Association of mitochondrial DNA copy number in peripheral blood leukocytes with risk of esophageal adenocarcinoma

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Materials and methods

Introduction

Esophageal cancer is among the leading causes of cancer death in the world. Although relatively rare in the USA, the incidence of esophageal cancer has been increasing during the past decade. In 2013, an estimated 17 990 new cases will be diagnosed and 15 210 patients will die from esophageal cancer (1). The two main types of esophageal cancer are esophageal squamous cell carcinoma, which typically occurs in the middle third of the esophagus, and esophageal adenocarcinoma (EAC), which predominately occurs in the lower third of the esophagus (2,3). The epidemiology of esophageal cancer varies significantly according to geographic location. In China and other countries in Asia, esophageal squamous cell carcinoma is the predominant cancer of the esophagus. In the USA and other Western countries, the incidence of EAC has increased at a rate exceeding any other cancers (4–6). Gastroesophageal reflux is a major cause of EAC (4). Other established risk factors of EAC include obesity and smoking. However, not all those who have been exposed to these risk factors develop EAC, suggesting that host genetic susceptibility and possibly gene–environment interactions may contribute to individual EAC risk (3–5).

Mitochondria are specialized organelles within cells that play a critical role in cellular energy metabolism, free radical generation and apoptosis. Mitochondrial DNA (mtDNA) is a circular, maternally inherited, double-stranded extrachromosomal DNA that is 16.5kb and contains 37 genes encoding polypeptides of the respiratory chain, transfer RNA and ribosomal RNA. MtDNA lacks introns and generally replicates at a high rate without an efficient DNA repair mechanism. Mutations in the mitochondrial genome or decreases in mtDNA copy number could lead to a deficiency in oxidative phosphorylation and an enhanced generation of adenosine triphosphate (ATP) by glycolysis (5). Decreased ATP generation by oxidative phosphorylation with concomitant enhanced glycolysis is often associated with cancer (6). Previous studies have reported that variations of mtDNA copy number in peripheral blood lymphocytes (PBLs) were associated with the risks of several cancers (7–18). There has been no such report in EAC. In this study, we used a case–control study to evaluate the association of mtDNA copy number in PBLs with the risk of EAC.

Study design

The study design and specimen collection methods have been described previously (2). Briefly, the MD Anderson Cancer Center EAC case–control study was initiated in October 2004. Cases were identified by reviewing the pathology reports of all patients who reported to the Department of Gastrointestinal Medical Oncology for clinic visits. Only patients who were diagnosed within the past 12 months were enrolled. Participation was not restricted on the basis of age, sex or disease stage. Eligible controls were selected from a pool of control subjects in ongoing case–control studies during the same time period (past 12 months). Briefly, healthy controls were identified and recruited using random digit dialing (19). Control subjects, who have had no prior history of cancer (except non-melanoma skin cancer), were frequency matched to the cases by age (±5 years), sex and race/ethnicity. The participation rate was 91.4% for cases. For controls, the overall response rate was ~51% and among those who agreed to participate, the response rate was ~88%. Written informed consent was obtained from all subjects, who were interviewed to elicit information on demographic characteristics, occupational history, tobacco and alcohol use, medical history and family cancer history. At the end of the interview, a 40 ml blood sample was obtained from each participant and delivered to the laboratory for processing. This study was approved by the institutional review board of MD Anderson Cancer Center.

Determination of mtDNA copy number via real-time PCR

Genomic DNA was extracted from whole-blood samples via QIAamp DNA Mini Kits (Qiagen, Valencia, CA). The mtDNA copy number was determined using a quantitative real-time PCR-based method as reported elsewhere with some modifications (20,21). Briefly, we used two pairs of primers in two steps of relative quantification of mtDNA content. One primer pair was used for the amplification of the ND1 gene in mtDNA. The primer sequences were as follows: forward primer (ND1-F), 5′-CCCTAAAACCCCGCCACATCT-3′; reverse primer (ND1-R), 5′-GAGCGATGGTAGTGAAGCTAAGGT-3′. Another primer pair was used for the amplification of the single-copy nuclear human globulin (HGB) gene. The primer sequences were as follows: forward primer (HGB-1), 5′-GTGCCACTTGACTCTGAGGAGA-3′, reverse primer (HGB-2), 5′-CCTTGATACCAACCTGCCCAG-3′.

