p53 Gene Mutation Spectrum in Hepatocellular Carcinoma

Tatsuya Oda, Hitoshi Tsuda, Aldo Scarpa, Michile Sakamoto, and Setsuo Hirohashi

ABSTRACT

In order to clarify the significance of mutation of the p53 tumor suppressor gene in the genesis and development of human hepatocellular carcinoma (HCC) in an aflatoxin B1 low-exposure area, the spectrum, i.e., incidence, type, and site, of p53 gene mutations was examined in 169 tissue samples resected mainly from Japanese patients using single-strand conformation polymorphism analysis and direct sequencing. Forty-nine tumors (29%) showed a p53 mutation (39 point mutations and 10 frame shifts). The point mutations comprised 18 transitions, only 4 of which occurred at CpG sites, and 21 transversions. Two evolutionarily conserved domains, IV and V, contained 65% of all mutations and codon 249 was the most frequent mutation site (7/49). The spectrum of p53 mutation did not differ among HCCs in relation to the type of hepatitis virus infection, sex, age, and background liver disease of patients, tumor size, or presence of metastasis, but incidence and site were significantly associated with the degree of differentiation of cancer cells. In poorly differentiated HCC, p53 mutation was frequent (54%) and clustered on domains IV and V, whereas in well or moderately differentiated HCC, the mutation was less frequent (21%) and equally distributed on domains II to V. Restriction fragment length polymorphism analysis revealed loss of heterozygosity on chromosome 17p in 55 (69%) of 80 informative cases and in 34 (95%) of 36 cases with p53 mutation. Therefore, p53 gene mutation is suggested to occur independently of the type of viral infection or status of preexisting liver disease and to occur preferentially in moderately and poorly differentiated HCC in association with or after loss of another p53 allele as a late event of HCC progression.

INTRODUCTION

Genetic alterations occurring endogenously or caused by exogenous factors play a fundamental role in the multistage processes of tumorigenesis and progression of malignancy (1). These include integration of viral genomes (2, 3), activation of protooncogenes (4), and inactivation of tumor suppressor genes (5).

HCC is one of the most common cancers in Asia and Africa (6). Various studies have confirmed its association with hepatitis B or C viral infection (70% of all cases) (7, 8). While activation of known protooncogenes does not seem to play an important role (9, 10), frequent allelic loss on specific chromosomal arms (1, 4q, 5q, 11p, 13q, 16p, 16q, and 17p) (11-16) indicates that dysfunction of diverse tumor suppressor genes located on these chromosomal arms is involved in the development of HCC. Among these, the p53 gene (17), located on chromosome 17p13, has been well analyzed, and frequent loss of one allele and mutations in the other allele have been reported to occur in diverse human cancer types including HCC (18-21).

Study of the mutational types of tumors seems to be a powerful way of assessing the contribution of endogenous versus exogenous sources of DNA damage if carcinogens leave their "footprints." Specific carcinogens are, in fact, known to produce specific and reproducible types of damage in the DNA molecule, mainly point mutations (22). For example, N-nitroso-N-methyleurea and dimethylnitrosoimine have been shown to induce transition from G to A and transversion from A to T in the c-Ha-ras gene in rat and mouse, respectively (23, 24). Transversion from G to T is caused by both exogenous carcinogens (25) and endogenous processes such as free radical damage arising from normal biochemical reactions in mouse and monkey (26). On the other hand, a number of mutations in different genes occur with a high frequency at particular sites; e.g., CpG sites are preferential targets for point mutations in mammalian cells during the process of DNA replication, presumably due to the spontaneous deamination of methylated cytosine residues (27). Examples of p53 mutations due to either exogenous or endogenous mechanisms can be found in different human cancers. In colon carcinoma and Burkitt-type malignant lymphoma, 50-65% of mutations are represented by C to T transitions at CpG sites. On the other hand, in non-small cell lung cancer, 57% of point mutations are G to T transversions, suggesting a specific form of direct DNA damage due to the presence of benzo(a)pyrene in tobacco (18).

