

Mitochondrial DNA G10398A Polymorphism and Invasive Breast Cancer in African-American Women

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Abstract

Mitochondria generate oxygen-derived free radicals that damage mitochondrial DNA (mtDNA) as well as nuclear DNA and in turn promote carcinogenesis. The mtDNA G10398A polymorphism alters the structure of Complex I in the mitochondrial electron transport chain, an important site of free radical production. This polymorphism is associated with several neurodegenerative disorders. We hypothesized that the 10398A allele is also associated with breast cancer susceptibility. African mitochondria harbor the 10398A allele less frequently than Caucasian mitochondria, which predominantly carry this allele. Mitochondrial genotypes at this locus were therefore determined in two separate populations of African-American women with invasive breast cancer and in controls. A preliminary study at Vanderbilt University (48 cases, 54 controls) uncovered an association between the 10398A allele and invasive breast cancer in African-American women, [odds ratio (OR), 2.90; 95% confidence interval (95% CI), 0.61-18.3; $P = 0.11$]. We subsequently validated this finding in a large, population-based, case-control study of breast cancer, the Carolina Breast Cancer Study at the University of North Carolina (654 cases, 605 controls). African-American women in this study with the 10398A allele had a significantly increased risk of invasive breast cancer (OR, 1.60; 95% CI, 1.10-2.31; $P = 0.013$). The 10398A allele remained an independent risk factor after adjustment for other well-accepted breast cancer risk factors. No association was detectable in white women (879 cases, 760 controls; OR, 1.03; 95% CI, 0.81-1.31; $P = 0.81$). This study provides novel epidemiologic evidence that the mtDNA 10398A allele influences breast cancer susceptibility in African-American women. mtDNA polymorphisms may be underappreciated factors in breast carcinogenesis. (Cancer Res 2005; 65(17): 8028-33)

Introduction

Mitochondria play a central role in cellular energy production. About 90% of the cellular ATP is produced by the electron transport chain embedded in the inner mitochondrial membrane (1-3). This chain of multisubunit protein complexes consists of gene products from both the mitochondrial and nuclear genomes. Mitochondrial DNA (mtDNA) encodes 13 subunits of the electron transport chain, as well as a distinct set of rRNAs and tRNAs (1, 4). Under physiologic conditions, as many as 2% of electrons leak from

the mitochondrial electron transport chain and reduce oxygen to superoxide anion, triggering the formation of a cascade of free radicals that indiscriminately damage biological macromolecules (3, 5-7). Mitochondria are therefore a major source of oxygen-derived free radicals, also collectively known as reactive oxygen species (ROS). Increased production of ROS and the resultant damage to both mtDNA and nuclear DNA have long been thought to play a key role in carcinogenesis (1, 8-12). Mitochondria are especially susceptible to damage by ROS because the mitochondrial genome lacks introns and has limited reparative capability (1). A continuing cycle of worsening mitochondrial dysfunction with increasing ROS production might be expected to result from such damage to mtDNA. Mitochondrial dysfunction is relevant to the genesis of many cancers and to the maintenance of the malignant phenotype (13, 14). Susceptibility to the effects of mitochondrial dysfunction may be particularly important in estrogen-inducible cancers, such as breast cancer, because the normal metabolism of estradiol through redox-cycling intermediates may also generate local ROS and oxidative injury in the breast that facilitates neoplastic transformation (15-18).

