Mitochondrial DNA Sequence Variation and Risk of Pancreatic Cancer

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Abstract

Although the mitochondrial genome exhibits high mutation rates, common mitochondrial DNA (mtDNA) variation has not been consistently associated with pancreatic cancer. Here, we comprehensively examined mitochondrial genomic variation by sequencing the mtDNA of participants (cases = 286, controls = 283) in a San Francisco Bay Area pancreatic cancer case-control study. Five common variants were associated with pancreatic cancer at nominal statistical significance (P < 0.05) with the strongest finding for mt5460g in the ND2 gene [OR = 3.9; 95% confidence interval (CI), 1.5–10; P = 0.004] which encodes an A331T substitution. Haplogroup K was nominally associated with reduced pancreatic cancer risk (OR = 0.32; 95% CI, 0.13–0.76; P = 0.01) when compared with the most common haplogroup, H. A total of 19 haplogroup-specific rare variants yielded nominal statistically significant associations (P < 0.05) with pancreatic cancer risk, with the majority observed in genes involved in oxidative phosphorylation. Weighted-sum statistics were used to identify an aggregate effect of variants in the 22 mitochondrial tRNAs on pancreatic cancer risk (P = 0.02). While the burden of singleton variants in the HV2 and 12S RNA regions was three times higher among European haplogroup N cases than controls, the prevalence of singleton variants in ND4 and ND5 was two to three times higher among African haplogroup L cases than in controls. Together, the results of this study provide evidence that aggregated common and rare variants and the accumulation of singleton variants are important contributors to pancreatic cancer risk. Cancer Res; 72(3); 1–10. ©2011 AACR.

Ref. 3. Mitochondria produce most the cellular energy, generate reactive oxygen species (ROS), and regulate apoptosis. The primary site of ROS and free radical production during oxidative respiration is the inner mitochondrial membrane (4–6), where the mitochondrial DNA (mtDNA) resides. If increased mitochondrial ROS production increases cancer risk, then mtDNA mutations that partially inhibit electron transport and increase ROS production might also increase cancer risk. Although a complete elucidation of the "Warburg effect" has not been achieved, several mechanisms have been proposed to explain this phenomenon (7). In turn, cancer cells have been shown to exhibit multiple alterations in mitochondrial content, structure, function, and activity (8–10).

The mtDNA is a circular double-stranded DNA molecule of 16,569 bp in humans that encodes 13 essential polypeptides of the oxidative phosphorylation (OXPHOS) system and the necessary RNA machinery for their translation within the mitochondria. MtDNA does not recombine, is maternally inherited (11), and has a unique organization in that its structural genes lack introns, intergenic spaces, and 5′ and 3′ noncoding sequences. Each human cell contains hundreds of mitochondria and thousands of copies of mtDNA with the number of copies being dependent upon the cell type. Somatic mtDNA mutations have been identified in many human tumors and are common in pancreatic cancers (12–15). Jones and colleagues (12) sequenced the complete mtDNA in 15 pancreatic cancer cell lines and xenografts and identified somatic mtDNA mutations and novel variants in nearly all
samples. Kassauei and colleagues (14) sequenced the mtDNA in 15 primary pancreatic cancers and nonneoplastic tissue and all pancreatic cancers showed at least one somatic mutation with the number of mutations per case ranging from 1 to 14. Somatic mtDNA mutations present in primary tumors were also detected in pancreatic juice from the same patients (15).

