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Mitochondrial DNA copy number and risk of gastric cancer: a report from the Shanghai Women's Health Study

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Abstract

Background—Mitochondrial DNA (mtDNA) are approximately 16,000bp circular double-stranded DNA molecules that are prime targets of oxidative damage. Several somatic mutations in mtDNA have been observed in gastric tumors suggesting an involvement in gastric cancer risk and progression. mtDNA copy number in leukocyte DNA has also been linked to several other cancers, although the temporal relationship between mtDNA and cancer has not been adequately explored.

Methods—Using a nested case-control study design, we examined the association between mtDNA copy number in 162 GC cases and 299 matched controls within the Shanghai Women's Health Study, a large population-based prospective cohort. Relative mtDNA copy number was measured in triplicate by a quantitative real-time PCR assay in peripheral leukocytes.

Results—mtDNA copy number levels were comparable among cases and controls, with a median of 1.04 (interquartile range, 0.87–1.25) and 1.06 (interquartile range, 0.88–1.29), respectively. Overall, mtDNA was not associated with GC risk. However, the association differed when stratified by the time between sample collection and cancer diagnosis. An association between low levels of mtDNA copy number (<median) and GC risk was apparent among earlier diagnosed cases, in particular, those diagnosed within two years of sample collection (odds ratio=5.32; 95%CI:1.03–27.60). This association was not present as the time between sample collection and cancer diagnosis increased.

Conclusions and Impact—Our findings suggest that there is no association between leukocyte mtDNA copy number and risk of GC; however, we observed a possible early disease effect on mtDNA copy number levels.

Keywords

Mitochondrial DNA copy number; mtDNA; gastric cancer; cohort study

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Introduction

Mitochondria are organelles found in all nucleated cells. The key role of mitochondria is to generate cellular ATP through oxidative phosphorylation.(1) Each cell contains several hundred to a thousand mitochondria and each of the mitochondria carry about 2–10 copies of mitochondrial DNA (mtDNA), resulting in approximately several hundred to thousand copies of mtDNA per cell. mtDNA are approximately 16,000 bp circular double-stranded DNA molecules that are a prime target of oxidative damage due to their proximity to the electron transport chain.(2) Compared with nuclear DNA, a higher rate of mutations occurs in the mtDNA. This has been ascribed to the high rate of reactive oxygen species generated nearby, the lack of protective histones and limited DNA repair capacity.(3)

Several somatic mutations in the mtDNA have been observed in gastric tumors, including a very large deletion of 4,977 bp and mutations in the D-loop region.(4, 5) The D-loop is a non-coding region of mtDNA that controls replication and transcription of mtDNA. Mutations in the D-loop may thus cause a decrease in mtDNA copy number or altered mtDNA gene expression.(6) It has been hypothesized that mutations or decreases in mtDNA copy number could lead to a deficiency in oxidative phosphorylation and enhanced generation of ATP by glycolysis.(6) *H. pylori* infection, one of the main risk factors for gastric cancer, also seems to induce mtDNA mutations in gastric cells.(7, 8) Several studies have reported a depletion in mtDNA copy number in gastric tumors compared to non-tumorous gastric tissues.(9–11)

Given the findings from experimental studies, we conducted a nested case-control study within a large prospective cohort of women residing in Shanghai, China, to evaluate whether mtDNA copy number measured in leukocyte DNA could be a potential indicator of risk of gastric cancer.

Materials and Methods

Study population

The Shanghai Women's Health Study is a population-based prospective cohort study of women residing in Shanghai, China. A detailed description of the study methodology is available.(12) In brief, 74,942 women aged 40–70 years residing in Shanghai were recruited into the study from 1996 to 2000. At baseline, detailed in-person interviews were conducted by trained interviewers to collect questionnaire information, yielding a response rate of 93%. Data were collected on demographic characteristics, personal habits, dietary habits, water drinking, physical activity, residential history, occupational history, family history of cancer, disease and surgery history, menstrual history, reproductive history and hormone use, and weight history. Body measurements were also taken at baseline. *H. pylori* infection was determined using *H. pylori* enzyme-linked immunosorbent assay kits (Biohit ELISA kit, Finland) to detect serum IgG antibodies. In follow-up surveys, interviewers were able to interview and follow-up with 99.8% (2000–2002), 98.7% (2002–2004), and 96.7% (2004–2007) of cohort members or their next of kin.

