

Evaluation of Candidate Genes *MAP2K4*, *MADH4*, *ACVR1B*, and *BRCA2* in Familial Pancreatic Cancer: Deleterious *BRCA2* Mutations in 17%¹

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ABSTRACT

It is estimated that familial aggregation and genetic susceptibility play a role in as many as 10% of pancreatic ductal adenocarcinomas. To investigate the role of germ-line mutations in the etiology of pancreatic cancer, we have analyzed samples from patients with pancreatic cancer enrolled in the NFPT for mutations in four tumor suppressor candidate genes: (a) *MAP2K4*; (b) *MADH4*; (c) *ACVR1B*; and (d) *BRCA2* by direct sequencing of constitutional DNA. These genes are mutated in clinically sporadic pancreatic cancer, but germ-line mutations are either not reported or anecdotal in familial pancreatic cancer. Pancreatic cancer patient samples were selected from kindreds in which three or more family members were affected with pancreatic cancer, at least two of which were first-degree relatives. No mutations were identified in mitogen-activated protein kinase kinase 4 (0 of 22), *MADH4* (0 of 22), or *ACVR1B* (0 of 29), making it unlikely that germ-line mutations in these genes account for a significant number of inherited pancreatic cancers. *BRCA2* gene sequencing identified five mutations (5 of 29, 17.2%) that are believed to be deleterious and one point mutation (M192T) unreported previously. Three patients harbored the common 6174delT frameshift mutation, one had the splice site mutation IVS 16–2A > G, and one had the splice site mutation IVS 15–1G > A. Two of the five *BRCA2* mutation carriers reported a family history of breast cancer, and none reported a family history of ovarian cancer. These findings confirm the increased risk of pancreatic cancer in individuals with *BRCA2* mutations and identify germ-line *BRCA2* mutations as the most common inherited genetic alteration yet identified in familial pancreatic cancer.

INTRODUCTION

Pancreatic cancer is the fifth leading cause of cancer death in North America. Despite advances in surgery, chemotherapy, and radiation therapy, the prognosis remains poor, with only a 4% 5-year survival (1). This low survival rate is, in large part, attributable to the advanced disease state at clinical presentation. By the time they are diagnosed, only ~20% of patients are candidates for surgical resection (2, 3). The identification of individuals at risk for pancreatic cancer would aid in targeting individuals who might benefit most from cancer surveillance strategies (4).

Although the majority of pancreatic cancer cases appear to be sporadic, ~10% of cases are believed to be caused by inherited genetic factors (5, 6). The first line of evidence for a genetic component to the disease comes from case reports in the literature describing families with multiple individuals affected with pancreatic cancer (7–12). Secondly, several case control studies have demonstrated that a family history of pancreatic cancer is an important risk factor for the disease, conferring an ~3-fold increased risk (13–15).

The third line of evidence supporting the role of genetic susceptibility in the development of pancreatic cancer is two prospective analyses of

at-risk relatives in kindreds in which there has been pancreatic cancer (16, 17). Tersmette *et al.* (17) followed families with at least a pair of first-degree relatives with pancreatic cancer and demonstrated an 18-fold increased risk of pancreatic cancer among apparently healthy first-degree relatives of patients with pancreatic cancer. In a subset of kindreds with three or more affected family members, there was a 57-fold increased risk of pancreatic cancer. The fourth line of evidence comes from recent segregation analyses performed on families in which there has been a pancreatic cancer. Klein *et al.* performed segregation analysis on 287 kindreds in which there was a pancreatic cancer and was able to mathematically reject nongenetic transmission models ($P < 0.0001$), whereas Mendelian Models provided a good fit for the data.³

