K-*ras* Mutation and *p16* and Preproenkephalin Promoter Hypermethylation in Plasma DNA of Pancreatic Cancer Patients:

In Relation to Cigarette Smoking

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Abstract

Objectives—To examine the profiles of K-*ras* mutations and *p16* and *preproenkephalin* (*ppENK*) promoter hypermethylation and their associations with cigarette smoking in pancreatic cancer patients.

Methods—In plasma DNA of 83 patients with untreated primary pancreatic ductal adenocarcinoma, DNA hypermethylation was determined by methylation-specific polymerase chain reaction and K-*ras* codon 12 mutations by enriched-nested polymerase chain reaction followed by direct sequencing. Information on smoking exposure was collected by in-person interview. Pearson χ^2 test and Fisher exact test were used in statistical analysis.

Results—K-*ras* mutations, *ppENK*, and *p16* promoter hypermethylation were detected in 32.5%, 29.3%, and 24.6% of the patients, respectively. Sixty-three percent (52/83) of patients exhibited at least one of the alterations. Smoking was associated with the presence of K-*ras* mutations ($P = 0.003$). A codon 12 G-to-A mutation was predominantly observed in regular smokers and in heavy smokers (pack-year of smoking ≥36). Smoking was not associated with *p16* or *ppENK* hypermethylation.

Conclusions—These preliminary observations suggest that plasma DNA might be a useful surrogate in detecting genetic and epigenetic alterations of pancreatic cancer. The findings on the association between K-*ras* mutation and smoking were in consistency with previous studies. Further studies on environmental modulators of epigenetic changes in pancreatic cancer are warranted.

Keywords

plasma DNA; K-*ras* mutation; DNA methylation; p16; preproenkephalin; cigarette smoking; pancreatic cancer

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Adenocarcinoma of the pancreas is the most common type of pancreatic cancer and is a highly lethal disease among all types of malignancies.[1] The profile of genetic and epigenetic alterations of pancreatic cancer has been unveiled by many studies.[2,3] Because cigarette smoking is a well established risk factor for pancreatic cancer, elucidation of the relationship

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between molecular alterations and such environmental factor will not only advance our understanding on the etiology of pancreatic cancer, but will also have implications for the clinical management of this disease.

Mutation activation of the K-*ras* oncogene is a well established molecular alteration in the pathogenesis of pancreatic ductal adenocarcinoma.[4] Aberrant hypermethylation of 5′ CpG islands of tumor suppressor genes has been identified as an alternative mechanism to mutation or deletion in gene inactivation in different types of cancers.[5] Alteration of the *p16* gene, which is responsible for controlling cell growth, has been well characterized in pancreatic carcinogenesis. The *p16* is inactivated in approximately 95% of pancreatic adenocarcinomas and approximately 15% of those were attributable to aberrant promoter methylation.[6] The *p16* gene is aberrantly methylated in 27% of pancreatic cancer cell lines.[7] The *preproenkephalin* (*ppENK*) gene encodes the opioid growth factor, which induces apoptosis in lung cancer cell lines, delays cell cycle progression, and exerts a negative growth regulatory effect on various kinds of cancers, including pancreatic cancer.[8] The *ppENK* gene has been shown to be aberrantly methylated in more than 90% of pancreatic carcinomas.[3]

Both genetic and epigenetic changes can be modulated by environmental factors. Several studies have found that the frequency of K-*ras* mutations in pancreatic tumor is associated with cigarette smoking[9] or alcohol consumption.[10] An accumulated body of evidence has shown that smoking affects the methylation of genes involved in cancer development,[11,12] but none pertains to pancreatic cancer.

There is strong evidence showing tumor cells release their DNA into circulation and that tumor-derived DNA has potential value in the early detection, monitoring, and prognosis of cancer.[13-15] A significantly higher level of plasma cell-free DNA has been found in pancreatic cancer patients.[16] Clinically, because of the late appearance of the disease, it is very difficult to accrue pancreatic tumor tissues because curative surgery is only feasible in a small proportion (∼20%) of patients. It would be very valuable if circulating DNA could be used as a surrogate for tumor tissues in detecting molecular markers. Detection of K-*ras* mutations in circulating DNA has a low sensitivity, but specificity was about 90% for the diagnosis of pancreatic cancer.[17] DNA hypermethylation has been detected in the pancreatic juice of patients with pancreatic ductal adenocarcinoma.[18] However, to our knowledge, there are no published reports pertaining to the detection of aberrant methylation in the plasma DNA of pancreatic cancer patients.

