Jpn. J. Cancer Res. 84, 961–965, September 1993

Identification of K-*ras* Oncogene Mutations in the Pure Pancreatic Juice of Patients with Ductal Pancreatic Cancers

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Pancreatic cancer is detected on the basis of morphological changes delineated by means of various image-diagnostic methods. However, differentiation between chronic pancreatitis and pancreatic cancer, especially at the early stage, is not always simple when based upon the morphological changes alone. Therefore, we attempted to elucidate K-*ras* mutations in the sediment of pure pancreatic juice (PPJ) containing exfoliated ductal pancreatic cancer cells. PPJ was collected endoscopically from 20 patients with pancreatic cancer (PC) and 18 patients with chronic pancreatitis (CP). Polymerase chain reaction and allele specific oligonucleotide dot blot hybridization for K-*ras* mutations were performed with the DNA extracted from these samples. A K-*ras* mutation at codon 12 was identified in the PPJ of 11/20 (55%) of the patients with PC. On the other hand, the same mutation was not identified in the PPJ of any patient with CP. Moreover, K-*ras* mutations at codons 13 and 61 were not recognized in the PPJ of any patient with either PC or CP. These findings suggested that the presence of a K-*ras* mutation at codon 12 in PPJ would be useful in confirming the diagnosis of PC.

Key words: Pancreatic juice — Mutation of K-*ras* — Pancreatic cancer — Endoscopic aspiration

Pancreatic cancer (PC) is the fourth or fifth most common malignancy in this country and the mortality rate is eight to nine per hundred thousand population. In contrast to other digestive malignancies, most patients with PC are not diagnosed until the advanced stage, which accounts for the poor prognosis. New markers capable of reliably detecting PC at an earlier stage, or distinguishing PC from chronic pancreatitis (CP) are needed.

Most pancreatic cancers are adenocarcinomas derived from the ductal epithelium of the pancreas, and therefore some proliferating cancer cells of the ductal nest may pass into the pancreatic juice. Based upon this, pure pancreatic juice (PPJ) has been noninvasively examined by cytological means.[1-4] However, the diagnostic rate for PC has not been high, since this procedure tends to be inaccurate due to the cell injury and degradation induced by the various digestive enzymes, such as proteases and amylase, in PPJ.

Recently, analysis of oncogenes has revealed that a point mutation of K-*ras* codon 12 occurs in about 90% of post-operative PC tissues.[5] Furthermore, this mutation does not occur in normal pancreatic tissue. Therefore, this mutation may be regarded as having high specificity for PC. This study was undertaken to elucidate the extent to which the point mutation of the K-*ras* oncogene may occur in PPJ sediments, for the purpose of detecting PC.

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MATERIALS AND METHODS

Subjects We studied 38 patients diagnosed and treated at the Department of Internal Medicine of the Cancer Research Institute Hospital at Kanazawa University, or its affiliated medical facilities. Their informed consent for this study was obtained. The group consisted of 20 patients with PC and 18 with CP. PC was located at the head (8 cases), body (9 cases), and tail (3 cases). In 7 patients with PC, the diagnosis was confirmed histopathologically at the time of operation or autopsy; the remaining 13 patients were diagnosed by endoscopic retrograde cholangiopancreatography (ERCP) in addition to ultrasonography (US), computed tomography (CT) and a compatible clinical course. All patients with PC had advanced carcinoma at clinical stages IV, except for two patients at stage II with a tumor that was less than 2 cm in diameter. The characteristics of all patients with PC are listed in Table I. The stage of pancreatic cancer was determined according to the General Rules for Cancer of the Pancreas by the Japan Pancreas Society.[6] On the other hand, the diagnosis of CP was based on the clinical diagnostic criteria of the Japanese Society of Gastroenterology[7] and all the cases corresponded to the definite criteria of CP.

Preparation of DNA from PPJ samples PPJ was endoscopically collected through an inserted cannula from the orifice of the papilla Vater under secretory stimulation by the intravenous administration of secretin (1 U/kg), using a duodenal fiberscope (JF-10: Olympus,

Table I. Clinical Data of the Cases with Pancreatic Cancer and K-*ras* Point Mutation at Codon 12