The evolution of human mtDNA is characterized by the emergence of distinct lineages associated with the major global ethnic groups. Mitochondrial haplogroups, and in some cases specific nonsynonymous (NS) single-nucleotide polymorphism (SNP), have been correlated with cancer development (16–24), suggesting that individuals who inherit certain variants might be more prone to cancer. Results to date do not support a significant involvement of common inherited mtDNA variation as a risk factor for pancreatic cancer (25, 26). However, these previous studies did not comprehensively examine sequence level mtDNA variation including rare variants and singletons (variants unique to a single participant). Because human mtDNA has a mutation rate that is 10 to 20 times higher than that of nuclear DNA (27–29) and approximately one third of sequence variants found in the general population may be functionally important (30), it is likely that most of the mtDNA variation that impacts function is rare in frequency and only detectable by direct sequencing. Population-based mtDNA resequencing (31) has identified more than 140 mtDNA polymorphisms with more than 1% allele frequency and a total of 64 tagging SNPs that efficiently capture all common variation in the coding regions exclusively. In the present study, we sequenced the entire mtDNA genome (>16.5 kb) using the Affymetrix Mitochondrial Resequencing Array 2.0 (MitoChip) to assess the role of common and rare mtDNA sequence variation in pancreatic cancer in nearly 600 case and control participants from a large population-based study of pancreatic cancer in the San Francisco Bay Area.

Materials and Methods

Study participants

A population-based case–control study of 532 pancreatic cancer cases and 1,701 controls was conducted between 1994 and 2001 in the San Francisco Bay Area in California (32). Participants who were on blood thinning medications, had a bleeding disorder, had a portacath in place, or had other contraindications to blood draw were not eligible to participate in the optional laboratory portion of the study. Cases with primary adenocarcinoma of the exocrine pancreas were identified using cancer registry rapid case ascertainment. Eligible cases were newly diagnosed from 1995 to 1999, 21 to 85 years old, and a San Francisco Bay Area resident. Patient diagnoses were confirmed by participants’ physicians and by the Surveillance, Epidemiology, and End Results abstracts that included histologic confirmation of disease. Of 794 eligible cases who were alive at first contact and able to complete an interview in English, 532 (67%) participated, 19% were too ill, 9% refused, and 6% had moved or had physician-indicated contraindications to contact. Of the 532 case participants, 452 (85%) were eligible for venipuncture, 12% had moved out of the area, and 3% had a portacath or were taking blood thinning medications.

Of the 452 case participants who were eligible for venipuncture, 309 (68%) provided a blood sample, 16% refused (needle phobia or physician-indicated contraindications), 7% were too ill, 3% had died, and 6% were not asked or blood draws were unsuccessful. The demographic characteristics of the 309 study cases who provided blood samples for analyses were similar to the 223 who did not (P = 0.30 for age at diagnosis, sex, white race, and Hispanic ethnicity). Of the 309 cases who provided a blood sample, 297 had DNA available for the mtDNA analysis and 12 did not have DNA available.

Among 1,588 eligible controls identified by random digit dial, 1,066 (67%) participated in the study, 29% refused, and 4% were too ill. Among 1,211 eligible Medicare controls identified from random sampling of the Health Care Financing Administration lists and recruited by mail, 635 (52%) participated in the study, 31% refused, 12% could not be located/had moved, and 5% were too ill. Of the 1,701 control participants, 1,634 (96%) were eligible for venipuncture (lived in the area). Of the 1,634 controls eligible for venipuncture, 964 gave blood (59%), 10% were not asked for a blood sample as the laboratory portion of the study had closed to recruitment, 11% refused for no specific reason, 15% refused for reasons of needle phobia, confidentiality, bad veins or other reasons, 4% were too ill or blood draws were unsuccessful, and 1% were lost to follow-up. Control participants who provided a blood sample were similar by age and Hispanic ethnicity (all P ≥ 0.81) but were more likely to be white (P = 0.002) and male (P = 0.002) when compared with those who did not provide blood. We had previously selected 301 random controls with sufficient DNA for inclusion in a genome-wide association study, and to create a comprehensive data set, we selected these same 301 controls for mtDNA analysis. A total of 286 pancreatic cancer cases and 283 controls yielded sequence data of sufficient quality for analysis (>95% success rate).

Interviews

Detailed in-person interviews were conducted in the homes of the participants or at a location of their choice. The University of California San Francisco Committee on Human Research approved the study protocols and procedures. Written informed consent was obtained from each study participant before interview. Race/ethnicity was based on self-report and was broadly defined as Caucasian, black/African-American, Asian, Hispanic (black or white), or “other race/ethnicity.”

