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Peripheral blood mitochondrial DNA content, A10398G polymorphism, and risk of breast cancer in a Han Chinese population

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Key words

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It has been reported that quantitative alterations and sequence variations of mtDNA are associated with the onset and progression of particular types of tumor. However, the relationship between mtDNA content, certain mtDNA polymorphisms in peripheral blood leukocytes and breast cancer risk remain obscure. This study was undertaken to investigate whether mtDNA content and the A10398G polymorphism in peripheral blood leukocytes could be used as risk predictors for breast cancer in Han Chinese women. Blood samples were obtained from a total of 506 breast cancer patients and 520 matched healthy controls. The mtDNA content was measured by using quantitative real-time PCR assay; A10398G polymorphism was determined by PCR-RFLP assay. There was no statistically significant difference between cases and controls in terms of peripheral blood mtDNA content or A10398G polymorphism. However, further analysis suggested that the risk of breast cancer was associated with decreased mtDNA content in premenopausal women ($P = 0.001$; odds ratio = 0.54; 95% confidence interval, 0.38–0.77), with increased mtDNA content in postmenopausal women ($P = 0.027$; odds ratio = 1.49; 95% confidence interval, 1.05–2.11). In addition, the associations between mtDNA content and several clinicopathological parameters of cases such as age, menopausal status, and number of pregnancies and live births were observed. This case–control study indicated that the peripheral blood mtDNA content might be a potential biomarker to evaluate the risk of breast cancer for selected Chinese women.

Mitochondria are semiautonomous organelles in eukaryotic cells that play an important role in cellular energy metabolism, generation of reactive oxygen species (ROS), calcium homeostasis, and apoptosis.[1] They consume oxygen to generate approximately 80–90% of the energy supply for the cell in the form of ATP and much of the ROS through oxidative phosphorylation (OXPHOS). Human mitochondrial DNA (mtDNA) is a 16 569-bp, maternally inherited, double-stranded circular DNA encoding 13 core polypeptide subunits of the respiratory chain complexes, two ribosomal RNAs, and a set of 22 transfer RNAs required for mitochondrial protein synthesis.[2] Compared to nuclear genomic DNA, mtDNA reveals high mutation rates caused by constant exposure to mutagenic oxygen radicals and lacks the protective mechanisms of DNA repair. These properties of mtDNA suggest their potential importance in apoptosis, ageing, and especially carcinogenesis.[3,4]

Human cellular mtDNA content varying across different tissues and cell types[5,6] is precisely regulated according to cellular physiological circumstances and may undergo significant changes under different internal or external microenvironments, such as hypoxia and steroid hormone stimulation.[7,8] Abnormal content may be a marker for mitochondrial dysfunction, a suspected contributor to mitochondrial-related diseases including kinds of cancers. In recent years, the identification of increased or reduced mtDNA copy number has been increasingly reported in a broad range of primary human cancers. These findings strongly indicate that mtDNA copy number alterations may exert a crucial role in the pathogenic mechanisms of tumor development.[9] For breast cancer, it was reported that mtDNA content in tumor tissues was less than adjacent normal tissues, and reduced copy number of mtDNA may be involved in breast neoplastic transformation or progression.[10–13] However, the results of studies regarding breast cancer risk and mtDNA content in peripheral blood leukocytes (PBLs) were not consistent. A retrospective case–control study showed decreased mtDNA copy number associated with increased breast cancer risk.[14] However, other studies showed different results, namely that breast cancer patients had a statistically significantly higher mtDNA copy number than matched control subjects.[15,16] Consequently, it remains to be determined whether mtDNA content in peripheral blood samples could be used as a risk predictor for breast cancer.

