**Carcinogenesis** vol.35 no.8 pp.1760–1764, 2014
doi:10.1093/carcin/bgu093
*Advance Access publication April 17, 2014*

# Increased leukocyte mitochondrial DNA copy number is associated with oral premalignant lesions: an epidemiology study

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**Although changes in the mitochondrial DNA (mtDNA) copy number in peripheral blood leukocytes (PBLs) have been linked to increased susceptibility to several cancers, the relationship between the mtDNA copy number in PBLs and the risk of cancer precursors has not been investigated. In this study, we measured the relative mtDNA copy number in PBLs of 143 patients with histologically confirmed oral premalignant lesions (OPLs) and of 357 healthy controls that were frequency-matched to patients according to age, sex and race. OPL patients had a significantly higher mtDNA copy number than the controls (1.36 ± 0.74 versus 1.11 ± 0.32; $P < 0.001$). In analyses stratified by sex, race, alcohol consumption and smoking status, the mtDNA copy number was higher in the OPL patients than in the controls in all the strata. Using the median mtDNA copy number in the control group as a cutoff, we found that individuals with a high mtDNA copy number had significantly higher risk of having OPLs than individuals with a low mtDNA copy number (adjusted odds ratio, 1.93; 95% confidence interval, 1.23–3.05, $P = 0.004$). Analysis of the joint effect of alcohol consumption and smoking revealed even greater risk for OPLs. Our results suggest that high mtDNA copy number in PBLs is significantly associated with having OPLs. To our knowledge, this is the first epidemiologic study to show that the mtDNA copy number may indicate the risk of cancer precursors.**

## Introduction

Mitochondria are double-membrane organelles that perform multiple cellular functions, including energy production, cell proliferation and apoptosis. Mitochondria possess their own DNA (mtDNA), which is susceptible to damage caused by high levels of reactive oxygen species owing to the lack of introns and protective histones and to its close proximity to the electron transport chain (1). Cells from different tissue origins differ significantly in number of mtDNA copies—from several hundreds to >10 000 copies per cell (2). The copy number of mtDNA is related to the energy demands of sustaining normal functions (3) and is affected by both endogenous and exogenous factors such as age, smoking, hormones, dietary factors, stress, environmental oxidants/antioxidants, ionization irradiation, chemotherapeutic agents and cellular reactions to oxidative damage (4–10).

mtDNA alterations, including copy number changes and mutations, can result in cancer (11). Previous studies have shown changes in the mtDNA copy number and somatic mutation in a variety of cancer types (12–16). Accumulating evidence also suggests that changes in the mtDNA copy number in peripheral blood lymphocytes (PBLs) are associated with an increased risk for cancer, including kidney cancer (17,18), colorectal cancer (19,20), lung cancer (21), pancreatic cancer (22), breast cancer (23), non-Hodgkin lymphoma (24) and soft tissue sarcoma (25). Some of the studies were conducted prospectively with prediagnostic specimens (20–22,24), indicating that mtDNA copy number alteration could serve as a biomarker for some cancer types. However, many of these studies were retrospective case controls studies and it is unclear whether such alterations occurred before the onset of cancer or as a result of the disease. In addition, it remains unknown whether the mtDNA copy number in PBLs differs between individuals with cancer precursors and healthy controls. Such comparison would lead to a better understanding of the role of mtDNA alteration in carcinogenesis.

Oral premalignant lesions (OPLs), one such cancer precursor, are white (leukoplakia) or red (erythroplakia) mucosal patches in the oral cavity or oropharynx (26). Individuals with OPLs are at high risk for oral cavity cancer (OCC) because OPLs have been associated with OCC epidemiologically, geographically and clinically (27,28). The global prevalence of OPLs was estimated to be 2.6% in 2003 (29), and an estimated 41 000 new cases of OCC will occur in the USA in 2013 (30). Tobacco smoking and alcohol consumption are established risk factors for OPLs (31,32). However, one study in white people found that ~17% of individuals with OPLs were not smokers or alcohol consumers (33), indicating that other unknown factors might be involved in the development of OPLs. Although the mechanism of the development of OCC from OPLs has not been well established (34), the fact that OCC usually arises from OPLs suggests that identifying more people at high risk of OPLs and preventing OPLs will help reduce OCC.

