

K-*ras* Point Mutations in the Supernatants of Pancreatic Juice and Bile Are Reliable for Diagnosis of Pancreas and Biliary Tract Carcinomas Complementary to Cytologic Examination

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In order to clarify whether DNA analysis for K-*ras* mutation can be used to diagnose cancers in supernatants of pancreatic juice and bile, samples from 29 cases of pancreatic, biliary tract, gastric, and neuroendocrine carcinomas, 1 malignant lymphoma case, 2 cases of pancreatic adenoma, 9 cases of chronic pancreatitis and 21 other non-cancer cases were examined. Polymerase chain reaction (PCR) products for K-*ras* gene codons 2 to 97 of exons 1 and 2 were generated with 33/33 (100%) pancreatic juice and 41/41 (100%) bile samples. By the single strand conformation polymorphism (SSCP) method, point mutations were detected in the pancreatic juice or bile supernatants of 13/13 (100%) pancreas cancer cases, 5/14 (35.7%) biliary tract cancer cases, 1/2 (50.0%) pancreatic adenoma cases and 3/9 (33.3%) chronic pancreatitis cases. Direct sequencing confirmed identical point mutations in the supernatants, malignant cells of cytologic smears of pancreatic juice or bile and cancer tissues. The DNA analysis demonstrated the presence of K-*ras* point mutations in 3 cases of pancreatic carcinomas with false-negative cytologic diagnoses. This novel method allows simultaneous testing for genetic abnormalities in supernatants of pancreatic juice and bile, after removing cells for cytologic diagnosis and screening for pancreatic and biliary tract tumors.

Key words: K-*ras* — Pancreatic juice — Bile — Supernatant — Pancreas cancer

Pancreatic juice and bile materials are generally used for cytologic diagnoses but differentiation of neoplastic from reactive epithelial cells is a frequent problem.^{1–6} DNA analysis with polymerase chain reaction (PCR) amplification has been introduced for detection of K-*ras* gene mutations in pancreatic juice^{4–11} and feces,^{12,13} and for microsatellite and *p53* suppressor gene changes in urine and sputum.^{14–16} However, these analyses have been performed basically with cells scraped from slides or in sediment. Material is scarce when cytologic diagnosis is applied, and when DNA analysis is performed with cytologic smears, material is lost owing to the processes necessary for extraction of DNA. Furthermore, this approach normally requires considerable time before results are obtained.

Recently, however, we have developed a novel method for detection of K-*ras* mutations in the supernatants of body fluid, including ascites and pleural effusions, for diagnosis complementary to cytologic examination.¹⁷ With this method using the supernatant, the PCR products for K-*ras* gene codons 2 to 97 of exons 1 and 2 were generated with 41 (93%) of 44 body cavity fluid samples. By

the single strand conformation polymorphism (SSCP) method, point mutations were detected in the ascites supernatants of 8 (89%) of 9 cases of pancreatic carcinoma. All of the point mutations were confirmed by direct sequencing. Further, this DNA analysis of supernatant of ascites fluid showed a K-*ras* point mutation in 3 cases of false-negative cytologic diagnosis (2 cases of pancreatic carcinoma and 1 case of colorectal carcinoma). This method allows simultaneous testing for genetic abnormalities in supernatants of body fluid, after removing cells for cytologic diagnosis. In the present study, we ascertained whether DNA analysis using PCR amplification is feasible and reliable using supernatants of pancreatic juice and bile, after centrifugation to remove cells.

MATERIALS AND METHODS

Patients and histological diagnosis Fresh samples of pancreatic juice and/or bile in 62 cases (esophagitis, 1; gastric ulcer, 1; chronic hepatitis, 1; cholecystitis [gallbladder stone], 18; chronic pancreatitis, 9; pancreatic adenoma, 2; pancreatic adenocarcinoma, 13 [head, 8; body, 3; tail, 2]; biliary tract adenocarcinoma, 14 [Vater's papilla, 3; extrahepatic bile duct, 7; intrahepatic bile duct, 1; gallbladder, 3]; gastric carcinoma, 1; malignant lymphoma of duodenum, 1; neuroendocrine carcinoma of the pancreas-

