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Original Article

Length heteroplasmies in human mitochondrial DNA control regions and breast cancer risk

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Abstract: It has been proposed that the presence of heteroplasmy in the hypervariable (HV) regions of the mitochondrial DNA (mtDNA) may be an indicator of mitochondrial genome instability, mtDNA dysfunction, and, thus, may be associated with increased cancer risk. However, whether heteroplasmy in the HV regions of mtDNA could be a risk predictor of oxidative stress-related human cancers, such as breast cancer, remains to be determined. To explore the role of heteroplasmy in the HV regions of mtDNA in breast cancer etiology, we analyzed heteroplasmy in the HV regions of mtDNA in whole blood from 103 patients with breast cancer and 103 matched control subjects. Both cases and controls displayed heteroplasmies in both of the HV1 and HV2 regions. Closer examination of the prevalence of length heteroplasmy indicated that the prevalence of heteroplasmies in both of the HV1 and HV2 regions was much higher in the cases than in the controls (HV1: 68% vs 49%, P=0.007; HV2: 46% vs 25%, P=0.002). The presence of length heteroplasmies in both of the HV1 and HV2 regions was associated with 2.18- and 2.49-folds increased risk of breast cancer, respectively, (HV1: OR=2.18, 95% CI: 1.19 - 4.00; HV2: OR=2.49, 95% CI: 1.32 - 4.69). Interestingly, we observed that the controls with length heteroplasmies in both HV1 and HV2 had statistically significantly lower copy number of mtDNA than the ones without heteroplasmies. These results suggest that the length heteroplasmy in the HV regions of mtDNA could be associated with a risk of breast cancer, perhaps through affecting the copy number of mtDNA.

Keywords: Mitochondrial DNA, length heteroplasmies, copy number, breast cancer

Introduction

Human mitochondrial DNA (mtDNA) is a closed circular duplex molecule. mtDNA molecules have a noncoding control region that includes a unique displacement loop (D-loop) containing replication and transcription controls [1-3]. This control region of human mtDNA has been shown to be highly polymorphic, probably due to high mutation rate [4]. Because mtDNA is maternally inherited and the mitochondria in the ovum are randomly dispersed among the daughter cells, the coexistence of wild-type and mutant mtDNA in the same cell and tissue, namely heteroplasmy, has been widely observed [5-7]. Studies have reported marked mtDNA length heteroplasmy in the control region among many cell types, including individual granulocytes and CD34 cell clones obtained from adult bone marrow and peripheral blood [3, 7]. The control region includes two hypervariable (HV) regions (HV1 and HV2), so called because of their high incidence of nucleotide variations [3]. Many mtDNA length heteroplasmies are localized in the HV2 homopolymeric cytidine (poly-C) tract that lies between nucleotide (nt) 303–315. Another poly-C tract variant is located between nt 16,184 and 16,193 in the HV1 region. A common mutation in these areas changes the thymidine (T) to a cytodine (C) and produces a long stretch of C's (called the C-stretch), which has been noted to be difficult to sequence beyond [8].

The presence of heteroplasmy in the HV regions may be an indicator of mitochondrial genome instability and mtDNA malfunction and, thus, may be associated with increased cancer risk. This hypothesis is supported by recent observation that a severe frameshift in the 303-315 poly-C tract my cause the impairment of mtDNA replication in hematopoietic tissue [9]. Previous

studies of mtDNA variation and cancer risk have focused on single nucleotide polymorphisms (SNPs) or point mutations [10-18]. The association between mtDNA length heteroplasmies in the HV regions and cancer risk is unclear. Heteroplasmy in the HV regions and genetic instability in mtDNA might be extremely relevant to breast cancer because mitochondrial linked oxidative stress has been suggested to play a significant role in breast cancer etiology [19-24]. So far, whether heteroplasmies in the HV regions in nonneoplastic tissues, such as whole blood (leukocytes), is a possible risk factor for breast cancer is unknown. We thereby investigated heteroplasmies in the HV regions measured in whole blood DNA as a possible biomarker for risk of breast cancer.

Materials and methods

Study population

Anonymised biospecimens and questionnaire data used in this study were made available through the Roswell Park Cancer Institute's (RPCI) Data Bank and BioRepository (DBBR) [25]. The DBBR is a Cancer Center Shared Resource, and is a biorepository of blood samples collected, processed and stored in a rigorous, standardized manner and linked to clinical and epidemiological data. Patients are enrolled through site-specific clinics prior to surgery and/or chemotherapy, and controls are individuals who are free from cancer and 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, and lifestyle habits including diet, use of dietary supplements, smoking, physical activity and alcohol intake. Additionally, demographic data and height and weight from young adulthood to present are collected for each participant. Blood samples are drawn in phlebotomy and transferred to the DBBR laboratory where specimens are processed and aliquoted into 0.5ml 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, a total of 103 women with breast cancer and 103 cancer-free women were included in the analysis.