The mtDNA G10398A polymorphism results in a nonconservative amino acid substitution of threonine (encoded by the A allele) for alanine (encoded by the G allele) within the NADH dehydrogenase (ND3) subunit of Complex I. The clinical significance of this polymorphism has emerged from recent research in neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, Friedreich's ataxia, and amyotrophic lateral sclerosis, as well as from studies of longevity (19-26). These epidemiologic studies have suggested that the 10398A allele is associated with the degenerative phenotype, whereas the 10398G allele is usually protective. Although the precise biochemical effects of the mtDNA G10398A polymorphism are not yet known, several lines of evidence suggest that it results in increased ROS production (oxidative stress) due to altered Complex I function (19, 26). Free radical production at Complex I, a key component of the mitochondrial electron transport chain, is believed to be important in the pathophysiology of neurodegenerative disorders such as Parkinson's disease (19, 27-29). Mitochondrial cybrid studies have shown that increased ROS production, increased expression of antioxidant proteins, and changes in mitochondrial morphology occur in conjunction with Complex I impairment (30, 31). Environmental toxins that display carcinogenic effects in animal models have also been found to impair Complex I function (12, 32).

Approximately 80% of whites possess the 10398A allele, whereas the prevalence of this allele in individuals of African heritage is reported to be ~5% (33). A small or modest effect of the 10398A allele on breast cancer risk, if present, would therefore be more readily detectable in African-American women. A pilot study of mitochondrial G10398A genotypes in 48 African-American women

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with primary invasive breast cancer and in 54 cancer-free controls suggested that this polymorphism might modulate breast cancer risk. These results led us to perform a much larger study that was appropriately powered to validate our initial finding. The results of both studies are reported here. We hypothesized that the mtDNA G10398A polymorphism impairs the function of Complex I in the mitochondrial electron transport chain, resulting in increased oxidative stress and breast cancer susceptibility.

Patients and Methods

Vanderbilt pilot study. The pilot study was carried out in 59 African-American women with primary invasive breast cancer who were treated at Vanderbilt University Medical Center between 1982 and 1996 (cases) and in 59 age- and race-matched women without any cancer who were hospitalized at Vanderbilt during the same period (controls). Details of the ascertainment and analysis of these patients have been previously published (34–36). All patients gave informed consent for genetic testing, and the study was approved by the Vanderbilt Institutional Review Board.

Carolina Breast Cancer Study. The Carolina Breast Cancer Study (CBCS) provided the validation sample for our study. This population-based, case-control study, conducted in 24 counties of central and eastern North Carolina, recruited women with primary invasive or *in situ* breast cancer (37). Incident cases of breast cancer were identified using a Rapid Case Ascertainment System in cooperation with the North Carolina Central Cancer Registry (38). Controls were selected from Division of Motor Vehicles (women younger than 65 years of age) and U.S. Health Care Financing Administration lists (women 65 years of age or older). In-person interviews were conducted to obtain blood samples and information on potential breast cancer risk factors. Procedures for recruiting and enrolling study participants were approved by the Institutional Review Board of the University of North Carolina School of Medicine, and informed consent was obtained from each participant.

Cases of primary invasive breast cancer were enrolled in two phases (phase 1, 1993–1996; phase 2, 1996–2001) with over-sampling of African-American and younger women (39). Controls were frequency matched to cases based on age (± 5 years) and self-reported race using randomized recruitment (40). Less than 2% of participants reported their race as Native American or “other”; these women were classified as white in the dichotomous categorization of race. Phase 2 recruitment also included cases of *in situ* breast cancer and age- and race-matched controls, but the number of African-American participants in the *in situ* study (106 cases, 70 controls) was insufficient for analysis of mtDNA G10398A genotypes. Therefore, only the analysis of invasive breast cancer cases in the CBCS is reported here.

A total of 1,808 women with invasive breast cancer (788 African-Americans, 1,020 whites) and 1,564 controls (718 African-Americans, 846 whites) participated in the CBCS (39, 41). Overall recruitment rates were 76% for cases and 55% for controls. The proportion of women who consented to phlebotomy and DNA extraction was similar among cases and controls (88% and 91%, respectively). DNA samples were available for mtDNA G10398A genotyping in 1,594 cases (679 African-Americans and 915 whites) and 1,419 controls (626 African-Americans and 793 whites). The prevalence of well-accepted breast cancer risk factors did not differ significantly between individuals who provided DNA samples and those who did not (39, 41). The histology of all breast cancer cases was reviewed by two study pathologists at the University of North Carolina, and cancers were staged according to standard criteria of the American Joint Committee on Cancer (AJCC; ref. 42).

