Mutations of p53 in Gallbladder Carcinomas in High-Incidence Areas of Japan and Chile

Naoyuki Yokoyama,1 Jiro Hitomi, Hidenobu Watanabe, Yoichi Ajikota, Martha Pruyas, Ivan Serra, Yoshio Shirai, and Katsuyoshi Hatakeyama

First Department of Pathology [N. Y., H. W., Y. A.], First Department of Surgery [N. Y., Y. S., K. H.], and Department of Anatomy [J. H.], School of Medicine, Niigata University, Niigata 951, Japan; Sotero Del Río Hospital, Concha Y Toro 3459, Santiago, Chile [M. P.]; and School of Public Health, Faculty of Medicine, University of Santiago, Melver 541, Santiago, Chile [J. S.]

Abstract

Gallbladder adenocarcinomas from patients in two high-prevalence areas, Niigata (Japan) and Santiago (Chile), were analyzed for acquired mutations in exons 5–8 of the p53 tumor suppressor gene, and the characteristics of p53 alterations in the two groups were compared. Of 42 alterations in the two groups were compared. Of 42 tumors, 22 (52.4%) harbored 25 alterations identified by PCR amplification and direct sequencing (13 of 22 tumors from Niigata and 12 of 20 tumors from Santiago). All alterations were single base pair substitutions, 20 (80%) leading to an amino acid substitution or a chain-terminating mutation. Missense mutations correlated highly with overexpression of the p53 protein (93.4%). Mutations of p53 leading to an amino acid substitution or a chain-terminating signal, and 5 (20%) were silent. Immunohistochemically, 55 of 84 cases (65.5%) showed overexpression of p53 protein, with no significant difference in frequency between the two areas. Missense mutations occurred in all four exons examined, most commonly in exon 5, but in no particular “hot spot.” In base-change spectra, all 12 mutations from Santiago showed transitions, with 4 arising at the CpG dinucleotide (33.3%). In contrast, no such transition was found at CpG sites in Niigata, and 4 of 13 mutations (30.8%) were transversions. The data indicated that p53 mutations are highly important in carcinogenesis in the gallbladder. In addition, the difference in p53 mutational spectra in Niigata and Santiago indicated a likely regional difference in mutagenesis.

Introduction

Although BTCs2 are relatively uncommon worldwide, their incidence shows considerable geographic variations. WHO has reported that Chile’s standardized mortality ratio for BTC was the highest in the world for both males and females from 1981 to 1986, occurring in marked excess in all regions of the country (1). Japanese standardized mortality ratios for BTCs were the world’s second highest for males and fifth highest for females with a steady increase in incidence (2). Among the 47 prefectures of Japan, Niigata represents a distinct area with the highest incidence of BTC (3, 4). Many epidemiological and clinical studies on GBC and extrahepatic bile duct cancer have been reported from both Chile and Niigata, but molecular aspects of the disease in these regions have not been described in detail.

Recent studies have proven that alteration of the p53 tumor suppressor gene is important in the development of various human cancers, with frequencies or spectra of p53 mutations varying between cancer types (5). Specific features have been displayed in patient populations geographically or occupationally at high risk for specific neoplasms. The most characteristic p53 mutational spectrum was derived from analysis of hepatocellular carcinomas linked to dietary aflatoxin exposure in high-incidence areas, particularly Qidong, China (6, 7). Such geographic variation have been seen in esophageal, breast, and other tumors (8, 9).

Along with other investigators, we have demonstrated frequent abnormal accumulation of p53 protein in adenocarcinomas of the gallbladder (10–12). However, studies of actual alterations of p53 have been limited in number and confined to Japan (13, 14). Therefore, features of the p53 mutational pattern in GBC remain elusive. In the present study, we examined 84 adenocarcinomas of the gallbladder from patients residing in Niigata, Japan or Santiago, Chile, describing the alterations of p53 in 42 cases, to disclose characteristics of p53 mutations in GBC and to identify any distinct mutational pattern or regional variation that might reflect a difference in mutagenesis of p53.

