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Detection of K-*ras* Point Mutations at Codon 12 in Pancreatic Juice for the Diagnosis of Pancreatic Cancer by Hybridization Protection Assay: A Simple Method for the Determination of the Types of Point Mutations

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The present study was undertaken to detect K-*ras* oncogene point mutations at codon 12 in pure pancreatic juice (PPJ) by the hybridization protection assay (HPA) method for the diagnosis of pancreatic cancer (PC). This assay can be carried out within 30 min and can determine not only the presence of a mutation, but also the mutational type of K-*ras* at codon 12. The minimal ratio of mutant DNA detectable by the HPA was 5–10% of the total DNA. PPJ was collected through a cannula under duodenal fiberscope control from 20 patients with PC and 20 patients with chronic pancreatitis (CP). Analysis of PPJ by the HPA revealed that the incidence of K-*ras* point mutations at codon 12 was 55% (11/20) in patients with PC and 0% (0/20) in those with CP. Mutational types of K-*ras* at codon 12 in PC were aspartic acid (Asp) in nine cases, both Asp and cysteine in one case, and arginine in one case. Analysis of K-*ras* point mutations at codon 12 in PPJ using the HPA method seems promising as a new genetic test for the diagnosis of PC, because the HPA method is simple, and can easily determine the mutational type.

Key words: Hybridization protection assay — Acridinium ester — Genetic diagnosis — K-*ras* mutation — Pancreatic cancer

Intraductal and echo-guided pancreatic biopsies for the qualitative diagnosis of pancreatic cancer (PC) have not yet come into wide use because of the technical difficulty and uncertainty in picking up the target lesions and the risk of iatrogenic complications. Instead of biopsy, cytological examination has been performed with aspirated duodenal fluid in the Pancreozymin-Secretin test or with pure pancreatic juice (PPJ) collected endoscopically.[1,2] However, the diagnostic rate for PC has not been high since this procedure tends to be inaccurate due to cell injury and degradation induced by the various digestive enzymes such as proteases and amylase present in PPJ.

The incidence of K-*ras* point mutations at codon 12 in PC tissue in surgical or autopsy specimens has been reported to be extremely high, ranging from 75% to over 90%.[3-8] With this point in mind, we attempted to identify K-*ras* point mutations in the precipitate of PPJ containing exfoliated ductal pancreatic cancer cells. Polymerase chain reaction (PCR) and allele-specific oligonucleotide (ASO) probe hybridization identified K-*ras* point mutations at codon 12 in the PPJ of 11/20 (55%) patients with PC.[9] On the other hand, the same mutation was not identified in the PPJ of any patient with chronic pancreatitis (CP). Moreover, we reported previously that a PCR-restriction fragment length polymorphism (RFLP) method for detection of K-*ras* point mutations at codon 12 increased the diagnostic rate (up to 81%) of K-*ras* mutant in the PPJs from patients with PC, although this method could not determine the mutational types of K-*ras* at codon 12.[10] However, these methods are too labor-intensive and time-consuming for clinical management.

Recently, several hybridization assay formats involving acridinium-ester-labeled DNA probes have been developed by Arnold *et al.*[11] This hybridization protection assay (HPA) technique was reported to discriminate sensitively between two closely related target sequences in 10^{-17} to 10^{-18} mol of target at the level of single-base differences and to be a simple and easy method. Therefore, we attempted to make use of the HPA method to detect K-*ras* point mutations at codon 12 in PPJ for the diagnosis of PC. This is the first report of clinical application of the HPA method using an acridinium-ester-labeled DNA probe for diagnosing cancer by identifying oncogene mutations.

MATERIALS AND METHODS

Subjects We studied 40 patients diagnosed and treated at the Department of Internal Medicine of the Cancer Research Institute Hospital at Kanazawa University, or