The mtDNA copy number increases as an early molecular event in human cells in response to endogenous or exogenous oxidative stress through cell-cycle arrest (35). To investigate whether patients with OPLs exhibit altered mtDNA copy number in PBLs and to determine whether such alteration is associated with having OPLs, we performed a case–control study comprising 143 participants with OPLs and 357 healthy controls. To our knowledge, this study is the first to investigate the association between mtDNA copy number alteration in PBLs and the risk of precursors of OCC.

**Abbreviations:** CI, confidence interval; mtDNA, mitochondrial DNA; OCC, oral cavity cancer; OR, odds ratio; PBL, peripheral blood leucocyte.

## Materials and methods

### *Study participants*

This study included patients identified with OPLs at The University of Texas MD Anderson Cancer Center between September 1997 and June 2010. We enrolled patients with clinical manifestation of OPLs (leukoplakia or erythroplakia) and a biopsy showing one or several of the following histopathologic features: hyperkeratosis; hyperplasia; mild, moderate or severe dysplasia and OCC *in situ*. Excluded from the study were patients with a history of cancer and any chemotherapy or radiation therapy. Epidemiologic data of OPL patients were gathered from a self-administered questionnaire used to collect epidemiological data, which was described previously (36). Questionnaire data included demographical information and tobacco and alcohol use history. Most of OPL patients were participants of a chemoprevention trial (37) and only those aged ≥18 years were enrolled.

Healthy individuals were identified from a database of controls and had been recruited in collaboration with the Kelsey–Seybold Clinic in Houston, TX. Controls were recruited at a similar time frame as the cases. Individuals from the control database with no history of cancer were frequency-matched by age, sex and race to the OPL patients. Epidemiologic questionnaire data were obtained through in-person interview for the controls by our trained staffs. For our analysis, a participant who had never smoked or who had smoked <100 cigarettes in his or her lifetime was defined as a never smoker. A participant who had smoked ≥100 cigarettes in his or her lifetime but who had quit more than 12 months before the OPL diagnosis (for patients) or the interview (for controls) was considered a former smoker. Current smokers were those who were currently smoking or who had quit <12 months before the diagnosis (for patients) or the interview (for controls). Ever smokers included former smokers and current smokers. A participant who reported never drinking an alcoholic beverage in his or her lifetime was considered a never consumer;

otherwise, anyone who drank an alcoholic beverage at any time was regarded as an ever consumer.

For both cases and controls, written, informed consent was collected from each participant and approval for conducting human subject research was obtained from the MD Anderson and the Kelsey–Seybold Institutional Review Boards. Once the interview was completed, the participants were asked to donate 40 ml blood and the blood collected into a heparinized tube was sent to the laboratory for molecular analysis.

*mtDNA copy number by quantitative real-time PCR*

We used QIAamp DNA mini kits (Qiagen, Valencia, CA) to extract genomic DNA from the participants' whole blood. The relative mtDNA copy number was measured by a quantitative real-time PCR-based method as described previously (17). Briefly, two pairs of primers were used in the two steps of relative quantification of mtDNA copy number. One primer pair (ND1-R and ND1-F) was used for the amplification of the *ND1* gene in mtDNA. Another primer pair was used for the amplification of the single-copy nuclear gene human globulin (*HGB*).

In the first step, the ratio of the copy number of *ND1* gene in mtDNA to the *HGB* copy number was determined for each sample from standard curves. This ratio is proportional to the mtDNA copy number in each cell. The ratio for each sample was then normalized to a calibrator DNA in order to standardize between different runs. A genomic DNA sample from a healthy control was used as the calibrator DNA to compare results of different independent assays. The PCR mixture, a total volume of 14 µl, contained 1× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 215 nM ND1-R (or HGB-1) primer, 215 nM ND1-F (or HGB-2) primer and 4 ng of genomic DNA. The thermal cycling conditions for the mtDNA (*MT-ND1* gene) amplification were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min; for the HGB amplification, the cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 56°C for 1 min. All samples were assayed in duplicate on a 384-well plate with an Applied Biosystems 7900HT Sequence Detection System. The PCRs for mtDNA and *HGB* were performed on separate 384-well plates with the same samples in the same well positions to avoid possible position effect.