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head, 1) were collected in Kitasato University East Hospital. Samples were obtained by endoscopic retrograde cholangioscopic or pancreatoscopic methods (ERC or ERP) in 49 cases, by percutaneous transcholeductal drainage (PTCD) in 7 cases and from surgically removed materials in 6 cases. When pancreatic juice and bile were obtained from identical cases, care was taken to avoid contamination, as follows: (1) localization of the tip of ERC or ERP was strictly confirmed; (2) the endoscopic tubes were washed with normal saline each time; (3) a new disposable syringe was used for each sampling; (4) early flowing fluid was discarded. Histopathologic diagnosis was used as the preferred criterion for inclusion in the study. In cases in which histologic materials were not available, cytologic diagnoses were accepted in conjunction with the results of laboratory examinations, including endoscopic retrograde cholangiopancreatography (ERCP), echography, computed tomography (CT), gastrofiberscopy and/or colonofiberscopy. All cytologic slides were reviewed, and malignant cell clusters for DNA extraction were designated by marking on the slides. When malignant cells were not available, epithelial cell clusters were selected for DNA extraction and molecular analysis of the cellular components by the microdissection method.^{4,18)} Primary tumor and non-tumor tissue specimens for molecular analysis were obtained from 10% buffered (pH 7.2) formalin-fixed, paraffin-embedded tissue blocks.

DNA extraction DNA extraction from the supernatants of pancreatic juice and bile, formalin-fixed, paraffin-embedded tissues and cellular components from cytologic smears was basically performed according to routine procedures.¹⁸⁻²¹⁾ Approximately 5 ml samples of pancreatic juice and bile were collected in 15 ml centrifuge tubes, diluted 2- to 5-fold with phosphate-buffered saline (pH 7.6), and then separated into supernatant and sediment components by centrifugation at 2,000 rpm for 10 min. Five milliliter 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 milliliter aliquots of supernatant were transferred into 1.5 ml centrifuge tubes, mixed with 100 μ l 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), 4.5% polysorbate 20 (Tween-20), 20 mM disodium dihydrogen ethylenediaminetetraacetate dihydrate (EDTA-2Na), and incubated at 37°C overnight after preincubation at 55°C for 3 h.²¹⁾ Each sample was then purified with phenol-chloroform, precipitated with ethyl alcohol, and resuspended in 50 μ l of distilled water. To avoid the possibility of cross-contamination, DNA extractions from tissue, cells on the cytologic smears, and supernatants were performed in separate rooms on different days.

Multiplex PCR Two microliter aliquots of DNA were amplified with a thermal-cycler (Takara Shuzo Co., Shiga), in a total volume of 50 μ l, the reaction mixture containing 50 mM KCl, 10 mM Tris-HCl pH 8.7, 3 mM MgCl₂, 0.1% Triton X-100, 6.25 pM of each of the primer pairs, 200 μ M of each dATP, dGTP, dCTP, and dTTP, and 2.5 U of recombinant-*Taq* polymerase (Toyobo Co., Osaka), overlaid with 50 μ l of mineral oil. The primers used for multiplex amplification were 5'-ACTGAATA-TAACTTGTGG-3', 5'-GCTATTGTTGGATCAATATTC-3' (Takara Shuzo Co.), 5'-GATTCCTACAGGAAGCAAGT-3' and 5'-CTATAATGGTGAATATCTTC-3' yielding 108 base pair (bp) and 185 bp amplified DNA fragments, in K-ras gene codons 2 to 37 of exon 1, and 38 to 97 of exon 2, respectively. The cycle conditions were an initial denaturation at 94°C for 3 min, two cycles of annealing at 56°C for 1 min, extension at 72°C for 1 min, denaturation at 94°C for 30 s and then 35 cycles of 45 s at 56°C, 30 s at 72°C, 20 s at 94°C, followed by final extension at 72°C for 3 min. Following amplification, 10 μ l reaction mixture samples were separated on 4% agarose gels and exposed to UV light (365 nm) after staining with ethidium bromide. In cases in which amplification could not be confirmed, 5 μ l of the 100-fold PCR product was reamplified (second PCR) with fresh reagents under the same conditions as used in the first amplification. For all PCR runs, distilled water was used instead of the DNA sample as a negative control.