Finally, it has been observed that pancreatic cancer occurs in excess of expected frequencies in several familial cancer syndromes, which are associated with specific germ-line gene mutations. These syndromes include Peutz-Jeghers syndrome (mutation in the *STK11/LKB1* gene), familial pancreatitis (mutations in the cationic trypsinogen gene, *PRSS1*), and hereditary nonpolyposis colorectal cancer (mutations in DNA mismatch repair genes; reviewed in Ref. 6). Familial breast cancer (mutations in *BRCA2*) is another syndrome in which an increased frequency of pancreatic cancer has been observed. Previous analysis of breast ovarian cancer families with *BRCA2* mutations demonstrated a 3.5-fold increased risk of pancreatic cancer (18). The final syndrome, familial atypical multiple mole-melanoma syndrome, involves mutations in the *p16* gene. Despite extensive study, germ-line *p16* mutations have not been found in the absence of any manifestation of familial atypical multiple mole-melanoma. These five clinically defined syndromes (including borderline syndromes, such as families carrying a *p16* gene mutation that have a single instance of melanoma and *BRCA2* mutation in families that have a single instance of breast cancer) do not, however, account for many cases of familial pancreatic cancer. Aside from these syndromes (and borderline syndromes), no causative germ-line mutations have yet been reported in familial pancreatic cancer. This contrasts with the case of sporadic pancreatic cancer, in which *BRCA2* mutations (apparently serving as low-penetrance alleles) have been found (5, 6). Thus, the major gene(s) responsible for the inheritance patterns of pancreatic cancer remains to be identified.

Here, we analyzed constitutional DNA isolated from pancreatic cancer patients from well-defined pancreatic cancer kindreds in whom three or more members were affected with pancreatic cancer, at least two of which were first-degree relatives. None of the kindreds satisfied the criteria for assignment to other clinically defined familial cancer syndromes. Four genes were analyzed: (a) *MAP2K4*;⁴ (b) *MADH4* (*SMAD4/DAC4*); (c) *ACVR1B* (*ALK4*, activin receptor type 1B); and (d) *BRCA2*. Each of these tumor suppressor genes is known to undergo germ-line or somatic genetic inactivation in sporadic pancreatic cancer and, thus, they constituted a set of candidate genes for a mutational survey in these families (19–23).

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⁴ The abbreviation used is: NFPT, National Familial Pancreas Tumor Registry.

MATERIALS AND METHODS

Subjects. The NFPTR was established at The Johns Hopkins Medical Institutions in 1994 to study the role of genetic factors in the development of pancreatic cancer. As of November 1, 2001, >780 families have enrolled in this registry. Patients complete an extensive questionnaire that relates medical and family history and exposure to possible pancreatic cancer-related risk factors, such as smoking and industrial chemicals. The questionnaire is based on a well-established hereditary colon cancer questionnaire that has undergone extensive verification testing. Where possible, cancers in the kindred are confirmed by review of pathology reports, death certificates, medical records, and/or diagnostic histopathologic material. The registry has been described in detail elsewhere (6, 24).

For this study, patients with pancreatic cancer were selected from kindreds enrolled in the NFPTR containing three or more cases of pancreatic cancer, where at least two of the affected persons were first-degree relatives. A total of 31 samples representing 29 kindreds were analyzed in this study. All of the subjects identified themselves as Caucasian. Of the 29 kindreds analyzed, 6 identified themselves as being of Ashkenazi descent, 10 as not Jewish, and 13 subjects did not specify. None of the kindreds satisfied the published criteria for other familial cancer syndromes. Informed consent was obtained from all persons according to an Institutional Review Board-approved protocol. Sample numbers have been coded to ensure confidentiality.

Samples. Peripheral blood samples from patients with pancreatic cancer were stored both as mononuclear cell pellets at -80°C and as EBV-immortalized lymphoblastoid cells. DNA was isolated from either immortalized cells or frozen peripheral blood mononuclear cell pellets using QIAamp DNA Isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

PCR and Gene Sequencing. Each exon of *MAP2K4* (25), *MADH4* (26), and *ACVR1B* (22) was amplified by PCR using primers and conditions reported previously. PCR products were visualized by 1% agarose gel electrophoresis and purified using QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions. Purified PCR products were subjected to cycle sequencing using the Big Dye terminator method and analyzed using an ABI Prism 3700 (Perkin-Elmer, Inc.). Sequences were examined using Sequencer software (Gene Codes Corp., Inc.).