A standard curve of a serially diluted reference DNA, one negative control and one calibrator DNA were included in each run. For each standard curve, one reference DNA sample was serially diluted 1:2 to produce a 7-point standard curve between 0.3125 and 20 ng of DNA. The $R^2$ for each standard curve was ≥0.99. Standard deviations for the cycle of threshold value were accepted at 0.25. If the result was out of the acceptable range, the test was repeated. To assess intraassay variation, we assayed nine blood DNA samples from healthy control subjects 3 times on the same day. To further evaluate interassay variation, we evaluated the same blood DNA samples from the nine control subjects on different days. In this study, the intraassay coefficient of variation was 4.5% for all samples and the interassay coefficient of variation was 5.5%. The intraclass correlation coefficient was 0.940 [95% confidence interval (CI), 0.919–0.955] for mtDNA assay and 0.830 (95% CI, 0.772–0.873) for HGB assay. All the lab technicians were blinded to the case–control status of the DNA samples.

*Statistical analysis*

All statistical analyses were done with the Stata 10.1 statistical software package (StataCorp, College Station, TX). The Pearson $\chi^2$ test was used to assess the differences in the distribution of host characteristics (i.e. sex, race, smoking status and alcohol consumption) between the patients and the controls. Student's *t*-test was used for analyzing continuous variables (age and mtDNA copy number). Unconditional multivariate logistic regression analysis was conducted to calculate odds ratios (OR) and 95% CI as estimates of OPL relative risk in relation to the mtDNA copy number, based on cutoff points at the median value in the controls, with the adjustment for potential confounding variables such as age, sex, race, smoking status and alcohol consumption where appropriate. All statistical tests were two-sided, and statistical significance was set at $P < 0.05$.

## Results

The characteristics of the study population are summarized in Table I. The 143 OPL patients and 357 healthy controls did not significantly differ in age, sex or race. There were more ever smokers in the OPL group than in the control group; the difference was borderline significant (58.0% versus 49%; $P = 0.068$). However, the OPL group contained significantly more alcohol consumers than did the control group (61.6% versus 30.3%; $P < 0.001$). Two-thirds of OPL patients had pathologic confirmation of oral dysplasia at various grades.

**Table I.** Distribution of selected characteristics between patients with OPLs and control participants

| Variables | OPL patients ($n = 143$) | Controls ($n = 357$) | $P^a$ |
|---|---|---|---|
| Age, mean (SD) | 57.3 (12.4) | 58.5 (11.4) | 0.300 |
| Sex, *n* (%) | | | |
| Male | 87 (60.8) | 215 (60.2) | |
| Female | 56 (39.2) | 142 (39.8) | 0.899 |
| Race, *n* (%) | | | |
| Caucasians | 126 (88.1) | 323 (90.5) | |
| Others | 17 (11.9) | 34 (9.5) | 0.430 |
| Black | 2 (1.4) | 11 (3.1) | |
| Hispanic | 7 (4.9) | 17 (4.8) | |
| Unknown | 8 (5.6) | 6 (1.7) | |
| Smoking status, *n* (%) | | | |
| Never | 60 (42.0) | 182 (51.0) | |
| Former | 48 (33.6) | 136 (38.1) | |
| Current | 35 (24.5) | 39 (10.9) | <0.001 |
| Ever | | | |
| Former and current | 83 (58.0) | 175 (49.0) | 0.068 |
| Alcohol consumption, *n* (%) | | | |
| Never | 53 (37.1) | 249 (69.7) | |
| Ever | 85 (59.4) | 108 (30.3) | <0.001 |
| Unknown | 5 (3.5) | | |
| Histology grade of OPLs, *n* (%) | | | |
| Hyperkeratosis | 14 (9.8) | | |
| Hyperplasia | 23 (16.1) | | |
| Mild dysplasia | 55 (38.5) | | |
| Moderate dysplasia | 26 (18.2) | | |
| Severe dysplasia | 12 (8.4) | | |
| Carcinoma *in situ* | 13 (9.1) | | |

[a]*P* value was determined by the Pearson $\chi^2$ test for sex, race, smoking status and alcohol consumption, and was determined by the Student's *t*-test for age.