The major aims of the current study were to (1) determine whether hypermethylation changes can be detected in plasma DNA and to (2) examine whether selected genetic change (K-*ras* codon 12 mutations) and epigenetic alterations (*p16* and *ppENK* gene hypermethylation) are affected by cigarette smoking. Their relationships to selected clinical characteristics were also investigated.

MATERIALS AND METHODS

Study Subjects and Biologic Specimens

Patients with pancreatic cancer were consecutively recruited from the Gastrointestinal Cancer Clinic at The University of Texas M. D. Anderson Cancer Center from February 2000 to January 2004. Subjects consisted of patients with pathologically confirmed primary pancreatic ductal adenocarcinoma (International Classification of Diseases for Oncology code C25.3; World Health Organization, 2000) who had not received any radiotherapy or chemotherapy before the recruitment. Untreated patients were selected because there is a concern that treatment might affect the biomarkers under investigation. All patients had no prior cancer history. There was no restriction on age, sex, or race. An informed consent was signed for the

in-person interview and blood donation. The institutional review board at The University of Texas M. D. Anderson Cancer Center approved the research protocol.

In 9 of 83 patients enrolled in this study, paired pancreatic tumor tissue sections were retrieved from the Department of Pathology at The University of Texas M. D. Anderson Cancer Center. Tumor DNA derived from these sections was analyzed in parallel with plasma DNA as a standard to compare the results generated from these 2 sources.

Data Collection

A 20-minute in-person interview was conducted to elicit detailed information on the patient's demographics, medical history (such as diabetes and pancreatitis), family history of cancer (first-degree relatives), and lifetime use of cigarette products. The data collection procedure has been described elsewhere.[19] Briefly, patients who have smoked at least 100 cigarettes in their lifetime were classified as ever smokers, otherwise as never smokers. Regular smokers (RSs) were defined as patients who smoked on a regular basis. Never smokers and ever smokers who did not smoke on a regular basis were classified as nonregular smokers (NRSs). Cumulative smoking was evaluated by pack-year, which is a product of the number of packs smoked per day and the number of years of smoking. Light and heavy smokers were classified by the median pack-years of RSs. All patients completed the in-person interview. Information on clinical tumor stage, that is, localized, locally advanced, and metastatic, was obtained from patients' medical records.

Microdissection of Tumor Cells and DNA Isolation

Two 20-μm serial sections of formalin-fixed, paraffin-embedded tissue from 9 patients were deparaffinized with xylene for 30 minutes (xylene was changed every 10 minutes) and dehydrated in series of ethanol. Slides were then stained with eosin to facilitate microdissection. Pancreatic ductal adenocarcinoma was identified under a microscope by a pathologist (J. Zhu), and the areas of interest were manually microdissected.[20] An estimated 10% to 20% of the cells collected were surrounding non-ductal cells. Nontumor tissue was collected in a separate tube. Microdissected tissues were transferred to a 1.5-mL microcentrifuge tube containing 50 μL of $1 \times$ TK buffer (0.05 mmol/L Tris-HCl, pH 8.9; 2 mmol/L EDTA; 1 mmol/L NaCl; 0.5% Tween 20; and 0.2 mg/mL proteinase K) and incubated at 56°C overnight. The tubes were placed in a 100°C block for 10 minutes to inactivate proteinase K (Roche Applied Sciences, Indianapolis, Ind). Genomic DNA was extracted using the Puregene DNA isolation kit following the protocol for DNA isolation from 5 to 10 mg paraffin-embedded tissue (Gentra, Minneapolis, Minn). DNA was quantified by ultraviolet spectrophotometry and stored at 4°C.

Plasma DNA Isolation

Plasma was isolated from freshly drawn blood (within 2 hours of blood collection) by Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ) density gradients at 2000 rpm for 25 minutes and stored at -80°C until use. Before DNA isolation, plasma was thawed at room temperature and subjected to centrifugation at 1200 rpm for 10 minutes twice. Two milliliters of supernatant was used for DNA isolation using the QIAamp DNA Midi kit (Qiagen, Valencia, Calif) according to the blood and body fluids protocol. The resulting DNA was eluted in 300 μL of sterile double distilled H_2O and then concentrated to 100 μL and stored at -20°C.

