

Inherited Predisposition to Pancreatic Adenocarcinoma: Role of Family History and Germ-Line *p16*, *BRCA1*, and *BRCA2* Mutations¹

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ABSTRACT

Susceptibility to pancreatic adenocarcinoma appears to be linked to germ-line mutations in genes causing various familial cancer syndromes. The objectives of this study were to estimate the proportion of unselected pancreatic cancer patients belonging to hereditary cancer syndrome families and to determine the frequency of *p16*, *BRCA1*, *BRCA2*, *hMSH2*, and *hMLH1* germ-line mutations in patients with a personal or family history of cancer. The study population consisted of 102 patients with histologically verified pancreatic adenocarcinoma, unselected for age, sex, family history, or ethnic origin. Patients completed a family history questionnaire and provided blood for mutation analysis. Three-generation pedigrees were constructed and classified as high risk/familial, intermediate risk/familial, intermediate risk/nonfamilial, or low risk according to defined criteria. High- and intermediate-risk cases were analyzed for germ-line mutations using a combination of methods. Thirty-eight of 102 (37%) patients were characterized as high or intermediate risk, and the remainder were classified as low risk. Germ-line mutations were identified in five (13%) of these cases [one in *p16* (I49S); one in *BRCA1* (5382 insC); and three in *BRCA2* (6174delT)]. The *BRCA1* and *BRCA2* mutations were identified in Ashkenazi Jewish patients. Four of the mutation carriers had strong family histories of the syndromes associated with the mutated genes. No mutations were identified in patients in whom the sole risk factor was a family history of pancreatic cancer, and only one mutation was found among patients with early-onset disease. We conclude that known causes of genetic predisposition are an important risk factor in a small proportion of pancreatic cancer patients, especially if associated with a strong family history of syndromes associated with the disease. The lack of detectable germ-line mutations in most high- and intermediate-risk cases suggests that there are probably additional, as yet unidentified genes predisposing to this disease.

INTRODUCTION

Pancreatic adenocarcinoma is the fourth leading cause of cancer deaths in both men and women in North America. Despite recent advances in surgery, chemotherapy, and radiation therapy (1), lifetime probabilities of developing and dying of this disease remain nearly equal (2, 3). These poor survival statistics are largely attributable to advanced disease at diagnosis because only ~20% of pancreatic carcinomas are resectable at presentation (4). Moreover, the majority of patients with resected pancreatic cancers develop local recurrences and metastases and die of their disease within a short time after surgery (5). Pancreatic cancer is associated with various environmental and lifestyle risk factors (6), occupational exposures (7), and medical conditions (8–10). How-

ever, the etiology of this nearly uniformly fatal disease still remains poorly understood.

Two lines of evidence have recently emerged to suggest that susceptibility to pancreatic cancer development may be inherited. The first line of evidence comes from case reports describing multiple individuals with pancreatic cancer in the same family (11). Several epidemiological studies have also demonstrated that a family history of pancreatic cancer is an important risk factor for the disease. In a case control study, Falk *et al.* (12) showed an increased risk of pancreatic cancer among persons reporting the occurrence of any cancer in a close relative (odds ratio = 1.86; 95% CI,³ 1.42–2.44). This risk was highest when the reported cancer was a pancreatic tumor (odds ratio = 5.25; 95% CI, 2.08–13.21). In a different case-control study in the French-Canadian population, 7.8% of cases but only 0.6% of controls reported a family history of pancreatic cancer (13). Furthermore, in an Italian study, a family history of pancreatic cancer was associated with a relative risk of 2.8 (95% CI, 1.3–6.3) for the disease, even after adjustment for smoking, alcohol, dietary factors, and medical history (14).

The second line of evidence comes from the observation that pancreatic cancer occurs in excess of expected frequencies in several familial cancer syndromes, which are associated with germ-line mutations in various cancer predisposing genes. The familial atypical multiple mole melanoma (3) syndrome is characterized by the development of multiple nevi and malignant melanomas (15). Germ-line *p16* mutations have been shown to cosegregate with melanoma in a subset of familial atypical multiple mole melanoma kindreds showing linkage to 9p21 (16, 17). Pancreatic adenocarcinoma is the second most common cancer in these families (18), and kindreds with *p16* mutations that impair protein function have a 13-fold increased risk of pancreatic cancer (19). An excess of pancreatic cancer also occurs in breast-ovarian cancer families with *BRCA1* and *BRCA2* mutations (20–24) and in kindreds with HNPCC (25–29), which is caused by germ-line mutations of *hMSH2*, *hMLH1*, and other mismatch repair genes (30–37).

It is estimated that ~5–10% of pancreatic cancers are associated with a strong familial predisposition (13, 38), but the proportion of pancreatic cancers attributable to inherited mutations of the above-mentioned genes remains largely unknown. Therefore, the present study was undertaken to determine the contribution of familial cancer predisposing genes to pancreatic cancer susceptibility. The specific objectives were twofold: (a) to estimate the proportion of unselected pancreatic cancer cases that belong to families with hereditary cancer syndromes; and (b) to estimate the frequency of germ-line mutations of the *p16*, *BRCA1*, *BRCA2*, *hMSH2*, and *hMLH1* genes in pancreatic cancer patients with a personal or family history of cancer.

Received 7/7/99; accepted 11/12/99.

