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A nested case–control study of leukocyte mitochondrial DNA copy number and renal cell carcinoma in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial

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Mitochondrial DNA (mtDNA) is vulnerable to mutations, and the number of copies of mtDNA per cell may increase to compensate for DNA damage. Case–control studies have reported associations between altered mtDNA copy number and risk of renal cell carcinoma (RCC); however, this association has not been investigated prospectively. We conducted a nested case–control study (252 cases and 504 controls) of RCC risk in relation to pre-diagnostic leukocyte mtDNA copy number in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial. mtDNA copy number was measured in triplicate using a fluorescence-based quantitative PCR assay; samples from 22 cases and 36 controls could not be assayed, leaving 230 cases and 468 controls for analysis. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using unconditional logistic regression. High mtDNA copy number was associated with an increased risk of RCC, both overall (highest quartile versus lowest: OR = 2.0, 95% CI = 1.2–3.2; P_{trend} = 0.002) and among cases diagnosed ≥6 years after blood collection (OR = 2.6, 95% CI = 1.4–5.0; P_{trend} = 0.003). These findings did not differ significantly by sex, body mass index, history of hypertension or smoking status ($P_{interaction}$ ≥ 0.3). Results of this study suggest that high pre-diagnostic leukocyte mtDNA copy number, a suspected marker of oxidative DNA damage and mitochondrial dysfunction, is associated with increased future RCC risk.

Introduction

Mitochondria supply eukaryotic cells with energy by synthesizing adenosine triphosphate through oxidative phosphorylation; they also play a critical role in apoptosis and are a primary source and target of intracellular reactive oxygen species (ROS) generated as by-products in the electron transport chain (1). Each mitochondrion has 2–10 copies of its own genome, mitochondrial DNA (mtDNA), which consists of a 16.6 kb double-stranded DNA molecule (2). mtDNA is vulnerable to mutations due to the lack of protective histones and the chromatin structure, limited DNA repair mechanisms and high exposure to ROS (3); the mutation rate of mtDNA is ~10-fold that of nuclear DNA (2).

It has been hypothesized that the number of copies of mtDNA per cell may increase to compensate for mtDNA damage or mitochondrial dysfunction (4). Studies of renal cell carcinoma (RCC) and other cancers have measured mtDNA copy number in peripheral blood leukocytes as a suspected marker of ROS-mediated DNA damage and mitochondrial dysfunction (reviewed in ref. 4); elevated mtDNA copy number in prospectively collected blood specimens has been associated with an increased risk of several malignancies including non-Hodgkin lymphoma (5), lung cancer (6) and pancreatic cancer (7). Two case–control studies of RCC have reported inverse associations with leukocyte mtDNA copy number (8,9); however, an inherent limitation of such studies with retrospective sample collection is the potential for disease- or treatment-related effects on measurements of leukocyte mtDNA content.

To our knowledge, no prospective studies have evaluated RCC risk in relation to mtDNA copy number in peripheral blood leukocytes. To address this research gap, we conducted a nested case–control study of pre-diagnostic leukocyte mtDNA copy number and risk of RCC in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial.

Abbreviations: BMI, body mass index; CIs, confidence intervals; mtDNA, mitochondrial DNA; ORs, odds ratios; RCC, renal cell carcinoma; ROS, reactive oxygen species.

Materials and methods

Enrollment and specimen collection procedures in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial have been described (10). Briefly, ~155 000 participants between 55 and 74 years of age were enrolled through study centers in 10 USA cities between 1993 and 2001. Half of the participants were randomized to the screening arm of the trial; these participants provided non-fasting blood samples at six annual medical examinations. Samples were processed and frozen within 2 h of collection and stored at −70°C. In addition to the annual screening examinations for trial disease outcomes during the first 6 years of follow-up, questionnaires were mailed annually to participants to follow-up for all cancer diagnoses, which were pathologically confirmed through medical record abstraction. The trial was approved by institutional review boards at the National Cancer Institute and the 10 study centers, and all participants provided written informed consent.

We identified 252 histologically confirmed incident cases of RCC (ICD-O-2 C64.9) diagnosed on or before 18 August 2009 that had an available pre-diagnostic source of peripheral leukocyte DNA. Controls (N = 504) were individually matched to each case (2:1 ratio of controls to cases) on sex, age at baseline (5 year categories), race, date of phlebotomy (4 month categories), study year of specimen collection and DNA source material (buffy coat, whole blood, previously extracted DNA). We selected the earliest available specimen for analysis (baseline samples for 84.9% of the selected cases and controls).