Materials and Methods

Tumor Specimens. From 49 adenocarcinomas of the gallbladder surgically resected from July 1989 to April 1994 at Sotero Del Río Hospital, Santiago, 20 cases were successfully extracted and amplified for DNA analysis (Chilean cases). Similarly, 22 surgical specimens were extracted and amplified among 37 adenocarcinomas of the gallbladder resected in hospitals in the Niigata prefecture from June 1982 to September 1996 (Japanese cases). The age and sex of patients are summarized in Table 1. All tissues studied were fixed in formalin and embedded in paraffin. Histological diagnosis and DNA preparation were performed in representative sections of the tumors. Histopathological diagnosis was made by examining 3-μm H&E sections according to the General Rules for Pathological Studies on Cancer of Biliary Tract of the Japanese Society of Biliary Surgery (15).

DNA Preparation. DNA extraction from paraffin sections was performed as follows. Tissues from five serial 10-μm sections were dewaxed for 5 min in two changes of xylene and rehydrated for 5 min in two changes of alcohol. Tissue dissec-

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1 To whom requests for reprints should be addressed, at First Department of Pathology, School of Medicine, Niigata University, Asahimachi-dori 1-757, Niigata 951, Japan. Fax: 81-25-223-0283.

2 The abbreviations used are: BTC, biliary tract cancer; GBC, gallbladder cancer.
tion was performed under a microscope to avoid contamination by noncancerous epithelium and stromal cells. More than 100 cells, which consisted of at least 50% carcinoma cells, were applied for DNA extraction using a DNA isolator PS kit (Wako, Osaka, Japan) according to the manufacturer’s instructions. The tissue was placed in a 0.5-ml microcentrifuge tube with 18 pA of sterile water. Alcohol successively with ethanol and was finally dissolved in 3 pA of DNA sample. The mixture was overlaid with mineral oil and subjected to PCR amplification. After 1 cycle of 94°C for 1 min, 55°C for 1 min 30 s, and 72°C for 1 min, 35 cycles of 94°C for 1 min, 60°C for 1 min 30 s, and 72°C for 1 min, a 7-min extension step proceeded at 72°C using the Program Temp Control System PC-700 (ASTEC, Fukuoka, Japan).

The product of the first PCR was used as the template for a second PCR, which was performed under the same conditions as the first, but one primer of each set was biotinylated at the 5’ end (Takara). In each experiment, control reactions containing no DNA sample or human placental DNA (Oncogene Science, Uniondale, NY) were performed simultaneously with sample reactions. The products of the second PCR were precipitated with ethanol and purified from 3% agarose gels (Nusieve 3:1 agarose; FMC, Rockland, ME) with a SUPREC 01 filter (Takara). In each experiment, control reactions containing no DNA sample or human placental DNA (Oncogene Science, Uniondale, NY) were performed simultaneously with sample reactions. The products of the second PCR were precipitated with ethanol and purified from 3% agarose gels (Nusieve 3:1 agarose; FMC, Rockland, ME) with a SUPREC 01 filter (Takara).

**Direct Sequencing.** All products of the second PCR were sequenced directly, using an Auto Load Solid Phase sequencing kit (Pharmacia Biotech, Uppsala, Sweden) and an automated laser fluorescent sequencer (A.L.F. DNA Sequencer II; Pharmacia). The same oligonucleotides as inner PCR primers were labeled fluorescently at their 5’ ends (Takara) and used for sequencing primers. To confirm the findings, we analyzed all samples at least twice, in sense and antisense directions.

**Immunohistochemistry.** Accumulations of p53 were detected immunohistochemically using mouse monoclonal antibody PAb1801 (Oncogene Science). A 3-μm-thick section from each case was stained by the streptavidin-peroxidase complex method. Cells positive for p53 were defined as those with a brown-stained nucleus, regardless of staining intensity. Four staining patterns were identified: diffuse, positive cells in most of the lesion; nested, positive cells aggregated in a focal area of the lesion; scattered, small numbers of isolated positive cells were scattered throughout the lesion; and negative. Overexpression of p53 protein was defined as diffuse or nested patterns, according to a previous study (10).