Genetic analyses. Genomic DNA extraction was done in the pilot study as previously described (34–36). In CBCS participants, DNA was extracted from peripheral blood lymphocytes by standard methods using an automated ABI-DNA extractor (Applied Biosystems Nuclei Acid Purification System) in the University of North Carolina Specialized Program of Research Excellence Tissue Procurement Facility. DNA samples were each assigned an anonymous identification number and transported on ice to

the Center for Human Genetics Research at Vanderbilt University Medical Center.

The mtDNA G10398A genotyping in both the pilot and validation studies was done by PCR using a fluorogenic 5' nuclease allelic discrimination TaqMan assay and primers that have been used in previous studies (18). Primer and probe sequences are as follows: 10398 forward primer: CTA CAA ACA ACT AAC CTG CCA CTA ATA G; 10398 reverse primer: GGG CAT TTG GTA AAT ATG ATT ATC A; TaqMan MGB probe for G allele: VIC-AGA CTG AGC CGA ATT; TaqMan MGB probe for A allele: 6FAM-TAG ACT GAA CCG AAT TG. Mitochondrial genotypes were analyzed using the ABI 7900 HT Sequence Detection System version 2.1 (Applied Biosystems, Foster City, CA) software. Forty-seven (1.5% of total samples) randomly selected, blinded duplicate samples were tested and had 100% concordance. Throughout this article, we refer to the mtDNA polymorphism as G10398A, reflecting the African-American perspective that the G allele is more common. Genotypes were classified as undetermined if PCR amplification failed with the specified set of probes and primers.

Statistical analysis. The mtDNA G10398A genotype frequencies were calculated as the proportion of cases or controls that carried A or G alleles. This polymorphism is generally homoplasmic (only one allele is present in a given individual). Genotype frequencies in cases and controls were compared using χ^2 tests. Tests for statistical significance were two sided with an α level of 0.05. The Vanderbilt University pilot study was analyzed using the STATA statistical software package (version 8.1; College Station, TX). In the analysis of the CBCS data, unconditional logistic regression was used to calculate odds ratios (ORs) for breast cancer and corresponding 95% confidence intervals (95% CI). The PROC GENMOD statement in SAS (version 8.2; SAS Institute, Cary, NC) was used to incorporate offsets derived from sampling probabilities developed to identify both eligible participants and age category (classified as an 11-level ordinal variable that reflected 5-year age categories; ref. 40). A crude (unadjusted) OR was calculated in the pilot study (in which cases and controls were frequency matched on age) due to limited power; the ORs reported for the CBCS study population were adjusted for age and sampling probabilities (offsets). β coefficients for mtDNA G10398A genotype did not differ after adjustment for the following factors: age at menarche, age at first full-term delivery, parity, family history of first-degree relative(s) with breast cancer, alcohol consumption, smoking, body mass index, oral contraceptive use, breastfeeding, high-dose chest irradiation, occupational radiation exposure, vitamin use, age at menopause, use of aspirin and other nonsteroidal anti-inflammatory medications, fruit consumption, vegetable consumption, and hormone replacement therapy.

The size of the confirmatory sample was determined based on the following estimations. We considered a 50% excess in the proportion of 10398A allele carriers in the invasive breast cancer case group compared with the control group to be reportable (half of the excess seen in the pilot study). Assuming a level of significance of 0.05 and a power of 0.80 to detect an effect of this magnitude, we determined that a sample of 974 (487 cases and the same number of controls) would meet our objectives (43). With the sample size of African-American women available from the CBCS, we had power of nearly 1.00 to detect the difference outlined above (43).

