

Detection of *ras* Gene Mutations in Pancreatic Juice and Peripheral Blood of Patients with Pancreatic Adenocarcinoma

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Abstract

Pancreatic adenocarcinomas are known to have a high incidence of *K-ras* gene mutations. Differential diagnosis of pancreatic cancer and chronic pancreatitis sometimes presents a clinical dilemma. We recently developed a highly sensitive and specific polymerase chain reaction capable of detecting 3–30 copies of mutant *K-ras* genes harboring codon 12 single base changes in the presence of 300,000 normal copies. Mutant *ras* genes were detected in DNA purified from pancreatic juice from all 6 cases of pancreatic adenocarcinoma and 1 case of intraductal papillary neoplasms of the pancreas. In 2 of 6 other cases with pancreatic adenocarcinoma, circulating metastatic cells were detected in DNA purified from peripheral blood. Activated *ras* genes were not found in pancreatic juice of three control cases (chronic pancreatitis and choledocholithiasis) or in the peripheral blood of two patients with insulinomas. Notable conclusions of this study are that there can be significant levels of shed tumor cells in peripheral blood and an even higher number in pancreatic juice. In addition, two different *K-ras* mutations were found in some patients.

Introduction

Over 90% of pancreatic adenocarcinomas contain mutated *ras* genes and the site of mutation is restricted to codon 12 of *K-ras* gene (1–3). Since mutant *K-ras* is not found in nonmalignant disease such as chronic pancreatitis, detection of mutant *K-ras* in pancreatic disease provides a definitive diagnosis of pancreatic adenocarcinoma (3, 4). Solid organ tumors are known to shed cells. Only an extremely small fraction of these cells have been shown to establish metastatic disease. Several laboratories have reported using the PCR² to detect metastatic cells in peripheral blood. These studies made use of the amplification of tissue-specific mRNA of tyrosine hydroxylase, tyrosinase, and prostate-specific antigen for neuroblastoma (5), melanoma (6), and prostate cancer (7), respectively, and relied on multiple copies of mRNA in each cell. We chose to target DNA rather than RNA for our studies to circumvent the restrictions on sample collection and preservation of cellular RNA. Sidransky *et al.* (8) recently described a method using PCR and phage cloning coupled to a radioactive probe-based detection strategy to identify mutant *ras* genes from shed colorectal tumor cells in feces. Unfortunately the described procedure is technically challenging since it requires sophisticated molecular biological methods.

The multiple PCR procedures that have been developed for the detection of point-mutated genes in an excess of unaltered genes have been reviewed (9). These procedures have compromised specificity or use incorrect statistical considerations for providing estimates of discrimination. We recently developed a highly sensitive PCR to specif-

ically amplify target mutant oncogenes in the presence of a vast excess of normal genes.³ This method was used to analyze the DNA samples extracted from the pancreatic juice of patients with pancreatic adenocarcinoma. On the basis of the dramatic increase in sensitivity, we also analyzed DNA samples from peripheral blood to determine if circulating metastatic cells containing a *K-ras* mutation could be detected.

Materials and Methods

Pancreatic juice was collected from ten Japanese patients at our hospitals during endoscopic retrograde cholangiopancreatography with injection of secretin (10). Two to 8 ml of pancreatic juice were obtained and examined by cytology. In two cases, pancreatic juice was divided in half and DNA was extracted separately. For extraction of DNA, 0.5 to 1 ml of pancreatic juices was centrifuged to collect cells; then the pellets were washed with PBS and resuspended in 100 μ l of 10 mM Tris (pH 8.0), 0.9% polyoxyethylene 10 lauryl ether (Sigma), and 0.05 mg/ml proteinase K. The mixtures were incubated at 56°C for 1 h, heated at 95°C for 10 min, and centrifuged briefly, and then the DNAs were collected using a Centricon 100 (Amicon). DNAs were resuspended in 40 μ l of 10 mM Tris (pH 8.0)-0.1 mM EDTA. Ten μ l of each sample, containing 5 to 100 ng of DNA as determined by the method of Walsh *et al.* (11), were used for PCR.