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its affiliated medical facilities. Informed consent for this study was obtained from all patients. The study population consisted of 20 patients with PC and 20 with CP. Tumors were located at the head (10 cases), body (6 cases), or tail (4 cases) of the pancreas. In 9 pancreatic carcinomas, the diagnosis was confirmed histopathologically at the time of operation or autopsy. The remaining 11 pancreatic tumors which were not examined histologically were diagnosed as pancreatic carcinomas based on the results of various imaging methods, such as endoscopic retrograde cholangiopancreatography, ultrasonography, computed tomography, endoscopic ultrasonography and a compatible clinical course. The stage of pancreatic cancer was determined according to the General Rules for Cancer of the Pancreas by the Japan Pancreas Society[12] as follows: stage I, tumor apparently localized in the pancreas and tumor size less than 2 cm; stage II, tumor apparently localized in the pancreas and metastases to regional lymph nodes; stage III, suspected invasion into surrounding tissues (serosa, retroperitoneum, portal venous and arterial systems), apparent invasion into duodenum, intrapancreatic bile duct and metastases to regional lymph nodes; and stage IV, distant metastases (liver metastasis, peritoneal implants, etc.) or manifest invasion into surrounding tissues (serosa, retroperitoneum, portal venous and arterial systems). All the patients with PC had advanced carcinoma of clinical stage III or IV, except for one patient with stage II tumor. Two patients had TS_1 tumors less than 2 cm in diameter (TS: tumor size). On the other hand, diagnosis of CP was based on the clinical diagnostic criteria of the Japanese Society of Gastroenterology.[13] Definite diagnosis was based on meeting any of the following four requirements: 1) confirmed findings of CP in the pancreatic tissue; 2) apparent calcification in the pancreas; 3) manifest dysfunction of exocrine pancreas; 4) confirmed findings of CP in pancreatography or other imaging studies. The 20 patients with CP were classified into the definite group based on the clinical diagnostic criteria listed above.

Collection of PPJ PPJ was collected endoscopically through a cannula inserted from the orifice of the papilla Vater under secretory stimulation by intravenous administration of secretin (1 U/kg), using a duodenal fiberscope (JF-230 or JF-1T20: Olympus, Tokyo) as described previously.[14–16]

DNA extraction from PPJ Genomic DNA was prepared from the PPJ obtained from patients with PC and CP according to a modification of the method described by Bowtell[17] and Jeanpiérrè.[18] Subsequently, genomic DNA was extracted with a phenol-chloroform mixture and precipitated with ethanol. The quantity of DNA was assessed from the absorbance at 260 nm and the quality was evaluated from the 260/280 absorbance ratio.

PCR amplification for HPA The exon 1 region of the K-*ras* gene containing codon 12 (162 bps) was amplified using PCR[19] for HPA as follows: 200 ng of genomic DNA; 50 pmol each of the appropriate primers (5′-GGCCTGCTGAAAATGACTGA-3′ and 5′-GTCCTGCACCAGTAATATGC-3′) synthesized on a Cyclone Plus DNA synthesizer (Milligan/Biosearch, Milford, MA); 200 μM of each dNTP (Takara, Kyoto); 6.0 units of Deep Vent DNA Polymerase (exo^-) (New England Biolabs, Beverly, MA); and 10 μl of 10× Vent buffer (New England Biolabs) were used in a 100 μl PCR reaction (10 m*M* Tris-HCl, pH 8.3-50 m*M* KCl-1.5 m*M* $MgCl_2$). After the initial 120 s denaturation (94°C), amplification was performed with 40 cycles of 90 s denaturation (94°C), 60 s annealing (55°C), and 15 s extension (72°C) with a thermal cycler (Thermal Sequencer TSR300; Iwaki, Tokyo). All PCR experiments were done with at least two template-negative tubes as controls.

Templates containing only wild-type K-*ras* sequences, derived from pancreatic cancer cell lines, were included as controls and used in HPA to help exclude PCR-induced mutation as an explanation for positive results (data not shown).

Preparation of acridinium-ester-labeled DNA probes Oligonucleotides for detecting the wild type and six point mutations of K-*ras* at codon 12 were synthesized with a DNA synthesizer (Cyclone Plus DNA synthesizer, Milligan/Biosearch) using standard phosphoramidite chemistry. The chemical labeling of the DNA probes with acridinium ester was achieved by using alkylamine linker-arms, which were introduced during DNA synthesis, and an N-hydroxysuccinimide ester of a methyl acridinium phenyl ester according to the method of Arnold *et al.*[11] The acridinium-ester-labeled DNA probes were purified and their chemiluminescence was detected with a Leader I luminometer (Gen-Probe, Inc., San Diego, CA).