Results

Vanderbilt pilot study. The African-American women genotyped in this preliminary pilot study had no statistically significant differences detected between cases and controls with regard to age, menopausal status, and occurrence of breast cancer in first degree relatives. Among cases, stages I to III accounted for 44 of 48 (92%) of the invasive breast cancers. DNA was available in 52 of 59 cases and in 56 of 59 controls in this study, and mtDNA G10398A genotyping was successful in all samples except 4 cases and 2 controls that failed to amplify. The unadjusted risk of invasive breast cancer was higher in African-American women with the 10398A allele as compared with women who carried the 10398G allele (OR 2.90; 95% CI, 0.61–18.3; $P = 0.11$). Although the increase in

risk was not statistically significant in this small sample, we were encouraged to further explore this finding in a population-based study that met our power requirements.

Carolina Breast Cancer Study. The demographic and clinical characteristics of the CBCS population stratified by race are summarized in Table 1. A total of 1,682 African-American women were enrolled in the CBCS. As mentioned, those 176 women enrolled in the Carcinoma *In Situ* part of the CBCS were not analyzed in this study. Therefore, there are 1,506 African-American women (788 cases and 718 controls) enrolled in the part of the CBCS studying invasive breast cancer. Of these, DNA samples were obtained on 679 cases and 626 controls. Of this group with DNA samples ($n = 1,305$), 1,259 (97%) were successfully genotyped at the mtDNA G10398A locus, 654 cases and 605 controls.

Genotype frequencies and ORs for mtDNA G10398A and breast cancer are presented in Table 2. The mt10398A allele was associated with a significantly increased risk of invasive breast cancer in African American women (OR, 1.60; 95% CI, 1.10-2.31; $P = 0.013$). Stratification by menopausal status revealed that the effect of the mt10398A allele was similar in premenopausal (OR, 1.65; 95% CI, 0.92-2.98; $P = 0.10$) and postmenopausal women (OR, 1.53; 95% CI, 0.96-2.46; $P = 0.08$). Stratification by stage at diagnosis of invasive breast cancer did not reveal any significant differences in

Table 1. Characteristics of cases of invasive breast cancer and controls in the CBCS stratified by race

Variable	Cases	Controls
African-Americans	$N = 654$	$N = 605$
Age at diagnosis (cases) or selection (controls)		
Mean	51.1	51.8
Median	49.5	50.0
Range	23-74	26-74
Menopausal status		
Premenopausal	293 (45%)	275 (45%)
Postmenopausal	361 (55%)	330 (55%)
Breast cancer in first-degree relative		
Yes	99 (16%)	65 (11%)
No	536 (84%)	512 (89%)
AJCC stage at diagnosis (cases)		
I	216 (35%)	
II	302 (49%)	
III	72 (12%)	
IV	27 (4%)	
Whites	$N = 879$	$N = 760$
Age at diagnosis (cases) or selection (controls)		
Mean	50.9	52.2
Median	48.0	49.0
Range	24-74	21-74
Menopausal status		
Premenopausal	447 (51%)	351 (46%)
Postmenopausal	432 (49%)	409 (54%)
Breast cancer in first-degree relative		
Yes	149 (17%)	100 (14%)
No	708 (83%)	640 (86%)
AJCC stage at diagnosis (cases)		
I	393 (48%)	
II	349 (42%)	
III	69 (8%)	
IV	16 (2%)	

Table 2. Genotype frequencies and ORs for mt10398A and breast cancer in women with invasive breast cancer, Vanderbilt Pilot Study and the CBCS

