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Peripheral blood mitochondrial DNA copy number, length heteroplasmy and breast cancer risk: a replication study

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Abstract

Oxidative stress has consistently been linked to breast carcinogenesis, and mitochondria play a significant role in regulating reactive oxygen species generation. In our previous study, we found that increased levels of mitochondrial DNA (mtDNA) copy number and the presence of mitochondrial length heteroplasmies in the hypervariable (HV) regions 1 and 2 (HV1 and HV2) in peripheral blood are associated with increased risk of breast cancer. In current study with 1000 breast cancer cases and 1000 healthy controls, we intended to replicate our previous findings. Overall, levels of mtDNA copy number were significantly higher in breast cancer cases than healthy controls (mean: 1.17 versus 0.94, $P < 0.001$). In the multivariate linear regression analysis, increased mtDNA copy number levels were associated with a 1.32-fold increased risk of breast cancer [adjusted odds ratio (OR) = 1.32, 95% confidence interval (CI) = 1.15–1.67]. Breast cancer cases were more likely to have HV1 and HV2 region length heteroplasmies than healthy controls ($P < 0.001$, respectively). The existence of HV1 and HV2 length heteroplasmies was associated with 2.01- and 1.63-folds increased risk of breast cancer (for HV1: OR = 2.01, 95% CI = 1.66–2.42; for HV2: OR = 1.63, 95% CI = 1.34–1.92). Additionally, joint effects among mtDNA copy number, HV1 and HV2 length heteroplasmies were observed. Our results are consistent with our previous findings and further support the roles of mtDNA copy number and mtDNA length heteroplasmies that may play in the development of breast cancer.

Introduction

Mitochondria play a vital role in cellular energy metabolism, apoptosis and reactive oxygen species (ROS) generation (1). Investigation of the mitochondria is of particular interest to breast cancer because oxidative stress has been deemed as an important player in breast carcinogenesis (2–4). It is hypothesized that genetic variations in mitochondrial DNA (mtDNA) could have adverse effect by increasing the generation of ROS and consequently increasing the individual's cancer risk (5). The genome of the mitochondria is complex, and different types of variations have been observed and investigated in terms of their relationships with human diseases (6,7).

mtDNA copy number is significantly varied (8). Levels of mtDNA copy number may be affected by both inherited genetic factors and levels of oxidative stress. In terms of breast cancer, the sources of oxidative stress may include a variety of endogenous and exogenous factors, such as hormones, age, dietary and environmental oxidants/antioxidants, reaction to oxidative damage etc. (2–4,9–11). In our previous study with 103 breast cancer cases and 103 healthy controls, we investigated the relationship between mtDNA copy number and breast cancer risk (12). We found that increased mtDNA copy number levels were associated with increased risk of breast cancer. Our results were further confirmed by two other studies (13,14). Similar association has also been observed in other types of cancer, from non-Hodgkin lymphoma, chronic lymphocytic leukemia, lung cancer, renal cell carcinoma, pancreatic cancer, colorectal cancer to melanoma (15).

mtDNA also shows high degree of heteroplasmies, defined as the occurrence of two or more types of molecules within the mtDNA population of the same individual (16,17). The most

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Abbreviations

BMI	body mass index
CI	confidence interval
HV	hypervariable
mtDNA	mitochondrial DNA
OR	odds ratio
ROS	reactive oxygen species

studied mtDNA heteroplasmies are two length heteroplasmies in hypervariable (HV) regions 1 and 2 (HV1 and HV2), both of which have been observed in various cell types and different types of populations (18–22). Previous studies suggest that mtDNA HV1 heteroplasmy is related to lower birth weight, diabetes mellitus and dilated cardiomyopathy (23–25). mtDNA HV2 heteroplasmy exists in a conserved region that may control mtDNA replication and transcription, so this region was a 'hot spot' for somatic mutations in a variety of cancers (26,27). To date, few studies have investigated the association between HV1 and HV2 length heteroplasmies and cancer risks. In our previous analysis, we found that the presence of HV1 and HV2 length heteroplasmies was associated with increased risk of breast cancer (19).

In this study, we attempted to replicate our previous findings in a large breast cancer case–control study with 1000 Caucasian American breast cancer cases and 1000 Caucasian American healthy controls.

