Genetic and Epigenetic Alterations of Familial Pancreatic Cancers

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Abstract

Background: Little is known about the genetic and epigenetic changes that contribute to familial pancreatic cancers. The aim of this study was to compare the prevalence of common genetic and epigenetic alterations in sporadic and familial pancreatic ductal adenocarcinomas.

Methods: DNA was isolated from the microdissected cancers of 39 patients with familial and 36 patients with sporadic pancreatic adenocarcinoma. KRAS2 mutations were detected by BstN1 digestion and/or cycle sequencing. TP53 and SMAD4 status were determined by immunohistochemistry on tissue microarrays of 23 archival familial pancreatic adenocarcinomas and in selected cases by cycle sequencing to identify TP53 gene mutations. Methylation-specific PCR analysis of seven genes (FoxE1, NPTX2, CLDN5, P16, TFPI-2, SPARC, ppENK) was done on a subset of fresh-frozen familial pancreatic adenocarcinomas.

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States. In 2008, an estimated 37,680 Americans will be diagnosed with pancreatic cancer and 34,290 individuals will die from this cancer (1). Pancreatic cancer is so lethal largely because most patients present with advanced inoperable disease. Detecting pancreatic cancers at an early stage could be the best way to reduce the mortality from this disease. Because the lifetime incidence of pancreatic cancer is <1% in the general population, early detection strategies are best directed at individuals with an increased risk of developing the disease.

Epidemiologic studies have helped to identify groups of individuals at risk of developing pancreatic cancer. A major risk factor for pancreatic cancer is having an inherited susceptibility to developing the disease. Approximately 5% to 10% of patients with pancreatic cancer have a family history of the disease, and prospective studies have shown that a person’s risk of developing pancreatic cancer increases with the number of affected first-degree relatives (2-4). In addition, having a germline mutation in the BRCA2, p16/CDKN2A, PRSS1, STK11, hMLH1, or FANCC genes predisposes to the development of pancreatic cancer (2, 5-9). However, these known inherited mutations account for only a small portion (<20%) of the familial clustering of pancreatic cancer, making likely the existence of more pancreatic cancer susceptibility genes that have yet to be identified (10, 11). Risk prediction tools have been developed to help target screening to individuals at highest risk based on their family history (12), but other risk factors such as cigarette smoking, obesity, diabetes mellitus, and vitamin B12 deficiency (13-17) may not increase pancreatic cancer risk enough to justify early detection strategies targeting these risk groups.

The recognition that individuals with an extensive family history of pancreatic cancer and those carrying a mutation in one of the pancreatic cancer susceptibility genes are at increased risk of developing pancreatic cancer has led to screening efforts to identify early...
pancreatic neoplasia in these high-risk individuals (18-21). These efforts have been successful at identifying early-stage pancreatic cancers and preinvasive pancreatic neoplasias that are curable with pancreatic resection (18, 19). For example, the CAPS2 study screened mostly individuals with a family history of pancreatic cancer in three or more blood relatives, one of whom was a first-degree relative of the person screened, using endoscopic ultrasound and computed tomography. Intraductal papillary mucinous neoplasms were identified in ~10% of the individuals screened (18). These results prompted an ongoing multicenter CAPS3 study (clinicaltrials.gov NCT00438906) that is evaluating endoscopic ultrasound, computed tomography, and magnetic resonance cholangiopancreatography in high-risk individuals with similar family history criteria to those of CAPS2. In addition, our institutional CAPS4 study is screening individuals from familial pancreatic cancer families who have a single first-degree relative with pancreatic cancer, using endoscopic ultrasound and magnetic resonance imaging/magnetic resonance cholangiopancreatography.

Molecular-based early detection strategies are also under investigation. These strategies rely on the detection of mutations or abnormal methylation patterns that are found in neoplastic cells and not in normal tissues (22-25). Although the genes and approaches chosen for these molecular-based screening tests are based on the known genetic alterations in sporadic pancreatic adenocarcinomas and their precursors, it is not known if similar alterations occur in familial pancreatic cancers. Pancreatic intraepithelial neoplasia from patients with familial pancreatic cancer have been found to harbor KRAS2 mutations just as sporadic pancreatic intraepithelial neoplasia (26), and familial pancreatic cancers have broadly similar allelic loss patterns to sporadic pancreatic cancers (27); nonetheless, molecular analysis of familial pancreatic cancers remains difficult because few familial pancreatic cancers are resected each year at any one institution. For example, assuming that 15% to 20% of patients present with resectable disease and 5% to 10% of patients have a family history of pancreatic cancer, only 1 or 2 of every 100 patients diagnosed with pancreatic adenocarcinoma will have a family history of the disease and undergo surgical resection of their cancer (28). The limited knowledge of the molecular alterations of familial pancreatic cancer has left investigators relying on the molecular profiles of sporadic pancreatic cancer as the basis of early detection strategies.