The 10 398 nucleotide position in the human mitochondrial genome is highly polymorphic. The polymorphism results in a

non-conservative amino acid substitution of threonine (encoded by the A allele) for alanine (encoded by the G allele) within the nicotinamide adenine dinucleotide dehydrogenase subunit 3 (ND3) of complex I. The precise mechanism of the adverse effect of the 10398A or 10398G allele is unknown. However, research results suggested that it may alter ROS generation by affecting function of complex I, which is a key component of the mitochondrial electron transport chain, and that it is important in the pathophysiology of various disorders, including cancers.[17–20] Previous population-based studies have reported the association between A10389G polymorphism and breast cancer risk, but the results are conflicting. For instance, mtDNA 10398A, which defines haplogroup N, has been reported to increase breast cancer risk among African-American women[17] and North Indians.[21] Whereas Setiawan *et al.* did not find any such association in the African-American population,[22] just like in the white women who were enrolled in the study by Canter *et al.*[17] In contrast, 10398G was found to increase sporadic breast cancer risk in European Americans[23] and Polish women.[24] So the relation between the mtDNA A10398G polymorphism and breast cancer risk remains controversial. More studies on ethnically diverged populations are required to quantify the level of association.

Based on this background, it is rational to hypothesize that the copy number and polymorphisms of mtDNA may play important roles in carcinogenesis concurrently, and lead to different susceptibilities to breast cancer. In this case–control epidemiologic study, we detected the peripheral blood mtDNA content and A10398G polymorphism concurrently to assess the associations between them and breast cancer risk in Han Chinese women, a group that, so far as we know, has not been investigated. Additionally, the correlations between mtDNA content and distribution of the A10398G genotype with clinicopathological parameters of cases were investigated. We aimed to provide certain kinds of insights into the risk prediction of breast cancer, to identify the high-risk populations that are most likely to develop breast cancer.

Materials and Methods

Study population. We carried out this study with available blood samples of breast cancer patients who were diagnosed at Zhongnan Hospital of Wuhan University and Hubei Cancer Hospital (both Wuhan, China) between 2010 and 2013. None of the cases had previous cancer history, or experience of surgery, chemotherapy, radiotherapy, or other treatments for breast cancer. The blood samples of matched controls were obtained from healthy female volunteers who had no history of cancer or metabolic diseases. All cases and controls were interviewed about their age, menopausal status, place of birth and residence, history of disease, and family history of disease before blood was drawn. The controls were frequency-matched to the cases in terms of age and residential. All participants were Han Chinese. Data including age, menopausal status, number of pregnancies and live births, histological type, pathological type, TNM stage, estrogen receptor (ER) status, progesterone receptor (PR) status, and human epidermal growth factor receptor 2 (Her2) status of breast cancer cases were collected from medical records. Each patient and volunteer whose blood samples were included in this study was informed orally before the samples were collected.

Laboratory methods. *Total DNA extraction.* Total DNA of each individual was extracted from 800 μL EDTA-Na2 anticoagulated peripheral blood samples according to the manufacturer's instructions of a commercial DNA isolation kit, TIANamp Blood DNA Kit (Tiangen Biotech, Peking, China). The extracted DNA was eluted in 100 μL TE buffer and stored at −20°C in a dedicated area that was only used for PCR assembly. Its concentration was determined using a NanoDrop ND-2000 Spectrophotometer from Thermo Fisher Scientific (Waltham, MA, USA).

Determination of mtDNA copy number by quantitative real-time PCR. Mitochondrial DNA content was assessed by quantification of a unique mitochondrial fragment in the human mitochondrial genome NC_012920 region relative to a single copy region of the nuclear gene β2M using a real-time PCR assay. The primers were: NC_012920, forward 5′-CTTCTGGC CACAGCACTT AAAC-3′ and reverse 5′-GCTGGTGTTAGG G TTCTTTGTTTT-3′ (64-bp product); and β2M, forward 5′-GCTGGGTAGCTCTAAA CAATGTATTCA-3′ and reverse 5′-CCATGTACT AACAAATGTCT AAAATGGT-3′ (93-bp product). Real-time PCR was carried out on an iQTM 5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with a total volume of 25 μL reaction mixture containing 100 ng DNA template (2 μL), 12.5 μL QuantiTect SYBR Green PCR Master Mix (TaKaRa, Otsu, Japan), and 0.5 μL each primer, 0.5 μL ROX Reference Dye II (TaKaRa), and 9 μL double-distilled H_2O. The cycling reaction condition was: initial "Hot Start" activation step for 5 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C.[25] Each sample was detected in duplicate, and calculating the mtDNA content was based on the mean of Ct (cycles of threshold) values.

