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Prevalence of Germline Mutations in Cancer Predisposition Genes in Patients with Pancreatic Cancer

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Abstract

Background & Aims—We investigated the prevalence of germline mutations in *APC*, *ATM*, *BRCA1*, *BRCA2*, *CDKN2A*, *MLH1*, *MSH2*, *MSH6*, *PALB2*, *PMS2*, *PRSS1*, *STK11*, and *TP53* in patients with pancreatic cancer.

Methods—The Ontario Pancreas Cancer Study enrolls consenting participants with pancreatic cancer from a province-wide electronic pathology database; 708 probands were enrolled from April 2003 through August 2012. To improve precision of *BRCA2* prevalence estimates, 290 probands were randomly selected from 3 strata, based on family history of breast and/or ovarian

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cancer, pancreatic cancer, or neither. Germline DNA was analyzed by next-generation sequencing using a custom multiple-gene panel. Mutation prevalence estimates were calculated from the sample for the entire cohort.

Results—Eleven pathogenic mutations were identified: 3 in *ATM*, 1 in *BRCA1*, 2 in *BRCA2*, 1 in *MLH1*, 2 in *MSH2*, 1 in *MSH6*, and 1 in *TP53*. The prevalence of mutations in all 13 genes was 3.8% (95% confidence interval, 2.1%–5.6%). Carrier status was significantly associated with breast cancer in the proband or first-degree relative (P<.01), and colorectal cancer in the proband or first-degree relative (P<.01), but not family history of pancreatic cancer, age of diagnosis, or stage at diagnosis. Of patients with a personal or family history of breast and colorectal cancer, 10.7% (4.4%–17.0%) and 11.1% (3.0%–19.1%) carried pathogenic mutations, respectively.

Conclusions—A small but clinically important proportion of pancreatic cancer is associated with mutations in known predisposition genes. The heterogeneity of mutations identified in this study demonstrates the value of using a multiple-gene panel in pancreatic cancer.

Keywords

cancer risk; familial pancreatic cancer; pancreatic cancer genetics

Introduction

Pancreatic cancer (PC) is a deadly malignancy. Since symptoms generally signal advanced disease[1], only about 20% present with localized disease that may be amenable to curative surgical resection[2]. As a result, PC carries a bleak prognosis; the five-year survival rate is 6%, only a slight improvement from 2% in the 1970s[3]. Approximately 40,000 Americans will die of PC in 2014, making this malignancy the fourth most common cause of cancer death in men and women[3]. New screening and treatment strategies to reduce deaths from PC are urgently needed.

A subset of PC is caused by highly penetrant germline mutations that cause well-characterized cancer syndromes. Hereditary breast and ovarian cancer syndrome is caused by heterozygous germline mutations in *BRCA1* and *BRCA2*. In addition to breast and ovarian cancer, *BRCA1* and *BRCA2* mutation carriers also face an increased risk of prostate cancer and PC[4, 5]. The risk of PC among carriers of *BRCA1* and *BRCA2* mutations has been estimated to be between four- and seven-fold greater than the general population[4-6].

Lynch syndrome, also called hereditary nonpolyposis colorectal cancer, is caused by heterozygous germline mutations in the DNA mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, and *PMS2*. MMR mutation carriers face an elevated risk of colorectal, duodenal, endometrial, urogenital tract, brain, hepatobiliary, and PC[7-9]. MMR gene mutation carriers have been estimated to develop PC at approximately eight times the rate of the general population[10].

Other familial cancer syndromes and genes impart increased risk of PC. These include familial adenomatous polyposis, familial atypical multiple mole melanoma syndrome, hereditary pancreatitis, Peutz-Jeghers syndrome, and Li-Fraumeni syndrome, which are caused by mutations in *APC*, *CDK2NA*, *PRSS1*, *STK11*, and *TP53*, respectively[1, 11].

Recently, *ATM* and *PALB2*, two genes that increase risk of breast cancer, were associated with familial PC[12, 13].

