Aberrations of the Tumor Suppressor p53 and Retinoblastoma Genes in Human Hepatocellular Carcinomas

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ABSTRACT

Aberrations of the p53 gene in 43 primary hepatocellular carcinomas (HCCs) were examined by single-strand conformation polymorphism analysis of polymerase chain reaction products. Of these hepatocellular carcinomas, 22 were advanced HCCs, and 21 were early HCCs. Structural abnormalities of the p53 gene were observed in eight of the 22 advanced HCCs, but in none of the early HCCs. Of the eight tumors with an abnormal p53 gene, seven had lost one of the two p53 alleles and, in the seventh tumors with identifiable mutations, point mutations were found in four tumors and deletions of several nucleotides were observed in two tumors. The remaining one retained both alleles and carried two point mutations. In addition to the aberrations of the p53 gene, loss of the retinoblastoma gene or loss of heterozygosity at chromosome 13q was observed in six of seven informative cases of eight tumors carrying a mutated p53 gene. These results suggest the involvement of at least two tumor suppressor genes in a late stage of hepatocarcinogenesis.

INTRODUCTION

HCC is one of the most common human tumors throughout the world and particularly in certain areas of Africa and Asia including Japan (1, 2). Epidemiological studies have indicated the carrier state of hepatitis B virus or intake of aflatoxin B1 as possible causative agents (3), but the molecular mechanisms of development of HCCs are still unclear.

Recently, many studies have shown multiple genetic changes in cancer cells, including activation of oncogenes, loss of distinctive chromosomal regions, and mutation of tumor suppressor genes (4). However, aberrations of oncogenes including the ras genes have been found in only a small proportion of HCCs (5). On the other hand, frequent allelic losses at loci on chromosomes 4q, 16q, and 17p have been reported (6-8), suggesting the involvement of mutations of tumor suppressor genes in development of HCC.

One of the tumor suppressor genes, p53, which is located on chromosome 17p, has been shown to be mutated in a wide variety of human tumors (9, 10) including HCC (11, 12). Most of the mutations of the p53 gene so far reported were loss of one allele and a subtle structural change of the remaining allele (9, 13). These two types of aberrations can be detected simultaneously by PCR-SSCP, a method that we developed recently (14, 15).

Therefore, in this study, we used this method to analyze DNAs from surgical specimens of HCC and detected mutations of the p53 gene in 8 of 22 cases of advanced HCC but in none of the cases of early HCC examined. We also found abnormal...
COABERRATIONS OF THE p53 AND RB GENES IN HUMAN LIVER TUMORS

Table 1 Primers used for amplification of the p53 and RB genes

<table>
<thead>
<tr>
<th>Amplified fragment</th>
<th>Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 gene</td>
<td>A</td>
<td>5' TGGAT CTTTC CTCAG CAGCC 3'</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5' CAGTG GTACCC ACTCAC CAGTT 3'</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>5' AACCT GTTTC CCTAC CAGAA 3'</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>5' ACCTA CAGTC CCCCT TGGCG 3'</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>5' GCAAC TGACC GTGCA AGTCA 3'</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>5' GCAAC TGACC GTGCA AGTCA 3'</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>5' ATGG TGCAAG CACAG CACAG 3'</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>5' GGCTC CTGAC CTGGA 3'</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>5' TTTC AGTCC TGAAG CCGGC 3'</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>5' TGGCG GTCTG CTAGG TTGGC 3'</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>5' GCAAC TGACC GTGCA AGTCA 3'</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>5' ATGG TGCAAG CACAG CACAG 3'</td>
</tr>
<tr>
<td>RB gene</td>
<td>25U</td>
<td>5' ATGGA AAGAG CTCTA GTTAC 3'</td>
</tr>
<tr>
<td></td>
<td>25D</td>
<td>5' TAAAG AAGAG CTCTA GTTAC 3'</td>
</tr>
</tbody>
</table>

has been reported by Buchman et al. (22). The fragments with Mobilities 1 and 2 carried a proline (CCC) and an arginine (CGC) codon, respectively, at position 72. The polymorphism revealed that Patients 8, 3, 1, 5, and 7 (Fig. 2a, Lanes JN, 2N, 3N, 5N, and 7N, respectively, at codon 72. The polymorphism (CAT) at position 49 and a proline codon at position 72, while the other had an aspartic acid codon (GAT) at position 49 and an arginine codon at position 72.