Bisulfite Treatment and Methylation-Specific Polymerase Chain Reaction

In brief, 500 ng to 1 μg of DNA was first denatured by sodium hydroxide and modified with sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison, Wis), treated with sodium hydroxide again, precipitated with ethanol, and resuspended in H_2O.[21] We used methylation-specific polymerase chain reaction (PCR) to

detect the *p16* and *ppENK* gene promoter hypermethylation following the methods described previously.[21,22] The primer sequences for the *p16* gene methylated reactions were as follows: 5′-TTA TTA GAG GGT GGG GCG GAT CGC-3′ (sense) and 5′-GAC CCC GAA CCG CGA CCG TAA-3′ (antisense), and for the unmethylated reaction, 5′-TTA TTA GAG GGT GGG GTG GAT TGT-3′(sense) and 5′-CAA CCC CAA ACC ACA ACC ATA A-3′(antisense). The primer sequences for the *ppENK* methylated reaction were as follows: 5′-TGT GGG GAG TTA TCG AGC-3′ (sense) and 5′-GCC TTC GCG AAA AAA ATC G-3′ (antisense), and for the unmethylated reaction, 5′-TTG TGT GGG GAG TTA TTG AGT-3′ (sense) and 5′-CAC CTT CAC AAA AAA AAT CAA TC-3′ (antisense). The methylation-specific PCR (MSP) amplification was carried out in a final volume of 50 μL, containing 3 μL sodium bisulfite-modified DNA, primers (300 ng each), deoxynucleotide triphosphates (dNTP, each at 2.5 mmol/L), $MgCl_2$ (1.5 mmol/L), 2-mercaptoethanol (1 mmol/L), ammonium sulfate (16.6 mmol/L), Tris (6.7 mmol/L, pH 8.8). The reaction was subjected to hot-start at 94°C for 4 minutes before the addition of 1.25 units of Taq polymerase (PGC Scientifics, Frederick, Md). The PCR for the *p16* reaction was followed by 55 cycles of 94°C for 45 seconds, the specific annealing temperature for 30 seconds, 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. Annealing temperature was 65°C for the methylated reaction and 60°C for the unmethylated reaction. After hot-start, the PCR for the *ppENK* reaction was followed by 45 cycles of 95°C for 20 seconds, 62°C for 30 seconds (annealing temperature was the same for the methylated and unmethylated reaction), 72°C for 30 seconds, and a final extension at 72° C for 3 minutes. All PCR reaction was performed using a MJ thermalcycler (MJ Research, Inc, Waltham, Mass). Ten microliters of each PCR product was loaded directly onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under an ultraviolet illuminator (Fig. 1). The sample was termed methylated if an MSP yielded a visible methylated band. The sample was termed unmethylated if no visible methylated band was present. Negative controls (no DNA loaded), unmethylated allele (human normal lymphocyte DNA) and methylated alleles (CpG genome universal methylated DNA, Chemicon International, Temecular, Calif) were included in each reaction. The unmethylated band was always present, which indicates the presence of normal DNA in plasma. The PCR reaction was conducted at least twice on each modified DNA template to determine the reproducibility of the assay. When inconsistency occurred (ie, methylated band was not consistently visible in duplicates), the assay was repeated starting from the bisulfite treatment step. Two concordant results of 3 measurements were accepted for demonstrating reproducibility. The sensitivity of MSP for *p16* methylation was determined using CpG genome universal methylated DNA that was serially diluted in water, mixed with normal lymphocyte DNA, then bisulfite converted and amplified by MSP. The lowest detection limit was 20 ng of universal methylated DNA in 1000 ng lymphocyte DNA, that is, 1 methylated genome in 500 unmethylated genomes (data not shown).

Analysis of K-*ras* Codon 12 Mutations

K-*ras* codon 12 mutations were detected by enrichednested PCR-restriction fragment length polymorphism and direct DNA sequencing analysis as previously described with little modification.[23] Two sets of primers were used for the first PCR and the second PCR amplification. The primer sequence for the first PCR were: 5′-ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT-3′ (sense) and 5′-TCA AGG AAT GGT CCT GGA CC-3′ (antisense). For the second PCR, the sequence of sense primer was the same as the first PCR and the sequence for the antisense primer was 5′-CTC TAT TGT TGG ATC ATA TT-3′. The first PCR was performed in a total volume of 50 μL containing: 39.75 μL of plasma DNA, 0.1 μmol/L of each primer, 200 μmol/L of each dNTP, 1.25 units of DNA polymerase (PGC Scientifics, Frederick, Md), 1 × PCR buffer with 1.5 mmol/L of $MgCl_2$. The PCR conditions consisted of 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The product was digested with 20 units of *BstN*I (New England Biolab, Beverly, Mass) under 65°C for 7