HPA After heat denaturation at 100°C for 10 min, 10 μl aliquots of PCR product were mixed and hybridized at 65°C for 15 min with 50 μl of acridinium-ester-labeled DNA probe corresponding to a point mutation of K-*ras* at codon 12 in 0.1 mol/liter lithium succinate buffer, pH 5.2, containing 100 g of lithium lauryl sulfate, 2 mmol of EDTA, and 2 mmol of ethylenebis(tetraacetic acid) per liter. Furthermore, 250 μl of differential hydrolysis buffer [sodium tetraborate buffer (0.15–0.20 mol/liter), containing 10–50 ml of Triton X-100 surfactant per liter, at pHs ranging from 7.0 to 8.5] was added and the mixture was incubated at 65°C for 5 min to eliminate the chemiluminescence of nonhybridized acridinium-ester-labeled DNA probe. The samples were cooled in ice-water and their chemiluminescence was measured with the Leader I luminometer using an automated reagent-injection method involving two detection reagents. Injection of

Table I. K-*ras* Mutations at Codon 12 in Pancreatic Cancer Cell Lines Detected by HPA (RLUs)

Cell lines	K-*ras* at codon 12	P-WT	P1	P2	P3	P4	P5	P6
HPAF	Gly, Asp	29,721	637	383	980	58,296	975	957
PANC-1	Gly, Asp	24,751	539	611	534	12,450	1,196	488
PaCa-2	Cys	480	812	733	61,043	347	858	512
BxPC-3	Gly	56,536	1,164	805	727	808	1,312	525

Cell lines, pancreatic cancer cell lines; P-WT, acridinium-ester-labeled DNA probe (wild type, GGT, Gly); P1, (AGT, Ser); P2, (CGT, Arg); P3, (TGT, Cys); P4, (GAT, Asp); P5, (GCT, Ala); P6, (GTT, Val).

detection reagent I (0.1% H_2O_2, vol/vol; 1 mmol/liter nitric acid) was followed, after a 1 s delay, by injection of detection reagent II (1 mol/liter NaOH). The resulting chemiluminescence was integrated for 2 s and measured in relative light units (RLUs). All of these steps were performed in a single 12×75 mm polypropylene tube.[11] The results of HPA were obtained within approximately 30 min after PCR amplification.

Sensitivity test for the mutant probes of K-*ras* at codon 12 by the HPA Two cancer cell lines, PaCa-2 (TGT, Cys) and BxPC-3 (GGT, Gly, wild type), were selected for determination of the minimal detection ratio of mutant DNA. The K-*ras* mutant DNA of PaCa-2 was mixed with the wild-type DNA of BxPC-3 at a constant ratio (100%, 50%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%) and then the mixtures were used as templates for PCR. The PCR products were then assayed by HPA.

The fragment including the wild-type K-*ras* at codon 12 was inserted into the plasmid pBluescript II SK(+) (Stratagene Cloning Systems, La Jolla, CA) by directional cloning. Six mutant plasmids corresponding to the six possible mutants at codon 12 of K-*ras* were generated by site-directed mutagenesis[20] using a "Transformer" Site-Directed Mutagenesis Kit (Clontec Labs, Palo Alto, CA) according to the recommendations of the manufacturer. Briefly, denatured target plasmid DNA containing K-*ras* wild type at codon 12 was annealed with each mutagenic primer and the selection primer, which was designed by incorporating two base changes within the unique restriction site target. The second strand was synthesized with T_4 DNA polymerase and sealed with T_4 DNA ligase. Most parental DNA was digested by the restriction enzyme *Afl* III (New England Biolabs) and the mixture of mutated and nonmutated plasmids was transformed into *Escherichia coli* BMH 71-18 *mut*S bacterial strain (defective in mismatch repair). Transformants were pooled, and the plasmid DNA was prepared from the mixed bacterial population. The isolated DNA was then subjected to secondary digestion with *Afl* III. The second transformation using the thoroughly digested DNA resulted in highly efficient recovery of the plasmids with the desired mutations of K-*ras* at codon 12. Finally, DNA sequences of the wild type and six

Table II. Minimal Detectable Ratio of K-*ras* Mutant at Codon 12 by HPA (RLUs)

X: PaCa-2 (%)	Probe WT (Gly)	Probe 3 (Cys)
100	1,776	149,415
50	108,498	79,377
10	181,833	8,282
5	207,070	7,285
1	134,068	3,220
0.5	195,192	2,278
0.1	207,165	1,141
0.05	233,844	1,044
0.01	235,911	1,223

$100a + 100b = 1{,}000 \quad X(\%) = b/(a+b) \times 100$
a: BxPC-3 (WT) 100 ng/μl
b: PaCa-2 (Cys) 100 ng/μl

mutant plasmids were confirmed with a DNA sequencer (370A DNA Sequencer, Applied Biosystems, Foster City, CA). Each K-*ras* mutant plasmid DNA was mixed with the wild-type plasmid DNA at a constant ratio (50%, 10%, 5%, 1%, 0.5%) and then these mixtures were used as template DNAs for PCR. The resultant PCR products were assayed by HPA.

