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K-*ras* and *p53* gene mutations in noncancerous biliary lesions of patients with pancreaticobiliary maljunction

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Abstract

Background/Purpose. We investigated the molecular mechanisms of carcinogenesis in the biliary epithelium in patients with pancreaticobiliary maljunction.

Methods. Point mutations of the K-*ras* gene and the *p53* gene, and the overexpression of *p53* gene products were examined in the cancerous and noncancerous biliary epithelium of 37 patients with pancreaticobiliary maljunction, with or without biliary dilatation.

Results. In the gallbladder epithelium of 5 patients with pancreaticobiliary maljunction associated with biliary carcinoma, K-*ras* gene mutations were detected in 3 (60%), *p53* gene mutations in 3 (60%), and the overexpression of p53 gene products in 4 (80%), while in the bile duct epithelium of these patients, these features were found in 2 of 3 (66.7%), in all of 3 (100%), and none of 3 (0%) specimens, respectively. In the gallbladder epithelium of patients with pancreaticobiliary maljunction without biliary carcinoma, K-*ras* gene mutations were detected in 8 of 24 (33.3%) specimens, *p53* gene mutations were detected in 16 of 27 specimens (59.3%), and the overexpression of p53 protein was detected in 5 of 27 (18.5%) specimens, while in the bile duct epithelium of these patients, these features were found in 10 of 25 (40%) specimens, 14 of 25 (56%) specimens, and 6 of 24 (25%) specimens, respectively.

Conclusions. These results suggest that noncancerous lesions of the biliary epithelium in patients with pancreaticobiliary maljunction have mutations of the K-*ras* gene and/or the *p53* gene, which provides genetic evidence that biliary epithelium has high carcinogenic potential.

Key words K-*ras* and *p53* gene mutations · Pancreaticobiliary maljunction · Biliary carcinogenesis

Introduction

Although pancreaticobiliary maljunction (PBM), an anomalous junction of the biliary duct and pancreatic duct located outside the duodenal wall, has been shown to carry a high risk for the development of biliary tract carcinoma, the precise mechanisms of carcinogenesis are still not known. Reflux of pancreatic juice into the bile duct and bile stasis have been considered to play important roles in carcinomas in the biliary tract.[1–4] Many investigators have attempted to elucidate the mechanisms of carcinogenesis and various mechanisms have been hypothesized, including irritation of biliary epithelium by pancreatic enzymes, changes in bile acid fractionation, and the involvement of mutagenic agents in the contents of the biliary tract.[5–10] However, the mechanism of carcinogenesis may not be so simple, because many animal experiments using a model of reflux of pancreatic fluid into the biliary tree failed to show the development of biliary carcinomas without the concomitant administration of carcinogenic agents.[11] To directly confirm the high carcinogenic potential of biliary epithelium in patients with PBM, we employed molecular biological techniques and elucidated the mechanism of carcinogenesis in cancerous and noncancerous biliary epithelium of patients with PBM. Using these techniques, we previously found, for the first time, that genetic alterations occurred in cancerous and even in noncancerous biliary epithelium of patients with PBM, regardless of the presence or absence of biliary dilatation.[13,25] In the present study, we collected a number of patients with PBM and analyzed the relationship between K-*ras* gene mutation and *p53* gene mutation and the types of mutations of these genes to test the hypothesis that multiple tumor-related gene mutations occur, particularly in various noncancerous lesions of the background biliary epithelium, in patients with PBM.

Materials and methods

Patients and pathological specimens

Tissues of the biliary tract epithelium were obtained from patients at the Department of Surgery, Fujita

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Health University hospital. Formalin-fixed paraffin-embedded tissue specimens obtained from 37 patients with PBM were used. Seven of these 37 patients had carcinoma of the gallbladder and bile duct, while the remainder did not have biliary carcinoma. As a control, biliary epithelium was also obtained from 6 patients with benign biliary diseases (3 patients with gallstone and 3 with cholesterol polyp of the gallbladder); these patients had no evidence of PBM.

K-ras *and* p53 *gene mutations*

Cancerous and noncancerous tissues were identified under a microscope and they were selectively scraped out from 10-μm paraffin-embedded tissue sections stained with hematoxylin and eosin, and the DNA was extracted using a Sepa-gene kit (Sankyo Junyaku Tokyo, Japan). Using these samples, we analyzed K-*ras* and *p53* gene mutations by a polymerase chain reaction — single strand conformation polymorphism (PCR-SSCP) method.[14] A series of primers for PCR, to amplify regions containing four coding exons, from exons 5 through 8, were synthesized and used for the nested PCR. When abnormally mobilized bands were detected on the SSCP gel, direct genomic sequencing of the oligonucleotides was conducted to determine the substitutions of the nucleotides and the types of K-*ras* gene and *p53* gene mutations. Patients with a single mutation or multiple mutations detected in any exons from 5 through 8 of the *p53* gene were defined as positive for *p53* gene mutations.