Measurements of relative mtDNA copy number were performed in triplicate as described previously (11). Briefly, a fluorescence-based quantitative PCR assay was used to determine the threshold cycle number of a mitochondrial gene (*ND1*) and a nuclear gene (β-globin gene, *HBB*). The ratio of *ND1* and *HBB* threshold cycle numbers, estimated using a linear regression model, is proportional to the mtDNA copy number in each cell. The overall coefficient of variation for mtDNA copy number was 5.3%, and the intraclass correlation coefficient was 0.96.

We were unable to assay samples from 22 cases and 36 controls, leaving 230 cases and 468 controls in our analysis. Potential determinants of natural log-transformed mtDNA copy number were evaluated among controls using bivariate analyses (t-tests for binary variables and analysis of variance for variables with three or more categories) and multivariate linear regression models. For our main analyses, we report the estimated odds ratios (ORs) and 95% confidence intervals (CIs) from unconditional logistic regression models including all cases and controls with valid mtDNA measurements. In addition to controlling for matching factors, we also adjusted for history of hypertension, smoking status and body mass index (BMI) in our final models. We also performed conditional logistic regression on all matched sets with a case and at least one control (223 cases and 436 controls). Subjects were categorized into quartiles based on the distribution among controls, and tests for trend were performed using the median value for each quartile. We performed analyses stratified by sex, age at blood collection, BMI, history of hypertension and smoking status; likelihood ratio tests were used to evaluate multiplicative interaction. To assess whether our findings may reflect disease-induced effects, we fit polytomous logistic regression models to compute ORs with mtDNA copy number for RCC case subgroups defined by time from blood collection to case diagnosis (<6 years, ≥6 years; this cut-point reflects the approximate median time after blood collection among cases). Analyses restricted to RCC cases (N = 213) with clear cell histology (ICD-O-2-M 8310 or 8312) were also performed.

All statistical analyses were performed using Stata version 11.0 (StataCorp LP, College Station, TX). Findings were considered statistically significant if two-sided *P*-values were <0.05.

Results

Cases and controls were comparable in terms of age, sex, race and smoking status, and cases were more likely than controls to be obese and to have a history of hypertension (Table I). Among controls, we observed decreasing mtDNA copy number with increasing age at blood collection ($P = 0.005$ in the multivariate analysis) and higher mtDNA copy number among women than among men ($P = 0.019$; Supplementary Table 1, available at *Carcinogenesis* Online).

The median mtDNA copy number was higher among cases than among controls (131 and 120.5, respectively; $P = 0.008$, Wilcoxon rank-sum test; Table I). High mtDNA copy number was associated with an increased risk of RCC (highest quartile versus lowest: OR = 2.0, 95% CI = 1.2–3.2; $P_{trend} = 0.002$; Table II), and this association remained among cases diagnosed ≥6 years after blood collection (OR = 2.6, 95% CI = 1.4–5.0; $P_{trend} = 0.003$). We found some evidence of stronger associations between mtDNA copy number and RCC among subjects who were male, <65 years of age at blood collection, overweight or obese (BMI ≥ 25 kg/m^2), and those without a history of hypertension, although tests of interaction by these factors were not statistically significant ($P_{interaction} \geq 0.1$; Table III). Associations with RCC for these established risk factors were similar before and after adjusting for mtDNA copy number in the statistical models (data not shown). Findings were unchanged after restricting to cases and controls with DNA newly extracted from buffy coat (>90% of selected subjects), RCC cases with clear cell histology (93% of selected cases) and RCC cases with higher grade tumors (grades 2–4, 57% of selected cases; Supplementary Table 2, available at *Carcinogenesis* Online). Similar results were observed from conditional models of matched case–control sets (Supplementary Table 2, available at *Carcinogenesis* Online).

Discussion

Results of this study, to our knowledge the first prospective investigation of leukocyte mtDNA copy number and RCC risk, suggest that high pre-diagnostic mtDNA copy number is associated with increased future risk of RCC. This association remained after restricting to RCC cases diagnosed ≥6 years after blood collection, which suggests that increased mtDNA copy number may be a biomarker of risk related to the etiology of RCC rather than an effect of as yet undiagnosed disease. Our findings are not consistent with those of two retrospective case–control studies that reported inverse associations between leukocyte mtDNA copy

Table I. Distribution of selected characteristics of cases and controls[a]

