Sporadic *TP53* Transition Mutations in Chronic Cholecystitis Are Possibly Linked to Gallbladder Carcinogenesis

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Abstract. *Background/Aim: Gallbladder cancers are well known to frequently exhibit TP53 as well as K-ras gene mutations. This study performed a TP53 gene investigation by PCR-SSCP and direct sequencing using both bile supernatants and tissue samples from cholecystectomy specimens lacking gallbladder cancer, in order to investigate gallbladder carcinogenesis. Materials and Methods: Eighteen out of 294 cases, mainly of cholecystitis, were extracted by screening SSCP of bile supernatants for TP53 mutations, and investigation of their tissue samples both SSCP and direct sequencing. Results: Non-neoplastic mucosal samples demonstrated shifted bands in 11 cases (61%), and mutations were confirmed in 7 cases (64%). Unexpectedly, no cases showed identical point mutations in both bile and tissue samples. G:C to A:T transitions, thought to be sporadic mutations, predominated (77%). Conclusion: Sporadic TP53 transition mutations were demonstrated in non-neoplastic lesions such as severe cholecystitis, indicating an importance for a chronic cholecystitis-carcinoma sequence in gallbladder carcinogenesis.*

Gallbladder carcinoma is a malignancy known to have a poor prognosis because of the difficult in detection at early stages. Serum CA19-9 and computed imaging (such as magnetic resonance imaging) are often helpful but cannot be applied to determine a diagnosis. In particular, flat type carcinomas are almost impossible to diagnose (1). This study therefore developed a novel method for detection of *K-ras* mutations in supernatants of body fluids (ascites, pleural effusions, pancreatic juice and bile), pointing to utility for diagnosis complementary to cytologic examination (2, 3). *TP53* gene mutations are also well known to be frequent in gallbladder carcinomas (4, 5). Therefore, it is important to detect both gene mutations.

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Key Words: Gallbladder carcinoma, PCR-SSCP, direct sequencing, *TP53,* transition mutations, cholecystitis.

Recent studies have indicated a close relationship between chronic inflammation and neoplasia. Examples include inflammatory bowel disease in the colon (6), *Helicobacter pylori* (Hp)-associated gastritis in the stomach (7), and Hashimoto's disease with papillary thyroid carcinoma development (8). Gallbladder carcinomas are also frequently observed with a background of chronic inflammation, this being the case for 50% of *in situ* lesions as reported by Albores-Saavedra *et al*. (9). p53 and $p21^{WAF1}$ protein overexpression has been previously demonstrated in mucosal epithelial cells of severe chronic cholecystitis as well as background mucosa of gallbladder carcinoma, further suggesting that DNA damage caused by oxidative stress with inflammation is closely associated with gallbladder carcinogenesis (10). In addition, it has been reported that microsatellite instability (MSI) may already be present in severe chronic cholecystitis, with possible alteration in the mismatch repair system (for example in enzymes such as hMLH1 and hMSH2) (11). Other studies have demonstrated that MSI may often be evident not only in tumor tissues but also in non-neoplastic conditions, including intestinal metaplasia (12) and chronic gastritis (13) ulcerative colitis (14, 15) and chronic pancreatitis (16), suggesting a chronic inflammation link. Therefore, analysis of the DNA status in non-neoplastic gallbladder mucosa, especially in severe chronic cholecystitis, is relevant for gallbladder carcinogenesis.

The present study investigated *TP53* gene mutation in bile supernatant and samples of gallbladder mucosae, especially non-neoplastic epithelial cells, in order to investigate their significance for early gallbladder tumorigenesis.

