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Research Article

Association of Leukocyte Mitochondrial DNA Copy Number with Colorectal Cancer Risk: Results from the Shanghai Women's Health Study

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Abstract

Background: Mitochondria play an important role in cellular energy metabolism, free radical production, and apoptosis, and thus may be involved in cancer development.

Methods: We evaluated mitochondrial DNA (mtDNA) copy number in peripheral leukocytes in relation to colorectal cancer risk in a case–control study of 444 colorectal cancer cases and 1,423 controls nested in the Shanghai Women's Health Study, a population-based, prospective cohort study. Relative mtDNA copy number was determined by a quantitative real-time PCR assay using peripheral leukocyte DNA samples collected at the time of study enrollment, before cancer diagnosis.

Results: We found that baseline mtDNA copy number was lower among women who subsequently developed colorectal cancer [geometric mean, 0.277; 95% confidence interval (CI), 0.269–0.285] than among women who remained cancer-free (geometric mean, 0.288; 95% CI, 0.284–0.293; $P = 0.0153$). Multivariate adjusted ORs were 1.26 (95% CI, 0.93–1.70) and 1.44 (95% CI, 1.06–1.94) for the middle and lower tertiles of mtDNA copy number, respectively, compared with the upper tertile (highest mtDNA copy number; $P_{trend} = 0.0204$). The association varied little by the interval between blood collection and cancer diagnosis.

Conclusions: Our data suggest that mtDNA copy number measured in peripheral leukocytes may be a potential biomarker useful for colorectal cancer risk assessment.

Impact: If confirmed, mtDNA copy number measured in peripheral leukocytes may be a biomarker useful for colorectal cancer risk assessment. *Cancer Epidemiol Biomarkers Prev; 23(11); 2357–65. ©2014 AACR.*

Introduction

Colorectal cancer is the third most common cancer worldwide among men and the second most common cancer among women, with more than 1.36 million new cancer cases and 694,000 deaths in 2012 (1). During the past two decades, colorectal cancer incidence and mortality have slightly declined in most Western countries, yet more than 140,000 new cases and 50,000 deaths are estimated to have occurred in the United States in 2013 (2). In Asian countries, including China, Japan, South Korea, and Singapore, incidence of colorectal cancer has demonstrated a 2- to 4-fold increase in recent decades (3). Therefore, studies to identify biomarkers, including genetic factors, would help with prevention and early detection of colorectal cancer by identifying populations at high risk for colorectal cancer.

Mitochondria are eukaryotic organelles that have their own genome, that is, mitochondrial DNA (mtDNA). Human mtDNA is a double-strand circular molecule consisting of 16,569 base pairs and containing 37 genes encoding 22 tRNA and 2 rRNA for translation machinery and 13 subunits of the respiratory complexes I, III, IV, and V essential for electron transport chains (4). In humans, the number of copies of mtDNA varies widely with cell type, presenting at 100 to 10,000 copies per cell (except in egg and sperm cells; 5). However, mtDNA copy number is tightly regulated, and specific tissues or individual cells contain a fairly constant number of mtDNA molecules (6, 7). The mechanism regulating mtDNA copy number is largely unknown, but it is presumably correlated with mitochondrial function and energy requirements (8–11). Consistent with this notion, alterations to mtDNA copy number are frequently observed in certain types of cancer and other mitochondria-associated diseases that present oxidative

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stress and metabolic abnormalities resulting from mitochondrial dysfunction (12–15).

Few studies have investigated the association of mtDNA copy number in peripheral leukocytes with cancer risk (16–21), and two studies that focused specifically on colorectal cancer (18, 21) had inconsistent results. To evaluate the association of leukocyte mtDNA copy number with colorectal cancer risk, we conducted a prospective study of 444 women diagnosed with colorectal cancer and 1,423 controls nested within the Shanghai Women's Health Study (SWHS).

