Splicing Mutations of the p53 Gene in Huma Hepatocellular Carcinoma

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ABSTRACT

Point mutations in exons of the tumor suppressor p53 gene occur frequently in many human tumors including hepatocellular carcinoma and are extensively studied. However, intronic point mutations are rare and are totally unknown for hepatocellular carcinoma. By reverse transcription and polymerase chain reaction amplification of p53 RNA from hepatocellular carcinoma tissues of 45 Taiwanese patients, we found amplified complementary DNA fragments of abnormal size in 4 (9%) tumors. Sequence analysis of these complementary DNA products revealed aberrant retention of intron 7 in one sample, insertion of 49 base pairs of the 3′ end of intron 6 in 2 samples, and deletion of exon 4 in the other sample. Direct sequencing of their genomic DNA revealed relevant point mutations at consensus sequence at either the 5′ or 3′ splice site of intron 7, 6, 6, and 3, respectively. The splicing mutations produced p53 mutants with truncation of COOH-terminus that are identical to those found in lung cancers. Three of the 4 patients with splicing mutations were younger and had huge tumors. The results suggest a possible role of these p53 mutants in the development of human cancers.

INTRODUCTION

HCC is a common malignancy in Africa and Asia (1). In Taiwan, chronic hepatitis B and hepatitis C virus infections are the major etiological factors, and dietary contamination of aflatoxin B, may also contribute to this malignancy (2–5). The molecular mechanisms leading to HCC formation, however, remained poorly understood. Recently, the structure and function of the p53 tumor suppressor gene have been extensively studied and found to be the most commonly mutated gene in human cancers (6–8). The majority of p53 mutations identified are missense changes in one of the four “hotspots” within exons 5 to 8 of the gene (6–8). For HCC collected from various geographical locations in the world, mutations in the p53 gene have been found in 18–58% of the tumors (9–15); most of the mutations are also missense point mutations, and some are microdeletions or base insertions in the coding region (11, 14). Codon 249 is a mutational hotspot for HCC from southern Africa and China, which is probably related to high aflatoxin B, exposure (9, 10, 12). In Taiwan, 20% of p53 mutations in HCC are codon 249 mutations (15). These results document that p53 mutations in HCC occur in exons of p53. Intrinsic point mutations of the gene which can lead to abnormal pre-mRNA splicing and defective p53 protein (16–20), however, have not been addressed in HCC before.

To study whether splicing mutations contribute to inactivation of the p53 gene in HCC, by RT-PCR of RNA and direct sequencing, we identified abnormal p53 RNA splicing in several HCC tissues and traced these mutations to relevant sites at the introns of genomic DNA. The association of this rare and unique mutation with some young patients with HCC in our study may be significant in hepatocarcinogenesis and warrants further studies.

MATERIALS AND METHODS

Liver Tissues and Nucleic Acids Preparation. Fresh paired tumor and nontumor liver tissues from 45 patients with HCC were studied. These patients consisted of 32 males and 12 females, with ages ranging from 12 to 74 years (48 ± 19, mean ± SD). Twenty-eight, 11, and 2 patients were positive for HBsAg, the antibody to hepatitis C virus, and both markers, respectively, and the remaining 4 patients were negative for both hepatitis markers. The liver samples were collected immediately after surgical resection. They were snap-frozen in liquid nitrogen and stored at -70°C until use. Total cellular RNA was prepared by using acid guanidinium thiocyanate-phenol-chloroform extraction method (21). Genomic DNA was extracted by phenol-chloroform method as described before (22).

Screening of Abnormal p53 RNA by RT-PCR. To synthesize p53 cDNA, 1 to 5 µg of the total RNA extracted from tumor and nontumor liver tissues were mixed with 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) and 500 ng random hexanucleotide primers (Promega, Madison, WI) in a total reaction volume of 20 µl (23). To amplify p53 cDNA covering the entire p53 coding region by polymerase chain reaction (24), 2 pairs of primers were used. The first pair centered nt positions 59–621 (sense primer, nt -59 to -40, 5′-TCCAGCAGGTCAGCAGCTTC-3′; antisense primer, nt 621–602, 5′-CATCCAAAATACTCCACACCGA-3′) and the second covered nt positions 565 to 979 (sense primer, nt 565–584, 5′-GCCCCTCTCTACAGCTCTAT-3′; antisense primer, nt 979–960, 5′-ATTCCTCACAGGTTCTC-3′) of the p53 cDNA. The cDNA was mixed with 10 pmol of the appropriate primers, buffer, and Taq polymerase (2.5 units) and then subjected to 35 cycles of amplification through denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, followed by additional extension for 7 min in a thermal cycler (Perkin Elmer Cetus, Emeryville, CA). The amplified p53 cDNA fragments were subjected to 1.8% agarose gel electrophoresis for analysis and then purified by electrophoresis in a 5% polyacrylamide gel (23).