Full gene sequencing of *BRCA2* in both the forward and reversed directions, covering ~10,200 bp comprising 26 exons and ~900 adjacent bp in the noncoding intervening sequence, was performed by Myriad Genetic Laboratories, Inc. (Salt Lake City, UT).

RESULTS

In an attempt to identify germ-line mutations that produce an increased risk for pancreatic cancer, we selected patients with pancreatic cancer from the NFPTR using stringent criteria: within the kindred, three or more individuals had to be affected with pancreatic cancer, two of which had to be first-degree relatives. In a previous prospective study, even the apparently healthy members of such kindreds on follow-up had a 57-fold increase in risk for pancreatic cancer as compared with that expected based on the Surveillance, Epidemiology, and End Results program data (17). Twenty-nine kindreds were analyzed in the present study. In 2 kindreds, samples were available from two family members affected with pancreatic cancer. Thus, the total number of samples analyzed was 31. Twenty-two samples from 20 kindreds satisfying the above criteria were originally selected and tested for mutations in *MAP2K4*, *MADH4*, *ACVR1B*, and *BRCA2*. An additional nine samples satisfying the above criteria became available after the *MAP2K4* and *MADH4* analyses were completed and analyzed for *ACVR1B* and *BRCA2* mutations only.

Table 1 describes the samples analyzed. The number of individuals affected with pancreatic cancer in these kindreds ranged from three to six, averaging 3.8 affected individuals/kindred. The age of onset of disease in these patients ranged from 39 to 82 years with an average of 66.7 years. This is virtually identical to the age of onset of other patients in the NFPTR with familial pancreatic cancer (minimally defined as a pair of first-degree relatives with pancreatic cancer; 66.8 years) and is not significantly different from the age of onset for the

nonfamilial NFPTR cases (minimally constituting a pancreatic cancer but no pair of first-degree relatives; 64.9 years).

No coding alterations in *MAP2K4* (0 of 22), *MADH4* (0 of 22), or *ACVR1B* (0 of 29) were identified in any of the samples tested. Two silent gene changes which do not result in amino acid substitutions, G997A in *MAP2K4* (patient 10-1) and G354A in *MADH4* (patient 6-1), were identified. A sibling of patient 6-1 with pancreatic cancer (patient 6-2) did not carry the same *MADH4* alteration, indicating that this alteration was not responsible for the pancreatic cancer in this kindred. Although silent changes could result in alterations in mRNA half-life or stability, these silent changes do not appear to be selected for during the evolution of pancreatic carcinomas. Thus, such a role remains speculative, and these changes are considered to be of doubtful clinical significance.

Sequencing of the *BRCA2* gene revealed that 5 patients from the 29 kindreds tested (17.2%) had *BRCA2* gene mutations which appeared to be deleterious. These are listed in Table 2. The pedigrees for these kindreds are shown in Fig. 1. Three patients (3-1, 12-1, and 26-1; Fig. 1, A-C, respectively) harbored the *BRCA2* 6174delT frameshift mutation, which results in premature truncation of the *BRCA2* protein and is found in ~1% of Ashkenazim (27). All 3 of these patients report themselves to be of Ashkenazi Jewish descent. Within the study population, a total of 6 patients identified themselves as being of Ashkenazi descent. Thus, the proportion of Ashkenazi with *BRCA2* mutations in this study (3 of 6) is greater than the ~1% expected by chance ($P < 0.001$, proportions test).