Genotyping at mtDNA A10398G. Genotyping mtDNA A10398G was carried out using the PCR-RFLP assay. DNA samples were amplified using primers located between mtDNA 10284–10484 np, forward primer 5′-CAAACAACTAACCTGC CAC-3′ and reverse primer 5′-ATGAGGGGC ATTTGGTA-3′ (201-bp product). The PCR was carried out in a total volume of 25 μL containing 100 ng genomic DNA, 2.5 μL of 10 × buffer, 0.5 μmol/L primers, 0.15 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, and 1 U *Taq* DNA polymerase (Tiangen Biotech). The PCR was started with an initial denaturation step for 5 min at 94°C, 32 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C, followed by a final extension step for 5 min at 72°C. All PCRs were carried out in an Applied Biosystems Model No. 9902 Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA).

Reaction conditions and restriction enzymes used were described previously.[26] In brief, the PCR products were digested by 2 U *DdeI* (New England Biolabs, Ipswich, MA, USA) at 37°C for 4 h, in which the mtDNA A10398G allele A yielded two fragments including 128 bp and 73 bp, allele G yielded three fragments including 90 bp, 73 bp, and 38 bp. The digestion products were analyzed directly by a 2% agarose gel, as published in our previous paper.[27] To confirm the accuracy of the method used, the genotypes were confirmed by DNA sequencing analysis, and, at the same time, approximately 10% of the samples were randomly selected to repeat the assays. The results were 100% concordant.

Statistical analyses. All data were analyzed using the SPSS 18.0 statistical program for Windows (PASW Statistics, SPSS Inc., Chicago, IL, USA). All tests were two-sided, and a *P*-value below 0.05 was considered statistically significant. The mtDNA copy number of each sample was log-transformed. Data were described as the mean ± SD for normal distributions and as

median and interquartile range for abnormal distribution. Student's *t*-test was used to determine the differences of the mtDNA content between breast cancer cases and healthy controls, and the differences between two subgroups. When the number of subgroups was more than two, one-way ANOVA was used to evaluate the differences of mtDNA content, followed by the least significant difference test. With adjustments for age, odds ratios (ORs) and confidence intervals (CIs) were calculated using logistic regression models. Pearson's χ^2-test was used where necessary to calculate the *P*-values and corresponding ORs with 95% CIs to determine the differences of the distribution of age and menopausal status between cases and controls, and the associations between mtDNA A10398G genotypes and breast cancer risk or clinicopathological variables.

Results

This study was carried out in 506 Han Chinese women with primary breast cancer who were diagnosed between 2010 and 2013 (cases) and in 520 women matched for age, residence and ethnicity without any cancer during the same period (controls). All women enrolled in this study had no metabolic diseases or family history of cancer. Baseline characteristics of both cases and controls are reported in Table 1. These two groups were well matched on sex, race, age ($P = 0.879$) and menopausal status ($P = 0.661$). Overall, there were no statistically significant differences between cases and controls in terms of mtDNA content (1.92 ± 0.34 *vs* 1.94 ± 0.33, $P = 0.508$) and A10398G polymorphism in the mitochondrial *ND3* gene ($P = 0.545$) in the Han Chinese population (Table 1).

As shown in Tables 2 and 3, we compared the mtDNA content and A10398G genotype distribution stratified by host characteristics. The mean mtDNA content was statistically significantly lower in young cases (aged ≤44 years) with breast cancer than matched controls (1.79 ± 0.28 *vs* 2.01 ± 0.32, $P = 0.000$), but higher in aged cases (≥60 years) than controls from the same age group (2.13 ± 0.29 *vs* 1.81 ± 0.30, $P = 0.000$). The mtDNA content of cases in the premenopausal period was lower than that of controls in the same period (1.88 ± 0.32 *vs* 1.98 ± 0.33, $P = 0.000$). Nevertheless, the trend was reversed in the postmenopausal subgroup (1.96 ± 0.34 *vs* 1.89 ± 0.32, $P = 0.012$). In addition, the case–control difference was not significant in the mtDNA 10398A and 10398G subgroups ($P = 0.133$ and $P = 0.706$, respectively). No modulating effect of age, menopausal status, or mtDNA content on A10398G genotype distribution in cases or controls was identified (the *P*-value ranged from 0.396 to 0.906). Further analysis (Table 4) suggested that the risk of breast cancer was associated with decreased mtDNA content in premenopausal women ($P = 0.001$; OR = 0.54; 95% CI, 0.38–0.77), and with increased mtDNA content in postmenopausal women ($P = 0.027$; OR = 1.49; 95% CI, 1.05–2.11).