Mitochondrial DNA sequencing

The entire mitochondrial genome was first amplified in 2 long-range PCR reactions. Mitochondrial fragments were amplified and prepared for array hybridization according to the Affymetrix protocol for GeneChip CustomSeq Resequencing Array. Briefly, long-range PCR reactions were carried out using LA PCR Kit (Takara Bio U.S.A) for each sample using 2 sets of overlapping primers. Five microliters of 5 ng/μL template genomic DNA were mixed with 0.1 μL HS LA Taq polymerase (5 U/μL), 2.5 μL 10× LA Taq buffer, 4 μL 2.5 mmol/L dNTPs, 5 μL of 3 μmol/L primer pair, and 8.4 μL dh20 for a total reaction volume of 25 μL. The PCR program was as follows: (i) 1 cycle, 94°C for 2 minutes; (ii) 30 cycles, 94°C
for 15 seconds and 68°C for 16 minutes; (iii) 1 cycle, 94°C for 21 minutes; and (iv) hold, 4°C. The resulting PCR products were assessed qualitatively by 1% agarose gel electrophoresis. The PCR product was purified using a Clontech Clean-Up plate. The purified DNA was quantified by PicoGreen and for selected samples, confirmed by NanoDrop measurements. The ampli-
cons were pooled at equimolar concentrations. Chemical
fragmentation was conducted and products confirmed to be
in the size range of 20 to 200 bp by 20% PAGE with SYBR Gold
staining. The IQ-EX control template, a 7.5-kb plasmid DNA,
was used as positive control. The samples were labeled with
TdT and hybridized to the array in a 49°C rotating hybridiza-
tion oven at 16 hours. Finally, streptavidin phycoerythrin
(SAPE) and then antibody staining were conducted. The
microarrays were processed in the GeneChip Fluidic Station
and the GeneChip Scanner. Signal intensity data were output
for all 4 alleles ("a," "c," "g," and "t"), permitting quantitative
estimates of allelic contribution. The allelic contribution was
assessed using the raw data from the individual signal inten-
sities by deriving the ratio of expected allele (REA), which is the
log ratio of the raw signal intensity of the expected allele at any
site (as defined by the mtDNA reference sequence) to the
average raw signal intensity of the other 3 alleles, at each site
for every individual.

The MitoChip uses standard Affymetrix GeneChip Sequence
Analysis Software (GSEQ). The output of the GeneChip DNA
Analysis (GDAS) included a report of the individual and total
number of SNPs as well as a case-by-case list of genotype
variations as determined by comparison to the mtDNA refer-
dence sequence. Most of the 13,452 mtDNA loci tiled on the array
were duplicated via independent probe sets allowing a test of
within-chip error rates of 0.0025% and 0.01% were obtained (detailed in Results).

The MitoChip reported within-chip error rates of 0.0025% and
was duplicated via independent probe sets allowing a test of
the proportions of African- and Asian-American participants in the study.

Statistical analysis

To examine mtDNA sequence variation for associations with
pancreatic cancer, we analyzed variants from the following
categories: Common haplogroups and individual variants
[minor allele frequency (MAF), ≥ 5%]; rare variants (MAF,
<5%); and singletons (occurring in a single participant—in the
case of haplogroup-specific analyses, singletons are variants
occurring in a single participant within the haplogroup).

variations as determined by comparison to the mtDNA refer-
ence sequence) to the

Unconditional logistic regression was used to obtain ORs as
estimates of relative risks (hereafter called risk) and 95%
confidence intervals (CI) for analyses involving haplogroups
and common variants. Allele frequencies and rare variants
(excluding singletons) were compared between cases and
controls using χ² tests. The major European mitochondrial
haplogroups were defined using variants identified from Phy-
loTree (35) and included subgroups H, V, J, T, U, K (B, F), and
(A, I, W, X, Y). To account for confounding by ancestry, we
defined eigenvectors using principal components analysis
(PCA) using the complete mtDNA genotype data (36). This
method has been shown to outperform haplogroup-stratified
or adjusted association analyses with no loss in power for
detection of true associations (36). All models were adjusted for
age in 5-year groups, sex, and the first 6 eigenvectors of
mitochondrial genetic ancestry derived from PCA. Models
examining individual common variants were restricted to
haplogroup N as the number of cases and controls are equi-
alent. We did not examine associations for haplogroups L and
M as the sample sizes are small and the numbers of cases and
controls reflect the proportions of African- and Asian-American
participants in the study.