hours. The product was resolved on 3% agarose gel. The second PCR reaction was performed in a total volume of 50 μL containing 1 μL of first PCR reaction product, 0.1 μmol/L of each primer, 200 Kmol/L of each dNTP, 1.25 units of DNA polymerase, and $1 \times$ PCR ×buffer with 1.5 mmol/L of $MgCl_2$. The PCR condition consists of 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The PCR product was digested with 20 units of *BstN*I under 65°C for 7 hours. The second PCR product (enriched PCR product) was subjected to direct sequencing after being excised from 2% 3:1 Nusieve agarose gel (Cambrex, Rockland, Me) using the Qiaquick Gel Extraction Kit following the manufacture's instructions (Qiagen, Valencia, Calif). The excised PCR product was sequenced using Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB, Cleveland, Ohio).[33] P-labeled terminators were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Before sequencing, the reaction product was subjected to denaturing at 72°C for 7 minutes. After electrophoresis, the gel was dried and exposed to the film for 24 hours. The sensitivity of detection was 1 mutant allele in 100 normal alleles.[23] The analysis was randomly duplicated in 5% of samples, beginning from the PCR step, depending on the availability of DNA. The samples that harbored mutations were verified by duplicate direct sequencing (Fig. 2).

Statistical Analysis

STATA intercooled 9.0 software (Stata Corporation, College Station, Tex) was used for data analysis. Two-tailed $P \leq 0.05$ were considered statistically significant. Pearson χ^2 test was used to evaluate the association between each marker and other variables. Fisher exact test was used when the number in any cell was less than 5. K-*ras* codon 12 status was categorized as 0 (wild type), 1 (G-to-A mutation), 2 (G-to-T mutation), and 3 (G-to-C mutation). Mutants were defined by combing 3 types of mutations together. Some biomarker assays were unsuccessful because of inadequate quality or quantity of plasma DNA. The missing data rate was 7.2% (n = 6), 9.6% (n = 8), and 31.3% (n = 26) for K-*ras* mutation, *ppENK*, and *p16* methylation, respectively. The missing data rate was high for the *p16* gene because it was the last marker to be evaluated, at which point the DNA had been exhausted for some samples. Forty-three patients (51.8%) had a complete set of all 3 biomarkers informative.

RESULTS

Table 1 shows the major characteristics of the 83 patients. The median age was 61 years (range, 35-79 years). Non-Hispanic whites comprised 85.5% of the patients. A total of 61.4% of the patients reported having a family history of cancer among their first-degree relatives; 25.3% and 10.8% of the patients, respectively, reported having a history of type II diabetes or pancreatitis; 19.3%, 44.6%, and 36.1% of the patients had localized, locally advanced, and metastatic disease, respectively. The median value of DNA yield was 250 ng/mL (range, 28.5-857.75 ng/mL) plasma. The DNA yield did not differ significantly by tumor stage. Hypermethylation of the *p16* and *ppENK* genes was detected in 60% (3/5) and 80% (4/5) of the plasma samples taken from patients whose tumors harbored the same methylation, respectively. No methylated band was present in the plasma sample of the patients whose corresponding tumor DNA had no methylation in the *p16* and *ppENK* promoter, which suggested that the specificity of methylation detection using plasma DNA was 100%.

Table 1 shows the frequency of 3 alterations in patients by different characteristics. The frequency of K-*ras* mutations and *ppENK* and *p16* hypermethylation was 32.5%, 29.3%, and 24.6%, respectively. A total of 62.6% (52/83) of patients expressed at least one of these alterations. None of the patients harbored all 3 alterations. There was no significant difference in the frequency of these alterations by age, sex, family history of cancer, history of pancreatitis or type II diabetes, and tumor stage. The prevalence of *p16* gene hypermethylation was not significantly different between patients who were younger than 61 years and those older than 61 years ($P = 0.21$, Fisher exact test). However, the frequency of K-*ras* mutation differed by

race. None of the Hispanic patients and 3 of 4 African American patients carried the codon 12 mutations ($P = 0.03$). K-*ras* mutations were more frequently detected among patients with metastatic tumor than among patients with localized tumor (42.3% vs 20.0%). The occurrence of *p16* hypermethylation was more frequently found among patients with localized tumors than those with metastatic tumors (45.4% vs 13.0%). However, these differences were not statistically significant (Table 1).

