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p53 Mutation in Gallbladder Carcinoma with an Anomalous Junction of the Pancreaticobiliary Duct

Kazutoshi DATE[1], Hidenobu WATANABE[1], Youichi AJIOKA[1], Yoshio SHIRAI[2], Naoyuki YOKOYAMA[2], Tatuya YOSHIKAWA[3], Kazuhiko KASUYA[4], Yasuhisa KOYANAGI[4] and Katsuyoshi HATAKEYAMA[2]

[1]Divison of Molecular and Diagnostic Pathology, [2]The First Department of Surgery, Niigata University School of Medicine, Niigata; [3]Department of Gastroenterology Surgery, Tokyo Women's Medical College; [4]Third Department of Surgery, Tokyo Medical College, Tokyo, Japan

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Summary. **The purpose of this study is to clarify whether or not *p53* gene mutations differ between gallbladder carcinoma (GBC) with an anomalous junction of the pancreaticobiliary duct (AJPBD) and GBC without AJPBD. We examined 17 GBCs with AJPBD and 22 GBCs without AJPBD. Immunohistochemically, the frequency of p53 protein over-expression was 64.7% in GBCs with AJPBD and 77.3 % in GBCs without AJPBD. *p53* mutations were found in 47.1% of GBCs with AJPBD and 50.0% of GBCs without AJPBD. The mutations were distributed from exons 5 to 8 without a preponderance in certain exons or specific mutational hot spots in either group. All mutations of GBCs with AJPBD were G: C to A: T transition, and 27.3% occurred at the CpG site. For GBCs without AJPBD, 30.3% of mutations were transversion, and mutations did not occur at the CpG site. These results indicate differences in the mutagenesis of *p53* gene alteration between GBCs with AJPBD and GBCs without AJPBD.**

Key words—anomalous junction of the pancreaticobiliary duct, gallbladder carcinoma, *p53*.

INTRODUCTION

An anomalous junction of the pancreaticobiliary duct (AJPBD) is suspected to be a high risk factor for gallbladder carcinoma (GBC) and bile duct carcinoma in Japan[1,2]. Moreover, the mean age of GBC-patients with AJPBD is approximately 10 years younger than that of GBC-patients without AJPBD[3]. In AJPBD, the junction of the pancreatic and common bile duct is present outside the duodenal wall. Consequently, pancreatic juice continuously refluxes into the biliary tract and injures the biliary tract epithelia with subsequent reactive epithelial hyperplasia of the gallbladder, which is one of the characteristic changes in AJPBD[4-6]. According to previous studies, this epithelial change is accompanied with increased cellular kinetics and correlates with the development of gallbladder carcinoma[7-11]. Moreover, *K-ras* point mutations are higher in GBCs with AJPBD than in GBCs without AJPBD[8,10,12,13].

Recent studies have revealed that some groups of cancers show specific frequency and alternation patterns of the *p53* gene. Specific characteristics of *p53* mutations have been found in hepatocellular, esophageal, breast, and other carcinomas. These *p53* mutation spectra are strongly linked to specific exogenous and endogenous carcinogens[14].

Several studies have reported frequencies and alteration patterns of *p53* in GBCs without AJPBD[15,16]. For GBCs with AJPBD, two studies were done to investigate the frequencies of *p53* mutations by utilizing polymerase chain reaction-single strand conformation polymorphism analysis (SSCP)[17,18]. Only one study investigated the alteration pattern of *p53* mutations by performing direct deoxynucleotide sequencing[18]. In this study, we compared *p53* mutations in GBCs with and without AJPBD.

MATERIALS AND METHODS

Materials

The samples were 17 surgically resected GBCs with AJPBD and 22 GBCs without AJPBD. These were

Correspondence: Kazutoshi Date, Division of Molecular and Diagnostic Pathology, Niigata University School of Medicine, Asahimachi-dori 1, Niigata 951-8122, Japan.

Table 1. Clinical backgrounds of patients and results of p53 analysis

	GBC with AJPBD	GBC without AJPBD	*P*
Total numbers of cases	17	22	
Age (yr)	59.5±10.3	68.7±8.6	<0.01
Male: Female	2 : 15	7 : 15	NS
P53 overexpression (%)	64.7 (11/17)	77.3 (17/22)	NS
p53 gene alteration (%)	47.1 (8/17)	50.0 (11/22)	NS

NS, Not significant; GBC, gallbladder carcinoma; AJPBD, anomalous junction of the pancreaticobiliary duct.

obtained from the archives of the First Department of Pathology, Niigata University School of Medicine, Department Gastroenterology Surgery, Tokyo Women's Medical College, and Third Department of Surgery, Tokyo Medical College, from June 1982 to September 1996. All patients underwent preoperative endoscopic retrograde cholangiopancreatography to diagnose AJPBD. The age and sex of patients are summarized in Table 1. The 22 GBCs without AJPBD have been reported previously[16].