Peripheral blood (5 ml) from eight different patients was collected in the presence of EDTA. The samples were centrifuged, the supernatants were discarded, and the pellets were resuspended in 4 ml of PBS at 4°C before 8 ml of cold distilled water were added to lyse RBC. After centrifugation and washing with PBS, the pellets were digested with proteinase K followed by phenol-chloroform extraction (12). The DNAs were then quantitated by UV absorption. About 100 μ g of DNA were extracted and 1 μ g of DNA in 10 μ l of 10 mM Tris (pH 8.0)-0.1 mM EDTA was used for each PCR. The highly sensitive and specific PCR method used is described separately.³ Since only three kinds of mutations at codon 12 of *K-ras* gene (CGT, GTT, or GAT) were reported in cases of Japanese patients with pancreatic adenocarcinoma (3), three different sense primers were used, the 3'-nucleotide of which was complementary to one of the mutant codons (*e.g.*, CGT, GTT or GAT). The sequences of the primers were: R1 = GGAGTTGGAGCTC; R2 = GTAGT-TGGAGCTGT; R3 = GTAGTTGGAGCTGA; and the antisense primer, R4 = CTATTGTTGGATCATATTTCG, respectively. The primers direct amplification of an 88- or 89-base pair product from exon 1. Three amplification reactions were performed for each sample; primer combinations used were R1–R4, R2–R4, and R3–R4. Briefly, up to 1 μ g of sample DNAs was used in 50 μ l amplification reactions containing 20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 50 μ M concentrations of each dATP, dCTP, dGTP, and TTP, 0.25 μ M concentrations of each primer, and 5 units of AmpliTaq DNA polymerase, Stoffel Fragment (Perkin-Elmer) incorporating AmpliWax (Perkin-Elmer) for greater specificity (13). For the primer combinations R1–R4 and R2–R4, PCR was performed for 45 cycles (95°C for 10 s, 56°C for 10 s) and for R3–R4, 35 cycles were used (95°C for 10 s, 56°C for 5 s) using a GeneAmp PCR system 9600 (Perkin Elmer).³ The thermal cycler was precycled (at least 5 cycles) to ensure accurate temperature control for the initial annealing steps.

Following amplification, 10 μ l of the reaction mixtures were loaded on 8% acrylamide gels and stained with ethidium bromide.

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² The abbreviations used are: PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

³ M. Tada, R. Saiki, M. Omata, M. Ohto, and J. J. Sninsky. Improved single nucleotide discrimination by PCR: *K-ras* codon 12 mutations, submitted for publication.

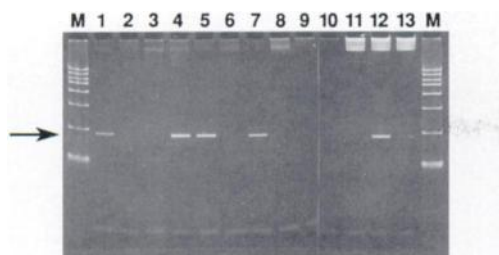


Fig. 1. Detection of GTT type mutation in pancreatic juice by PCR. Lanes 1–10 correspond to patients 1–10 (Table 1); Lane 11, negative control (1 µg of human placental DNA); Lanes 12 and 13, positive control [0.1 (calculated as approximately 30 copies of mutant *ras* genes) and 0.01 ng, respectively, of previously characterized tumor DNA in 1 µg of human placental DNA]; M, GelMarker (Research Genetics) with bands at 1000, 700, 500, 400, 300, 200, 100, and 50 base pairs. Arrow, position of 88-base pair PCR product.

Results

Following a systematic and comprehensive survey of reaction-conditions, thermal cycling parameters, alternative DNA polymerases, and the sequestering of reaction components prior to the initial denaturation step, we identified a highly sensitive and specific PCR protocol for the identification of rare cancer cells harboring a single nucleotide alteration.³ The optimized PCR assay provides for the detection of 0.01 ng of mutant DNA (equivalent of 3 copies of activated *K-ras* genes) for either CGT or GTT codons in 1 µg of DNA (equivalent of 150,000 cells or 300,000 copies of *K-ras* genes) that did not contain an activated oncogene. Detection of the GAT codon requires 10-fold more copies in 1 µg of DNA.³

Pancreatic juices were initially analyzed cytologically. Although there were no cases that proved malignant by cytology, mutations of *K-ras* codon 12 were detected in all 6 cases with pancreatic adenocarcinoma and 1 case with papillary neoplasm of the pancreas, whereas 3 control cases (2 cases with chronic pancreatitis, 1 case with choledocholithiasis) were not found to contain mutations (Fig. 1; Table 1). Of the 7 cases, 2 cases contained a GAT mutation, 2 cases showed a GTT mutation, 2 cases showed both GAT and GTT mutations, and 1 case showed both GAT and CGT mutations (Table 1). Two DNA samples (from patients 4 and 5) were divided in two and the separated fractions were extracted independently; the results were the same (data not shown).