In this study, the associations between peripheral blood mtDNA content, A10398G polymorphism, and traditional clinicopathological parameters, such as age, menopausal status, age at menarche, number of pregnancies, number of live births, tumor size, lymph node metastasis, histological types, ER status, PR status, and Her2 status were analyzed (Tables 5 and 6). For the breast cancer patients, the differences in mtDNA content were statistically significant among subgroups of age, menopausal status, number of pregnancies, and number of live births ($P = 0.000$, 0.006, 0.001, and 0.000, respectively), but not significant among the other subgroups stratified by age at menarche, tumor size, lymph node metastasis, histological types, ER status, PR status, Her2 status, and mtDNA A10398G polymorphism (*P*-values ranged from 0.273 to 0.999). Cases who were in the premenopausal period or pregnant less than four times had decreased mtDNA content (1.88 ± 0.32 *vs* 1.96 ± 0.34, $P = 0.006$; 1.89 ± 0.34 *vs* 2.00 ± 0.32, $P = 0.001$). In addition, the mean value of mtDNA content increased significantly with increasing age and number of live births ($P = 0.000$ and 0.000, respectively). In addition, the distribution of mtDNA 10398A and 10398G carriers was different in the subgroups, but the differences were not significant in most of the subgroups (*P*-values ranged from 0.052 to 0.977), with the exception of the histological types and Her2 status subgroup ($P = 0.006$, 0.016). There were also no differences between cases with triple-negative breast cancer and non-triple-negative

Table 1. Baseline characteristics of breast cancer cases ($n = 506$) and matched healthy controls ($n = 520$)

Variable	Cases, *n* (%)	Controls, *n* (%)	*P*-value
Sex, *n* (%)			
Female	506 (100.0)	520 (100.0)	–
Ethnicity, *n* (%)			
Han Chinese	506 (100.0)	520 (100.0)	–
Age, years, *n* (%)			
Median (range)	50 (24–81)	51 (25–81)	0.879†
≤44	161 (31.8)	147 (28.3)	
45–59	226 (44.7)	234 (45.0)	
≥60	119 (23.5)	139 (26.7)	0.344‡
Menopausal status, *n* (%)			
Pre	257 (50.8)	257 (49.4)	0.661‡
Post	249 (49.2)	263 (50.6)	
Mitochondrial DNA content			
Mean ± SD	1.92 ± 0.34	1.94 ± 0.33	0.508§
Genotype, *n* (%)			
10398A	246 (48.6)	243 (46.7)	0.545‡
10398G	260 (51.4)	277 (53.3)	

†Mann–Whitney *U*-test was used to evaluate the difference in ages between cases and matched controls. ‡The χ^2-test was used to examine differences. –, no *P* value was calculated. §Student's *t*-test was used to examine the difference of mtDNA content between two groups.

Table 2. Analysis of the association between mtDNA content and breast cancer risk stratified by selected variables

	mtDNA content		*P*-value†
	Cases, mean ± SD	Controls, mean ± SD	
Age, years			
≤44	1.79 ± 0.28	2.01 ± 0.32	0.000
45–59	1.91 ± 0.35	1.96 ± 0.33	0.069
≥60	2.13 ± 0.29	1.81 ± 0.30	0.000
Menopausal status			
Pre	1.88 ± 0.32	1.98 ± 0.33	0.000
Post	1.96 ± 0.34	1.89 ± 0.32	0.012
Genotype			
10398A	1.93 ± 0.33	1.97 ± 0.30	0.133
10398G	1.92 ± 0.34	1.90 ± 0.35	0.706

†Student's *t*-test was used to examine the differences of mtDNA content between two groups.