Immunohistochemical staining of p53 *gene product*

Immunohistchemical staining of p53 gene products was performed by the streptavidin-biotin peroxidase method, using a mouse monoclonal antibody against *p53* protein (DO-7; Novo Castra, New Castle, UK). The p53 gene product was defined as positive when any of the nuclei in the tissues were clearly stained. This criterion is based on the finding that, because carcinogenesis starts in a single cell and develops monoclonally, epithelial cells clearly stained for p53 protein appear to be important for carcinogenesis, despite the small number of positive cells.

Results

K-ras *gene mutations*

Representative samples of the SSCP analysis of exon 1 of the K-*ras* gene are shown in Fig. 1. While the K-*ras* gene mutation was detected in none of the gallbladder epithelia of patients without PBM, abnormal bands that corresponded to a mutant allele were detected not only in cancerous epithelium but also in hyperplastic, metaplastic, and inflammatory epithelium of the gallbladder and/or common bile duct epithelium in patients with PBM.

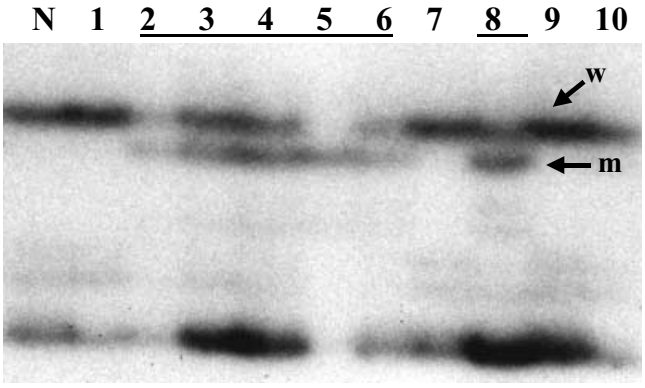

Fig. 1. Detection of the K-*ras* oncogene mutation at codon 12 in single-strand conformation polymorphism (SSCP) analysis in cancerous, hyperplastic and/or metaplastic, and inflammatory biliary epithelium in patients with and without pancreaticobiliary maljunction (PBM). *Lane N*, Control; *lane 1*, normal bands; *lanes 2 through 6*, mutation bands; *lane 7*, normal bands; *lane 8*, mutation bands; *lane 9*, normal bands; *lane 10*, normal bands. *Arrows* indicate the positions of the mutant (*m*) and wild-type (*W*) bands. Representative samples only are presented

Point mutations in the K-*ras* gene were found at codon 12, substituting glycine (GGT) for aspartic acid (GAT); and at codon 13, substituting glycine (GGC) for arginine (CGC) (Fig. 2, Table 1). K-*ras* point mutations at codon 12 were found in 3 of 5 (60.0%) gallbladder carcinomas, 2 of 3 (66.7%) bile duct carcinomas, 8 of 24 (33.3%) of the gallbladder epithlia, and 10 of 25 (40%) of the bile duct epithelia in patients with PBM without carcinoma, while no point mutations of the K-*ras* gene were detected in the gallbladder epithelium of patients without PBM (Table 2). K-*ras* gene mutations were examined in 7 cancerous and 68 noncancerous lesions in 37 patients with PBM. K-*ras* point mutations were detected in 3 of 5 (60%) cancerous lesions, 7 of 19 (36.8%) hyperplastic and metaplastic lesions, and 6 of 19 (31.6%) inflammatory lesions in gallbladder epithelium. In bile duct epithelium, by contrast, K-*ras* point mutations were found in 2 of 3 (66.7%) cancerous lesions, 2 of 7 (28.6%) hyperplastic and metaplastic lesions, and 10 of 22 (45.5%) inflammatory lesions (Table 3).

p53 *immunohistochemical staining*

The findings for p53 immunohistchemical staining performed on the histological sections are shown in Table 1. Positive staining for p53 was observed not only in the nuclei of the cancer cells but also in those of noncancerous cells of patients with PBM. Diffuse and strong positive p53 staining was seen in the cancerous tissues

Table 1. K-*ras* and *p53* gene mutations, and the overexpression of p53 gene product in cancerous and noncancerous biliary epithelium in patients with pancreaticobiliary maljunction (PBM)