Non-radioisotopic single strand conformation polymorphism analysis (non-RI-SSCP) and non-radioisotopic direct sequencing (non-RI-direct sequencing) PCR product samples of 1 μ l were diluted with 49 μ l of gel-loading buffer (95% formamide, 4 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and heated at 94°C for 3 min, followed by quenching on ice. Then 3 μ l aliquots were immediately loaded onto 18% polyacrylamide gels containing 10% formamide, 4% glycerol, 2.5% sucrose, 0.5 \times Tris-HCl boric acid EDTA (TBE) buffer. Electrophoresis was carried out at 550 V for 4 h followed by silver staining with the aid of a Silver Stain Plus kit (Bio-Rad Lab., Hercules, CA).²¹⁾

Direct sequencing was performed with non-radioisotopic detection using chemiluminescence. PCR products (10 μ l), including exonuclease I and shrimp alkaline phosphatase (USB Co., Cleveland, OH), in 22 cases (cases 28-31, 33-46, 49, 51, 53 and 57), in which abnormal bands were detected and 40 cases (cases 1-27, 32, 47, 48, 50, 52, 54-56, 58-62), where wild-type bands were seen with SSCP analysis were incubated for 30 min at 37°C and heated at 80°C for 10 min, according to the procedure of Werle *et al.*,²²⁾ with slight modifications. Non-radioisotopic detection using chemiluminescence was carried out with a Sequencing High Plus kit (Toyobo Co.) according to the manufacturer's recommendations and the above-

described non-labeling primers. The cycle sequencing steps were initial denaturation at 94°C for 3 min, 35 cycles (56°C 15 s, 60°C 3 min, 94°C 20 s), and final extension at 60°C for 10 min.

RESULTS

Extractable DNA and PCR products of exons 1 and 2 of the *K-ras* gene were detected in all supernatants of pancreatic juice (33 cases) and bile (42 cases) samples after dilution 2- to 5-fold with PBS and centrifugation at 2,000 rpm for 10 min, in accordance with the previously reported data¹⁷⁾ for ascites supernatant (Fig. 1, A and B). Epithelial cell counts in the sediments ranged from 34 to 112,095/ml for the cytologic smears.

SSCP analysis of tumor tissues and/or epithelial cell clusters of cytologic smears revealed abnormally shifted bands in 1 (11.1%) (case 28) of 9 cases of chronic pancreatitis and 19 cases of pancreatic or biliary tract tumors (cases 31, 33-46, 49, 51, 53, and 56), including 13/13 pancreatic adenocarcinoma cases (100%), 1/2 pancreatic adenoma cases (50%), 4 of 11 bile duct adenocarcinoma cases (36.4%) and 1 of 3 gallbladder adenocarcinoma cases (33.3%) (Table I). As for controls, normal bands of SSCP were confirmed in the supernatant and sedimentary cells of pancreatic juice and/or bile and tissue samples in 3 cases of gastric carcinoma, pancreatic neuroendocrine carcinoma and duodenal malignant lymphoma, 18 cases of cholecystitis, one of esophagitis, one of gastric ulcer and one of chronic hepatitis.