Table 3. Analysis of the association between mtDNA A10398G genotype distribution and breast cancer risk stratified by selected variables

	Controls		Cases		*P*-value†	OR (95% CI)‡
	10398A	10398G	10398A	10398G		
Age, years, *n* (%)						
≤44	65 (44.2)	82 (55.8)	73 (45.3)	88 (54.7)	0.843	0.96 (0.61–1.50)
45–59	108 (46.2)	126 (53.8)	112 (49.6)	114 (50.4)	0.465	0.87 (0.60–1.26)
≥60	70 (50.4)	69 (49.6)	61 (51.3)	58 (48.7)	0.885	0.97 (0.59–1.57)
Menopausal status, *n* (%)						
Pre	118 (45.9)	139 (54.1)	122 (47.5)	135 (52.5)	0.724	0.94 (0.66–1.33)
Post	125 (47.5)	138 (52.5)	124 (49.8)	125 (50.2)	0.607	0.91 (0.65–1.30)
Mitochondrial DNA content,§ *n* (%)						
Low	105 (41.8)	146 (58.2)	118 (45.6)	141 (54.4)	0.396	0.86 (0.61–1.22)
High	138 (51.3)	131 (48.7)	128 (51.8)	119 (48.2)	0.906	0.98 (0.70–1.38)

†The χ^2-test was used to examine differences. ‡Odds ratio (OR) was estimated using subjects with 10398G genotype as reference. §Mitochondrial DNA content was dichotomized based on the mean value in the controls as the cut-off point. CI, confidence interval.

Table 4. Association between mtDNA content and breast cancer risk in premenopausal and postmenopausal women

Menopausal status	Mitochondrial DNA content†	Cases, *n* (%)	Controls, *n* (%)	*P*-value	OR (95% CI)‡
Pre	Low	145 (56.4)	106 (41.2)		
	High	112 (43.6)	151 (58.8)	0.001	0.54 (0.38–0.77)
Post	Low	114 (45.8)	145 (55.1)		
	High	135 (54.2)	118 (44.9)	0.027	1.49 (1.05–2.11)

†Mitochondrial DNA content was dichotomized based on the mean value in the controls as the cut-off point. ‡Odds ratio (OR) was calculated using logistic regression models with adjustments for age. CI, confidence interval.

breast cancer in the mtDNA content ($P = 0.405$) and the distribution of A10398G alleles ($P = 0.401$).

Discussion

Mitochondria are organelles that produce ATP through OXPHOS in eukaryotic cells. Many researchers have provided evidence that content and somatic mutations of mtDNA play an important role in initiation and progression of various cancers.[3] However, to the best of our knowledge, this is the largest epidemiological study into the associations between peripheral blood mtDNA content, A10398G polymorphism, and risk of breast cancer in a Han Chinese population, and the first study showing the distinct change trend of mtDNA content in different age subgroups and menopausal status subgroups.

Previous evidence suggested that mtDNA copy number decreases in tumor tissues of breast cancer,[10–13] advanced gastric cancer,[28] hepatocellular carcinoma,[29,30] Ewing's sarcoma,[31] non-small-cell lung cancer,[32] but increases in head and neck cancers,[33] ovarian cancer,[34] prostate cancer,[35] esophageal squamous cell carcinoma,[36] and colorectal carcinoma.[37] Then abnormal mtDNA content in breast tissues may induce dysfunction of mitochondrion, which plays an important role in tumorigenesis. The peripheral blood mtDNA that was in a similar internal environment may be used as a sensitive marker for mitochondrial function and energy metabolism of cells, and a predictor for the cancer risk. However, the results of studies about peripheral blood mtDNA content in breast cancer were inconsistent.