All resected specimens were fixed in 10% buffered formalin solution. In all cases, the entire gallbladder was cut stepwise in 3~5 mm-thick slices and embedded in paraffin according to the General Rules for Pathological Studies on Cancer of the Biliary Tract of the Japanese Society of Biliary Surgery[19]. After histological examination, one or two representative paraffin blocks of each tumor were selected for DNA preparation.

Immunohistochemical staining of p53 protein

Three serial 3 μm thick sections, 5~10 serial 10 μm sections, and a last 3 μm section were cut from the representative paraffin blocks. The first three sections were stained with hematoxylin-eosin (H-E), p53-protein and Ki-67 immunostaining respectively. The middle section was prepared for DNA extraction, and the last section was stained with H-E. The p53 and Ki-67 immunostains employed mouse monoclonal antihuman p53 protein antibody PAB-1801 (Oncogene Science Inc., Manhasset, NY, USA) and Ki-67 mouse monoclonal antibody MIB-1 (Immunotech S.A., Marseille, France), respectively, with the streptavidin-peroxidase complex method. The Ki-67 positive cells were well preserved in inner control areas such as the germinal center of the lymph follicles and the metaplastic epithelium of the gallbladder. Thus, these selected sections were evaluated as allowing the value of p53 immunostaining. There were four staining patterns for p53 immunohistochemistry: diffuse (most of the tumor cells were positive in the lesion), nested (positive cells were aggregated in focal area(s) of the lesion), scattered (small numbers of isolated positive cells were scattered throughout the lesion), and negative (no positive cells in the lesion). Diffuse or nested staining patterns were regarded as indicative of p53 protein over-expression according to our previous study[20].

DNA preparation

DNA was extracted from the 5-10 serial 10 μm thick paraffin sections cut between the first three sections and the final section. Tissue dissection was performed under a microscope to avoid contamination by noncancerous cells and stromal cells and to obtain p53 over-expression areas in cases with a p53 nested pattern. More than 100 cells, which consisted of at least 50% carcinoma cells, were applied to DNA extraction using a DNA isolator PS kit (Wako, Osaka, Japan) following the manufacturer's protocol. Extracted DNA was finally dissolved in 30 μl of sterile water.

Polymerase chain reaction (PCR)

Four fragments of the *p53* gene, exon 5 (codons 126-186), exon 6 (codons 187-224), exon 7 (codons 225-261), and exon 8 (codons 262-306), were amplified by nested PCR using two sets of primers for each exon as described previously[16]. A second set of PCR reactions was performed using one primer biotinylated at the 5' end (Takara Shuzo Co., Ltd., Otsu, Japan.). In each PCR run, samples without DNA and human placental DNA (Oncogene Science, Uniondale, NY, USA) were used as negative and positive controls, respectively. The products of the second PCR reaction were purified using a Mermaid Kit (Bio 101, La Jolla, CA) or precipitated with ethanol and purified using a SUPREC-01 (Takara), and dissolved in 50 μl distilled water.

Direct sequencing

All products of the second PCR reaction were sequenced directly, using an Auto Load Solid Phase sequencing kit (Phalmacia Biotech, Uppsala, Sweden) and an automated laser fluorescent sequencer (A.L.F. DNA Sequencer II; Phalmacia). The same oligonucleotides at the inner PCR primers were labeled fluorescently at their 5' ends (Takara). For confirmation of the results, all samples were analyzed at least twice, in sense and antisense directions.

RESULTS

Clinical background

Patients with GBCs with AJPBD were significantly younger than those without AJPBD (Mann-Whitney's U test). The male: female ratio of GBC-patients without AJPBD was higher than those with AJPBD (trend by Fisher's exact test) (Table 1).