Materials and Methods

Study participants

All colorectal cancer cases and controls were participants of the SWHS, a population-based, prospective cohort study. Details on the methodology for the SWHS have been published previously (22). In brief, from 1996 to 2000, 74,941 Chinese women ages 40 to 70 years and residing in seven urban communities in Shanghai were recruited into the cohort study (response rate, 92.7%). Baseline data for all participants were collected by conducting in-person interviews and completing questionnaires. Interviewers measured participants' body weight, standing and sitting height, and waist and hip circumferences and collected a blood sample from 75.8% of participants and a urine sample from 87.7% of participants. The blood sample was collected in an ethylenediaminetetraacetic acid (EDTA) vacutainer tube (Becton, Dickinson and Company). After collection, the samples were kept in a Styrofoam box with ice packs (0°C–4°C), and processed within 6 hours for long-term storage at −80°C. The cohort has been followed using a combination of biennial home visits and annual record linkage to cancer incidence and mortality data collected by the Shanghai Cancer Registry and death certificate data collected by the Shanghai Vital Statistics Unit. Nearly all cohort members were successfully followed (response rates for in-person follow-up surveys were more than 96%). All possible incident cancer cases were verified by home visits. For cohort members who were diagnosed with cancer, medical charts were reviewed to verify the diagnosis and detailed information regarding pathologic characteristics of the cancer was obtained. The study protocol was approved by the Institutional Review Boards of Vanderbilt University and the Shanghai Cancer Institute, and all participants provided written informed consent before they were interviewed.

This analysis is part of a broader study evaluating associations of leukocyte mtDNA copy number with multiple cancers, including colorectal cancer, breast cancer, endometrial cancer, ovarian cancer, and pancreatic cancer. The incidence-density sampling method was used to select one control for each participant diagnosed with breast cancer and two controls for each participant diagnosed with other types of cancer. Cases and controls were individually matched on age (≤730 days), date (≤30 days) and time (morning or afternoon) of sample collection, time interval since the last meal (≤2 hours), antibiotic use during the week before sample collection (yes/no), and menopausal status at the time of the sample collection. After excluding controls whose mtDNA copy-number assays failed ($n = 7$), the initial analysis of the nested case–control data included 444 incident colorectal cancer cases identified during follow-up through December 2009, and 881 individually matched controls. To maximize statistical power, we also conducted analyses, including mtDNA data from all controls selected for all five types of cancer included in the broader study ($n =$ 1,423). The results from these two rounds of analyses were similar; thus, herein, we report the results of the analyses that included data from all 1,423 controls.

Laboratory measurements

Genomic DNA was extracted from buffy coats using a QIAamp DNA Blood Mini Kit (Qiagen), following the manufacturer's protocol. On the basis of a quantitative real-time PCR (qRT-PCR) analyses, the relative mtDNA copy number was designated as a ratio of an mtDNA gene (*MT-ND1* or *ND1*) to a nuclear gene (*BRCA1*). The sequences of the *ND1* gene chosen for the primers targeting mtDNA are single-copy in the mtDNA genome and not similar to any regions in the human genomic DNA sequences (23). A nonpolymorphic region of the *BRCA1* locus, which is present in only two copies in a diploid genome, was chosen for the primers' targeting region. For *ND1* gene fragment amplification, the primers were 5′-ACGCCATAAAACTCTTCACCAA-3′ (forward) and 5′-GGTTCGGTTGGTCTCTGCTA-3′ (reverse). The TaqMan MGB probe was 5′-VICAGAACACCTCTGATTACTCMGBNFQ-3′ (23). For *BRCA1* gene fragment amplification, the primers were 5′-AAACATGTTCCTCCTAAGGTGCTTT-3′ (forward) and 5′-ATGAAACCAGAAGTAAGTCCACCAGT-3′ (reverse). The TaqMan MGB probe was 5′-6FAMACACAGCTAGGACGTMGBNFQ-3′. The total reaction volume was 10 μL and contained: 5 μL 2×TaqMan Genotyping Master Mix (Life Technologies), 5-ng template DNA, 110 nmol/L primers and 100 nmol/L probe for *ND1*, and 440 nmol/L primers and 400 nmol/L probe for *BRCA1* amplification. The qRT-PCR for *ND1* and *BRCA1* measurement was run in the same well with a thermal cycling program as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles consisting of 95°C for 30 seconds and 60°C for 1 minute. The RT-PCR was performed in an ABI Prism 7900HT Sequence Detection System (Life Technologies), generating amplicons of 437 bp in length for *ND1* and 119 bp in length for *BRCA1*. Data analyses were based on measurement of the cycle threshold by using SDS 2.3 software. Each sample was assayed in triplicate. The coefficient of variation (CV) was calculated from the ratios of *ND1* to *BRCA1* in each triplicate assay. The assays were repeated if the CV was more than 15%. CEPH 1347-02 DNA was used as a reference and was serially diluted to make standard curves from 250 to 0.122 ng. The amount of *ND1* and *BRCA1* was calculated according to the standard curves,