The relation between mtDNA content in PBLs and breast cancer risk has been examined in only two studies. Shen *et al.* reported that high mtDNA copy number (above the median in controls) in an individual's PBLs was associated with a statistically significant increased risk of breast cancer, compared with low copy number (OR = 4.67; 95% CI, 2.45–8.92), with a statistically significant dose–response relationship in trend analysis ($P < 0.01$).[15] Nevertheless, in the present case–control study, we found that the mtDNA content was negatively correlated with the risk of breast cancer. Results of our research showed that distribution of mtDNA content was contrary in the different menopausal status population. These findings are not consistent with those of Shen *et al.* Further analysis of Shen's study suggested that the difference of the median mtDNA index between cases and controls was significant in white group, but not in black group. So, the reason why the results of Shen's and the present study were not consistent may be that the case and control subjects in Shen's study included both white and black women, but the study population of ours was Han Chinese. It indicated the racial differences of mtDNA content distribution.

In the current study, the mtDNA content of aged patients (≥60 years) was higher than that of matched controls. This result is in agreement with the findings from another recent study including 183 breast cancer cases and 529 individually matched controls in a Singaporean Chinese population aged 45–74 years, which showed that increased mtDNA content was positively associated with breast cancer risk (P for trend = 0.01).[16] However, we found that reduced mtDNA content was associated with risk of breast cancer in young women (≤44 years old). Moreover, no significant correlation was found in women aged 45–59 years. The results in premenopausal women were also inconsistent with these in the postmenopausal subgroup. This may be attributed to different levels of endogenous oxidants and antioxidants,[15] proteins encoded by nuclear genes such as *p53*, mtDNA polymerase γ

Table 5. Mitochondrial DNA content of cases stratified by selected clinicopathological parameters

	MtDNA content		*P*-value
	n (%)	Mean ± SD	
Age, years			
≤44	161 (31.8)	1.79 ± 0.27	0.000†
45–59	226 (44.7)	1.91 ± 0.35	
≥60	119 (23.5)	2.13 ± 0.29	
Menopausal status			
Pre	257 (50.8)	1.88 ± 0.32	0.006‡
Post	249 (49.2)	1.96 ± 0.34	
Age at menarche (years)			
≤13	268 (53.0)	1.91 ± 0.34	0.273‡
>13	238 (47.0)	1.94 ± 0.34	
Number of pregnancies			
≤3	348 (68.8)	1.89 ± 0.34	0.001‡
>3	158 (31.2)	2.00 ± 0.32	
Number of live births			
0	18 (3.6)	1.74 ± 0.44	0.000†
1	282 (55.7)	1.89 ± 0.32	
≥2	206 (40.7)	1.99 ± 0.33	
Tumor size, cm			
≤2	130 (25.7)	1.90 ± 0.33	0.451‡
>2	322 (63.6)	1.93 ± 0.34	
Unknown	54 (10.7)		
Lymph node metastasis			
Negative	223 (44.1)	1.91 ± 0.33	0.661‡
Positive	259 (51.2)	1.93 ± 0.35	
Unknown	24 (4.7)		
Histological types			
Non-invasive carcinoma	29 (5.7)	1.89 ± 0.32	0.566†
Invasive ductal carcinoma	380 (75.1)	1.92 ± 0.34	
Invasive lobular carcinoma	16 (3.2)	2.02 ± 0.32	
Other invasive carcinoma	61 (12.1)	1.95 ± 0.34	
Unknown	20 (4.0)		
ER status			
Negative	201 (39.7)	1.93 ± 0.33	0.806‡
Positive	268 (53.0)	1.92 ± 0.35	
Unknown	37 (7.3)		
PR status			
Negative	234 (46.2)	1.92 ± 0.32	0.999‡
Positive	235 (46.4)	1.92 ± 0.35	
Unknown	37 (7.3)		
Her2 status			
0–1	268 (53.0)	1.93 ± 0.34	0.506‡
2–3	196 (38.7)	1.91 ± 0.34	
Unknown	42 (8.3)		
Types			
TNBC	92 (18.2)	1.94 ± 0.32	0.405‡
Non-TNBC	374 (73.9)	1.92 ± 0.34	
Unknown	40 (7.9)		
Genotype			
10398A	246 (48.6)	1.93 ± 0.33	0.581‡
10398G	260 (51.4)	1.92 ± 0.34	

†*P*-values were obtained using one-way ANOVA on the rank followed by the least significant difference test. ‡Student's *t*-test was used to examine the differences in mtDNA content between two groups. ER, estrogen receptor; Her2, human epidermalgrowth factor receptor-2; PR, progesterone receptor; TNBC, triple negative breast cancer.