Identifying germline mutations in genes that increase risk of PC has important ramifications for the patient and their blood relatives. PC patients carrying these mutations may benefit from experimental and targeted therapies, as proposed with PARP inhibitors and platinum-based chemotherapy in PC patients with *BRCA1* and *BRCA2* mutations[14, 15]. Unaffected relatives who also carry mutations may be candidates for investigational PC screening protocols[16, 17]. Most importantly, patients and relatives who carry mutations can benefit from well-established targeted extra-pancreatic cancer screening protocols and interventions, such as prophylactic mastectomy and salpingooopherectomy in *BRCA1* and *BRCA2* carriers[18, 19], and more intensive colorectal cancer screening and management in Lynch and familial adenomatous polyposis syndromes[20].

The goal of this study was to determine the prevalence of germline mutations in genes that increase risk of PC in a population-based cohort of PC patients and to determine which clinical characteristics are associated with mutation carrier status.

Methods

Patients

Probands were selected from the Ontario Pancreas Cancer Study (OPCS), which has been described previously[21]. The OPCS is a population-based registry that contacts all patients in Ontario with a pathological diagnosis of pancreatic ductal adenocarcinoma from a province-wide electronic pathology reporting system. Consenting participants answer questionnaires, agree to a review of medical records, and provide biospecimens (blood, saliva, and access to biopsies and resections). OPCS probands recruited between April 2003 and August 2012 with available blood or saliva samples were included in this study. The research ethics board of Mount Sinai Hospital approved this study.

Patient sampling procedure

We aimed to detect at least one mutation for any gene with mutations in at least one percent of pancreatic cancer. To achieve this goal with at least 95% power, a sample size of 290 patients was selected. The subset of 290 probands was randomly selected from the OPCS using a stratified random sampling strategy to maximize the precision for the population estimate of *BRCA2* mutation prevalence, since *BRCA2* was the only gene that we expected to be frequently mutated. Family history of pancreatic, breast, or ovarian cancer was expected to modulate the prevalence of *BRCA2* mutations, so probands were stratified according to family history of cancer. The three strata were: PC in a first, second, or third degree relative; breast or ovarian cancer in a first, second or third degree relative without family history of PC; or no family history of pancreatic, breast, or ovarian cancer in a first, second or third degree relative. The sampling weight for each strata was defined such that it minimized the variance estimate of the *BRCA1* and *BRCA2* mutation prevalence estimates following the approach proposed by Choi and Briollais[22]. Probands with known mutations

were included in the randomization. A control subject without a history of cancer was sequenced along with the 290 PC probands to assist with variant filtering.

Next-generation sequencing (NGS) and bioinformatics

Genomic DNA was extracted from peripheral blood lymphocytes using organic solvent isolation or column-based purification methods. A custom targeted capture kit was designed using NimbleDesign (NimbleGen Inc., Madison, U.S.A.) that targeted the exonic and splice site regions of 385 genes previously associated with cancer for use in the clinical laboratory at Mount Sinai Hospital. Libraries were created using the SeqCap EZ Library (NimbleGen Inc., Madison, U.S.A.) and KAPA Library Preparation Kits (Kapa Biosystems, Inc., Wilmington, U.S.A.) according to the manufacturers' protocols. NGS was performed on Illumina HisSeq 2500 platforms (Illumina Inc., San Diego, U.S.A.). Bases were called with default settings using Illumina BCLFAST2 Conversion Software (v.1.8.4, Illumina Inc., San Diego, U.S.A). Sequencing reads were aligned to the reference genome hg19 using the Burrows-Wheels Aligner (v.0.6.2-r126)[23]. Picard (v1.79, http://picard.sourceforge.net, accessed 1 February 2014) removed duplicate reads. The Genome Analysis Toolkit (GATK) (v2.0-25-gf27c683)[24] was used to detect single nucleotide substitutions and small insertions and deletions, using best practices from the GATK website (http://www.broadinstitute.org/gatk/, accessed 1 February 2014). To maximize sensitivity to detect variants, no variant quality filters were applied. ANNOVAR[25] and in-house scripts annotated variants.