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which were made in each assay plate. For all standard curves, the R^2 was 0.99 or greater. In addition, each plate contained one negative control (water) and one or more quality controls (CEPH 1347-02, 5 ng), which were later used to normalize the different batches (plates) for statistical processing. Representative PCR amplification plots and standard curves are presented in the Supplementary Fig. S1.

Statistical analysis

Means and frequency distributions for selected baseline characteristics were calculated for cases and controls, and differences were compared using *t* tests (for continuous variables) or χ^2 tests (for categorical variables). mtDNA copy number for each sample was first normalized to the quality control DNA (CEPH 1347-02) to minimize batch (plate) effects. The standardized mtDNA copy number was then log-transformed to achieve normal distribution. We compared differences in the geometric means of mtDNA copy number between cases and controls by using ANOVA with adjustment for assay batch (plate) and age at blood collection.

To estimate associations of mtDNA copy number with colorectal cancer risk, cases were categorized according to mtDNA copy number into tertiles based on the distribution among controls. ORs and 95% confidence intervals (CI) were estimated by using logistic regression models with adjustment for assay batch (plate) and age at blood collection. Adjustment for matching variables (age, date and time of sample collection, antibiotic use, and menopausal status), exercise (ever/never), and total meat intake did not change the association results, and thus, we only presented the results without those adjustments in the article. Tests for linear trend were estimated using the median value for each mtDNA copy-number tertile. The linear association per SD of decrease in mtDNA level was also estimated.

To evaluate nonlinear associations between colorectal cancer risk and mtDNA copy number, the continuous, log-transformed mtDNA copy number and nonlinear terms created by using a restricted cubic spline function with four knots were included in the logistic regression model. The linear effect, nonlinear effect, and overall effect of mtDNA copy number were assessed with the likelihood ratio test.

Analyses of the association of colorectal cancer risk with mtDNA copy number were stratified by the time interval between blood collection and cancer diagnosis to evaluate the consistency of the association over time. Associations of mtDNA copy number with colon cancer and rectal cancer were analyzed separately to evaluate possible heterogeneity of the association.

Results

The baseline demographic characteristics and major risk factors for colorectal cancer cases and controls are presented in Table 1. Because we included all available controls in the analysis, cases were older and more were postmenopausal compared with controls. Cases and controls were comparable in education level, body mass index (BMI), waist-to-hip ratio, fruit and vegetable intakes, and meat intake. Compared with controls, cases were more likely to participate in regular exercise. In addition, cases were more likely to have a family history of colorectal cancer or other cancer and to have a personal

Table 1. Baseline characteristics of colorectal cancer cases and controls, the SWHS (1996–2000)