Materials and methods

Study participants

De-identified genomic DNA samples and questionnaire data used in this study were obtained from the Roswell Park Cancer Institute's (RPCI) Data Bank and BioRepository (DBBR). Detailed description of DBBR has been published previously (28). The DBBR is a Cancer Center Shared Resource and is a biorepository of blood samples collected, processed and stored in a rigorous, standardized manner, linked with clinical and epidemiological data. Patients are enrolled prior to surgery and/or chemotherapy, and controls are individuals who are free from cancer and who are visitors or family members of patients. Relationships between patients and controls are carefully annotated, so that we avoid overmatching patients to their own family or friends. Patients and controls are consented to provide a non-fasting blood sample and to complete a questionnaire that collects data on family history of cancer, medical history, smoking history, menstrual and reproductive history; lifestyle habits including diet, use of dietary supplements, smoking, physical activity, alcohol intake; and demographic data and height and weight from young adulthood to present. Blood samples are drawn in phlebotomy and transferred to the DBBR laboratory through the pneumatic tube system. In the laboratory, specimens are processed and aliquoted into 0.5 ml straws that are labeled with barcoded ID number and frozen. All samples are stored in liquid nitrogen and are available for use by RPCI and other researchers with Institutional Review Board (IRB)-approved protocols. Genomic DNA was extracted from whole blood for all the samples by use of Gentra Puregene Blood Kit (Qiagen, Valencia, CA). In this study, we included 1000 women with breast cancer as cases and 1000 cancer-free women as controls. The cases and controls were frequently matched on age, menopausal status and time of blood drawn. The study was approved by MD Anderson Cancer Center IRB. The study subjects included in our previous study (12) (103 breast cancer cases and 103 healthy controls) were not included in this study.

Quantification of mtDNA copy number

The method for determining mtDNA copy number was detailed in our previous publication (12,29) and was shown to have high interassay reliability. In brief, two pairs of primers were used in the two steps of relative quantification for mtDNA content. One primer pair was used for the amplification of the *MT-ND1* gene in mtDNA. Another primer pair was used for the amplification of the single-copy nuclear gene human globulin. In the first step, the ratio of mtDNA copy number to human globulin copy number, which is also referred as mtDNA index, was determined for each sample from standard curves. This ratio is proportional to the mtDNA copy number in each cell and, for each sample, was normalized to a calibrator DNA (DNA sample from healthy control) in order to standardize between different runs. All samples were assayed in triplicate on a 96-well plate with a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The PCR for ND-1 and human globulin was always performed on separate 96-well plates with the same samples in the same well positions to avoid possible position effects. In each run, a standard curve of a reference DNA and a negative control was included. The R^2 for each standard curve was at least 0.99.

Determination of HV region length heteroplasmies

The method for determining HV region length heteroplasmies was detailed in our previous publication (19). ABI-Prism 3100 Genetic Analyzer (Applied Biosystems) at MD Anderson DNA Analysis Core Facility and Gene Scan Analysis Software (version 3.1) were used to determine the fragment length of the specific PCR product. To safeguard accuracy and consistency, each sample was run in duplicate. To further confirm the variant alleles, we randomly selected 10% of the DNA samples that exhibited length heteroplasmies in HV1 and HV2 regions and performed sequencing analysis. The same PCR primers from gene scan analysis (without Hex fluorescent dye) were used, and the same PCR was carried out. The amplified PCR products were sequenced with an ABI-PRISM 3730xl sequencer (Applied Biosystems) in the MD Anderson DNA Analysis Core Facility.