Variant characterization

Variants in *APC, ATM, BRCA1, BRCA2, CDKN2A, MLH1, MSH2, MSH6, PALB2, PMS2, PRSS1, STK11*, and *TP53* were considered for analysis if they were: a) called as non-reference by GATK; b) predicted to affect protein sequence or the splice site (*i.e.* +/- 5 base pairs); c) had an allele frequency of less than 1% in the 1000 Genome project[26], dbSNP138[27], and NHLBI Exome Variant Server ESP6500 dataset (http://evs.gs.washington.edu/EVS/, accessed 1 February 2014); and d) were not present in the control subject who was sequenced simultaneously with the cohort.

Rare nonsynonymous variants were classified as either pathogenic, benign, or variants of unknown significance (VUS). For *BRCA1, BRCA2, MLH1, MSH2, MSH6* and *PMS2*, variants were first classified according to the databases generated by Vallée *et al.*[28], and the InSIGHT consortium[29], accessed through the LOVD websites (http://chromium.liacs.nl/LOVD2/colon_cancer/home.php? and http://brca.iarc.fr/LOVD/home.php?; accessed 1 February 2014, respectively). These groups have classified large numbers of variants in *BRCA1, BRCA2, MLH1, MSH2, MSH6* and *PMS2* according to the International Agency for Research on Cancer system based on available information from the literature. Classes 1 and 2 were considered benign; class 3 VUS; and classes 4 and 5 pathogenic. Rare nonsynonymous variants not in these databases were classified based on their predicted effect on the protein product. Nonsense and no-stop variants, variants changing the canonical splice sites (*i.e.* +/-2 base pairs), and frameshift insertions and deletions were considered pathogenic unless they occurred in the last exon. The remaining rare nonsynonymous variants were classified as VUS. Classification of each pathogenic variant was assessed based on a literature review performed independently by a clinical geneticist

(J.L.E.). Variants classified as pathogenic mutations that were detected in the NGS data were inspected using the Integrative Genomics Viewer[30], and variants that appeared to be true calls were verified with Sanger sequencing as previously described[31]. For MMR mutation carriers, immunohistochemistry was performed on available pancreatic tumors by our clinical lab for the MLH1, MSH2, MSH6 and PMS2 proteins as previously described[32].

Statistical analysis

The percentage of mutation carriers in the OPCS and the associated 95% confidence intervals were estimated from the random sample by an inverse-weighting strategy using the Horvitz-Thompson estimator with a finite population correction[33]. To assess the association between clinical characteristics and mutation prevalence, the sample was divided into subpopulations based on history of pancreatic, breast, and colorectal cancer in the proband or first-degree relative, as well as age at diagnosis (younger than 65 *versus* 65 and older, the sample median) and stage at diagnosis (Stage 2A or less *versus* Stage 2B and greater). Mutation prevalence estimates in these subpopulations were computed from the Horvitz-Thompson estimator incorporating sample weights and compared using Rao and Scott's Pearson chi-squared statistic[33]. Statistical significance level was set at $p<0.05$. Statistical analysis was performed in the R Environment for Statistical Computing (http://www.r-project.org/) using the “survey” package[34].

Results

Patient selection and clinical characteristics

Seventy-one patients were randomly selected from 136 OPCS patients with a family history of PC, 39 from 85 patients with a family history of breast or ovarian cancer but no family history of PC, and 180 from 487 OPCS patients without family history of pancreatic, breast or ovarian cancer. Patient characteristics are displayed in Table 1.