Baseline characteristics	Cases ($n = 444$)	Controls ($n = 1,423$)	P^a
Age (y), mean (SD)	58.6 (8.7)	55.2 (9.1)	<0.0001
Postmenopausal, %	77.0	61.4	<0.0001
BMI, mean (SD)	24.6 (3.4)	24.4 (3.5)	0.2804
Waist-to-hip ratio, mean (SD)	0.820 (0.053)	0.816 (0.054)	0.1652
Education ≥ high school, %	32.0	36.8	0.0668
Family history of colorectal cancer, %	3.2	2.6	0.5326
Family history of any cancer, %	30.6	26.6	0.0939
Personal history of inflammatory bowel disease, %	0.9	0.6	0.5526
Personal history of diabetes, %	7.9	6.6	0.3543
Ever smoked regularly, %	2.5	3.2	0.4194
Ever drank alcohol regularly, %	3.2	2.7	0.6478
Ever exercised regularly, %	47.1	39.1	0.0030
Ever used aspirin regularly, %	2.5	2.5	0.9509
Ever used hormone replacement therapy, %[b]	3.8	4.2	0.7329
Fruit and vegetable intake, g/d, mean (SD)	558.0 (292.6)	569.9 (296.4)	0.4595
Meat intake, g/d, mean (SD)	48.9 (41.4)	52.1 (50.2)	0.1414

[a]*P* values (except for age) were derived from regression models with adjustment for age at sample collection.
[b]Among postmenopausal women only.

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history of inflammatory bowel disease or diabetes. However, the differences were not statistically significant. Very few women in our cohort regularly smoked cigarettes, drank alcoholic beverages, or took aspirin or hormone replacement therapy.

No associations were observed between mtDNA copy number and age or menopausal status (Table 2), nor were any associations observed for mtDNA copy number and major colorectal cancer risk factors (Table 2). There was a significant ($P < 0.0001$) negative correlation ($r = -0.123$) between age at sample collection and mtDNA copy number.

Baseline mtDNA copy number was lower among women who subsequently developed colorectal cancer than among women who remained cancer free. When comparing the original mtDNA copy number (before standardization), the median (Q1 and Q3) was 0.284 (0.225–0.341) for cases and 0.287 (0.238–0.347) for controls ($P < 0.0001$). The geometric means (95% CIs) were 0.277 (0.269–0.285) for cases and 0.288 (0.284–0.293) for controls ($P = 0.0153$; Table 3).

The risk of developing colorectal cancer increased as mtDNA copy number decreased (Fig. 1) and the association exhibited a linear dose–response pattern ($P_{trend} = 0.0200$). Tests for nonlinearity were not significant ($P = 0.4266$). The ORs were 1.26 (95% CI, 0.93–1.70) for the middle mtDNA copy-number tertile and 1.44 (95% CI, 1.06–1.94) for the lower tertile compared with the upper tertile (highest number of copies of mtDNA; Table 4). The observed association was slightly more evident for colon cancer than for rectal cancer (Table 4). The ORs per SD decrease in mtDNA copy number were 1.15 (95% CI, 1.02–1.30; $P = 0.0204$) for colorectal cancer, 1.16 (95% CI, 1.01–1.34; $P = 0.0430$) for colon cancer, and 1.12 (95% CI, 0.94–1.34; $P = 0.2021$) for rectal cancer (Table 4).

Blood samples were collected from 1 to 65 months before colorectal cancer diagnosis, and additional analyses were conducted to evaluate colorectal cancer risk by the time interval between blood collection and cancer diagnosis. Cases were categorized into two strata: <5 years and ≥5 years between blood collection and colorectal cancer diagnosis. The geometric mean was significantly lower among cases diagnosed ≥5 years after blood collection when compared with controls ($P = 0.0349$; Table 3). The geometric mean was also lower among colorectal cancer cases diagnosed <5 years after blood collection, although the difference for this group was not statistically significant ($P = 0.2313$; Table 3). The ORs per each SD decrease in mtDNA copy number were 1.16 (95% CI, 1.00–1.34; $P = 0.0463$) for cases diagnosed ≥5 years after blood collection and 1.12 (95% CI, 0.94–1.33; $P = 0.2176$) for cases diagnosed <5 years after blood collection (Table 4).