Variants also were grouped and tested jointly to assess the
contribution of multiple variants to the pancreatic cancer risk
using weighted-sum statistics computed as described in the
work of Madsen and Browning (37). The weighted-sum method
is designed for resequencing data where rare mutations are
observed directly and generally has higher power than alter-
native methods for these types of analyses (37). The weighted-
sum method is based on the common disease-rare variant
hypothesis wherein variants with lower frequencies in the
unaffected individuals were weighted more heavily; approxi-
mately 70% of all rare missense mutations are reported to be
deleterious (38). Thousand permutations of case–control status
were conducted to obtain one-sided P values, testing the
hypothesis that most rare mutations are deleterious and
associated with disease status. Variants from genes encoding
the 4 mtDNA-encoded OXPHOS complexes, rRNA, tRNA, and
hypervariable regions were assessed using the weighted-sum
method (37) among haplogroup N participants only. All
weighted-sums were computed using custom Perl scripts.
For singletons, the total number of gene- or region-specific var-
iants was compared between pancreatic cancer cases and
controls using Fisher exact test as was the number of individ-
uals harboring singleton variants unique to cases or controls.
Because of the potential for confounding by mtDNA ancestry,
all singleton analyses were conducted for each major hap-
logroup L, M, and N. Bonferroni correction for multiple testing
took into account the number of haplogroups (n = 8) and
genes/regions examined for singleton analysis (n = 17) and the
number of common and rare variants discovered and analyzed
detailed in Results).

In silico prediction methods were used to examine mtDNA
nucleotide conservation and the impact of nonsynonymous
coding substitutions on amino acid protein sequence. PhastCons (39) is a hidden Markov model–based method that estimates the probability that each nucleotide belongs to an evolutionary conserved element. On the basis of a multispecies sequence alignment, the method considers the conservation of sites flanking the base of interest when producing base-by-base conservation scores. The PhastCons scores range from 0 to 1 and represent probabilities of negative selection. PhyloP (40) separately measures conservation at individual nucleotides, ignoring the effects of their neighbors. Also based on a multispecies sequence alignment, this method is more appropriate for evaluating signatures of selection at particular nucleotides. PhyloP scores represent \(-\log P\) values under a null hypothesis of neutral evolution. Sites predicted to be conserved are assigned positive scores and sites predicted to be fast-evolving are assigned negative scores. For PhastCons and PhyloP, a higher value indicates a more conserved position. The effects of nonsynonymous coding substitutions (amino acid changes) on protein function were assessed using PolyPhen2 (41). PolyPhen2 is a tool that predicts the possible impact of an amino acid substitution on the structure and function of a human protein. Predictions are based on information that includes 8 sequence-based and 3 structure-based features characterizing the substitution. As a final output, PolyPhen2 calculates a posterior probability that a mutation is damaging and qualitatively reports it as benign, possibly damaging, or probably damaging.

**Results**

Sequencing of 16,543 mtDNA bases (positions 12–16,555) from 286 pancreatic cancer cases and 283 controls participants yielded a cumulative total of 2,169 variants including: 66 common variants (MAF, ≥5%); 251 low frequency variants (MAF, 1%–5%); and 1,859 rare variants (MAF, <1%) including 1,393 haplogroup-specific (L, M, or N) singletons unique to either cases or controls. Distributions of age, education level, race, ethnicity, sex, smoking status, and major haplogroups for cases and controls are presented in Table 1. The case and control participants were similar by white/non-white race (\(\chi^2, P = 0.74\)) and Hispanic ethnicity (\(\chi^2, P = 0.58\)). In general, cases were somewhat less educated, were more likely to be current smokers, and a greater proportion were African-American. Haplogroup distributions largely overlapped with self-identified race for African-Americans (97%) and European-Americans (96%). Self-identified Asian-Americans were distributed between haplogroups M (55%) and N (43%). While major haplogroup M is largely unique to Asia, the minor Asian haplogroups are descended from both major haplogroups M (e.g., haplogroups C, D, and G) and N (haplogroups A, B, and F). Participants who self-identified as Hispanic were distributed between haplogroups N (75%) and M (19%). The results for the Asian and Hispanic participants are not unexpected as the mtDNA traces the maternal lineage exclusively and may not reflect an admixed genetic or self-identified ancestry.