Patient no.	Carcinoma	BD	Age (years)	Sex	K-*ras* gene		K-*ras* gene mutation	product		p53 gene		*p53* gene mutation
					GB	CBD	Exon:codon,nucleotide substitution	GB	CBD	GB	CBD	Exon:codon,nucleotide substitution
1	−	+	34	M	●	NA	E1:12, GGT-GAT	○	NA	○	NA	
2	−	+	47	F	●	●	E1:12, GGT-GAT	○	●	●	●	GB,E6:207GAT-GAC
3	−	+	36	F	NA	●	E1:12, GGT-GAT	○	○	●	●	CBD,E8:278CCT-CTT GB,E7:247AAC-AGC E7:249AGG-AAG
4	−	+	25	F	NE	●	E1:12, GGT-GAT	○	●	○	●	CBD,E5:192CAG-TAG
5	−	+	60	F	○	○		○	○	○	●	CBD,E8:275TGT-AGT
6	−	+	26	F	●	○	E1:12, GGT-GAT	●	●	●	●	GB,E8:275TGT-AGT E6:195CAG-TAG CBD,E6:217,218GTG-GTT E8:275TGT-AGT
7	−	+	34	M	○	●	E1:12, GGT-GAT	○	○	●	○	GB, E8:278CCT-TCT, GB,E5:156CGC-Cdel, E5:135TGC-TAC E5:148GAT-AAT
8	−	+	42	M	●	NA	E1:12, GGT-GAT	○	NA	●	NA	GB,E5:144CAG-AAG E7:233CAC-CGAC(Ginser)
9	−	+	67	M	NA	NE		NA	○	NA	○	
10	−	+	36	M	○	○		○	○	○	●	CBD,E5:156CGC-Gcdel E7:244GGC-GGT E8:278CCT-CTT
11	−	+	25	F	○	○		○	NE	○	●	
12	−	+	29	F	○	○		●	○	○	○	
13	−	+	41	M	○	○		○	●	●	○	
14	−	+	75	F	NE	NA		○	NA	●	NA	GB,E5:156CGC-Gcdel
15	−	+	44	M	○	○		○	○	●	●	CBC,E5:156CGC-Gcdel GBC,E5:144CAG-AAG
16	−	+	27	F	○	○		○	○	○	●	
17	−	+	61	F	○	○		○	○	○	○	
18	−	+	21	F	○	●	E1:12, GGT-GAT	○	○	○	○	
19	−	+	32	M	●	●	E1:12, GGT-GAT	○	○	○	●	CBD,E6:193CAT-CCT
20	−	−	41	F	NA	●	E1:12, GGT-GAT	NA	○	NA	●	

21	−	−	63	F	●	●	E1:12, GGT-GAT	●	○	●	●	GB,E6:205TAT-TAC E8:275TGT-AGT CBD,E5:157GTC-GAC
22	−	−	26	F	○	○		○	○	●	○	GB,E8:277TGT-TAT
23	−	−	23	F	NA	○		NA	○	NA	●	CBD,E6:212TTT-TTA
24	−	−	47	F	●	●	E1:12, GGT-GAT	○	○	●	●	CBD,E8:208CCT-CTT GB,E8:282CGG-CAG
25	−	−	49	M	○	NE		○	NE	●	NE	
26	−	−	50	F	○	●	E1:12, GGT-GAT	○	○	●	○	GB,E5:135TGC-TAC, E5:140ACC-ATC
27	−	−	34	M	○	○		●	●	○	○	
28	−	−	79	F	○	○		●	NA	●	NA	GB,E8:278CCT-CTT
29	−	−	37	F	○	○		○	○	●	○	
30	−	−	44	F	●	○	E1:12, GGT-GAT	○	●	●	○	GB,E6:214CAT-TAT
31	BDC	+	35	F	●	●	E1:12, GGT-GAT E1:13, GGC-CGC	○	○	●	●	CBC,E8:274GTT-GCT
32	BDC	+	70	F	○	○		○	○	●	●	CBC,E8:276GCC-GTC
33	GBC	+	71	F	○	NA		●	NA	○	NA	
34	GBC	+	55	F	○	○		●	○	●	●	GBC,E5:148GAT-CAT E8:272GTG-Gdel GB,E5:156CGC-Gcdel, E5:154GGC-AGC, E5:141TGC-TAC, E5:135TGC-TAC
35	GBC	−	62	F	●	NA	E1:12, GGT-GAT	●	NA	●	NA	GBC,E6:205TAT-TGT
36	GBC	−	57	F	●	NA	E1:12, GGT-GAT E1:13, GGC-CGC	●	NA	●	NA	
37	GBC,BDC	−	57	F	●	●	E1:12, GGT-GAT	○	○	○	●	

BD, Biliary dilatation; GB, gallbladder epithelium; CBD, common bile duct epithelium; GBC, gallbladder cancer; BDC, bile duct cancer; NE, not examined; NA, specimen not available. Circles, negative expression of *p53* gene product or K-*ras* gene and *p53* gene mutation; dots, positive expression of *p53* gene product or K-*ras* gene and *p53* gene mutation; underline, transversion type of *p53* gene mutation