PCR-RFLP and ASO-dot blot hybridization (DBH) PCR-RFLP using *Bst*N I for the detection of K-*ras* mutations at codon 12 was performed as we reported previously.[10] Radioisotopic ASO-DBH was also performed as we described previously.[9]

RESULTS

Detection of K-*ras* point mutations by HPA To confirm the sensitivity and specificity of this method, DNAs extracted from four pancreatic cancer cell lines (HPAF, PANC-1, PaCa-2 and BxPC-3) and synthesized oligonucleotides corresponding to wild type (GGT) and six possible mutations of K-*ras* at codon 12 were examined. Table I shows that HPAF and PANC-1 had K-*ras* mutation from GGT (glycine, Gly) to GAT (aspartic acid, Asp) in one allele because high RLUs of chemiluminescence were recognized both in Gly and Asp. On the other hand, PaCa-2 had mutations from GGT to TGT

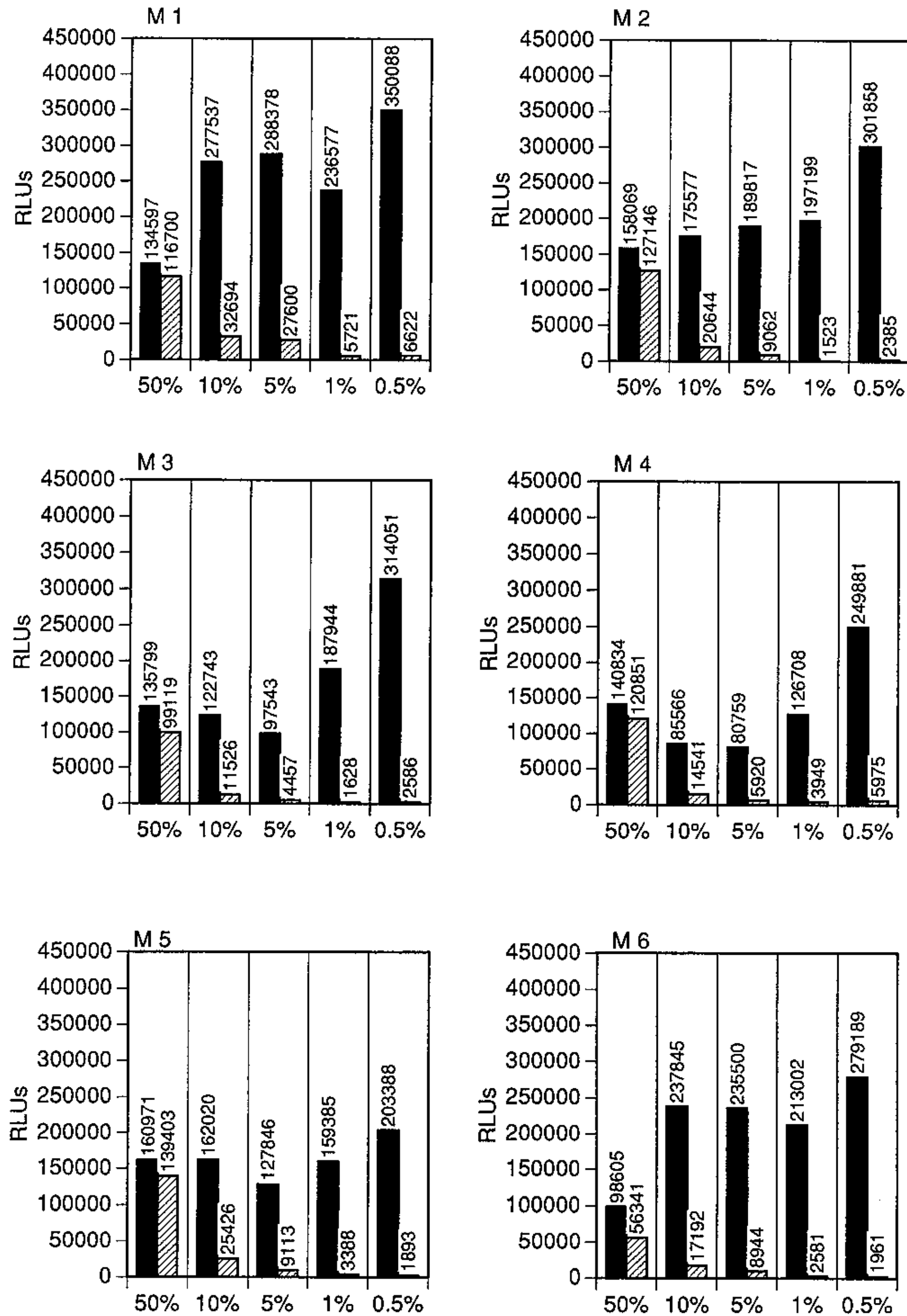

Fig. 1. HPA was performed for the PCR products with mixtures of each mutant plasmid and wild-type plasmid DNAs at a constant ratio using the six acridinium-ester-labeled DNA probes (M1: AGT, Gly; M2: CGT, Arg; M3: TGT, Cys; M4: GAT, Asp; M5: GCT, Ala; M6: GTT, Val). The fragment including the K-*ras* wild type at codon 12 was inserted into the plasmid pBluescript II SK(+) by directional cloning. Six mutant plasmids corresponding to the six possible K-*ras* mutants at codon 12 were generated by site-directed mutagenesis. Each K-*ras* mutant plasmid DNA was mixed with the wild-type plasmid DNA at a constant ratio (50%, 10%, 5%, 1%, 0.5%) and used as the template for PCR. The PCR products were then assayed by HPA. Chemiluminescence of the hybridization products was measured using a Leader I luminometer. The sensitivity of the HPA was sufficient to detect 5 to 10% of each K-*ras* mutant DNA mixed with the wild-type sequence. ■ WT probe, ▨ M probe.

(cysteine, Cys) in both alleles of K-*ras* at codon 12 because high RLUs of chemiluminescence were recognized only in Cys, and the other DNA probes, including the wild type, had very low RLUs. BxPC-3 had no mutations of K-*ras* at codon 12 because high RLUs were recognized in the wild type. These results agree with those of previous reports.[9, 10)]