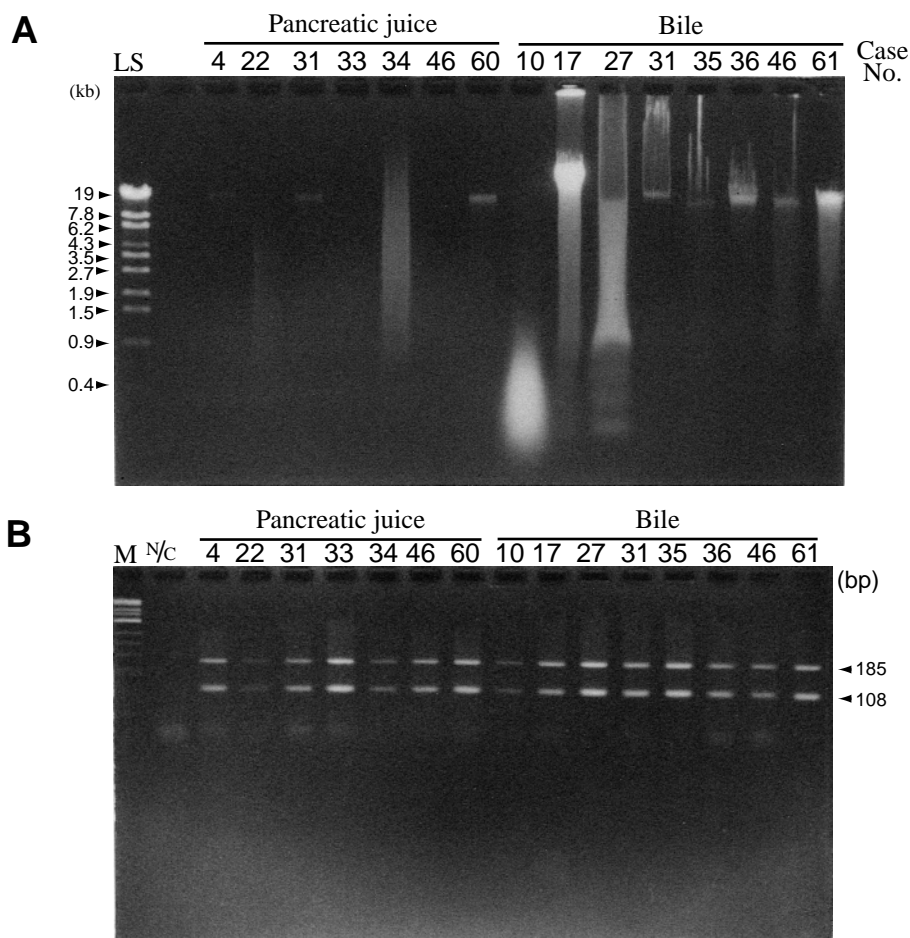


Fig. 1. A: Demonstration of extractable DNA in the supernatants of pancreatic juice (cases 4, 22, 31, 33, 34, 46 and 60) and bile (cases 10, 17, 27, 31, 35, 36, 46 and 61). Ten microliter aliquots of DNA were electrophoresed in an agarose gel and stained with ethidium bromide. Lane LS, molecular marker of *SlyI* digested λ DNA. B: Multiplex PCR detection of the *K-ras* gene exons 1 and 2 in the supernatants of pancreatic juice (cases 4, 22, 31, 33, 34, 46 and 60) and bile (cases 10, 17, 27, 31, 35, 36, 46 and 61). Lane M, *HinfI*-digested ϕ X174 DNA as molecular markers; lane N/C, distilled water as a negative control. Arrows indicate the amplified prod-