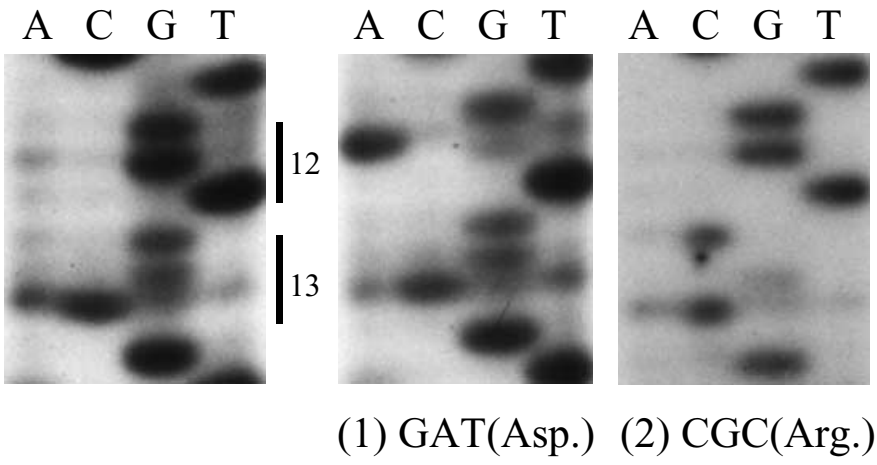

Fig. 2. Direct sequencing of the K-*ras* gene at codons 12 and 13. (*1*) A mutation at codon 13, substituting GGT (glycine) for GAT (aspartic acid), found in a patient with gallbladder carcinoma. (*2*) A mutation at codon 13, substituting GGC (glycine) for CGC (arginine), found in a patient with bile duct carcinoma