Variable	Cases	Controls	OR (95% CI)
Vanderbilt pilot study			
African-American race	$N = 48$	$N = 54$	
mtDNA 10398A	7 (15%)	3 (6%)	2.90 (0.61-18.29)
mtDNA 10398G	41 (85%)	51 (94%)	Reference
CBCS			
African-American race	$N = 654$	$N = 605$	
mtDNA 10398A	84 (13%)	52 (9%)	1.60 (1.10-2.31)
mtDNA 10398G	570 (87%)	553 (81%)	Reference
White race	$N = 879$	$N = 760$	
mtDNA 10398A	700 (80%)	601 (79%)	1.03 (0.81-1.31)
mtDNA 10398G	179 (20%)	159 (21%)	Reference

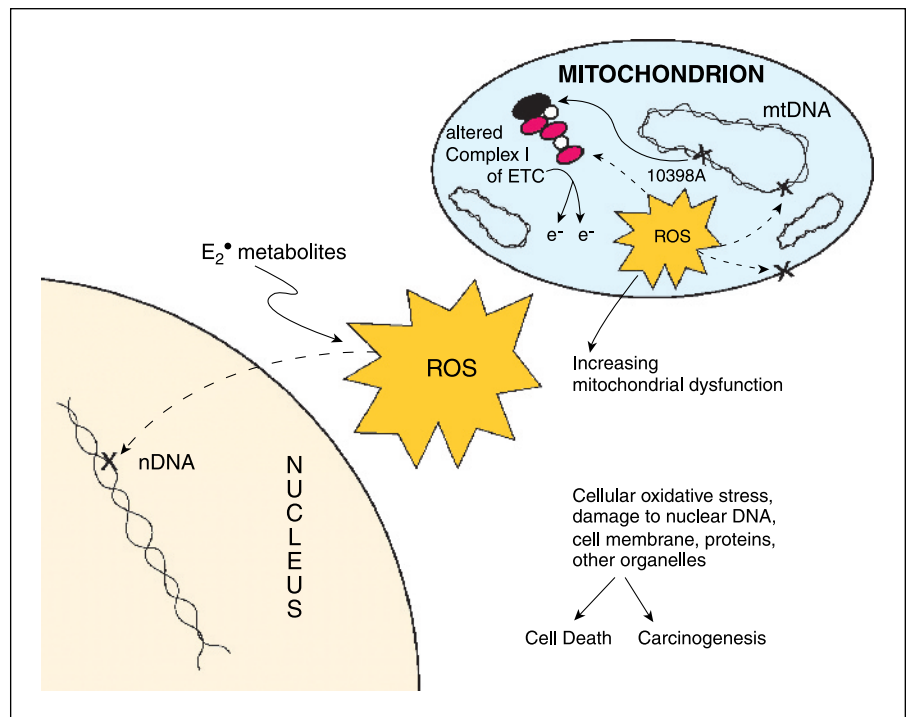
the frequency of the 10398A allele. The frequency of the 10398A allele by disease stage was as follows: stage I, 0.17; stage II, 0.12; stages III and IV, 0.11 ($P = 0.20$). Tumor expression of ER, PR, HER2, and P53 mutations was previously determined in cases enrolled in the CBCS (39, 41, 42). No significant differences were detected between mtDNA G10398A polymorphism allele frequencies with regard to the expression of any of these tumor markers.

Finally, we also successfully genotyped 879 white invasive breast cancer cases and 760 white controls in the CBCS (98% of available samples) for the mtDNA G10398A polymorphism. We did not show an association between the 10398A allele and invasive breast cancer in white women (OR, 1.03; 95% CI, 0.81-1.31; $P = 0.81$). ORs were 1.08 (95% CI, 0.76-1.55; $P = 0.66$) and 0.97 (95% CI, 0.70-1.35; $P = 0.87$) in premenopausal and postmenopausal white women, respectively.