A specific type of p53 mutation has also been demonstrated in HCCs of subjects exposed to food contaminated with aflatoxin B1, in which most mutations are G to T transversions occurring at codon 249 (19, 20). This type of mutation is similar to that caused by the same substance in mutagenesis experiments in Escherichia coli (28). On the other hand, judging from studies on the limited number of liver cancers occurring in different geographic areas, the types and sites of p53 mutations seem to show a random distribution (21, 29, 30). However, comprehensive information on the incidence, recessive or dominant-negative role (31), and clinical implication of p53 mutations in primary HCC is lacking. In particular, it would be of interest to determine whether any special type of p53 mutation is associated with HBV and HCV infection, or with histological features of preexisting liver disease. Moreover, the p53 mutation spectrum in an aflatoxin low-exposure area like Japan should be analyzed (29).

In the present study, we examined the spectrum, i.e., incidence, type, and site, of p53 gene mutations and loss of heterozygosity on chromosome 17p in a large series of HCCs. Furthermore, we analyzed the association between p53 mutation and clinical parameters including the type of hepatitis virus infection.

MATERIALS AND METHODS

Patients and Pathological Samples. One hundred sixty-nine frozen tissue samples of HCC were obtained from 140 patients (128 Japanese, 6 Korean, 4 Indonesian, and 2 Taiwanese) who underwent surgical treatment at the National Cancer Center Hospital, Tokyo, Japan. All of
these were primary HCC tumor nodules: 76 were from patients with a single HCC nodule; 90 were from 61 patients with multiple HCC nodules; and the remaining 3 were recurrent HCC tumors obtained at a second operation in three different patients (Table 1). Tumors diagnosed pathologically and/or genetically as metastatic in origin were excluded from the present series (32, 33). The 169 tumors were classified into 9 early HCCs corresponding to the in situ or microinvasive stage of cancer, 35 well differentiated HCCs, 84 moderately differentiated HCCs, and 41 poorly differentiated HCCs (32, 34).

Seroserological tests for serum HBsAg in all 140 patients and serum anti-HCV antibody in 128 patients were performed using the reverse passive hemagglutination procedure (SERODIA-HBs, Fujiirebio Inc., Tokyo, Japan), and an enzyme-linked immunosorbant assay test system using anti-HCV antibody (Ortho, Raritan, NJ), respectively. Serum HBsAg and anti-HCV antibody were judged as positive in 30 and 67 patients, respectively, whereas 31 patients were negative for both.

PCR-SSCP Analysis and Direct DNA Sequencing. DNA was extracted according to standard procedures (35). Inasmuch as 98% of p53 gene mutations in diverse types of cancer have been found in exons 5–8 (18), we focused our study on these exons. The PCR-SSCP method described by Orita et al. (36) was used to detect the p53 gene mutation.

### Table 1 Components of HCC nodules analyzed

<table>
<thead>
<tr>
<th>Component</th>
<th>Single nodule</th>
<th>Multiple nodule</th>
<th>Recurrent</th>
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<tbody>
<tr>
<td>Patients</td>
<td>76</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>Nodules</td>
<td>76</td>
<td>38</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>140</td>
<td>169</td>
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</table>

### Table 2 p53 gene mutations in hepatocellular carcinoma and clinico-pathological parameters

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Pathological parameters of tumors</th>
<th>p53 gene mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Background</td>
<td>Liver</td>
<td>Virus</td>
</tr>
<tr>
<td>Sex/Age</td>
<td></td>
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</table>

* - Both HBV and HCV negative.
+ - Negative; +, microscopically positive; ++, macroscopically positive.
The SSCP analysis and direct DNA sequencing were performed using the same procedure as that reported previously (33). Briefly, each exon 5–8 of the p53 gene was amplified by 35 cycles of PCR using 5'-end-labeled primers and Taq polymerase (Perkin Elmer/Cetus, Norwalk, CT) and analyzed on 6% polyacrylamide gels after denaturation of the PCR products by heating. Then, abnormally shifted bands detected by SSCP analysis were eluted from the gel, and single-strand templates for sequencing were obtained by 55 cycles of asymmetrical (20:1 primer ratio) PCR. The sequencing was performed using a 7-deaza-GTP Sequenase version 2 kit (United States Biochemicals, Cleveland, OH) and analyzed on 8% polyacrylamide gel containing 5 M urea.