DNA analysis

p53 mutations were found in 47% of GBCs with AJPBD and 50% of GBCs without AJPBD. All mutations were single base pair substitutions (Table 2). The mutations were found throughout exons 5 to 8 in GBCs with and without AJPBD. No specific mutational hot spot was found in either group. All of the GBCs with AJPBD showed transition type mutations alone. However, GBCs without AJPBD revealed 69% with transition type and 31% with transversion type mutations. G: C to A: T transition type mutations were found in all of the GBCs with AJPBD and in 39% of GBCs without AJPBD. A mutation at the CpG dinucleotide was detected in three cases with AJPBD (27%), but not in any of the GBCs without AJPBD. In case W9, three base pair substitutions were detected at exon 7 and exon 8 (Fig 1). The base substitutions of two cases (W1 and W2) and one of the substitutions in case W9 were mutations without amino acid substitution (silent mutation). All of the other base substitutions of GBCs with and without AJPBD were missense mutations.

Immunohistochemistry

Immunohistochemically, the frequency of p53 protein over-expression did not significantly differ between GBCs with AJPBD (52.9%) and GBCs without AJPBD (77.3%) (Table 1). Out of 11 p53-overexpressing cases of GBCs with AJPBD, 6 cases showed missense mutations, 3 cases showed silent mutations (one case had missense mutations in other areas), and 5 cases showed no mutations in the exons 5~8 (Table 2). Two of six p53-nonoverexpressing GBCs with AJPBD had missense mutations, and the remaining 4 did not show mutations in exons 5~8. In GBCs without AJPBD, there were 17 p53-overexpressing cases. Ten of these exhibited missense mutations, but no mutations were in the remaining 7 (Table 2). Of the 5 p53-nonoverexpressing cases, one revealed missense mutations, but the remaining 4 did not reveal any mutations.

DISCUSSION

AJPBD is thought to be a high risk factor for GBC in Japan[1]. Furthermore, GBCs with AJPBD and GBCs without AJPBD are each reported to arise from different background non-neoplastic mucosa. Specifically, more than half of all GBCs without AJPBD originate from metaplastic mucosa[21,22,23] and GBCs with AJPBD mainly arise from hyperplasia of the native gallbladder epithelium[4,5]. The proliferative activity of non-neoplastic mucosa is noted to be higher in hyperplastic mucosa of the gallbladder with AJPBD than in the gallbladder mucosa without AJPBD[9,10]. These results suggest that GBCs with AJPBD probably originate from hyperplastic epithelium of the native epithelium. These hyperplastic changes with increased cellular kinetics may play an important role in the carcinogenesis of the gallbladder with AJPBD.

AJPBD causes a free reflux of pancreatic juice into the biliary tract with a subsequent mixture of pancreatic juice and bile that produces substances hazardous to the gallbladder epithelium. However, specific mutagens in GBCs with and without AJPBD have yet to be determined. Pancreatic enzymes (e.g., phospholipase A2, elastase 1, and trypsin) and secondary bile acids, which are elevated in the bile of the biliary duct and gallbladder and injurious to their epithelial cells, are activated under AJPBD conditions[24,25]. Shimada et al.[23] indicated that the concentration of lysophosphatidylcholine (LPC) in the bile was much higher in the presence of AJPBD. LPC, which has a cytotoxic effect on epithelial cells, was produced by phospholipase A2 in refluxing pancreas juice. Sugiyama et al.[25] reported that lysolecithin in the phospholipid, which is produced from lecithin by activated phospholipase A2 in refluxing pancreas juice, was significantly elevated in the presence of AJPBD. These previously published studies have not clarified the mechanism of genetic alterations under these hazardous substances. There are, however, a

Table 2. p53 mutation in exon 5-8 in adenocarcinomas of gallbladder with AJPBD and without AJPBD