Variant detection and characterization

The average number of sequencing reads per patient was 20,763,412 (range 6,438,944-75,459,926), of which on average 66% (range 35-88%) were unique reads that aligned to the target. The average depth of unique aligned reads covering each base within the target was 354 (range: 65 to 1328). On average, 10 and 50 unique sequencing reads covered 98% (range:91-99) and 95% (range:75-98) of the target in each sample, respectively.

Twenty-one mutations detected by NGS were predicted to be pathogenic (Supplementary Table 1). Of these, one appeared to be a false call based on very low variant allele fraction in the NGS data. Another 8 variants were false calls based on Sanger sequencing. Sanger sequencing primers could not be designed to verify a splice site single-nucleotide substitution in *PRSS1* (c.40+1G>A) called in one sample. This patient had no history of pancreatitis. Therefore, eleven pathogenic variants were successfully verified with Sanger sequencing (Table 2).

There were 144 other rare nonsynonymous variants detected in the 13 genes that were not deemed pathogenic. Of these, 37 were benign according to the IARC databases. One of the remaining 107 VUS was a frameshift deletion in the last exon of *BRCA2* (c.2806_2809del) that introduces a stop codon 172 bases from the reference stop codon. On average, each proband carried 1.2 VUS (range 0-6). The number of VUS identified per gene was strongly correlated with the number of bases sequenced per gene (correlation coefficient=0.88; p<0.001) (Figure 1).

Pathogenic mutation carriers

Eleven of the 290 probands carried a pathogenic mutation (Table 2), which corresponded to a 3.8% (95% confidence interval: 2.1-5.6%) mutation carrier prevalence in the OPCS (Figure 2). Three mutations were in *ATM*, one in *BRCA1*, two in *BRCA2*, one in *MLH1*, two in *MSH2*, one in *MSH6*, and one in *TP53*. The youngest age of onset for PC in all patients carrying mutations was 50 years. While most patients carrying mutations had some family history of cancer, none met the Amsterdam II criteria for Lynch Syndrome[8]. Only one patient had a first-degree relative with PC. This relative did not carry the same *ATM* mutation as the proband, as described in a previous report[31]. Two other probands had relatives with DNA available to determine whether the mutations were transmitted or were de novo. A sister of the *MSH2* c.942+3A>T carrier also carried the mutation. She had a history of colon cancer, ductal breast cancer, bladder and transitional cell carcinoma of the ureter, and sebaceous carcinoma. A niece of the *MSH6* p.F569fs carrier also carried the mutation. Her father (the proband's brother) died of colorectal cancer. The two MMR mutation carriers with pancreatic tumor specimens available for MMR immunohistochemistry were immunodeficient for the respective protein encoded by the gene with the germline mutation. There was no family or personal cancer history in the *TP53* nonsense carrier, who developed PC at age 65. No other biospecimens were available from the probands or relatives for segregation or tumor analysis.

Clinical characteristics and mutation carrier status

There was a significant association between mutation carrier status and the following: breast cancer in the proband or a first-degree relative (10.7% (4.4-17.0) *versus* 2.1% (0.7-3.6), p<0.001), and colorectal cancer in the proband or a first-degree relative (11.1% (3.0-19.1) *versus* 2.8% (1.2-4.3), p=0.002) (Figure 3). There was no significant association between mutation carrier status and first-degree relatives with PC, age at diagnosis, or stage at diagnosis.

Discussion

In our study, thirteen genes that substantially increase risk of PC were sequenced using a multiple-gene panel in 290 patients sampled from a population-based cohort of pathologically confirmed PC patients. Eleven germline mutations in seven genes were identified, the majority in genes associated with inherited breast and colorectal cancer. The mutation carrier prevalence was 3.8% (2.1-5.6%), corresponding to approximately 1 in 26 probands. The mutation carrier prevalence approached 1 in 10 when the proband or a first-

degree relative had a history of breast or colorectal cancer. Family history of PC, age of diagnosis, and stage were not associated with mutations in the genes sequenced.