Table I. Clinicopathologic Data and K-ras Gene Mutations

Case No.	Clinical diagnoses	Materials ^{a)}	Method	Tumor location	Histologic and/or cytologic diagnoses	Conclusive stage	Tumor size (mm)	Cytologic examination	K-ras mutation		
									Supernatants	Cells	Tissues
1	Esophagitis	P	ERP		Esophagitis			Negative ^{b)}	Wild (GGT)	Wild (GGT)	Wild (GGT)
2	Gastric ulcer	B	ERC		Gastric ulcer			Negative	Wild	Wild	Wild
3	Chronic hepatitis	P	ERP		Chronic hepatitis			Negative	Wild	Wild	Wild
4-9	Gallbladder stones	P	ERP		Cholecystitis			Negative	Wild	Wild	Wild
10-16		B	ERC					Negative	Wild	Wild	Wild
17-21		B	Surgical					Negative	Wild	Wild	Wild
22-26	Chronic pancreatitis	P	ERP		Chronic pancreatitis			Negative	Wild	Wild	NA
27		B	ERC					Negative	Wild	Wild	NA
28		P	ERP					Negative	GAT(Asp):12	Wild	GAT(Asp):12
29, 30		B	ERC					Negative	Wild	Wild	NA
31	Pancreatic cysts	P	ERP	Pancreas head	Intraductal papillary adenoma		42	Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
32		B	ERC					Negative	Wild	Wild	Wild
33		P	ERP					Negative	Wild	Wild	Wild
34		B	ERC					Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
35		P	ERP					Negative	Wild	Wild	Wild
36		B	ERC					Negative	Wild	Wild	Wild
37		B	ERC					Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
38		B	PTCD					Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
39		B	PTCD					Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
40		P	ERP					Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
41		B	ERC					Negative	Wild	Wild	Wild
42		P	ERP	Pancreas body	Ductal adenocarcinoma		27	Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
43		B	ERC					Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
44		P	ERP	Pancreas tail			70	Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
45		B	ERC					Negative	Wild	Wild	Wild
46	Common bile duct tumors	P	ERP	Extrahepatic bile duct	Adenocarcinoma		25	Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
47		B	ERC					Negative	Wild	Wild	Wild
48		B	PTCD					Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
49		B	ERC					Negative	Wild	Wild	Wild
50		B	ERC					Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
51		B	PTCD					Negative	Wild	Wild	Wild
52		B	PTCD					Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
53	Cholangioepithelial carcinomas	P	ERP	Intrahepatic duct	Adenocarcinoma		15	Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
54	Vater's papilla carcinomas	P	ERP	Vater's papilla	Adenocarcinoma		40	Negative	Wild	Wild	Wild
55		B	ERC					NA	Wild	Wild	Wild
56		B	PTCD					Negative	Wild	Wild	Wild
57	Gallbladder polyps	P	ERP	Gallbladder	Adenocarcinoma		3	Negative	Wild	Wild	GAT(Asp):12
58		B	ERC					Negative	GAT(Asp):12	GAT(Asp):12	GAT(Asp):12
59	Gallbladder carcinomas	B	Surgical					Negative	Wild	Wild	Wild
60	Stomach carcinoma	P	ERP	Stomach	Adenocarcinoma		12	Negative	Wild	Wild	Wild
61	Pancreatic tumor	P	ERP	Pancreas head	Neuroendocrine carcinoma		110	Negative	Wild	Wild	Wild
62	Malignant lymphoma of duodenum	P	ERP	Duodenum	Malignant lymphoma		110	Negative	Wild	Wild	Wild

a) P: pancreatic juice, B: bile. NA: not available.

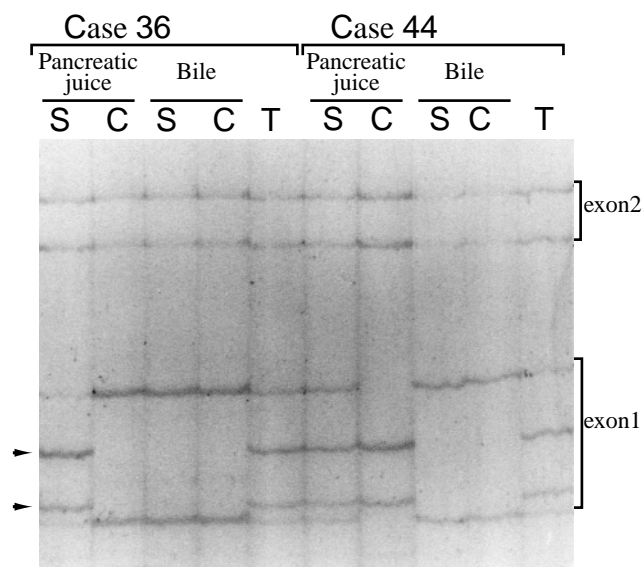


Fig. 2. Non-RF-SSCP analysis of amplified products of exons 1 and 2 of the *K-ras* gene from the supernatants of pancreatic juice and bile, microdissected malignant and/or non-malignant cells of cytologic smears and primary tumor tissues in cases 36 and 44. Lane S, supernatant specimens; lane C, microdissected malignant and/or non-malignant cells of the cytologic smear; lane T, microdissected primary tumor tissue from a histology section. Arrows indicate mutant allele bands.