	Age	Sex	Hist. type[a]	Depth[b]	p53 overexp.	Codon/Exon	Base change	Amino acid	at CpG site
GBC with AJPBD									
W1	57	F	wel	ss	Diffuse	138(5)	GCC to GCT	Ala to Ala	
W2	63	M	pap	ss	Diffuse	294(8)	GAG to GAA	Glu to Glu	
W3	57	F	wel	m	Diffuse	175(5)	CGC to CAC	Arg to His	+
W4	67	F	pap-wel	ss	Diffuse	273(8)	CGT to TGT	Arg to Cys	+
						279(8)	GGG to AGG	Gly to Arg	
W5	57	F	pap, wel	ss	Diffuse	173(5)	GTG to ATG	Val to Met	
W6	73	F	wel	ss	Diffuse	No mutation			
W7	46	F	mod	ss	Diffuse	No mutation			
W8	68	F	pap, wel	ss	Diffuse	No mutation			
W9	45	F	pap, wel	ss	Diffuse	244(7)	GGG to AGC	Gly to Ser	+
						272(8)	GTG to ATG	Val to Met	
						299(8)	CTG to CTA	Leu to Leu	
W10	38	F	pap	mp	Nested	No mutation			
W11	64	F	wel	m	Nested	No mutation			
W12	74	F	pap-wel	ss	Negative	258(7)	GAA to AAA	Glu to Lys	
W13	75	M	wel	ss	Negative	208(6)	GAC to AAC	Asp to Asn	
W14	49	F	pap-wel	ss	Negative	No mutation			
W15	62	F	wel	m	Negative	No mutation			
W16	60	F	wel	m	Negative	No mutation			
W17	57	F	pap-wel	ss	Negative	No mutation			
GBC without AJPBD									
WO1	74	F	wel	ss	Diffuse	132(5)	AAG to GAG	Lys to Glu	
WO2	76	F	wel	ss	Diffuse	163(6)	CAT to AAT	His to Asn	
WO3	78	F	mod	ss	Diffuse	140(5)	ACC to ATC	Thr to Ile	
						166(5)	TCA to ACA	Ser to Thr	
WO4	70	F	wel	m	Diffuse	276(8)	GCC to CCC	Ala to Pro	
WO5	76	F	por	ss	Diffuse	280(8)	AGA to AAA	Arg to Lys	
WO6	71	M	mod	ss	Diffuse	280(8)	AGA to AAA	Arg to Lys	
WO7	70	M	wel	ss	Diffuse	238(7)	TGT to CGT	Cys to Arg	
WO8	79	F	por	ss	Diffuse	271(8)	GAG to AAG	Glu to Lys	
WO9	69	M	mod	ss	Diffuse	231(7)	ACC to ATC	Thr to Ile	
WO10	58	F	mod	ss	Diffuse	160(5)	ATG to GTG	Met to Val	
						220(6)	TAT to AAT	Tyr to Asn	
WO11	79	F	wel	ss	Diffuse	No mutation			
WO12	61	M	wel	ss	Diffuse	No mutation			
WO13	60	F	pap	ss	Diffuse	No mutation			
WO14	80	M	pap	ss	Diffuse	No mutation			
WO15	53	M	wel	ss	Diffuse	No mutation			
WO16	76	F	por	ss	Diffuse	No mutation			
WO17	71	F	pap	m	Diffuse	No mutation			
WO18	49	F	pap	ss	Scattered	No mutation			
WO19	62	M	por	ss	Negative	205(6)	TAT to TGT	Tyr to Cys	
WO20	62	F	pap	ss	Negative	No mutation			
WO21	66	F	pap	ss	Negative	No mutation			
WO22	71	F	wel	ss	Negative	No mutation			

[a]: Hist., histological; pap, papillary adenocarcinoma; wel, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated. [b]: m, mucosa; mp, tumor invasion.

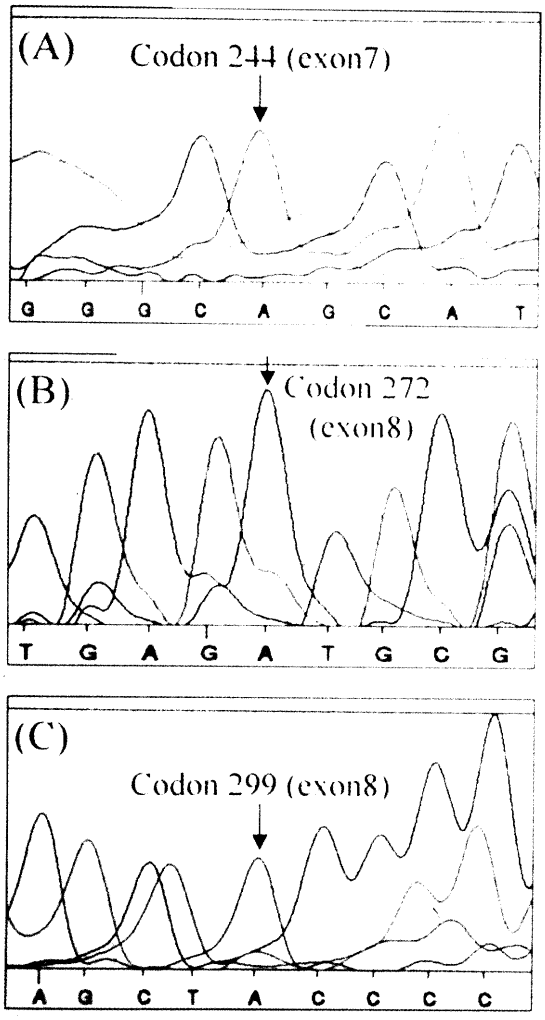

Fig. 1. Electrophoretograms of tumor sequencing. Multiple different *p53* alterations occur within the same domain of case W9. A missense mutation is detected at codon 244 (**A**) and 272 (**B**). A silent mutation is detected at codon 299 (**C**).