Materials and Methods

A total of 325 cholecystectomy specimens without gallbladder cancer (including 104 gallstone or severe cholecystitis requiring surgical resection, 5 pancreatico-biliary malformations (PBMs), and a large number of other malignancies such as gastric, hepatocellular and pancreatic cancer) presenting at Kitasato University East Hospital from 1999-2002, were investigated. Before opening the specimens, bile was collected by aspiration syringe. In hematoxylin-eosin sections, the degree of mucosal chronic inflammation was classified using a modification of

previously published inflammation score (IS), defined as follows: IS0, no edematous change observed, but a few lymphocytes present in the lamina propria; IS1, mild lymphocytic infiltration with edematous change in the lamina propria; IS2, moderate lymphocytic infiltration, but lymph follicles not observed in the lamina propria; IS3, two or more lymph follicles per 5 mm of mucosa in length (10).

DNA extraction. DNA extraction from the supernatants of bile was performed according to routine procedures (17). Approximately 5 ml samples of bile were collected in 15 ml centrifuge tubes, diluted 2- to 5-fold with PBS (pH 7.6), and then separated into supernatant and sediment components by centrifugation at 2,000 rpm for 10 min. Five ml aliquots of supernatant were gently mixed with 5 ml of ethyl alcohol to prevent DNA degradation. From each sample, a precipitated pellet was then produced by centrifugation, followed by DNA extraction after addition of 1 ml of distilled water. One ml aliquots of supernatant were transferred to 1.5 ml centrifuge tubes, mixed with 100 μl of lysis buffer (100 mM Tris-HCl pH 8.7, 500 mM KCl, 3 mg/ml protein kinase K, 4.5% NONIDET P-40 (Nacalai Tesque, Kyoto, Japan), 4.5% polysorbate 20 (Tween-20), 20 mM EDTA-2Na), and incubated at 37˚C overnight after preincubation at 55˚C for 3 hours. Each sample was then purified with phenol-chloroform, precipitated with ethyl alcohol, and resuspended in 50 μl of distilled water.

DNA extraction from formalin-fixed, paraffin-embedded tissue was as follows; after deparaffinization, mucosal epithelial cells were microdissected from three serial 15-μm-thick sections under a stereomicroscope, and cellular DNA was extracted through proteinase K/phenol-chloroform treatment. Hyperplastic mucosal lesions with or without nuclear atypia were primarily dissected.

PCR-SSCP and direct sequencing. The entire coding region (from exon 5 to exon 9) of human genomic *TP53* was amplified by single PCR with the FastStart High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany). The primer sets were as follows: exon 5, forward 5'-TTCCTCTTCCTGCAGTACTC-3' and reverse 5'-GCCCCAGCTGCTCACCATCGCTA-3'; exon 6, 5'-GCCTCTGATTCCTCACTGATTG-3' and 5'-AGTTGCAAACCAG ACCTCAG-3'; exon 7, 5'-CCTCATCTTGGGCCTGTGTTATC-3' and 5'-CAAGTGGCTCCTGACCTGGAGTC-3'; exon 8, 5'-CCTAT CCTGAGTAGTGGTAA-3' and 5'-GTCCTGCTTGCTTACCTCGC-3'; exon 9, 5'-CCTTTCCTTGCCTCTTTCCTAG-3' and 5'-CCACTTGATAAGAGGTCCCAAGAC-3', respectively. PCR procedures were performed with 35 cycles of denaturation at 96˚C for 30 s, annealing at 55˚C for 60 s and extension at 72˚C for 60 s, with a pre-denaturing time of 5 min and a final extension time of 5 min. As a negative control, distilled water was used instead of template DNA for each examination. Three percent agarose gels were employed to determine amplified product amounts and artifact bands. The PCR products were denatured at 96˚C for 15 min and electrophoresed in gels for SSCP and visualized by silver staining (employing a Gene Gel Excel 12.5/24 kit and a silver staining kit, Amersham Pharmacia Biotech AB, Uppsala, Sweden). For screening SSCP, partially multiplex PCR (exons 8 and 9) was applied. All cases with shifted bands, as well as some cases without by SSCP, were selected for DNA direct sequencing, using a BigDye Terminator ver. 3.1 Cycle Sequencing Kit and a 3100-Avant Genetic analyzer (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's protocol. All *TP53* point mutations were confirmed with both forward and reverse primer pairs at least twice.