In addition to the loss of one allele, the tumor from Patient 7 showed a mobility shift of Fragment C from the remaining allele (Fig. 2a, Lane 7C). This shift suggested the presence of a mutation in exon 4 of the gene.

The mobility of the separated strands of Fragment C from Patient 8 was slightly different from those of others (Fig. 2a, Lane 1). Nucleotide sequence analysis revealed that this difference was due to a second nucleotide substitution in codon 49, as shown in Fig. 3a, in addition to the DNA polymorphism in codon 72 described above. One allele carried a histidine codon (CAT) at position 49 and a proline codon at position 72, while the other had an aspartic acid codon (GAT) at position 49 and an arginine codon at position 72.

Aberrations were also observed in Fragments D (Fig. 2b) and F (Fig. 2c), carrying the regions of exons 5 and 7, respectively. The signal corresponding to the normal sequence was greatly reduced in the fragment from tumors of Patients 13 (Fig. 2b, Lane 1C), 2, and 5 (Fig. 2c, Lanes 1C2 and 2C2, respectively), suggesting loss of one p53 allele. The mobility shift of fragments from the remaining allele in these tumors indicated the presence of a subtle structural change in exon 5 of the gene in the tumor from Patient 13 and in exon 7 in the tumors from Patients 2 and 5. The livers of Patients 2 and 5 contained multiple tumors, but some of these multiple tumors did not show the mobility shift of Fragment F described above (Fig. 2c, Lanes 1C1 and 2C1). The results indicated that different genetic events occurred independently in these tumors in the same liver.

Nucleotide Sequence Analysis of the Remaining Allele of the p53 Gene. To elucidate the structural changes causing the observed mobility shifts, we determined the nucleotide sequences of the fragments showing mobility shifts. Results for Fragment C from Patient 7, Fragment D from Patient 13, and Fragment F from Patients 2 and 5 are shown in Fig. 3, b, c, and d, respectively. Comparison of the sequence ladders of Fragment C of DNAs from the tumor and normal tissue of Patient 7 revealed that, in the tumor DNA, the arginine codon (CGT) at position 110 in exon 4 of the p53 gene was mutated to a cysteine codon (TGT) by a C to T transition at the first letter of the codon (Fig. 3b). Mutation of the 110th codon, which is not conserved among species, has not been reported previously.

Nucleotide sequence analyses of Fragments F from Patients 2 and 5 (Fig. 3d) clearly demonstrated mutation of the methionine codon (ATG) to a valine codon (GTG) at position 246 in exon 7 of the p53 gene and mutation of the glycine codon (GGC) to a serine codon (AGC) at position 244, respectively. These two positions are within regions of the p53 protein that are conserved among species.

Fig. 3c shows that the mobility shift observed in Fragment D in the tumor from Patient 13 was not due to a nucleotide substitution but to deletion of 31 base pairs from codon 168 to 178 in exon 5. The mutation resulted in deletion of 10 amino acids and a shift of the reading frame of the p53 gene.

Fragments A to H from other HCCs were similarly analyzed, and the aberrations of the p53 gene observed in 8 of 43 HCCs including those described above are summarized in Table 2. In 6 of 8 tumors with the mutated p53 gene, loss of one of the two alleles and a point mutation or deletion of a short nucleotide sequence in the remaining alleles were observed, while no mutation was detected in at least exons 2 to 11 in the remaining allele of the tumor from Patient 1 (Table 2). In Patient 19, both alleles of the p53 gene were retained, and two mutations, one in exon 5 and one in exon 8, were detected (Table 2). We did not determine which each allele had one mutation. In addition to these mutations, two polymorphic nucleotide substitutions, one of which was described above (Figs. 2a and 3a), were newly found and are indicated in Table 2.