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¹ Supported in part by the National Cancer Institute of Canada [S. A. N., H. O., M. R., S. G.] with funds provided by the Canadian Cancer Society; and by the Edward Christie Stevens Fellowship and the Joseph M. West Family Memorial Fund (Department of Postgraduate Medicine awards to [G. L.]).

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³ The abbreviations used are: CI, confidence interval; HNPCC, hereditary nonpolyposis colorectal cancer; PTT, protein truncation test; MSI, microsatellite instability.

MATERIALS AND METHODS

Study Design

Patient Accrual and Questionnaires

Patients with newly diagnosed and histologically confirmed pancreatic adenocarcinoma were eligible for study entry. Potentially eligible patients were approached in surgeons' offices and medical and radiation oncology clinics at four tertiary care institutions in downtown Toronto (Mount Sinai Hospital, The Toronto Hospital, Princess Margaret Hospital, and Wellesley-Central Hospital) between 1995 and 1999. Of 113 eligible patients, 11 were subsequently excluded because they withdrew participation (5 patients), died before enrollment was complete and their spouse declined participation (3 patients), or were lost to follow-up (3 patients). The remaining 102 (90%) patients form our final study population.

All patients were asked to provide a blood sample (20 ml) for mutation analysis and complete a self-administered family history questionnaire to document the current ages, ages at death, causes of death, and ages at diagnosis of any cancer and the types of cancer found in any member of their first-, second-, or third-degree relatives. Patients were also asked to indicate their ethnic origin. Questionnaires were most often completed by patients at home and mailed back to the investigators. This was necessary given the poor health of most patients and the need to contact other relatives to obtain information. A personal or telephone interview with the patient (or spouse, if the patient died during the course of the study) was occasionally conducted to complete the questionnaires. Information provided in the questionnaires was verified, and missing information was completed in a second telephone interview with all patients. Every attempt was made to verify cancer diagnoses in relatives through examination of hospital records and/or pathology reports. The relative or a proxy was approached for consent to obtain this information from medical records departments. Data thus obtained was used to construct a three-generation pedigree.

All blood samples and questionnaire data were obtained after informed consent was obtained in accordance with protocols approved by the University of Toronto Human Ethics committee.

Risk Classification

Pedigrees/cases were classified based on the criteria outlined in Table 1. Pedigrees were classified as high risk/familial or intermediate risk/familial if they satisfied criteria for various inherited cancer syndromes, namely, familial melanoma, familial breast-ovarian cancer syndrome, HNPCC, or familial pancreatic cancer. Patients were classified as intermediate risk/nonfamilial if they developed pancreatic cancer before 50 years of age or if they had multiple primary cancers without an associated family history of cancer. If the pedigrees did not meet any of these criteria, they were considered low risk and excluded from further genetic testing. Our Mount Sinai criteria for HNPCC are modified from the Amsterdam criteria (26) and include: (a) three individuals in at least two successive generations with at least one colorectal cancer and two others with either gastrointestinal, genitourinary, or gynecological cancers with no age limit for the cancer diagnosis; or (b) any colorectal cancer patient diagnosed at <35 years of age with or without a family history of cancer; or (c) any individual with multiple primary cancers of the sites associated with HNPCC with or without a family history of cancer; and (d) a clinical diagnosis of familial adenomatous polyposis is excluded (39).

Mutation Analysis

The mutation detection strategy is outlined in Table 2. All high-risk and intermediate-risk patients underwent testing for *p16* and *BRCA2*. The former is a small gene (3 exons) and is technically simple to screen (40). Previous studies suggest a role for *BRCA2* in pancreatic cancer even in the absence of a family history of breast and/or ovarian cancer (41, 42). We have previously screened 39 Jewish pancreatic cancer patients for the *BRCA1* 185delAG founder mutation and found that, unlike *BRCA2* 6174delT, *BRCA1* germ-line mutations probably do not contribute to disease in patients with sporadic pancreatic cancer.⁴ Hence, *BRCA1* testing was confined to patients with family histories suggestive of breast-ovarian cancer syndrome. Likewise, mismatch repair gene expression was analyzed only in patients with a personal or family history suggestive of HNPCC.

Molecular Experiments

DNA/RNA Extraction and cDNA Preparation

WBC pellets were separated from fresh blood using Ficoll (Amersham Pharmacia Biotech), and DNA was prepared from these pellets by phenol:chloroform extraction after overnight proteinase K digestion (43). Total RNA was also isolated from the pellets using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. cDNA was synthesized in a 20- μ l reaction mixture containing 1–5 μ g of RNA, 4 μ l of 5 \times first strand buffer, 2 μ l of 0.1 M DTT, 1 μ l of 10 mM deoxynucleotide triphosphate mix, 10 units of RNasin RNase inhibitor (Promega, Madison, WI), 1 μ l of random hexamer (1 μ g/ μ l; Pharmacia Biotech, Quebec, Canada), and 150 units of Superscript II RNase H⁻ Reverse transcriptase (Life Technologies, Inc.). The mixture was incubated at 45°C for 60 min, denatured at 95°C for 5 min, and stored at -70°C.