**Results**

**Clinical Characteristics.** Chilean patients were younger than Japanese patients, and the male:female ratio in Santiago was lower than in Niigata (Table 1). These differences were statis-
mal cells in tumor tissue samples or heterozygous mutations in the Japanese and Chilean groups, respectively. Some of the raw data showed that most of the mutations were missense or nonsense mutations, and 20 cases (20%) were mutations resulting in no amino acid change. Base changes were distributed throughout all exons examined, with 11 mutations (44%) concentrated in exon 5.

Table 2  

<table>
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<th>Base change</th>
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Niigata

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*Hist., histological; pap., papillary adenocarcinoma; wel., well-differentiated adenocarcinoma; mod., moderately differentiated adenocarcinoma; por., poorly differentiated adenocarcinoma.
*mRASSs, tumor extends to subserosal layer along Rokitanski-Aschoff Sinus; ss, tumor invades to subserosal layer; se, tumor invades beyond serosa; hinf1, tumor invades to liver extension 2 cm or less.

All 25 mutations were single base pair substitutions. Among them, 20 cases (80%) were missense or nonsense mutations, and 5 cases (20%) were mutations resulting in no amino acid change. Base changes were distributed throughout all exons examined, with 11 mutations (44%) concentrated in exon 5. However, we could not find any organ-specific or geographically specific mutational hot spots.

Table 3 shows the spectrum of detected mutations. Four of 13 mutations in the Niigata group (30.8%) were of transversion type, and no cases of transition were found at CpG sites. In contrast, all 12 mutations of the Santiago group were transitions, and 4 (33.3%) arose at the CpG dinucleotide. The difference in the frequency of transition at CpG sites between the two groups was statistically significant (P < 0.05, Fisher’s test).
p53 Mutations in Gallbladder Carcinomas

In the present study, we investigated p53 mutations in adeno-

Discussion

with nonsense mutations were negative for staining.

showed a diffuse staining pattern (Fig. 2), whereas both cases

depression in the 47 and 37 cases from each respective area (Table

Table 3  Base-change spectrum of p53 in adenocarcinoma of gallbladder

\[
\begin{array}{ccccccccc}
\text{Transition at CpG site} & & & & & & & & \\
\text{G:C} & \text{A:T} & \text{A:T} & \text{G:C} & \text{G:C} & \text{A:T} & \text{C:G} & \text{A:T} & \text{A:T} & \\
\text{Santiago, Chile (20)} & 55.0 & 10 & 2 & 4 & 33.3 & 0 & 0 & 0 & 0 \\
\text{Niigata, Japan (22)} & 50.0 & 5 & 4 & 0 & 0 & 0 & 0 & 0 & 0 \\
\text{Fuji et al.: Japan (23)} & 78.3 & 3 & 6 & 1 & 1 & 0 & 2 & 0 & 0 \\
\text{Takagi et al.: Japan (16)} & 31.3 & 1 & 2 & 0 & 0 & 1 & 0 & 0 & 0 \\
\end{array}
\]

\[\text{Numbers in parentheses, number of cases analyzed.}\]

\[\text{Not significantly different by } \chi^2 \text{ test.}\]

\[\text{Significantly different at the probability level of } P < 0.05 \text{ by Fisher's test.}\]

Immunohistochemistry. Immunohistochemically, we ob-
served p53 overexpression (diffuse or nested patterns) in 55 of
84 cases (65.5%). No significant difference was seen in fre-
quency of overexpression between tumors from Niigata
(73.0%) and Santiago (59.6%). Of the 42 cases with DNA
analysis, 28 (11 from Santiago and 17 from Niigata) showed
p53 overexpression, in line with overall frequency of overex-
pression in the 47 and 37 cases from each respective area (Table
1). Fifteen of 16 cases with a missense mutation of p53 (93.4%)
showed a diffuse staining pattern (Fig. 2), whereas both cases
with nonsense mutations were negative for staining.