The mutations detected in this study have clinical implications for the probands and their relatives who carry the mutation. BRCA mutation carriers may benefit from tamoxifen and prophylactic mastectomy and/or salpingo-opherectomy for primary prevention, and more rigorous breast cancer surveillance for secondary prevention[18, 19, 35, 36]. Carriers of MMR mutations may benefit from daily aspirin as a preventative measure, prophylactic hysterectomies, and earlier and more frequent colonoscopies[37]. The clinical implications of the mutations discovered in *ATM* are less clear, although some experts would recommend augmented breast cancer surveillance[38]. Furthermore, all mutation carriers represent a high-risk subgroup of patients for PC researchers to consider for screening studies and for experimental targeted therapies.

Previous studies that have sequenced genes known to increase risk of PC have focused on high-risk subsets of PC patients, for example, based on ethnicity or family history of cancer[39-53]. Estimates of *BRCA1* and *BRCA2* mutation prevalence in select subgroups of PC patients based on Ashkenazi Jewish ethnicity or family history of breast, ovarian and/or PC ranged from 0 to 24% [40, 41, 43-46, 49, 50, 52, 53]. Only two small studies have sequenced MMR genes in cohorts of PC patients selected for ethnicity or family history[51,47]. The rate of MMR gene mutations in our cohort, comparable to that in colorectal cancer cohorts, warrants further investigation. Our cohort had no *PALB2* or *CDK2NA* mutation carriers, which is consistent with previous studies that found very low rates of *PALB2* and *CDKN2A* mutations in PC [12, 39, 49, 50, 54-56]. The lack of mutations in *APC, PRSS1*, and *STK11* does not eliminate the possibility that these genes cause a small proportion of PC.

Our study has several limitations. First, our approach to classifying variants was conservative; many rare nonsynonymous variants were classified as VUS. If some VUS are pathogenic, we may have underestimated the prevalence of mutation carriers. There was a significant correlation between the number of VUS and bases sequenced per gene, which supports a more benign role for most of these variants. Second, we only assessed single nucleotide substitutions and small insertions and deletions, not other potential sources of pathogenic genetic variation, which would again lead to conservative estimates of mutation carrier prevalence. Finally, there are important limitations to the OPCS as a population-based registry. Given the poor prognosis of PC, it is difficult to recruit PC patients using traditional epidemiologic strategies, including rapid case ascertainment afforded by our Ontario Cancer Registry[57]. During the study period, the OPCS recruited from all of Ontario based on pathological diagnosis of PC, which ensures no contamination with patients with other diagnoses. Unfortunately, this neglects patients with advanced disease who never receive a pathological diagnosis. Among patients with a pathological diagnosis, the OPCS has a response rate around 25%[21]. Participants tend to be younger, surgical patients treated in academic centers[21]. If the factors affecting inclusion in the OPCS are related to mutation carrier status, our results may not accurately reflect the population of Ontario. We did not find age or stage to be associated with carrier status; future adequately powered studies should explore these potential associations.

Technologic advances have made the cost of multiple-gene panels comparable to individual gene testing[58]; however, the added value of simultaneously sequencing many genes remains uncertain[59] and likely depends on the clinical setting. Multiple gene panels have been evaluated in breast and ovarian cancer cohorts[60-62], leading to the recent development of guidelines that recommend multiple-gene panels for breast cancer patients when family history is consistent with mutations in a range of genes[63]. We detected a wide breadth of mutations in our cohort of PC patients. This supports the use of multiple gene panels in PC, and emphasizes the importance of ongoing efforts to catalogue and characterize variants in genes that predispose to PC[28, 29] and to identify new genes that cause PC. Interestingly, probands with personal or family history of breast or colorectal cancer were most likely to carry mutations, but classic personal and family history was absent in several cases. This raises questions with important clinical implications. With falling sequencing costs, which patients should be referred for multiple-gene panel sequencing? What is the relevance of mutations in probands with atypical personal and family history? Studies suggest that familial factors modify tissue-specific cancers risks for germline mutations in the BRCA and MMR genes[64-66]. Do mutations in pancreatic cancer probands with atypical family history impart a different cancer risk? Addressing these questions will require innovative approaches and extensive genetic characterization of large cohorts. Ongoing collaborative international sequencing efforts of well-characterized PC patients[67, 68] form the foundation to answer these challenging questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MMR	mismatch repair
NGS	next-generation sequencing
OPCS	Ontario Pancreas Cancer Study
PC	pancreatic cancer
VUS	variants of unknown significance