We therefore examined a series of well-characterized adenocarcinomas of the pancreas resected from individuals with a family history of pancreatic cancer for molecular alterations. We analyzed these cancers for evidence of mutations in the KRAS2, TP53, and SMAD4 genes, as well as for methylation of FoxE1, NPTX2, CLDN5, P16, TFFP-2, SPARC, and ppENK (29). These genes were chosen because they are targeted in sporadic adenocarcinomas of the pancreas (30-39), and some have also been shown to be useful in screening for early noninvasive pancreatic neoplasms (22, 23, 34, 36).

Materials and Methods

Patients and Samples. These studies were carried out with the approval of the Johns Hopkins Institutional Review Board. We analyzed infiltrating ductal adenocarcinomas from 45 patients with familial pancreatic cancer including 36 surgical resection specimens and 9 samples obtained at autopsy (40). These patients were chosen by identifying patients in our familial pancreatic cancer database who had undergone surgical resection at our institution as well as patients from our database of patients who had undergone pancreaticoduodenectomy who had a family history of pancreatic cancer. We then enrolled subjects into the study if they had sufficient pancreatic cancer and normal tissue. Patients were defined as having a familial pancreatic cancer if they reported at least one first-degree relative with pancreatic cancer. Family history information was obtained either from the family’s enrollment in the National Familial Pancreas Tumor Registry (ref. 4; n = 39) or from a review of their medical records. All of the familial cases had a minimum of two family members affected with pancreatic cancer and seven of the cases reported three or more family members with pancreatic cancer. Detailed family history information was available for all of the individuals enrolled in the registry and all of these patients had familial pancreatic cancer as defined by having at least one first-degree blood relative with pancreatic cancer. Twelve of the familial patients had been tested for germline BRCA2 mutations. One patient with familial pancreatic cancer carried the Ashkenazi Jewish BRCA2 6174delT mutation (5). One patient was found to have a polymorphism of uncertain significance. The inherited genetic basis of the other patients’ familial cancers was not known. Fresh-frozen cancer tissue was available for DNA analysis from 16 patients (7 female; 88% Caucasian; mean age 67 y; range, 45-83 y) with primary familial pancreatic ductal adenocarcinoma who had undergone surgical resection at the Johns Hopkins Hospital from 1990 to 2004. Formalin-fixed paraffin-embedded archival tissues were available from 29 of the patients (12 female; 83% Caucasian; mean age at diagnosis 66 y; range, 40-83 y) and were used to create tissue microarrays using previously described methods (41). These tissue microarrays included 9 of the 16 cases from whom frozen cancer tissue was available. DNA sufficient for KRAS2 gene mutation analysis was isolated from the tissue microarray cores from 14 of these patients. Pancreatic cancer tissues were obtained at autopsy from nine patients who had volunteered to give their tissues to the Johns Hopkins Pancreatic Cancer Rapid Autopsy program (40). These patients had a similar demographic profile to the surgical cases (5 females; age 69 ± 13.3 y). One of the familial pancreatic cancers was cultured as a xenograft, and this xenograft was used as the source of DNA. The demographic profile of our patient population did not differ significantly from that of the familial and sporadic pancreatic cancer kindreds enrolled in the National Familial Pancreas Tumor Registry. For example, the average age at diagnosis for familial pancreatic cancer kindreds enrolled in the National Familial Pancreas Tumor Registry is 66 (SD ± 11.5 y), with 50% of these familial pancreatic cancer patients female and 93% Caucasian. The average age of the individuals with apparently sporadic pancreatic cancer enrolled in the registry is 67 y, with 48% female and 93% Caucasian.

To compare the findings in familial pancreatic cancers with those in sporadic pancreatic cancer, we analyzed...
infiltrating ductal adenocarcinomas from 36 patients with primary sporadic pancreatic ductal adenocarcinoma and the xenografts of 5 other surgically resected sporadic pancreatic cancers who had undergone pancreaticoduodenectomy during the study period. These patients were similar in demographics to the familial cases (13 females; 92% Caucasian; mean age 67 y; range, 38-82 y) and included only patients with resectable stage cancers. None of these patients reported a family history of pancreatic cancer, and none of the patients reported a mutation in one of the known pancreatic cancer susceptibility genes (based on a review of their medical record). Frozen tissues from the six primary pancreatic cancers that were used to create these pancreatic cancer xenografts were microdissected to compare the KRA52 gene sequencing results of the primary pancreatic cancers with their corresponding xenografts.

