

Advances in Brief**A Prospective Study of K-ras Mutations in the Plasma of Pancreatic Cancer Patients¹**

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Abstract

K-ras mutations are frequently found in primary pancreatic adenocarcinomas. In this prospective study, we looked for K-ras mutations in the plasma of patients with pancreatic cancer. We isolated plasma DNA from 21 pancreatic cancer patients using a simple and rapid extraction technique and detected K-ras alterations with a PCR assay and subsequent product sequencing. Patients were followed up to determine their clinical outcome. We found K-ras mutations in the plasma of 17 patients (81%). In cases in which both plasma and pancreatic tissue were available, DNA mutations were similar in corresponding plasma and tissue samples. Plasma DNA alterations were found 5–14 months before clinical diagnosis in four patients. Mutant DNA was not found in the plasma of two patients with chronic pancreatitis or in five healthy controls. Our results indicate that K-ras mutations are often found in DNA isolated from the plasma of pancreatic cancer patients and that a noninvasive plasma-based assay may provide qualitative diagnostic information to clinicians in the future. Larger studies are required to further assess the relevance of our findings to clinical practice.

Introduction

Pancreatic adenocarcinoma is the fourth most common cause of cancer death in the Western world. Median survival after diagnosis is less than 6 months (1), and long-term survival rates are less than 5% (2), partly because symptoms usually occur only at a late pathological stage. Even when symptoms are

present, pancreatic adenocarcinoma can be difficult to differentiate from other conditions on the basis of clinical features and imaging investigations. Thus, a false positive diagnosis is common (3–5), whereas fine-needle aspiration or biopsy yields a false negative result in 10–40% of cases (6). Serum markers have been developed to complement conventional diagnostic tests (2), but even the most promising quantitative assays rely on a cutoff point above or below which a diagnosis of pancreatic cancer is more or less likely.

The K-ras gene is mutated in over 90% of pancreatic cancers (7). These mutations are well defined, reliably detected by DNA amplification assays, and occur early in the genesis of pancreatic cancer (8), indicating that mutant K-ras might serve as a qualitative marker for pancreatic cancer (9–12). K-ras alterations have been found in pancreatic cancer tissue obtained by fine-needle aspirate (9, 13) and in pancreatic duct secretions obtained endoscopically (10, 11). Mutant DNA has also been extracted from pancreatic cancer cells found circulating in peripheral blood (12). However, this phenomenon seems to be restricted to intraoperative and early postoperative periods (14), making it unsuitable for diagnostic purposes.

In addition to its presence within circulating cells, DNA is also found circulating in blood plasma. Nanogram quantities are present in the plasma of normal subjects (15, 16), and increased quantities circulate in patients with chronic autoimmune disorders such as systemic lupus erythematosus (16). Cancer patients have even higher quantities of serum or plasma DNA (17–20); the highest levels are found in those with pancreatic cancer (21). DNA extracted from the plasma of cancer patients also displays neoplastic characteristics such as decreased strand stability (22). Studies using PCR have identified microsatellite alterations in the plasma and serum DNA of patients with head and neck cancer (23) and lung cancer (24). In addition, ras gene mutations have been detected in the plasma of patients with colorectal cancer (25) and hematological malignancies (26). Finally, Sorenson *et al.* (27) found mutant plasma DNA in three patients with pancreatic cancer. In our prospective study, we isolated DNA from the plasma of patients with a suspected diagnosis of pancreatic cancer and analyzed this DNA for K-ras mutations.

Materials and Methods

Patients. This study included 21 patients with pancreatic cancer (17 with a firm diagnosis at the time of plasma sampling and 4 who were undergoing investigations for possible pancreatic cancer at the time of sampling). The median age of the study population was 60 years (range, 41–88 years; 9 males and 12 females). Diagnosis was established by a combination of pancreatic biopsy or aspirate, abdominal ultrasound, computed tomography, and endoscopic retrograde cholangiopancreatography. All patients had unresectable disease at diagnosis and were treated by endoscopic biliary stenting and/or chemotherapy and radiotherapy. Patients were followed for a median of 5.5 months (range, 1.2–16.7 months) and follow-up ended in March 1997.

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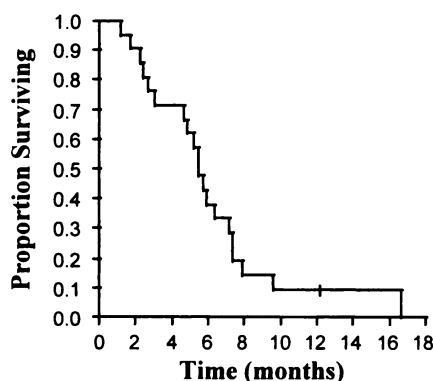


Fig. 1 Survival of 21 patients with advanced pancreatic cancer.

