agents (3, 4, 29). In certain HCCs, the heterogeneity within a single tumor represents distinct areas of heterogeneous differentiation (8–10). In this study, 3 of 34 cases of HCC containing different subpopulations also showed molecular heterogeneity of the *p53* gene within each tumor. In addition, the acquisition or accumulation of *p53* mutations was detected in the lesions with a higher differentiation in these three cases. Our results first suggested the possibility that, in certain HCC cases at least, a subclone which has acquired the *p53* mutation might progress to the lower differentiated cell population and, thus, result in the formation of tumor heterogeneity. Clinical reports have suggested that poorly differentiated cells might grow more rapidly than well-differentiated ones in HCC (30). HCC progression could be associated with a clonal expansion of cells which have acquired a mutation in the *p53* gene.

Table 2 *p53 mutations and histological differentiations of HCC lesions*

Grade[a]	No. of lesions or mutations/total
I	0/8 (0)[b]
II	5/29 (17.2)
III	8/26 (30.8)
IV	3/5 (60.0)

[a] The histological grade of tumor differentiation was assigned according to the grading system of Edmondson and Steiner (18).
[b] Numbers in parentheses, percentage.

Recently, the significance of *p53* mutations has been emphasized in the carcinogenesis of numerous human malignancies (12). Mutations in the *p53* gene have two unique aspects relating to carcinogenesis. The mutated *p53* genes fail to act as a tumor suppressor gene since wild-type *p53* can suppress the growth of carcinoma cells (31, 32). Otherwise, a mutant *p53* can act as an oncogene which is able to immortalize some cells and to cooperate with the activated *ras* oncogene to transform rat cells (33, 34). This oncogenic potential is recognized as acting in a "dominant negative" manner, without either loss or mutation of the other allele (35). Its transforming activity is not equal among the various types of *p53* mutations (36); for example, a mutant of arginine to 273-histidine cooperates with activated *ras* to transform cells from 3 to 10 times less than that of arginine to 175-histidine does (12). Bischoff *et al.* (37) proved that the former is recessive but the latter is dominant to wild-type *p53* in a system using fission yeast, and they also explained that the transforming activity of mutant *p53* is reflected by its dominance to wild-type *p53*. However, the oncogenic activities of the various kinds of *p53* mutants have not

yet been thoroughly studied. Therefore, the oncogenic activities of the mutated *p53* detected in tumor should be individually assessed.

In addition, we demonstrated one HCC (Case 1) carrying double missense mutations in the lower differentiated lesion. In several reports, double missense mutations of *p53* have been detected in individual tumors (7, 11) or cell lines (21). In Case 1, because the allele of the wild-type *p53* gene was lost in their HCC lesions, the double mutations would thus be found in the remaining allele. If the accumulation of *p53* mutations may be closely associated with clonal expansion, then there is a possibility that the additional mutation in *p53* might increase the progressive potential composed of a single mutation. However, the significance of these double mutations still remains to be clarified.

Our study has suggested that *p53* mutations might play a role in clonal expansion within the HCC tumor. Further study of the transforming activity of the individual *p53* mutations, detected here, including double mutations, may eventually help us clarify the role of the mutation of *p53* in the progression of malignancies.

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