Role and Mutational Heterogeneity of the p53 Gene in Hepatocellular Carcinoma

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ABSTRACT

The mutational spectrum of the p53 gene was analyzed in 53 hepatocellular carcinomas. Somatic mutations of the p53 gene were detected in 17 cases (32%). Among these 17 mutations, 9 were missense mutations; the other 8 cases were nonsense mutations, deletions, or mutations at the intron-exon junctions. These mutations were found in a wide region stretching from exon 4 to exon 10 without any single mutational hot spot. G:C to T:A transversions were predominant, suggesting the involvement of environmental mutagens in the mutagenesis of the p53 gene in a subset of the hepatocellular carcinoma cases. Mutations of the p53 gene occurred frequently in advanced tumors, although several tumors in the early stages also showed mutations. A deletion map of chromosome 17 was constructed by using 10 polymorphic probes and was compared with the p53 gene mutation in each case. Loss of heterozygosity (LOH) on chromosome 17p was observed in 49% of the cases (24 of 49), and two commonly deleted regions were detected (around the p53 locus and at 17p13.3 to the telomere). Sixteen of the 17 cases with p53 gene mutations showed LOH around the p53 locus, and mutations were rare in hepatocellular carcinomas without LOH. However, no mutations were detected in 8 cases with LOH on 17p, suggesting the possibility that an unidentified tumor suppressor gene(s) located on 17p may have also been involved in hepatocarcinogenesis.

INTRODUCTION

Increasing evidence has supported the fact that the p53 gene acts as a tumor suppressor gene (1), and mutations of the p53 gene are frequently found in a variety of cancers (2). Bressac et al. (3) reported an abnormal structure and expression of the p53 gene in HCC derived cell lines. In addition, a mutational hot spot at codon 249 of the p53 gene was found in patients in China and South Africa, which was considered to be closely associated with dietary aflatoxin B1 intake (4–6). In contrast, no such mutational hot spot has been reported thus far in Japanese HCC cases (7, 8), suggesting the involvement of different etiological factor(s). Therefore, an intensive analysis with a large number of samples is required to understand the mutational mechanism underlying the p53 gene during hepatocarcinogenesis.

A study on colorectal carcinomas demonstrated that mutations of the p53 gene were closely associated with LOH on 17p, and vice versa (9). We have also reported that the LOH on 17p in HCCs was strongly correlated with the aggressiveness of the tumor (10), suggesting a role for p53 gene inactivation in the progression of HCCs.

Several recent studies, however, have shown that the LOH on chromosome 17p was not necessarily associated with mutations of the p53 gene in tumors such as malignant gliomas, ovarian cancers, and neuroectodermal tumors (11–13). Allelotypic studies on HCCs and breast cancers have also revealed a common deleted region other than the p53 locus, which suggests that an unidentified tumor suppressor gene might be located on 17p (14, 15). Furthermore, other tumor suppressor genes such as NF-1, nm23, and the prohibitin gene were mapped to the proximal region of chromosome 17q (16–18). Therefore, it may be interesting to compare mutations of the p53 gene with the deletion map of chromosome 17 obtained from the same HCC samples.

In this present study, we have investigated the mutational profile of the p53 gene in 53 Japanese HCC cases by PCR-SSCP analysis and by direct genomic sequencing over the entire coding region, and compared these mutations with several clinical parameters. We also deletion mapped chromosome 17 by using 10 RFLP probes in the same tumor samples, and analyzed the relationship between mutations of the p53 gene and LOH.

MATERIALS AND METHODS

Samples. We used 53 HCC patients, with their informed consent, in this study. The patients underwent surgery at Kyoto University Hospital, and the tumors and surrounding noncancerous tissues were frozen immediately after surgical removal and stored at −80°C until the DNA isolation. The clinical stage of each patient was determined according to the classification scheme in the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (19). Histological studies were performed at the Clinical Pathology Department of the hospital, and histological grades were assigned according to Edmondson’s grading system (20). Among the cases analyzed, 40 were males and 13 were females. Furthermore, 7 patients were positive for serum hepatitis B virus surface antigen, and 46 were negative. Eight cases were at clinical stage I, 7 were at stage II, 15 were at stage III, and 23 cases were at stage IV.