We also evaluated whether mtDNA copy number is associated with colorectal cancer tumor stage among 351 colorectal cancer cases that have tumor stage information. No associations were observed between mtDNA copy number and TNM stage. The geometric means (95% CIs) were 0.286 (0.273–0.300), 0.262 (0.250–0.276), and 0.270 (0.244–0.299) for stages I/II, III, and IV, respectively ($P = 0.1508$).

Discussion

In this prospective study, we found that prediagnostic mtDNA copy number in peripheral leukocytes was significantly and inversely associated with subsequent colorectal cancer risk among Han Chinese women in a dose-dependent manner. This association was observed for both colon cancer and rectal cancer, although the difference was not statistically significant for rectal cancer, most likely due to the smaller sample size of this subgroup. The association varied little by the interval between blood collection and cancer diagnosis, suggesting that the observed association is unlikely a result of undiagnosed colorectal cancer present at the time of blood sample collection. Our study supports the hypothesis that mtDNA copy number is associated with colorectal cancer risk.

A previous case–control study conducted among 320 Chinese men and women with colorectal cancer and matched controls found that mtDNA copy number in peripheral leukocytes was higher among cases compared with controls (21). However, a case–control study cannot exclude the possibility that alterations in mtDNA copy number are actually a sign of colorectal cancer itself or a consequence of diagnostic processes or treatment for colorectal cancer. Recently, a prospective study, including 422 colorectal cancer cases, showed a U-shaped association between mtDNA copy number and risk for colorectal cancer (18). Among the 168 cases who provided a prediagnosis blood sample, when compared with the second mtDNA copy-number quartile, women in the lowest quartile had a higher OR for colorectal cancer risk (OR, 4.83; 95% CI, 1.42–16.38) than did men in the lowest quartile (OR, 3.72; 95% CI, 1.02–13.54), whereas men in the highest quartile had a higher OR for colorectal cancer risk (OR, 8.74; 95% CI, 2.52–30.34) than did women in the highest quartile (OR, 2.69; 95% CI, 0.75–9.69; ref. 18). The sample size for that analysis was small. In our study, women with lower mtDNA copy number had increased risk for colorectal cancer. Our finding is consistent with a report from Liao and colleagues (20) that decreased prediagnostic mtDNA copy number is associated with increased gastric cancer risk.

Lower mtDNA copy number has been reported in colorectal cancer tissue. Lee and colleagues (24) found lower mtDNA copy number in 28% (7/25) of colorectal cancer cases (24). Lin and colleagues (25) further showed that mtDNA depletion was correlated with decreased expression of mitochondrial transcription factor A (TFAM) and β-F1-ATPase among 153 patients with colorectal cancer. Moreover, mtDNA copy number and TFAM and β-F1-ATPase expression were disease-stage dependent. Chang and colleagues (26) measured mtDNA copy number in 194 patients with colorectal cancer and found

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Table 2. mtDNA copy number by selected baseline characteristics of colorectal cancer cases and controls