Germ-Line Mutation Analysis

p16. PCR was performed in a 20- μ l reaction mixture containing 200 ng of DNA, 2 μ l of 10 \times PCR buffer [1 \times PCR buffer = 10 mM Tris-HCl (pH = 8.3), 50 mM KCl, and 0.01% gelatin], 1.25–3.0 mM MgCl₂, 1 μ l of 10 mM deoxynucleotide triphosphates, 0.5 μ l each of forward and reverse primers (20 μ M), 8.8 μ l of 12% DMSO, and 1 unit of AmpliTaq polymerase (Perkin-Elmer, Branchburg, NJ). Exons 1 α and 2 of *p16* were amplified using previously described primers (X1.31F and X1.26R for exon 1, and X2.62F and X2.42R for exon 2; Ref. 17). In some cases, it was necessary to amplify exon 1 in two smaller fragments using a new set of internal primers: 1AR, 5'-TCGGTGC GTTGGGCAGCG-3'; and 1BF, 5'-CGGGGTCGGGTAGAG-GAG-3'. PCR was performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer, Foster City, CA) as follows: denaturation at 94°C for 4 min; 30 cycles each of denaturation at 94°C for 15 s, annealing at 55–67°C for 20 s, and extension at 72°C for 20 s; and a final extension at 72°C for 10 min. PCR products were electrophoresed on 2% agarose gels and purified using the Qiaex II gel extraction kit (Qiagen, Mississauga, Ontario, Canada). Purified PCR products were cycle sequenced to directly screen for mutations using the Thermosequase radiolabeled dideoxy chain termination kit (Amersham Life Science, Cleveland, OH). ³²P-labeled sequencing products were separated by electrophoresis on 6% denaturing polyacrylamide gels (80 W, 1.5–3 h), dried (80°C for 1 h), and exposed to autoradiographic film (BioMax; Eastman Kodak, Rochester, NY) overnight at room temperature.

BRCA1 and BRCA2

PTT. PTT was used to screen for mutations in these large genes. About 60% of *BRCA1* and *BRCA2* are encoded by a large exon 11 (44, 45), and this was screened first by amplifying overlapping fragments from genomic DNA. If no truncated protein was observed in exon 11, the remaining coding region

⁴ H. Ozcelik, unpublished data.

Table 1 Classification criteria for pedigrees/cases

Risk category	Criteria
High risk/familial ^a	
Familial melanoma	≥2 melanoma(s) among 1 st , 2 nd , or 3 rd -degree relatives
Breast-ovarian cancer syndrome	>2 breast cancers at age ≤60 years or ovarian cancers at any age among 1 st , 2 nd , or 3 rd -degree relatives or ≥1 male breast cancer
HNPCC	Amsterdam criteria; Ref. 26
Familial pancreatic cancer	≥2 pancreatic cancers among 1 st , 2 nd , or 3 rd -degree relatives
Intermediate risk/familial ^a	
Familial melanoma	1 melanoma among 1 st , 2 nd , or 3 rd -degree relatives
Breast-ovarian cancer syndrome	2 breast cancers at age ≤60 years or ovarian cancers at any age among 1 st , 2 nd , or 3 rd -degree relatives
HNPCC	Mount Sinai criteria (see text; Ref. 39)
Familial pancreatic cancer	1 pancreatic cancer among 1 st , 2 nd , or 3 rd -degree relatives
Intermediate risk/nonfamilial ^b	
Early-onset pancreatic cancer	Pancreatic cancer diagnosed at age ≤50 years
Multiple primary cancers	Pancreatic cancer and a personal history of breast, ovarian, colon, endometrial, or genitourinary cancer or melanoma
Low risk	None of the above

^a There was overlap among various familial cancer syndromes. However, if any high-risk criteria were satisfied, the patient was classified as high risk.

^b If patients satisfied these criteria but also had an associated family history of cancer, they were classified as belonging to the high-risk/familial or intermediate-risk/familial groups.

was examined by reverse transcription-PCR and PTT, using cDNA as template. PCR was performed using previously described overlapping primers and conditions in a 50-μl reaction volume containing 100 ng of DNA as template (44). For reverse transcription-PCR, 150 ng of cDNA were substituted for DNA, and the reaction volume was reduced to 30 μl.

Protein products were subsequently synthesized in a coupled *in vitro* transcription/translation reaction (TnT/T7 coupled rabbit reticulocyte lysate system; Promega) in a reaction mixture containing 2.4 μl of PCR product, 0.24 μl of TNT buffer, 0.12 μl of amino acid minus methionine, 4.8 units of RNasin, 0.12 μl of T7 polymerase, 0.40 μl of [³⁵S]methionine (Amersham Life Sciences), and 2.6 μl of rabbit reticulocyte lysate. The mixture was incubated at 30°C for 2 h. Proteins were separated by SDS-PAGE (13% SDS-PAGE, 280 V, 3 h). Gels were fixed (70% water, 20% methanol, 10% acetic acid) for 30 min, dried at 60°C for 2 h, and exposed to autoradiographic film as described above. All putative mutations were confirmed by cycle sequencing of an independent PCR product, as described above.

Heteroduplex Analysis. In addition to PTT, heteroduplex analysis was used to rapidly screen appropriate cases (patients of Ashkenazi Jewish descent) for known founder mutations, namely, *BRCA2* 6174 delT, *BRCA1* 185delAG, and *BRCA1* 5382 insC. PCR was performed using previously described primers and conditions (42, 46, 47) in a 50-μl volume containing 100 ng of DNA as template.