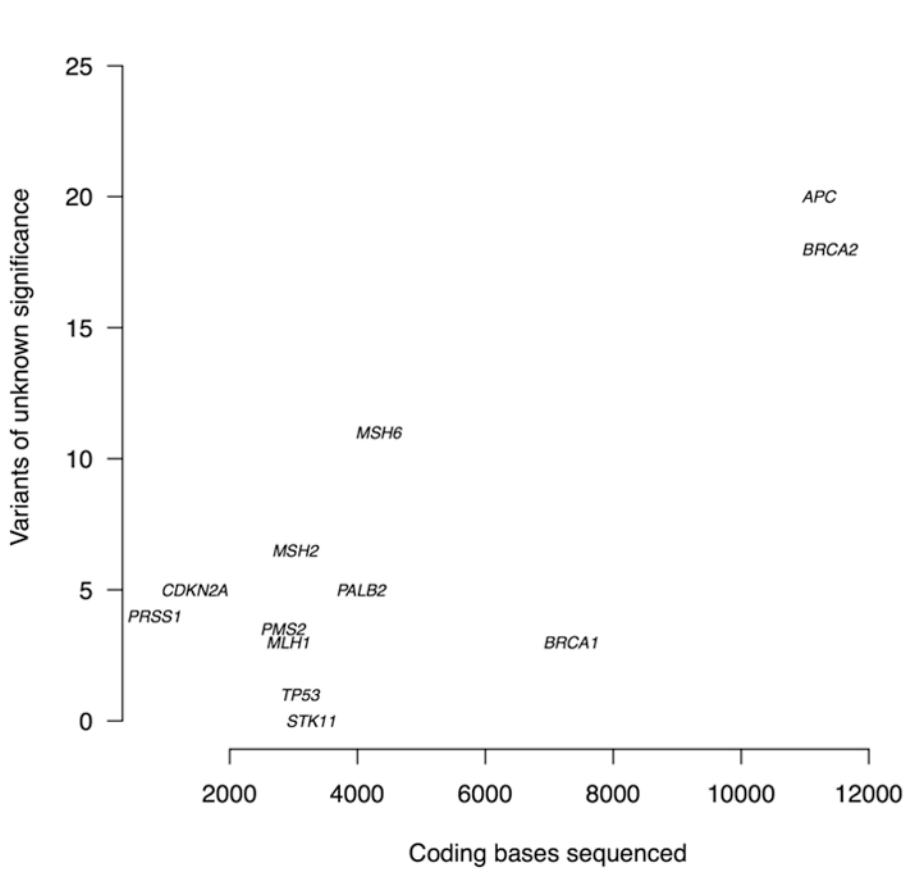


Figure 1.

The number of variants of unknown significance detected in each gene *versus* bases sequenced of each gene (correlation coefficient=0.88; $p<0.001$).

