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

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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 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-termination 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 correlated highly with overexpression of the p53 protein (93.4%). Mutations of *p53* 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 indicate a likely regional difference in mutagenesis.

Introduction

Although BTCs[2] 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 Rio 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. Histopathololological 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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[2] The abbreviations used are: BTC, biliary tract cancer; GBC, gallbladder cancer.

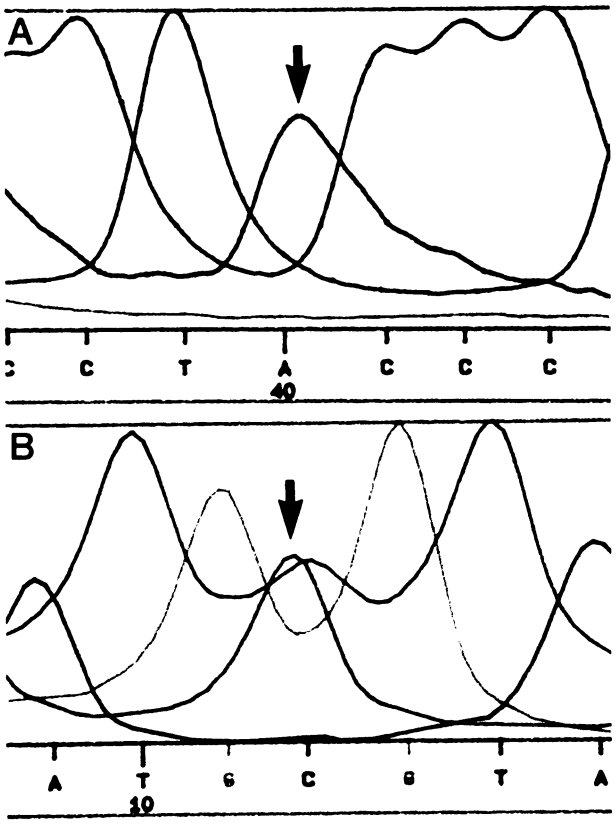

Fig. 1. Electrophoreograms of tumor sequencing. *A*, missense mutation of CH6; Santiago, at codon 141 (TGC→TAC) is shown with a prominent mutant signal. *B*, wild-type signal with mutant signal are seen in missense mutation of JP15; Niigata, at codon 238 (TGT→CGT).

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 μl of enzyme reaction solution and heated at 90°C for 10 min. After Proteinase and Enzyme Activator were added, it was incubated for 20 min to 2 h at 50°C. Enzyme reaction solution, Proteinese and Enzyme Activator are contents of the DNA isolator kit. Extracted DNA was precipitated with isopropyl alcohol successively with ethanol and was finally dissolved in 30 μl of sterile water.

PCR. Oligonucleotide primers were synthesized by Takara (Tokyo, Japan). Four fragments of DNA, including exons 5–8 of *p53*, were amplified by nested PCR using two sets of primers for each exon. The primers for the first PCR were as follows: exon5, 5′-AACTCTGTCTCCTTCCTCTT-3′ and 5′-AACCAGCCCTGTCGTCTCTC-3′; exon6, 5′-GCCTCTGATTCCTCACTGAT-3′ and 5′-TTAACCCCTCCTCCCAGAGA-3′; exon7, 5′-AGGTCTCCCCAAGGCGCACT-3′ and 5′-CAG GGT GGC AAG TGG CTC CT-3′; and exon8, 5′-TTCCTTACTGCCTCTTGCTT-3′ and 5′-AGGCATAACTGCACCCTTGG-3′. The following were the primers the second PCR: exon5, 5′-GTCTCCTTCCTCTTCCTACA-3′ and 5′-TGTCGTCTCTCCAGCCCCAG-3′; exon6, 5′-TCTGATTCCTCACTGATTGC-3′ and 5′-TCCTCCCAGAGACCCCAGTT-3′; exon7, 5′-AAGGCGCACTGGCCTCATCT-3′ and 5′-GGTGGCAAGTGGCTCCTGAC-3′; and exon8, 5′-CTTACTGCCTCTTGCTTCTC-3′ and 5′-CATAACTGCACCCTTGGTCT-3′.

The first PCR proceeded in a 50-μl reaction mix containing 50 mM potassium chloride, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 1.5 mM magnesium chloride, and 20 mM of each nucleotide (dATP, dCTP, dGTP, nad dTTP; Takara), 20 pM of each primer, 2.5 units of Taq polymerase (Promega, Madison, WI), and 3 μl 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, 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).

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).