few reports of a case of GBC with AJPBD that had high levels of bile interleukin-6 after biliary drainage[26] and that interleukin-6 signaling activated the *ras* gene[27].

The mutation of *K-ras* codon 12 was found higher in the majority of GBCs with AJPBD than in GBCs without AJPBD[8]. A specific point mutation of GGT to GAT transition was frequently observed in codon 12 and 13 of *K-ras* in gallbladder mucosa with AJPBD[28]. These observations suggest that some endogenous mutagenic substances induced by the mixing of bile and pancreatic juice may contribute to specific *K-ras* mutations. Wistuba et al.[29] reported that *ras* point mutations were a rare and late event in GBC, probably related to tumor progression. There might be another pathway of carcinogenesis in the gallbladder with AJPBD such as via hyperplastic mucosa with *K-ras* mutations.

In our present study, GBCs with AJPBD and GBCs without AJPBD revealed 65% and 77% *p53* protein over-expression and 47% and 50% *p53* mutations, respectively. There were not any statistically significant differences between the two groups. Hanada et al. reported similar data with 4 (67%) and 3 (50%) of 6 Stage I GBCs with AJPBD that had *p53* protein over-expression and p53 mutations, respectively[17]. Matubara et al. reported that *p53* mutations were found in 4 (80%) of 5 biliary carcinomas and in 10 (39%) of 29 noncancerous biliary lesions associated with AJPBD[18]. These results suggest that *p53* mutations may contribute to the early stage of carcinogenesis in the gallbladder mucosa with AJPBD and without AJPBD.

This present study showed a low concordance (51.3%, 20/39) between p53 protein over-expression and *p53* mutations. In p53-overexpressing cases, the concordance was 50.0% (14/28, corresponding to actual amino acid substitutions) and the discordance was 50.0% (14/28, corresponding to actual amino acid substitutions) and the discordance was 50.0% (14/28), while in p53-nonoverexpressing cases it was 72.7% (8/11) and 27.3% (3/11), respectively (Table 2). Two cases of GBCs with AJPBD in discordance between p53 over-expression and silent mutations and 7 cases in discordance between p53 over-expression and no mutations may have other missense mutations at other exons or different pathways that cause the accumulation of p53 proteins in muclei[32,33]. Three p53 -nonoverexpressing cases bearing missense mutations may be explained by the degeneration of the p53 protein secondary to irritation from bile or by the formation of severely truncated p53 protein structures that were non-reactive with the antibody used[34].

The *p53* mutational spectrum in human malignant tumors is expected to provide information about endogenous and exogenous mutagens because these mutagens lead to specific kinds of base substitutions at proper sites[14]. The representative features of the *p53* mutations due to endogenous biological processes are transitions at the CpG dinucleotide as found in colon cancer and brain tumors. G to T transversions are the most frequent substitutions observed in cancers of the lung, breast, esophagus and liver, and are more likely to be due to exogenous carcinogens that injure DNA[35].

There are reports on the frequency of transition,

Table 3. Base change spectrum of p53 within adenocarcinomas of gallbladder

	% of p53 mutation	% of transition at CpG site		% of transversion	% of deletion/insertion
GBC with AJPBD					
Present study, Japanese specimens (17)	47(8)	100(8)	27(3)	0	0
GBC without AJPBD;					
Takagi et al; Japanese specimens (16)[35]	31(5)	60(3)	0	20(1)	20(1)
Yokoyama et al. Japanese specimens (22)[16]	50(11)	69(9)	0	31(4)	0
Fujii et al. Japanese specimens (23)[15]	70(16)	50(8)	6(1)	69(11)	0
Hanada et al; Japanese specimens (32)[30]	34(11)	27(3)	9(1)	91(10)	0
flat type (16)	44(7)	29(2)	14(1)	86(6)	0
polypoid type (16)	25(4)	25(1)	0	100(4)	0
GBC (? with or withuot AJPBD)					
Jonas et al; German specimens (7)[36]	29(2)	100(2)	0	0	0
Yokoyama et al. Chilean specimens (20)[16]	55(11)	100(11)	33(4)	0	0

( ): Number of cases analyzed.