Immunohistochemistry. A streptavidin-biotin immunoperoxidase complex method using a commercial kit (LSAB2/HRP, DAKO, Carpinteria, CA, USA) was applied. Histologic sections (4 μm thick) were deparaffinized and heated in citrate buffer solution (0.01 M, pH=6.0) for five 3-min cycles using a microwave-oven to retrieve antigens. Endogenous peroxidase activity was inhibited by incubation with 0.3% H_2O_2 in methanol for 30 min at room temperature. After non-specific reactions were blocked with 10% normal porcine serum, the sections were incubated with the primary antibodies, polyclonal anti-Ki-67 (1/200 diluted; DAKO, Glostrup, Denmark) and monoclonal anti-p53 (clone DO7; 1/200 diluted, Novocastra Laboratories, Newcastle upon Tyne, UK) at 4˚C overnight. After washing in 0.01M PBS, the slides were incubated with a biotinylated second antibody for 30 min, and finally incubated with streptavidin-peroxidase for 30 min. For color development, 0.05% 3-3' diaminobendizine was used as the final chromogen and nuclear counter staining was achieved with 0.3% methyl green solution.

Statistical analysis. The frequencies of *TP53* mutation and clinico-pathological factors were tested with the χ^2 test method. $P<0.05$ was regarded as statistically significant. All statistical analyses were performed on a personal computer using Statview software version 5 (Abacus Concepts, Berkeley, CA, USA).

Ethics. Samples were obtained from Kitasato University East Hospital with written informed consent of all patients, and the design was approved by the Medical School and University Hospital Ethics Committee (No. B01-26).

Results

Of the 325 investigated bile samples, *TP53* could be amplified successfully by PCR in 294 cases. Screening SSCP showed shifted bands in 18 (6%) of these. These corresponding 18 cholecystectomy specimens were formalin-fixed to give paraffin embedded sections and 1 to 25 mucosal lesions in each case (10.3±6.2 lesions, average±standard deviation) were microscopically dissected and investigated. As a positive control, identical *TP53* point mutations in both bile and tissue samples were confirmed in 2 out of 5 gallbladder carcinomas with diffuse p53 overexpression (Table I, Figure 1). In addition, five normal mucosal samples from five cholecystectomy specimens without any shifted bands by screening bile SSCP were applied as negative controls. A summary of the results is shown in Table I. The five PBMs, except one, were negative for mutation by screening bile SSCP. Second SSCP was able to detect *TP53* mutations in 11 (61%) out of 18 bile supernatants. In addition, direct sequencing showed various *TP53* point mutations in 5 (45%) of these cases. In tissue samples, shifted bands were detected in 11 (61%) out of 18 cases by SSCP, and in 7 (64%) of these cases, mutations were confirmed by direct sequencing. Therefore, *TP53* point mutations were found in 2% (7/294) of the cholecystectomy specimens. All point mutation cases demonstrated by direct sequencing consistently showed aberrant shifted bands by SSCP.

Table I. *Summary of TP53 mutation of bile supernatants and cholecystectomy specimens.*