The distribution of age, sex, race, and tumor stage did not differ significantly between RSs and NRSs (data not shown). Table 2 presents the frequency of alterations by smoking status. Three types of K-*ras* mutations were detected in 25 of 77 patients, including 18 G(G)T to G(A)T mutation (Gly to Asp), 5 G(G)T to G(T)T mutation (Gly to Val), and 2 (G)GT to (C)GT mutation (Gly to Arg), which accounted for 72.0%, 20.0%, and 8.0% of all mutations, respectively. The K-*ras* mutation spectrum was significantly different between the RSs and the NRSs ($P = 0.003$, Fisher exact test). The frequency of the G(G)T to G(A)T transition mutation was 31.9% in the RSs and 10.0% in the NRSs, whereas the G(G)T to G(T)T transversion mutation was exclusively detected in the NRSs. The K-*ras* mutation spectrum was also associated with pack-years of smoking. The G(G)T to G(A)T mutation was detected in 10.0%, 27.3%, and 36.0% of the NRSs, the RSs who had less than 36 or equal to or greater than 36 pack-years of smoking, respectively ($P = 0.03$, Fisher exact test). The frequency of *ppENK* and *p16* hypermethylation was comparable between the RSs and the NRSs and also did not differ by pack-years of smoking (all $P \geq 0.5$). There was no significant association between these alterations and current smoking status (never, former, and current; data not shown).

DISCUSSION

In the current study, we have shown that gene promoter hypermethylation can be detected in the plasma DNA of patients with pancreatic cancer. We confirmed that K-*ras* codon 12 mutations were associated with cigarette smoking in patients. However, we failed to show any association between smoking and *p16* or *ppENK* hypermethylation. These observations support a role of cigarette smoking-induced gene mutation in pancreatic carcinogenesis and that plasma DNA might have a value as a surrogate for tumor tissues in detecting genetic and epigenetic alterations in pancreatic cancer.

The frequency of K-*ras* mutations tended to be higher in the patients who smoked regularly than the patients who did not. A series of studies have shown that K-*ras* mutation detected in paraffin-embedded pancreatic tumor tissue can be modulated by environmental factors,[24] including coffee drinking,[25] organochlorine compounds,[26] occupational exposure to hydrocarbon solvents,[27] and alcohol consumption.[10] These studies indicated that environmental factors could play a role in pancreatic carcinogenesis through modulation of K-*ras* oncogene activation. In particular, cigarette smoking, the only established environmental risk factor of pancreatic cancer, has been linked to the presence of K-*ras* mutation in 89 tumor tissues[9] and in nonneoplastic exocrine pancreatic lesions of 39 cancer-free male heavy smokers.[28] Some studies, however, did not show that K-*ras* mutation rate differed in ever smokers and never smokers.[29] In the current study, we also found the association between smoking status and K-*ras* mutation spectrum. The G-to-A transition mutation at codon 12 was predominantly detected in the RSs. This observation supports the etiologic role of the *N*-nitroso compounds in smoking-related pancreatic cancer as suggested by animal studies.[30,31] Aromatic amine and alkylating agents can activate K-*ras* through G-to-A transition.[32,33] Interestingly, patients who had high cumulative exposure to smoking tended to have a higher frequency of G-to-A mutations than those who had lower exposure. However, this observation was based on a small sample size and needs to be confirmed.

The current study failed to show that cigarette smoking was associated with either *p16* or *ppENK* hypermethylation. In contrast, both experimental studies[34-36] and clinical studies using human tissue sample[37-40] have shown significant associations between carcinogen exposure, including tobacco compounds and their derivatives, and gene promoter methylation in cancer patients. In 1995, Costa[41] proposed a model for the epigenetic mechanism of nickel compounds. Based on the model, nickel compounds induce an increase in chromatin condensation, causing neighboring genes (such as tumor suppressor genes) that are actively expressed in euchromatin to be condensed into heterochromatin. The involvement of DNA cytosine methyltransferase causes subsequent gene promoter hypermethylation, which eventually silences the genetic activity that might be essential for maintenance of genomic stability.[41] Given existing evidence, it is still important to investigate the effect of environmental exposure on gene promoter hypermethylation in pancreatic cancer. The lack of association in our study may be explained by non-differential misclassification of methylated or unmethylated alleles in the RSs and the NRSs. Because plasma DNA is a mixture of neoplastic and nonneoplastic DNA, MSP might not be sensitive enough to pick up the methylated allele when copy number is low. In addition, the bisulfite modification can result in DNA breakage and thus lowering the sensitivity of MSP relative to ordinary PCR.[18] However, there is no reason to believe that the false-negative results were related to the smoking status of the patients because the exposure status was unknown to the laboratory personnel doing the assay. Such non-differential misclassification would bias the association toward null. Alternatively, our results suggest that mechanisms other than smoking, such as intrinsic tumor microenvironment and dietary factors, may play a role in determining the profile of gene promoter hypermethylation.