Two of the deleterious *BRCA2* mutations identified involved alterations of nucleotides that are essential for proper mRNA processing. Patient 2-1 (Fig. 1D) had a nucleotide substitution at the splice acceptor site of intron 16 (IVS16-2A > G). Although the effect of this particular mutation has not been established definitively in other families, similar mutations in other genes are usually deleterious. Comparable mutations in *BRCA1* and *BRCA2* have also been demonstrated through biochemical analysis to result in nonfunctional proteins. Patient 11-2 (Fig. 1E) harbored a mutation at the splice donor site of intron 15 (IVS15-1G > A). This mutation has only been reclassified recently as a splice site-inactivating mutation from its previous classification as a presumed polymorphism. This change affects five families (of which this patient is one) that have been evaluated by Myriad Genetic Laboratories.

The average age of onset of disease in patients with deleterious *BRCA2* mutations (66 years) was essentially the same as that of the study group (66.7 years). One of the patients with a deleterious *BRCA2* mutation reported a family history of breast cancer (Fig. 1D), and 1 reported both a personal and family history of breast cancer (Fig. 1E). None of the *BRCA2* mutation carriers reported a family history of ovarian cancer.

One *BRCA2* alteration of unknown significance was identified, M192T (patient 18-1). This alteration has not been observed previously by Myriad Genetic Laboratories and has not been reported in the Breast Cancer Information Core database. The location of this alteration does not lie within the known functional domains of *BRCA2* (28). It is difficult to predict the potential ramifications of this missense mutation because the three-dimensional structure of *BRCA2* has not yet been elucidated. The functional significance of this mutation remains undefined, although questionable, because as of yet, no *BRCA2* missense mutation has been firmly established as deleterious.

Three patients (6-2, 6-3, and 7-1) from the 29 kindreds (10.3%) had the *BRCA2* K3326X variant (also known as 3326ter), which causes loss of the final 93 amino acids of the *BRCA2* protein. This alteration has been identified in ~1–2% of European and United States control groups. Thus, the observed frequency of this alteration in this study group was somewhat above expected. Evidence suggests that this variant does not significantly increase susceptibility to breast cancer

Table 1 Characteristics of samples selected from the NFPTR

Sample ^a	Sample type ^b	No. with pancreatic cancer in kindred	Sex	Relationship of other family members with pancreatic cancer to the patient tested	Age at diagnosis
1-1	CL	5	F	Sister, father, aunt, grandfather	66
2-1	CL	3	F	Mother, father	51
3-1	Leuk	4	M	Brother, 2 cousins	74
4-1	CL	3	M	Father, brother	77
5-1	CL	7	F	Daughter, sister, brother, cousin, aunt, uncle	62
6-1	CL	6	F	2 brothers, sister, mother, father	75
6-2	CL	6	M	2 sisters, brother, mother, father	67
7-1	Leuk	5	M	2 sisters, mother, nephew	68
7-2	CL	5	M	2 aunts, uncle, grandmother	39
8-1	Leuk	4	M	Mother, grandmother, cousin	82
9-1	CL	3	F	Father, uncle	69
10-1	CL	4	F	2 brothers, sister	69
11-1	CL	3	F	Father, brother	58
12-1	Leuk	3	F	Father, uncle	75
13-1	Leuk	4	M	Mother, aunt, grandfather	73
14-1	Leuk	4	M	Father, uncle, cousin	68
15-1	Leuk	3	F	Father, grandmother	51
16-1	CL	4	F	Father, sister, brother	74
17-1	Leuk	3	M	Brother, nephew	70
18-1	Leuk	3	M	Father, grandfather	79
19-1	CL	3	M	Father, uncle	49
20-1	Leuk	3	M	Mother, aunt	72
21-1	Leuk	3	F	Father, grandfather	54
22-1	CL	3	F	Brother, uncle	72
23-1	CL	5	F	Son, uncle, cousin, grandfather	69
24-1	CL	3	F	2 uncles	55
25-1	CL	3	F	Father, brother	71
26-1	Leuk	3	F	Sister, mother	69
27-1	Leuk	4	F	Sister, mother, aunt	73
28-1	CL	6	F	2 brothers, father, aunt, niece	77
29-1	CL	3	M	Mother, father	57

^a Initial number is the family code; second number specifies the individual.