Gene scan analysis for the determination of HV region length heteroplasmies

In order to examine the qualitative and quantitative profiles of the poly-C mtDNA length heteroplasmies in the HV1 and HV2 regions, the forward primer 5'-CTTTCCACACAG ACATCATAAC-3' and reverse primer 5'-ATCTGGTTAGGCTGGTGT TAG-3' along with the forward primer 5'-CTTGACCACCTGTAGTACATA-3' and reverse primer 5'-GGAGT TGCAGTTGATGTGTGA-3' were used, respectively. Each forward primer was labeled with Hex fluorescent dye. mtDNA products encompassing the poly-C tracts were amplified in a 50 μl reaction mixture containing 1 ng of total DNA, 0.8 mM primer pairs, 400 mM of each dNTP, 1 U *Taq* DNA polymerase (Promega, WI), and 5 μl of 10x buffer and distilled water. For PCR, the 1 min initial denaturation at 96°C was followed by 32 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. After the completion of PCR, 1 μl of each PCR product and 0.5 ml of the gene scan internal size standard labeled with fluorescent dye ROX (Applied Biosystems) were added to 20 μL of deionized formamide. Denaturation was then conducted at 96°C for 10 min, followed by a cooling step at -20°C for 2 min. The denatured PCR products were separated *via* CE using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) and Gene Scan Analysis Software (version 3.1). With this software, one can automatically size the fragment length of the specific PCR product as well as quantify its amount by calculating the area under the fragment peak. Each sample was analyzed by at least two different PCR reactions in order to ensure accurate and repeatable results.

Sequencing analysis

To further confirm the variant alleles, we performed sequencing analysis in the DNA samples which exhibited heteroplasmy in the hypervariable (HV) regions. The same PCR primers (without Hex fluorescent dye) used in the gene scan analysis were used for sequencing. The same PCR reactions were carried out. The am-

plified PCR products were sequenced using the dideoxynucleotide chain termination method. Both strands of the amplified PCR products were sequenced with an ABI-PRISM 3730xl Autosequencer (Applied Biosystems, USA) in the Roswell Park Cancer Institute (RPCI) Biopolymer Core. For quality control, random duplicate samples (5%) were run for each sequence analysis.

Statistical analysis

The distributions of demographic variables between the cases and controls were compared using the Chi-square (χ^2) test for categorical data and the rank-sum test for continuous variables. For HV region length heteroplasmies, the frequency of each length heteroplasmy was calculated. The Chi-square (χ^2) 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. To assess the strength of the association between breast cancer risk and HV region length heteroplasmies, we calculated the odds ratio (OR) and its 95% confidence interval (CI) using unconditional logistic regression analysis. Potential confounders were tested at the p=0.10 level and only age significantly affected risk and was included in the model. Mann–Whitney U tests were utilized for the determination of statistical differences in the mtDNA copy numbers between the two groups (the group without HV region length heteroplasmies and the group with HV region length heteroplasmies). All P values were two-sided. Associations were considered statistically significant at $P<0.05$.

Results

The characteristics of the study population are summarized in **Table 1**. The case patients and control subjects were well-matched on ethnicity (*P*=1.00), menopausal status (*P*=1.00), and age (*P*=0.68). There were no statistically significant differences between the case patients and the controls subjects in terms of daily alcohol intakes (*P*=0.65), smoking history (*P*=0.78), family history of breast cancer (*P*=0.57), current fruit intake (*P*=0.56), current vegetable intake (*P*=0.54), current exercise (*P*=0.74), and BMI (*P*=0.32).

The distribution of HV region length heteroplasmy was statistically significantly different between breast cancer cases and controls (P=0.038 for the Poly-C tract in HV1 region and P=0.022 for the Poly-C tract in HV2 region) (**Table 2**). Consistent with the literatures, 5CT4C was the most frequent Poly-C tract in the HV1 region in both cases and controls. More controls (50%) than cases (31%) displayed only one pattern of the poly-C tract in HV1 region (P=0.007), namely they didn't exhibit length heteroplasmy. Interestingly, all of them displayed the 5CT4C tract. About 50% of the controls and 69% of the cases exhibited length heteroplasmy in the HV1 region (more than one pattern of Poly-C tract). A total of 10 different patterns of length heteroplasmies were observed, including 5CT4C, 5CT4C+5CT3C, 9C+10C+11C, 3CT4C+3CT3C, 3CT6C+3CT5C, and 5 others.