### Table 1. Characteristics of pancreatic cancer cases and controls in a population-based study in the San Francisco Bay Area, California (1995–1999)

<table>
<thead>
<tr>
<th></th>
<th>Cases ((n = 286))</th>
<th>Controls ((n = 283))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, y</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>65 (11)</td>
<td>64 (12)</td>
</tr>
<tr>
<td><strong>Education, y</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>1–12</td>
<td>120 (42)</td>
<td>91 (32)</td>
</tr>
<tr>
<td>&gt;12–16</td>
<td>111 (39)</td>
<td>120 (42)</td>
</tr>
<tr>
<td>&gt;16</td>
<td>55 (19)</td>
<td>72 (25)</td>
</tr>
<tr>
<td><strong>Self-reported race/ethnicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White, non-Hispanic</td>
<td>226 (79)</td>
<td>223 (79)</td>
</tr>
<tr>
<td>White, Hispanic</td>
<td>16 (6)</td>
<td>20 (7)</td>
</tr>
<tr>
<td>Black</td>
<td>22 (8)</td>
<td>11 (4)</td>
</tr>
<tr>
<td>Asian</td>
<td>17 (6)</td>
<td>22 (8)</td>
</tr>
<tr>
<td>Other</td>
<td>5 (2)</td>
<td>7 (2)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>153 (54)</td>
<td>152 (54)</td>
</tr>
<tr>
<td>Women</td>
<td>133 (46)</td>
<td>131 (46)</td>
</tr>
<tr>
<td><strong>Cigarette smoking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoked</td>
<td>82 (29)</td>
<td>106 (39)</td>
</tr>
<tr>
<td>Former smoker</td>
<td>131 (48)</td>
<td>129 (48)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>64 (23)</td>
<td>35 (13)</td>
</tr>
<tr>
<td><strong>Haplogroup N</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>246 (87)</td>
<td>248 (88)</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Haplogroup L</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>27 (10)</td>
<td>14 (5)</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Haplogroup M</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>11 (4)</td>
<td>21 (7)</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Common haplogroups and individual variants**

Risk of pancreatic cancer among 8 European sub-haplogroups is reported in Table 2. No haplogroup met statistical significance after adjustment for multiple comparisons (8 haplogroups, critical \(\alpha = 0.006\). Carriers of haplogroup K had a nominally reduced pancreatic cancer risk compared with the most common European haplogroup H (OR = 0.32; 95% CI, 0.13–0.76; \(P = 0.01\)). There also were no individual common variants that met statistical significance after multiple comparisons adjustment [66 common variants detected by sequencing (MAF, ≥5%), critical \(\alpha = 0.0008\)]. Of the 66 common variants, 5 reached nominal statistical significance \((P < 0.05) and 2 yielded a strong (statistically nonsignificant) association with pancreatic cancer risk: mt5460g \((P = 0.004)\) and mt1811g \((P = 0.008)\). The mt5460g variant is associated with an increased risk of pancreatic cancer \((OR = 3.9; 95\% CI, 1.5–10; \(P = 0.004)\) encodes an A331T substitution in the ND2 gene. All analyses were adjusted for age, sex, and 6 eigenvectors of mitochondrial genetic ancestry derived from PCA. Restricting the haplogroup and common variant analyses to self-identified white/non-Hispanic did not alter the results (data not shown).