DNA Isolation and Southern Blot Analysis. High molecular weight DNA was isolated from the tumor and surrounding noncancerous tissues as previously described (10). Restriction endonuclease digestion, agarose gel electrophoresis, Southern blot hybridization, probe labeling by nick-translation, and autoradiography were also all performed as previously described (10).

RFLP Probes. The polymorphic probes used in this study are shown in Table 1. Probes pYNH37.3, pYNZ22, pMCT35.1, pHF12-1, p10.5, p1A10-41, pHH1152, pCM86, and pTH159 were a gift from Dr. Y. Nakamura. Probe prR4-2 was a gift from Dr. C. W. Miller. Further details of these probes can be found in Human Gene Mapping 11 (21).

PCR-RFLP Analysis. In addition to BgrII RFLP detected by Southern blot analysis with the prR4-2 probe, we also analyzed the BstUI (AccII) polymorphism at codon 72, which can be detected by PCR-RFLP analysis (22). The fourth exon of the p53 gene was amplified by using a pair of primers which were the same as those used in the PCR-RFLP analysis. The amplified DNA fragments were then digested with 5 units of BstUI for 4 h and electrophoresed on 2% agarose gels.

PCR-SSCP and Direct Sequencing. Each sample was screened for mutations of the p53 gene by the PCR-SSCP method, as described by Toguchida et al. (23). For SSCP, each pair of primers was designed to cover the entire coding region and the intron-exon junctions. One hundred ng of genomic DNA was amplified in a buffer containing 0.1 μl [α-32P]dCTP (10 mCi/ml). Thirty cycles of PCR were performed, with 75 s of denaturation at 94°C, 90 s of annealing at 52 or 60°C, and 120 s of polymerization at 71°C. The PCR products were diluted 8- to 10-fold with 0.1% sodium dodecyl sulfate and 10 mM EDTA, and then mixed with the same volume of dye solution (95% formamide; 20 mM...
 EDTA; 0.05% xylene cyanol; 0.05% bromphenol blue). This was followed by denaturation at 80°C for 3 min, and then the samples were applied to 6% neutral polyacrylamide gels with or without 10% glycerol, and electrophoresed at 5 W for 12 to 16 h with cooling by a desk top fan.

Samples which showed altered mobility from the normal controls on the PCR-SSCP gels were further analyzed by direct sequencing. A single-strand DNA fragment was amplified by asymmetric PCR, purified by SUPREC-02 radiolabeled at the 5' end by using a MEGALABEL kit (Takara), and then sequenced directly by using a T7-Sequencing kit (Pharmacia). The sequencing primer was one of a pair of PCR primers, and was neutral polyacrylamide gels with or without 10% glycerol, and electrophoresed to yield representative examples of the PCR-SSCP analysis and direct sequencing of the mutant samples.

RESULTS

Mutational Spectrum of p53 Gene in HCC and Corresponding Clinical Stage. By PCR-SSCP analysis and direct sequencing, 17 different mutations were detected in 53 tumors (32%). Fig. 1 shows representative examples of the PCR-SSCP analysis and direct sequencing of the mutant p53 genes. A summary of the p53 mutations, with the clinical profile from each patient, is shown in Table 2. The following types of mutations were detected: 9 cases of missense mutations, 4 cases of nonsense mutations, 2 cases of mutations at the intron-exon junction, and 2 cases of deletions (one with a 1-base pair deletion and the other with a 58-base pair deletion) (Table 2). Five of the missense mutations (cases 8, 19, 26, 28, and 53) were within the conserved domains, whereas 2 missense mutations (cases 5 and 48) were observed at the conserved amino residues within exons 6 and 10. Except for these subtle mutations, we could not detect any structural alterations with Southern blot analysis, using the pR4-2 probe.