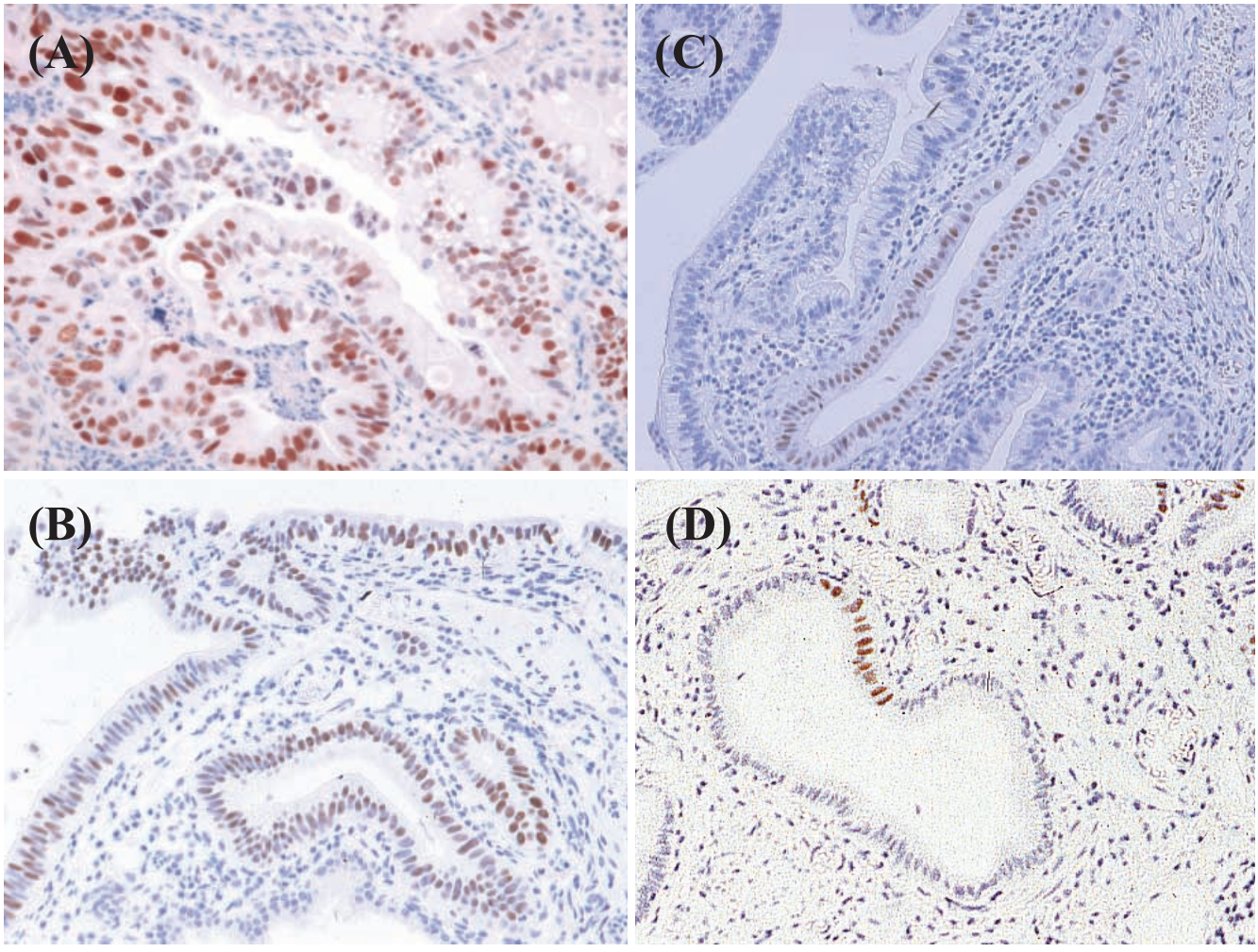

Fig. 3A–D. Immunohistchemical staining of *p53* gene product in cancerous and noncancerous gallbladder epithelium obtained from a patient with pancreatiobiliary maljunction (PBM). Diffuse and strongly stained pattern in a cancerous lesion (**A,B**). Positive staining was seen in limited glandular structures in noncancerous lesions (**C**). Weakly positive staining was detected in some areas of a single granular structure (**D**). A, ×200; B, C, D, ×100

Table 2. Mutations of the K-*ras* gene and the *p53* gene, and the overexpression of p53 gene product in biliary carcinoma, and in noncancerous lesions without the association of biliary carcinoma in patients with PBM

		K-*ras* gene	*p53* gene	p53 protein
With carcinoma ($n = 7$)[a]	GB	3/5 (60.0%)	3/5 (60.0%)	4/5 (80.0%)
	CBD	2/3 (66.7%)	3/3 (100%)	0/3 (0.0%)
Total		5/8 (62.5%)	6/8 (75%)	4/8 (50%)
Without carcinoma ($n = 31$)	GB	8/24 (33.3%)	16/27 (59.3%)	5/27 (18.5%)
	CBD	10/25 (40.0%)	14/25 (56.0%)	6/24 (25.0%)
Control	GB	0/6 (0%)	0/6 (0%)	0/6 (0%)

GB, Gallbladder epithelium; CBD, common bile duct epithelium
[a] One patient had gallbladder and bile duct carcinoma

Table 3. Incidence of mutations of the K-*ras* gene and the *p53* gene in cancerous and noncancerous biliary epithelia of patients with PBM

	Gallbladder			Common bile duct			
	Inflammatory	Hyperplasia and metaplasia	Carcinoma	Inflammatory	Hyperplasia and metaplasia	Carcinoma	Total
K-*ras* gene mutation	6/19 (31.6%)	7/19 (36.8%)	3/5 (60%)	10/22 (45.5%)	2/7 (28.6%)	2/3 (66.7%)	30/75 (40%)
p53 gene mutation	8/23 (34.8%)	10/21 (47.6%)	3/5 (60%)	13/22 (59.1%)	4/9 (44.4%)	3/3 (100%)	40/83 (48.2%)
p53 gene product	2/24 (8.3%)	7/21 (33.3%)	4/5 (80%)	4/23 (17.4%)	2/7 (28.6%)	0/3 (0%)	19/83 (22.9%)

(Fig. 3A,B), whereas positive staining was seen in limited glandular structures in the noncancerous lesions (Fig. 3C). Weakly positive staining was detected in some areas of a single granular structure (Fig. 3D). Overexpression of p53 protein was found in 4 of 5 (80%) cancerous lesions of the gallbladder, but in none of 3 (0%) lesions of common bile duct carcinoma. In patients with PBM without carcinoma, overexpression of p53 protein was found in gallbladder epithelium in 5 of 27 patients (18.5%) and in bile duct epithelium in 6 of 24 patients (25%) (Table 2).

p53 gene mutations

p53 gene mutations were detected in 3 of 5 (60%) gallbladder carcinomas and in 3 of 3 (100%) common bile duct carcinomas. In patients with PBM without carcinomas, *p53* gene mutations were found in 16 of 27 (59.3%) gallbladder epithelia and in 14 of 25 (56.0%) bile duct epithelia (Table 2). While *p53* gene mutations were not detected in the gallbladder epithelia of patients without PBM, abnormal bands that corresponded to a mutant allele were detected in both cancerous and noncancerous epithelia of gallbladder and/or common bile duct epithelium in patients with PBM (Fig. 4). *p53* gene mutations were found in 20 of 83 (24.1%) lesions in exon 5, 12 of 83 (14.5%) in exon 6, 3 of 83 (3.6%) in exon 7, and in 20 of 83 (24.1%) in exon 8 in cancerous and noncancerous biliary epithelia (Table 4). Genetic alterations of the *p53* suppressor gene were examined in 83 lesions in the 37 patients with PBM, using PCR-SSCP.

The results of direct genomic sequence analysis of the *p53* gene mutations are shown in Fig. 5. Of 45 mutations in which these sequences were determined, 37 were point mutations (82.2%), 7 were deletion mutations (15.6%), and 1 was an insertion mutation (2.2%). Of the 37 point mutations determined, 26 (70.3%) were A:T—G:C transition types, and 11 (29.7%) were A:T—C:G transversion types (Table 1).