^b CL, EBV-immortalized lymphoblastoid cells lines; Leuk, peripheral blood leukocytes.

(29). We have investigated previously the role of this alteration and found that in patients harboring the K3326X alteration, loss of heterozygosity of the *BRCA2* locus was not observed in their pancreatic tumors.⁵ In addition, cosegregation of K3326X with pancreas cancer was not observed in a family with multiple pancreatic cancers. This alteration is therefore considered to be a genetic variant of limited or no clinical significance.

In addition to the alterations described above, two *BRCA2* polymorphisms were identified in the study population: (a) V2728I (patient 7-2); and (b) T598A (patient 28-1, who also harbored the K3326X variant).

DISCUSSION

We have used a candidate gene approach to screen for mutations in four tumor suppressor genes: (a) *MADH4*; (b) *ACVR1B*; (c) *MAP2K4*; and (d) *BRCA2*, in patients likely to harbor genetic susceptibility(s) to pancreatic cancer. *MADH4* (*DPC4*) functions in the transforming growth factor- β /activin suppressive pathway. Previous studies identifying genetic inactivation of genes in these signaling pathways in human tumors have confirmed the importance of these pathways for tumor suppression. *MADH4*, a major tumor suppressor in pancreatic cancer, is somatically inactivated in 55% of pancreatic carcinomas and a lesser proportion of other cancer types (19, 21). Inherited *MADH4* gene mutations cause juvenile polyposis, but as yet, germ-line mutations have not been implicated in familial pancreatic cancer. Somatic gene mutations in the activin receptor type 1B, *ACVR1B*, in pancreatic tumors have been described recently, but this gene had not been evaluated previously in the setting of familial cancer predisposition (22). In the current study, germ-line mutations of *ACVR1B* or *MADH4* were not identified in any of the familial pancreatic cancer kindreds tested. This result is consistent with a previous finding

demonstrating an absence of germ-line *MADH4* mutations in a less rigorously selected set of familial pancreatic cancer patients (26).

The *MAP2K4* gene codes for a component of a stress and cytokine-induced signal transduction pathway, functioning downstream of the Ras protein and upstream of c-Jun (30, 31). The *MAP2K4* gene was selected for study because it is somatically inactivated in ~4% of pancreatic carcinomas, 6% of biliary, and 5% of breast carcinomas, and it has not been evaluated previously in the setting of familial cancer predisposition (23, 25). In the current study, germ-line mutations of the *MAP2K4* gene were not identified in any of the familial pancreatic cancer kindreds tested. Thus, it is unlikely that germ-line mutations of *MAP2K4*, *ACVR1B*, or *MADH4* account for a significant number of inherited pancreatic cancers.

BRCA2 is a tumor suppressor gene whose protein product is thought to function in DNA repair, although its exact role in neoplasia is not well developed (28). Mutations in the *BRCA2* gene are associated with familial breast cancer syndrome and may be involved in up to half of hereditary breast cancer. In addition, germ-line *BRCA2* gene mutations result in an increased lifetime risk of ovarian, pancreas, and prostate cancer (18).

Previous studies have demonstrated that an excess of pancreatic cancer is observed in some breast and/or ovarian cancer families with *BRCA2* mutations; however, the incidence of pancreatic cancer in these families appears to be relatively low (32–35). We now rigor-

Table 2 Germ-line *BRCA2* variants of deleterious or uncertain significance identified in familial pancreatic cancer

Patient	Mutation	Interpretation
2-1	IVS16-2A > G, K3326X	Suspect deleterious (IVS16-2A > G)
3-1	6174delT	Deleterious
11-1	IVS15-1G > A	Deleterious
12-1	6174delT	Deleterious
18-1	M192T	Uncertain significance
26-1	6174delT	Deleterious

⁵ E. Rozenblum, M. Goggins, C. J. Yeo, R. H. Hruban, S. E. Kern, unpublished data.