Table 6. Distribution of mtDNA A10398G genotype among subgroups of breast cancer cases stratified by selected clinicopathological parameters

	MtDNA 10398 genotype		*P*-value†
	10398A, *n* (%)	10398G, *n* (%)	
Age, years			
≤44	73 (29.7)	88 (33.8)	0.576
45–59	112 (45.5)	114 (43.8)	
≥60	61 (24.8)	58 (22.3)	
Menopausal status			
Pre	122 (49.6)	135 (51.9)	0.600
Post	124 (50.4)	125 (48.1)	
Age at menarche (years)			
≤13	128 (52.0)	140 (53.8)	0.683
13	118 (48.0)	120 (46.2)	
Number of pregnancies			
≤3	161 (65.4)	187 (71.9)	0.116
>3	85 (34.6)	73 (28.1)	
Number of live births			
0	7 (2.8)	11 (4.2)	0.652
1	136 (55.3)	146 (56.2)	
≥2	103 (41.9)	103 (39.6)	
Tumor size, cm			
≤2	64 (26.0)	66 (25.4)	0.977
>2	159 (64.6)	163 (62.7)	
Unknown	23 (9.3)	31 (11.9)	
Lymph node metastasis			
Negative	99 (40.2)	124 (47.7)	0.052
Positive	138 (56.1)	121 (46.5)	
Unknown	9 (3.7)	15 (5.8)	
Histological types			
Non-invasive carcinoma	10 (4.1)	19 (7.3)	0.006
Invasive ductal carcinoma	203 (82.5)	177 (68.1)	
Invasive lobular carcinoma	5 (2.0)	11 (4.2)	
Other invasive carcinoma	21 (8.5)	40 (15.4)	
Unknown	7 (2.8)	13 (5.0)	
ER status			
Negative	105 (42.7)	96 (36.9)	0.337
Positive	128 (52.0)	140 (53.8)	
Unknown	13 (5.3)	24 (9.2)	
PR status			
Negative	114 (46.3)	120 (46.2)	0.677
Positive	119 (48.4)	116 (44.6)	
Unknown	13 (5.3)	24 (9.2)	
Her2 status			
0–1	120 (48.8)	148 (56.9)	0.016
2–3	110 (44.7)	86 (33.1)	
Unknown	16 (6.5)	26 (10.0)	
Type			
TNBC	42 (17.1)	50 (19.2)	0.401
Non-TNBC	189 (76.8)	185 (71.2)	
Unknown	15 (6.1)	25 (9.6)	
Mitochondrial DNA content‡			
Low	118 (48.0)	141 (54.2)	0.159
High	128 (52.0)	119 (45.8)	

†The χ^2-test was used to evaluate the differences. ‡Mitochondrial DNA content was dichotomized into high and low groups using the mean value in the controls as the cut-off point. ER, estrogen receptor; Her2, human epidermalgrowth factor receptor-2; PR, progesterone receptor; TNBC, triple negative breast cancer.

(*POLG*), and mitochondrial single-stranded DNA binding protein (*mtSSB*), the feedback mechanism of mtDNA biogenesis,[9] because it is confirmed that the modulations on the association of mtDNA content with breast cancer risk are multifactorial and multifaceted. However, it is unclear which one plays the most important role. Further studies are needed to investigate the mechanisms, so that we can develop individualized strategies for both premenopausal and postmenopausal women.

Xia *et al.*[14] detected the peripheral blood mtDNA content of 60 patients with breast cancer and 51 age-matched healthy individuals. The results indicated that the content of mtDNA in stage I breast cancer patients was significantly lower than in other stages, and the quantitative level of mtDNA was not associated with clinicopathological parameters except menopausal status. However, mtDNA content was associated with age, number of pregnancies, and number of live births in the present study. The moderate sample size may limit the statistical power of the previous study to evaluate the relation between mtDNA content and selected clinical variables. Consequently, additional studies are warranted to further elucidate these associations and the molecular mechanisms.