Discussion

The primary aim of the present study was to precisely analyze the incidence and the types of gene mutations of the K-*ras* gene and the *p53* gene and the overexpression of p53 gene product in a large series of cancerous and noncancerous biliary lesions of patients with PBM. The results of the present study clearly demonstrated a high incidence of K-*ras* gene mutations and *p53* gene mutations, not only in cancerous lesions but also in noncancerous lesions in the biliary epithelium of patients with PBM, which further confirmed the find-

Table 4. *p53* gene mutations in exon 5 through exon 8 in cancerous and noncancerous biliary epithelia of patients with PBM

	Cancerous		Noncancerous		
Exon	GB	CBD	GB	CBD	Total
Exon 5	2/5 (40%)	1/3 (33%)	10/43 (23.3%)	10/32 (31.3%)	20/83 (24.1%)
Exon 6	1/5 (20%)	0/3 (0%)	6/43 (14.0%)	5/32 (15.6%)	12/83 (14.5%)
Exon 7	0/5 (0%)	0/3 (0%)	1/43 (2.3%)	2/32 (6.3%)	3/83 (3.6%)
Exon 8	2/5 (40%)	3/3 (100%)	8/43 (18.6%)	7/32 (21.9%)	20/83 (24.1%)

GB, Gallbladder epithelium; CBD, common bile duct epithelium

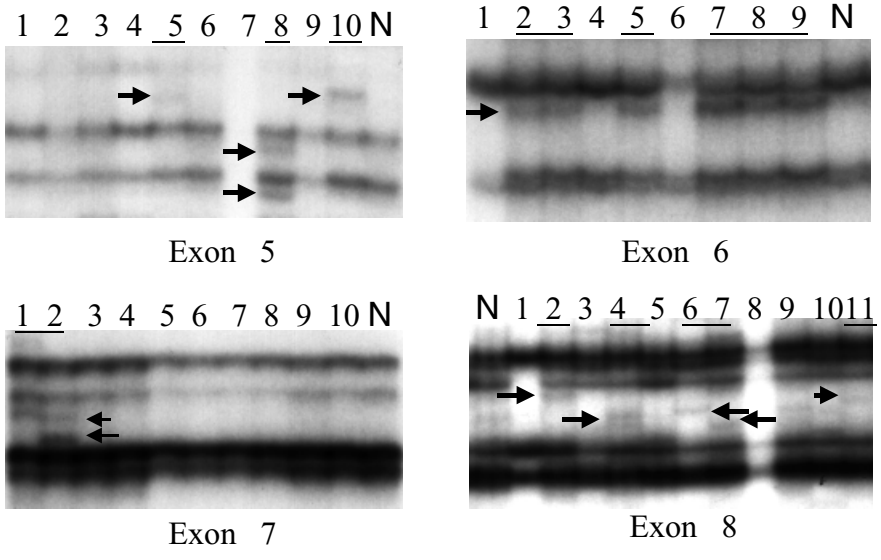

Fig. 4. Detection of mutations in exon 5 through exon 8 of the *p53* gene by polymerase chain reaction (PCR)-SSCP analysis in cancerous and noncancerous biliary epithelia in patients with PBM and in other patients without PBM. *Lane N*, Control; *underlined lane numbers*, mutation bands. *Arrows* indicate the positions of the mutant. Part of the experimental result is presented