Table 1 Backgrounds of patients and results of *p53* analysis

	Santiago (Chile)	Niigata (Japan)	*P*
Total numbers of cases	47	37	
Age (yr)	59.7 ± 13.8	68.2 ± 9.8	<0.05
Male:female	3:44	13:24	<0.01
p53 overexpression (%)	59.6 (28/47)	73.0 (27/37)	NS[a]
No. of DNA-analysis cases	20	22	
Age (yr)	59.3 ± 13.2	68.1 ± 8.7	<0.05
Male:female	1:19	8:14	<0.05
p53 overexpression (%)	55.0 (11/20)	77.3 (17/22)	NS
p53 gene alteration (%)	55.0 (11/20)	50.0 (11/22)	NS

[a] NS, not significant.

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-

Table 2 *p53* mutations in exon 5-8 in adenocarcinoma of gallbladder from Santiago and Niigata

	Age	Sex	Hist. type[a]	Depth[b]	p53 overexp.	Codon/Exon	Base change	Amino acid
Santiago								
CH1	47	F	por	ss	Diffuse	248 (7)	CGG → TGG	Arg → Trp
CH2	63	F	mod	ss	Negative	192 (6)	CAG → TAG	Gln → stop
CH3	64	F	mod	ss	Negative	213 (6)	CGA → TGA	Arg → stop
CH4	28	F	por	ss	Diffuse	151 (5)	CCC → CCT	Pro → Pro
						184 (5)	GAT → GAC	Asp → Asp
CH5	68	F	por	ss	Diffuse	No mutation		
CH6	85	F	por	ss	Diffuse	141 (5)	TGC → TAC	Cys → Tyr
CH7	62	F	wel	ss	Diffuse	No mutation		
CH8	63	F	wel	ss	Diffuse	280 (8)	AGA → AAA	Arg → Lys
CH9	47	F	mod	se	Diffuse	175 (5)	CGC → CAC	Arg → His
CH10	56	F	por	se	Negative	181 (5)	CGC → CGT	Arg → Arg
CH11	62	F	por	ss	Diffuse	127 (5)	TCC → TCT	Ser → Ser
CH12	71	F	mod	hinf1	Diffuse	141 (5)	TGC → CGC	Cys → Arg
CH13	62	M	por	ss	Diffuse	No mutation		
CH14	56	F	por	se	Negative	No mutation		
CH15	62	F	mod	ss	Diffuse	248 (7)	CGG → TGG	Arg → Trp
CH16	67	F	pap	se	Negative	No mutation		
CH17	34	F	mod	se	Negative	No mutation		
CH18	76	F	mod	ss	Scattered	No mutation		
CH19	62	F	por	hinf1	Negative	No mutation		
CH20	50	F	mod	ss	Negative	No mutation		
Niigata								
JP1	71	F	pap	mRASss	Diffuse	No mutation		
JP2	74	F	wel	ss	Diffuse	132 (5)	AAG → GAG	Lys → Glu
JP3	62	F	pap	ss	Negative	No mutation		
JP4	76	F	wel	ss	Diffuse	193 (6)	CAT → AAT	His → Asn
JP5	78	F	mod	ss	Diffuse	140 (5)	ACC → ATC	Thr → Ile
						166 (5)	TCA → ACA	Ser → Thr
JP6	70	F	wel	mRASss	Diffuse	276 (8)	GCC → CCC	Ala → Pro
JP7	71	F	wel	ss	Negative	No mutation		
JP8	79	F	wel	ss	Diffuse	No mutation		
JP9	66	F	pap	ss	Negative	No mutation		
JP10	60	F	pap	ss	Diffuse	No mutation		
JP11	80	M	pap	ss	Diffuse	No mutation		
JP12	63	M	pap	ss	Diffuse	294 (8)	GAG → GAA	Glu → Glu
JP13	49	F	pap	ss	Scattered	No mutation		
JP14	71	M	mod	ss	Diffuse	280 (8)	AGA → AAA	Arg → Lys
JP15	70	M	wel	ss	Diffuse	238 (7)	TGT → CGT	Cys → Arg
JP16	53	M	wel	ss	Diffuse	No mutation		
JP17	79	F	por	ss	Diffuse	271 (8)	GAG → AAG	Glu → Lys
JP18	62	M	por	ss	Negative	205 (6)	TAT → TGT	Tyr → Cys
JP19	61	M	wel	ss	Diffuse	No mutation		
JP20	69	M	mod	ss	Diffuse	231 (7)	ACC → ATC	Thr → Ile
JP21	58	F	mod	ss	Diffuse	160 (5)	ATG → GTG	Met → Val
						220 (6)	TAT → AAT	Tyr → Asn
JP22	76	F	por	ss	Diffuse	No mutation		

[a] Hist., histological; pap, papillary adenocarcinoma; wel, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma.