Discussion

mtDNA is a rich template for genetic variation that exhibits exclusively maternal transmission (1, 4). We focused our attention on the mtDNA G10398A polymorphism because recent research in neurodegenerative diseases, particularly Parkinson's disease, has implicated the 10398A allele in the degenerative phenotype (18-22). Whereas our study provides evidence of a relationship between the mtDNA G10398A polymorphism and breast cancer susceptibility, the mechanism underlying this relationship remains to be elucidated. The nonconservative amino acid change that results from this polymorphism in the ND3 subunit of Complex I may increase the rate of electron leakage and ROS generation at this site, contributing to mtDNA and nuclear DNA mutations and cumulative mitochondrial dysfunction (Fig. 1; refs. 12, 44, 45). Proposed mechanisms for the adverse effects of the 10398A allele in other diseases involving oxidative stress include less efficient functioning of Complex I in individuals whose mitochondria carry this allele or, alternatively, reduced ability of cells in these individuals to constrain an oxidative challenge (18, 19). The effect of the 10398A allele may not be sufficiently severe to affect reproductive fitness, however, because its deleterious effects in late-onset diseases such as Parkinson's disease predominantly occur after the reproductive years. For this reason, some mitochondrial polymorphisms have been relatively protected from

Figure 1. Alterations in mitochondrial Complex I structure can increase cellular oxidative stress and lead to carcinogenesis. mtDNA polymorphisms such as G10398A may lead to decreased efficiency of the electron transport chain (ETC) and an increased leakage of electrons (e^-) at this site. This results in increased generation of mitochondrial and cellular ROS, nuclear DNA (nDNA) and mtDNA mutations, and damage to lipid membranes and other cellular constituents. In the breast, redox-cycling estrogen metabolites (E_2^*) may also contribute to cellular oxidative stress and carcinogenesis.



selective forces. Increased mitochondrial free radical production may therefore be an important shared feature in the pathogenesis of neurodegenerative and neoplastic phenotypes (19).

There are also epidemiologic parallels between Parkinson's disease and breast cancer with respect to mitochondrial polymorphisms. Geographic variation in the prevalence of Parkinson's disease seems to reflect differences in the distribution of mtDNA G10398A genotypes. Approximately 80% of whites express the 10398A allele, and the prevalence of Parkinson's disease is also highest in this group (46, 47). Similarly, the age-adjusted incidence of breast cancer is lower in African-American women than in white women for reasons that have been unclear (120.8 per 100,000 versus 142.0 per 100,000; refs. 48, 49). The significantly lower overall prevalence of the mitochondrial 10398A allele in African-Americans offers one possible explanation for this disparity. In our sample, however, we did not detect an increased risk of breast cancer in white women who carried the 10398A allele as compared with white women with the G allele. This discrepancy may be due to the interaction of the 10398A allele in African-American women with other as-yet-unidentified genetic and environmental risk factors.

In this study, we found that variation in the mitochondrial genome modulates the risk of invasive breast cancer in African-American women. To our knowledge, this study is the first to show an association between a mitochondrial Complex I polymorphism and breast cancer in humans. Our findings complement the results recently reported by Petros et al. (14). These investigators introduced the pathologic mtDNA mutation T8993G into a PC3 prostate cell line by cybrid transfer and showed that tumors in nude mice derived from these cells were significantly larger than tumors derived from cells that did not carry this mutation (14). In separate analyses described in the same study, both somatic and germ line variations in the gene encoding the cytochrome oxidase subunit of mitochondrial Complex IV (COI) were also shown to affect the development of prostate cancer in men. The authors concluded that

polymorphisms in this gene that are prevalent in African-Americans may contribute to the increased predisposition of African-American men to prostate cancer; however, the statistical power to confirm this hypothesis was lacking (14). We similarly hypothesized that genetic polymorphisms in key subunits of the electron transport chain encoded by mtDNA, along with other genetic and environmental factors, lead to increased ROS production, intracellular oxidative stress, and breast carcinogenesis. The CBCS provided a large, population-based sample of breast cancer cases and controls with adequate power to test our hypothesis. In African-American women in the CBCS, the 10398A allele conferred a significantly increased risk of invasive breast cancer regardless of menopausal status, suggesting that mitochondrial polymorphisms and any resulting changes in mitochondrial function may play a role in the etiology of this disease.