During the process of ROS-associated OXPHOS, certain mtDNA mutations may generate increased superoxide and nitric oxide and lead to aberrant mitochondrial biogenesis. It was reported that mtDNA A10398G (Thr–Ala) polymorphism in the *ND3* gene influenced breast cancer susceptibility in African-American women.[17] The researchers proposed that the 10398A allele may be deleterious in African-American populations because African mitochondrial haplotypes are prone to generate more ROS than mitochondria in other ethnic populations, or because coexistent mutations in other mitochondrial or nuclear genes decrease cellular capacity to manage oxidative stress. However, results of later research regarding the A10398G polymorphism were contradictory, as mentioned above.[21–24] The present study found that the mtDNA A10398G polymorphism was not associated with the risk of breast cancer in Chinese women, even stratified by selected variables. The A10398G genotype varied with histological types and Her2 status of breast cancer patients, but was not associated with other clinical parameters such as age, menopausal status, age at menarche, number of pregnancies, number of live births, tumor size, lymph node metastasis, or hormone receptor status. It seems that both 10398A and 10398G are not deleterious alleles for breast cancer risk in Han Chinese women. A recent meta-analysis, which did not include any research into Chinese populations, showed a similar negative overall correlation between this polymorphism and breast cancer risk.[38]

It was hypothesized that levels of mtDNA copy number might be affected by the intracellular and extracellular environment. Previous studies about tissues of various cancers, including breast cancer, suggested that decreased mtDNA copy number was associated with the occurrence of somatic point mutations located close to the replication origins of the heavy-strand and/or those at the D310 homopolymeric C-stretch (C-tract) in the D-loop region.[10,31,39] On the contrary, some researchers reported that the decreasing process of mtDNA copy number may neither contribute to the shift of homoplasmic/heteroplasmic state of point mutation in mtDNA nor to the decrease in the proportion of mtDNA with 4977-bp deletions in cancer cells.[40] In the present study, the A10398G polymorphism, which was proposed to be involved in function complex I and increased ROS production, was investigated in Chinese women. Unfortunately, the association between the A10398G polymorphism and mtDNA content was not found in the breast cancer cases. The results reflected that this allele might not be a major factor in modulating mtDNA copy number of PBLs in Han Chinese populations. However, it does not mean that the 10 398-bp mutation could not affect mtDNA content in other cancers or other populations. More studies should be carried out to evaluate the relation between these two factors and the mechanism by which the content of mtDNA is precisely modulated under various somatic mutations including point mutations, insertions, and large-scale deletions in the mtDNA genome.

Strengths of our study included its large sample size, and all the participants were enrolled from Hubei province and adjacent areas. This region is highly attractive for carrying out population-based research because of the low mobility rate, which could greatly reduce the potential confounding effects of the heterogeneous participant characteristics in most case–control studies. Moreover, the blood samples of cases were obtained before they underwent surgery, chemotherapy, radiotherapy, or other treatments, so we can exclude treatment-induced effects on somatic mutation and content of mtDNA. However, our study has unavoidable limitations. A limitation intrinsic to our study design is that it was restricted to Han Chinese women; the results of other ethnic groups need further evaluation. Like other case–control studies, we cannot determine whether the association between risk of breast cancer, clinical parameters, and peripheral blood mtDNA copy number reflects an etiologic mechanism.

In conclusion, our case–control study offers evidence that peripheral blood mtDNA content might be useful as a tool to estimate risk of breast cancer for selected Chinese women. The risk of breast cancer was associated with decreased mtDNA content in premenopausal women. In contrast, increased mtDNA content was associated with breast cancer risk in aged and postmenopausal women. Our data also showed that the mtDNA content in PBLs is associated with several clinical variables such as age, menopausal status, number of pregnancies, and live births in breast cancer cases. More research is warranted to further elucidate the molecular mechanisms of these associations.

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Disclosure Statement

The authors have no conflict of interest.

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