Exon	Forward primer	Reverse primer
1	CTCCGTTAGTTTCCCGAGAC	TGTGCGGTGGCTGGACTCAA
3	CCATAGAATTTGCGACTACAC	CTCCCAAAGTACTGGGATTAC
4	TTGTGAGCTGCTTGGATCATC	GCTATGTCCTATTTTCCCAAC
12	AGTACTAGGCAGATCTTAATC	AACTTTTTGATCTCTGACTTG
15	TTCTCTCCACACAGGACACTG	GTGAGCGAAGCACCAGAAATG
17	ACCTGTCCCAGCAGAAACTG	

Table III. *FANCA* primers

Sequences for the forward and reverse primers for specific exons are shown. The remaining primers were previously documented [29].

MATERIALS AND METHODS

Subjects

The GenBank reference sequence and version number NM_000135.1 was used in this study**.** The DNA variant numbering is based on the cDNA sequence. Position +1 corresponds to the A of the ATG translation initiation codon. Protein sequences are numbered with the initiator methionine as codon 1.

Lymphocyte DNA was analyzed from 44 patients with familial pancreatic cancer enrolled in the National Familial Pancreatic Tumor Registry (NFPTR) [15]. Upon identification of the c.2574C>G variant in exon 27, we examined this exon from 110 additional patients with familial pancreatic cancer. Patients with pancreatic cancer were selected if they had at least 2 or more first degree relatives with pancreatic cancer. The

familial pancreatic cancer samples were from patients with a mean age of 66.7 +/- 12.3 years, males 50.3%.

To determine the carrier frequency of the c.2574C>G variant, we analyzed three control populations: healthy spouses of patients with familial pancreatic cancer (115 samples from spouses with a mean age of 66.9 +/- 11.3 years, males 43.1%), patients who had undergone cholecystectomy (65 samples matched in age with sporadic cases) for non-malignant disease at Johns Hopkins Hospital, and individuals undergoing routine screening colonoscopy (668 samples) at the Mayo Clinic. The colonoscopy controls included patients with a similar age to individuals with pancreatic cancer (mean age of 59.3 +/- 12.3 years, males 52.9%).

Lymphocyte DNA from spousal controls and colonoscopy controls, and DNA obtained from formalin-fixed paraffin-embedded gallbladder specimens of our hospital controls was isolated and analyzed. None of the controls had a prior history of pancreatic cancer or colorectal cancer. We also determined if the c.2574C>G variant contributed to apparently sporadic pancreatic cancer by analyzing the germline DNA from 124 individuals with apparently sporadic pancreatic cancer (patients with pancreatic cancer but without a known family history of pancreatic cancer). The mean age for the sporadic cases is 66.23 +/- 10.3 years, males 48%. This study was approved by the Johns Hopkins Joint Committee for Clinical Investigation and the Mayo Clinic IRB

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PCR Analysis

PCR was performed using Amplitaq Gold as per the manufacturer's protocol (Applied Biosystems). Primer sequences are shown in Table III. Several of the primers

used have been reported elsewhere [29]. For heteroduplex analysis, PCR products were diluted 1:5 in 1X Amplitaq PCR buffer (Applied Biosystems) and 2.5mM $MgCl_2$. Diluted samples were then loaded onto 96-well PCR plates (PGC Scientifics) and overlaid with mineral oil. To form heteroduplexes, samples were thermocycled using the following program: 95ºC for 3 min, 95ºC-80ºC for 3ºC/min, 80ºC-50ºC for 1ºC/min, 50ºC for 20min, 50ºC-45ºC for 1ºC/min, 45ºC-25ºC for 2ºC/min, 4ºC hold. Heteroduplexed samples were subjected to temperature gradient capillary electrophoresis (TGCE) using a SCE 9610 capillary sequencer adapted to perform TGCE (SpectruMedix Corporation, State College, PA). Up to 5 injections with varying temperatures were performed on each plate. Variants were identified with the Revelation version 2.4 mutational discovery software (SpectruMedix Corporation, State College, PA). For cycle sequencing, PCR products were purified using the Qiaquick PCR clean-up kit and sequenced with an ABI 3700 automated capillary sequencer or an ABI 377 automated sequencer. Sequences were analyzed with Sequencher (Gene Codes Corp.). Sequence variants were confirmed by sequencing independent PCR products in both directions. Blood samples from 668 colonoscopy controls from the Mayo Clinic were evaluated for the presence of the c.2574C>G variant by dHPLC analysis (Transgenomics Inc., Carpentaria, CA) of PCR products generated using PCR primers and conditions described above. PCR products were heteroduplexed, loaded onto a Transgenomic WAVE dHPLC system, and analyzed using product specific melting temperatures [30]. When altered peaks were identified on the dHPLC analysis, the original genomic DNA was re-amplified by PCR and sequenced in both directions to confirm the presence of the variant.

REFERENCES

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