	Cases ($n = 444$)		Controls ($n = 1,423$)		All Participants ($n = 1,867$)	
Variable	**No.**	**Geometric mean (95% CI)**	**No.**	**Geometric mean (95% CI)**	**No.**	**Geometric mean (95% CI)**
Age						
≤60 years	194	0.268 (0.247–0.292)	886	0.288 (0.280–0.297)	1,080	0.285 (0.278–0.293)
>60 years	250	0.265 (0.248–0.284)	537	0.293 (0.281–0.305)	787	0.287 (0.277–0.297)
P		0.8355		0.6276		0.8538
Postmenopausal						
No	102	0.271 (0.245–0.300)	549	0.295 (0.284–0.305)	651	0.290 (0.280–0.300)
Yes	342	0.265 (0.251–0.280)	874	0.287 (0.280–0.295)	1,216	0.284 (0.277–0.290)
P		0.7239		0.3453		0.3730
BMI						
<25	263	0.268 (0.255–0.283)	859	0.291 (0.285–0.297)	1,122	0.287 (0.282–0.293)
≥25	180	0.263 (0.247–0.280)	564	0.288 (0.281–0.295)	744	0.284 (0.278–0.290)
P		0.5428		0.5078		0.4081
Family history of colorectal cancer						
No	430	0.268 (0.255–0.280)	1,386	0.290 (0.286–0.295)	1,816	0.286 (0.282–0.291)
Yes	14	0.238 (0.200–0.282)	37	0.278 (0.254–0.304)	51	0.268 (0.248–0.290)
P		0.1692		0.3477		0.1101
Family history of any cancer						
No	308	0.269 (0.256–0.283)	1,045	0.290 (0.285–0.296)	1,353	0.287 (0.282–0.292)
Yes	136	0.259 (0.243–0.277)	378	0.289 (0.280–0.297)	514	0.283 (0.276–0.290)
P		0.2572		0.7178		0.3121
Personal history of inflammatory bowel disease						
No	440	0.266 (0.254–0.279)	1,414	0.290 (0.285–0.295)	1,854	0.286 (0.282–0.290)
Yes	4	0.305 (0.223–0.415)	9	0.272 (0.226–0.327)	13	0.284 (0.242–0.332)
P		0.3927		0.4929		0.9174
Personal history of diabetes						
Yes	35	0.258 (0.230–0.290)	94	0.276 (0.260–0.293)	129	0.273 (0.260–0.288)
No	409	0.267 (0.255–0.280)	1,329	0.291 (0.286–0.296)	1,738	0.287 (0.283–0.291)
P		0.5607		0.0851		0.0696
Ever smoked regularly						
Yes	11	0.255 (0.210–0.309)	46	0.301 (0.277–0.326)	57	0.291 (0.270–0.314)
No	433	0.267 (0.254–0.280)	1,377	0.290 (0.285–0.294)	1,810	0.286 (0.282–0.290)
P		0.6371		0.3813		0.6148
Ever drank alcohol regularly						
Yes	14	0.302 (0.254–0.359)	39	0.297 (0.272–0.325)	53	0.301 (0.278–0.326)
No	430	0.265 (0.253–0.278)	1,384	0.290 (0.285–0.295)	1,814	0.285 (0.281–0.290)
P		0.1356		0.5817		0.1867
Ever exercised regularly						
Yes	209	0.265 (0.249–0.280)	557	0.289 (0.282–0.297)	766	0.285 (0.279–0.292)
No	235	0.268 (0.254–0.283)	866	0.290 (0.285–0.296)	1,101	0.286 (0.281–0.292)
P		0.6738		0.7921		0.7800
Ever used aspirin regularly						
Yes	11	0.283 (0.233–0.344)	36	0.286 (0.260–0.314)	47	0.288 (0.265–0.313)
No	433	0.266 (0.254–0.279)	1,387	0.290 (0.285–0.295)	1,820	0.286 (0.282–0.290)
P		0.536		0.7610		0.8678
Ever used hormone replacement therapy[a]						
No	329	0.268 (0.255–0.282)	837	0.285 (0.278–0.291)	1,165	0.281 (0.275–0.288)
Yes	13	0.311 (0.257–0.376)	37	0.302 (0.275–0.331)	50	0.304 (0.280–0.330)
P		0.1313		0.2144		0.0657

P values were derived from the two-way ANOVA *F* test by using log-transformed data.

[a]Among postmenopausal women.

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Table 3. Baseline mtDNA copy number for cases and controls and mtDNA copy number stratified by time since blood collection

	Cases	Controls	P^a
Colorectal cancer			
Number	444	1,423	
Geometric mean (95% CI)	0.277 (0.269–0.285)	0.288 (0.284–0.293)	**0.0153**
Colon cancer			
Number	276	1,382	
Geometric mean (95% CI)	0.276 (0.267–0.286)	0.288 (0.284–0.293)	**0.0323**
Rectal cancer			
Number	168	1,252	
Geometric mean (95% CI)	0.278 (0.266–0.291)	0.287 (0.282–0.292)	0.1971
Time since blood collection (colorectal cancer)			
<5 years			
Number	177	1,229	
Geometric mean (95% CI)	0.281 (0.269–0.293)	0.289 (0.284–0.294)	0.2313
≥5 years			
Number	267	1,372	
Geometric mean (95% CI)	0.277 (0.267–0.288)	0.289 (0.285–0.294)	**0.0349**