Genetic association studies like this one have potential limitations that we expressly attempted to avoid. Failure to replicate findings, particularly findings obtained in small or highly selected population samples, is a common problem in association studies (50). First, we replicated our findings in two geographically separate populations. Second, the size of the CBCS sample provided adequate statistical power to detect a clinically important increase in breast cancer risk and validate our initial observation. Sample size requirements will be continue to pose a challenge in other ethnic groups in which the magnitude of the effect may be smaller. Third, we used a population-based study design that substantially decreases the effect of selection bias. We also focused our analysis on a single, self-reported racial group and therefore minimized the confounding effect that unidentified population substructure might present. Finally, we chose to target a single-nucleotide polymorphism that has already been implicated as having a role in ROS production in other diseases. Mounting evidence of a role for the mtDNA 10398A allele in mitochondrial dysfunction lends further biological plausibility to its involvement in breast carcinogenesis.

Indeed, many factors other than the mtDNA G10398A polymorphism are likely to be important in the development and progression of invasive breast cancer in African-American women (51). The mortality rate for premenopausal breast cancer is twice as high in African-American women as in white women (48). Although socioeconomic disparities often lead to delayed breast cancer diagnosis, other environmental factors may also be involved. Dietary differences and genetic variations in lipid and iron metabolism may modify the carcinogenic effects of mitochondrial polymorphisms like G10398A in different racial/ethnic groups (36, 41, 52). Other investigators have shown that environmental factors can compromise mitochondrial Complex I function, resulting in increased production of ROS (32, 44, 53). In the CBCS, analysis of established risk factors for breast cancer was consistent with the published literature (49), but the 10398A allele remained an independent predictor of breast cancer risk when we adjusted for these other factors.

It is possible that the 10398A allele is in linkage disequilibrium with a more important causative polymorphism. Clusters of mitochondrial polymorphisms constitute haplogroups that have been used extensively to track human migrations and, more recently, to explore variation in disease phenotypes (54–57). Besides underlying polymorphisms such as mtDNA G10398A, there may be other mtDNA polymorphisms that impair the efficiency of mitochondrial electron transport. One recent study showed that the risk of Parkinson's disease and progression of Parkinsonian dementia was associated with the number of nonsynonymous substitutions in genes encoding the ND subunits of mitochondrial Complex I (58).

Clearly, an important next step will be to further define and characterize polymorphisms in the mtDNA that influence mitochondrial ROS generation, and potentially, carcinogenesis. Individual nonsynonymous polymorphisms may also alter mitochondrial function by mechanisms other than increased electron leakage and oxidative stress. Therefore, epistatic interactions between individual loci

within the mitochondrial genome as well as nuclear-mitochondrial gene interactions should be investigated (59). Niemi et al. (60) recently reported that mtDNA G10398A genotype modified the effects of a polymorphism in the noncoding region of the mitochondrial genome on longevity in both the Finnish and Japanese populations.

In summary, this study provides new evidence that variation in the mitochondrial genome contributes to breast cancer susceptibility and that it may underlie differences in the incidence of breast cancer between African-American and white women. The magnitude of the risk associated with the 10398A allele that we report from the CBCS suggests that this polymorphism is an important new risk factor to consider in the etiology of breast cancer in African-American women. Future studies involving breast cancer in African-American women will need to take into account the distribution of the mitochondrial DNA 10398A allele as a population substructure within this self-identified racial category. Recent reviews of the genetic epidemiology of breast cancer have not mentioned the mitochondrial genome (61, 62). As we contemplate genome-wide association studies of breast cancer that encompass the 6,000,000,000 bp in the human diploid nuclear genome, it is worth noting that humans have two genomes. Variations in the 16,569 bp mitochondrial genome should not be overlooked in the search to uncover factors important in breast carcinogenesis.

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