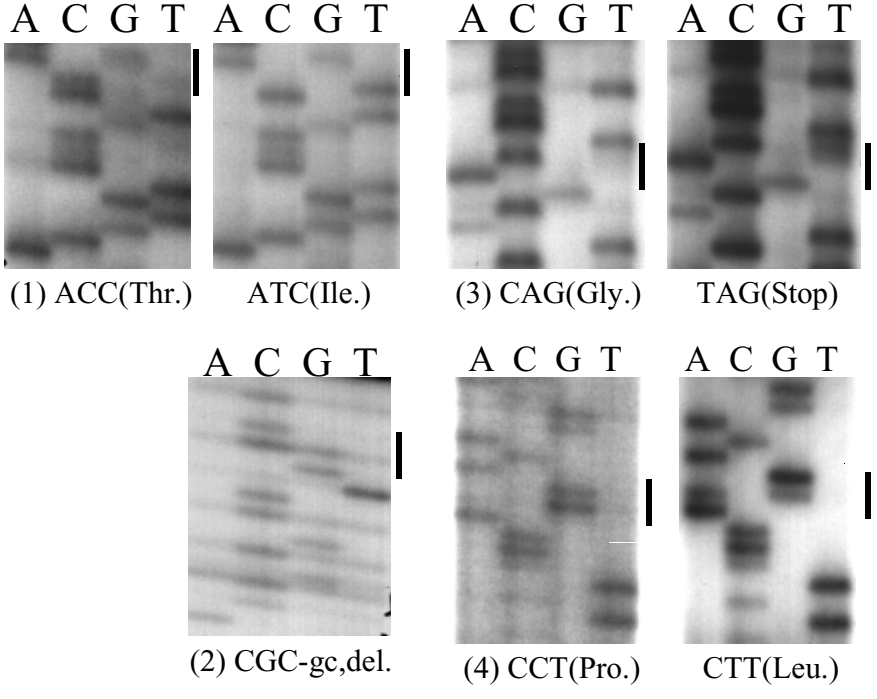

Fig. 5. Direct sequencing of *p53* suppressor oncogene at codons 140, 156, 195, and 278. A mutation at codon 140, substituting threonine (ACC) for isoleucine (ATC), found in a patient with no carcinoma (*1*). A mutation at codon 156, substituting arginine (CGC) for GC deletion, found in a patient with no carcinoma (*2*). A mutation at codon 195, substituting glutamic acid (CAG) for stop codon (TAG), found in a patient without carcinoma (*3*). A mutation at codon 278, substituting proline (CCT) for leucine (CTT), found in a patient with no carcinoma (*4*). No mutation of the *p53* suppressor oncogene was found in the nonneoplastic gallbladder and bile duct epithelia of six patients without PBM

Table 5. Relationship between K-*ras* gene mutation and *p53* gene mutation in carcinoma and in noncancerous lesions without biliary carcinoma

With carcinoma	K-*ras* gene mutation			Without carcinoma	K-*ras* gene mutation		
p53 gene mutation		+	−	*p53* gene mutation		+	−
	+	4	2		+	12	11
	−	0	1		−	2	3

ings in our previous studies.[12,13,25] These results suggest that multiple gene mutations are accumulated in the various noncancerous lesions that occur in the biliary epithelium in patients with PBM, which provides further evidence to suggest that these noncancerous lesions are in a precancerous state.

The results of the present study clearly demonstrated a high incidence of K-*ras* gene mutations in various noncancerous epithelial lesions, as well as in carcinoma. We have previously demonstrated a high incidence of K-*ras* gene mutations, not only in biliary carcinoma but also in noncancerous lesions of the biliary epithelium in patients with PBM.[12,13] K-*ras* gene mutations were detected in 5 of 8 (62.5%) gallbladder and bile duct carcinomas and in 18 of 49 (36.7%) biliary epithelia without carcinoma in patients with PBM in the present series. The incidence of K-*ras* gene mutations in cancerous and noncancerous lesions in a large series of patients with PBM was thus similar to that reported previously.[13] The incidence of K-*ras* point mutation in biliary carcinoma in patients with PBM has been reported to range from 41% to 83%,[14–19] and it is generally agreed that K-*ras* gene mutation occurs in biliary carcinoma in patients with PBM, despite some differences in the incidence from report to report.[14–19]

In contrast, the incidence of K-*ras* gene mutation in biliary epithelium of noncancerous lesions varies from report to report, and also from lesion to lesion. To our knowledge, the K-*ras* gene mutation in benign biliary lesions was first reported by Ohta et al.,[20] in 1993. They noted a point mutation at codon 12 of the K-*ras* gene in biliary papillomatosis. Tanno et al.[18] reported that K-*ras* point mutations at codon 12 were found in 2 of 15 patients (13%) with hyperplasia of gallbladder epithelium associated with PBM, but not in nonneoplastic gallbladder mucosa. Iwase et al.[17] also found K-*ras* point mutations at codon 12, in 5 of 14 patients (36%) with hyperplasia associated with PBM. In contrast, Itoi et al.[16] demonstrated no K-*ras* gene mutation in inflammatory, metaplastic, or hyperplastic biliary epithelium. This difference in the incidence of K-*ras* gene mutations in various noncancerous lesions could also be caused by differences in the methods employed in each study. Nonetheless, the results of the present study support the presence of positive K-*ras* gene mutations in noncancerous lesions in patients with PBM.

Whether K-*ras* gene mutation in the biliary epithelium in patients with PBM is congenital or whether it arises as an influence of environmental factors remains unknown. If K-*ras* mutations are caused by chronic irritation of bile fluid mixed with pancreatic juice, they should appear as a long-term event. However, Tomishige et al.[19] recently reported that K-*ras* gene mutation was detected in a 1-month-old infant with congenital biliary dilatation, suggesting that a genetic alteration occurs in an early phase of life. They also mentioned that there was no correlation among age, incidence of K-*ras* gene mutation, and incidence of noncancerous lesions in the biliary epithelium of choledochal dilatation. They also demonstrated K-*ras* gene mutation in inflammatory, hyperplastic, and metaplastic lesions in the biliary tract of pediatric patients with congenital biliary dilatation. Therefore, it appears that the K-*ras* gene mutation in patients with PBM may not simply be caused by an environmental factor alone. Nevertheless, because the results of the present study showed K-*ras* gene mutations in noncancerous biliary epithelium of patients with PBM, and because K-*ras* gene mutation is involved in an early phase of tumor progression, the noncancerous biliary lesions are considered to be in a precancerous state.