The survival of the patient population is shown in Fig. 1. Blood was taken from five healthy subjects and three patients with chronic pancreatitis for use as negative controls. The study was approved by the East London and City Health Authority Ethics Committee.

DNA Preparation and Detection of K-ras Alterations. Peripheral blood (10 ml) was collected in heparinized tubes from informed and consenting patients and centrifuged at $2000 \times g$ for 12 min. Plasma and lymphocytes were stored separately at -70°C until further use. After biopsy, approximately 1 mm^3 of biopsy material was taken for K-ras testing. DNA was extracted from tissues, lymphocytes, and blood plasma as described previously (24, 25).

Plasma and tumor DNA samples were examined separately for K-ras codon 12 alterations using a two-stage RFLP (RFLP-PCR) assay (25, 28). The initial $50\text{-}\mu\text{l}$ reaction included 50 mM KCl, 20 mM Tris-HCl, (pH 8.3), 200 μM each nucleotide, 1.5 mM MgCl_2 , 5 μM each primer P1 (Table 1; antisense) and P2 (sense), and 1.5 units of AmpliTaq DNA polymerase. After a hot-start and initial denaturation of 10 min, 30 cycles (96°C for 1 min, 55°C for 1 min, and 73°C for 30 s) were performed, followed by a 7-min extension at 73°C . PCR products were digested with the enzyme *MvaI* (Biofinex, Praroman, Switzerland) according to manufacturer's instructions. Wild-type DNA was used as a negative control, and DNA from SW480 cells was used as a positive control for PCR product digestions. The first amplification product was reamplified using primers P3 (Fig. 1; antisense) and P2 (sense), and reaction conditions were identical to those used in the first amplification. After *MvaI* digestion, products were visualized on a high-resolution electrophoresis system (Sea 2,000; Elchrom Scientific, ChAM, Switzerland). All RFLP-PCR products were also analyzed by direct sequencing using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, OH).

PCR experiments were performed at least twice on each sample, and genomic DNA from cancer patients, plasma DNA from chronic pancreatitis patients, and DNA from healthy subjects served as negative controls. DNA from the SW480 cell line, which is homozygous for the K-ras codon 12 GTT mutation, was used as a positive control.

Table 1 Primers used in RFLP-PCR reactions

Primer	Sequence (5'→3')
1	TCAAAGAATGGTCCTGGACC
2	ACTGAATATAAACTTGTGGTAGTTGGACCT
3	TAATATGTCGACTAAAACAAGATTTACCTC

Results

Adequate amounts of DNA were extracted from the plasma of all patients and controls. RFLP-PCR analysis and gel electrophoresis showed that the wild-type K-ras gene was present in all tumor and plasma specimens and that only mutant DNA was present in the SW480 cell line (Fig. 2). K-ras alterations were not found in DNA extracted from the plasma of three patients with chronic pancreatitis or from five normal subjects.

PCR product sequencing identified K-ras mutations in the plasma of 17 of 21 cancer patients (81%). All mutations were situated at the first or second base of codon 12 (Table 1). The most common alteration was from GGT (wild type) to GTT (valine). Alterations from GGT to TGT (cysteine), GAT (aspartic acid), and CGT (arginine) were also found, and a combination of two mutations was seen in the plasma of patient 4 at initial testing. Biopsy tissues were available for 10 patients, and plasma and tumor DNA alterations corresponded in each case in which mutations were present (Table 2).

In this prospective study, we detected K-ras alterations in DNA isolated from the plasma of four cancer patients before diagnosis (Table 2, patients 1–4). Patient 1 presented with abdominal pain and jaundice. Chronic pancreatitis was diagnosed after radiological tests and a laparotomy, and an endoscopic biliary stent was inserted. The patient was followed-up in clinic, and during the course of follow-up, a plasma sample was taken for K-ras analysis 1 year later. DNA extracted from this sample harbored a GTT mutation at codon 12. The patient remained well for an additional 14 months before being admitted with weight loss. Imaging showed a mass in the pancreatic head with associated liver metastases that were biopsied. These contained poorly differentiated adenocarcinoma, and extracted DNA had an identical K-ras mutation to that found at initial plasma testing.