[b] 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.

tically significant (Fisher's test and Mann-Whitney's *U* test). The same tendencies persisted when comparisons were limited to the 42 tumors with DNA analysis.

DNA Analysis. Of the 42 cases analyzed, at least one *p53* mutation was found in 11 cases of Niigata and in 11 from Santiago (Table 2). The total frequency of *p53* mutations was 52.4%. No significant differences were evident in frequency between the two areas by a χ^2 test (50.0% for Niigata and 55.5% for Santiago). Numbers of mutations were 12 and 13 for the Japanese and Chilean groups, respectively. Some of the raw data showed mutant base signals with normal sequences at an approximately equal level, resulting from the presence of normal cells in tumor tissue samples or heterozygous mutations (Fig. 1).

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).

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

	% of mutation	Transition			Transversion				Deletion/Insertion
		G:C → A:T	A:T → G:C	at CpG site	G:C → T:A	G:C → C:G	A:T → C:G	A:T → T:A	
Santiago, Chile (20)[a]	55.0[b]	10	2	4 (33.3%)[c]	0	0	0	0	0
Niigata, Japan (22)[a]	50.0[b]	5	4	0 (0%)[c]	1	1	0	2	0
Fujii *et al.*: Japan (23)[a]	78.3	3	6	1 (4%)	4	4	6	2	0
Takagi *et al.*: Japan (16)[a]	31.3	1	2	0 (0%)	0	1	0	0	1

[a] Numbers in parentheses, number of cases analyzed.
[b] Not significantly different by χ^2 test.
[c] Significantly different at the probability level of $P < 0.05$ by Fisher's test.

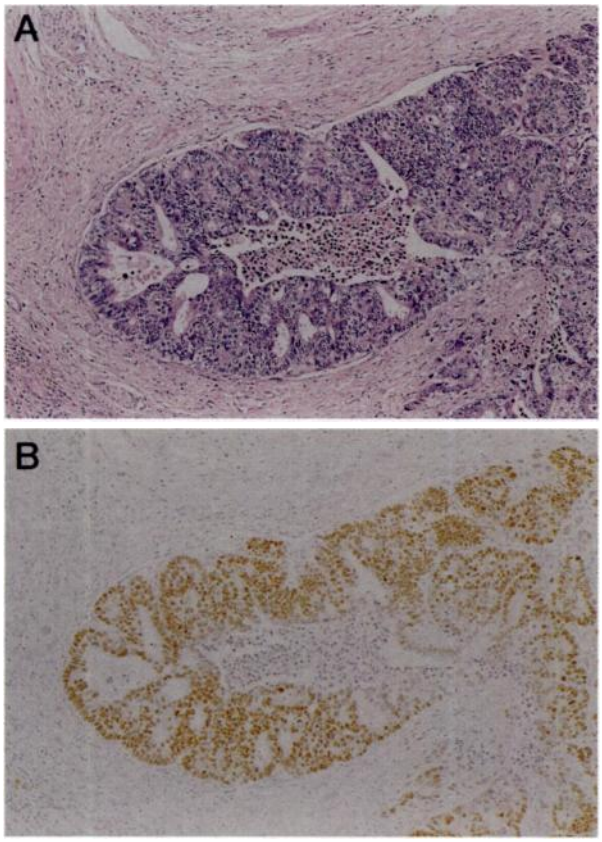

Fig. 2. Representative case of overexpressed p53 protein with diffuse pattern (CH15; Santiago, moderately differentiated adenocarcinoma). *A*, H&E staining, and *B*, immunostaining, of *p53* protein.

Immunohistochemistry. Immunohistochemically, we observed p53 overexpression (diffuse or nested patterns) in 55 of 84 cases (65.5%). No significant difference was seen in frequency 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 overexpression 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 adenocarcinomas of the gallbladder from two geographic areas with 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 representative of the total 84 cases from the viewpoints of age, male:female ratio, and frequencies of p53 overexpression (Table 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 compatible 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 factors for GBC, *e.g.*, prevalence of cholelithiasis or typhoid carrier in Chile and some bile juice contents in Niigata, definite 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 occurrences (4 of 13), with 46.2% (6 of 13) of all Niigata mutations 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 Japanese adenocarcinoma of the gallbladder in comparison to other cancers, such as colorectal, gastric, and hepatocellular carcinomas (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 spontaneous 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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