High Frequency of p53 Gene Mutation in Adenocarcinomas of the Gallbladder

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Abstract

p53 mutations in adenocarcinomas of the gallbladder were analyzed by deoxynucleotide sequencing of the gene. Of 23 cases, 16 (70%) harbored 18 missense mutations in exon 5, 6, or 8 of the p53 gene. The characteristics of the p53 mutation spectrum observed in gallbladder carcinomas were (a) frequent mutations at an A:T pair (10 of 42 mutations, or 48% of the p53 mutations), (b) high transversion incidence (12 of 18 mutations, or 67% of the p53 mutations), and (c) only one mutation at the CpG site. The immunohistochemical study revealed that 36 (55%) of 65 cases showed an abnormal accumulation of p53 immunoreactivity, and 12 (52%) of 23 cases had p21 expression. No statistical correlation was observed between p53 and p21 immunoreactivity. These results suggest that p53 mutations may confer the carcinogenesis of the adenocarcinoma of the gallbladder with the specific mutation spectrum of p53.

Introduction

It is known that the p53 tumor suppressor gene product plays a pivotal role in normal cell growth and differentiation (1, 2). Inactivation of the p53 gene by allelic loss and point mutation have been found in a variety of human malignancies (3, 4). We also have demonstrated that allele loss and mutation of the p53 gene are frequently associated with gastric cancer and occur in an early stage of human stomach carcinogenesis (5–7). Differences in the deoxynucleotide base-substitution spectrum of p53 mutation have been clarified in several malignancies (8). Transitions, especially at CpG dinucleotides, predominate in colorectal carcinomas (9) and brain tumors (3), whereas G→T transversions are frequently found in breast (3) and esophageal carcinomas (10). Selective G→T transversions at codon 249 are reported in hepatocellular carcinomas (11). Moreover, we found that the characteristics of the p53 mutation spectrum observed in primary gastric carcinomas were (a) frequent mutation at an A:T pair and (b) high transversion incidence (6). The epidemiological significance of these accumulating mutational spectra of the p53 gene is that specific mutagenic effects of exogenous or endogenous carcinogens might be linked with the risk factors of these malignancies (12).

Previous studies demonstrated that the incidence of gallbladder carcinoma was more frequent in Japan than in the West (13). Although accumulations of abnormal p53 product have been reported to be seen often in gallbladder carcinomas (14, 15), there is almost no analysis for p53 mutations performed directly by deoxynucleotide sequencing. The aims of this study are to examine p53 mutations in adenocarcinomas of the gallbladder and to determine whether p53 mutation or accumulation correlates with the expression of p21 protein, which is induced by wild-type p53.

Materials and Methods

Tissues. A total of 65 surgically resected adenocarcinomas of the gallbladder were used. Nineteen patients were men, and 46 were women. The tissues were fixed routinely in 10% formalin and embedded in paraffin wax. The definitions of histological classification and stage grouping were made according to the WHO International Histological Typing of Tumors (16) and the International Union against Cancer (17), respectively. Informed consent was obtained from all subjects.

DNA Preparation. Genomic DNA was prepared from formalin-fixed and paraffin-embedded tissue by the proteinase K-phenol-chloroform extraction method (18) with some modifications. Briefly, paraffin blocks were sectioned at 10μm in thickness and attached to glass slides, and then tumor parts were excised from each specimen under a microscope. Paraffin was eliminated by three extractions with xylene, tissues were then immersed in ethanol and incubated in 500μl of lysis buffer (50mM Tris·HCl pH 9.0) containing proteinase K to a final concentration of 0.5 mg/ml at 55°C for 8 h. After phenol-chloroform extraction, genomic DNA was precipitated with ethanol. All possible precautions were taken to avoid contamination.

DNA Sequencing. Genomic DNA was dissolved in a total volume of 50 μl of solution containing 1X PCR buffer, 0.2 mM of each deoxynucleotide triphosphate, 1 μM of each primer, and 2.5 units of Taq polymerase. Templates were denaturated for 5 min at 94°C followed by 35 cycles of PCR with incubations of 1 min at 55°C, 2 min at 72°C, and 1 min at 94°C. Primers covering conserved portions of exons 5–8 of the human p53 gene for the PCR amplification of the p53 gene used in this study were as follows: exon 5, 5′-TCTCTTCTTACAGTACTC-3′ and 5′-CAGCTGCTCAACATCGCTA-3′; exon 6, 5′-CAGTGTGTTTCTAGTGCAG-3′ and 5′-GGTGCAAACCAGACCTCAG-3′; exon 7, 5′-CTTGGCCACAGGCTCT-3′ and 5′-GGGTTCTCGTCGCCTCAG-3′; and exon 8, 5′-TTGGAGTAGAGGGGAGGCTG-3′ and 5′-GTG-CTTGCTTCGTCGCTTA-3′. All amplifications included

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negative controls featuring the amplification mixture and deionized water added in place of template DNA.