Case	Age & sex	Location	Size of pancreatic adenocarcinoma (cm)	Stage	K-*ras* mutation[c]	Type of mutation
1	59 M	Head	1.8[a]	II	+	Asp
2	56 M	Body	6.0[a]	IV	+	Cys
3	82 M	Head	5.0[b]	IV	−	
4	68 M	Body	8.0[a]	IV	+	Arg
5	60 M	Head	3.8[b]	IV	+	Asp
6	62 M	Tail	6.1[b]	IV	−	
7	76 F	Head	3.6[b]	IV	+	Asp
8	73 M	Body	4.6[b]	IV	−	
9	73 M	Body & Tail	6.0[a]	IV	+	Asp
10	47 M	Body	4.1[b]	IV	+	Arg
11	61 M	Head	4.0[b]	IV	+	Ala
12	44 F	Body	1.8[a]	II	−	
13	60 F	Head	4.0[b]	IV	−	
14	62 M	Tail	3.5[b]	IV	−	
15	51 M	Head	4.0[b]	IV	−	
16	64 F	Body	4.0[b]	IV	−	
17	58 M	Body	8.0[a]	IV	+	Asp
18	51 M	Head	6.0[a]	IV	−	
19	82 M	Tail	6.2[b]	IV	+	Asp
20	86 F	Body & Tail	8.0[b]	IV	+	Val

a) Resected tumor size.
b) Measurement by ultrasonography, computed tomography, and/or endoscopic ultrasonography.
c) K-*ras* mutation at codon 12.

Tokyo) as we previously reported.[8,9] Subsequently, PPJs were ultracentrifuged (1200 rpm, 10 min) and the sediment was collected. DNA was extracted according to the modified method of Bowtell[10] and Jeanpiérrè.[11] Subsequently, DNA was extracted by phenol-chloroform mixture. DNAs were also extracted from four PC cell lines for control studies; HPAF (provided by Kyowa Medex, Tokyo), PANC-1 and BxPC-3 (purchased from Dainippon Pharmaceutical Co., Osaka) and PaCa-2 (provided by Japanese Cancer Research Resources Bank-Cell, Tokyo).

Polymerase chain reaction The polymerase chain reaction (PCR) primers were 5′-GACTGAATATAAACTTGTGG-3′ (sense) and 5′-CTATTGTTGGATCATATTCG-3′ (antisense) that flank codons 12/13 of the c-K-*ras* gene, and also 5′-TTCCTACAGGAAGCAAGTAG-3′ (sense) and 5′-CACAAAGAAAGCCCTCCCCA-3′ (antisense) that flank codon 61 of the c-K-*ras* gene. The PCR reaction mixure contained 0.2 μg of genomic DNA, 1.0 μM of each primer, 200 μM of each dNTP (dATP, dCTP, dTTP, dGTP), 10 μl of 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 0.1% gelatin), 2.5 units *Taq* polymerase, and double-distilled water to give a final volume of 100 μl. On the top of the sample, 100 μl of high-quality mineral oil (Sigma, St. Louis, MO) was layered to prevent evaporation. The samples were subjected to PCR (denaturation, 94°C, 1 min; annealing, 52°C, 2 min, extension, 72°C, 1 min, 40 cycles) using a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CA). DNA corresponding to K-*ras* codons 12/13 and 61 was amplified by PCR, essentially following the method described by Saiki *et al.*[12] using a PCR cycler and a kit (Perkin Elmer Cetus).

Dot blot hybridization Three microliters of each amplified DNA was added to 15 μl of 0.4 M NaOH, and 25 mM EDTA, then the mixture was heated at 95°C for 2 min, spotted on to a Hybond-N nylon filter (Amersham Japan, Tokyo) and fixed using a UV cross-linker (120 mJ/cm^2). Dot blot hybridization using allele specific oligonucleotide (ASO) probe was performed as described by Verlaan-de Vries *et al.*[13] A series of specific synthetic 20mer antisense single-stranded DNA probes (Takara, Kyoto) corresponding to the possible point mutations at codons 12 and 61, and those (Clontech, Palo Alto, CA) at codon 13 were radiolabeled at the 5′ terminal using [γ-^{32}P]ATP (>5000 Ci/mmol, 10 mCi/ml) (Amersham) and T_4 polynucleotide kinase (Takara). The nylon filters were finally washed with 3 M tetramethylammonium chloride, 50 mM/HCl (pH 7.5), 2 mM EDTA and 0.1% sodium dodecyl sulfate at 61°C. Subsequently, autoradiography was performed at −80°C for 12 h using an intensifying screen.

RESULTS

We performed PCR and ASO probe dot hybridizations to detect K-*ras* mutations at codons 12, 13 and 61 with extracted DNA from PPJ samples.

In a control study using four PC cell lines, three of them contained K-*ras* mutations at codon 12, from GGT to GAT (aspartic acid) in HPAF and PANC-1, to TGT (cysteine) in PaCa-2. No mutations of K-*ras* at codons 12, 13 or 61 were recognized in BxPC-3 (data not shown).

