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# Mitochondrial DNA copy number and lung cancer risk in a prospective cohort study

**H.Dean Hosgood III*, Chin-San Liu[1], Nathaniel Rothman, Stephanie J.Weinstein, Matthew R.Bonner[2], Min Shen, Unhee Lim[3], Jarmo Virtamo[4], Wen-ling Cheng[1], Demetrius Albanes† and Qing Lan†**

Division of Cancer Epidemiology and Genetics, National Cancer Institute, 6120 Executive Boulevard, EPS 8118, MCS 7240, Bethesda, MD 20892-7240, USA, [1]Department of Neurology and Vascular and Genomic Research Center, Changhua Christian Hospital, Changhua 500, Taiwan, [2]Department of Social and Preventative Medicine, University at Buffalo the State University of New York, Buffalo, NY 14226, USA, [3]Epidemiology Program, Cancer Research Center of Hawaii, Honolulu, HI 96813 and [4]Department of Chronic Disease Prevention, National Institute for Health and Welfare, Helsinki 00271, Finland

*To whom correspondence should be addressed.Tel: +1 301 594 4649; Fax: +1 301 402 1819
Email: hosgoodd@mail.nih.gov

**Mitochondria are eukaryotic organelles responsible for energy production. Mitochondrial DNA (mtDNA) lack introns and protective histones, have limited DNA repair capacity and compensate for damage by increasing the number of mtDNA copies. As a consequence, mitochondria are more susceptible to reactive oxygen species, an important determinant of cancer risk, and it is hypothesized that increased mtDNA copy number may be associated with carcinogenesis. We assessed the association of mtDNA copy number and lung cancer risk in 227 prospectively collected cases and 227 matched controls from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Conditional logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs), adjusting for age at randomization, smoking years and number of cigarettes smoked per day. There was suggestion of a dose-dependent relationship between mtDNA copy number and subsequent risk of lung cancer, with a prominent effect observed in the highest mtDNA copy number quartile [ORs (95% CI) by quartile: 1.0 (reference), 1.3 (0.7–2.5), 1.1 (0.6–2.2) and 2.4 (1.1–5.1); $P_{trend} = 0.008$]. This is the first report, to the best of our knowledge, to suggest that mtDNA copy number may be positively associated with subsequent risk of lung cancer in a prospective cohort study; however, replication is needed in other studies and populations.**

## Introduction

Mitochondria are the eukaryotic organelles responsible for energy production through the synthesis of adenosine triphosphate. In normal cells, mitochondria have 2–10 copies of their genomes [mitochondrial DNA (mtDNA)] (1). mtDNA is a circular molecule that lacks introns and protective histones. As a consequence, the mutation rate for mtDNA is substantially greater than that of nuclear genomic DNA (1). Further, mitochondria have limited DNA repair capacity and compensate for damage by increasing the number of mtDNA copies. In some cancers, evidence suggests that mtDNA may also undergo mutations, insertions or deletion in response to oxidative stress (2,3).

Reactive oxygen species (ROS) are an important determinant of cancer risk. Tobacco smoke is one exposure that induces oxidative stress by creating ROS within the human body (4,5). Biomarkers of oxidative stress have been found to be substantially increased in smokers relative to non-smokers (6–8). Given that mitochondria are highly susceptible to ROS (9), mtDNA copy number may serve as a biomarker for exogenous and endogenous exposures that are associated with subsequent tobacco-related cancer risk.

To reduce the possibility that the latent disease might have altered mtDNA copy number, we evaluated mtDNA copy number in peripheral white blood cell DNA and risk of developing lung cancer in a prospective cohort study, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study.

**Abbreviations:** CI, confidence interval; mtDNA, mitochondrial DNA; OR, odds ratio; ROS, reactive oxygen species.

†These authors contributed equally to this work.

## Materials and methods

The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study cohort has been previously described (10). Briefly, 29 133 male smokers, aged 50–69 years, were recruited from 1985 through 1988 from Southwest Finland. Subjects were randomized to receive α-tocopherol and/or β-carotene supplements. The study was approved by Institutional Review Boards at the US National Cancer Institute and the National Public Health Institute of Finland. All participants provided written informed consent.

The Finnish Cancer Registry and the Register of Causes of Death, which provides ∼100% of cancer case ascertainment in Finland, was used to identify incident lung cancers ($n = 229$) through 30 April 2002 (11). Fewer than 50% of cases had clear classification as squamous cell carcinoma ($n = 74$) or adenocarcinoma ($n = 34$). Controls were selected from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study cohort members who were alive, free of cancer at the time of the case diagnosis, and were individually matched to cases on date of birth (±5 years).