SSCP analysis of supernatants from pancreatic juice showed abnormally shifted bands in 14 cases, including 3 (cases 28–30) of 9 cases (33.3%) of chronic pancreatitis, 1 (case 31) of 2 (50%) pancreatic adenomas, 9 (cases 33–36, 41–45) of 9 (100%) pancreatic adenocarcinomas and 1 (case 53) of 3 (33.3%) bile duct adenocarcinomas (Table I). According to pathological examination and/or CT, echography and ERCP, biliary tract involvement by pancreatic carcinoma was detected or suspected in all cases in which abnormal SSCP bands were observed in bile samples from ERC. The supernatants of bile showed abnormally shifted bands in 10 cases, including 6 (cases 35, 37–40 and 42) of 8 (75%) pancreatic adenocarcinomas, 3 (cases 46, 49, 51) of 10 (30%) bile duct carcinomas and 1 (case 57) of 3 (33.3%) gallbladder carcinomas (Table I). Identically shifted bands in supernatants of both pancreatic juice and bile or tumor tissues or tumor cell clusters of cytologic smears were detected in 22 cases (cases 28–31, 33–46, 49, 51, 53 and 57). The point mutations responsible were confirmed to be in codon 12 (GGT-GAT, Gly-Asp in cases 28, 31, 33, 36, 37, 40, 42–44, 46, 51, 53 and 57; GGT-GTT, Gly-Val in cases 35, 38, 39, 41 and 45; GGT-TGT, Gly-Cys in cases 34 and 49) by direct sequencing (Fig. 2, A and B). The same results of SSCP and direct sequencing for *K-ras* point mutation in the supernatant and pancreatic tissue were obtained in one case of chronic pancreatitis (case 28), and in micro-

Table II. Comparison of *K-ras* Gene Mutations and Cytologic Diagnosis with Pancreatic Juice and Bile

Histologic and/or cytologic diagnoses	Cytologic diagnoses	<i>K-ras</i> gene mutations								
			Pancreatic juice or bile						Tissues (primary tumor or non-tumor)	
			Supernatants	Malignant cells or non-malignant epithelial cells						
Esophagitis, gastric ulcer, chronic hepatitis and cholecystitis	(n=21) Positive	0	Mutant type	0	Mutant type	0	Mutant type	0	Mutant type	0
	Negative	21	Wild type	21	Wild type	21	Wild type	21	Wild type	21
Chronic pancreatitis	(n=9) Positive	0	Mutant type	3	Mutant type	0	Mutant type	1	Mutant type	1
	Negative	9	Wild type	6	Wild type	9	NA	8	Wild type	8
Pancreatic adenomas	(n=2) Positive	0	Mutant type	1	Mutant type	1	Mutant type	1	Mutant type	1
	Negative	2	Wild type	1	Wild type	1	Wild type	1	Wild type	1
Pancreatic adenocarcinomas	(n=13) Positive	10	Mutant type	13	Mutant type	10	Mutant type	9	Mutant type	9
	Negative	3	Wild type	0	Wild type	3	NA	4	Wild type	4
Bile duct or gallbladder adenocarcinomas and Vater's papilla adenocarcinomas	(n=14) Positive	8	Mutant type	5	Mutant type	5	Mutant type	4	Mutant type	4
	Negative	6	Wild type	9	Wild type	9	NA	8	Wild type	8
Gastric carcinoma, neuroendocrine carcinoma and malignant lymphoma	(n=3) Positive	0	Mutant type	0	Mutant type	0	Mutant type	0	Mutant type	0
	Negative	3	Wild type	3	Wild type	3	Wild type	3	Wild type	3