LOH on Chromosome 17p. Ninety-five cases, including all cases showing p53 gene mutation, were analyzed for LOH on chromosome 17p by restriction fragment length polymorphism analysis using a pYNZ22 (D7S5) probe (37), as described previously (13, 14). In 68

Fig. 1. PCR-SSCP analysis of p53 mutation in HCC. (A) Representative cases of SSCP analysis for p53 gene mutations in HCC. PCR-amplified fragments which encompass exons 5–8 of the p53 gene were electrophoresed independently on 6% polyacrylamide gels. Cases were judged positive for mutation when bands showing different mobility shifts from normal controls were observed. The bands showing mobility shifts indicated by arrowheads were then subjected to DNA sequencing analysis. (B) Direct DNA sequencing of PCR-amplified fragments. Representative sequences of abnormally shifted bands from each of exons 5–8 in SSCP analysis are shown. The sequences of the coding strands are shown for exons 5 and 6, and of the non-coding strands for exons 7 and 8. N, normal controls; R1, D4-a, S4, M15, and D1-a, case numbers (see Table 2).
cases, extensive study with five additional DNA probes, p144D6 (D17S34), pMCT35-1 (D17S31), pHF12-2 (D17S1), p10-3 (MYH2), and p10-41 (D17S71) (37), was performed.

RESULTS

Mutations of the p53 gene were detected in 46 (33%) of the 140 patients and 49 (29%) of the 169 HCC nodules. The results of the genetic study and clinicopathological profiles of the p53 mutation-positive HCC patients are summarized in Table 2. Representative results of SSCP analysis and direct sequencing are presented in Fig. 1.

Specific Mutation Spectrum of p53 in HCC. The mutations were represented by point mutation in 39 cases (80%), microdeletion in 9 cases (18%), and one-base insertion in one case (Table 3). Nineteen point mutations were at G:C and 20 at A:T base pair sites. Transition (in which a purine is substituted for a pyrimidine or vice versa) was detected in 21.

The sites of p53 mutations are represented schematically in Fig. 2. Mutations were clustered at two domains (38). One was domain IV, covering codons 234–258 in exon 7, where 23 (47%) of 49 mutations were detected: 20 of 36 point mutations; and 3 of 10 frameshift mutations. The other was domain V, covering codons 270–286 in exon 8, where 9 (18%) of the mutations (8 point mutations and 1 microdeletion) were detected. The most frequent mutation site in HCC was codon 249, demonstrating 7 mutations accounting for 14% of all mutations and 33% of the transversions. Four of them were from Japanese patients (S-7, D-10-b, P-1-b, and M-9; see Table 1) with G to T transversion at the second position in 3 cases and at the third position in 1 case. The other three were from non-Japanese patients, two being from Taiwanese (D-9 and M-8) with A to T transversion at the first position and one from an Indonesian (S-8) with G to T transversions at the third position.