**Immunohistochemistry.** Five-micrometer sections were cut from the constructed tissue microarrays and deparaffinized by routine techniques followed by steaming in a Target Retrieval Buffer (Dako) for 20 min at 90°C to 100°C. Slides were then cooled for 20 min and incubated for 60 min with a SMAD4 primary antibody diluted 1:200 (clone B8; Santa Cruz Biotechnology) using the DAKO autostainer. Labeling was detected with the DAKO Envision Plus Detection Kit following the protocol suggested by the manufacturer and counterstained using hematoxylin. The conditions for p53 immunohistochemistry were similar to those described above apart from incubating sections overnight with a primary prediluted p53 antibody (clone DO-7; DAKO). The results observed in the familial cases were compared with previous studies of sporadic pancreatic cancers (42-45).

**Microdissection.** Fresh-frozen samples were embedded in optimal controlled temperature media and 8-μm-thick sections were cut in a cryostat at −20°C and mounted on glass slides. Every second section was stained with H&E and covered with a glass coverslip for diagnostic purposes. The fresh-frozen sporadic pancreatic cancer samples were microdissected by hand as previously described (36). The familial pancreatic cancer samples were subjected to laser capture microdissection. Tissue section slides that underwent laser capture microdissection were fixed with 75% ethanol for 30 s, rinsed in distilled water for 30 s, and stained in HistoGene Staining Solution (Arcturus Engineering, Inc.) for 20 s. After rinsing with distilled water for 30 s, the slides were treated with increasing concentrations of ethanol up to 100% ethanol for 3 min to dry the sample followed by xylene treatment for 5 min and air drying. The stained slides were microdissected within 2 h using the Pixcell II LCM system (Arcturus Engineering, Inc.) using the CapSure HS LCM Caps (Arcturus Engineering, Inc.). Usually ~2,000 to 5,000 cells were microdissected per lesion from one or more slides with an estimated neoplastic cellularity of 90%. The formalin-fixed paraffin-embedded tissues were microdissected from tissue microarray cores. Four cores containing cancer were microdissected per case to ensure that at least ~200 cancer cells were sampled per case (estimated neoplastic cellularity of 20-50%).

**DNA Isolation.** DNA was isolated from the frozen microdissected tissues and the xenograft samples using the DNeasy Tissue Kit (Qiagen). DNA was isolated from formalin-fixed tissues using the Qiagen QIAmp DNA micro kit.

### Table 1. Genetic alterations in familial versus sporadic pancreatic cancers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Familial cancers*</th>
<th>Sporadic cancers*</th>
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<tbody>
<tr>
<td>KRA5</td>
<td>31/39 (80%)</td>
<td>28/36 (78%)</td>
</tr>
<tr>
<td>P53</td>
<td>13/23 (56.5%)</td>
<td>71/117 (60.7%)</td>
</tr>
<tr>
<td>SMAD4</td>
<td>14/23 (60.9%)</td>
<td>52-55%</td>
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*There is no significant difference between the familial and the sporadic groups.

1. Walter et al. (45).

2. Wilentz et al. (42), Tascilar et al. (43), and Infante et al. (44).

**KRA52 and TP53 Mutation Detection.** Mutations in KRA52 were determined by one of two methods, BstN1 digestion and cycle sequencing. The BstN1 method is more sensitive than cycle sequencing for detecting low concentrations of KRA52 gene mutations (46), and so it was used for the formalin-fixed tissues with lower neoplastic cellularity. KRA52 analysis of codons 12 and 13 in fresh-frozen cancer DNAs was done by cycle sequencing. We also confirmed the KRA52 mutations of a subset of the frozen-tissue DNAs by the BstN1 method. PCR amplification of the KRA52 gene was done using an upstream KRA52 primer (5'-ACTGAATATAAACCTTGTGAGTTGGACCT-3') that encoded a G to C substitution at the first position of codon 11. The downstream wild-type primer was 5'-TCAAAAGAATGTCCCTGCACC-3'. Fifty-microliter PCRs were done in 96-well PCR plates with Platinum Taq polymerase. The sequence of the upstream KRA52 primer generated a BstN1 restriction enzyme site (CCTGG) overlapping the first two nucleotides of codon 12. PCR amplification of the first exon of KRA52 using the primers described above generated a DNA fragment of 157 nucleotides. Upon incubation with BstN1, the fragments containing the wild-type codon 12 sequences were cleaved, resulting in two bands of 128 and 29 nucleotides. Fragments containing mutations at either the first or second positions of codon 12 were not cleaved. Codons 12 and 13 of the KRA52 gene were sequenced in 25 and the coding region of the TP53 gene was sequenced in 8 of the familial pancreatic cancers using methods as previously described (26, 40).