Case	Age (years)		Clinical diagnosis	Gallstone	IS	Bile supernatant			Tissue	
						Screening SSCP	2nd SSCP	Sequence	SSCP	Sequence
1	65	M	Cholecystitis	+	1	Exon 9	+	-	-	-
2	64	M	Cholecystitis	+	1	Exon 8	-	-	-	-
3	31	F	Cholecystitis with PBM	-	1	Exon 8	-	-	+	-
4	48	M	Cholecystitis	+	3	Exon 8	-	-	-	-
5	60	M	Hepatocellular ca.	-	1	Exon 7	+	-	+	248: CGG-TGG (Arg-Trp)
6	29	F	Cholecystitis	+	1	Exon 8	+	282: CGG-TGG (Arg-Trp)	+	-
7	59	M	Gastric ca.	-	1	Exon 8	+	282: CGG-TGG (Arg-Trp)	+	-
8	54	M	Cholecystitis	-	1	Exon 9	-	-	-	-
9	75	M	Liver meta. (leiomyosarcoma)	-	1	Exon 7	+	-	-	-
10	43	F	Cholecystitis	+	3	Exon 6	+	221: GAG-AAG (Glu-Lys)	+	217: GTG-ATG (Val-Met)
11	49	F	Cholecystitis	+	1	Exon 7	+	-	+	243: ATG-ACG (Met-Thr)
12	67	M	Cholecystitis	+	3	Exon 5	-	-	+	-
13	58	M	Liver meta.(rectal ca.)	-	0	Exon 9	+	329: ACC-ATC (Thr-Ile)	+	318: CCA-CTA (Pro-Leu)
14	89	M	Cholecystitis	+	3	Exon 7	+	-	+	various (see Figure 4)
15	70	M	Hepatocellular ca.	-	1	Exon 7	+	248: CGG-TGG (Arg-Trp)	+	247: AAC-AGC (Asn-Ser) 255: ATC-ATT (silent)
16	58	F	Cholecystitis	+	2	Exon 5	+	-	+	142: CCT-TCT (Pro-Ser) 165: CAG-CGG (Gln-Arg)
17	32	M	Cholecystitis	+	2	Exon 6	-	-	-	-
18	74	F	Rectal ca.	-	1	Exon 7	-	-	-	-
							11/18 (61%)	5/18 (28%)	11/18 (61%)	7/18 (39%)
C1	51	F	Gallbladder ca. (focal)	-		-	-	-	-	-
C2	80	F	Gallbladder ca. (-)	+		-	-	-	-	-
C3	64	F	Gallbladder ca. (diffuse)	-		-	-	-	-	-
C4	67	M	Gallbladder ca. (diffuse)	-		Exon 6	+	194: CTT-CGT (Leu-Arg)	+	194: CTT-CGT (Leu-Arg)
C5	59	F	Gallbladder ca. (diffuse) (p53 overexpression)	+		Exon 7	+	236: TAC-TGC (Tyr-Cys)	+	236: TAC-TGC (Tyr-Cys)

IS: Inflammatory score; PBM: pancreatico-biliary malformation.

However, the SSCP results did not always exhibit consistency between bile and tissue samples, and unexpectedly, identical point mutations could not be detected in both of them in any case. The detected *TP53* point mutation codons compared with International Agency for Research on Cancer (IARC) *TP53* mutation database (http://www-p53.iarc.fr) are shown in Figure 2. In the present study, exon 7 point mutations accounted for 65% (17/26). Seven were in hotspots (codons 245, 248, 249 and 282) including CpG islands (codons 248 and 282). All were missense except for 6 silent mutations. G:C to A:T transitions predominated (20/26, 77%), with only 2 cases of transversion mutations (Figure 3).

Slightly serrated or compact tubular proliferated gallbladder mucosal foci, with or without mild nuclear atypia, occasionally showed *TP53* mutations by SSCP or direct sequencing (Figure 4). However, the modified ISs between the 18 cases and the other cases were not significant (1.5±0.9 and 1.4±0.8, respectively, p=0.777). Gallbladder epithelium in inflamed mucosae usually showed a few scattered positive reactions for Ki-67 and p53 immunohistochemically (Figure 5), and mutated lesions did not exhibit significantly elevated labeling indices (data not shown). Dissected lesions with hotspot mutations did not show any particular histological features, and there was no correlation between *TP53* status and Ki-67/p53 immunoreactions.

Discussion

It has been previously demonstrated that *K-ras* SSCP with supernatants of peritoneal or pleural effusions and pancreatic juice could add supplementary information to cytologic examination for malignancy (2, 3). Especially in pancreatic carcinomas, SSCP analysis identified *K-ras* point mutations in addition to cytologic diagnoses of malignancy in pancreatic juice as well as in ascites (3). Furthermore, use of supernatants has the advantage that cytologic examination is also possible. From the present results this method is also useful with bile supernatants and may help detect even flat dysplasia of the gallbladder, a pre-cancerous lesion almost undetectable by computed imaging.