Aberrations of the p53 Gene in Advanced HCCs. As shown in Table 3, 21 of the 43 HCCs analyzed were early HCCs with very well-differentiated histology. No aberration of the p53 gene was observed in these early HCCs. Of the 22 advanced HCCs, 3, 11, and 8 were well, moderately, and poorly differentiated, respectively. The mutations of the p53 gene detected were all in these advanced HCCs, 4 in moderately differentiated (36%) and 4 in poorly differentiated (50%) HCCs, indicating the involvement of aberrations of the p53 gene in less differentiated and advanced tumors (Table 3). Grouping the HCCs analyzed according to size indicated that almost all the aberrations of the p53 gene were present in tumors of more than 3 cm in diameter (7 of 19; 37%). The mutation was less frequent in tumors of 2 to 3 cm in diameter (one of 6; 14%) and was not found in 18 tumors of less than 2 cm in diameter. These results also indicated the involvement of p53 gene mutations in advanced HCCs.

No relationship of aberrations of the p53 gene with the age or sex of patients or with chronic liver disease was observed.

Aberrations of the RB Gene in HCCs. We also analyzed aberrations of another tumor suppressor gene, RB, in HCCs. Recently, Yandell and Dryja (23) reported a nucleotide sequence polymorphism of a T to A transversion in intron 25 at a position 10 base pairs upstream from the 5' end of exon 26 of the RB gene. We tested for loss of an RB allele as the absence of this polymorphism detected by the PCR-SSCP method. Representative results are shown in Fig. 4. Mobility shift due to the polymorphism is obvious in slower moving strands of the fragment carrying intron 25, and Patients 9, 1, 5, and 13

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are heterozygous (Fig. 4, Lanes 1 to 4, respectively). Loss of heterozygosity was observed in tumors from Patients 1, 5, and 13 (Fig. 4, Lanes 2C, 3C2, and 4C, respectively), indicating loss of one of the RB alleles in these tumors. Loss of heterozygosity at the RB locus was observed in 4 of 21 informative cases of HCCs by this analysis. Tumor DNAs were also subjected to RFLP analysis using D13S1 DNA as a probe, and loss of heterozygosity on chromosome 17p was observed in two other tumors (data not shown). Including these two tumors, loss of heterozygosity at the RB locus was detected in 6 of 7 informative cases of 8 tumors with a mutated p53 gene, but not in 17 informative cases without a p53 gene mutation as summarized in Table 2. These results suggested that mutations in both the p53 and RB genes were involved in development of HCCs.

DISCUSSION

Aberrations of the p53 gene include deletion of the whole gene and subtle structural changes of the gene. As described previously, PCR-SSCP analysis can detect these two aberrations simultaneously (12, 24). Therefore, we used this method for detecting aberrations of the p53 gene in HCCs and found aberrations in 8 of 22 advanced HCCs, but not in any of the 21 early HCCs examined. As SSCP analysis might miss some mutations, the number observed is a minimum estimate for frequency of mutation. Of the 8 tumors with aberrations of the p53 gene, 7 had lost one of the two alleles. The remaining one retained both alleles and carried two point mutations, although it remained to be determined whether each allele had one point mutation. Of the 7 tumors in which one p53 allele was lost, point mutations in the remaining alleles were observed in 4 tumors, and deletions of several nucleotides were observed in two others. The seventh tumor did not show a mobility shift in any fragment analyzed, suggesting that a mutation, if present, was in a region outside the coding sequence.

All 6 point mutations detected in this study resulted in amino acid substitutions, and 5 of the 6 mutations were located in regions of the gene that are highly conserved among species (9). One point mutation was found in codon 110 (Table 1). In this codon, a C to T transition of the first letter resulted in the replacement of arginine by the remotely related amino acid cysteine. The amino acid at this position is not conserved among species. It is arginine in humans (25), histidine in mice (26), and glutamine in Xenopus (27). An amino acid change in a nonconservative region of the p53 gene is rare in a variety of human tumors, but the mutation must be oncogenic.