Similar patterns were observed for the Poly-C tract in the HV2 region. As expected, 7CT6C was the most frequent pattern of the Poly-C tract in both cases and controls. More controls (75%) than the cases (54%) displayed only one pattern of the Poly-C tract (7CT6C) in the HV2 region (P=0.002), namely they didn't exhibit length heteroplasmy. About 25% of the controls and 46% of the cases exhibited length heteroplasmy in the HV2 region (more than one pattern of poly -C tract). A total of 6 different patterns of length heteroplasmies were observed, namely 7CT6C, 7CT6C+8CT6C, 8CT6C+9CT6C, 8CT6C+9CT6C+10CT6C, 9CT6C+10CT6C+11CT6C, and 7CT6C +6CT6C. The samples which showed length heteroplasmies in either HV regions were further confirmed in the sequencing analysis.

In the breast cancer risk analysis presented in **Table 3**, subjects who exhibited length heteroplasmies in the HV1 region in comparison to those who did not, had a 2.18-fold increased risk of breast cancer (OR = 2.18, 95% CI: 1.19 to 4.00) after adjustment for age. In further stratified analysis to investigate the associations between the specific patterns of length heteroplasmies and breast cancer risk, we found that the risk was increased to 3.98 (95% CI: 1.18, 9.01) for those who exhibited 3CT6C+3CT5C length heteroplasmies. Similar findings were observed for the HV2 region. Using individuals who didn't exhibit length heteroplasmies in the HV2 region as the reference

Table 1. Distribution of selected characteristics of cases and controls

Characteristics	Subjects Cases (n=103) N(%) Median	Controls (n=103) N (%) Median	P Value
Ethnicity			
White	93 (90.3)	93 (90.3)	1.00[1]
Black	10 (9.7)	10 (9.7)	
Menopausal Status			
Premenopausal	31 (30.1)	32 (31.1)	
Postmenopausal	72 (69.9)	71 (68.9)	1.00[1]
Daily Alcohol Intake			
No drink per day	28 (27.2)	25 (24.3)	
0.5-1 drink per day	62 (60.2)	73 (70.9)	
> 1 drink per day	13 (12.6)	5 (4.9)	0.65[2]
Ever Smoked 100 cigarettes			
No	52 (50.5)	51 (49.5)	
Yes	50 (48.5)	49 (47.6)	
Don't' Know	0 (0)	2 (1.9)	0.78[2]
Family History of Breast Cancer			
No	84 (81.6)	88 (85.4)	
Yes	19 (18.4)	15 (14.6)	0.57[1]
Current Vegetable Intake			
< Once per week	6 (5.9)	5 (4.9)	
1-6 per week	54 (52.4)	55 (53.4)	
> 1 per day	41(39.8)	42 (40.8)	0.95[2]
Current Fruit Intake			
< Once per week	13 (12.6)	11 (10.7)	
1-6 per week	44 (42.7)	44 (42.8)	
> 1 per day	44 (42.7)	47 (45.7)	0.88[2]
Current Exercise (20 minutes)			
Never	33 (32)	37 (35.9)	
< Once per week	30 (29.1)	22 (21.4)	
1-2 per week	22 (21.4)	34 (33)	
3-4 per week	16 (15.5)	9 (8.7)	0.74[2]
Age (years)	58	56	0.68[3]
BMI	27.79	26.43	0.32[3]

[1.] Fisher Exact Analysis was used to examine differences. [2.] Chi square was used to examine differences. [3.] Mann Whitney U was used to examine difference.

group, those who exhibited length heteroplasmies in the HV2 region had a 2.49-fold increased risk of breast cancer (OR = 2.49, 95% CI: 1.32 to 4.69) after adjustment for age.

To explore the impact of HV region length heteroplasmies on the biological function of the mitochondrial, we compared the copy number of mtDNA between individuals with or without

Table 2. Distribution of poly-C length heteroplasmy in HV1 and HV2 regions of mtDNA of cases and controls