Direct Sequencing of p53 cDNA. Direct sequencing was performed by the dideoxy chain termination method (Sequenase Version 2.0, United States Biochemicals; dSanger Cycle Sequencing System, Bethesda Research Laboratories) according to the manufacturer’s instructions. The primers used for amplification of p53 cDNA were also used for sequencing. In addition, an antisense primer (nt 1139–1174, 5′-AGTTGGAAGATGTAGTTGGAGG-3′) was used to sequence the region uncovered by the aforementioned primers. The sequencing reaction products were analyzed on a 6% polyacrylamide gel containing 8 M urea.

Sequence Analysis of Genomic DNA. Genomic DNA was amplified by the same method as described above by using 100 ng of genomic DNA extracted from tissues containing abnormal p53 RNA as template. The amplified DNA was then subjected to direct sequencing. For studying the 5′ splice site consensus sequence of intron 7 of the p53 gene in tumor 60T, the genomic DNA were amplified with the sense primer (nt 565–584, 5′-GCCTCTCTCTACAGACTAT-3′) and the antisense primer (nt 979–960, 5′-ATTCCTACCATCCAGGTTCTC-3′). The sequencing primer used was a sense primer (nt 679–698, 5′-TCTGACGTATCCACCATCCA-3′). For analyzing the 3′ splice site of intron 6 of tumor 7T and 54T, the sense primer (nt 603–622, 5′-GCGTGGAAGATGTAGTTGGAGG-3′) and the antisense one (nt 782–763, 5′-CTTGGATTTCTCAGGGTGAT-3′) were used. The sequencing primer was the antisense primer (nt 782–763). For detecting the genomic DNA mutation for tumor 80T, sense primer (nt 11–30, 5′-GGCAATCTAGATTCTGCT-3′) and another antisense primer (nt 508–489, 5′-CTATGCTGCTGATT-3′) were used. The sequencing primer was an antisense primer (nt 200–181, 5′-GGCAATCTGAGGACTAT-3′).

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3 The abbreviations used are: HCC, hepatocellular carcinoma(s); RT-PCR, reverse transcription-polymerase chain reaction; RFLP, restriction fragment length polymorphism; HBsAg, hepatitis B surface antigen; cDNA, complementary DNA; nt, nucleotide(s).
Southern Blot Analysis. To detect loss of heterozygosity at locus near p53, RFLP study was performed. Ten µg of DNA from tumor and the corresponding nontumor tissues were digested with restriction endonuclease BamHI at 37°C overnight. They were electrophoresed in 1.0% agarose gel and transferred to a Zeta-probe blotting membrane (Bio-Rad, Richmond, CA). A probe pYNZ 22 (catalogue no. 57575, American Type Culture Collection), mapping near p53 (25), was labeled with [α-32P]dCTP by random primer extension. The procedure of prehybridization, hybridization, washing, and autoradiography was performed as described before (22). Allelic deletion was detected by absence of one of the alleles in the DNA from the corresponding nontumor tissue.

RESULTS

Detection of Abnormal Forms of p53 cDNA Amplified from RT-PCR. When the RNA from HCC were amplified by RT-PCR with primers covering the 5' half of p53 RNA, three tumor samples contained amplified fragments larger than that anticipated from the wild type (Fig. 1A, Lanes 1, 2, and 3). When it was amplified with primers covering the 3' half of p53 RNA, we found a smaller abnormal cDNA fragment in another HCC sample (Fig. 1B, Lane 4). Abnormal cDNA was not detectable in the RT-PCR-amplified products generated from nontumor counterparts of these four patients.

Sequence Analysis of Abnormal Forms of p53 cDNA and Identification of Splice Site Mutation. In order to characterize these aberrant p53 cDNA, we purified these fragments and did direct sequencing. In tumor 60, direct sequencing of the aberrant cDNA fragment (Fig. 1A, Lane 3) revealed retention of the entire intron 7 (Fig. 2A), while the normal-sized cDNA fragment revealed same sequence corresponding to the wild-type p53. The retention of intron 7 generated a TAG stop codon within the intron. To identify the location of mutation in genomic DNA, the tumor DNA were amplified and sequenced using primers described in “Materials and Methods.” A substitution of A for G at the position of 3' splice site consensus sequence of one of the alleles in the DNA from the corresponding nontumor tissue.

The larger cDNA fragments of the other two tumor samples, 7T and 54T (Fig. 1, Lanes 1 and 2), were found to contain aberrant insertion of 49 base pairs from the 3' end of intron 6 (Fig. 3A). The aberrant intron sequence created a TGA stop codon within exon 7. Sequence analysis of DNA from these 2 tumors revealed mutations with A to T or A to G substitutions, respectively, at the position of 3' splice acceptor site of intron 6 (Fig. 3C). A cryptic splice site upstream to the original was instead used which resulted in the aberrant retention of 49 base pairs of intron sequence (Fig. 3C).