The deletions of one and 31 nucleotides found in this study resulted in shifts of the reading frame. The deletion of one nucleotide (Table 2) resulted in a frame shift in the C-terminal region of the p53 protein with alteration of the last 12 amino acids to a polypeptide sequence of 22 amino acid residues. The role of the C-terminal region of the p53 protein is still unknown. The deletion of 31 nucleotides with shift of the reading frame (Table 2) resulted in a gross change of the amino acid sequence
of the p53 protein downstream from position 168 and created a stop codon in exon 7. This mutation should result in a truncated protein of 244 amino acid residues.

A G to T transversion at the third letter of codon 249 of the p53 gene causing replacement of arginine by serine has been observed at high frequency in HCCs of patients in China and southern Africa, where aflatoxin B₁ is a risk factor (28, 29). We detected the same mutation in codon 249 in the human hepatocellular carcinoma cell line, PLC/PRF/5, by the PCR-SSCP method with the same set of primers used in the present study (12). However, altered amino acids were scattered over evolutionary conserved and nonconserved domains of the p53 pro-

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Mutations</th>
<th>Loss of the p53 gene</th>
<th>Codon</th>
<th>Nucleotide alteration</th>
<th>Amino acid alteration</th>
<th>Loss of the RB gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced hepatocellular carcinoma</td>
<td>1C</td>
<td>+</td>
<td>246</td>
<td>ATG to GTG</td>
<td>Met to Val</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2C2</td>
<td>+</td>
<td>244</td>
<td>GCC to AGC</td>
<td>Gly to Ser</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5C2</td>
<td>+</td>
<td>110</td>
<td>CGT to TGT</td>
<td>Arg to Cys</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7C</td>
<td>+</td>
<td>381–382</td>
<td>1-base pair deletion</td>
<td>Frame shift</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>9C</td>
<td>+</td>
<td>168–178</td>
<td>31-base pair deletion</td>
<td>Frame shift</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>13C</td>
<td>–</td>
<td>175</td>
<td>GCC to AGC</td>
<td>Arg to Ser</td>
<td>NI*</td>
</tr>
<tr>
<td></td>
<td>19C</td>
<td>–</td>
<td>227</td>
<td>GCC to GAC</td>
<td>Lys to Asp</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>32C</td>
<td>+</td>
<td>276</td>
<td>GTG to ATG</td>
<td>Val to Met</td>
<td>+</td>
</tr>
<tr>
<td>14 other tumors</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Early hepatocellular carcinoma</td>
<td>All 21 tumors</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
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</table>

Polymorphic substitutions

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Nucleotide alteration</th>
<th>Amino acid alteration</th>
<th>Loss of the RB gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>8N</td>
<td>CAT to GAT</td>
<td>Asp to His</td>
<td>–</td>
</tr>
<tr>
<td>18N</td>
<td>GCC to GTC</td>
<td>Ala to Val</td>
<td>–</td>
</tr>
</tbody>
</table>

*ND, not detected; NI, not informative.
+ Loss of the RB gene was detected by RFLP analysis.
- Loss of the RB gene was not observed in 7 informative cases.
NI Loss of the RB gene was not observed in 10 informative cases.
In this work we found a novel nucleotide substitution in codon 49 in DNA from noncancerous tissue of a patient (Patient 8 in Fig. 3a and Table 1). This patient did not show any other aberration of the p53 gene in the tumor DNA except this substitution. For the following reasons, we tentatively propose that this polymorphism is a germinal mutation causing pro- nelessness to cancer. (a) The amino acid of codon 49 is relatively well conserved among species. It is aspartate in humans (25) and mice (26), glutamate in chickens (34), and asparagine in Xenopus (27), but the nucleotide substitution in this patient resulted in its replacement by a completely different amino acid, histi- dine. (b) An allele with a histidine codon at position 49 is very rare, not being observed in DNAs from the 84 individuals analyzed, including patients with HCCs, cholangiocarcinomas, melanomas, and several other tumors and normal individuals. (c) The patient had a family history of ovarian and breast cancers. The segregation of this allele in this family remains to be analyzed.

**REFERENCES**

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