The mean mtDNA copy number in PBLs was significantly higher in OPL patients than in the controls (1.36 ± 0.74 versus 1.11 ± 0.32; $P < 0.001$; Table II). We compared the mtDNA copy number stratified by demographic characteristics. No modifying effect of sex, age, race, smoking status, pack-years or alcohol consumption on mtDNA copy number was identified in either the OPL patients or the controls. Higher mtDNA copy number was consistently observed in OPL patients compared with controls by sex, age, race, smoking status, pack-years <20 and alcohol consumption ($P < 0.05$ for all strata). Only in the subgroup of pack-years ≥20, the case–control difference did not attain statistical significance.

We next performed unconditional logistic regression analysis to assess the association between mtDNA copy number and OPL risk (Table III). When the mtDNA copy number was dichotomized into high and low groups by the median (50th percentile) value in the controls as the cutoff point, we found that individuals with a high mtDNA copy number had a significantly increased risk of OPLs (OR = 1.93; 95% CI, 1.23–3.05), compared with those with a low mtDNA copy number after adjusting for age, sex, race, smoking status and alcohol consumption.

We further assessed the joint effects of the mtDNA copy number and alcohol consumption on the risk for OPLs (Supplementary Table 1, available at *Carcinogenesis* Online). OPL patients and controls were categorized into four groups by mtDNA copy number (low or high as dichotomized by the median value in controls) as well as by alcohol consumption (never or ever drinkers). Subjects who had neither of these risk factors (i.e. "low copy number and never drinkers") were used as the reference group. Compared with this neither risk factor group, 'high copy number and never drinkers', 'low copy number and ever drinkers' and 'high copy number and ever drinkers' groups showed a significant gradual increase of OPL risk with ORs of 2.92 (95% CI, 1.51–5.68), 6.88 (95% CI, 3.42–13.84) and 10.31 (95% CI, 5.12–20.76), respectively (*P* for trend <0.001). When we assessed the joint effect of mtDNA copy number and smoking status

**Table II.** mtDNA copy number by characteristics of OPL patients and control subjects

| Variables | OPL patients | | Controls | | $P^a$ |
|---|---|---|---|---|---|
| | *n* | mtDNA copy number, mean (SD) | *n* | mtDNA copy number, mean (SD) | |
| Overall | 143 | 1.36 (0.74) | 357 | 1.11 (0.32) | <0.001 |
| Sex | | | | | |
| Male | 87 | 1.43 (0.87) | 215 | 1.10 (0.33) | <0.001 |
| Female | 56 | 1.26 (0.47) | 142 | 1.13 (0.29) | 0.019 |
| $P^a$ | | 0.203 | | 0.312 | |
| Age[b] | | | | | |
| ≤59 | 72 | 1.41 (0.89) | 186 | 1.09 (0.27) | <0.001 |
| >59 | 71 | 1.32 (0.57) | 171 | 1.14 (0.36) | 0.004 |
| $P^a$ | | 0.446 | | 0.133 | |
| Race | | | | | |
| Caucasians | 126 | 1.33 (0.70) | 323 | 1.11 (0.32) | <0.001 |
| Others | 17 | 1.60 (1.02) | 34 | 1.10 (0.31) | 0.012 |
| $P^a$ | | 0.172 | | 0.846 | |
| Smoking status | | | | | |
| Never | 60 | 1.40 (0.81) | 182 | 1.09 (0.27) | <0.001 |
| Ever | 83 | 1.34 (0.69) | 175 | 1.13 (0.35) | 0.002 |
| $P^a$ | | 0.628 | | 0.216 | |
| Pack-years in ever smokers[c] | | | | | |
| <20 | 20 | 1.51 (1.00) | 92 | 1.10 (0.38) | 0.003 |
| ≥20 | 44 | 1.27 (0.54) | 83 | 1.17 (0.32) | 0.179 |
| $P^a$ | | 0.218 | | 0.251 | |
| Alcohol consumption[d] | | | | | |
| Never | 53 | 1.36 (0.56) | 249 | 1.11 (0.30) | <0.001 |
| Ever | 85 | 1.33 (0.76) | 108 | 1.12 (0.35) | 0.011 |
| $P^a$ | | 0.792 | | 0.892 | |

[a]All *P* values were determined by the Student's *t*-test.
[b]The median age in the control group was 59 years.
[c]Pack-years were not available for 19 ever smokers.
[d]In the OPL group, five patients did not answer the alcohol consumption question.