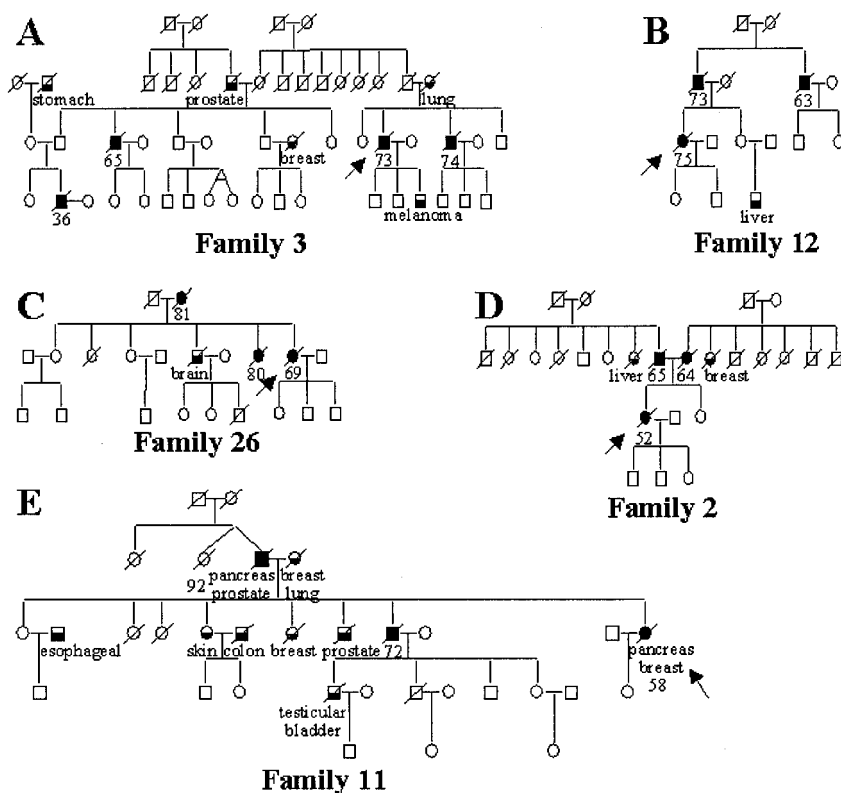


Fig. 1. Pedigrees from kindreds with deleterious *BRCA2* mutations. Arrows, the individuals that were tested. Filled symbols, individuals affected with pancreatic cancer. The age of onset for individuals with pancreatic cancer is displayed under the filled symbol. White symbols, unaffected individuals. Half-filled symbols, individuals affected with cancers other than that of the pancreas (specific type is noted). In families 3, 12, and 26 (A–C), the individuals with pancreatic cancer that were tested (arrow) have the *BRCA2* 6174delT mutation. In family 2 (D), the affected individual that was tested (arrow) has the *BRCA2* IVS16-2 A > G mutation. In family 11 (E), the affected individual that was tested (arrow) has the *BRCA2* IVS15-1G > A mutation.

ously study the role of germ-line *BRCA2* gene mutations within familial pancreatic cancer kindreds. We identified deleterious mutations in 5 patients (5 of 29, 17.2%) and one mutation of uncertain significance. It is possible that *BRCA2* alterations exist in additional patients because gene sequencing does not detect deletions of promoter regions, exons or entire genes, epigenetic changes, or primary errors of RNA transcript processing (36).

In contrast to our findings, a recent study by Lal *et al.* (37) failed to identify any *BRCA2* gene mutations in 4 pancreatic cancer patients classified as high-risk/familial pancreatic cancer (defined as more than or equal to two pancreatic cancers among first-, second-, or third-degree relatives) or in 12 patients classified as intermediate risk/familial pancreatic cancer (defined as one pancreatic cancer among first-, second-, or third-degree relatives). This discrepancy is likely to reflect the smaller sample size in their high-risk group and less stringent classification criteria in their intermediate risk group.