Of the eligible participants, approximately 75% donated a blood sample at baseline. A 10 ml blood sample was drawn into an EDTA Vacutainer tube. Samples were kept cold (0–4°C) and processed within 6 hours for long-term storage at −70°C. DNA was extracted from peripheral leukocytes by the standard phenol chloroform method. Included in this nested case-control study are 162 incident gastric cancer cases and 299 matched controls who provided a blood sample at the baseline survey. Incident gastric cancer cases were identified through in-person follow-up interviews and by linking to the Shanghai Cancer Registry and

the Shanghai Vital Statistics Unit. Controls were randomly selected from cohort members and matched to cases by age at sample collection (±2 years), menopausal status, time of sample collection (morning or afternoon), date of sample collection (±1 month) and time interval since last meal (±2 hours). Controls were also free of any cancer at the time of cancer diagnosis for their corresponding case. Given this sample size, our study had sufficient power (80%) to detect a minimum OR of 2.1.

Mitochondrial DNA Copy Number Assay

Relative mtDNA copy number levels were measured in samples by a quantitative real-time PCR assay.(13–15) In brief, relative mtDNA copy number is measured by determining the ratio of mitochondrial (Mt) copy number to single copy nuclear gene (S) copy number in experimental samples relative to reference DNA. The ratio is proportional to the mtDNA copy number in each cell. The single copy nuclear gene used in this study was human hemoglobin, beta (HBB). Pooled DNA from 30 randomly selected SWHS participants served as the reference DNA pool used to create in every Mt and S PCR run a fresh standard curve on which the system is calibrated, which ranged from 0.25 ng/μl to 20 ng/μl. All PCRs were performed on a 7900HT Fast real Time PCR System (Applied Biosystems, Foster City, CA). All samples were run in triplicate and the average of all three measurements was calculated. Reasonably high reproducibility was observed with this assay with a CV of 8% and an ICC of 81% on blinded duplicate samples.

Statistical Analyses

Differences between cases and controls, and levels of demographic and exposure characteristics were estimated using Wilcoxon non-parametric tests for continuous variables and ANOVA tests for categorical variables. To identify potential determinants of mtDNA copy number levels and/or factors that could modify the association between mtDNA copy number levels and gastric cancer risk, Wilcoxon non-parametric and ANOVA tests were used to evaluate differences among controls in relation to selected characteristics. The distribution of mtDNA copy number among controls was used to determine cut points for quartiles and median. Quartiles were collapsed to two categories, above and below the median, for stratified analyses due to small numbers. Conditional logistic regression, adjusted for potential risk factors for gastric cancer: age, body mass index (BMI: kg/m^2), education, fruit and vegetable intake, smoking status, recent NSAID use, and family history of gastric cancer, was used to estimate odds ratios (OR) and 95% confidence intervals (95% CI). Tests for trend were calculated by modeling a variable coded 0, 1, 2 and 3. Sensitivity analyses included additional adjustments for *H. pylori* and stratified analysis by time between blood sample collection and cancer development. All analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC).

Results

The characteristics of the 162 gastric cancer cases and 299 control subjects in this study are provided in Table 1. There were no significant differences between cases and controls for any of the selected characteristics. Few of the women smoked, drank alcohol, used non-steroidal anti-inflammatory drugs (NSAID) or multivitamins in this study. A large proportion of the women, both cases and controls, tested positive for *H. pylori* IgG antibodies. Overall, mtDNA copy number was not statistically different between gastric cancer cases and controls with medians of 1.04 and 1.06, respectively (Wilcoxon p-value=0.51).

We also evaluated whether mtDNA copy number levels differed by selected characteristics (Table 2). Overall, we found no differences in mtDNA copy number levels by smoking

status, alcohol use, obesity, physical activity, family history of gastric cancer, NSAID or multivitamin use, *H. pylori* status, age, fruit and vegetable or meat intake among the controls. However, several of these comparisons could be limited by small numbers in certain strata. When characteristics were evaluated continuously, only age was inversely correlated with mtDNA with a spearman correlation of −0.13 (p=0.02).

Overall, mtDNA copy number was not significantly associated with gastric cancer risk (Table 3). Compared to the highest quartile of mtDNA copy number, those with the lowest quartile of mtDNA copy number were associated with an OR of 1.05 (95% CI: 0.58–1.90; p-trend=0.69). Additional adjustment for *H. pylori* status (one of the main risk factors for gastric cancer), which was only available on a subset of the samples (n=378 subjects), did not change the effect estimates appreciably.

However, analysis stratified by the interval between blood collection and cancer diagnosis (reference date for controls) revealed a different pattern (Table 4). Among those diagnosed within 2 years of sample collection, low mtDNA was associated with an OR of 5.32 (95% CI: 1.03–27.60). Subsequent time periods did not reveal any significant associations between mtDNA copy number and risk of gastric cancer. After cases diagnosed within the first two years of blood sample collection were excluded, an association between mtDNA copy number and gastric cancer was no longer present.