**Table III.** Risk estimates of OPLs for mtDNA copy number

| mtDNA copy number | OPL patients (*n* = 143), *n* (%) | Controls (*n* = 357), *n* (%) | Adjusted OR (95% CI)[a] | *P* |
|---|---|---|---|---|
| By median | | | | |
| <1.08 | 56 (39.2) | 186 (52.1) | 1.00 (ref.) | 0.004 |
| ≥1.08 | 87 (60.8) | 171 (47.9) | 1.93 (1.23–3.05) | |

[a]OR and 95% CI determined by unconditional multivariate logistic regression, adjusted for age, sex, race, pack-years and alcohol consumption.

on risk, we observed a significant ($P < 0.001$) gradual increase of risk for never smokers with a high mtDNA copy number (OR = 2.52, 95% CI, 1.32–4.85), ever smokers with a low mtDNA copy number (OR = 2.79, 95% CI, 1.43–5.47) and ever smokers with a high mtDNA copy number (OR = 3.79, 95% CI, 1.96–7.33), compared with never smokers with a low mtDNA copy number. No significant interaction between alcohol consumption and mtDNA copy number ($P_{interaction} = 0.137$) and between smoking status and mtDNA copy number ($P_{interaction} = 0.158$) was detected.

## Discussion

We found that OPL patients had a significantly higher mtDNA copy number in PBLs than that of controls. When using the median mtDNA copy number in controls as a cutoff, we further found that individuals with a higher mtDNA copy number had a 2 times greater risk for having OPLs than those with a lower mtDNA copy number.

A growing body of evidence supports the association of mtDNA copy number alteration with carcinogenesis. Our study's results are consistent with those of previous studies that found that a high mtDNA copy number in PBLs was associated with an increased risk of several types of cancer, such as non-Hodgkin lymphoma (24), lung cancer (21) and colorectal cancer (19). However, a reverse correlation was also reported in other cancers, including renal cell carcinoma (17,18), breast cancer (23) and soft tissue sarcoma (25). Presumably, change in the mtDNA copy number may be regulated in a tumor specific manner during carcinogenesis (38), and the relationship between mtDNA copy number and cancer risk is multifactorial and multifaceted. The mtDNA copy number might elevate as an adaptive response to mitochondrial dysfunction, which is associated with oxidative stress, aging, immune response activation and response to environmental exposure. Furthermore, the mtDNA copy number might be affected by mutations in both the nuclear and mitochondrial genomes (3). During the process of reactive oxygen species–associated oxidative phosphorylation, certain mtDNA mutations may accumulate and gain mtDNA replicative advantage to the cell. For example, mutations in the D-loop of the mtDNA, which controls replication of mtDNA, may result in an increased copy number in some cancers (39–41) and decreased copy number in others (42,43). Some germ-line mutations are associated with mtDNA depletion (44,45). Total body irradiation could induce the 4977-bp 'common deletion' in the mitochondrial genome and result in increase of the mtDNA copy number in PBLs (5). Such deletion in mtDNA is also associated with aging (46) and might result from oxidative stress (47). The mtDNA copy number in PBLs may change as a reflection of the circulation oxidative stress derived from endogenous and exogenous factors, and it might increase as a compensatory mechanism for mtDNA damage (4).

Our results, based on mtDNA copy numbers in blood cells, also support a previous tissue-based study that showed an association between a high mtDNA copy number and the development of head and neck squamous cell carcinoma that progresses from benign lesions to premalignant intermediates to tumors (15). Such accordance between the results of blood cell-based studies and tissue-based studies can also be observed in other cancers. For example, our group recently reported that a low mtDNA copy number in PBLs is associated with increased risk of soft tissue carcinoma (25); this finding supports the results from tissue-based studies that showed a significantly lower mtDNA copy number in Ewing sarcoma (43) and osteosarcoma (12) than in normal tissue. We and others found high mtDNA copy number is associated with increased risk of renal cell carcinoma (17,18), which supported the findings from tissue-based study (48). Such accordance was also observed in studies of colorectal cancer (19,49,50). This consensus between the results from case–control studies of blood cells and the results of tissue studies of specific cancer sites is derived from indirect comparisons among independent studies. Taken together, these lines of evidence support the notion that the mtDNA copy number in PBLs could be a surrogate biomarker of risk for some cancers and cancer precursors.