Among the 15 base substitutions, G:C to T:A transversions were the most common changes observed (7 cases, 47%; see Table 3). In 6 of these transversions, a guanine residue on the non-transcribed strand was mutated. No mutations were detected in the histologically normal tissues surrounding the tumors that exhibited mutations.

The frequencies of p53 mutations in HCCs of each clinical stage were as follows: 9 of 23 (39%) in stage IV, 5 of 15 (33%) in stage III, 2 of 7 (29%) in stage II, and 1 of 8 (13%) in stage I (Fig. 2). This suggests a relationship between p53 mutations and the progression of the HCCs. No relationship was found between the p53 mutations and the serum hepatitis B virus surface antigen status (Table 2).

Deletion Map of Chromosome 17 and Mutations of p53 Gene. Table 1 summarizes the results from the LOH analyses on chromosome 17, and Fig. 3 shows the deletion map of chromosome 17. In some tumor samples, residual faint bands which might be caused by contamination of normal cells were observed. When it was difficult to determine whether there was loss, we performed densitometric analysis and only the cases in which the density of fainter band was <50% of normal tissue were determined as allelic loss. Among the 53 cases of HCC, 50 cases were informative with respect to at least one locus on chromosome 17, and LOH was detected in 24 cases (48%).

LOH on the short arm was observed in 24 of 49 cases (49%), whereas LOH on the long arm was found only in 3 of 25 cases (12%). Furthermore, all 3 of these cases also showed LOH on the short arm, suggesting the loss of the whole chromosome. Although most of the cases with LOH on 17p showed allelic losses at all informative loci on the short arm, 3 cases (cases 33, 45, and 54) showed LOH at the D17S5 locus yet retaining heterozygosity at the p53 locus. On the other hand, one case (case 22) showed LOH at the p53 locus without LOH on more distal loci (Fig. 3). These results indicate that two commonly deleted regions exist on 17p in HCCs: one around the p53 locus, and the other stretching from the D17S5 locus to the telomere.

![Fig. 1. Examples of PCR-SSCP analysis (a), and direct sequencing (b) in human HCCs. Arrows indicate bands with a shifted mobility detected in the SSCP analysis (a).](image)
LOH ON CHROMOSOME 17 AND MUTATION OF p53 GENE IN HCC

Table 2. Mutations of p53 gene in HCC and clinical profiles of patients

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age</th>
<th>Sex</th>
<th>HBsAg</th>
<th>Clinical stage</th>
<th>Codon</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>48</td>
<td>M</td>
<td>-</td>
<td>IV</td>
<td>110</td>
<td>C→T → A</td>
<td>Arg → Leu</td>
</tr>
<tr>
<td>5</td>
<td>62</td>
<td>M</td>
<td>-</td>
<td>III</td>
<td>337</td>
<td>C→G → T</td>
<td>Arg → Cys</td>
</tr>
<tr>
<td>8</td>
<td>63</td>
<td>M</td>
<td>-</td>
<td>III</td>
<td>173</td>
<td>G→T → T</td>
<td>Val → Leu</td>
</tr>
<tr>
<td>19</td>
<td>67</td>
<td>M</td>
<td>-</td>
<td>II</td>
<td>270</td>
<td>T→T → G</td>
<td>Phe → Val</td>
</tr>
<tr>
<td>26</td>
<td>65</td>
<td>M</td>
<td>-</td>
<td>III</td>
<td>177</td>
<td>C→C → G</td>
<td>Pro → Arg</td>
</tr>
<tr>
<td>28</td>
<td>60</td>
<td>F</td>
<td>-</td>
<td>III</td>
<td>132</td>
<td>A→A → T</td>
<td>Lys → Asn</td>
</tr>
<tr>
<td>48</td>
<td>64</td>
<td>M</td>
<td>+</td>
<td>IV</td>
<td>215</td>
<td>A→G → A</td>
<td>Ser → Asn</td>
</tr>
<tr>
<td>53</td>
<td>67</td>
<td>M</td>
<td>-</td>
<td>II</td>
<td>242</td>
<td>T→G → A</td>
<td>Cys → Ser</td>
</tr>
<tr>
<td>55</td>
<td>76</td>
<td>M</td>
<td>+</td>
<td>II</td>
<td>35</td>
<td>T→T → T</td>
<td>Leu → Phe</td>
</tr>
</tbody>
</table>