[a]*P* values were derived from the two-way (case–control status and assay batches/plates) ANOVA *F* test for the case–control difference by using log-transformed data. Bold indicates significantly significant at $P < 0.05$.

that low mtDNA copy number in tumor tissue was significantly associated with advanced tumor stage and higher p53 mutation. Lower mtDNA copy number was also correlated with microsatellite instability based on data derived from 50 colorectal cancer biopsy samples (27). Taken together, the evidence suggests that reductions in mtDNA copy number in colorectal tissue may play a role in the development of colorectal cancer.

It has been shown that exercise and exposure to cold may lead to higher mtDNA copy number (28, 29). Exposure to cigarette smoke and alcohol may decrease expression of peroxisome proliferator activated receptor-gamma (PPAR-γ)-coactivator 1 alpha (PGC-1α), which is involved in mitochondrial biogenesis (30, 31). Compared with light smokers (<30 pack-years), heavy smokers (≥30 pack-years) had lower mtDNA copy numbers in lung tissues ($P < 0.05$; ref. 32). Taken together, these findings indicate that environmental factors are involved in the regulation of mtDNA copy number. Xing and colleagues (17) have shown that mtDNA copy number in leukocytes is higher among smokers compared with never smokers and lower among men compared with women. However, Qu and colleagues (21) found no modulating effects for sex, age, BMI, smoking status, or alcohol consumption on mtDNA copy number in either colorectal cancer cases or controls (21). In our study, we also observed no association of mtDNA copy number with smoking status, alcohol consumption, aspirin intake, exercise, BMI, or other variables (Table 2). However, smoking, alcohol consumption, and aspirin use are all rare in our study population.

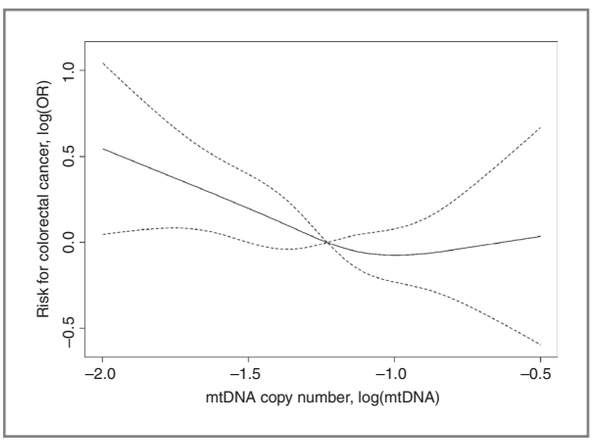

Figure 1. Association between baseline mtDNA copy number and subsequent risk for colorectal cancer. Solid line, ORs; dotted line, 95% CI. *P* values for tests of overall association, linearity, and nonlinearity were 0.0683, 0.0200, and 0.4266, respectively.

The potential mechanism by which lower mtDNA copy number leads to cancer development is unclear. mtDNA copy number in circulating blood cells and other tissues could be a biomarker of mitochondrial dysfunction (33). It has been suggested that reductions in mtDNA copy number may disrupt mitochondrial membrane potential and lead to mitochondrial dysfunction. Dysfunctional mitochondria could trigger retrograde signaling from the mitochondria to the nucleus, causing an adaptive response that results in alterations to the nuclear gene-expression profile and to cell physiology and morphology (34). Further studies are needed to elucidate the potential