To investigate the limit of detection of a K-*ras* mutant, we used DNAs from the two cancer cell lines, PaCa-2 (TGT, Cys) and BxPC-3 (GGT, Gly, wild type). As shown in Table II, the K-*ras* mutant DNA of PaCa-2 was mixed with the wild-type DNA of BxPC-3 at constant ratios of the wild-type DNA and used as the template for PCR. HPA using cancer cell lines was almost sensitive enough to detect 5% of the K-*ras* mutant DNA from PaCa-2 with a cut-off value of 7,000 RLUs. Six mutant plasmids which were generated by site-directed mutagenesis were mixed with the wild-type plasmid at a constant ratio of the wild-type plasmid as templates for PCR, as shown in Fig. 1. The sensitivity of HPA using mutant

Table III. Analysis for K-*ras* Mutations in Pure Pancreatic Juice Obtained from Patients with Pancreatic Cancer and Chronic Pancreatitis by HPA (RLUs)

	K-*ras* at codon 12	P-WT	P1	P2	P3	P4	P5	P6
PC-1	Gly, Asp	108,886	1,177	562	760	36,450	1,017	1,835
PC-2	Gly	105,341	956	528	6,505	8,368	975	5,957
PC-3	Gly, Asp	82,670	936	489	916	32,009	1,128	644
PC-4	Gly	75,652	946	525	5,492	4,782	966	3,230
PC-5	Gly, Asp	94,118	1,083	637	7,794	32,469	1,085	595
PC-6	Gly	86,868	994	484	637	1,236	1,048	461
PC-7	Gly	118,037	1,156	688	768	1,877	1,024	496
PC-8	Gly	112,380	921	694	839	1,940	1,295	500
PC-9	Gly, Asp	54,046	603	636	595	12,397	1,322	516
PC-10	Gly, Asp	104,279	1,208	575	6,521	65,894	1,128	599
PC-11	Gly, Asp	107,250	1,187	588	9,657	33,243	1,202	2,021
PC-12	Gly	87,041	1,015	525	712	1,662	1,145	549
PC-13	Gly, Asp	62,319	875	587	3,680	17,993	1,152	581
PC-14	Gly, Asp	136,630	2,307	56,687	1,812	3,742	1,364	706
PC-15	Gly, Asp	20,210	892	546	1,357	21,372	1,193	590
PC-16	Gly, Cys, Asp	26,531	860	546	21,301	17,974	1,223	644
PC-17	Gly	34,755	638	568	732	752	851	468
PC-18	Gly, Asp	89,302	980	657	613	58,130	906	641
PC-19	Gly	160,820	1,774	818	921	7,085	1,214	1,450
PC-20	Gly	18,307	756	639	647	720	1,042	570
CP-1	Gly	41,501	739	631	674	1,045	1,020	598
CP-2	Gly	178,268	1,247	875	1,394	3,714	1,088	823
CP-3	Gly	178,085	1,670	1,513	1,047	3,522	1,410	743
CP-4	Gly	81,240	914	816	915	3,630	1,287	683
CP-5	Gly	19,211	807	522	583	610	790	525
CP-6	Gly	39,210	916	553	646	5,625	926	556
CP-7	Gly	33,908	756	548	647	903	917	584
CP-8	Gly	13,891	592	517	558	763	1,041	611
CP-9	Gly	96,881	895	548	791	2,527	1,011	619
CP-10	Gly	146,665	1,315	981	1,083	2,880	898	661
CP-11	Gly	187,750	1,411	837	1,066	3,495	950	774
CP-12	Gly	69,822	875	567	659	1,482	944	926
CP-13	Gly	31,077	716	561	556	795	894	692
CP-14	Gly	79,404	754	525	655	1,468	840	2,107
CP-15	Gly	22,757	830	676	553	746	911	499
CP-16	Gly	18,220	778	676	610	726	902	542
CP-17	Gly	201,895	1,438	952	1,293	3,430	2,137	762
CP-18	Gly	30,156	851	739	724	881	1,173	641
CP-19	Gly	41,651	814	671	691	1,028	1,012	639
CP-20	Gly	78,593	1,692	688	650	1,681	1,267	609