Statistical analysis

Statistical analyses were performed using STATA statistical package (version 13, STATA, College Station, TX). For demographic characteristics, we used the Student's *t*-test for continuous variables, the chi-square test for two-level categorical variables and the Pearson chi-square for all other categorical variables to compare means and frequencies between cases and controls. Since the mtDNA copy number data were not normally distributed among the control group, we performed the analysis using both data with and without log transformation. We found no significant differences in the estimated associations with and without log transformation, and therefore, only data without log transformation were presented here. The Student's *t*-test was used to determine differences between cases and controls for the mean of mtDNA copy number associated with selected categorical characteristics. To determine the difference of mtDNA copy number by selected characteristics within either case or control groups, the Student's *t*-test and analysis of variance test were used for two-level categorical variables and variables with more than two levels, respectively. Odds ratio (OR) and 95% confidence interval (CI) were estimated with unconditional logistic regression for the main effect of mtDNA copy number on breast cancer risk. Potential confounders, namely age, menopausal status, smoking status and body mass index (BMI) category, were included in the model. The mtDNA copy number variable was examined as a continuous variable, a categorical variable based on quartile distributions in controls and a categorical variable divided by the median value. Cutoff points for all constructed categorical variables were determined based on the distribution within the control population. The dose response was tested for the quartile distribution of mtDNA copy number by inserting the median value of each quartile and then treating the variable as a continuous variable in the logistic regression model. For HV region length heteroplasmies, the frequency of each length heteroplasmy was calculated. The chi-square test was used to test the distribution differences of HV region length heteroplasmies between breast cancer cases and healthy controls. For the poly-C tract in HV1, those exhibiting only CCCCCTCCCC (5CT4C) were used as a reference group. For the poly-C tract in HV2, those exhibiting only CCCCCCCTCCCCCC (7CT6C) were used as reference group (19). Unconditional logistic regression analysis was used to determine the association between breast cancer risk and HV region length heteroplasmies. Potential confounders, namely age, menopausal status, smoking status and BMI category, were included in the model. Potential interactions among levels of mtDNA copy number, HV1 and HV2 length heteroplasmies were assessed. All *P*-values were two sided. Associations were considered statistically significant at $P < 0.05$.

Results

Table 1 summarizes the characteristics of the study population. The case and control groups were not differed by ethnicity ($P = 1.000$), age ($P = 0.683$), menopausal status ($P = 0.359$), cigarette smoking status ($P = 0.188$), current fruit intake ($P = 0.334$), current vegetable intake ($P = 0.563$) and BMI as continuous ($P = 0.323$) and categorical variables ($P = 0.544$). However, the case and control groups were differed by daily alcohol intake ($P < 0.001$), current exercise ($P < 0.001$) and family history of breast cancer ($P = 0.003$).

Overall, levels of mtDNA copy number were significantly higher in breast cancer cases than healthy controls (mean: 1.17 versus 0.94, $P < 0.001$) (Table 2). The difference in mtDNA copy number levels between cases and controls was not affected by age category, menopausal status, daily alcohol intake, family history of breast cancer, current vegetable intake, current fruit intake or BMI category. For cigarette smoking status, the significant difference was only evident in ever smokers ($P < 0.001$) but not in never smokers ($P = 0.153$). For current exercise, the significant difference was evident in study subjects who exercise regardless of the intensity but not in those who do not exercise at all ($P = 0.110$). When compared levels of mtDNA copy number within the case or control group by selected demographic characteristics, significant differences were found in relation to age (Supplementary Figure 1, available at *Carcinogenesis* Online), age category, menopausal status, cigarette smoking status, BMI (Supplementary Figure 2, available at *Carcinogenesis* Online) and BMI category. Among healthy controls, a significant trend of decreasing mtDNA copy number levels was observed with increasing age category from <50, 50–60, >60 years old ($P = 0.023$). However, similar trend was not observed among breast cancer cases. Postmenopausal breast cancer cases had statistically

Table 1. Distribution of selected characteristics of breast cancer cases and controls