Association of p53 Mutation with Cancer Cell Differentiation. The incidence of p53 mutation was significantly associated with cancer cell differentiation (Table 4). In poorly differentiated HCCs, the mutation was detected in more than one-half of the cases, whereas in well differentiated HCCs, the incidence of mutation was extremely low. Other clinicopathological parameters, such as patient sex and age, tumor size, type of associated liver damage, presence of portal vein tumor thrombi, and intrahepatic metastasis showed no specific correlation with the incidence of p53 mutation. Irrespective of the degree of differentiation of cancer cells, mutations in domains IV and V were the most common, but the pattern of distribution of p53 mutation sites differed between poorly differentiated HCC and well or moderately differentiated HCC (Fig. 3); in poorly differentiated HCC, 14 of 16 cases (88%) carried the point mutation in domain IV or V. On the other hand, in well and moderately differentiated HCC, point mutations of p53 were detected outside of domain IV or V in 9 (39%) of 23 cases. Thus, mutations in the regions outside domains IV and V were almost entirely (9 of 11) confined to the well and moderately differentiated groups.

Lack of Striking Difference in p53 Mutation Pattern between HBV- and HCV-positive Patient Groups. Among the 128 patients for whom data on both HBV and HCV infection were available, the incidence of p53 mutation was 33% (10 of 30) in HBsAg-positive cases, 30% (20 of 67) in anti-HCV antibody-positive cases, and 39% (12 of 31) in cases negative for both (Table 4). There was no significant difference in the incidence or type of mutations among these three groups. The sites of mutation showed that mutations in the HCV-associated group tended to be spread along the gene, whereas in the HBV group the mutations were clustered in domain IV (Fig. 4), although the difference was not significant. Moreover, different mutations were detected in a single patient, i.e., in three patients with multiple HCCs and chronic viral infection (D9, D10, and P1), the types and sites of mutations differed between two tumors (Table 2). The mutations in codon 249 numbered 3 (G to T transversions) in HCV-positive cases and 3 in HBV-positive cases (two A to T and one G to T) (Table 2).

Coincidence of p53 Mutation and LOH on 17p. Of the 95 cases examined, LOH on chromosome 17p was positive in 55 (69%) of 80 informative cases (Table 5). These 55 tumors were considered to carry only one allele of the p53 gene, and 34 of them also carried a p53 gene mutation in the remaining allele. The other 21 one-allele tumors and virtually all of the two-allele
Table 4 Incidence of p53 gene mutations

<table>
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<tr>
<th>Clinical parameters</th>
<th>Type of hepatitis viruses</th>
<th>Background liver disease</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Pathological parameters</th>
<th>Differentiation of cancer cells</th>
<th>Tumor size (mm)</th>
<th>Intrahepatic metastasis</th>
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<td>HBV (HBsAg positive)</td>
<td>Chronic hepatitis</td>
<td>≥49</td>
<td>Male</td>
<td>Analyzed</td>
<td>Early HCC</td>
<td>≤20</td>
<td>Positive</td>
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<td>Advanced HCC</td>
<td>21–49</td>
<td>Positive</td>
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<td></td>
<td></td>
<td>Well differentiated</td>
<td>50≤</td>
<td>Positive</td>
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<tr>
<td></td>
<td>HCV (anti-HCV antibody positive)</td>
<td>Precirrhosis</td>
<td></td>
<td></td>
<td></td>
<td>Moderately differentiated</td>
<td></td>
<td>Positive</td>
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<tr>
<td></td>
<td></td>
<td>Liver cirrhosis</td>
<td></td>
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<td></td>
<td></td>
<td>Fibrosis</td>
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No. of patients

<table>
<thead>
<tr>
<th>Clinical parameters</th>
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<th>p53 mutation positive (%)</th>
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<td>HBV</td>
<td>30</td>
<td>10 (33)</td>
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<tr>
<td>HCV</td>
<td>67</td>
<td>20 (30)</td>
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<tr>
<td>Both negative</td>
<td>31</td>
<td>12 (39)</td>
</tr>
<tr>
<td>Undetermined</td>
<td>12</td>
<td>4 (33)</td>
</tr>
<tr>
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<td>63</td>
<td>22 (35)</td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td>17</td>
<td>6 (35)</td>
</tr>
<tr>
<td>Precirrhosis</td>
<td>56</td>
<td>17 (30)</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>4</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Fibrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr) ≥49</td>
<td>20</td>
<td>5 (25)</td>
</tr>
<tr>
<td>50–59</td>
<td>45</td>
<td>12 (27)</td>
</tr>
<tr>
<td>60≤</td>
<td>75</td>
<td>29 (39)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>119</td>
<td>39 (33)</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>7 (33)</td>
</tr>
<tr>
<td>Total</td>
<td>140</td>
<td>46 (33)</td>
</tr>
</tbody>
</table>