PCR products were mixed with 6 μl of sucrose dye (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose in water), and heteroduplex formation was carried out by denaturing the PCR products at 95°C and slow cooling to room temperature. Products were separated by electrophoresis on 12% polyacrylamide or mutation detection enhancement gels (FMC Bioproducts, Rock-

land, ME; 300 V, 4 h), stained with ethidium bromide, and visualized with UV light.

hMSH2 and hMLH1

All available paraffin-embedded tumor tissues from pancreatic cancer patients with a family history suggestive of HNPCC were cut into 6-μm sections, and immunohistochemical analysis was carried out as described previously (48). Cancers were considered to have loss of hMSH2 or hMLH1 expression if there was no detectable nuclear staining of neoplastic cells. Intact nuclear staining of surrounding nonneoplastic epithelium, stromal cells, or lymphocytes was a prerequisite for evaluation of sections and served as an internal positive control.

All germ-line mutations were confirmed by repeat testing of an independent PCR product.

Statistical Analyses

SPSS software was used for statistical analyses. Mean ages at diagnosis in mutation-positive and -negative families are indicated with corresponding SDs and were compared using the Student's *t* test. Ethnic differences and mutation frequencies in the various risk categories were compared with the Fisher's exact test. All *P*s are two-sided, unless otherwise stated.

Table 2 Mutation detection strategy and results of mutation analysis

Category	Genes tested	No. of patients	Mutations
High risk/familial		8	3
Melanoma	<i>p16, BRCA2</i>	1	1(p16 149S) ^a
Breast-ovarian cancer	<i>BRCA1, BRCA2, p16</i>	3	2(BRCA2 6174delT) ^b
HNPCC	<i>hMSH2, hMLH1, p16, BRCA2</i>	0	—
Pancreatic cancer	<i>p16, BRCA2</i>	4	—
Intermediate risk/familial ^c		19	1
Melanoma	<i>p16, BRCA2</i>	2	—
Breast-ovarian cancer	<i>BRCA1, BRCA2, p16</i>	4	1(BRCA1 5382insC) ^d
HNPCC	<i>hMSH2, hMLH1, e p16, BRCA2</i>	9	—
Pancreatic cancer	<i>p16, BRCA2</i>	12	—
Intermediate risk/nonfamilial ^f		11	1
Early-onset disease	<i>p16, BRCA2</i>	10	1(BRCA2 6174delT) ^g
Multiple primary cancers	<i>p16, BRCA2</i>	1	—
Total		38	

^a This individual had early-onset disease, two primary cancers (pancreas-melanoma), and met high-risk criteria for familial melanoma as well as intermediate-risk criteria for familial pancreatic cancer.

^b One individual had two primary cancers (pancreas and male breast), satisfied high-risk criteria for breast-ovarian cancer syndrome, and also satisfied the Mount Sinai criteria for HNPCC. The other patient met high-risk criteria for breast-ovarian cancer syndrome.

^c There was overlap among the various familial cancer syndromes, particularly in the intermediate-risk group. Hence, in this group, the number of patients adds up to more than 19.

^d This patient satisfied three intermediate-risk/familial criteria (melanoma and breast-ovarian and pancreatic cancer) and also had early-onset disease.

^e *hMSH2* and *hMLH1* were tested by immunohistochemical analysis of gene expression in tumors. Adequate tissue for immunohistochemistry was available for only three patients.

^f If patients satisfied these criteria and also had an associated family history, they were classified as belonging to the high-risk/familial or intermediate-risk/familial groups.

^g This patient had an unknown family history but had early-onset disease and was of Ashkenazi Jewish descent.

RESULTS

Study Patients

The study population consisted of 65 males and 37 females with a mean age of 60.3 ± 12 years. All patients had adenocarcinoma of the pancreas, and the diagnosis was confirmed by review of pathology reports. Two patients had pancreatic cystadenocarcinomas. Fourteen of 102 (14%) patients reported that they were of Ashkenazi Jewish descent.

Risk Status

Twenty-seven of 102 patients (26%) satisfied criteria for various familial cancer syndromes. Of these, 8 patients were classified as high risk, and 19 were classified as intermediate risk. The distribution of study patients in various risk groups is shown in Fig. 1. The largest group was composed of 16 patients who satisfied our criteria for familial pancreatic cancer. Of these patients, 12 patients had one other affected relative with pancreatic cancer, 2 patients had two other affected relatives with pancreatic cancer, and 2 patients had three other affected relatives with pancreatic cancer. The next largest group was made up of nine patients fulfilling our Mount Sinai criteria for HNPCC. None of these patients satisfied the more stringent Amsterdam criteria (26). Seven patients fulfilled criteria for the breast-ovarian cancer syndrome. Four of these patients had two relatives with breast and/or ovarian cancers, and three patients had more than two relatives with breast and/or ovarian cancers. The smallest familial cancer syndrome group was comprised of three patients with a family history of melanoma. Only one patient had more than one relative affected with melanoma.

There was considerable overlap between the familial cancer syndromes and risk groups, particularly in the intermediate risk/familial category, with some patients satisfying multiple risk criteria. Of the eight high-risk patients, three had early-onset disease, and two had double primary cancers (pancreas-breast and pancreas-melanoma). Of the 19 patients in the intermediate-risk/familial group, 4 had early-onset disease, and 2 had double primary cancers (pancreas-breast and pancreas-colon).

In contrast, the intermediate-risk/nonfamilial group was made up of 10 patients who were diagnosed at age ≤ 50 years and 1 patient with two primary cancers (pancreatic cancer-melanoma), without an associated family history of cancer.