Fifty-five percent (11/20) of the patients with PC were identified as having K-*ras* mutation at codon 12, as shown in Fig. 1. Table I also shows the results of K-*ras* analyses at codon 12 and the characteristics of patients with PC. The positivity of K-*ras* mutation at codon 12 of PC in terms of location was 50% (4/8) in the head, 67% (6/9) in the body, and 33% (1/3) in the tail. In terms of the tumor size, the positivity was 50% (1/2) in T_1 (<2.0 cm in diameter), 43% (3/7) in T_2 (2.1–4.0 cm), 50% (3/6) in T_3 (4.1–6.0 cm), and 80% (4/5) in T_4 (>6.1 cm). Table II shows the spectrum of mutational patterns of K-*ras* oncogene in PPJ with PC. The mutations were from GGT (the corresponding amino acid is glycine) to GAT(aspartic acid) in six patients, to CGT (arginine) in two, to TGT(cysteine) in one, to GCT (alanine) in one, and to GTT(valine) in one patient. On the other hand, none of the patients with CP (18 patients) had K-*ras* mutations at codon 12. Moreover, no K-*ras* mutations at codons 13 and 61 were found in any patient with PC or CP.

The PC in Case 1 was located in the uncal portion of the pancreatic head and was 1.8 cm in diameter. The same K-*ras* mutation at codon 12 from GGT(glycine) to GAT(aspartic acid) was identified both in the PPJ and in surgical specimens from Case 1 (Fig. 2).

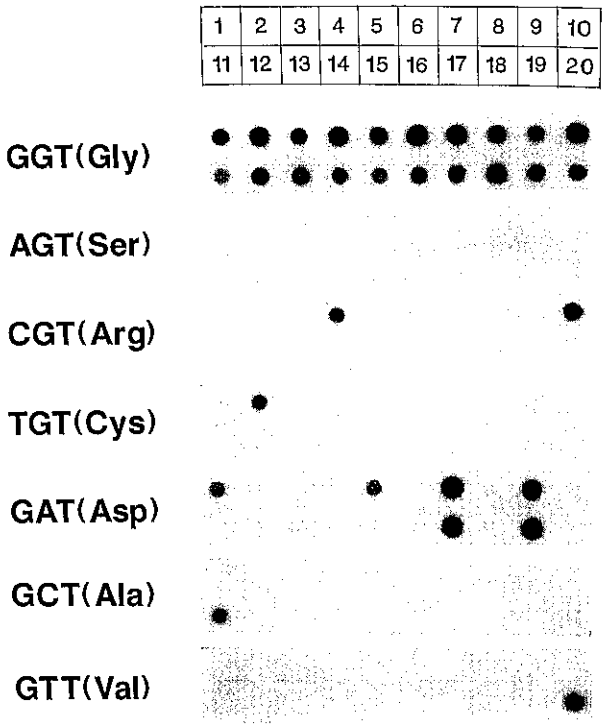

Fig. 1. Results of PCR and dot blot hybridization with ASO probes for the normal sequence GGT(Gly) of codon 12, and for the possible mutated sequences AGT(Ser), CGT(Arg), TGT(Cys), GAT(Asp), GCT(Ala), GTT(Val). Samples of PPJ were obtained from patients (1 to 20) with pancreatic cancer. The positive ratio of K-*ras* mutations at codon 12 was 55% (11/20) in PPJ samples from the patients with pancreatic cancer.

Table II. Comparison of the Incidence of K-*ras* Gene Mutations in PPJ Obtained from Patients with Pancreatic Cancer and Those with Chronic Pancreatitis

K-*ras* mutation	Pancreatic cancer (n=20)	Chronic pancreatitis (n=18)
Mutation at codon 12	11 (55%)	0 (0%)
AGT(Ser)	0	0
CGT(Arg)	2	0
TGT(Cys)	1	0
GAT(Asp)	6	0
GCT(Ala)	1	0
GTT(Val)	1	0
Mutation at codon 13	0 (0%)	0 (0%)
Mutation at codon 61	0 (0%)	0 (0%)

DISCUSSION

PC is currently diagnosed by various modalities such as US, endoscopic ultrasonography (EUS), ERCP, CT and magnetic resonance imaging (MRI), and by the presence of the serum tumor markers; CA 19-9, CEA, and so on. Although ERCP is regarded as the most reliable and sensitive method among them, there are many individuals in which the differentiation between the tumor-forming type of CP and PC is difficult based only on morphological changes of the pancreatic ducts. There are also many patients in which the detection of a small PC is difficult. Cytologic examinations of PPJ have been utilized to diagnose PC qualitatively. Skillful cytologists have reported that positive rates in PPJ from patients with PC were 52,[1] 60,[2] 33[3] and 76%.[4] Nevertheless, this examination is generally difficult to perform in the case of PC because of the cell injury and degradation induced by the various digestive enzymes in pancreatic juice, and its diagnostic efficacy has been disappointing.