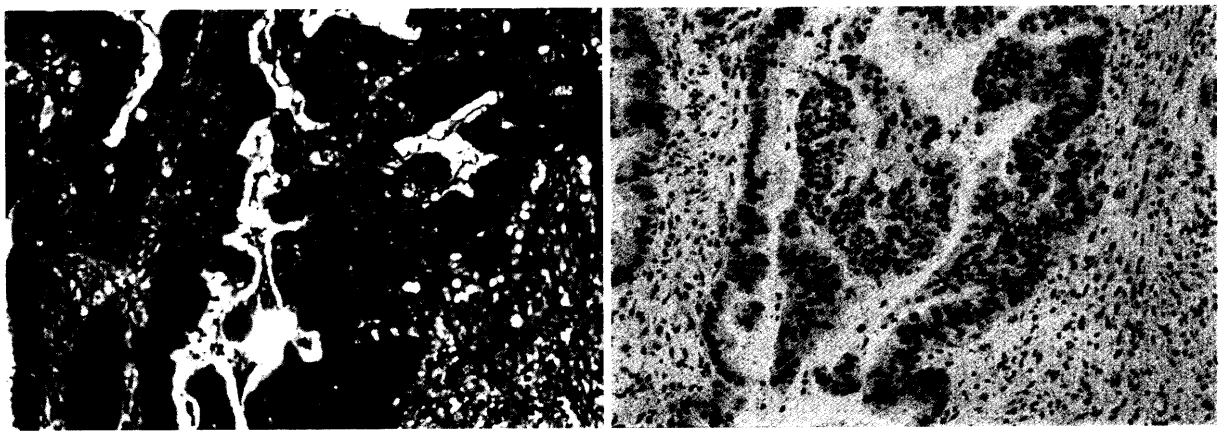

Fig. 2. Diffuse p53-staining pattern in GC with AJPBD (W9). **a.** H&E staining, and **b.** immunostaining of p53 protein (×400).

CpG site transition, and transversion of GBCs without AJPBD. In these reports, transitions were found in 27～69% of GBCs, with 0～9% arising at the CpG dinucleotide, and transversions were found in 20～91% of GBCs (Table 3). However, one of our previous papers on 20 Chilean GBCs showed a very different result. All of the p53 mutations detected were transitions with 33% arising at CpG dinucleotide. Unfortunately, we could not confirm whether or not these cases were associated with AJPBD.

In the present study, all mutational spectra of GBCs with AJPBD were transitions at the G: C pair. In contrast, mutations of GBCs without AJPBD comprised transitions at the G: C pair in 38.5% and transversions in 30.8%. Although the present series was relatively small, the mutational spectrum of GBCs with AJPBD was unique with very frequent transitions and mutations at G: C pairs. Furthermore, three G: C to A: T transitions at the CpG site were located in codons 175, 244 and 273. It is reported that G: C to A: T transitions at the CpG site were the most frequent substitutions observed in colorectal cancer and brain tumors[14]. Mutational hotspots of transition at CpG sites in codons 175, 245, 248, 273 and 282 are thought to reflect endogenous mutations caused by spontaneous deamination of 5-metyl-

cytosine[35]. In this study, all mutations at the CpG site were located at the above described hot spot or adjacent to it. The lower incidence of mutations at the CpG site in GBCs with AJPBD in comparison to that in colorectal cancers and brain tumors may be explained by the influence of other mutagens. The *p53* mutations of Chilean GBCs showed the same base-change spectra as seen with GBCs with AJPBD in this study[16]. It is speculated that the same biological transformation process of *p53* exists between Japanese GBCs with AJPBD and Chilean GBCs. Therefore, as a future step to expand this premise, Chilean patients with GBCs should be examined for whether or not AJPBD is an associated transformation factor.

In conclusion, different types of mutagenesis of *p53* may exist between GBCs with and without AJPBD. Furthermore, there must alternative genetic sequences in the tumor progression of GBCs with and without AJPBD because of the differences in histogenesis, incidence of *K-ras* mutations, and *p53* mutation patterns in gallbladder carcinoma.

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