Patient 2 was admitted with jaundice and abdominal pain. He had consumed over 70 units of alcohol/week for many years. Imaging showed a mass in the pancreatic head, and histological examination of biopsy tissue showed focal pancreatic fibrosis without cancer. Tissue DNA contained wild-type K-ras, but plasma DNA had a TGT mutation. The patient was well for an additional 5 months and then developed gastric outlet obstruction. A biopsy showed chronic pancreatitis associated with poorly differentiated adenocarcinoma. GAT and TGT alterations were found in both tissue and plasma DNA.

Patient 3 presented with weight loss, jaundice, and pain. Imaging showed a pancreatic mass. Biopsy tissues showed diffuse fibrosis without cancer. Tissue DNA had wild-type K-ras, and plasma DNA had a GTT mutation. A biliary stent was inserted, and the patient was discharged with a diagnosis of chronic pancreatitis. He deteriorated clinically over 7 months and died 1 month later, having refused a further pancreatic

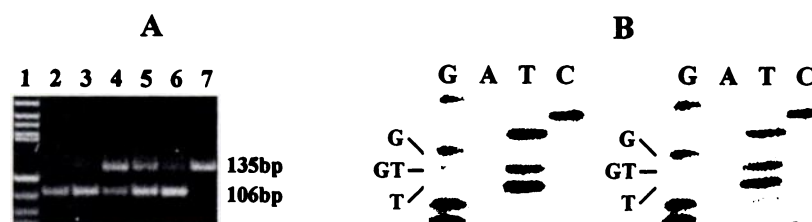


Fig. 2 A, detection of *K-ras* mutations by RFLP-PCR. *Lane 1*, molecular weight marker (pBR 322 DNA digested with *Hae*III); *Lane 2*, lymphocyte control; *Lane 3*, plasma DNA from patient with chronic pancreatitis (faint 135-bp band appearing in this lane results from partially undigested wild-type DNA as confirmed by sequencing); *Lane 4*, case 17 plasma DNA; *Lane 5*, case 7 plasma DNA; *Lane 6*, case 7 tumor DNA; *Lane 7*, SW480 DNA. B, sequence of the region flanking *K-ras* codon 12 in (left) tumor and (right) plasma DNA from patient 1 (GTT mutation present in both samples).

biopsy. Based on imaging investigations and the clinical course, the final diagnosis was pancreatic cancer.

Patient 4 presented with weight loss and abdominal pain. He had consumed over 100 units of alcohol/week for 25 years. Imaging identified a fullness in the pancreatic head, and a biopsy contained pancreatic tissue without cancer. DNA extracted from both biopsy tissue and from plasma had GTT and TGT mutations. He was well for 7 months, until he was admitted with jaundice. Imaging again identified a mass in the pancreatic head, and a biopsy showed fibrosis and infiltration by adenocarcinoma. Both tissue and plasma DNA had a TGT alteration.

Discussion

Our results show that DNA extracted from the plasma of patients with advanced pancreatic cancer often contains a *K-ras* mutation. These results are also the first to indicate that detection of mutant plasma DNA may precede conventional cancer diagnosis.

Serum from patients with pancreatic cancer contains about 650 ng DNA/ml compared with 270 ng/ml in colorectal cancer patients and 14 ng/ml in healthy subjects (21). We have previously extracted DNA from the plasma of cancer patients by phenol followed by centrifugation on a Cs_2SO_4 gradient (18, 25). In the present study, we used a simplified extraction protocol (24) that requires only 1–3 ml of plasma and that has reduced extraction times from 120 h (18, 25) to less than 4 h. The RFLP-PCR assay reliably detects *K-ras* mutations in gastrointestinal cancers (28) and has previously been used both by Sorenson's group (27) and by our group (25) to characterize mutant plasma DNA. The assay is sensitive, (25) and in contrast to techniques that require multiple PCR runs and mutant specific primers to amplify individual point mutations (25, 29), two RFLP-PCR runs can be used to screen for all codon 12 *K-ras* abnormalities. The RFLP-PCR technique could be simplified further by using high-resolution fragment analysis (Fig. 2) rather than PCR product sequencing to detect mutations. This would also reduce costs and assay times and allow plasma DNA mutations to be detected within 36 h of venesection.