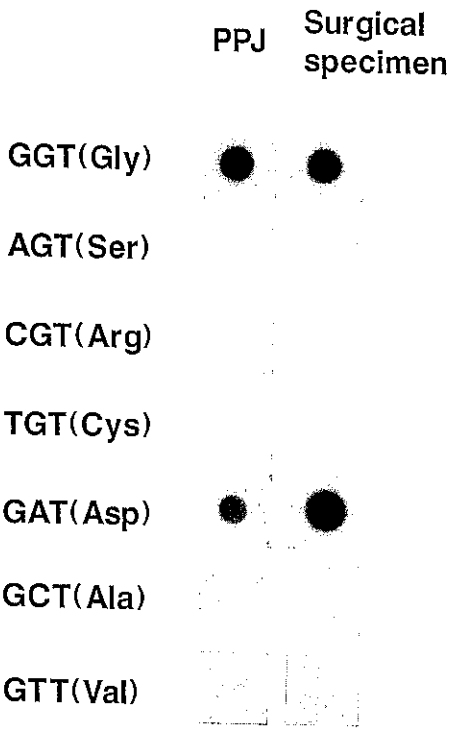

Fig. 2. DNA was extracted from the PPJ and a surgical specimen obtained from a patient (Case 1) with pancreatic adenocarcinoma (head, 1.8 cm in diameter) and analyzed by means of PCR and ASO dot blot hybridization. Both the PPJ sample and surgical specimen showed the same mutation from GGT(Gly) to GAT(Asp).

The incidence of the point mutations at codon 12 of K-*ras* oncogene in surgical specimens of PC is reportedly from 75 to over 90%.[14–18] However, K-*ras* point mutations at codons 13[17] or 61 in PC have also been rarely reported to our knowledge. This study revealed K-*ras* mutation at codon 12 in the PPJ sediments obtained from 55% of patients with PC and in none of those with CP, and mutations were not found at codons 13 or 61 in any patient with PC or CP. These results suggest that identification of a K-*ras* mutation at codon 12 in PPJ can be a useful approach for definitive diagnosis of PC. Furthermore, the ASO dot blot hybridization method for K-*ras* mutation is more objective and reliable than cytological examination of pancreatic juice for qualitative diagnosis of PC.

The incidence of K-*ras* mutation in PC (20 patients) by ASO probe dot blot hybridization was 55%. Among these, there was a patient with a T_1 cases (less than 2 cm in tumor diameter) at the uncal portion of the pancreatic head, whose PPJ sediments and surgical specimens have a K-*ras* mutation of glycine to aspartic acid at codon 12. Therefore, this method is able to detect a small PC.

On the other hand, Yanagisawa *et al.*[19] recently reported that mucous cell hyperplasias of pancreas with chronic inflammation frequently had K-*ras* point mutation at codon 12 (10/16 lesions; 62.5%) although these lesions were suggested to be neoplastic and/or precancerous in nature. However, Tada *et al.*[18] found no K-*ras* mutations in 9 tissue samples of CP obtained from surgery or autopsy. In the present study, no K-*ras* point mutations were found in the PPJ obtained from the 18 patients with CP. Although there remains a question as to exactly what stage of the malignant transformation of the pancreas K-*ras* mutation would be activated, K-*ras* mutation is now widely recognized to occur at a relatively early stage of multistep carcinogenesis of ductal pancreatic carcinoma,[20,21] in accordance with observations on colon carcinoma.[22–24] The question of how K-*ras* mutation at codon 12 happens even in chronic pancreatic inflammation without neoplastic lesion must await further study in many cases, but the detection of K-*ras* mutation in PPJ is considered to mean that malignant transformation of the ductal epithelium of the pancreas has occurred, suggesting that ours is a promising approach to the diagnosis of PC.

Generally, the ASO probe method is a reliable and definitive test but it cannot detect a mutation if it is present at a ratio below 5% of the total DNAs.[25] A higher positive rate would be obtained if specimens collected by endoscopic retrograde intraductal brushing cytology for tumorous lesions, as reported by Nakaizumi *et al.*,[4] were available for the detection of K-*ras* mutations at codon 12, since many cancer cells can be collected. Moreover, we are currently attempting to develop a more sensitive means of detecting K-*ras* mutations based upon the methods of PCR-restriction fragment length polymorphism (RFLP) with modified primers according to Haliassos *et al.*[25] or Levi *et al.*[26] If a more sensitive method is developed, it would increase the diagnostic rate of identification of K-*ras* mutation in the PPJs from patients with PC. Therefore, the presence of K-*ras* mutation at codon 12 in PPJ would become an important parameter for confirming diagnoses of PC, including early stages of PC, especially in patients in which differentiation from CP, including the tumor-forming type of CP, is difficult.

ACKNOWLEDGMENTS

We thank the Japanese Cancer Research Resources Bank (JCRB)-Cell for the gift of the pancreatic cancer cell line (PaCa-2). This work was supported in part by a Grant-in-Aid for Cancer Research (No. 05857062) from the Ministry of Education, Science and Culture of Japan.

(Received April 27, 1993/Accepted June 15, 1993)

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