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Table 4. Association of baseline mtDNA copy number and subsequent risk of colorectal cancer

	mtDNA copy number (tertiles)			
	3 (Highest)	**2**	**1**	**P_{trend}[a]**
Colorectal cancer				
Number of case/control	104/475	149/473	191/475	
OR (95% CI)	1.00 (reference)	1.26 (0.93–1.70)	1.44 (1.06–1.94)	
OR (95% CI)[b]		1.15 (1.02–1.30)		**0.0204**
Colon cancer				
Number of case/control	63/459	93/454	120/469	
OR (95% CI)	1.00 (reference)	1.27 (0.88–1.83)	1.46 (1.02–2.10)	
OR (95% CI)[b]		1.16 (1.01–1.34)		**0.0430**
Rectal cancer				
Number of case/control	41/404	56/416	71/432	
OR (95% CI)	1.00 (reference)	1.24 (0.80–1.93)	1.37 (0.88–2.13)	
OR (95% CI)[b]		1.12 (0.94–1.34)		0.2021
Time since blood collection (colorectal cancer)				
<5 years				
Number of case/control	40/410	61/412	76/407	
OR (95% CI)	1.00 (reference)	1.39 (0.89–2.16)	1.59 (1.02–2.48)	
OR (95% CI)[b]		1.12 (0.94–1.33)		0.2176
≥5 years				
Number of case/control	64/464	88/449	115/459	
OR (95% CI)	1.00 (reference)	1.18 (0.82–1.70)	1.32 (0.92–1.90)	
OR (95% CI)[b]		1.16 (1.00–1.34)		**0.0463**

[a]Conditional logistic regression models were used to derive *P* values for linear trends by modeling the log-transformed mtDNA content as a continuous variable.

[b]OR per SD decrement. Bold indicates significantly significant at $P < 0.05$.

molecular mechanisms by which lower mtDNA copy number is associated with increased colorectal cancer risk.

Our study has several strengths. First, participants were drawn from a large prospective cohort consisting of 74,941 women living in the same area and had a high response rate (92.7%). The study population is well-characterized, relatively homogeneous, and has low smoking and alcohol consumption rates (22), which reduces the effect of gender, lifestyle, and other confounding factors on mtDNA copy number. Second, the prospective study design reduces the possibility that lower mtDNA copy number is a consequence of colorectal cancer development or cancer treatments, such as chemotherapy or surgery, which have been reported to influence mitochondrial redox homeostasis and reduce mtDNA copy number (35–37). Third, we set up the qRT-PCR for measurement of the mtDNA and reference gene in the same tube. Amplification of the mtDNA and reference nuclear gene under the same conditions should reduce assay variation. Different reference nuclear genes, such as hemoglobin beta (38), 18s rDNA (18), and *RBCA1* (our study) were used in the mtDNA copy-number measurements, which should not affect the results of the association study. However, it would be difficult to compare the raw mtDNA copy-number data among different studies. Our study also has some limitations. First, although our study is the largest prospective study conducted to date addressing the association of mtDNA copy number with colorectal cancer risk, the sample size for some analyses was relatively small. In addition, our study was conducted among Chinese women. Further research is warranted to evaluate the association in large prospective studies, including both men and women and different racial/ethnic groups. Second, lifestyle changes over time could affect the association between mtDNA copy number and colorectal cancer risk and a single measurement may not reflect mtDNA copy number over a lifetime. However, Thyagarajan and colleagues (18) conducted a small study and found that mtDNA copy number has a relatively small within-person variation over a 2-month period. We also found no association between mtDNA copy number and major risk factors for colorectal cancer. Third, potential selection bias is another concern for epidemiologic studies. However, the potential selection bias should be small in this study, because of the very high response rate (92.7%) at baseline and the very low rate of loss to follow-up (<1%). Fourth, antioxidant capacity may play a role in mtDNA regulation. Further studies are needed to evaluate the joint effects of antioxidant capacity and mtDNA copy number in relation to colorectal cancer risk.

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In summary, this prospective study indicates that lower mtDNA copy number in peripheral blood leukocytes is associated with subsequent development of colorectal cancer. mtDNA copy number measured in peripheral blood leukocytes may be a potential biomarker useful for colorectal cancer risk assessment. Further studies are needed to confirm our findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Huang, W. Wen, B.-T. Ji, Q. Cai
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-T. Gao, B.-T. Ji, W. Zheng, Q. Cai
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