PC, pancreatic cancer; CP, chronic pancreatitis; P-WT, acridinium-ester-labeled DNA probe (wild type, GGT, Gly); P1, (AGT, Ser); P2, (CGT, Arg); P3, (TGT, Cys); P4, (GAT, Asp); P5, (GCT, Ala); P6, (GTT, Val).

Table IV. Results of HPA Compared with Those of PCR-RFLP and ASO-DBH in PPJ from Patients with Pancreatic Cancer

	K-*ras* mutations at codon 12		
	ASO-DBH	PCR-RFLP	HPA
PC-1	n.d.	+	Asp
PC-2	n.d.	+	–
PC-3	Asp	+	Asp
PC-4	n.d.	+	–
PC-5	n.d.	+	Asp
PC-6	–	–	–
PC-7	–	–	–
PC-8	–	–	–
PC-9	n.d.	+	Asp
PC-10	n.d.	+	Asp
PC-11	n.d.	+	Asp
PC-12	n.d.	–	–
PC-13	n.d.	+	Asp
PC-14	Arg	+	Arg
PC-15	Asp	+	Asp
PC-16	n.d.	+	Cys, Asp
PC-17	n.d.	+	–
PC-18	Asp	+	Asp
PC-19	n.d.	–	–
PC-20	n.d.	+	–

n.d., not done; Asp, aspartic acid; Cys, cysteine; Arg, arginine.

plasmids of K-*ras* at codon 12 was also sufficient to detect mutants at a ratio of 5–10% in the wild type. These results suggest that K-*ras* mutant probes with chemiluminescence over 10,000 RLUs would certainly be positive.

Incidence of K-*ras* point mutations in PPJ detected by HPA Table III shows the results of determination of K-*ras* mutations at codon 12 by HPA using the PPJ samples obtained from 20 patients with PC and 20 with CP. To prevent ambiguity in the present results, the presence of K-*ras* mutants was defined as positive when the chemiluminescence of the mutant DNA probe exceeded 10,000 RLUs. According to this definition, the incidence of K-*ras* mutations at codon 12 determined by HPA was 55% (11/20) in PC. With respect to the mutational types of these 11 cases, nine had a mutation from GGT to GAT (Asp), one had two mutations from GGT to GAT (Asp) and from GGT to TGT (Cys), and one had a mutation from GGT to CGT (Arg). On the other hand, the same mutations were not detected in CP by HPA. None of the 20 CP had chemiluminescence of the mutant DNA probe exceeding 7,000 RLUs. When 7,000 RLUs was set as the cut-off value, the incidence of K-*ras* mutations at codon 12 determined by HPA was 65% (13/20) in PC.