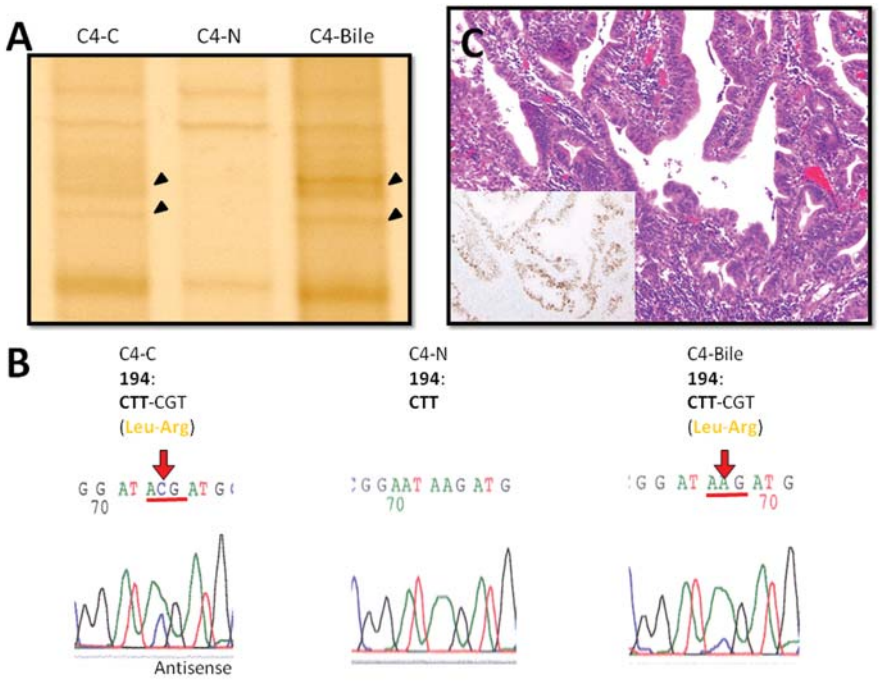

Figure 1. *TP53 SSCP (A), direct sequence (B) and histology (C) of gallbladder carcinoma case (C4) with diffuse p53 overexpression (inset, C). Both carcinoma tissue and bile sample showed identical point mutation. Normal control (C4-N) was extracted from muscularis propria and subserosal tissue of gallbladder.*

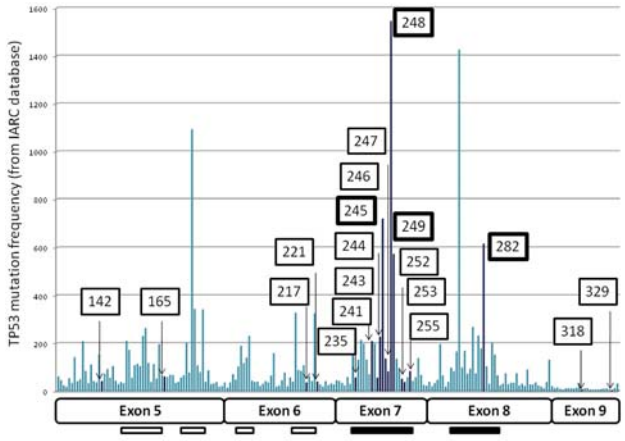

Figure 2. *Comparison of mutated codon distribution between our result and the IARC TP53 mutation database. Numbers indicate mutated codons, and bold boxes (codon 245, 248, 249 and 282) indicate hotspot codons. White and black underbars indicate 'mutation hotspots'.*