Mutation Analysis

The results of the mutation analysis are summarized in Table 2. **p16.** Thirty-eight high-risk and intermediate-risk cases were analyzed for mutations. One *p16* mutation was identified, a missense mutation that leads to an amino acid substitution, isoleucine to serine at codon 49 (I49S). This mutation was identified in a high-risk patient with a strong family history of melanoma. We have characterized the functional significance of this mutation in a yeast-two hybrid assay (49) and found that this variant has compromised binding to cdk4 when compared with wild-type p16 protein (50).

Another *p16* sequence variant, which leads to an alanine to threonine substitution at codon 148 (A148T), was identified in a patient with a family history of breast-ovarian cancer. This previously reported variant neither impairs p16 protein function nor segregates with disease in melanoma kindreds, and it is found in normal controls. It has therefore been designated a polymorphism (17, 51). No *p16* mutations were identified in the 11 patients who belonged to the intermediate-risk/nonfamilial group.

BRCA1 and BRCA2. The majority (80%) of mutations in these genes are nonsense or frameshift mutations that lead to truncated proteins (52). Hence, PTT was used to screen for mutations. Like *p16*, *BRCA2* was also tested in all high-risk and intermediate-risk patients. Only those patients with a personal or family history of breast and/or ovarian cancer who did not also have *BRCA2* mutations were screened for *BRCA1* alterations. Four mutations were identified [one in *BRCA1* (5382 insC) and three in *BRCA2* (all 6174delT)], all in patients of Ashkenazi Jewish descent. All these mutations are frameshifts and lead to truncated, nonfunctional proteins (52). The patient with the *BRCA1* mutation satisfied intermediate-risk criteria for breast-ovarian cancer. Two of the three patients harboring *BRCA2* mutations were classified as high risk for breast-ovarian cancer syndrome, and one was classified as intermediate risk/nonfamilial. This latter patient had early-onset pancreatic cancer (age at diagnosis, 35 years) but had been adopted, and family history was not available.

In addition, another *BRCA2* sequence variant, which leads to an alanine to threonine substitution at codon 2951 in exon 22 (A2951T), was identified in one patient with a family history of pancreatic cancer. This variant is a previously described polymorphism (53).

hMSH2 and hMLH1. Although there are six mismatch repair genes, only five (*hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, and *hMSH6/GTBP*) are mutated in the germ-line of HNPCC family members. Furthermore, the majority (>90%) of mutations in these kindreds involve either *hMSH2* or *hMLH1* (30–37). Because the inactivation of mismatch repair genes is associated with complete loss of corresponding mismatch repair protein expression, immunohistochemistry using monoclonal antibodies against mismatch repair gene products can be used to identify tumors with mismatch repair deficiency (54). We have recently demonstrated a high correlation between mismatch repair protein expression and germ-line mutation status in tumors from HNPCC patients (48). Hence, this technique was used to analyze patients satisfying HNPCC criteria.

Adequate tumor and normal tissue for immunohistochemistry was available for only three patients who met the criteria for HNPCC, and these were tested for hMSH2/hMLH1 expression. All three tumors demonstrated intact expression of these mismatch repair proteins. Therefore, these cases were not considered for direct germ-line mutation analysis of *hMSH2* and *hMLH1*.

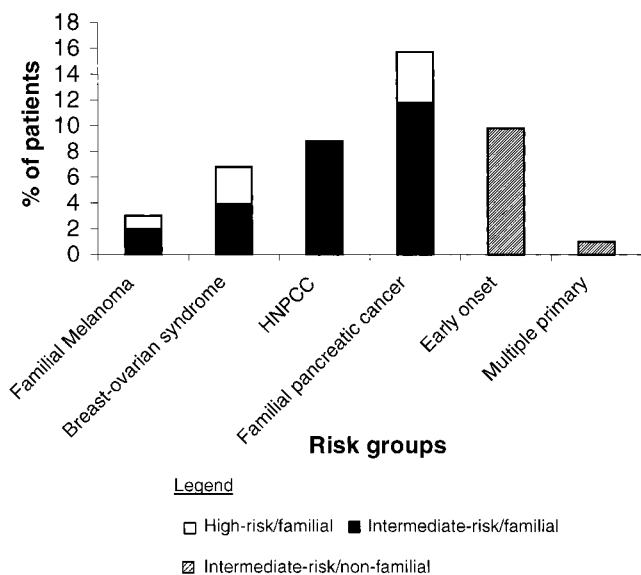


Fig. 1. Distribution of study patients in high-risk groups. Distribution of patients in the various risk groups is described in Table 1. The frequencies are expressed as a percentage of the total number of study patients. The first four bars represent patients at high risk and intermediate risk for familial cancer syndromes. The hatched bars represent intermediate-risk patients without an associated family history of cancer.