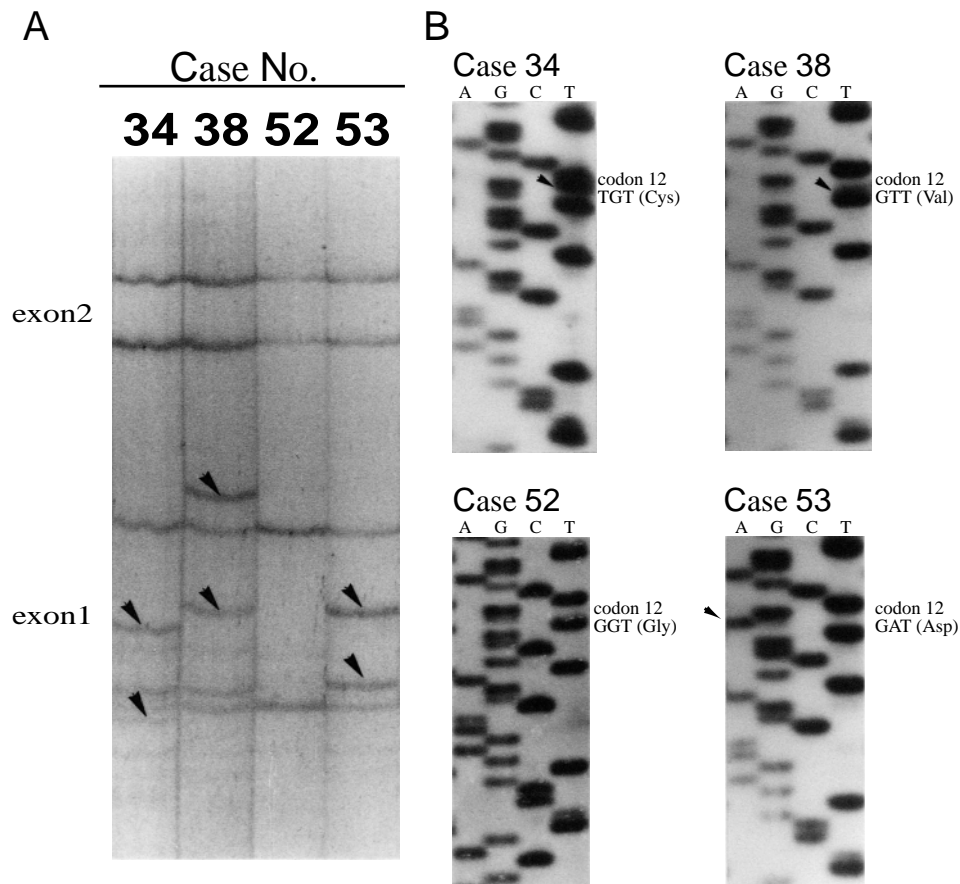


Fig. 3. A: Non-RF-SSCP analysis of amplified products of exons 1 and 2 of the *K-ras* gene from the supernatants of pancreatic juice (cases 34, 53) and bile (cases 38, 52). Arrows indicate mutant allele bands. B: Non-RF-direct sequencing analysis of PCR amplified products of *K-ras* gene exon 1 from the supernatants of pancreatic juice (cases 34, 53) and bile (cases 38, 52). Arrows indicate mutant allele bands.

dissected adenoma cell clusters of cytologic smear and tumor tissue in case 31 (intraductal papillary adenoma).

K-ras point mutations were detected in both supernatants and tumor cells of cytologic smears from either pancreatic juice or bile in 10 of 13 (76.9%) pancreatic adenocarcinomas (Fig. 3). Although malignant cells were not found in cytologic smears of pancreatic juice, *K-ras* point mutations were observed in the supernatants in the 3 remaining cases (cases 36, 43, 45) for which pancreatic adenocarcinomas were histologically confirmed (Tables I and II). Further, the bile supernatant demonstrated a *K-ras* mutation, although malignant cells were not detected in the cytologic smear of bile in case 42, in which a *K-ras* mutation was confirmed in the supernatant and malignant cells of cytologic smears derived from pancreatic juice (Fig. 3).

In 5 of 14 cases (35.7%) of biliary tract carcinoma, including bile duct and gallbladder carcinomas diagnosed

by cytology and/or histology, *K-ras* mutations were detected in both supernatants and tumor cells of cytologic smears of bile samples.

DISCUSSION

Our newly described method¹⁷⁾ was found to be reliable for DNA extraction from the supernatants of pancreatic juice and bile after dilution with phosphate-buffered saline (PBS) and centrifugation with 2,000 rpm for 10 min in all cases in the present study. As also found for ascitic or pleural fluid, the amount of extracted DNA from pancreatic juice or bile samples was small in comparison with those from serum or plasma.²³⁻²⁵⁾ However, PCR products for *K-ras* gene exons 1 and 2 could be generated from a minimum of 34 cells/ml in pancreatic juice or bile (case 40), although at least 250×10^3 cells/ml have been reported to be necessary with ascitic or pleural fluid.¹⁷⁾ Thus, PCR

products from pancreatic juice or bile are appropriate for *K-ras* gene analysis, independent of the cell numbers in the samples.

Direct sequencing analysis of *K-ras* gene exons 1 and 2 in the present study confirmed the abnormally shifted bands in SSCP and revealed identical abnormalities of codon 12 among the supernatants and tumor cell clusters of cytologic smears or primary tumors in 18 of 25 cases of pancreatic and biliary tract adenocarcinomas. The data indicate that analysis of DNA extracted from the supernatants of pancreatic juice or bile gives results of similar accuracy to those obtained with surgically obtained tumor tissue or tumor cell clusters in cytologic smears.