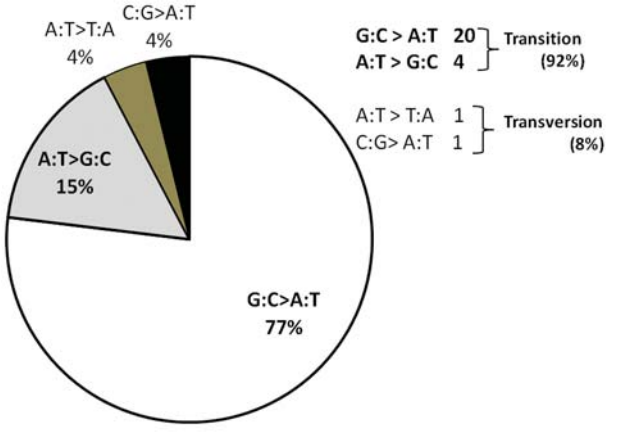

Figure 3. *TP53 point mutation spectra of the bile supernatants and cholecystectomy specimens. G:C to A:T transition mutation occupied 77% of all.*

It has been previously demonstrated that *K-ras* or *TP53* point mutations are often observed in non-neoplastic gallbladder mucosae (18), especially with PBMs (19, 20), as well as gallbladder carcinomas. Indeed, in the present study, it was possible to find *TP53* missense mutations in non-neoplastic gallbladder mucosae as well as in bile supernatants. While *K-ras* mutations were also investigated (data not shown), it proved difficult to assess the results because of extreme differences in sensitivity between SSCP and direct sequencing.

Figure 2 illustrates a comparison between the current results and the IARC *TP53* mutation database. With the exception of some codons (such as 245, 248, 249 and 282), both bile