In the present study, we found only wild-type *K-ras* in the plasma of three patients with chronic pancreatitis and in healthy controls. A number of previous studies have also noted that *K-ras* gene alterations are not present in benign pancreatic conditions, indicating a high specificity of mutations for the

presence of pancreatic cancer (9–12, 29–31). However, some recent studies have also demonstrated *K-ras* mutations in microdissected tissues taken from patients with pancreatic hyperplasia in either the presence or absence of chronic pancreatitis (32, 33). This has led to the suggestion that pancreatic cell hyperplasia may be a premalignant condition (32), analogous to the relationship between adenomatous polyp and adenocarcinoma in the large bowel. Although the presence of *K-ras* alterations in some cases of chronic pancreatitis may be important in elucidating pathways leading to pancreatic cancer, the significance for plasma DNA testing in these conditions is unknown. Leon *et al.* (17) found that levels of plasma DNA increased in line with cancer progression, with the highest levels being found in those with metastatic disease. In addition, Nawroz *et al.* (23) showed that mutant plasma DNA was found primarily in head and neck cancer patients with advanced disease. These results suggest that even if gene alterations are present in small numbers of hyperplastic cells, they are unlikely to be present at detectable levels in the plasma of such patients. However, further research on nonmalignant pancreatic diseases would be valuable to determine the true specificity of plasma DNA *K-ras* mutations for cancer.

We found mutant plasma DNA in 81% of our cancer patients, including four in whom the diagnosis had not been made at the time of plasma testing. In addition, we found that corresponding plasma and tumor *K-ras* alterations were similar when both tumor and plasma samples were available. Studies on other tumor types have also found a close correlation between plasma and tumor DNA mutations (23–27), indicating that a proportion of plasma DNA derives from the primary cancer. A *K-ras* mutation was present in the primary tumor but absent in the plasma of patient 8, perhaps as a result of PCR assay insensitivity to low levels of mutant plasma DNA. *K-ras* mutations were also absent in the plasma DNA of two cases in which tumor DNA was negative and in an additional case in which tumor tissue was unavailable for study. Although the sensitivity of the plasma DNA assay for cancer is high at 81%, it might be improved further still by a search for other gene alterations such as those of the p53 gene within plasma DNA.

K-ras alterations have previously been found in pancreatic cancer biopsies and in pancreatic duct brushings (29, 31). Mutant DNA has also been found in pure pancreatic juice, bile, duodenal juice, and even feces of pancreatic cancer patients, suggesting that genetic testing could supplement conventional

Table 2 Clinical features and K-ras mutations in 21 pancreatic cancer patients

No./sex/age (yr)	Tumor site	Tumor size (cm) ^a	Plasma K-ras	Tumor K-ras	Outcome	Follow-up (mo) ^b
1/F/49	Head	2.0 × 2.0	GTT	GTT ^c	DOD ^d	16.7
2/M/63	Head	4.0 × 3.5	TGT	TGT/GAT	DOD	7.3
3/M/70	Head	3.0 × 2.0	GTT		DOD	9.6
4/M/56	Head	2.0 × 2.0	GTT/TGT	GTT/TGT	AWD ^f	12.2
5/M/57	Head	3.0 × 3.0	TGT	TGT	DOD	2.4
6/M/73	Head	4.0 × 3.0	CGT	CGT	DOD	3.0
7/F/53	Body	4.0 × 3.5	GTT	GTT	DOD	6.4
8/F/75	Head	3.0 × 3.0		TGT	DOD	1.7
9/M/69	Head	5.0 × 4.0			DOD	4.7
10/F/56	Head	4.0 × 3.0			DOD	1.2
11/F/71	Body	4.5 × 3.5	GTT	NA ^g	DOD	7.2
12/F/75	Head	4.5 × 2.0	TGT	NA	DOD	5.7
13/F/48	Head	4.0 × 3.0	GTT	NA	DOD	7.3
14/F/88	Head	3.0 × 2.0	TGT	NA	DOD	4.8
15/M/51	Head	3.0 × 2.5	GAT	NA	DOD	5.5
16/M/50	Head	2.0 × 2.0	TGT	NA	DOD	5.2
17/F/60	Head	5.0 × 3.0	GTT	NA	DOD	2.2
18/F/41	Head	2.0 × 2.0	GTT	NA	DOD	7.9
19/F/74	Body	3.5 × 3.5	GAT	NA	DOD	2.7
20/M/74	Head	4.0 × 2.0	GTT	NA	DOD	5.5
21/F/52	Head	4.0 × 3.0		NA	DOD	5.9

^a Estimated from radiological data.

^b From time of plasma sample.

^c Tumor tissue obtained at biopsy of liver metastasis.

^d DOD, dead of disease.

^e No tumor tissue or mutant DNA found in initial pancreatic biopsy.

^f AWD, alive with disease.

^g NA, no biopsy tissue available for DNA testing.

diagnosis (10, 11, 30, 34). Our results indicate that a noninvasive plasma-based assay might also provide rapid and qualitative diagnostic information to clinicians in the future. Larger studies are required to assess the relevance of our findings to clinical practice.

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