The amplified PCR products were separated by electrophoresis with low-melting-point agarose and purified using Wizard PCR Prep (Promega, Madison, WI). Identification of mutation in the p53 gene was determined by direct sequencing of DNAs, using a Circum Vent Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs, Beverly, MA). The DNA preparation, PCR, and sequencing reaction were repeated at least three times to confirm the results.

**PCR-RFLP.** Allelic loss was examined using the PCR-RFLP technique with two different polymorphisms within p53. One marker was AccII (19), and the other was BamHI polymorphism within the p53 gene. Primers for this study were the following: AccII, 5'-TCTGGGAAGGGACAGAAGATGAC-3' and 5'-TTGCCGTCCCAAGCAATGGATGA-3'; BamHI, 5'-GAAGGCCCTCGGTATGGGTATACA-3' and 5'-TCAGAAGGAATGGAAAGACTCAG-3'.

**Immunohistochemistry.** p53 and p21 proteins were detected immunohistochemically by means of the avidin-biotin-peroxidase complex method using 10% formaldehyde-fixed, paraffin-embedded sections. Several blocks from each case were used for immunohistochemical staining. A mouse monoclonal antibody against human p53 protein (DO-7, Novocastra Laboratories, Newcastle, UK) and a mouse monoclonal antibody against p21 (RBI, Newmarket, UK) were used as the primary antibodies at the 1:100 and 1:300 dilutions, respectively. The immunoreactivity was graded as follows: −, almost no positive cells; +, 10–30% of tumor cells showed positive immunoreactivity; ++, 30–60% of tumor cells showed moderate immunoreactivity and/or 10–30% of tumor cells showed strong immunoreactivity; and ++++, more than 60% of tumor cells showed strong immunoreactivity.

**Statistics.** Statistical analysis was performed using the Kendall tau b correlation analysis, the signed rank test, the generalized Wilcoxon test, the Kaplan-Meier model, and the Cox proportional hazards model. A probability of $P < 0.05$ was considered statistically significant.

**Results**

**DNA Sequencing Analysis.** Of 23 cases, 16 (70%) harbored 18 missense mutations in exon 5, 6, or 8 of the p53 gene, as shown in Table 1. All cases with alterations in p53 gene had

<table>
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<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Histological type</th>
<th>Stage</th>
<th>Existence of galstone</th>
<th>Immunohistochemical study</th>
<th>Affected codon</th>
<th>Nucleotide substitution</th>
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<td>Pap</td>
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<td>Pro→Pro</td>
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<td>F</td>
<td>Poor</td>
<td>IV</td>
<td>−</td>
<td>p53</td>
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<td>CAT→CGT</td>
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<td>IV</td>
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<td>p53</td>
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<td>CAT→CGT</td>
<td>His→Arg</td>
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<tr>
<td>23</td>
<td>64</td>
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<td>Poor</td>
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<td>p53</td>
<td>190</td>
<td>CAT→CGT</td>
<td>His→Arg</td>
<td>−</td>
</tr>
</tbody>
</table>

* Histological typing was done according to Ref. 16.
* Staging was done according to Ref. 17.
* Pap., papillary; Well, well-differentiated; Poor, poorly differentiated.
* Not an informative case.
missense mutations, resulting in amino acid substitutions (Fig. 1). Missense mutations were detected in 10 (83%) of 12 papillary adenocarcinomas, in 3 (60%) of 5 well-differentiated adenocarcinomas, and in 3 (50%) of six poorly differentiated adenocarcinomas, respectively. Two papillary adenocarcinomas each showed two missense mutations (cases 1 and 2). In addition, six of the base substitutions were without amino acid substitution (silent mutation). Silent mutations were found to be near missense mutational points (Fig. 2, case 2). Silent mutation itself does not affect p53 protein. But it was accompanied with missense mutation in this study; therefore, its occurrence may be related to genetic instability. Statistically, there was no correlation between p53 mutations and clinicopathological features, including histology and clinical stage (data not shown).