	Cases (n = 1000)	Controls (n = 1000)	
Characteristics	N (%)	N (%)	P-value*
Ethnicity			
White	1000 (100.0)	1000 (100.0)	1.000
Menopausal status			
Premenopausal	301 (30.1)	320 (33.0)	
Postmenopausal	699 (69.9)	680 (68.0)	0.359
Daily alcohol intake			
No drink per day	280 (28.0)	250 (25.0)	
0.5–1 drink per day	620 (62.0)	730 (73.0)	
>1 drink per day	100 (10.0)	20 (2.0)	<0.001
Cigarette smoking status			
Never	685 (68.5)	712 (71.2)	
Ever	315 (31.5)	288 (28.8)	0.188
Family history of breast cancer			
No	841 (84.1)	887 (88.7)	
Yes	159 (15.9)	113 (11.3)	0.003
Current vegetable intake			
<1 per week	62 (6.2)	51 (5.1)	
1–6 per week	543 (54.3)	552 (55.2)	
>1 per day	395 (39.5)	397 (39.7)	0.563
Current fruit intake			
<1 per week	133 (13.3)	113 (11.3)	
1–6 per week	447 (44.7)	445 (44.5)	
>1 per day	420 (42.0)	442 (44.2)	0.334
Current exercise (20 min)			
Never	332 (33.2)	271 (27.1)	
<1 per week	302 (30.2)	220 (22.0)	
1–2 per week	221 (22.1)	341 (34.1)	
3–4 per week	145 (14.5)	168 (16.8)	<0.001
BMI category			
Normal weight	326	349	
Overweight	471	458	
Obese	203	193	0.544
Age (years)	58	56	0.683
BMI	27.8	26.4	0.323

*For categorical variables, chi-square test was used to examine the differences. For continuous variables, Student's t-test was used to examine the difference.

Table 2. Comparison of mtDNA copy number in breast cancer patients and controls

Characteristics	Cases (N = 1000)	Controls (N = 1000)	P*
Overall	1.17	0.94	<0.001
By age category			
<50	1.18	0.99	<0.001
50–60	1.16	0.92	<0.001
>60	1.15	0.88	<0.001
P**	0.673	0.023	
By menopausal status			
Premenopausal	1.12	0.96	0.01
Postmenopausal	1.23	0.92	<0.001
P**	0.013	0.506	
By daily alcohol intake			
No drinks per day	1.18	0.91	0.018
0.5–1 drinks per day	1.15	0.96	0.034
>1 drinks per day	1.17	0.96	0.028
P**	0.845	0.819	
Cigarette smoking status			
Never	1.07	0.89	0.153
Ever	1.3	1.01	<0.001
P**	0.021	0.042	
By family history of breast cancer			
No	1.15	0.93	<0.001
Yes	1.21	0.98	0.037
P**	0.472	0.725	
By current vegetable intake			
<1 per week	1.13	0.95	0.041
1–6 per week	1.15	0.97	0.007
>1 per day	1.24	0.91	<0.001
P**	0.412	0.583	
By current fruit Intake			
<1 per week	1.19	0.9	0.038
1–6 per week	1.13	0.91	0.006
>1 per day	1.16	0.97	<0.001
P**	0.349	0.802	
By current exercise (20 min)			
Never	1.15	0.89	0.11
<1 per week	1.12	0.95	<0.001
1–2 per week	1.19	0.98	0.003
3–4 per week	1.21	0.86	0.002
P**	0.872	0.354	
BMI category			
Normal weight	1.23	0.99	<0.001
Overweight	1.19	0.96	<0.001
Obese	1.14	0.94	<0.001
P**	<0.001	0.024	

*P-value comparing mean mtDNA copy number between cases and controls. Student's t-test was used to examine the difference.

**P-value comparing mean mtDNA copy number between groups defined by selected characteristics. Analysis of variance test was used to test examine differences within categories.

significantly higher levels of mtDNA copy number than premenopausal breast cancer cases ($P = 0.013$). But, the difference was not significant in healthy controls. Irrespective to case–control status, levels of mtDNA copy number were significantly higher in ever smokers than never smokers ($P = 0.021$ and 0.042, respectively). With the increasing of BMI category from normal weight, overweight or obesity, levels of mtDNA copy number significantly decreased in both cases and controls ($P < 0.001$ and 0.024, respectively).

Using mtDNA copy number as a continuous variable, in the multivariate linear regression analysis, we found that increased mtDNA copy number levels were associated with a 1.32-fold increased risk of breast cancer after adjusting for age, menopausal status, smoking status and BMI category (adjusted OR = 1.32, 95% CI = 1.15–1.67) (Table 3). When levels of mtDNA copy number were dichotomized into two groups (high or low) using median levels of mtDNA copy number in controls (0.94), high levels of mtDNA copy number were associated with a 2.25-fold increased risk of breast cancer after adjusting for age, menopausal status, smoking status and BMI category (adjusted OR = 2.25, 95% CI = 1.88–2.73). In further quartile analysis using 25%, 50% and 75% values of mtDNA copy number among control subjects as cutoff points, we found that study subjects in the second, third and fourth quartiles were at an increased risk of breast cancer (adjusted ORs for the second, third and fourth categories = 1.50, 95% CI = 1.11–2.13; 1.96, 95% CI = 1.27–2.87 and 2.65, 95% CI = 2.07–3.43, respectively) when compared with those with the lowest quartile of mtDNA copy number. A statistically significant dose–response trend was observed ($P < 0.001$).