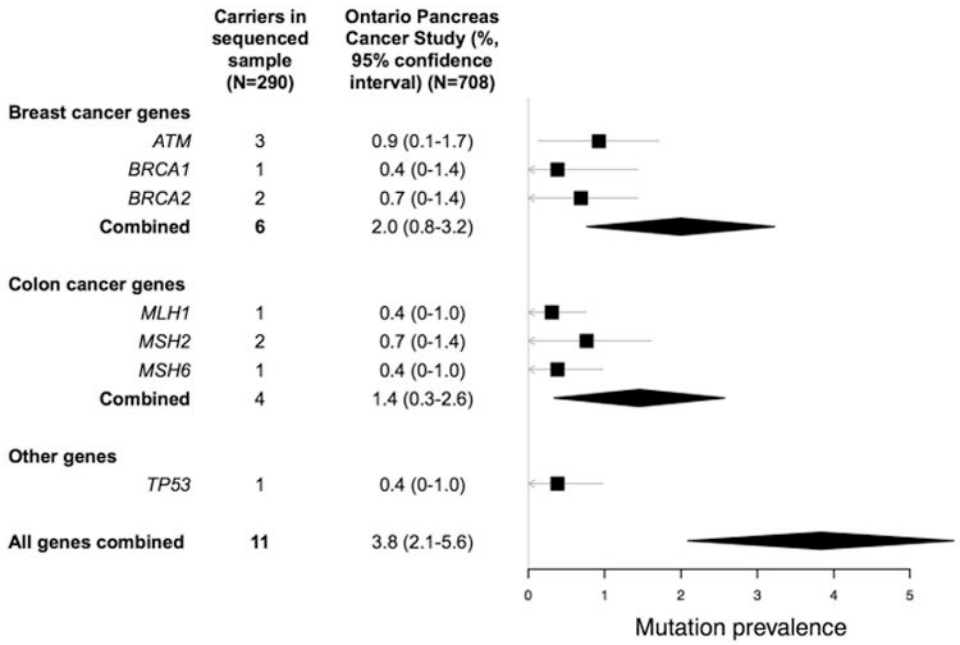
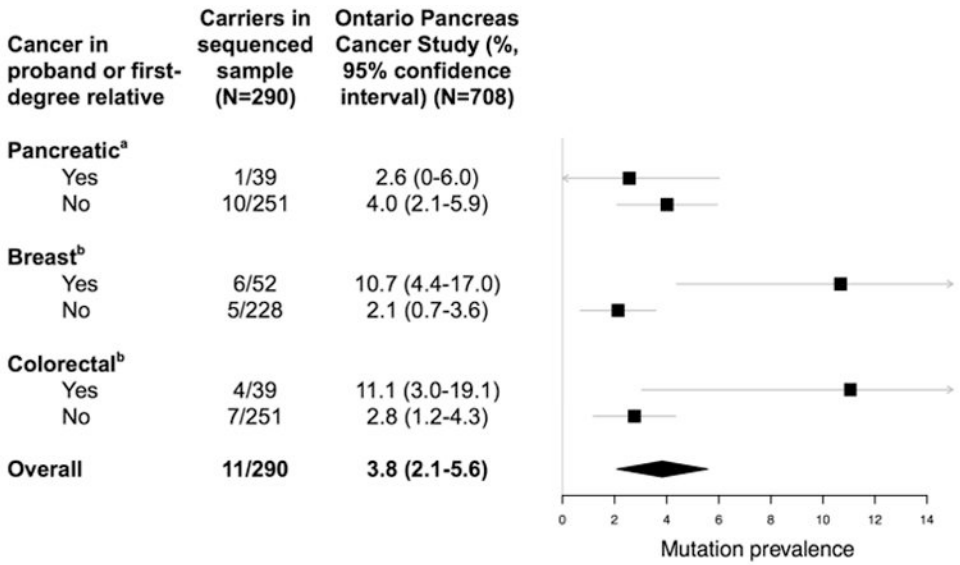


Figure 2.

The prevalence of germline mutations in thirteen genes sequenced in a population-based cohort of pancreatic cancer patients (N=708), estimated from a random sample (N=290), with genes grouped by the predominantly associated cancer. The squares reflect point estimates, the bands reflect 95% confidence intervals, and the diamonds reflect summary estimates and corresponding 95% confidence intervals.



[a] Pancreatic cancer in a first-degree relative (all probands had pancreatic cancer).