**Rare variants**

Of 710 low frequency (MAF, 1%–5%) and rare variants (MAF, <1%) detected by sequencing (excluding singletons), none
met statistical significance after adjustment for multiple comparisons (critical $\alpha = 0.0001$). A total of 19 haplogroup-specific variants yielded nominal statistically significant associations ($\alpha < 0.05$) with pancreatic cancer risk (Table 3). All were from either haplogroup N or L. Of these, 13 were detected from complex I, III, IV, and V coding regions, 2 from the 12S RNA, and 4 from the hypervariable (HV) or noncoding regions. Two of the coding region variants resulted in nonsynonymous substitutions: L555Q (mt14000) in ND5 and K6N (mt14763) in CytB. The L555Q substitution in ND3 was predicted to be probably damaging when assessed using PolyPhen2. The mt14763 variant underlying the K6N substitution shows strong evidence of belonging to a conserved sequence element (PhastCons = 1.00, PhyloP = 4.47).

Weighted-sum statistics

Multiple variants across the combined 22 mtDNA tRNA regions were statistically significantly associated with pancreatic cancer as determined by permutation of case–control status (one-sided $P = 0.02$). Our results also suggest that there was an excess of variants in complex III (CytB) among pancreatic cancer cases, although the effect was not statistically significant (one-sided $P = 0.06$).

Singletons

A total of 1,393 singleton mtDNA variants unique to cases or controls were identified across the coding, tRNA, rRNA, and hypervariable regions for the 3 major haplogroups L, M, and N. Two of these genes/regions harbored a significantly higher burden of singleton variants in cases than in controls after adjustment for multiple comparisons (18 mtDNA genes/regions, critical $\alpha = 0.003$). Specifically, the number of singleton variants among haplogroup N cases versus controls was statistically significantly higher in the HV2 region ($P = 0.006$) and nominally higher in the 12S RNA ($P = 0.03$) region. The frequency of HV2 and 12S RNA singletons was 3 times higher in cases than in controls: HV2 region, 9% cases and 3% controls; 12S RNA 7% cases and 2% controls. The frequency of ND4 and ND5 singletons was 2 to 3 times higher in cases than in controls: ND4, 41% cases versus 14% controls; ND5, 74% cases versus 36% controls (Fig. 2). In addition, singleton variants were observed among haplogroup L cases only for the COII and COIII genes. Among haplogroup M participants, cases had statistically significant fewer singleton variants in the 12S RNA ($P = 0.03$) region than in controls (Fig. 3).

Of the 1,393 singleton variants identified in the 3 major haplogroups, 625 were located within the 4 mtDNA-encoded OXPHOS complexes with 221 resulting in nonsynonymous coding substitutions (Supplementary Tables S1–S5). An excess of ND2 NS substitutions was observed among cases compared with controls ($P = 0.02$). Among the complex V genes, ATP6 and ATP8, approximately 25% of nonsynonymous substitutions in controls were predicted to be

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**Table 2. ORs and 95% CIs for pancreatic cancer associated with haplogroup N subgroups and common mtDNA variants among haplogroup N participants, San Francisco Bay Area, California (1995–1999)**

<table>
<thead>
<tr>
<th>Haplogroup N</th>
<th>Subgroups</th>
<th>Cases $(n = 246), n (%)$</th>
<th>Controls $(n = 248), n (%)$</th>
<th>OR (95% CI)$^a$</th>
<th>$P^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>ND2 mt5460g</td>
<td>20 (8)</td>
<td>6 (2)</td>
<td>3.9 (1.5–10)</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>COII mt9698c</td>
<td>10 (4)</td>
<td>24 (9)</td>
<td>0.44 (0.20–0.97)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>mt1811g</td>
<td>20 (7)</td>
<td>40 (14)</td>
<td>0.51 (0.29–0.91)</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>tRNA mt12307g</td>
<td>16 (7)</td>
<td>34 (14)</td>
<td>0.45 (0.24–0.85)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>HV2 mt150t</td>
<td>13 (5)</td>
<td>26 (11)</td>
<td>0.70 (0.49–0.99)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

$^a$All analyses were adjusted for age, sex, and 6 eigenvectors of mitochondrial genetic ancestry derived from PCA.

$^b$Nominally significant results for 5 common variants of 66 total common variants detected.