DNA was extracted from the whole blood using the phenol–chloroform method (12), and a fluorescence-based quantitative polymerase chain reaction (QPCR) determined mtDNA copy number (13). Cases and their matched controls were blindly assayed consecutively within each batch. Blinded quality control duplicate samples were interspersed in each batch to evaluate assay reproducibility. The overall coefficient of variation of this assay was 13%. mtDNA copy number data were available for 227 case–control pairs.

The correlation between age at randomization and mtDNA copy number was determined by the Spearman correlation coefficient. mtDNA copy number was categorized into quartiles based on the distribution among controls. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using conditional logistic regression models, adjusting for age at randomization, number of cigarettes smoked per day and number of years smoking. A test for trend was calculated using mtDNA copy number as a continuous variable. Further adjustment by other factors, such as body mass index, physical activity and caloric intake, did not change the beta-coefficient for mtDNA copy number ≥10%. The mtDNA copy number and smoking interactions were tested by including the interaction term of the dichotomous variables (based on the median among controls) in the model. All statistical analyses were performed using SAS (Cary, NC).

## Results

Cases and controls were similar with respect to age, alcohol and energy consumption, physical activity level and trial supplementation group (Table I). As expected, cases smoked more cigarettes per day and smoked for a longer period of time than controls.

Among controls, mtDNA copy number was positively associated with age ($r = 0.11$, $P = 0.08$). The median (±standard deviation) mtDNA copy number among cases and controls in the α-tocopherol (cases: 125.6 ± 45.4; controls: 122.2 ± 34.1; $P = 0.10$) and the β-carotene supplementation arms (cases: 125.6 ± 48.6; controls: 127.4 ± 38.2; $P = 0.63$) were similar. Men in the highest quartile of mtDNA copy number experienced a significantly increased risk of lung cancer (OR = 2.4; 95% CI = 1.1–5.1) compared with those in the lowest quartile (Table II). There was also evidence that risk of lung cancer increased in a dose-dependent manner with mtDNA copy number ($P_{trend} = 0.008$). The association between mtDNA copy number and lung cancer risk was evident among heavy smokers (≥20 cigarettes per day), but not light smokers (<20 cigarettes per day) (Table III); however, the interaction between mtDNA copy number and smoking was not significant. Similar results were seen when classifying heavy and light smokers on the median years of smoking among controls.

 

**Table I.** Baseline characteristics of lung cancer cases and individually matched controls

| | Cases ($n$ = 227) Mean (SD) | Controls ($n$ = 227) Mean (SD) | $P$-value[a] |
|---|---|---|---|
| Age at baseline (years) | 58.7 (5.0) | 58.4 (4.8) | 0.57 |
| Smoking | | | |
| Number of cigarettes per day | 18.8 (9.6) | 17.0 (9.6) | 0.06 |
| Years of smoking | 38.5 (7.1) | 35.8 (9.2) | <0.01 |
| Body mass index (kg/m$^2$)[b] | 25.6 (3.5) | 26.3 (3.9) | 0.03 |
| Alcohol consumption (g/day)[c] | 17.1 (18.5) | 18.7 (20.0) | 0.42 |
| Caloric consumption (kcal/day)[c] | 2696 (727) | 2648 (669) | 0.48 |
| | *N* | *N* | $P$-value[d] |
| Physical activity frequency (leisure) | | | 0.79 |
| <1 per week | 103 | 106 | |
| 1–2 per week | 73 | 76 | |
| ≥3 per week | 51 | 45 | |
| Supplementation group | | | 0.62 |
| Placebo | 52 | 64 | |
| Alpha-Tocopherol | 57 | 55 | |
| Beta-Carotene | 58 | 55 | |
| Alpha-Tocopherol and Beta-Carotene | 60 | 53 | |

[a]*t*-test.
[b]Data not available for one case.
[c]Dietary data unavailable for seven cases and 20 controls.
[d]chi-squared test.

**Table II.** ORs and 95% CI for mtDNA copy number and risk of lung cancer

| mtDNA copy number quartile | Cases ($n$ = 227) | Controls ($n$ = 227) | OR[a,] (95%CI)[a] |
|---|---|---|---|
| Quartile 1 (≤100.6) | 52 | 57 | 1.0 (reference) |
| Quartile 2 (>100.6–124.8) | 59 | 56 | 1.3 (0.7–2.5) |
| Quartile 3 (>124.8–151.5) | 43 | 57 | 1.1 (0.6–2.2) |
| Quartile 4 (>151.5) | 73 | 57 | 2.4 (1.1–5.1) |
| $P_{trend}$[b] | | | 0.008 |

[a]ORs and 95% CIs determined by conditional logistic regression, adjusted for age at randomization, number of years smoking and number of cigarettes per day.
[b]Trend determined by treating mtDNA copy number as a continuous variable, adjusted for age at randomization, number of years smoking and total number of cigarettes.