Table 3. Risk of breast cancer as estimated by mtDNA copy number

mtDNA index (relative copy number)	Number of cases (%)	Number of controls (%)	OR (95% CI)[a]
Continuous variable	1000 (100)	1000 (100)	1.32 (1.15–1.67)
Categorical variable			
By mean in controls			
<0.94	317	513 (51.3)	1.00
≥0.94	703 (70.3)	487 (48.7)	2.25 (1.88–2.73)
By quartile in controls			
First	142 (14.2)	256 (25.6)	1.00
Second	223 (22.3)	257 (25.7)	1.50 (1.11–2.13)
Third	274 (27.4)	245 (24.5)	1.96 (1.27–2.87)
Fourth	361 (36.1)	242 (24.2)	2.65 (2.07–3.43)
P for trend			<0.001

[a]ORs were adjusted by age, menopausal status, smoking status and BMI category.

The comparison of mtDNA HV region length heteroplasmy between breast cancer cases and healthy controls and the relationship between mtDNA HV region length heteroplasmy and risk of breast cancer are summarized in Table 4. The distributions of HV1 and HV2 length heteroplasmies were statistically significantly different between breast cancer cases and controls ($P < 0.001$, respectively). 5CT4C was the most common poly-C tract in the HV1 region with 63.4% of controls and 46.5% of cases displaying only 5CT4C. Ten different patterns of HV1 length heteroplasmies were observed, including 5CT4C, 5CT4C + 5CT3C, 9C + 10C + 11C, 3CT4C + 3CT3C, 3CT6C + 3CT5C and five others. For the poly-C tract in the HV2 region, 7CT6C was the most common with 72.2% of controls and 61.9% of cases exhibiting only 7CT6C. Six different patterns of HV2 length heteroplasmies were observed, including 7CT6C, 7CT6C + 8CT6C, 8CT6C + 9CT6C, 8CT6C + 9CT6C + 10CT6C, 9CT6C + 10CT6C + 11CT6C and 7CT6C + 6CT6C.

In relation to breast cancer shown in Table 4, study subjects who exhibited length heteroplasmies in the HV1 region had a 2.01-fold increased risk of breast cancer (OR = 2.01, 95% CI = 1.66–2.42) than those who did not, after adjustment for age, menopausal status, smoking status and BMI category. Similar findings were observed for the HV2 region. Study subjects who exhibited length heteroplasmies in the HV2 region had a 1.63-fold increased risk of breast cancer (OR = 1.63, 95% CI = 1.34–1.92) than those who did not after adjustment for age, menopausal status, smoking status and BMI category.

Finally, we examined the joint effects of mtDNA copy number and HV1/2 length heteroplasmies on breast cancer risk (Table 5). Compared with study subjects with low levels of mtDNA copy number and no HV1 or HV2 length heteroplasmies, those who had high levels of mtDNA copy number alone had 1.51-fold increased risk of breast cancer (OR = 1.51, 95% CI = 1.07–2.14). Those with both high mtDNA copy number and one HV length heteroplasmies (HV1 or HV2) had significantly increased risk of breast cancer than those with low levels of mtDNA copy number and no HV1 or HV2 length heteroplasmies (OR = 4.18, 95% CI = 2.36–6.79 and OR = 4.01, 95% CI = 2.33–6.43, respectively).