Discussion

In this prospective study, we found no overall association between mtDNA copy number and risk of gastric cancer. However, a positive association between low mtDNA copy number in blood drawn within the two years prior to cancer diagnosis and risk of gastric cancer was observed. These findings suggest that low levels of mtDNA copy number could be an indicator of impending gastric cancer diagnosis, but our results are preliminary given our relatively small sample size.

mtDNA copy number variations in leukocyte DNA have been observed in several types of cancer.(14, 16–19) Thus far, results from these five studies are somewhat mixed. The majority of these studies have reported an increased risk of cancer with higher mtDNA copy number, but at varying definitions/levels of “high” mtDNA copy number. Xing et al. was the single study that observed an association with lower mtDNA content and renal cell cancer risk.(14) Three of these studies have been conducted within case-control studies and two studies were nested within prospective cohort studies.(18, 19) The mechanism through which altered mtDNA copy number plays a role in carcinogenesis still remains unclear, but it has been demonstrated in several studies that mtDNA depletion can alter mitochondrial gene expression and lead to a deficiency in oxidative phosphorylation and enhanced generation of ATP by glycolysis.(11) The positive association we observed with lower levels of mtDNA copy number among cases diagnosed within two years of blood collection is consistent with the mtDNA depletion that has been reported in studies of gastric and other tumors.(9–11, 20) However, to our knowledge, data are not available on the correlation between mtDNA copy number in leukocytes and gastric tissue.

The association we observed between low mtDNA copy number and increased risk of gastric cancer appears to fade as the time between blood sample collection and cancer diagnosis increases. As the association appears to be particularly strong among cases diagnosed within 2 years of blood sample collection, it is possible that the association may be driven by low mtDNA copy number among undiagnosed gastric cancers at the time of blood sample collection. One could postulate that in a retrospective case-control study setting, a similar association to what we observed among those diagnosed within 2 years of

blood sample collection may be detected. The timing of when the blood sample was taken in relation to gastric cancer development seems to be an important issue in the measurement of mtDNA copy number.

To the best of our knowledge, this is the first study to evaluate mtDNA copy number measured in leukocyte DNA and risk of gastric cancer, as well as the first to examine the association prospectively by years between blood collection and cancer diagnosis. Strengths of our study include measurement of mtDNA copy number in triplicate and the collection of prospective blood samples from study subjects when they were healthy. In addition, there was a high proportion of follow-up (99.8%) and a large proportion of the cohort providing prediagnostic blood samples. Due to the relatively small size of our study, we were unable to evaluate potential interactions with other gastric cancer risk factors.

In conclusion, we observed no overall association between leukocyte mtDNA copy number levels and risk of gastric cancer. Through stratified analyses, we observed a possible indication of early disease with lower mtDNA copy number levels. As our study was limited by a small sample size, the relation between mtDNA copy number and gastric cancer should be further explored in a larger study with prospective samples in order to confirm these findings.

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Table 1

Distribution of selected characteristics for women from the Shanghai Women s Health Study

Characteristics	Cases (n= 162)	Controls (n= 299)	p-value
Ever smoking, N(%)	9 (5.6)	13 (4.4)	0.56
Ever alcohol use, N(%)	3 (1.9)	6 (2.0)	0.91
Body mass index (kg/m2), N(%))			
<25 kg/m2	88 (54.3)	186 (62.2)	
≥25 and <30 kg/m2	64 (39.5)	97 (32.4)	
≥ 30 kg/m2	10 (6.2)	16 (5.4)	0.26
Regular moderate or vigorous physical activity (> 3 MET h/day), N(%))	20 (26.3)	31 (24.2)	0.74
Education, N(%)			
High school or more	39 (24.1)	94 (31.4)	
Less than High school	123 (75.9)	205 (68.6)	0.10
Family history of gastric cancer, N(%)	14 (8.6)	19 (6.4)	0.36
NSAID use, within past year, N(%)	4(2.5)	7(2.3)	0.93
Regular multiple vitamin use, N(%)	11 (6.8)	22 (7.4)	0.82
H.pylori positive*, N(%)	129 (96.3)	226 (92.6)	0.16
Age, years, median (IQR)	61 (50–65)	61 (50–65)	0.80
Fruits/vegetables intake, g/d, median (IQR)	452.5 (297.2–635.1)	485.4 (348.6–697.7)	0.12
Fruits, g/d, median (IQR)	211.5 (92.4–320.3)	227.6 (120.4–352.4)	0.17
Vegetables, g/d, median (IQR)	242.1 (156.0–372.4)	250.0 (177.1–357.8)	0.32
Meat intake, g/d, median (IQR)	38.7 (21.7–59.4)	37.3 (23.9–56.3)	0.93
mtDNA copy number, median (IQR)	1.04 (0.87–1.25)	1.06 (0.88–1.29)	0.51