[b] $p<0.01$

Figure 3.

The prevalence of germline mutations in thirteen genes sequenced in a population-based cohort of pancreatic cancer patients (N=708), estimated from a random sample (N=290), with the cohort subdivided by the presence of pancreatic cancer in a first-degree relative, and the presence of breast or colorectal cancer in the proband or a first-degree relative. The squares reflect point estimates, the bands reflect 95% confidence intervals, and the diamonds reflect summary estimates and the corresponding 95% confidence intervals.

Table 1

Baseline characteristics of pancreatic cancer probands, compared to the overall population-based study from which they were sampled.

Clinical values	Sequenced sample of probands (%) (N=290)	Ontario Pancreas Cancer Study weighted % estimate (N=708) [a]
Female	144/290 (49.7)	49.1 (2.3)
Ashkenazi Jewish ethnicity	13/290 (4.5)	4.2 (0.9)
Cigarette smoking		
Never	133/280 (47.5)	46.6 (2.3)
Former	17/280 (6.1)	6.01(1.1)
Current	131/280 (46.8)	47.4 (2.3)
Cancer in first-degree relative		
Pancreas	39/290 (13.9)	10.5 (0.8)
Breast	57/290 (19.7)	18.5 (1.6)
Colorectal	36/290 (12.4)	12.2 (1.5)
Personal history of cancer		
Breast	6/290 (2.1)	1.9 (0.6)
Colorectal	7/290 (2.4)	2.4 (0.7)
Age at pancreas cancer diagnosis	64.5 (0.6)[b]	64.3 (0.5)[b]
Stage 2A or less at diagnosis	50/285 (17.5)	17.4 (1.8)

[a] Estimates of the percentage (standard error) of each baseline characteristics reflective of all probands in the Ontario Pancreas Cancer Study estimated from the sequenced sample.

[b] Mean (standard error).

Table 2

Clinical characteristics of the pancreatic cancer probands who carried pathogenic mutations in the sample of 290 probands who were sequenced for thirteen genes associated with pancreatic cancer risk.

Gene	Reference sequence	Nucleotide	Codon	Previous cancer	Cancer in first-degree relatives	Immunohistochemistry	Age of onset	Stage at diagnosis	Survival from diagnosis (months)
ATM	NM_000051	c. 1931C>A	p.S644X	None	1 pancreatic (non-carrier), 1 prostate, and 1 lymphoma	.	66	2A	7, alive
ATM	NM_000051	c.3801delG	p.E1267fs	None	1 breast and 1 prostate	.	50	4	6, deceased
ATM	NM_000051	c.8874_8877del	p.2958_2959del	None	1 lung and 1 prostate	.	58	4	18, deceased
BRCA1	NM_007294	c.4117G>T	p.E1373X	None	1 lung	.	59	1B	2, alive
BRCA2	NM_000059	c.2806_2809del	p.936_937del	None	1 breast, 2 multiple myeloma	.	62	4	6, deceased
BRCA2	NM_000059	c.5066_5067insA	p.A1689fs	None	1 colorectal and 1 kidney	.	67	2B	82, alive
MLH1	NM_000249	c.677+3A>G	.	CRC age 62	2 breast, 1 lymphoma	N/A	66	2B	10, deceased
MSH2	NM_000251	c.942+3A>T	.	None	2 colorectal, 1 breast, 1 ureter, 1 bladder, 1 kidney	Deficient	68	2B	13, alive
MSH2	NM_000251	c.1906g>c	p.A636P	CRC at age 24 and 43	1 breast, 1 colorectal	Deficient	53	3	19, deceased
MSH6	NM_000179	c.1707delC	p.F569fs	Gallbladder, CRC and lung at age 69	1 breast, 1 colorectal, 1 melanoma, 1 endometrial, 1 prostate	N/A	71	2B	7, deceased
TP53	NM_000546	c.916C>T	p.R306X	None	None	.	65	4	13, deceased