Table 4. Distribution of poly-C length heteroplasmy in HV1 and HV2 regions of mtDNA of cases and controls

	Reference sequence	Patterns	Cases, *N* (%)	Controls, *N* (%)	*P*-value[a]	OR[a] (95% CI)
HV1	CCCCCTCCCC (5CT4C)	5CT4C (no length heteroplasmy)	465 (46.5)	634 (63.4)		1
		5CT4C + 5CT3C	207 (20.7)	185 (18.5)		1.50 (1.21–1.90)
		9C + 10C +11C	173 (17.3)	160 (16.0)		1.45 (1.12–1.88)
		3CT4C + 3CT3C	32 (3.2)	21 (2.1)		2.03 (1.12–3.74)
		3CT6C + 3CT5C	34 (3.4)	0 (0.0)		NA
		Others	89 (8.9)	5 (0.0)	<0.001	NA
		Total variants	535 (53.5)	366 (36.6)		2.01 (1.66–2.42)
HV2	CCCCCCCTCCCCCC (7CT6C)	7CT6C (no length heteroplasmy)	619 (61.9)	722 (72.2)		1
		7CT6C + 8CT6C	243 (24.3)	182 (18.2)		1.53 (1.26–1.98)
		8CT6C + 9CT6C	102 (10.2)	68 (6.8)		1.72 (1.25–2.49)
		8CT6C + 9CT6C + 10CT6C	18 (1.8)	28 (2.8)		0.79 (0.36–1.43)
		9CT6C + 10CT6C + 11CT6C	9 (0.9)	0 (0.0)		NA
		7CT6C + 6CT6C	9 (0.9)	0 (0.0)	<0.001	NA
		Total variants	381 (38.1)	278 (27.8)		1.63 (1.34–1.92)

NA, not applicable.

[a]OR was adjusted by age, menopausal status, smoking status and BMI category only.

Table 5. Joint analysis of mtDNA copy number and heteroplasmies

mtDNA copy number	HV1	HV2	Cases, N (%)	Controls, N (%)	OR (95% CI)[a]
Low	WT	WT	72	162	1.00
High	WT	WT	183	273	1.51 (1.07–2.14)
Low	Variant	WT	96	166	1.30 (0.88–1.93)
Low	WT	Variant	84	136	1.39 (0.92–2.09)
High	Variant	WT	268	121	4.18 (2.36–6.79)
High	WT	Variant	126	63	4.01 (2.33–6.43)
Low	Variant	Variant	65	49	2.98 (1.83–4.87)
High	Variant	Variant	106	30	6.95 (3.74–11.45)

WT, wild-type.
[a]OR was adjusted by age, menopausal status, smoking status and BMI category only.

Furthermore, we found that the presence of high levels of mtDNA copy number and HV1 and HV2 length heteroplasmies was associated with 6.95-fold increased risk of breast cancer (OR = 6.95, 95% CI = 3.74–11.45). No significant two-way interaction was observed.

Discussion

In this study with 1000 breast cancer cases and 1000 healthy controls, we confirmed our previous findings that mtDNA copy number and HV region length heteroplasmies are associated with increased risk of breast cancer. Our findings on the relationship between mtDNA copy number and breast cancer risk are also in agreement with two other studies (13,14). Considered the close relationship between mitochondrial and oxidative stress, our results provide evidence for a possible role of oxidative stress in breast carcinogenesis.

In our previous study, we found a significant inverse relationship between mtDNA copy number and age in both cases and controls. In this study, we observed the similar inverse relationship. However, the trend was only significant in controls. Our finding is consistent with the results from Lemnrau *et al.* (13). In their study, they found an inverse association between mtDNA copy number and age at blood collection for both cases and controls. Our finding is also consistent with several previous reports in muscle cells, leukocytes and neurons (30–34). Lee *et al.* (35) reported that the mtDNA copy number in the leukocyte may have a positive correlation with age before an individual reaches age 50–60 years and then progressively shifts to negative correlation thereafter. Because the mean ages of our cases and controls are 58 and 56 years, our findings are expected. In addition, we observed that postmenopausal breast cancer cases had higher levels of mtDNA copy number than premenopausal breast cancer cases. However, similar difference was not observed in healthy controls. Considered postmenopausal women are generally older than premenopausal women, the unexpected relationship between levels of mtDNA copy number and menopausal status in breast cancer cases is baffling. More research is needed to clarify the relationship.