Characteristics	Cases (*N* = 230)	Controls (*N* = 468)
mtDNA copy number, median (IQR)	131 (102–159)[b]	120.5 (96–150.5)
Age at baseline[c], years		
55–59	67 (29.1)	135 (28.9)
60–64	82 (35.7)	165 (35.3)
65–69	51 (22.2)	114 (24.4)
70–74	30 (13.0)	54 (11.5)
Sex[c]		
Female	66 (28.7)	137 (29.3)
Male	164 (71.3)	331 (70.7)
Race[c]		
White, non-Hispanic	209 (90.9)	416 (88.9)
Black, non-Hispanic	10 (4.4)	26 (5.6)
Other	11 (4.8)	26 (5.6)
BMI, kg/m^2		
<25	50 (21.7)	140 (30.2)
25–30	95 (41.3)	212 (45.8)
>30	85 (37.0)	111 (24.0)
Missing	0	5
Smoking status		
Never	92 (40.0)	212 (45.3)
Former	117 (50.9)	209 (44.7)
Current	21 (9.1)	47 (10.0)
History of hypertension		
No	131 (57.2)	306 (66.0)
Yes	98 (42.8)	158 (34.1)
Missing	1	4
DNA source material[c]		
Buffy coat	208 (90.4)	422 (90.2)
Whole blood	2 (0.9)	6 (1.3)
Previously extracted DNA	20 (8.7)	40 (8.6)
Year of blood collection[c], median (range)	1997 (1993–2002)	1997 (1994–2002)
Season of blood collection[c]		
January–April	67 (29.1)	133 (28.4)
May–August	83 (36.1)	172 (36.8)
September–December	80 (34.8)	163 (34.8)
Study year of selected specimen[c]		
Baseline	197 (85.7)	394 (84.2)
Other[d]	33 (14.4)	74 (15.8)

IQR, interquartile range.
[a]Reported as frequency (%) unless otherwise noted. Missing values are excluded from percentages.
[b]$P = 0.008$, Wilcoxon rank-sum test.
[c]Cases and controls were matched on these selected characteristics.
[d]Specimens selected from a subsequent annual examination 1–5 years after baseline.

Table II. ORs for risk of RCC in relation to mtDNA copy number, overall and stratified by time from blood collection to case diagnosis[a]

mtDNA copy number[b]	Overall		<6 years to RCC diagnosis		≥6 years to RCC diagnosis	
	Ca/Co	OR (95% CI)	Ca/Co	OR (95% CI)	Ca/Co	OR (95% CI)
First quartile	42/116	1.0 (Ref)	22/116	1.0 (Ref)	20/116	1.0 (Ref)
Second quartile	47/118	1.0 (0.6–1.7)	16/118	0.6 (0.3–1.3)	31/118	1.5 (0.8–2.9)
Third quartile	64/117	1.5 (0.9–2.5)	30/117	1.3 (0.7–2.4)	34/117	1.7 (0.9–3.3)
Fourth quartile	77/117	2.0 (1.2–3.2)	37/117	1.4 (0.8–2.7)	40/117	2.6 (1.4–5.0)
		$P_{trend} = 0.002$		$P_{trend} = 0.078$		$P_{trend} = 0.003$

Ca, case; Co, control.
[a]Analyses were performed using unconditional models adjusted for matching factors (age, sex, race, year of blood draw, season of blood draw, study year and material type for DNA extraction) as well as history of hypertension, smoking status and BMI. Tests for trend were performed by assigning the within-category median for each quartile and analyzing these data as a continuous variable. Analyses stratified by time from blood collection to RCC diagnosis were performed using polytomous logistic regression models.
[b]Quartiles of mtDNA copy number were assigned based on the distribution among controls as follows: first quartile, ≤95; second quartile, 96–120; third quartile, 121–150; fourth quartile, ≥151.

Table III. ORs for risk of RCC in relation to mtDNA copy number, stratified by sex, age, BMI, history of hypertension and smoking status[a]