**Table III.** mtDNA copy number and risk of lung cancer stratified by smoking level

| mtDNA copy number | Light smokers[a] | | Heavy smokers[a] | | |
|---|---|---|---|---|---|
| | Cases/ controls | OR[b,] (95% CI)[b] | Cases/ controls | OR[b,] (95% CI)[b] | $P$-interaction |
| Low (≤124.79) | 68/83 | 1.0 (reference) | 43/30 | 1.6 (0.9–3.0) | |
| High (>124.79) | 75/94 | 1.1 (0.6–1.9) | 41/20 | 2.6 (1.2–5.5) | 0.37 |

[a]Based on median distribution of number of cigarettes smoked per day in controls (<20 cigarettes per day versus ≥20 cigarettes per day).
[b]OR and 95% CI determined by conditional logistic regression, adjusted for age at randomization and number of years smoking.

To evaluate if undiagnosed lung cancer cases at the time of blood sample collection may have influenced our findings, cases diagnosed within the 2 years of follow-up after blood sample collection were excluded from analyses. After exclusion, the results for the association between mtDNA copy number and lung cancer were similar ($P_{trend}$ = 0.001). Restriction to only squamous cell carcinoma cases and matched controls yielded similar but non-significant results. Stratified analyses by supplementation arms also yielded similar associations between lung cancer risk and median mtDNA copy number for subjects receiving α-tocopherol (OR = 1.2; 95% CI = 0.4–3.8) or β-carotene (OR = 1.3; 95% CI = 0.4–3.8).

## Discussion

Future risk of lung cancer was associated with mtDNA copy number in the present study. Our results suggest that this association may be particularly important among heavy smokers. This is the first study, to our knowledge, to evaluate the risk of lung cancer and mtDNA copy number in a prospective cohort.

Cigarette smoke is a complex mixture of >4000 substances, of which many are chemicals that may introduce high levels of ROS in the human body (14). The ROS burden among smokers is attributed to both oxidants and pro-oxidants found in tobacco smoke (4,5). As such, oxidative stress may play a substantial role in the pathogenesis of smoking-related cancer. Levels of 8-hydroxydeoxyguanosine (8-oxodG), a biomarker for oxidative damage, have been found to be elevated in the lung tissues and peripheral leukocytes of smokers (15,16). Similarly, levels of the oxidative stress biomarker $F_2$-isoprostanes, have also been found to be substantially increased in smokers relative to non-smokers (6–8). Thus, it is conceivable that heavy smokers would have higher internal doses of ROS than light smokers, supporting our findings that heavy smokers would have higher mtDNA copy numbers.

One functional consequence of ROS damage is the disruption of cellular structural elements, including the lipid membranes of mitochondria (17). ROS affect mitochondrial function by damaging mtDNA and impairing electron chain transport. Because mtDNA is in close proximity to the inner membrane of the mitochondria, where electron chain transport occurs, direct oxidative damage to mtDNA is greater than to nuclear DNA. In addition to defenses that scavenge ROS, mitochondria respond to oxidative stress by increasing mtDNA copy number (18–20). In lung tumor tissues, the degree of oxidative mtDNA damage has been found to be significantly associated with mtDNA copy number (21). In fact, mice exposed to tobacco smoke have increased mitochondrial damage compared with unexposed mice (22,23). Thus, our findings that mtDNA copy number is particularly important at predicting future risk of lung cancer among heavy smokers is biologically plausible.

A small case–control study (122 cases; 122 controls) carried out in Xuanwei, China is the only other investigation to have evaluated the risk of lung cancer and mtDNA copy number (24). Similar to our

results, higher mtDNA copy number was associated with lung cancer risk in Xuanwei; however, the retrospective design used in the past study and the evaluation of mtDNA in post-diagnostic biospecimen did not rule out the possibility that the clinical course of lung cancer or treatment might have increased mtDNA copy number in blood of cases compared with controls. It has been hypothesized that the association observed in Xuanwei is attributed to the ROS induced by the high level of inhalation exposures from in-home coal combustion by-products (25). This previous report in Asians, in concert with our findings in Caucasians, suggests that mtDNA may be important to lung cancer etiology.

In conclusion, our results suggest that mtDNA copy number is associated with future development of lung cancer among heavy smokers. The major strength of our study is that the biological samples were collected prospectively, before lung cancer diagnosis. Due to our moderate sample size, replication is needed in larger prospective studies, ideally with more diverse populations (i.e. including women, other races and non-smokers). Future research should also include assays and analyses to elucidate the potential modification of this association by genetic variation of the mitochondrial genome or interindividual variation of mitochondrial function efficiency.

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