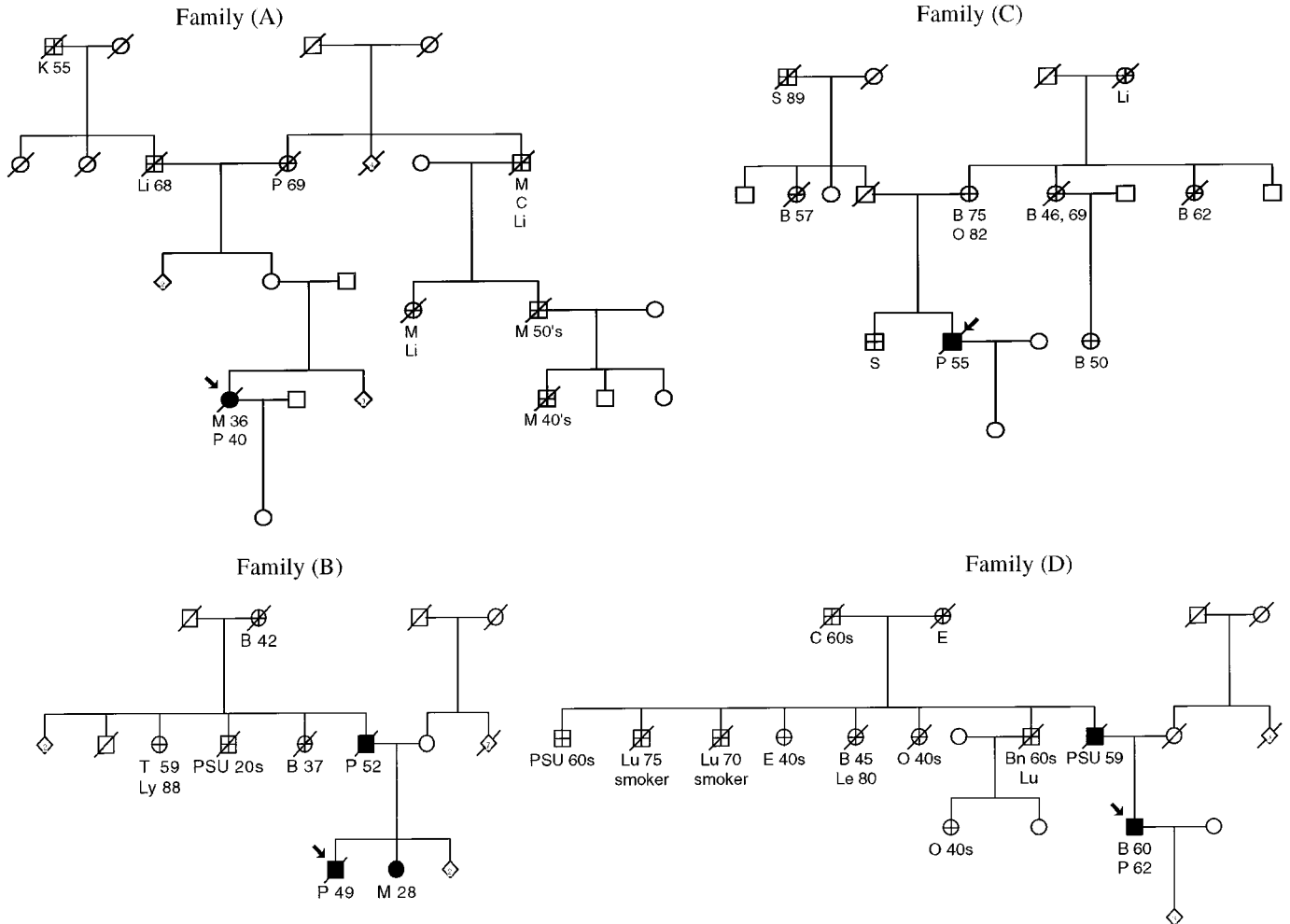


Fig. 2. Pedigrees for mutation-positive cases. Probands are indicated with an arrow. Affected individuals are designated by filled symbols (diagnosis verified by review of pathology or medical records) or + (unable to verify diagnosis). Age at diagnosis, if known, is indicated by numerals next to the cancer sites, which are designated as follows: B, breast; Bn, brain; C, colon; E, endometrial; K, kidney; Li, liver; Le, leukemia; Lu, lung; Ly, lymphoma; M, melanoma; O, ovary; P, pancreas; PSU, primary site unknown; S, skin cancer (nonmelanoma); and T, thyroid. To protect confidentiality, the birth order of siblings has been modified, and most unaffected family members are not shown. Germ-line mutations are as follows: Family (A), *p16 149S*; Family (B), *BRCA1 5382 insC*; Families (C) and (D), *BRCA2 6174delT*.

To summarize, 5 of 38 (13%) tested pancreatic cancer patients had germ-line mutations in three different cancer predisposing genes. Pedigrees of mutation carriers are shown in Fig. 2. Of the five mutations, three were identified in cases with high-risk family histories (3 of 8 tested, 38%). Of the remaining two mutations, one was identified in a patient with an intermediate-risk family history (1 of 19 tested, 5%), and the other was identified in a young Jewish patient with an unknown family history (1 of 11 tested, 9%). The mutation frequency was significantly different between the high-risk and intermediate-risk groups (3 of 8 versus 2 of 30; Fisher's exact $P = 0.05$). Although mutation carriers tended to be younger, there was no statistically significant difference in age at diagnosis between mutation carriers (48.2 ± 10.9 years) and noncarriers (58.7 ± 12.4 years).

DISCUSSION

Evidence for an inherited predisposition to pancreatic adenocarcinoma is based in part on a greater than expected frequency of the disease in kindreds with familial melanoma, breast-ovarian cancer syndrome, and HNPCC. Pancreatic cancer can also be found, albeit less commonly, in other familial cancer syndromes such as Peutz-Jeghers syndrome (55), Li-Fraumeni syndrome (56), and familial adenomatous polyposis (57). However, many of the previous studies

have methodological flaws, including the inability to histologically confirm pancreatic cancer in a consistent manner, ascertainment bias (in favor of the relevant syndrome), small study populations, and lack of prospectively obtained family histories. Thus, the role of germ-line mutations of cancer predisposing genes in pancreatic cancer susceptibility is not well-defined. To the best of our knowledge, the present study is the first to address these issues by estimating the frequency of germ-line mutations predisposing to various familial cancer syndromes in a population of pancreatic cancer patients with histologically verified adenocarcinoma and unselected for family histories.