Our finding of significantly higher mtDNA copy numbers in patients with OCC precursors than in healthy controls suggests that change in the mtDNA copy number might be an early event and a driving force in oral carcinogenesis. Although few studies have linked mtDNA copy number alteration to cancer precursor risk, the biological plausibility boosts the association of mtDNA copy number alteration with carcinogenesis. In one study, the mtDNA copy numbers progressively increased in tissues with the premalignant intermediate stages (mild, moderate and severe dysplasia) and in head and neck squamous cell carcinoma (15). Another tissue-based study showed that mtDNA mutation incidence increased with the grade of premalignant lesions from the upper aerodigestive tract (51). Frequent mtDNA mutations could also be detected in mucosal cells from ulcerative colitis, a premalignant condition that can lead to colorectal cancer (52). These lines of evidence indicate that the mtDNA alteration exhibited in cancer precursors may function in the early steps of carcinogenesis. However, such alteration might not necessarily result in clinically significant cancer, such as invasive OCC, because additional and cumulative environmental stimulus and genetic alterations are also involved in tumor progression. These factors could partially explain why oral dysplasia can regress or disappear after the elimination of a stimulus (such as smoking cessation) (53), although the mechanism of transformation of OPLs into OCC remains unknown.

We did not observe significant interactions between high mtDNA copy number and alcohol consumption and between high mtDNA copy number and smoking status, suggesting that these two factors might affect OPL risk independently of mtDNA copy number. These results were consistent with previous studies showing no significant interaction between smoking and mtDNA copy number on the risk of other tobacco-related cancers (18,21,22). Alternatively, our sample size may not be sufficient to detect a statistically significant interaction between smoking and mtDNA copy number as well as drinking and mtDNA copy number. The interactions among diverse OPL risk factors needed to be tested in a larger prospective study. When calculating the joint effect of alcohol consumption and mtDNA copy number, we found that alcohol consumers with a high mtDNA copy number had a 10 times higher risk of OPLs than non-consumers with a low mtDNA copy number. These data suggest that measuring mtDNA copy numbers in PBLs, in addition to environmental risk factors, would help to identify individuals at high risk of OPLs.

Our study had a few limitations. First, 'reverse causation' is always a concern for retrospective case–control studies, although we studied a precancerous condition. A prospective study is needed to ascertain the causal–effect relationship. Second, we could not adjust for grams/day of drinking and there might be residual confounding by the intensity of alcohol consumption. Third, our study was too small to detect a statistically interaction between smoking and mtDNA copy number as well as drinking and mtDNA copy number. Apart from these limitations we could not determine whether OPL patients with a high mtDNA copy number would more likely develop OCC than would patients with a low copy number because of the limited sample size and also because many of the OPL patients were later involved in various chemoprevention trials. The overall malignant transformation rates for OPLs range from 1.1 to 17.5%, depending on the length of follow-up (54). Only a prospective study with sufficient OPL patients enrolled and long-term observation of the natural progression of the disease would enable this question to be addressed.

In conclusion, our study showed that high mtDNA copy number in PBLs is associated with having OPLs. The main purpose of targeting people at high risk of OPLs is to provide personalized prevention of the disease and reduce its subsequent transformation into malignancy. Therefore, further work is necessary to identify genetic and environmental factors that facilitate the malignant progression of OPLs.

## Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

## Funding

The National Cancer Institute Specialized Center of Research Excellence (P50 CA097007 to The University of Texas MD Anderson Cancer Center; S.M.L., principal investigator; X.W., project leader); the MD Anderson Cancer Center Start-Up Fund (to J.G.).

*Conflict of Interest Statement:* None declared.

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*Received November 24, 2013; revised March 31, 2014;*
*accepted April 10, 2014*