During the first step, the relative mtDNA copy number and HGB copy number were determined for each sample from standard curves. We then determined the ratio of mtDNA to HGB copy number, which was proportional to the mtDNA copy number in each cell. Each PCR in a total volume of 14 μl contained 1× SYBR Green Mastermix (Applied Biosystems; Foster City, CA), 215 nM ND1-F (or HGB-1) primer, 215 nM ND1-R (or HGB-2) primer and 5ng of genomic DNA. The thermal cycling conditions for the mtDNA ND1 gene amplification were 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The thermal cycling conditions for the HGB amplification were 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All samples were assayed in duplicates in a 384 well plate with an Applied Biosystems 7900 Sequence Detection System. The PCRs for mtDNA and HGB were always performed on separate 384 well plates with the same samples in the

Abbreviations: ATP, adenosine triphosphate; CI, confidence interval; EAC, esophageal adenocarcinoma; mtDNA, mitochondrial DNA; OR, odds ratio; PBL, peripheral blood lymphocyte; RCC, renal cell carcinoma.

*These authors contributed equally to this work.
same well positions to avoid possible position effect. A standard curve of a diluted reference DNA, one negative control and one calibrator DNA were included in each run. Each plate contained randomly selected samples to have equal representation of cases and controls. The ratio for each sample was normalized to a calibrator DNA to standardize different runs. The calibrator DNA was a genomic DNA sample from a healthy control subject that was used to compare the results from different independent assays. For each standard curve, one reference DNA sample was serially diluted 1:2 to produce a seven-point standard curve between 0.3125 and 20ng of DNA. The R² for each standard curve was 0.99 or greater; Standard deviations for the cycle of threshold values were accepted at 0.25 or less. Otherwise, the sample was repeated. To assess intra-assay variation, we assayed nine blood DNA samples from healthy control subjects three times on the same days. To evaluate interassay variation, we evaluated the same blood DNA samples from nine control subjects on different days. The average intra-assay and interassay coefficient of variance was 4.5 and 5.5%, respectively (8). All laboratory personnel performing the experiments described above were blinded to the case–control status of all DNA samples.

**Statistical analysis**

Statistical analysis was performed using STATA 10.0 software (Stata, College Station, TX). Differences in host characteristics between patients and control subjects were assessed via the chi-square test for categorical variables and Student’s t-test for continuous variables. Multiple logistic regression modeling was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for EAC risk. Potential confounders, including age, sex and smoking, were adjusted in the model. The mtDNA copy number was categorized in two and four groups, using the median and quartiles among control subjects as cutoff points.

Never smokers were defined as those who had smoked fewer than 100 cigarettes during their lifetimes. Former smokers were defined as those who had smoked >100 cigarettes but had quit >1 year prior to diagnosis (patients) or interview (control subjects). Current smokers were defined as those who were currently smoking or had stopped smoking <1 year prior to diagnosis (patients) or interview (control subjects). Ever smokers included both former and current smokers.

### Results

A total of 218 pairs of patients and controls were included in the analysis. The patients and controls were similar in distributions with regard to age, sex and race/ethnicity, as they were frequency matched on these variables. The proportion of ever smokers was significantly lower in controls than in patients; however, there were no statistically significant differences between the cases and controls in terms of pack-years of smoking. The relative mean mtDNA copy number was significantly lower in patients (1.16 ± 0.30) than in controls (1.24 ± 0.41). The median values among cases and controls were 1.13 (range = 0.35–2.27) and 1.22 (range = 0.46–4.24), respectively (Table I).