The peak incidence of pancreatic cancer is in the seventh and eighth decades of life, and males are at a slightly higher risk than females. The mean age of our patients was 60.6 years (range, 30–87 years). Although pancreatic cancer is rare before age 50 years (2), patients diagnosed at ≤ 50 years of age and males are overrepresented in our study population and constitute 17% and 64% of the total series, respectively. Furthermore, our series has a greater proportion of Jewish patients when compared to the general population of Ontario (14% of total versus $<2\%$; Ref. 58). Although certain populations such as New Zealand Maoris and Hawaiians are characterized by the highest incidence rates for pancreatic cancer (59), the incidence of this disease in Israeli Jews is comparable to that in several developed

countries (60). Therefore, our study population probably reflects the referral patterns inherent in the institutions from which our patients were recruited because all eligible patients were approached for the study, regardless of age, sex, ethnicity, or family history. As a result, the results of the present study should be cautiously extrapolated to other populations.

A large proportion of patients (26%) was classified as being at increased risk for familial cancer syndromes. This is not surprising given our broad criteria, some of which are less stringent than most published definitions (17, 23, 26). Germ-line mutations were identified in 5 of 38 (13%) tested cases. Four of the five mutations were identified in patients with strong family histories consistent with the corresponding cancer syndrome, with many affected relatives, and satisfying multiple high- and intermediate-risk criteria (Table 2; Fig. 2). The exception was a young Jewish patient who had been adopted. The presence of a *BRCA2* 6174delT mutation in this patient highlights the value of studying such individuals even in the absence of a known family history.

Our findings are in keeping with previous studies in which germ-line *p16* mutations have only been reported in pancreatic cancer patients with an associated family history of melanoma (15, 19). Of 38 high- and intermediate-risk patients tested, only 1 had a strong family history of melanoma, and this individual carried a *p16* mutation. Two other patients with a single relative affected with melanoma, 24 patients with family histories of other cancer syndromes, and 11 patients with early-onset disease and double primary cancers did not harbor *p16* mutations.

A family history of pancreatic cancer has been noted to predict the presence of a *BRCA2* mutation (23, 61), and pancreatic cancers are more frequent in breast cancer families with *BRCA2* mutations than in families without mutations (23). Although other studies have suggested a role for germ-line *BRCA2* mutations in the development of sporadic pancreatic cancer (41, 42), all *BRCA2* mutations and the single *BRCA1* mutation in our series were identified in patients with strong family histories for breast-ovarian cancer (with the exception of the adopted patient discussed earlier). Moreover, all these individuals were of Ashkenazi Jewish descent, although the *BRCA2* 6174delT mutation has independent origins in both Ashkenazi Jewish and non-Jewish populations (62). No *BRCA2* mutations were identified in patients satisfying criteria for other inherited cancer syndromes, including familial pancreatic cancer, or in the remaining patients in the intermediate-risk/nonfamilial group. No *BRCA1* mutations were identified in four patients with histories suggestive of breast-ovarian cancer syndrome and lacking *BRCA2* mutations, in whom the entire *BRCA1* coding sequence was screened.

The *BRCA2* 6174delT founder mutation has been previously identified in 10% of Ashkenazi Jewish patients with apparently sporadic pancreatic cancers seen at our institution (42). Of the 14 Jewish (including 1 Sephardic Jewish) patients in the present series, 3 satisfied criteria for breast-ovarian cancer syndrome (2 were classified as high risk, and 1 was classified as intermediate risk). In contrast, only 4 of 88 non-Jewish patients had a family history of breast-ovarian cancer (3 of 14 versus 4 of 88; Fisher's exact $P = 0.05$). Of the remaining Jewish patients, one was characterized as high risk for familial pancreatic cancer, one was classified as intermediate risk for other familial cancer syndromes, two were classified as intermediate risk/nonfamilial, and seven were classified as low risk. Four of 13 (31%) Ashkenazi Jewish patients were mutation carriers (three *BRCA2* 6174delT mutations and one *BRCA1* 5382 insC mutation), and all those in whom family history was available met familial breast-ovarian cancer syndrome criteria. No *BRCA2* mutations were identified in the remaining high-, intermediate- and low-risk Jewish patients in whom the entire gene was screened. These patients also did

not harbor *BRCA1* 185delAG or 5382 insC mutations or *p16* mutations.

Although we were unable to test all cases satisfying Mount Sinai criteria for HNPCC, the finding of intact hMSH2/hMLH1 expression in the three screened tumors is not entirely unexpected because other studies have previously demonstrated that germ-line mismatch repair mutations are uncommon in families not satisfying the Amsterdam criteria for HNPCC (63, 64). Although there was no significant difference in age of onset between *p16*, *BRCA1*, and *BRCA2* mutation carriers and noncarriers, the former tended to be much younger, in keeping with other studies that noted that *BRCA2* mutations associated pancreatic cancers (the majority of mutations detected in our series) occur at a younger age than compared to the general population (23, 42).