Determining whether there is an association between the tested markers and clinical outcome was not the major goal of the current study because we had a limited number of patients who had localized tumors. Nevertheless, our study showed that K-*ras* mutations were more frequently detected in patients with late-stage disease than in patients with localized tumors. Several previous studies also found that the occurrence of K-*ras* mutation in circulating DNA was correlated with late tumor stage[42] and large tumor size.[43] Patients with metastatic disease may have tumors with a larger volume, and therefore, more abundant tumor DNA in circulation. Promoter hypermethylation of the *p16* and *ppENK* genes seems to be more frequently detected in patients with localized tumors than those with more advanced tumors. This is consistent with previous findings that *p16* alteration is an early event in pancreatic carcinogenesis.[44,45] Indeed, both *p16* and *ppENK* hypermethylation have been detected in precancerous lesions in the pancreas.[22] The prognostic value of promoter hypermethylation of tumor suppressor gene such as *p16* in pancreatic cancer is not clear and needs further investigation.

Early diagnosis is critical to improve the survival of patients. Because both K-*ras* mutations and gene methylation occur in the early stage of carcinogenesis, detection of these alterations in the plasma/serum of cancer patients may serve as an attractive noninvasive tool for early detection.[46] However, K-*ras* mutation in serum alone is not a satisfactory marker in early detection of pancreatic cancer.[47] Recently, Marchase et al[48] concluded that K-*ras* mutation is not useful in the early detection of pancreatic cancer because K-*ras* abnormality could not be identified in serum-derived DNA of patients whose tumors harbored the abnormalities. This observation was in contrast with many other previous studies[42,43,49] that detected K-*ras* mutation in circulating DNA with varied detection rates. The discrepancy may be caused by differences in study sample (plasma or serum), sample storage and process, sensitivity of the mutation detection assay, and patient group. Generally, plasma DNA is considered to be more representative of tumor status and is enriched for tumor DNA than serum DNA.[49-51] In the current study, the sensitivity for any one of the markers is low, with frequencies ranging between 24% and 32%. When combining the 3 markers, the detection rate increased to 63%. In a subset of 43 patients who had all 3 biomarkers measured, 67% (29/43) of the patients

expressed at least 1 alteration. Even in patients with localized tumors, 56% expressed at least 1 alteration. Therefore, combination of biomarkers representing different biologic pathways may be valuable in early detection of pancreatic cancer, thus warrants further investigation. Apparently, it is important to evaluate these markers in age-matched cancer-free controls or patients with chronic pancreatitis as well. In particular, *p16* promoter hypermethylation has been detected in normal human mammary epithelia.[52]

There are some limitations in this study. First, the use of plasma DNA has its own limitations. Although much attention has been focused on circulating DNA, its origin and mechanisms of release and clearance are unclear, and we have no solid evidence to show that plasma DNA comes from pancreatic tumors. Second, although comparison of *p16* methylation profiles in paired tumor and plasma DNA from 9 patients showed 70% sensitivity and 100% specificity in detecting methylated alleles using plasma DNA, we were unable to either retrieve more tumor samples or to include patients without cancer to validate these findings. Thus, the true sensitivity and specificity of the assay need to be further determined in future studies. Third, low sensitivity could be one of the reasons that we did not observe any association between methylation and smoking. Furthermore, missing data and small numbers in certain analyses may all contribute to the lack of association between smoking and methylation patterns. On the other hand, some significant observations may be caused by random bias. Nevertheless, the frequency of K-*ras* mutations in the plasma DNA of the current study (32.5%) is within the range reported in other studies using plasma DNA of patients with pancreatic cancer (25%-35%).[49,53] More advanced techniques and the enrichment of tumor-derived DNA may reduce the likelihood of both false-negative and false-positive results.[54] Recently, a method has been developed to detect mutant templates in a ratio of 1:10,000 wild-type alleles.[55] Such highly sensitive techniques, together with the application of multiple markers, may hold the hope of early detection of pancreatic cancer using circulating DNA.

In summary, we detected K-*ras* codon 12 mutations and aberrant hypermethylation using plasma DNA in a series of pancreatic cancer patients. We confirmed the previous observation showing that cigarette smoking was significantly associated with K-*ras* mutation. In addition, we found that G(G)T to G(A)T transition mutation is more frequently seen in the RSs and those with a higher level of cigarette exposure than in the NRSs. This finding agrees with animal studies that suggest that the *N*-nitroso compounds in cigarette smoke can induce specific activating mutation in the K-*ras* gene and play a role in human pancreatic carcinogenesis. On the other hand, we failed to show any association between cigarette smoking and *ppENK* and *p16* gene methylation. Our observation supports the feasibility of using plasma DNA as a surrogate to detect molecular alterations in pancreatic cancer patients. Future research is needed to demonstrate the interaction between environmental modulators and epigenetic events.