Discussion

In the present study, we investigated p53 mutations in adeno-
carcinomas of the gallbladder from two geographic areas with

Fig. 2  Representative case of overexpressed p53 protein with diffuse pattern (CH15: Santiago, moderately differentiated adenocarcinoma) \(A\), H&E staining, and \(B\), immunostaining of p53 protein.

exceptionally high prevalence and compared features of the two
groups. Although case numbers were decreased by difficulties
with DNA preparation, the 20 informative specimens from
Santiago and 22 informative cases from Niigata were repre-
sentative of the total 84 cases from the viewpoints of age,
male:female ratio, and frequencies of p53 overexpression (Ta-
ble 1).

In the two regions, p53 alterations were observed equally
frequently (55.0% for Santiago and 50.0% for Niigata), as in
most other digestive tract carcinomas reported thus far (5).
Immunohistochemically detected p53 overexpression was com-
patible in frequency to a previous study (10). Tumors with p53
mutations corresponding to actual amino acid substitutions
showed a high correlation (93.4%) with overexpression of p53
protein. Therefore, our data suggested that p53 mutations are
markedly associated with the carcinogenesis of gallbladder in
both countries. Thirteen cases with a silent mutation or no
mutation in exons 5–8 showed overexpression of the protein,
which may have resulted from missense mutations in other
exons or from different pathways causing accumulation of p53
proteins in nuclei (16).

Although several studies have proposed possible risk fac-
tors for GBC, \(e.g.,\) prevalence of cholelithiasis or typhoid
carrier in Chile and some bile juice contents in Niigata, define
causes for the high incidence of this disease in Niigata and
Chile have not been clarified (1, 4, 17). From our study, we also
found neither specific mutational sites nor spectrum that could
be associated with any particular exogenous carcinogen in
either country. However, notable geographic differences with
etiological interest were seen in mutational spectra of p53.
Mutations in Niigata comprised transversions in 30.8% of oc-
currences (4 of 13), with 46.2% (6 of 13) of all Niigata muta-
tions taking place at the A:T pair. No Japanese case showed
G:C to A:T transition at CpG sites. These findings (Table 3) are
largely consistent with data reported previously by Fujii et al.
(14) and Takagi et al. (13). Therefore, common features, \(i.e.,\)
relatively frequent mutation at the A:T pair and infrequent
transition at CpG sites may be seen in \(p53\) mutations of Japa-
nese adenocarcinoma of the gallbladder in comparison to other
cancers, such as colorectal, gastric, and hepatocellular carcino-
mas (5, 14). In contrast, although the present case series is
relatively small, the mutational spectrum for the Santiago cases
is unique with a very high incidence of transitions (12 of 12)
and of mutations at G:C pairs (10 of 12). Furthermore, G:C to
A:T transition at CpG sites was relatively frequent (4 of 12).
Frequent transitions, especially at CpG sites, are features of
mutational spectra found in cancers not strongly linked to
specific exogenous carcinogens (5, 18). Transitions at CpG
sites are thought to be endogenous mutations caused by sponta-
neous deamination of 5-methylcytosine (19, 20). Among 39
CpG dinucleotides in the human \(p53\) coding region, codons
175, 213, 245, 248, 273, and 282 are known as hot spots resulting from endogenous mutational processes (7), and all four mutations at CpG from Santiago were found in these hot spots (Table 2). Therefore, geographic variation in mutagenesis of p53 might exist in adenocarcinoma of the gallbladder between Niigata patients and those from Santiago, considering also that the two groups differ in age and male:female ratio. Such geographic differences may reflect variation in carcinogenesis of the gallbladder.

In conclusion, p53 alterations evidently are important in the development of gallbladder carcinomas in the different high-prevalence areas, Niigata and Santiago, but differences in p53 mutational spectra between the two areas suggest regional variations in mutagenesis. Additional studies of more cases are needed in both areas or other areas of the world with high or low prevalence of gallbladder carcinomas.

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