Similar to other studies, the average age of disease onset of disease in patients with deleterious *BRCA2* mutations was not different from that of patients with sporadic disease. Previous work in our lab demonstrated that inactivation of *BRCA2* by loss of heterozygosity in pancreatic ductal lesions is a relatively late event in pancreatic tumorigenesis, implying a necessity for earlier genetic alterations before bi-allelic inactivation of *BRCA2* (38). This may explain why, unlike other hereditary cancers, there is a late age of onset of hereditary pancreatic cancers, an age similar to that seen in sporadic disease.

Two of the 5 subjects in which a deleterious *BRCA2* mutation was identified reported a family history of breast cancer (Fig. 1, D and E). Interestingly, the mutations in these families were alterations at splice junctions in introns 15 and 16. Despite the fact that the 6174delT mutation is strongly implicated in the development of breast cancer, none of the subjects with a 6174delT mutation reported a family history of breast cancer (Fig. 1, A–C). It is possible that the absence of breast cancer in the 6174delT mutation kindreds is simply attributable to a relative paucity of females in these families and is not

greater than expected by chance. However, two of the three *BRCA2* mutation carriers are female themselves and were >60 years old at the time of diagnosis. Family 26 is particularly striking, because all 3 of the affected patients were female and ≥ 69 years of age at the time they were diagnosed with pancreatic cancer. Germ-line *BRCA2* gene mutations have been reported previously in 7% of unselected patients and 10% of Ashkenazi Jewish patients with apparently sporadic pancreatic cancer (no first-degree relatives with pancreatic cancer) in the absence of a family history of breast, pancreatic, and/or ovarian cancer (20, 27). It is possible that small family sizes in these prior studies and/or the low penetrance of *BRCA2* gene mutations made it difficult to identify an inheritance pattern in these cases.

The importance of *BRCA2* mutations in familial pancreatic cancer is also supported in a recent case report that describes a family in which a *BRCA2* mutation is associated with a high penetrance of pancreatic cancer, a breast cancer, and atypical breast epithelial changes (39). In that report, a 2-bp deletion (6819del TG) was identified in a family in which there were four cases of pancreatic cancer over two generations (fulfilling the criteria used in this study). Similar to the 6174delT mutation, the 6819del TG mutation results in protein truncation in exon 11.

Although a genotype-phenotype correlation between the location of the mutation within *BRCA2* and the risk for ovarian *versus* breast cancer has been demonstrated previously, it is currently unclear whether *BRCA2* mutations that lead to an increased incidence of pancreatic cancer are site specific. Clearly, the location of the *BRCA2* mutation is not the only factor influencing cancer type, because the 6174delT and other truncating mutations in exon 11 have been associated with breast cancer predisposition. It is likely that additional genetic (such as additional predisposing genes) and/or environmental factors influence the cancer phenotype. Additional studies are necessary to fully define the risks of breast, ovarian, and pancreatic cancer associated with germ-line *BRCA2* mutations.

The relative risk of pancreatic cancer in subjects with *BRCA2*

mutations was estimated previously to be 3.5 within a population of breast ovarian families (18). Such a low relative risk, however, could not account for the 7–10% rate of germ-line mutations observed in apparently sporadic pancreatic cancer (20, 27), nor the 57-fold increased risk of pancreatic cancer in familial pancreatic cancer kindreds with three or more affected family members (17), nor the results presented here. Again, it is necessary to define additional covariates to determine the *BRCA2*-associated risk of pancreatic cancer within different populations. Taken together, however, the data strongly suggest that germ-line *BRCA2* mutations can be associated with a relatively high proportion of the total (sporadic and familial) incidence of pancreatic cancers, making it the most common inherited genetic predisposition to pancreatic cancer identified to date.

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