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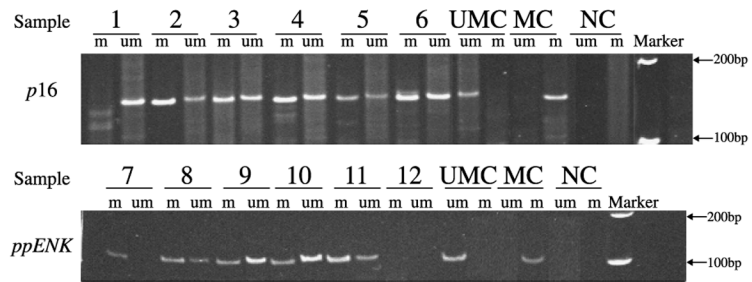
FIGURE 1.
Examples of aberrant methylation of p16 and ppENK genes in plasma DNA of pancreatic cancer patients. m indicates methylated; MC, methylated allele control; NC, negative control, distilled water; um, unmethylated; UMC, unmethylated allele control.
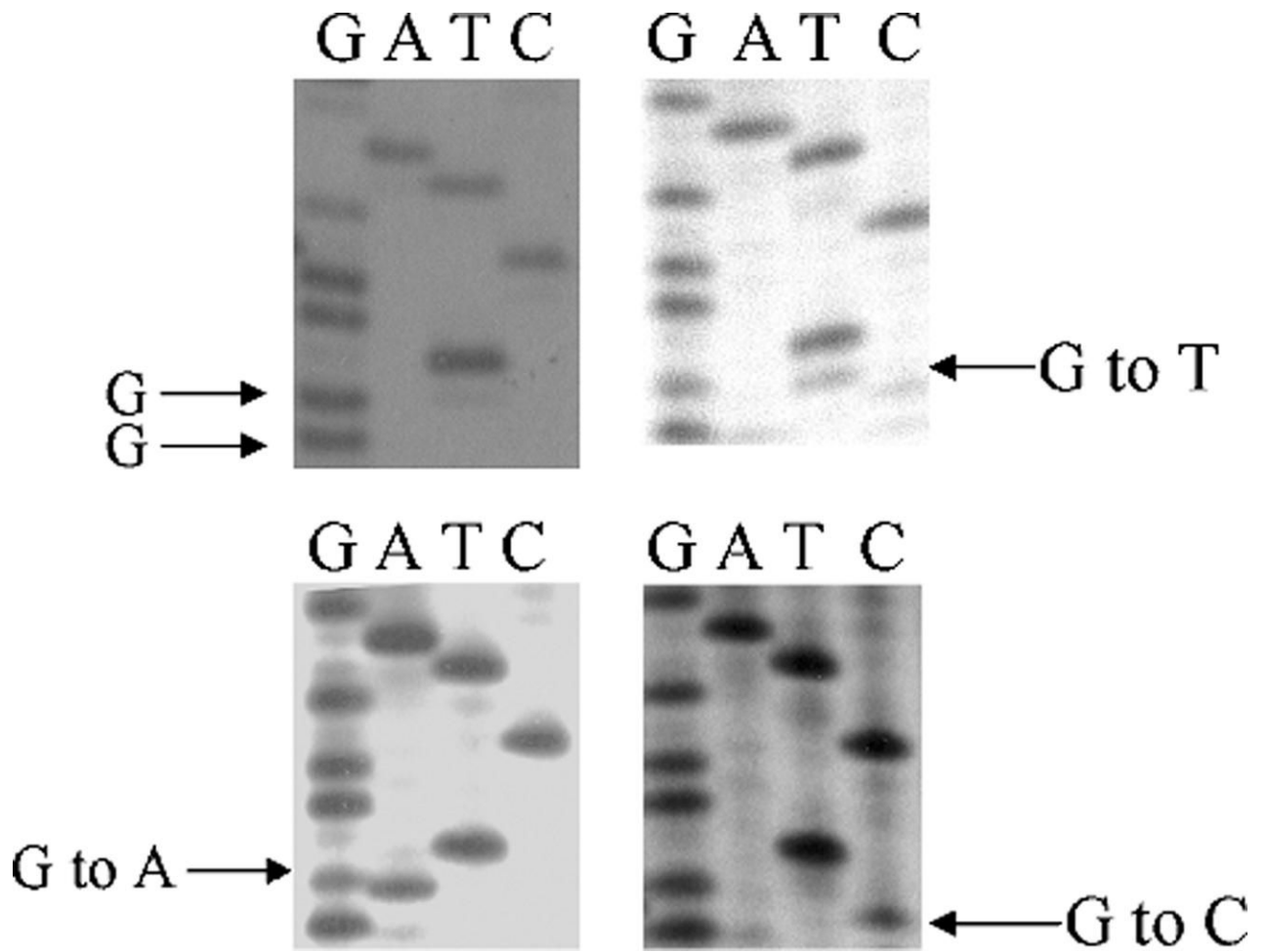
FIGURE 2.
Autoradiograph shows K-*ras* codon 12 mutations as detected by direct sequencing. Because plasma DNA contains normal DNA, the wild-type allele is present.