K-ras mutation detection rates for the supernatants, 9/9 (100%) in pancreatic juice and 6/8 (75%) in bile, in cases of pancreatic adenocarcinoma were higher than the malignant cells detection rates with cytologic smears (7/9, 77.8% in pancreatic juice and 5/8, 62.5% in bile) or the *K-ras* mutation detection rates with cell clusters from cytologic smears (6/8, 75% in pancreatic juice and 4/7, 57.1% in bile). Further, supernatant *K-ras* mutations were detected in 3 cases of pancreatic adenocarcinomas for which malignant cells were not noted in cytologic smears. Thus, all 13 cases of pancreatic adenocarcinomas proved positive in the present study, showing that *K-ras* mutation analysis of supernatants from pancreatic juice or bile can overcome the problem of false-negative diagnosis based on cytology or *K-ras* mutation analysis of microdissected cell clusters of cytologic smears. In 5 of 14 cases (35.7%) of biliary tract carcinomas, including bile duct and gallbladder carcinomas, *K-ras* mutation was detected in both the supernatants and cell clusters of cytologic smears of bile or pancreatic juice. The *K-ras* mutation detection rates of pancreas and biliary tract carcinomas obtained in the present study are within the previously reported ranges (50–100% in pancreas carcinoma^{1–3,7–13,17,26–29}) and 29.0–56.5% in biliary tract carcinoma.^{30–33}) In several pancreatic adenocarcinomas, *K-ras* mutations were detected in bile as well as pancreatic juice. Since great care was taken with bile sampling in order to rule out the possibility of contamination with pancreatic juice, this might point to biliary tract involvement, as observed with surgically obtained materials or CT and/or echography or ERC.

Regarding the *K-ras* mutation detection sensitivity with supernatant samples, the lowest rate of tumor cells to total cells to sediment was 6% in cytologic smears. The finding of two cases in which the same *K-ras* point mutation was

detected in the supernatant as in tumor tissue, although tumor cells were not found in the cytologic smears, suggests that the detection sensitivity is sufficiently high to be essentially independent of the number of tumor cells within the pancreatic juice or bile samples. Further, all of the mutated *ras* genes found with PCR-SSCP were confirmed by direct sequencing. The present analysis using the supernatant with first or second PCR-SSCP showed almost equivalent or greater detection sensitivity as compared to previous studies^{7,34}) using the cell pellet or sediment with the enriched-PCR method. Since apoptosis of cancer cells is enhanced, together with cell proliferation, compared to that of normal cells,^{34–36}) nucleic acids originated from cancer cells shed from ducts may appear at relatively high concentration and be well preserved in the supernatant of pancreatic juice and bile. This is convenient for oncogene analysis using the PCR method, as in the case of ascites or pleural effusions.¹⁷⁾

K-ras point mutations in sediments of pancreatic juice have been detected in 6–31% of chronic pancreatitis and 25–89% of intraductal adenoma cases, according to previous reports.^{2,13,28,34}) Similarly, in the present study, 3/9 (33.3%) of chronic pancreatitis and 1/2 (50%) of intraductal adenoma cases were positive. Since mutations of *K-ras* oncogene occur not only in pancreatic adenocarcinoma, but also in other benign lesions, the possibility of false positives in chronic pancreatitis or ductal papillary hyperplasia or intraductal papillary adenoma^{2,29,32,34,37}) must be taken into account. Accordingly, before adopting our new method for routine laboratory use, it is still necessary to study more cases of benign pancreatic disorders and to define the assay limitations from the standpoint of cytologic examination. Although the above-described points remain to be clarified, analysis of *K-ras* point mutations in the supernatant of pancreatic juice and bile could be a good screening method for pancreas and biliary tract tumors.

In conclusion, *K-ras* gene analysis of supernatants from pancreatic juice and bile is a useful tool with the following merits: 1) tests can be used for screening for pancreas and biliary tract tumors; 2) tests can be performed with no loss of cytologic smear samples; 3) comparison of oncogene changes allows a check to be made for false-negatives in cytology diagnoses.

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