**Methylation-Specific PCR.** The methylation status of seven genes (FOLK1, NPI2, CLDN5, p16, TP52, SPARC, ppENK) that were previously identified as aberrantly methylated in sporadic pancreatic cancers (34) was analyzed in 14 familial pancreatic cancers and compared with the results we previously obtained in patients with sporadic pancreatic cancer. Two familial pancreatic cancer samples were not included because of insufficient DNA. The methylation status of each gene was determined by methylation specific PCR. Primers were designed to detect the sequence differences between methylated and unmethylated DNA after bisulfite modification and each primer pair contained at least three CpG sites to provide for optimal specificity. To control for assay performance, we included positive and negative controls for each gene (cell lines with...
known methylation status). As a further control for assay performance, we also assayed six sporadic pancreatic cancers and found the prevalence of methylation in these six sporadic pancreatic cancers was similar to the prevalence we reported previously for these seven genes (average 76.2% versus 74.5%; refs. 34, 35).

Statistics. Descriptive statistics were used for most comparisons. The Kruskal-Wallace test was used to compare the mean percentage of methylated genes in the familial versus the sporadic cancers. P < 0.05 was regarded as statistically significant.

Results

**KRA$S_2$ Analysis.** Thirty-one of the 39 familial pancreatic cancers (80%) tested for $KRA$S2 gene mutations harbored a mutation detected by BstN1 digestion and/or cycle sequencing. In one individual both a cancer sample and a xenograft sample were available for study and the same mutation was identified in both the cancer sample and the xenograft sample. Eight of nine (89%) of the tissues obtained at autopsy harbored a $KRA$S2 gene mutation, 23 of the 30 (77%) samples obtained from surgical candidates contained a $KRA$S2 gene mutation, and there was no significant difference in the detection of $KRA$S2 mutations using archival versus frozen tissues. Two of the mutations detected by cycle sequencing occurred in codon 13 (GGC to GAC and GGC to CGC). The sporadic pancreatic cancers had almost an identical prevalence of $KRA$S2 gene mutations as the familial pancreatic cancers, with 28 of 36 cancers harboring a $KRA$S2 gene mutation (78%). In five of these cases, both a primary pancreatic cancer sample and a xenograft from the same cancer were available for analysis. In one paired sample, both cancer and xenograft were found to be wild type. In the remaining four paired samples, both cancer and xenograft sample harbored a $KRA$S2 gene mutation. Interestingly, in one of the primary cancer/xenograft pairs the primary cancer had a codon 13 mutation and the corresponding xenograft had a codon 12 mutation. In the sporadic cases, 26 of the observed mutations occurred in codon 12 and 2 in codon 13. Previous studies have noted $KRA$S2 gene mutations at codon 12/13 in ~75% to 90% of primary pancreatic ductal adenocarcinomas (47) and our findings in the sporadic and in the familial carcinomas are consistent with these results (Table 1).

**Immunohistochemistry.** $SMAD_4/DPC_4$ is a tumor suppressor gene that has been shown to be genetically inactivated in ~55% of sporadic pancreatic cancers (48). Because mutations in the $SMAD_4$ gene lead to ubiquitination and degradation of Smad4 protein products, loss of Smad4 protein is a reliable indicator of mutational inactivation of $SMAD_4$ (42). We therefore examined Smad4 protein expression in tissue microarrays of familial pancreatic cancers. Loss of Smad4 labeling was observed in 61% (14 of 23 evaluable cases) of familial pancreatic cancer cases (Fig. 1A and B). This finding is similar to our previous reports of loss of Smad4 observed in between 52% and 55% of surgically resectable pancreatic cancers (42-44). In addition, all nine autopsy samples showed loss of Smad4 labeling.

$TP_53$ gene mutations are found in ~70% of invasive pancreatic cancers. Most $TP_53$ gene mutations are missense mutations and result in retention of the mutant p53 protein. The resulting p53 protein overexpression is a reliable indicator of the presence of a $TP_53$ gene missense mutation. P53 positive immunolabeling was observed in 57% (13 of 23 cases) of the familial pancreatic cancers (Fig. 1C and D). This prevalence is not significantly
different from the 61% (71 of 117 cases) we had previously reported for sporadic pancreatic adenocarcinomas ($P = 0.8167$; ref. 45). We also sequenced eight familial pancreatic cancers and found $TP53$ gene mutations in seven of the cancers (W53Ter, Y205D, R249M, H179R, R175H, R273H, and a splice donor mutation in intron 6).