The incidence of the overexpression of p53 gene product in biliary carcinoma in patients with PBM reported previously ranges from 67% to 100%,[21–25] but the incidence in noncancerous lesions has not been documented. Hanada et al.[21] reported that four of six (67%) stage 1 gallbladder cancers in patients with PBM were positive for p53 overexpression, which may then have contributed to early-stage carcinogenesis of the gallbladder mucosa. In contrast, Yamamoto et al.[22] demonstrated p53 overexpression in all three (100%) gallbladder cancers at an advanced stage, but not in epithelial hyperplasia. They thought that the overexpression of p53 gene product might be a late event in gallbladder carcinogenesis in PBM, based on the their results that the incidence of the overexpression was dependent on the grade of neoplastic change. However, the results of the present study indicated no direct correlation of overexpression of p53 gene product with the grade of neoplastic change, thus being inconsistent with their report. These differences could arise from differences in the methodology and in the criteria for positive

p53 immunohistochemical staining. Our criterion for positive p53 staining is based on the finding that carcinogenesis occurs in a single cell, and that the monoclonal expansion of a cell cluster that originates from a single p53-positive cell then occurs.[25] With this criterion, positive p53 staining is detected in any neoplastic lesions of biliary epithelium, and is not dependent on the histopathological grade of neoplastic changes. This criterion could be validated by the finding that weakly positive staining was detected in some areas of a single glandular structure, a finding that is consistent with the phenomena of monoclonal proliferation of p53-positive cells.

The results of the present study indicated that patients with *p53* gene mutation did not necessarily correspond to those with overexpression of p53 product, and vice versa. This dissociation has also been reported in other types of cancers,[26] and there are a number of possible explanations. One is that positive p53 staining is related to stabilization of the protein retained on the histological section by means of some mechanisms other than mutation, such as protein complex formation, or by inactivation of the enzyme degradation pathway.[27] Recent studies of the regulation of p53 protein molecules have indicated that the p53 gene product may be regulated by mdm2 and/or p14ARF,[28] which are considered to be important for the determination of functional aspects of the p53 protein; these could be a fairly important research target in the future.

The types of *p53* gene mutations have been shown to be clues to the carcinogenic process. In the present study, the transversion type of *p53* gene mutation was seen in 29.7% of the 37 point mutations. The transversion type of *p53* gene mutation has been shown to occur frequently in circumstances in which a certain carcinogenic agent is involved in the carcinogenic process.[29] Therefore, the high incidence of the transversion type of *p53* gene mutation is consistent with the idea that certain mutagens may be involved in the carcinogenic process in patients with PBM.

In the present study, 4 of 7 patients (57.1%) with PBM with carcinoma were positive for both K-*ras* gene and *p53* gene mutations (Table 5). This is not surprising, because mutations of multiple tumor-related genes have been shown to be encountered frequently in various human gastrointestinal carcinomas. However, to our knowledge, this is the first report of mutations of multiple genes in noncancerous biliary lesions in patients with PBM. In the present study, 12 of 28 patients (42.9%) without biliary carcinoma were positive for mutations of both the K-*ras* gene and the *p53* gene (Table 5). We have taken advantage of having a large number of histological specimens of various noncancerous lesions of the bile duct without biliary carcinoma available for gene analyses, because exclusive resection of the extrahepatic duct has been adopted for the past 20 years once PBM has been diagnosed, regardless of whether there is an association of biliary carcinoma, and regardless of whether there is dilatation of the bile duct.[6] The high incidence of gene mutations of multiple tumor-related genes detected in the noncancerous lesions of patients with PBM without biliary carcinoma would justify our surgical strategy of resection of the common bile duct combined with so-called "diversion surgery", which separates bile flow from pancreatic juice to prevent carcinogenesis, in patients with PBM without biliary carcinoma. However, the extent of resection of the common bile duct is still controversial, and no consensus has been reached in terms of the extent of resection of the bile duct that is necessary to effectively prevent biliary carcinoma. Moreover, there is no clinical evidence that strongly indicates that a particular area of biliary epithelium has high carcinogenic potential.

In summary, the present study has clearly demonstrated that multiple gene mutations are accumulated in various noncancerous lesions in the background biliary epithelium in patients with PBM. This provides, for the first time, strong evidence that the background biliary epithelium of patients with PBM is in a precancerous state. Although the alterations of these multiple genes may provide some explanation of the high incidence of biliary carcinogenesis in patients with PBM, further evidence of alterations of other tumor-related genes, and further accumulation of patients with PBM, may be required to explain the entire spectrum of molecular events that occur in biliary carcinogenesis.

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