Location of poly-C tract	Reference sequence	Patterns	Cases, N (%)	Controls, N(%)	P value[1]
nt 16184-16193 in HV1	CCCCCTCCCC (5CT4C)	5CT4C (no length hetero-plasmy)	32 (31%)	51 (50%)	
		5CT4C + 5CT3C	20 (19%)	19 (18%)	
		9C + 10C +11C	13 (13%)	15 (14%)	
		3CT4C + 3CT3C	10 (10%)	5 (5%)	
		3CT6C + 3CT5C	10 (10%)	4 (4%)	
		others	18 (17%)	9 (9%)	0.038
nt 303-315 in HV2	CCCCCCCTCCCCCC (7CT6C)	7CT6C (no length hetero-plasmy)	56 (54%)	77 (75%)	
		7CT6C + 8CT6C	24 (23%)	18 (17%)	
		8CT6C + 9CT6C	12 (12%)	6 (6%)	
		8CT6C + 9CT6C + 10CT6C	6 (6%)	2 (2%)	
		9CT6C + 10CT6C + 11CT6C	3 (3%)	0 (0%)	
		7CT6C + 6CT6C	2 (2%)	0 (0%)	0.022

[1]. Chi square was used to examine differences.

Table 3. Breast cancer risk estimates of poly-C length heteroplasmy in HV1 and HV2 regions of mtDNA

Location of poly-C tract	Patterns	Cases, N (%)	Controls, N(%)	OR[1] (95%CI)
nt 16184-16193 in HV1	5CT4C (no length heteroplasmy)	32 (31%)	51 (50%)	Reference
	5CT4C + 5CT3C	20 (19%)	19 (18%)	1.68 (0.72, 3.88)
	9C + 10C +11C	13 (13%)	15 (14%)	1.38 (0.53, 3.57)
	3CT4C + 3CT3C	10 (10%)	5 (5%)	3.19 (0.88, 12.86)
	3CT6C + 3CT5C	10 (10%)	4 (4%)	3.98 (1.02, 18.63)
	others	18 (17%)	9 (9%)	3.19 (1.18, 9.01)
	total length hetero-plasmy	71 (69%)	52 (50%)	2.18 (1.19, 4.00)
nt 303-315 in HV2	7CT6C (no length heteroplasmy)	56 (54%)	77 (75%)	Reference
	7CT6C + 8CT6C	24 (23%)	18 (17%)	1.83 (0.86, 3.94)
	8CT6C + 9CT6C	12 (12%)	6 (6%)	2.75 (0.88, 9.43)
	others	11 (11%)	2 (2%)	7.56 (1.54, 71.97)
	total length hetero-plasmy	47 (46%)	26 (25%)	2.49 (1.32, 4.69)

[1].Odds ratio was adjusted by age only.

Table 4. Correlations between poly-C length heteroplasmy in HV1 and HV2 regions of mtDNA and mtDNA copy number

		Median mtDNA copy number	
	Patterns	cases	controls
nt 16184-16193 in HV1	5CT4C	1.22	0.89
	variant allele	1.3	0.7
	P value	0.34[1]	0.027[1]
nt 303-315 in HV2	7CT6C	1.19	0.88
	variant allele	1.34	0.72
	P value	0.18[1]	0.032[1]

[1]Mann Whitney U was used to examine difference.

length heteroplasmies (**Table 4**). The mtDNA copy number was obtained from our previous publication [26]. Compared to those without length heteroplasmies in the HV1 region, subjects with length heteroplasmies in the HV1 region had a statistically significantly lower copy number of mtDNA (0.89 vs 0.70, P=0.027). Similarly, compared to those without length heteroplasmies in the HV2 region, samples with length heteroplasmies in the HV2 region had a statistically significantly lower copy number of mtDNA (0.88 vs 0.72, P=0.032). No significant difference was observed in the breast cancer cases.

Discussion

To the best of our knowledge, this is the first study of the relationship between mtDNA HV region length heteroplasmies measured in peripheral whole blood DNA and the risk of breast cancer. In our study, we found that mtDNA HV region length heteroplasmy was associated with a statistically significantly increased risk of breast cancer. In addition, we observed that mtDNA HV region length heteroplasmy was associated with a decreased copy number of mtDNA, suggesting a role of mtDNA genetic instability in the determination of blood mtDNA copy number.

Although the impact of mtDNA HV region length heteroplasmy on human diseases is still unclear, deletions [27-28] or point mutations [29-30] in mtDNA are the most common genetic defects seen in individuals with mtDNA-associated diseases. For example, single mtDNA deletions are a common cause of sporadic mitochondrial disease; in these cases, an identical mtDNA deletion is detected in all cells within an affected tissue [31]. In another large group of individuals with mitochondrial disease, there are multiple mtDNA deletions in affected tissues, particularly in the muscle and the central nervous system [32-33]. In addition, there are numerous reports of mtDNA deletions in aged postmitotic tissues and individuals with neurodegenerative diseases [34-35]. It has been estimated that approximately 50% of all mtDNA molecules contain an mtDNA deletion [34-35].