Table II shows, in general, that mtDNA copy numbers were higher among controls than among cases across all categories by sex, age, race/ethnicity and smoking status, although the difference in female, ever smokers and non white did not reach statistical significance. We also analyzed the correlation between mtDNA copy number and age among controls and did not find significant correlation ($r^2 = 0.001, P = 0.984$).

We next analyzed the association between mtDNA copy number and the risk of EAC (Table III). Using the median mtDNA copy number in controls as the cutoff point, we found that lower mtDNA copy number was associated with a significantly greater risk of EAC, with an OR of 1.55 (95% CI, 1.05–2.29). Analysis of the data by the quartile distribution of mtDNA copy number in controls revealed a dose–response association between mtDNA copy number and EAC risk. Compared with individuals within the highest quartile of mtDNA copy number, those in the higher mid, lower mid and lowest quartiles had an OR of 0.88 (95% CI, 0.49–1.59), 1.14 (95% CI, 0.65–2.02) and 1.77 (95% CI, 1.03–3.04), respectively ($P$ for trend, 0.017). We also performed stratified analyses and observed that the association between lower mtDNA copy number and EAC risk reached significance in men, younger subjects and never smokers but not in women, older subjects and ever smokers (Table IV). However, there was no significant heterogeneity of the ORs across different strata of these variables in the test of heterogeneity ($P$ = 0.164, 0.945 and 0.739 for sex, age and smoking strata, respectively).

### Discussion

In this case–control study, we found that lower mtDNA copy number in PBLs was associated with a significantly increased risk of EAC. To the best of our knowledge, this is the first molecular epidemiological study that has evaluated mtDNA copy number in PBLs as a susceptibility biomarker for EAC.
Mitochondrial DNA and esophageal cancer risk

Somatic mtDNA mutation and mtDNA copy number reduction are common phenomena in human cancers (22–26). Recently, many studies have investigated the association of constitutive mtDNA copy number in PBLs with the risk of cancers (7–18). Several studies reported that low mtDNA copy number in PBLs was significantly associated with an increased risk of cancer, including renal cell carcinoma (RCC) (8,14), hepatocellular carcinoma (17), soft tissue sarcoma (16) and Ewing’s sarcoma (18), whereas others showing that high mtDNA copy number in PBLs was associated with increased risks of lung cancer (9), breast cancer (10), pancreatic cancer (12), colorectal cancer (13) and non-Hodgkin lymphoma (7). More interestingly, a recent prospective study reported a U-shaped association between mtDNA copy number in PBLs and colorectal cancer risk: both the lowest quartile and highest quartile conferred significantly increased cancer risks compared with the second quartile (15). These heterogeneous results are likely due to small sample sizes, different study designs and heterogeneous study populations. It is also possible that mtDNA copy number modulates cancer risks in a cancer type-specific manner.

Table IV. Association of EAC with mtDNA copy number stratified by selected host characteristics