The mutations detected in pancreatic juice correlated with those shown by directly analyzing tumor samples (3). Tumor samples from four of seven patients (Table 1) had been analyzed previously by amplification of exon 1 of *K-ras* gene followed by direct sequencing (3). In 3 cases, a point mutation shown by direct sequencing matched the results from the single nucleotide discriminatory PCR method. In another patient, only a GAT mutation was demonstrated by direct sequencing; both GAT and GTT mutations were detected by the optimized PCR.

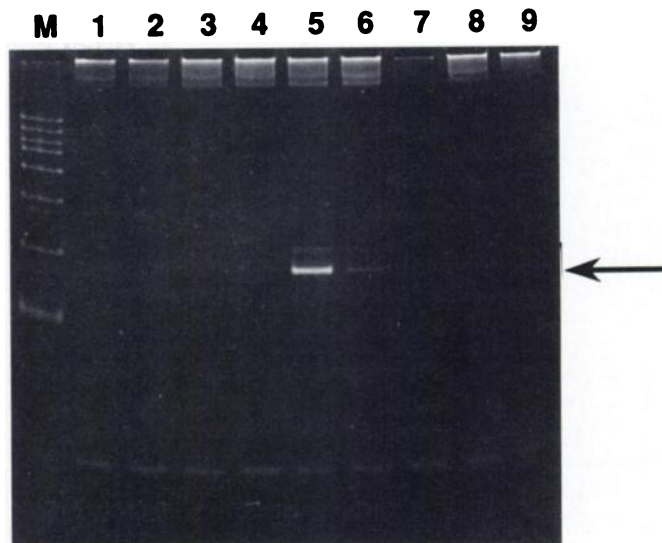


Fig. 2. Detection of GTT type mutation in peripheral blood. Lanes 1–8 correspond to patients 11–18 (Table 2); Lane 9, negative control (1 µg of human placental DNA); M, GelMarker (Research Genetics) (molecular weights as indicated in legend to Fig. 1).

Our success in detecting activated oncogenes in pancreatic juice suggested that this procedure may be capable of detecting metastatic cells in peripheral blood. GTT mutations were detected in 2 of the 6 cases with pancreatic adenocarcinoma (Fig. 2; Table 2). The *ras* mutations were detected in peripheral blood from one patient with evidence of metastasis to remote organs and from another that had no evidence of metastatic spread (Table 2). Mutations were not detected in two cases of insulinoma.

Discussion

The clinical diagnosis of pancreatic adenocarcinoma has improved but there are still cases in which a definitive diagnosis of pancreatic cancer from chronic pancreatitis cannot be made. These two diseases may share similar clinical and radiological characteristics (14). In such cases, pathological diagnosis is required for specific diagnosis. Fine needle aspiration cytology or biopsy have been used for specific diagnosis of pancreatic adenocarcinoma (10). Because of the location of the pancreas, biopsy is not as straightforward as gastrointestinal tract tumors of the stomach and colon. In addition, a risk that tumor cells may spread following biopsy has been reported (15). Cytological examination of cells in samples of pancreatic juice is another option (10); however, the earlier reports that showed high sensitivity for diagnosis have not been repeated (16). Although the PCR method, as used in this study, is not quantitative, comparison to a panel of copy number controls and extrapolation permitted estimation of the approximate number of activated oncogenes detected. As much as 1–10% of

Table 1 Detection of *ras* gene mutations in pancreatic juices

Patient	Age (yr)	Sex	Diagnosis	Tumor size (cm)	Cytology	Mutant in pancreatic juice	Tumor mutation ^a
1	60	M	Pancreatic adenocarcinoma	5 × 8	Class 1	GTT	GTT
2	61	M	Pancreatic adenocarcinoma	4 × 4	Class 2	GAT	GAT
3	72	M	Pancreatic adenocarcinoma	4 × 4	Class 2	GAT	GAT
4	76	M	Intraductal papillary neoplasms	N/A	Class 2	GAT/GTT	N/A ^b
5	57	M	Pancreatic adenocarcinoma	4 × 5	Class 2	GAT/GTT	GAT
6	71	F	Pancreatic adenocarcinoma	3 × 4	Class 2	GAT/CGT	N/A
7	75	F	Pancreatic adenocarcinoma	3 × 4	Class 2	GTT	N/A
8	73	M	Chronic pancreatitis		Class 2	N/D	N/A
9	40	F	Chronic pancreatitis		N/A	N/D	N/A
10	53	M	Choledocholithiasis		N/A	N/D	N/A