Pathological parameters

<table>
<thead>
<tr>
<th>Analyzed</th>
<th>p53 mutation positive (%)</th>
</tr>
</thead>
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<td></td>
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</tbody>
</table>

Fig. 3. p53 mutation spectrum in HCC classified according to cancer cell differentiation. Downward arrows, point mutations; upward arrows and sequential horizontal bars, frameshift mutations and damaged areas.

Well differentiated HCC

Moderately differentiated HCC

Poorly differentiated HCC

Carcinogens can induce mutations by direct adduct-driven mutagenesis or by triggering increased cellular turnover (= mitogenesis), which increases the mutation rate indirectly (39). In the present study, no specific type of mutation indicative of an association with particular carcinogens was found in Japanese HCCs, at variance with the specific predominance of G—T transversion in non-small cell lung cancers. Transition of C—T at CpG sites, which results from a spontaneous error of DNA replication observed in cancers such as colon cancer, was also infrequent. The presence and type of hepatitis virus infection were unrelated to the spectrum of p53 mutation. Moreover, in multiple primary cases both with and without viral infection, p53 mutations were different in each tumor in terms of presence, types, and sites. These data suggest that various carcinogens are involved in the genesis of p53 gene mutation, or if one or more specific carcinogens are associated, their effect is indirect, most probably due to DNA polymerase infidelity linked to mitogenesis. In this regard, hepatitis virus, which increases cellular turnover by inflammation, could be considered an indirect mutagen linked to mitogenesis.

It has been reported that G to T transversion at the third base of codon 249 is a specific feature of aflatoxin-related endemic HCCs (19, 20, 29). Moreover, mutation at codon 249 has also been preferentially observed in HCCs from HBV-infected patients in aflatoxin high-exposure areas (40), and mutations at codon 249 have never been identified in non-HBV-related HCCs (18). Thus, it is also suggested that, in addition to aflatoxin intake, HBV infection is necessary for its induction. In tumors showed no p53 mutation upon SSCP analysis. Conversely, all except two tumors with p53 mutations also carried LOH on 17p.

DISCUSSION

In total, 33% of HCC patients showed p53 mutations, and four noteworthy features of the mutation spectrum in HCC were revealed: (a) almost equal incidence of transitions and transversions; (b) low incidence of mutations at CpG sites (8%); (c) high frequency of frameshift mutations (20%); (d) preferential (65%) clustering of mutations at two "hot spots," domains IV and V.

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p53 MUTATION SPECTRUM IN HCC

Fig. 5. Genetic model of multistage hepatocarcinogenesis. Chronic effect of hepatitis virus infection or alcohol intake induce chronic liver damage. Still unknown genetic or chemical alterations cause development of early or well differentiated HCCs. Thereafter, p53 gene mutations and preceding LOH on 17p are known genetic or chemical alterations cause development of early or well differentiated HCCs. Moreover, morphological examination of the well differentiated cases with p53 mutation revealed that a significant proportion of the tumor was composed of Edmondson Grade II cells. Therefore, these genetic abnormalities may occur largely at the stage of progression from well to moderately or to poorly differentiated HCC.

Our comprehensive analysis of this large series of HCCs suggests a role for p53 gene mutation and LOH on 17p in the late stage of the multistage process (45) of hepatocarcinogenesis. A tentative model of progression of HCC is shown in Fig. 5. However, the earliest and the latest genetic events in hepatocarcinogenesis remain undetermined, and identification of such genetic alterations seems to be important for further understanding the molecular basis of multistage hepatocarcinogenesis.

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