In both cases and controls, we also found that levels of mtDNA copy number were significantly higher in ever smokers than never smokers. Similar relationship is not observed in previous breast cancer studies, including ours (12–14). However, the relationship between mtDNA copy number levels and cigarette smoking was reported previously in a lung cancer study (36). In study subjects from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study, Hosgood *et al.* (36) reported that heavy smokers had higher levels of mtDNA copy number than lighter smokers. Cigarette smoke contains many substances that may lead to high levels of ROS in the human body (37). Levels of 8-hydroxydeoxyguanosine (8-oxodG) have been found to be elevated in the peripheral leukocytes of smokers (38). Likewise, levels of F2-isoprostanes have shown markedly elevated in smokers relative to non-smokers (39,40). Thus, it is plausible that ever smokers would have higher ROS endogenously than never smokers, and correspondingly ever smokers would have higher levels of mtDNA copy number than never smokers.

Additionally, we observed a significant trend of decreasing levels of mtDNA copy number with the increasing of BMI category from normal weight, overweight or obesity in both cases and controls. The relationship was not observed in our previous study and other similar studies (12–14). Adipose tissue is the main source of cytokines and adipokines that increase systemic oxidative stress (41,42); thus, obesity may decrease mitochondrial function (43). The relationship between obesity-related phenotypes and mtDNA copy number levels has been explored in a few studies (44,45). Consistent with our findings, Lee *et al.* (45) found that visceral fat area was independently inversely associated with mtDNA copy number levels in 94 healthy young participants ($P < 0.01$). In 144 postmenopausal women, Kim *et al.* (44) found that the levels of blood mtDNA copy number were lower in study subjects with metabolic syndrome than in those without metabolic syndrome ($P < 0.01$). Clearly, more research is needed to help better understand the roles of these factors that may modify the levels of mtDNA copy number.

Very few studies have ever investigated the relationship between mtDNA heteroplasmy in blood DNAs and cancer risk. In our previous analysis in a small case–control study, we found the presence of mtDNA HV1 and HV2 length heteroplasmies was associated with increased risk of breast cancer (19), which was confirmed by this study. Shin *et al.* (18) found that mtDNA HV region length heteroplasmies from blood cells occur frequently in healthy subjects in a Korean population study. Using next-generation sequencing, Payne *et al.* (46) observed low-level heteroplasmic variance in all tested healthy individuals. mtDNA is exclusively maternally inherited (16). Currently, our knowledge on how heteroplasmic mtDNAs contribute to human disease is still poorly understood. In our previous study, we observed that mtDNA HV region length heteroplasmy was associated with a decreased copy number of mtDNA (19). However, in this study, we cannot find correlation between mtDNA copy number and length heteroplasmies, either in cases, controls or all subjects, suggesting mtDNA copy number and length heteroplasmies may play different roles in breast cancer development and do not have interaction with each other.

The major strength of this study includes the large sample size, detailed epidemiologic questionnaire data and analysing mtDNA copy number and mtDNA HV region length

heteroplasmy together. The main weakness is our study is cross-sectional in nature, which does not allow us to infer casual relationships among mtDNA copy number, mtDNA HV region length heteroplasmy and breast cancer risk. We did not have repeated measures of mtDNA copy number, and a single measurement may not reflect mtDNA copy number over a lifetime. Lemnrau *et al*. (13) found that mtDNA copy number showed large temporal variation after comparing mtDNA copy number from two blood samples collected ~6 years apart from 91 women. Blood cell composition may be varied individually, and fluctuations in blood cell composition could be a confounding factor behind the observed differences. Oxygen level will affect ROS levels, and in turn, will be a stimulant to the increased biogenesis of mitochondria. In this study, blood oxygenation levels were not measured. In addition, we did not have matched tumor or normal breast tissues to compare mtDNA copy number and mtDNA HV region length heteroplasmies between target and surrogate tissues. He *et al*. (47) found the frequency of heteroplasmic variants varied significantly by tissues. Nevertheless, our study provides evidence to support the role of mtDNA copy number and mtDNA HV region length heteroplasmies in the etiology of breast cancer. Further research is needed to clearly define the predictive value of mtDNA copy number in breast cancer screening and early detection and to prospectively understand the role of mtDNA HV region length heteroplasmies in breast cancer risk.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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