**DNA Methylation.** The methylation pattern of seven genes previously shown to undergo frequent aberrant methylation in sporadic pancreatic cancers was analyzed using methylation specific PCR in frozen pancreatic cancer tissues from 14 of the patients with familial pancreatic cancer. Their methylation profile was compared with results we have previously reported in sporadic pancreatic cancer tissues and is presented in Table 2. The average methylation of the seven genes in the familial pancreatic cancers was 68%, which was not statistically different from that which was observed in the sporadic pancreatic cancers ($P = 0.242$).

**Clinical Correlations.** There were no significant clinicopathologic differences identified between the familial pancreatic adenocarcinomas and the sporadic pancreatic adenocarcinomas. For example, the average tumor size of the frozen familial pancreatic adenocarcinomas used for DNA methylation analysis ($2.9 \pm 0.9$ cm) is similar to the mean tumor size of pancreatic adenocarcinomas that undergo pancreaticoduodenectomy at Johns Hopkins Hospital ($3.1 \pm 1.4$ cm; ref. 28).

**Discussion**

In this study, we examined the prevalence of genetic and epigenetic alterations in familial pancreatic adenocarcinomas and found a similar prevalence of mutations of $KRAS2$ and inactivation of the $TP53$ and $SMAD4$ genes in familial as in sporadic pancreatic ductal adenocarcinomas. We also found a similar prevalence of aberrant methylation of a gene panel in both familial and sporadic pancreatic adenocarcinomas.

These findings provide evidence that familial pancreatic adenocarcinomas undergo similar genetic and epigenetic alterations as are found in sporadic pancreatic adenocarcinomas. Although further characterization of other genetic and epigenetic alterations in an expanded set of familial pancreatic adenocarcinomas is needed to extend these observations, these results provide evidence for the use of our sporadic pancreatic cancer molecular marker panel to help screen individuals with a strong family history of pancreatic cancer.

In the absence of molecular profiles of familial pancreatic cancers, the screening tests being evaluated to detect molecular changes of pancreatic neoplasia in individuals with a family history of familial pancreatic cancer have relied on the premise that the molecular alterations common to sporadic pancreatic neoplasms will similarly also be characteristic of familial pancreatic neoplasms. For example, activating $KRAS2$ gene mutations seem to be an early event in the development of pancreatic ductal adenocarcinoma and because the majority of mutations occur in one codon (codon 12), numerous studies have evaluated its potential use as a marker for pancreatic cancer. Indeed studies have assessed its effectiveness in detecting pancreatic cancer by evaluating the $KRAS2$ gene mutation rate in the stool (49, 50), cyologic brushings (51), and plasma (52) of patients with pancreatic cancer and comparing the mutation rate with that of healthy individuals and individuals with chronic pancreatitis (24, 25, 53-56).

Because $KRAS2$ gene mutation detection has some predictive value for distinguishing pancreatic cancer from other noncancerous conditions of the pancreas and because it is frequently mutated in both familial and sporadic pancreatic cancers, the quantification of $KRAS2$ gene mutations could potentially serve as one of a panel of molecular markers that could be used to help predict the presence of an underlying pancreatic neoplasm. Similarly, because the $TP53$ gene is mutated in $\sim 75\%$ of sporadic pancreatic adenocarcinomas and in a significant fraction of familial pancreatic cancers, it too is a potential molecular marker of pancreatic neoplasia (57-59).

We also observed that familial pancreatic cancers harbored aberrant methylation of a panel of genes commonly methylated in sporadic pancreatic adenocarcinomas (35, 60, 61), supporting the utility of investigating DNA methylation detection strategies to detect familial pancreatic neoplasms (22, 23).

In conclusion, we show that the common genetic and epigenetic alterations that occur in sporadic pancreatic adenocarcinomas are also observed at a similar prevalence in familial pancreatic adenocarcinomas. These findings add to our growing body of knowledge regarding familial pancreatic adenocarcinomas and will be important when developing tests to diagnose early pancreatic cancer.

**Disclosure of Potential Conflicts of Interest**

M. Goggins has a licensing agreement with Oncomethylome Sciences. The other authors disclosed no potential conflicts of interest.

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**References**

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