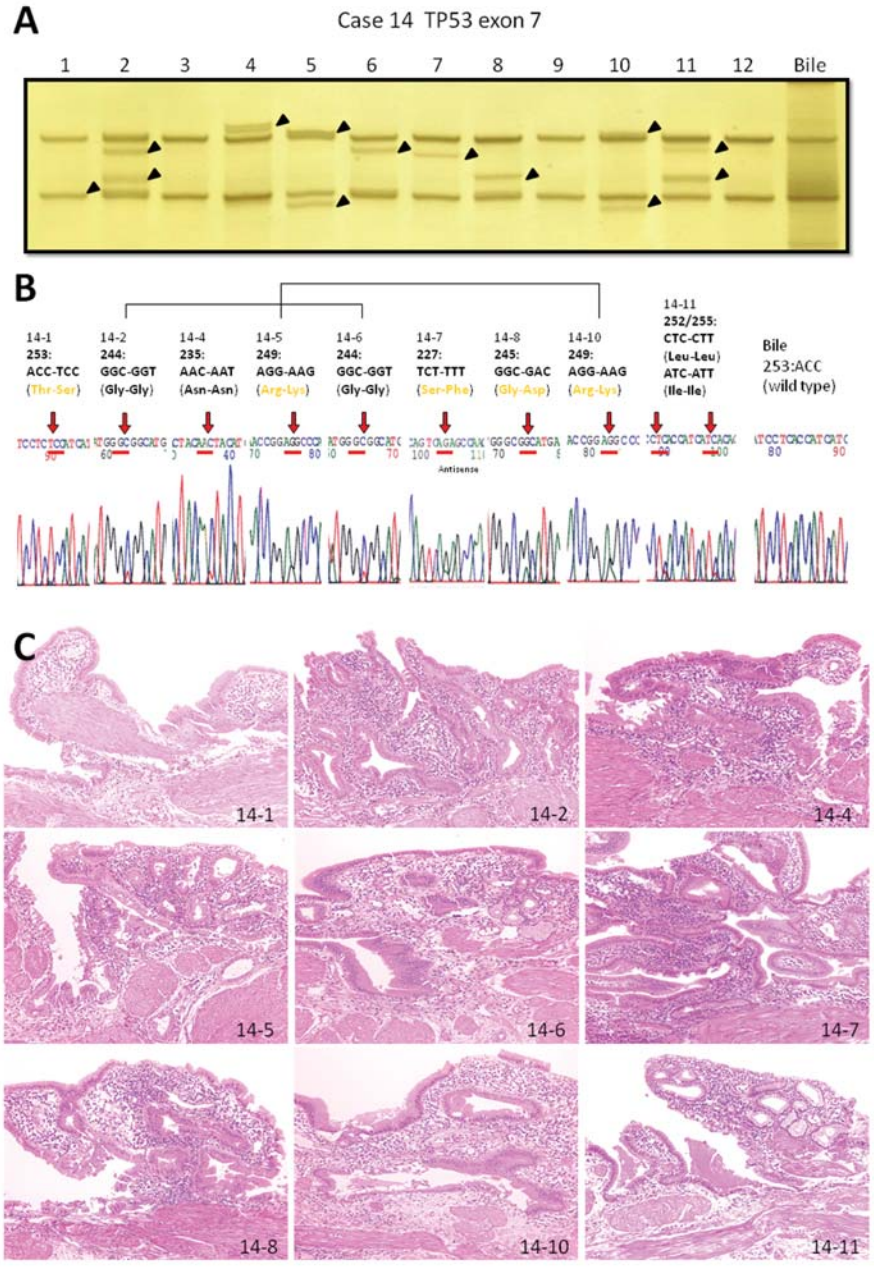

Figure 4. *TP53 SSCP (A), direct sequence (B) and histology (C) of both bile supernatants and cholecystectomy specimen. Case 14 showed various point mutations of exon 7 in tissue samples of cholecystitis by both SSCP (A) and direct sequence (B), but not by direct sequence in bile supernatant (B). Mutation results were confirmed by repeating at least two times, and confirmed by forward and reverse primer pairs. Histologies of lesions with TP53 mutations: Slightly serrated or compact tubular proliferation in gallbladder mucosa with or without mild nuclear atypia (C).*

supernatants and tissues showed point mutations that were not regarded as belonging to mutation hotspots. A previous study pointed out that more than 50% of the rare spot *TP53* mutations different from hotspots (21), indicating that they may not be important for functions. Indeed, in this study it was not possible to find a significant correlation between the mutations and immunoreactions for Ki-67 and p53. Therefore, rare spot *TP53* point mutations might not be associated directly with development of gallbladder neoplasia, and chronic inflammation-associated carcinogenesis might require initiators like exogenous carcinogens.

Moreno *et al*. demonstrated *TP53* point mutation of the gallbladder not only carcinomas but also non-neoplastic mucosae in Chilean patients (18). They also described G:C to

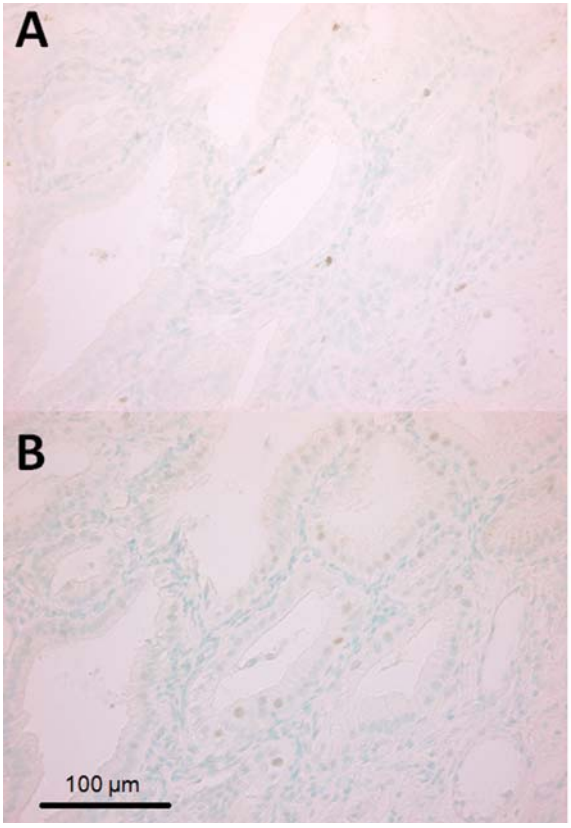

Figure 5. *Immunoreactivity for Ki-67 (A) and p53 (B) in serial sections of gallbladder mucosa (Case 5) with TP53 exon 7 point mutation. Sporadic positive cells are present (×200).*