$^c$Multiple comparisons adjusted $P$ value ($\alpha = 0.006$) for 8 haplogroups ($\alpha = 0.0006$) and 66 common variants ($\alpha = 0.0008$).
nonconserved whereas all nonsynonymous substitutions in cases were conserved ($P = 0.02$).

**Discussion**

In this study, we sequenced the entire mtDNA in a large population-based case–control study of pancreatic cancer to examine the role of haplogroups and common genetic variants, rare sequence variants, and singletons. We observed inverse associations with pancreatic cancer risk for participants from European haplogroup K when compared with the most common European haplogroup H. The haplogroup K association was not seen in a previous study of pancreatic cancer that included replication (26). The low frequency of haplogroup K participants in this study and lack of consistency with the earlier study likely mean that our finding is a false-positive.

**Table 3.** Rare, haplogroup-specific mtDNA variants associated with pancreatic cancer, San Francisco Bay Area, California (1995–1999)

<table>
<thead>
<tr>
<th>Region</th>
<th>Gene</th>
<th>Site</th>
<th>Cases</th>
<th>Controls</th>
<th>Haplogroup</th>
<th>Common allele</th>
<th>Rare allele</th>
<th>Amino acid position</th>
<th>PhastCons</th>
<th>PhyloP</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>ND4L</td>
<td>10,586</td>
<td>7</td>
<td>1</td>
<td>L</td>
<td>tct</td>
<td>tca</td>
<td>S39S</td>
<td>0.00</td>
<td>−7.02</td>
<td>0.046</td>
</tr>
<tr>
<td>ND5</td>
<td>12,810</td>
<td>6</td>
<td>0</td>
<td>L</td>
<td>tct</td>
<td>tgg</td>
<td>W158W</td>
<td>0.00</td>
<td>−0.58</td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td>ND5</td>
<td>12,954</td>
<td>4</td>
<td>0</td>
<td>N</td>
<td>gct</td>
<td>gcc</td>
<td>A206A</td>
<td>0.00</td>
<td>−2.44</td>
<td>0.040</td>
<td></td>
</tr>
<tr>
<td>ND5</td>
<td>13,485</td>
<td>6</td>
<td>0</td>
<td>L</td>
<td>ata</td>
<td>atg</td>
<td>M383M</td>
<td>0.00</td>
<td>−4.18</td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td>ND5</td>
<td>14,000</td>
<td>6</td>
<td>0</td>
<td>L</td>
<td>cta</td>
<td>cag</td>
<td>L555Q$^a$</td>
<td>0.00</td>
<td>−0.50</td>
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**NOTE:** Nonsynonymous substitutions are indicated in bold.

$^a$Nonsynonymous variant predicted to have a damaging effect on the resulting protein using PolyPhen2 (41).
result. Common haplogroup N variants (MAF, >5%) in complex I (ND2), complex IV (COIII), 16S, tRNA, and HV2 genes/regions also yielded nominally significant associations with pancreatic cancer risk. This includes an A331T substitution in the ND2 gene that was present in 20 cases (8%) and 6 controls (2%).

Research to identify genetic factors that contribute to complex phenotypes such as the development of cancer must be sensitive to the ways that genes and genetic perturbations operate. For example, it is now widely recognized that common genetic variants play a much smaller role in mediating phenotypic expression and disease risk than initially thought (42–45) and that identification of causative variants requires comprehensive resequencing of genomic loci in multiple subjects (46). In this study, we identified numerous low frequency (MAF, 1%–5%) and rare variants (MAF, <1%) from haplogroups N and L that were associated with pancreatic cancer risk. Nearly 70% of these variants were observed in the OXPHOS complexes including 2 nonsynonymous variants from the ND5 and CytB genes. The ND5 substitution was predicted to have a damaging effect on the resulting protein, whereas the CytB substitution showed evidence of belonging to a conserved sequence element. However, focusing on nonsynonymous variants may not be overly informative as nonsynonymous SNPs and synonymous SNPs (SNPs) share similar likelihood and effect size for disease association and synonymous SNPs are just as likely to be involved in disease mechanisms (47). The remaining variants occurred in the 12S RNA, hypervariable, and noncoding regions. Several of the coding and noncoding variants were predicted to belong to evolutionary conserved regions and may play important roles in mtDNA copy number (48) and genome transcription (49, 50) possibly causing severe alterations in mitochondrial function (17, 50–53). In the present study, mt296 was observed in 38% of haplogroup L cases and no controls, possibly reflecting a risk factor related to mtDNA copy number. Previous
studies have described a variant located at mt295 that defines Caucasian haplogroup J and that has been found to change mitochondrial copy number (48), which may partially account for observations that haplogroup J is overrepresented in long-lived people and centenarians from several populations (54–56).