Mitochondria have been theorized to perform a critical role in cancer development. The mtDNA mutation rate is at least ten-fold higher than that of nuclear DNA. This higher mutation rate has been attributable to the lack of protection by histones, less efficient DNA repair capacity, proximity to reactive oxygen species generated by the electron transport chain, and unique structural characteristics which may favor mutational events [36]. Thus, considering the potential role of oxidative stress in breast carcinogenesis, our finding that mtDNA HV region length heteroplasmies were associated with an increased risk of breast cancer is not surprising at all. Reports with regard to an accumulation of mtDNA mutations in the blood have, so far, proven contradictory. Some studies have found that the numbers of deletions and point muta-

tions in blood cells tends to be substantially smaller than the numbers found in postmitotic tissues [3]. However, in a Korean population study, Shin *et al* found that mtDNA HV region length heteroplasmies occur frequently in healthy subjects [37]. Our results are consistent with their observations.

In the HV1 region, a homopolymeric cytidine (poly-C) tract is seen in the nt 16,184–16,193 poly-C region, in which the thymidine (T) at nt 16,189 becomes a cytodine (C), thereby generating a stretch of ten or more C's. Previous studies suggest that this polymorphism may confer a predisposition to diabetes mellitus, lower birth weight, and dilated cardiomyopathy [3]. However, a significant number of individuals display this particular substitution; for example, the Federal Bureau of Investigation database indicates that about 9.8% of Caucasians and 12.6% of African Americans exhibit this C-stretch. Consistent with these findings, we observed that 13% of breast cancer cases and 14% of healthy controls have this C stretch (indicated as 9C+10C+11C in this study). Additionally, we observed several other types of variant patterns in this poly-C stretch, including deletion and insertion, etc. Although we don't know the exact biological significance of this poly-C tract, we observed a significant difference in pattern distribution between breast cancer cases and healthy controls (P=0.038).

The nt 303–315 poly-C stretch in the HV2 region is composed of 12–18 C bases, which is usually interrupted by a T base at position 310. This region exhibits polymorphic length variations among different individuals, as well as heteroplasmic variation within an individual [2, 38]. Moreover, this region has been determined to be a "hot spot" for somatic mutations in a variety of cancers [39-40]. Since the 303–315 poly-C region resides in a conserved sequence block believed to control mtDNA replication and transcription, it is possible that the 303–315 poly-C tract length variations may play a role in the regulation of mtDNA metabolism. Closer examination of the HV2 length heteroplasmies found in our study subjects indicated that 46% of breast cancer cases and 25% of healthy controls exhibited length heteroplasmies and we observed a significant difference in the pattern distribution between breast cancer cases and healthy controls (P=0.022). This suggests that length heteroplasmy might lead to changes in mtDNA function and ultimately modify breast cancer risk.

In a recent publication [26] using the same study population, we observed that mtDNA copy number is significantly higher in breast cancer cases than in controls. The copy number of mitochondria reflect the net results of gene-environmental interactions between unknown hereditary factors and the levels of oxidative stress (an imbalance between reactive oxygen species (ROS) production and the antioxidant capacity), caused by a variety of endogenous and exogenous factors. Therefore, the case-control difference in mtDNA copy number might reflect the possible case-control difference of oxidative stress. In this study, we observe that the cases exhibiting length heteroplasmies in both HV1 and HV2 had slightly higher copy number of mtDNA than the ones without length heteroplasmies, although the difference didn't reach statistical significance. In contrast, we noticed that the controls exhibiting length heteroplasmies in both HV1 and HV2 had statistically significantly lower copy number of mtDNA than the ones without length heteroplasmies. Considering that the length heteroplasmies in the HV regions might lead to genomic instability in the mitochondria, our observation might suggest that mtDNA HV region length heteroplasmies results in decreased mtDNA copy number and consequently reduces the cell's ability to combat oxidative stress resulting in an increase in the risk of breast cancer.

In summary, our study provides the first evidence that mtDNA HV region length heteroplasmies is statistically significantly higher in breast cancer cases compared to the healthy controls. Results also indicate the role of mtDNA HV region length heteroplasmies in the determination of mtDNA copy number. Because the study is relatively small, and also the nature of the case-control study design, replication in a large study is necessary, preferably in prospective cohort studies. Nevertheless, our study indicates that mtDNA HV region length heteroplasmies might be a potentially useful tool in studying the etiology of breast cancer. The knowledge from this study will have the potential for clinical application because it provides a means of identifying a subgroup that are most likely to develop breast cancer. Such individuals may then be targeted for specific intervention programs such as chemoprevention and dietary modification.

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