Continuous variables are displayed as medians (Interquartile range) and frequencies are displayed as counts (percentage)

Comparison of cases and controls conducted using chi-square test for categorical variables and Wilcoxon non-parametric test for continuous variables

*Available on only 378 subjects

Table 2

mtDNA copy number levels by selected characteristics

		Controls				
Characteristic	**N**	**Mean**	**25%**	**Median**	**75%**	**p-value**
Smoking status						
Never	286	1.13	0.88	1.07	1.28	
Ever	13	1.02	0.83	0.96	1.36	0.54
Alcohol use						
Never	293	1.12	0.88	1.06	1.28	
Ever	6	1.41	0.95	1.38	1.53	0.23
Body mass index (kg/m2)						
<25 kg/m2	186	1.15	0.90	1.08	1.30	
≥25 and <30 kg/m2	97	1.08	0.84	1.04	1.23	
≥ 30 kg/m2	16	1.09	0.90	1.01	1.36	0.39
Regular moderate or vigorous physical activity						
≤ 3 MET h/day	97	1.12	0.86	1.05	1.27	
>3 MET h/day	31	1.08	0.92	1.01	1.21	0.58
Family history of gastric cancer						
No	280	1.13	0.88	1.06	1.29	
Yes	19	1.13	0.97	1.08	1.39	0.45
NSAID use, within past year						
No	292	1.12	0.88	1.06	1.29	
Yes	7	1.23	1.05	1.16	1.30	0.27
Regular multiple vitamin use						
No	277	1.13	0.88	1.06	1.28	
Yes	22	1.11	0.91	1.16	1.31	0.78
H.pylori positive *						
No	18	1.10	0.76	1.04	1.28	
Yes	226	1.16	0.90	1.12	1.33	0.28
Age at baseline, years						
<50	68	1.16	0.89	1.10	1.36	

	Controls					
Characteristic	**N**	**Mean**	**25%**	**Median**	**75%**	**p-value**
50–60	70	1.19	0.86	1.16	1.34	
>60	161	1.08	0.87	1.02	1.23	0.14
Fruits/vegetables intake, g/d						
<median	149	1.12	0.87	1.03	1.25	
≥ median	150	1.13	0.89	1.08	1.31	0.39

P-value comparing mtDNA copy number between different levels of characteristic (Anova and wilcoxon)

*H. pylori status available on 244 controls only

Table 3

Odds ratio and 95% Confidence Interval for mtDNA copy number and gastric cancer

				Unadjusted		Adjusted*		Adjusted†	
Quartile	**Range**	**Cases**	**Controls**	**OR**	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**
4	≥1.29	33	62	1.00		1.00		1.00	
3	1.06-<1.29	38	62	0.91	0.52–1.59	0.91	0.51–1.60	1.02	0.54–1.91
2	0.88-<1.06	32	62	1.22	0.69–2.16	1.19	0.66–2.14	1.51	0.78–2.91
1	<0.88	31	63	1.09	0.61–1.95	1.05	0.58–1.90	1.25	0.65–2.40
p-trend					0.59		0.69		0.35

*Adjusted for smoking, age, BMI, fruit and vegetable intake, education, family history of gastric cancer, and NSAID use.

†Same adjustments as above but additionally adjusted for H. pylori statu within 378 subjects (134 cases, 244 controls)

Table 4

Association between mtDNA Copy Number Levels and Risk of Gastric Cancer Stratified by Time Between Sample Collection and Cancer Diagnosis

Time	Median	Range	Cases	Controls	OR*	95% CI
<2 years	2	≥1.06	10	32	1.00	
	1	<1.06	18	18	**5.32**	**1.03–27.60**
2–4 years	2	≥1.06	22	46	1.00	
	1	<1.06	26	41	1.61	0.64–4.06
4–6 years	2	≥1.06	19	32	1.00	
	1	<1.06	15	31	0.71	0.27–1.83
>6 years	2	≥1.06	23	39	1.00	
	1	<1.06	29	60	0.86	0.39–1.90

*Adjusted for smoking, age, BMI, fruit and vegetable intake, education, family history of gastric cancer, and NSAID use.