A:T transitions to be the most common mutation, in line with the current results. Interestingly, previous reports of *TP53* point mutations in gallbladder cancer similarly referred to many transition mutations (67-100%) rather than transversion mutations (4, 5, 18, 22, 23). In contrast, lung tumors of smokers frequently demonstrate the latter transversion mutation (24, 25), suggesting a link with exogenous carcinogen exposure. G:C to A:T transition mutations might be endogenous *TP53* mutations caused by spontaneous deamination of 5-methylcytosine (26). Yoshida *et al*. previously found polyclonal *TP53* mutations in regenerative crypts and low-grade dysplasia, but monoclonal changes in high-grade dysplasia or carcinoma in cases of long-standing ulcerative colitis (27). Interestingly, they also described G:C to A:T transition mutations to account for the vast majority. Furthermore, Hp-associated gastritis features multiple *TP53* point mutations, mainly involving transition mutations at exons 7 and 8 (28). Taking the current results into consideration, it is strongly suggested that chronic inflammation cause polyclonal transition mutations. Non-neoplastic lesions may not have stable *TP53* abnormalities, differing from malignant tumors.

In the present study, various serrated hyperplastic mucosae or compact tubular lesions occasionally showed *TP53* mutations. Although there was no clear association between inflammatory scores and *TP53* mutations, sporadic *TP53* point mutations were found in the cholecystectomy specimens diagnosed mainly as gallstones or severe cholecystitis. Additionally, it has previously been demonstrated that even non-neoplastic mucosa in severe chronic cholecystitis demonstrate MSI as well as high p53 and $p21^{WAF1}$ overexpression, indicating a strong chronic inflammation-carcinoma link in the gallbladder (10, 11). Recently, two carcinogenesis models for gallbladder have become established: the flat dysplasia-carcinoma and the adenoma-carcinoma sequences. It has previously been argued that gallbladder adenomas differ from carcinomas from beta-catenin mutation analyses (29), indicating that flat dysplasia may be more important for gallbladder carcinogenesis. Additional reports from morphological and molecular studies have supported this theory (30). The method using bile supernatant might be useful to detect flat tumors of the gallbladder even in pre-cancerous stages.

The discrepancy in the results between bile supernatants and tissues were unexpected. One possibility for the discrepancy is that responsible non-neoplastic epithelial cells might be shed or eliminated quickly by biological self-defense systems. A previous study reported that *K-ras* and *TP53* mutations in both biopsies and bile were detected only in biliary tract carcinomas but not in non-neoplastic lesions (4).

The discrepancy in the results between SSCP and directing sequence also require discussion in this context. All *TP53* point mutations found by direct sequencing were associated with aberrant shifted bands by SSCP, but shifted bands could not always be confirmed to reflect point mutations (bile 5/11; tissue 7/11, respectively). This is presumably due to differences in sensitivity. Previous authors have also demonstrated that SSCP was able to detect around 90% of DNA point mutations using human DNA samples (31). Additionally, the aberrant shifted bands that were found in SSCP in the current study often showed very weak densities.

In conclusion, *TP53* mutation analyses of bile supernatants using SSCP and direct sequence might predict pre-neoplastic lesions especially in severe chronic cholecystitis, and indicate risk of gallbladder carcinoma. This is the first reported analysis of a large number of non-neoplastic gallbladder mucosae, and sporadic *TP53* transition mutations were found to occur relatively frequently in severe cholecystitis. A chronic cholecystitis-carcinoma sequence appears to be the most important pathway in gallbladder carcinogenesis.

Acknowledgements

We would like to thank Ms. T. Tsuruta, Ms. Y. Numata and Mr. M. Yokozawa for technical assistance. This work was supported by a Grant-in-Aid for Young Scientists (No.18790253), by a Kitasato University Research Grant for Young Researchers (N. Yanagisawa) and by the Kanagawa Nanbyo Foundation (I. Okayasu).

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Received August 24, 2010
Revised October 14, 2010
Accepted October 15, 2010