Analysis	mtDNA copy number[b]				P_{trend}[c]	P_{int}[d]
	First quartile	Second quartile	Third quartile	Fourth quartile		
By sex						
Men						
Cases/controls	31/84	37/90	46/92	50/65	0.002	
OR (95% CI)	1.0 (Ref)	1.1 (0.6–1.9)	1.4 (0.8–2.4)	2.3 (1.3–4.2)		
Women						
Cases/controls	11/32	10/28	18/25	27/52		
OR (95% CI)	1.0 (Ref)	1.0 (0.4–3.0)	2.1 (0.8–5.9)	1.6 (0.6–4.1)	0.3	0.4
By age at blood collection						
55–64 years						
Cases/controls	20/66	32/67	42/68	48/82		
OR (95% CI)	1.0 (Ref)	1.5 (0.8–3.0)	2.1 (1.1–4.1)	2.2 (1.1–4.2)	0.03	
65–74 years						
Cases/controls	22/50	15/51	22/49	29/35		
OR (95% CI)	1.0 (Ref)	0.6 (0.3–1.3)	0.9 (0.4–2.0)	2.0 (0.9–4.2)	0.03	0.1
By BMI						
<25 kg/m^2						
Cases/controls	11/32	13/36	14/33	12/39		
OR (95% CI)	1.0 (Ref)	1.0 (0.4–2.7)	1.3 (0.4–3.6)	0.5 (0.2–1.5)	0.2	
≥25 kg/m^2						
Cases/controls	31/82	34/81	50/83	65/77		
OR (95% CI)	1.0 (Ref)	1.2 (0.7–2.1)	1.8 (1.0–3.1)	2.8 (1.6–5.1)	<0.001	0.1
By history of hypertension						
No hypertension						
Cases/controls	23/78	27/70	35/76	46/82		
OR (95% CI)	1.0 (Ref)	1.4 (0.7–2.7)	1.5 (0.8–2.9)	2.0 (1.1–3.8)	0.03	
Hypertension						
Cases/controls	19/36	20/47	28/40	31/35		
OR (95% CI)	1.0 (Ref)	0.6 (0.3–1.4)	1.2 (0.5–2.8)	1.5 (0.6–3.5)	0.1	0.7
By smoking status						
Never smoker						
Cases/controls	17/54	17/52	26/53	32/53		
OR (95% CI)	1.0 (Ref)	0.9 (0.4–2.1)	1.5 (0.7–3.2)	2.1 (1.0–4.7)	0.02	
Former smoker						
Cases/controls	19/51	24/56	34/51	40/51		
OR (95% CI)	1.0 (Ref)	1.2 (0.6–2.5)	1.9 (0.9–3.8)	2.4 (1.2–4.9)	0.009	
Current smoker						
Cases/controls	6/11	6/10	4/13	5/13		
OR (95% CI)	1.0 (Ref)	1.1 (0.1–9.2)	0.3 (0–3.2)	1.1 (0.1–11.4)	0.9	0.6

[a]Analyses were performed using unconditional models adjusted for matching factors (age, sex, race, year of blood draw, season of blood draw, study year and material type for DNA extraction) as well as history of hypertension, smoking status and BMI (with the exception of the stratifying variable in each analysis).
[b]Quartiles of mtDNA copy number were assigned based on the distribution among controls as follows: first quartile, ≤95; second quartile, 96–120; third quartile, 121–150; fourth quartile, ≥151.
[c]Tests for trend were performed by assigning the within-category median for each quartile and analyzing these data as a continuous variable.
[d]Evidence of multiplicative interaction was assessed using likelihood ratio tests.

number and RCC (8,9); however, these studies relied on measurements of mtDNA copy number in postdiagnostic specimens, which may be influenced by tumor- or treatment-induced effects. Unlike previous retrospective studies, our findings from this prospective investigation are unlikely to be explained by reverse causation bias.

In most prospective studies conducted to date, peripheral leukocyte mtDNA copy number has been positively associated with future risk of various malignancies including non-Hodgkin lymphoma (5), lung cancer (6) and pancreatic cancer (7), but not gastric cancer (12). In the study of gastric cancer by Liao *et al.* (12), a strong inverse association was observed in the first 2 years of follow-up after blood collection but not thereafter, suggesting that reduced mtDNA content in peripheral blood may be an early disease effect for this malignancy. In a recent study involving both prevalent and incident cases of colorectal cancer, a U-shaped association with mtDNA copy number was observed (13).

The specific biologic mechanisms by which increased leukocyte mtDNA copy number may be related to renal carcinogenesis, either as a marker of global mtDNA content or as a surrogate for mtDNA copy number in kidney epithelial cells, are not fully understood. High mtDNA content in peripheral blood leukocytes may be indicative of increased oxidative stress and ROS-mediated DNA damage, and it is possible that mtDNA copy number may increase to compensate for mtDNA damage and mitochondrial dysfunction, as hypothesized previously (4). Oxidative stress may alter cellular composition and has been shown to increase mtDNA content *in vitro* (14). Evidence from animal models suggests that oxidative stress and resultant lipid peroxidation can contribute to the development of RCC by directly damaging proximal renal tubule cells, the progenitor cells for most forms of RCC (15,16). Similarly, a high level of oxidative stress is commonly observed in RCC, with higher levels correlating to worse prognosis (17).

Strengths of this study include its prospective design, with specimens collected from RCC cases up to 13 years prior to diagnosis, and the uniform procedures for specimen collection and processing among screening arm participants in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial. A limitation of this study was the relatively modest sample size for analyses stratified by established risk factors for RCC (e.g. BMI, hypertension and smoking status).

In conclusion, in this study, we observed an increased risk of RCC in relation to high pre-diagnostic leukocyte mtDNA copy number. These findings support a role for ROS-mediated oxidative DNA damage and mitochondrial dysfunction in the etiology of RCC. As this study is, to our knowledge, the first prospective investigation of the

association between mtDNA copy number and RCC, confirmation of these findings in other studies with prospectively collected samples is needed. Additional research is also warranted to investigate the role of circulating markers of oxidative stress in the etiology of RCC.

Supplementary material

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

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