Inherited predisposition to cancer is often manifested by early age at onset or multiple primary tumors in the same individual (65). We therefore analyzed the *p16* and *BRCA2* genes in an additional 11 patients with early-onset disease or multiple primary cancers without associated family histories of cancer (intermediate risk/nonfamilial). Only one patient (unknown family history) had a mutation, suggesting that these variables, in isolation, are probably not associated with an increased inherited risk of pancreatic cancer related to germ-line mutations in these two genes. A similar pattern has been noted for early-onset colorectal cancers (66), but not for early-onset breast cancers (67, 68).

There are several possible explanations for the absence of detectable germ-line mutations in most high- and intermediate-risk cases. First, the methods used for mutation screening may not be adequately sensitive. Direct sequencing has been shown to accurately detect essentially all types of genetic variations (69) and was used to screen for *p16* mutations. Exon 3 was not analyzed because no disease-associated germ-line mutations have been reported in this small fragment to date (15, 70). PTT (used to screen *BRCA1* and *BRCA2*) may miss some missense mutations, gross chromosomal aberrations, and regulatory region silencing mutations where only the wild-type allele would be amplified or the mutant RNA is unstable. Although not as sensitive as sequencing the complete coding sequence, PTT has nevertheless been shown to be a rapid and efficient method of screening for mutations in the large *BRCA1* and *BRCA2* genes (44).

More than 80% of tumors from patients with HNPCC are characterized by the presence of deficient mismatch repair as seen by the presence of alterations in simple repetitive DNA sequences called MSI. Because only 10–15% of sporadic colorectal cancers demonstrate MSI, examination of tumors for MSI is a useful complementary screening test to identify patients with possible germ-line mismatch repair gene mutations (31, 71–73). However, tumor samples from many pancreatic cancer patients consist only of fine-needle aspiration biopsies (with no normal tissue for comparison) or have poor neoplastic cellularity (less than 50%) and are hence unreliable for MSI testing (74). Therefore, immunohistochemistry for hMSH2 and hMLH1 expression was used to screen tumor samples from patients that met our HNPCC criteria for mismatch repair deficiency. We have recently shown immunohistochemistry to have 97% sensitivity and 100% specificity for identifying cancers with hMSH2 and hMLH1 inactivation (48).

Taken together, although some patients may harbor mutations in exon 3 of *p16* or in other mismatch repair genes, the low frequency of germ-line mutations in intermediate-risk patients in our series is unlikely to be due solely to the methods used for mutation screening.

Second, our family history classification may suffer from recall bias because patients with cancer diagnoses tend to recall familial cancers at a higher frequency than noncancer patients. They also selectively assign the primary site of their cancer to tumors in relatives, leading

to the suggestion of an inherited cancer syndrome, when, in fact, none exists (14). In our study, efforts were made to minimize this source of bias by confirming reported cancers in relatives. Third, the apparent familial aggregation of cancers in some families may represent chance association of sporadic cases. Fourth, young age of onset and multiple primary cancers by themselves are not likely to be important risk factors. Fifth, these families may harbor mutations in other genes predisposing to pancreatic cancer such as *STK11* (75) and the *cationic trypsinogen* gene (76), which were not tested in the present study. Sixth, and most likely, there are probably as yet unidentified genes predisposing to pancreatic cancer, particularly in patients meeting only familial pancreatic cancer criteria.

In summary, we report that 13% of our pancreatic cancer cases with personal or family histories suggestive of inherited predisposition occur in association with germ-line mutations of the *p16*, *BRCA1*, and *BRCA2* genes. Importantly, 80% (four of five) of mutations were identified in Jewish patients, three of whom had family histories of breast-ovarian cancer, whereas only one *p16* mutation was identified in a non-Jewish patient with a family history of melanoma. We conclude that known causes of genetic susceptibility are an important risk factor in a small proportion of pancreatic cancer patients, particularly when associated with a strong family history of familial cancer syndromes. The absence of identifiable mutations in a large proportion of seemingly high- and intermediate-risk cases suggests that there are as yet unidentified genes predisposing to this disease.

Our findings have several possible clinical implications. First, they stress the importance of a prospectively obtained family history in characterizing inherited cancer risk. Second, the ability to identify a population of individuals at increased risk for pancreatic cancer based on inherited mutations affords the opportunity to develop and validate novel screening protocols that would allow early detection and intervention at a potentially curable stage of disease, thereby improving survival. Based on our findings, we would suggest genetic screening only in those patients with significant family histories of familial cancer syndromes known to be associated with pancreatic adenocarcinoma. Genetic testing can also be offered to relatives because determining the carrier status of at-risk family members may lead to a significant reduction in anxiety for noncarriers. Furthermore, mutation carriers can undergo screening for melanoma and breast cancer according to current guidelines (77, 78). However, it is important to emphasize that there are currently no established clinical protocols for pancreatic cancer screening; hence, there is at least a potential psychological risk associated with the identification of carriers predisposed to a nearly fatal disease.

As a result of the above and the fact that the lifetime risk of pancreatic cancer in mutation carriers has not been defined, we strongly believe that genetic and clinical screening of pancreatic cancer patients and family members should only be conducted in research settings with appropriate pre- and post-test counseling. Lastly, our study indicates that further work is necessary to characterize other unknown inherited susceptibility genes in pancreatic cancer.

ACKNOWLEDGMENTS

We thank the patients and families who agreed to participate in this study; the surgeons and medical and radiation oncologists for assistance in the initial identification of study patients; Cecilia Cabral-Bolarinho, John White, Xiu-Hong Shi, Susan Lau, and Hyeja Kim for technical advice and assistance; and Lisa Madlensky for helpful discussion.

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