Because collections of rare variants within genes or genomic regions are likely to influence phenotypic expression in important ways (45), examining the collective frequency of rare or singleton variants may reveal the role of specific genes in disease etiology. Our results provide evidence for a significant aggregate effect of sequence variants in the 22 mitochondrial tRNAs for pancreatic cancer risk and suggest that complex III (CytB) gene variants may also play a role. The burden of singleton variants among European haplogroup N cases was 3 times higher than in controls for the HV2 and 12S RNA regions, suggesting that these mtDNA regions may contribute to risk among persons of European descent. Furthermore, the burden of singleton variants among the African haplogroup L cases was higher than in controls for the entire mtDNA, in particular for OXPHOS complexes I and IV. More specifically, among the complex I genes ND4 and ND5, rare variants were 2 to 3 times more frequent in cases than in controls, whereas in the complex IV, COIII gene singleton variants occurred in 41% of cases and in no controls. Interestingly, these results are consistent with a study of prostate cancer where germ line mtDNA COI(complex IV) missense mutations were reported in 11% of prostate cancer cases compared with 2% of the no-cancer controls (17). This may be of particular importance as incidence and mortality of pancreatic cancer are 48% and 37% higher, respectively, in African-Americans relative to European-Americans (57), and cannot be attributed to racial differences in currently known risk factors (38).

The results of this study suggest that aggregated common and rare variants and the accumulation of singleton variants are important contributors to pancreatic cancer risk. This study had a number of strengths, including complete mtDNA sequencing allowing for an unbiased assessment of mitochondrial genomic variation; a well-characterized case–control study of pancreatic cancer; and an analytic approach that includes both aggregated and accumulated sequence variants. A few weaknesses are also acknowledged, including small sample sizes for the African and Asian ancestry samples; low power to detect effect of individual variants; possible survival and selection bias, and no validation study. Demographic characteristics of the study cases who provided blood samples for analyses were similar to those who did not whereas control participants who provided a blood sample were more likely to be white men. While the 13 mtDNA-encoded OXPHOS genes are essential to mitochondrial energy production and are considered the most functionally important (59), hundreds of nuclear DNA-encoded and dozens of mtDNA-encoded bioenergetics genes are distributed throughout both genomes (30, 60). Future studies of mitochondrial genetic variation will therefore need to account for a complex set of interactions involving the nuclear and mitochondrial genomes (61).

Disclosure of Potential Conflict of Interest

No potential conflicts of interests were disclosed.

Grant Support

The support was provided, in part, by grants CA133080 (G.J. Tranah, principal investigator); CA97076, CA108370, CA109767, and CA89726 (E.A. Holly, principal investigator) from the National Cancer Institute, NIH; by the Rombauer Pancreatic Cancer Research Fund and by the Hasbun Fund. E.T. Lam was supported in part by NIH training grant T32 GM007175. The collection of cancer incidence data for the UCSF study was supported by the California Department of Public Health as part of the statewide cancer reporting program; the National Cancer Institute’s Surveillance, Epidemiology and End Results Program under contract N01-PC-35136 to the Northern California Cancer Center; and the Centers for Disease Control and Prevention’s National Program of Cancer Registries.

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Received May 20, 2011; revised November 30, 2011; accepted December 3, 2011; published OnlineFirst December 15, 2011.

References

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Cancer Res Published OnlineFirst December 15, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-1682

Supplementary Material
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