Clinicopathological Features of the Patients. The clinical and pathological features of these 4 patients with splicing mutations of p53 were summarized in Table 1. It is noteworthy that they were all HBsAg carriers, and patients with splicing mutations resulting in truncation of the COOH-terminus of p53 protein (7T, T54, and T60) were younger (average age, 17 years) yet had larger tumors (average size, 16 cm).
p53 SPlicing MUTATIONS IN HEPATOCellular CARCinoma

5í SPLICING MUTATIONS IN HEPATOCELLULAR CARCINOMA

7N 7T-

ACGT ACGT

Bon Bot'

ACGT ACGT

M .- and B. sequence analysis of p53 cDNA of tumor 7T and 80T show aberrant insertion of 49 base pairs from 3' end of intron 6 and deletion of intron 4, respectively. In C, the diagram shows the splicing mutations in 7T, 54T, and 80T. * sequences analyzed that are derived from the abnormal forms of p53 cDNA in the tumors.

DISCUSSION

Splicing mutation of the p53 gene is uncommon and has only been reported in lung cancer, leukemia cell lines, and esophageal cancer (16-20). Previously, we found overexpression and exonic point mutations of p53 in about one-third of HCC tissues (15, 26). In this study, we found 4 (9%) out of 45 HCC samples had point mutations either at the 5' or 3' splice site consensus sequence of the p53 gene. Abnormal p53 gene transcripts resulting from aberrant splicing were identified and characterized.

It is intriguing that the abnormally spliced p53 products in HCC found in this study were identical with that reported in lung cancers (16-18). It strongly suggests that these p53 mutants may have unique biological properties. Recently, wild-type p53 is known to function as a transcription factor, with a transcription activation domain at its NH2 terminus and a DNA-binding domain at COOH-terminus (27-31). Intact COOH-terminal domain is not only required for transactivation function of wild-type p53, but also indispensable for transforming activity of activated p53 mutants through complex formation with wild-type p53 (dominant-negative effect) (32-34). In the present study, the splicing mutations would result in insertion of 49 base pairs of intron 6 and 344 base pairs of intron 7 to the coding region of the p53 gene. Stop codons would be generated within exon 7 of 7T and 54T and within intron 7 of 60T (17), respectively. Eventually, defective p53 proteins with truncated COOH-terminus must have been produced. These splicing mutants must lose their ability to oligomerize with wild-type p53. Since p53 mutations of 54T and 60T are in heterozygous forms, we suspect that these p53 splicing mutants might gain some unusual functions and contribute to the development of HCC.

The splice site mutation of HCC 7T was a homozygous change by genomic DNA sequence analysis and RFLP study (Fig. 4). However, we noted that a normal-sized band, although fainter than the aberrant one, appeared in the agarose gel when the RT-PCR products amplified from 7T RNA were electrophoresed (Fig. 1, Lane 1). Sequence analysis of the normal-sized p53 cDNA fragments did not disclose any mutation. We cannot rule out the possibilities of contamination of normal RNA from connective tissues in the tumor or the existence of heterogeneity of the tumor cell population in the same tumor. It is well known that both human and animal tumors are heterogeneic in many aspects such as their immunogenicity, karyotype, metastatic potential, drug sensitivity, etc. (35). Specifically, different clones of tumor cells in a single large HCC have actually been demonstrated by analyzing patterns of hepatitis B virus DNA integration (36).

All four HCC patients who had splicing mutations were HBsAg carriers and were younger as compared to the average age (52 years) of HBsAg-positive HCC patients in our series. Two of the four pa-

Table 1 p53 intronic mutations leading to abnormal RNA splicing in 4 HCC tissues

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>HBsAg/anti-hepatitis C virus</th>
<th>HCC size (cm)</th>
<th>53 mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>14</td>
<td>+/-</td>
<td>20 x 18</td>
<td>6 (3' SAS)</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>21</td>
<td>+/-</td>
<td>10 x 10</td>
<td>6 (3' SAS)</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>16</td>
<td>+/-</td>
<td>19 x 16</td>
<td>6 (3' SAS)</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>49</td>
<td>+/-</td>
<td>3.2 x 2.8</td>
<td>6 (3' SAS)</td>
</tr>
</tbody>
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a SAS, splice acceptor site; SDS, splice donor site.

b Heterozygous change.
patients did not have cirrhosis which usually coexisted with HCC in Taiwan (37). These findings also suggested that the splicing mutants of p53 might be associated with earlier occurrence of HCC in HBsAg carriers, even in those without liver cirrhosis. Further studies on the functions of these unique p53 splicing mutants are needed.

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REFERENCES


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