Results of HPA compared with those of PCR-RFLP and DBH on PPJ samples In this study, the incidence of K-*ras* point mutations at codon 12 in PPJ obtained from the patients with PC was determined as 55% (11/20) by HPA, 75% (15/20) by PCR-RFLP, and 57% (4/7) by ASO-DBH using radioisotope labeling, as shown in Table IV. The mutational types determined by HPA were in agreement with those of the ASO-DBH, although the numbers revealed by the latter method were small. Differences in the results between the HPA and the PCR-RFLP were recognized in four (PC-2, 4, 17, and 20) of 20 cases in PC. These 4 cases of PC had positive results by PCR-RFLP and negative results by HPA. However, the remaining 16 cases had consistent results in both HPA and PCR-RFLP. However, when 7,000 RLUs was set as the cut-off value, K-*ras* mutation in PPJ increased to 65% due to positive values in PC-2 and PC-19. PC-2 had positive results by both PCR-RFLP and HPA. On the other hand, PC-19 was positive only by HPA.

DISCUSSION

Previously, we reported the usefulness of detection of K-*ras* point mutations at codon 12 for the diagnosis of PC using DBH with an ASO probe, by which this mutation was detected in 55% (11/20) of PPJ samples from patients with PC, but not in 18 patients with CP.[9] Moreover, the diagnostic rate of PC with PCR-RFLP using modified primers increased subsequently to 81% (21/26).[10] The incidence of K-*ras* point mutations at codon 12 detected by the PCR-RFLP method was higher (81%) than that with DBH using the ASO probe (55%). However, K-*ras* mutation at codon 12 was detected in two of 32 patients with CP by PCR-RFLP, reducing its cancer specificity. Furthermore, PCR-RFLP cannot determine the mutational types of K-*ras* at codon 12. Generally, the radioisotope-labeled ASO probe method is the most reliable and definite but it cannot detect a mutant DNA if it is present in less than 5% of the cells studied.[21]

As chemiluminescence associated with methyl acridinium esters can be completely eliminated by hydrolysis of the phenyl ester, an acridinium-ester-labeled DNA probe system was developed using selective protection from hydrolysis of the acridinium ester bond located in the interior of the probe. The interior location, the so-called intercalation site, stabilizes the acridinium ester to hydrolysis.[22–24] Consequently, very pronounced differences occur in rates of hydrolysis between the hybridized and nonhybridized forms of acridinium-ester-labeled DNA probes. This assay could be easily carried out within 30 min, since it is unnecessary to separate the hybridized from the nonhybridized acridinium-ester-labeled DNA. Using this procedure, HPA was reported to have a limit of detection of about 5×10^{-19} mol.[11] Moreover, it is possible to detect single-base mismatches using acridinium-ester-labeled DNA probes, engineering

the probes to place the acridinium ester in the region of the mismatch. In this study, acridinium-ester-labeled DNA probes were developed for the wild type and six possible mutants of K-*ras* at codon 12. As shown in Fig. 1, each mutant DNA probe had a minimal level of detection of 5–10% K-*ras* mutant in the total DNA. Furthermore, as shown in Table III, the prevalence of K-*ras* mutation at codon 12 determined by the HPA (55%) in this study was the same as that with ASO-DBH (55%), as shown in our previous study.[9] If 7,000 RLUs is taken as the cut-off value, the prevalence of K-*ras* mutation at codon 12 determined by the HPA would be 65% (PC-2 and PC-19 also become positive). On the other hand, none of the 20 examined CP had over 7,000 RLUs of mutant DNA probes. These results suggest that the sensitivity of HPA is slightly higher than that of the ASO-DBH. Furthermore, the HPA method is superior to ASO-DBH with regard to simplicity and ease of performance. However, as shown in Table IV, PC-19 was positive only by HPA, not by PCR-RFLP, with a cut-off value of 7,000 RLUs. Our previous study[10] showed that PCR-RFLP was more sensitive than HPA in this study with regard to the minimal detection level of K-*ras* mutation. Consequently, 7,000 RLUs may not be suitable as a cut-off value, although there is a possibility of an invalid result in PCR-RFLP in PC-19.

Moreover, some samples in CP (CP-8, 13, 15, 16) as well as in PC (PC-20) showed low hybridization in total. These results were thought to be mainly attributable to insufficient amplification of K-*ras* at codon 12 by PCR. These poorly reactive samples would need re-examination by PCR and HPA to avoid false-negative results. At present, optimal conditions of PCR and hybridization are still being examined.

As shown in Table IV, the incidence of K-*ras* mutation in PC determined by the HPA (55%) was lower than that by PCR-RFLP (75%), and discrepant results were recognized in four cases. However, K-*ras* mutation at codon 12 was not found in any of 20 patients with CP by HPA, but was found in one with CP (CP-6) by PCR-RFLP in the present study and in two of 32 patients with CP by PCR-RFLP in our previous study,[10] suggesting that the HPA method is superior to PCR-RFLP with regard to cancer specificity from the viewpoint of semiquantitative evaluation with an appropriate cut-off value. CP-6 showed a positive result by PCR-RFLP and a negative one by HPA, but this sample showed a relatively high value (5,625 RLUs) of the Asp probe by HPA. These results suggest that there was a relatively large proportion of hyperplastic cells, which have been shown to contain K-*ras* mutations, in the PPJ obtained from this CP patient.