TABLE 1

Characteristics of Patients and Frequency of Alterations

Patients Characteristics (N = 83)	Frequency					
	K-ras* Mutations 25/77 (32.5)	P†	ppENK Methylation 22/75 (29.3)	P†	p16 Methylation 14/57 (24.6)	P†
Age at diagnosis (yrs)						
<54	8/19 (42.1)	0.79	6/20 (30.0)	0.70	4/12 (33.3)	0.20
54-61	7/20 (35.0)		6/16 (37.5)		5/12 (41.7)	
61-69	6/19 (31.6)		4/20 (20.0)		3/19 (15.8)	
<69	4/19 (21.0)		6/19 (31.6)		2/14 (14.3)	
Sex						
Women	12/31 (38.7)	0.46	9/30 (30.0)	1.00	5/21 (23.8)	0.92
Men	13/46 (28.3)		13/45 (28.9)		9/36 (25.0)	
Race						
Non-Hispanic white	22/65 (33.9)	0.03	19/64 (29.7)	0.42	11/49 (22.4)	0.17
Hispanic	0/8 (0.0)		2/8 (25.0)		3/5 (60.0)	
African Americans	3/4 (75.0)		1/3 (33.3)		0/3 (0.0)	
Family history of cancer in first-degree relatives						
No	8/30 (26.7)	0.39	7/28 (25.0)	0.61	7/21 (33.3)	0.24
Yes	17/47 (36.2)		15/47 (31.9)		7/36 (19.4)	
Type II diabetes						
No	19/58 (32.7)	1.00	16/56 (28.6)	0.78	12/45 (26.7)	0.48
Yes	6/19 (31.6)		6/19 (31.6)		2/12 (16.7)	
History of pancreatitis						
No	23/68 (33.8)	0.46	20/67 (29.8)	0.70	12/50 (24.0)	1.00
Yes	4/9 (44.4)		2/8 (25.0)		2/7 (28.6)	
Tumor stage						
Localized	3/15 (20.0)	0.33	3/14 (21.4)	0.48	5/11 (45.4)	0.12
Locally advanced	11/36 (30.6)		12/33 (36.4)		6/23 (26.1)	
Metastatic	11/26 (42.3)		7/28 (25.0)		3/23 (13.0)	

Values are expressed as n (%).

* Includes all types of mutations.

† P value for χ^2 test or Fisher exact test.

TABLE 2

Frequency of Alteration in Plasma DNA of Pancreatic Cancer Patients by Smoking Status

Biomarkers	All* (N = 83)	NRSs (n = 33)	RSs (n = 50)	$P^\dagger$	Pack-Yrs‡			$P^\dagger$
					0 (n = 33)	1-36 (n = 24)	≥36 (n = 26)	
K-*ras* codon 12								
Wild type	52 (67.5)	21 (70.0)	31 (66.0)	0.003	21 (70.0)	15 (68.1)	16 (64.0)	0.03
G-to-A mutation	18 (23.4)	3 (10.0)	15 (31.9)		3 (10.0)	6 (27.3)	9 (36.0)	
G-to-T mutation	5 (6.5)	5 (16.7)	0 (0.0)		5 (16.7)	0 (0.0)	0 (0.0)	
G-to-C mutation	2 (2.6)	1 (3.3)	1 (2.1)		1 (3.3)	1 (4.6)	0 (0.0)	
p16								
Unmethylated	43 (75.4)	15 (75.0)	28 (75.7)	0.60	15 (75.0)	11 (68.8)	17 (80.9)	0.80
Methylated	14 (24.6)	5 (25.0)	9 (24.3)		5 (25.0)	5 (31.2)	4 (19.1)	
ppENK								
Unmethylated	53 (70.7)	20 (69.0)	33 (71.7)	0.50	20 (69.0)	16 (72.7)	17 (70.8)	0.96
Methylated	22 (29.3)	9 (31.0)	13 (28.3)		9 (31.0)	6 (27.3)	7 (29.2)	

Values are expressed as n (%).

*The numbers do not add up because of missing data.

† *P* value for Pearson χ^2 test or Fisher exact test.

‡ Pack-year was categorized by the median of RSs.