The p53 mutational spectrum of gallbladder carcinoma observed in this study is summarized in Table 2. One (5%) was A:T→T:A transversion, three (17%) were A:T→G:C transitions, and six (33%) were A:T→C:G transversions; therefore, 10 (55%) of 18 mutations were detected at an A:T pair. In addition, transversions were found frequently in gallbladder adenocarcinomas studied [12 (66%) of 18 mutations: G:C to T:A, 11%; G:C to C:G, 17%; A:T to T:A, 5%; A:T to C:G, 33%]. Mutation at CpG dinucleotide was observed in only one case (case 9) at codon 202. No CpG→TpG transition at codons 175, 245, 248, 273, and 282 was found. Seven (39%) missense mutations were found in exon 5, five (28%) in exon 6, and six (33%) in exon 8. In contrast, there was no mutation in exon 7. Of 10 cases complicated with gallstones, 6 showed p53 mutation.

PCR-RFLP. We performed PCR-RFLP analysis for LOH detection at the p53 locus. LOH was detected in 2 (10%) of 20 informative cases by BamHI polymorphism (Fig. 3), but there was no LOH by AccII polymorphism. This frequency of LOH was lower than that of other malignant tumors (5). Two cases
Mutations in Gallbladder Cancer

Fig. 4. Immunostaining of p53 protein in human gallbladder carcinoma. Well-differentiated tubular adenocarcinoma at ×100 (case 13). Many tumor cells were positive for p53 protein within the nuclei.

Table 3 Immunohistochemical detection of p53 protein in human adenocarcinomas of the gallbladder

<table>
<thead>
<tr>
<th>Histological type</th>
<th>Positive cases</th>
<th>p53 immunoreactivity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Pap§</td>
<td>9/21 (43%)</td>
<td>4</td>
</tr>
<tr>
<td>Well</td>
<td>14/25 (56%)</td>
<td>2</td>
</tr>
<tr>
<td>Mod</td>
<td>4/5 (80%)</td>
<td>2</td>
</tr>
<tr>
<td>Poor</td>
<td>9/14 (64%)</td>
<td>3</td>
</tr>
<tr>
<td>Total (P = 0.3626)</td>
<td>36/65 (56%)</td>
<td>8</td>
</tr>
</tbody>
</table>

§ Histological typing was done according to Ref. 16.

Pos, papillary. Well, mod, poor; well-, moderately, and poorly differentiated.

Significant correlation was not detected.

(cases 9 and 20) with LOH at p53 locus had a missense mutation of the gene.

Immunohistochemical Analysis. Of 65 adenocarcinomas of the gallbladder, 36 (55%) showed positive immunoreactivity for p53 protein, regardless of histological type and staging. In all positive cases, p53 immunoreactivities were confined to the nuclei of the tumor cells (Fig. 4).

There was no significant correlation between the expression of p53 protein and histological type (Table 3). No obvious correlation was observed between p53 immunoreactivity and other clinical factors determined according to TNM classification (data not shown). In addition, there was no statistical relationship between survival rate of the gallbladder carcinoma patients and expression of p53 protein (data not shown). Four of 16 cases in which mutation could be detected by the DNA sequencing technique did not show p53 immunoreactivity (Table 1).

We next examined whether p21 expression is correlated with p53 expression. p21 (20), the expression of which is directly induced by wild-type p53, is an important mediator of p53-dependent tumor growth suppression (21). Twelve (52%) of 23 cases showed p21 immunoreactivity, which was seen mainly in the nuclei of the tumor cells. The expression of p21 immunoreactivity displayed heterogeneity of that level within the same tumor. p21 immunoreactivity was not related to any clinicopathological factors (data not shown).

Recently, it was reported that there is a strong negative correlation between the presence of p53 mutations and p21 expression (22). We expected that there would be a negative correlation between p21 and p53 expression of the gallbladder carcinomas. However, six cases with p21 expression showed p53 immunoreactivity, whereas five cases did not show either p21 or p53 immunoreactivity. In some cases, expressions of both were observed in the same area (Fig. 5). In this study, it was not clear whether there was any relationship between p21 and p53 expression.

Discussion
In gallbladder carcinoma, little is known about the molecular mechanism existing behind the tumor progression and metastasis. Recently, we reported that decreased expression of nm23 may play an important role in local invasion of this malignancy (23). Some investigators have suggested that p53 mutation was detected in high frequency with the use of immunohistochemical methods (70–90%, Refs. 14 and 15). However, there has been only one report (24) on the p53 mutation in gallbladder carcinoma by the deoxynucleotide sequencing method. To our...
knowledge, this is the first detailed report of the analysis of p53 mutations in adenocarcinomas of the gallbladder by deoxyribonucleotide sequencing.