<table>
<thead>
<tr>
<th>Host characteristics</th>
<th>Cases, N (%)</th>
<th>Controls, N (%)</th>
<th>Adjusted OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1.22</td>
<td>57 (32.95)</td>
<td>84 (48.55)</td>
<td>1 (reference)</td>
<td></td>
</tr>
<tr>
<td>≤1.22</td>
<td>116 (67.05)</td>
<td>89 (51.45)</td>
<td>1.85 (1.19–2.88)</td>
<td>0.006</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1.22</td>
<td>26 (57.78)</td>
<td>25 (55.56)</td>
<td>1 (reference)</td>
<td></td>
</tr>
<tr>
<td>≤1.22</td>
<td>19 (42.22)</td>
<td>20 (44.44)</td>
<td>0.86 (0.35–2.15)</td>
<td>0.750</td>
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<tr>
<td><strong>Age, years</strong></td>
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<tr>
<td>Age ≤ 60</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>&gt;1.22</td>
<td>39 (38.61)</td>
<td>56 (51.85)</td>
<td>1 (reference)</td>
<td></td>
</tr>
<tr>
<td>≤1.22</td>
<td>62 (61.39)</td>
<td>52 (48.15)</td>
<td>1.89 (1.06–3.38)</td>
<td>0.031</td>
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<tr>
<td>Age &gt; 60</td>
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</tr>
<tr>
<td>&gt;1.22</td>
<td>44 (37.61)</td>
<td>53 (48.18)</td>
<td>1 (reference)</td>
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<tr>
<td>≤1.22</td>
<td>73 (62.39)</td>
<td>57 (51.82)</td>
<td>1.38 (0.79–2.39)</td>
<td>0.256</td>
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<tr>
<td><strong>Smoking status</strong></td>
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<tr>
<td>Never smoking</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1.22</td>
<td>30 (44.12)</td>
<td>56 (58.33)</td>
<td>1 (reference)</td>
<td></td>
</tr>
<tr>
<td>≤1.22</td>
<td>38 (55.88)</td>
<td>40 (41.67)</td>
<td>1.90 (0.98–3.69)</td>
<td>0.058</td>
</tr>
<tr>
<td>Ever smoking</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1.22</td>
<td>53 (35.33)</td>
<td>53 (43.44)</td>
<td>1 (reference)</td>
<td></td>
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<tr>
<td>≤1.22</td>
<td>97 (64.67)</td>
<td>69 (56.56)</td>
<td>1.39 (0.85–2.28)</td>
<td>0.192</td>
</tr>
</tbody>
</table>

*Sex: adjusted for age, ethnicity and smoking status; age: adjusted for sex, ethnicity and smoking status; smoking status: adjusted for age, sex and ethnicity.

In conclusion, to our knowledge, our case–control study offers the first evidence that low mtDNA copy number in PBLs is associated with an increased risk of EAC with a significant dose–response effect. Our findings support an important role for mitochondria dysfunction in esophageal carcinogenesis.

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Conflict of Interest Statement: None declared.

References


Our study had a few limitations. First, we did not repeat mtDNA copy number measurement in the same subject and a single measurement may not reflect mtDNA copy number over a lifetime. However, Thiyagarajan et al. (15) conducted a small study and found mtDNA copy number is relatively stable over time. Second, although our sample size is sufficient for an overall association analysis, it is limited for stratified and interaction analyses. Large-scale validation is necessary to confirm and extend our observations. Third, we used the peripheral blood leukocytes to detect the relationship between mtDNA copy number and the risk of EAC. However, mtDNA copy number may be different among leukocyte subpopulations, such as neutrophils have low number of mtDNA copies (33). It is possible that the observed case–control differences may be partially due to systematic differences in leukocyte subpopulations. Future studies are needed to compare mtDNA copy number in different subpopulations of leukocytes between cases and controls. Finally, reverse causation is always a concern for retrospective case–control study evaluating an intermediate biomarker. Previous studies have shown examples of significant differences in cancer risk association with intermediate biomarkers by prospective versus retrospective studies (8,14,34–37). For example, the association of mtDNA copy number in PBLs with the risk of RCC was evaluated in three studies: two prospective case–control studies showed that lower mtDNA copy number conferred an increased risk of RCC (8,14), whereas a prospective nested case–control study demonstrated a significant association of higher mtDNA copy number with increased risk of RCC (37). Prospective study is always the preferred design when evaluating intermediate biomarkers for cancer risk association. In our current study, we only included newly diagnosed EAC cases and collected blood samples before any treatment, which should reduce the impact treatment on mtDNA copy number. We did not observe significant relationship between tumor stage and grade and mtDNA copy number (data not shown), arguing against the effect of disease status on mtDNA copy number. A previous classic twin study estimated a genetic heritability of 65% for mtDNA copy number in PBLs (8), supporting its usage as a risk assessment biomarker. Nevertheless, future prospective validation is warranted to confirm our observations.

In conclusion, to our knowledge, our case–control study offers the first evidence that low mtDNA copy number in PBLs is associated with an increased risk of EAC with a significant dose–response effect. Our findings support an important role for mitochondria dysfunction in esophageal carcinogenesis.

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