The mutational types of PC obtained by the HPA were in complete agreement with those by ASO-DBH. The mutational types of the K-*ras* mutants in 11 cases of PC were Asp in nine cases, Asp+Cys in one, and Arg in one, as shown in Tables III and IV. The mutational transition from GGT (Gly) to GAT (Asp) was observed in 10/20 cases (50%) of PC. The spectrum of K-*ras* mutations in this study was similar to that of previous reports based on analysis of K-*ras* mutations at codon 12 with PC tissue specimens after surgery or autopsy.[6, 7, 25, 26] One case had double mutations (Asp+Cys) of K-*ras* at codon 12 in PPJ. The relationship between the mutational patterns, including double mutations of K-*ras* at codon 12, in PPJ and the susceptibility to the occurrence of PC or the prognosis of patients with PC remains to be elucidated.

With regard to the investigation of K-*ras* mutations at codon 12 in PPJ, in addition to our previous studies,[9, 10] Tada *et al.*[27] recently reported detection of mutant K-*ras* genes in the PPJ from all of six cases with PC (100%) using the highly sensitive and specific PCR method. Miki *et al.*[28] also reported detection of K-*ras* point mutations in four of six PC cases (67%) using PCR coupled with RFLP. More recently, Kondo *et al.*[29] reported K-*ras* point mutations in six of nine cases of PC (67%) using PCR-RFLP followed by non-radioisotopic single-strand conformation polymorphism. On the other hand, the above three reports were in agreement that there were no K-*ras* mutations in PPJ obtained from patients with CP, based on analysis of 2, 16, and 10 cases, respectively. In the present study, among 20 patients with CP, none was positive for K-*ras* mutation at codon 12 as determined by semiquantitative HPA using a suitable cut-off value.

Recently, Yanagisawa *et al.*[30] and Caldas *et al.*[31] reported that mucous cell hyperplasias of the pancreatic ductal epithelium with chronic inflammation, which had been regarded as benign, showed K-*ras* point mutations at codon 12 in 63% (10/16) and 71% (5/7) of selectively microdissected specimens, respectively. These results suggest that hyperplastic mucous cells in CP could be shed and flow into PPJ, and K-*ras* point mutations at codon 12 would be detected in PPJ in some cases of CP. Actually, Tada *et al.*[32] reported detection of K-*ras* point mutations at codon 12 in 57% of PPJ samples in 7 CP cases and in 42% of 12 healthy cases using the highly sensitive and specific PCR method described in their previous report.[27]

However, previous analyses of specimens of total CP tissues showed no K-*ras* point mutations at codon 12.[8, 33] These results suggest that pancreatic ductal epithelium harboring hyperplasia represents only a small portion of the total CP tissue; i.e., any mutant DNA is diluted by normal DNA, and no mutant K-*ras* would be found if the mutant DNA were diluted to levels below the limit of detection. In fact, as we reported previously,[10] the prevalence of K-*ras* mutant at codon 12 in PPJ from patients with CP was only 6% (2/32) as determined by PCR-

RFLP, which has a maximum sensitivity of 0.2% K-*ras* mutant in PPJ. Furthermore, K-*ras* mutation was not detected in any patients with CP by ASO-DBH[9] or by HPA in the present study, in which the minimal detectable ratio of mutant DNA was 5 to 10% of the total DNA. It will be necessary to improve methods of detection of K-*ras* mutants in PPJ with regard to cancer specificity by determination of the optimal sensitivity of detection for K-*ras* mutations in PPJ or by the development of a quantitative detection method for K-*ras* mutations in PPJ as well as the setting of cut-off values as used for conventional serum tumor markers. This HPA method may be regarded as a candidate for such a procedure.

This HPA system is a fast, simple, sensitive, and precise method for the detection of not only the presence of point mutations, but also their types. The HPA system has also been used for determination of HLA typing in blood samples.[34] Therefore, this system has wide clinical applications and should be a useful genetic test for K-*ras*, H-*ras*, N-*ras*, and other suitable genes with fixed point mutations in PPJ, various biopsy specimens (sputum, urine, stools) and surgical specimens.

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