It has been described that overexpression of p53 protein correlates with the existence of missense mutations of the gene (25). From this point of view, the frequency of p53 missense mutations in gallbladder carcinomas reported previously by Takagi et al. (24) was quite low in comparison with the frequency of p53 overexpression by immunohistochemistry as reported previously (70–90%; Refs. 14 and 15) as well as that found in the present study (50%). On the other hand, the high frequency of p53 missense mutations (70%) correlated well with the frequency of abnormal accumulation of p53 protein found in this study. However, 4 of 16 gallbladder adenocarcinomas that were found to have missense mutations by the DNA sequencing technique were found negative for p53 protein immunohistochemically. This might be due to the degradation of p53 protein irritated by bile. Therefore, DNA sequencing should be performed for precise identification of p53 mutations in this malignancy.

Many investigators have reported that most p53 mutations have occurred in the regions of the gene that are highly conserved through evolution and presumably of functional importance, primarily between exons 5 and 8 (codons 126–306) (3). In our study, all 18 mutations were found in exons 5–8. A frequent mutational point, a so-called “hot spot,” has been reported in various tumors (4, 26). However, this study did not indicate the existence of a hot spot in gallbladder adenocarcinoma.

Several authors have proposed that p53 mutations are associated with the progression of various cancers (26). Point mutations of the p53 gene are observed frequently at the CpG dinucleotide site (4). Conversely, p53 mutation at the CpG dinucleotide site is a rare event of esophageal cancer (8). Taken together, the pattern of p53 mutations in tumor differs depending on cancer type, suggesting that mutational type may reflect the mutagen, which may cause specific base changes, sometimes of specific p53 codons (12). For example, benzo[a]pyrene is a mutagen in cigarette smoke that causes G→T transitions in bronchogenic carcinoma (27). In contrast, in the gastric carcinomas and adenomas, there were no specific changes in the p53 gene, but mutations at the A:T pair occurred frequently (6, 28).

The present study also revealed that a high incidence (55%) of mutation at A:T pair was observed in adenocarcinomas of the gallbladder. Especially, of 18 mutations, 3 (17%) were A:T→G:C transitions, and 6 (33%) were A:T→C:G transitions. Mutation at the CpG dinucleotide site was detected in one case at codon 202, which is not a hot spot. As discussed for esophageal cancer by Hollstein et al. (8, 10), a high incidence of p53 mutation at an A:T pair occurred by DNA depurination caused by irritants to mucosa, including ethanol or urethan contained in alcoholic beverages. In the case of gallbladder carcinoma, environmental factors or chemical carcinogens have not yet been determined. In this context, our results do suggest the involvement of some specific agent in bile, e.g., deoxycholic acid, lithocholic acid, and cholesterol, which irritates the mucosa of the gallbladder. However, to date, there has been no report that any component of bile itself is directly implicated in carcinogenesis. In addition, we investigated whether the existence of gallstones is related to p53 mutation, but there was no significant statistical relationship between p53 mutation and complications with gallstones.

In contrast to a high p53 mutation rate, frequency of LOH at p53 was very low (10%). In most malignant tumors, LOH at p53 occurs more frequently. We reported that LOH on 17p13.1 was observed in more than 60% of gastric carcinomas, regardless of histological type (5). At least, as each case showing LOH at the p53 locus had a missense mutation, the mechanism of p53 inactivation may be related to both mutation and LOH. p21 (sdi1/WAF1/CIP1), whose product binds to cyclin-cyclin-dependent kinase complexes and inhibits the function of cyclin-dependent kinases (29), encodes the wild-type p53-inducing transcript in mammalian cells (21). Recently, it was reported that overexpression of p21 suppresses proliferation and cell growth of tumors in vitro (30). Özçelik et al. (22) suggested that p53 mutation may reduce the ability of p53 to induce p21 in vivo. Taken together, we anticipated that gallbladder carcinomas would show no or lower levels of p21 expression. However, p21 expression was detected in 12 (52%) of 23 cases immunohistochemically [especially, ++ positive cases were 7 (31%); Table 1]. In addition, expression of both p21 and p53 was detected in six cases. Therefore, it was difficult to estimate whether there is a significant correlation between the expression of p21 and p53. Additional study with an increased number of cases on this issue would be required.

In conclusion, the data presented here indicate that p53 gene mutations with the specific p53 mutation spectrum is implicated in the development of gallbladder carcinomas.

Acknowledgments

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