Table 3. Nucleotide changes in p53 mutations found in Japanese HCC's

<table>
<thead>
<tr>
<th>Transversion</th>
<th>No. of Cases (%)</th>
<th>Transversion</th>
<th>No. of Cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G:C to A:T</td>
<td>7 (47)</td>
<td>G:C to T:A</td>
<td>3 (20)</td>
</tr>
<tr>
<td>G:C to G:C</td>
<td>3 (20)</td>
<td>A:T to G:C</td>
<td>0 (0)</td>
</tr>
<tr>
<td>A:T to T:A</td>
<td>1 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A:T to C:G</td>
<td>1 (7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

Mutations of the p53 gene have been demonstrated to be a common event in various types of human malignancies. The mutational profile of each type of tumor, however, seems to be different from other tumors (24). Even within a single type of tumor, the mutational spectrum might be affected by different genetic and/or environmental factors, and thus a comparative study may reveal important etiological factors behind the development of each tumor. In this paper, we have characterized mutations of the p53 gene in Japanese HCC patients, with special attention to the LOH on 17p.

The overall frequency of p53 gene mutation in the HCCs in this study was 32% (17 of 53), which is consistent with previously reported values in Japanese patients (7, 8). Kress et al. (25) also showed a lower frequency (15%) in HCC from Germany, and the frequency is consistently higher (50%) in HCC patients from groups where the codon 249 hot spot predominates (4, 5). This difference in frequency is probably due to different etiologies of hepatocarcinogenesis in different areas. In contrast to the hot spot observed in HCCs in China and south Africa, which we discuss later, no mutational hot spot was observed, and mutations were generally found in a wide region from exons 4 to 10. Most of the missense mutations were observed at residues within evolutionarily conserved domains, or at scattered but conserved amino residues, as reported in other types of cancers (24).

Unlike other cancers such as colorectal carcinomas, where missense mutations accounted for 94% of the cases (9), almost one-half of the mutations (8 of 17) in HCCs were not missense mutations. Among these 8 mutations, 4 were nonsense mutations and 2 were base substitutions at the intron-exon junctions, which had not been reported in previous studies using HCCs (4, 5, 7, 8). We do not have any clear explanation for this difference. However, different patient populations or higher sensitivity of our PCR-SSCP method might give rise to these different results.

As mentioned above, we did not detect any mutations at codon 249 which was previously reported as a mutational hot spot in HCCs in China and South Africa (4-6). This mutational hot spot was considered to be closely associated with aflatoxin B1, and therefore our results suggest the involvement of mutagens other than aflatoxin B1 in the development of HCCs in the Japanese population.

G:C to T:A changes were predominant among the base substitutions observed, including 6 G to T transversions on the non-transcribed strand, which may be attributable to preferential repair of the transcribed strand (26). This type of transversion in the p53 gene was frequently observed in cancers in both small cell and non-small cell lung cancers, which was considered to be associated with cigarette smoke (24, 27). Recent reports have shown that a variety of mutagenic
deletion mapping, however, support the idea that another tumor suppressor gene located on the short arm of chromosome 17 might be involved in the development of some HCCs. The fact that three cases with LOH on the distal portion of 17p still retained heterozygosity at the p53 locus and showed no detectable abnormalities in the p53 gene, and the fact that one case with LOH only at the p53 locus showed a p53 gene mutation further support the presence of two tumor suppressor genes on 17p, both of which may play a critical role in hepatocarcinogenesis.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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