^a The sequence of wild type is GGT. These sequences were determined by PCR followed by direct sequencing as described elsewhere (3).

^b N/A, not available; N/D, not detected.

Table 2 Detection of *ras* gene mutations in peripheral blood

Patient	Age (yr)	Sex	Diagnosis	Tumor size (cm)	Metastases to remote organ ^a	Mutant in peripheral blood
11	68	M	Pancreatic adenocarcinoma	6 × 7	+	Not detected
12	42	F	Insulinoma	3.5 × 3.5	-	Not detected
13	60	M	Insulinoma	3 × 4	+	Not detected
14	50	M	Pancreatic adenocarcinoma	4 × 4	+	Not detected
15	70	M	Pancreatic adenocarcinoma	2 × 4	-	GTT
16	56	M	Pancreatic adenocarcinoma	4 × 3	+	GTT
17	63	M	Pancreatic adenocarcinoma	4 × 3	+	Not detected
18	60	F	Pancreatic adenocarcinoma	6 × 9	-	Not detected

^a Liver, pleura, peritoneum, and distant lymph nodes.

the cells in the pancreatic juices from patients examined harbored point mutations. The basis for the discrepancy of the results of molecular biological and cytological methods is unclear but may be due to degenerative changes of tumor cells in pancreatic juice (16). Confirmation of these results with larger numbers of samples is required to resolve this question. This molecular approach provides a more sensitive and specific as well as more convenient diagnosis of pancreatic adenocarcinoma.

Three of the 7 cases were shown to have two different mutations at codon 12 of *K-ras* (Table 1). Previous analysis of tumor material from one of these cases (patient 5) using entire exon amplification and direct sequencing of PCR products detected only one of these mutations. The presence of two different mutations of *ras* genes from the same sample, although previously reported, are rare (17). Preliminary quantitation indicated that the GAT mutation was in approximately a 4–10-fold excess of other mutations. There are multiple possible explanations for the presence of multiple *ras* mutations. The *ras* mutations may not be the primary genetic insult of the oncogenic process in pancreatic cancer but instead may be a result of the genetic lability initiated by an earlier genetic alteration. Alternatively, the genetic instability introduced by the first *ras* mutation may increase the likelihood of a mutation in the other copy of the *ras* gene in the same cell with advanced tumors. It would be interesting to determine if both mutations are found in the same cell. The dramatic increase in sensitivity of this PCR method for activated oncogenes presumably is the reason for the surprising large proportion of samples with two different mutations. Additional studies with this highly sensitive procedure will be necessary to determine the prevalence and significance of this observation.

The optimized single nucleotide discriminatory PCR assay was capable of detecting mutant DNA in peripheral blood in a fraction of the pancreatic adenocarcinomas tested. When samples of peripheral blood were studied, a GAT mutation was not detected although this was the most common type of mutation in cases of Japanese pancreatic adenocarcinoma (3). Whether this represents a statistical anomaly or the fact that the GAT mutation is detected with 10-fold less sensitivity is unclear. By extrapolation, these data indicate that there may be as many as 2 million circulating metastatic cells in peripheral blood alone. This result suggested that large numbers of tumor cells were circulating in the blood stream, in some cases, which may reflect one reason for the poor prognosis of pancreatic adenocarcinoma. Unlike the PCR procedure that targets tissue-specific mRNA to detect metastatic cells in peripheral blood (5–7), the PCR method described here does not identify the affected tissue or organ. On the other hand, the detection of activated *ras* genes can be used for all cancers in which this oncogene participates in the carcinogenic process thereby reduc-

ing the number of different assays required. Increasing the sensitivity to detect GAT mutations and studying larger numbers of cases including early stage pancreatic adenocarcinoma should be carried out to confirm these results and, by comparison to clinical features, to determine utility beyond differential diagnosis.

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