Mitochondrial DNA Quantity Increases with Histopathologic Grade in Premalignant and Malignant Head and Neck Lesions

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ABSTRACT

Purpose: Mitochondria are highly susceptible to oxidative damage. Although mitochondrial function decreases with oxidative damage, overall mitochondrial DNA (mtDNA) content increases to compensate for general mitochondrial dysfunction. We performed quantitative polymerase chain reaction for genes specific to mitochondrial and nuclear genomes to investigate relative mitochondrial abundance in a spectrum of dysplastic head and neck lesions.

Experimental Design: DNA from mild, moderate, and severe dysplasias, as well as invasive tumors and normal mucosal cells, was extracted. Using quantitative polymerase chain reaction, mitochondrial to nuclear DNA ratios were determined by quantification of cytochrome c oxidase subunit 1 (CoxI) and \(\beta\)-actin genes.

Results: Mean CoxI/\(\beta\)-actin DNA ratios for mild, moderate, and severe premalignant lesions were 0.0529, 0.0607, and 0.1021, respectively. The mean ratio for the normal mucosal cells contained in saliva was 0.0537, whereas the mean ratio for tumors was 0.1667. As a whole, our experimental model demonstrated significance (\(P = 0.0358\)). Comparisons between individual categories showed borderline significance when compared with the normal group, with \(P\) values of 0.0673, 0.0747, and 0.0824 for moderate and severe dysplasia and invasive tumor, respectively.

Conclusions: Head and neck squamous cell carcinomas arise through premalignant intermediates and may be merely morphologic manifestations of accumulated genetic alterations. In keeping with this molecular tumor progression model, our study shows that mtDNA increases according to histopathologic grade, a phenomenon that may be a feedback mechanism that compensates for a generalized decline in respiratory chain function. Therefore, high mtDNA content may be another marker of genetic alteration, a measure of relative DNA injury, and a surrogate measure of histopathologic grade.

INTRODUCTION

The mitochondrial genome exhibits higher mutation rates than the nuclear genome and is highly susceptible to damage caused by reactive oxygen species (1, 2). The error-prone replication and repair of mitochondrial genes and the absence of histones, which normally serve to package and protect DNA from damage, contribute to the vulnerable nature of mitochondrial DNA (mtDNA). Recent investigations have shown that mtDNA alterations increase with age. This is believed to be a result of lifelong, cumulative oxidative damage coupled to the reduced efficiency of mtDNA repair systems (3–7). These age-related alterations in turn cause declines in mitochondrial respiratory function (8, 9). Acute oxidative damage by the application of hydrogen peroxide, like the chronic age-related accumulation, has also been shown to decrease mitochondrial function in cultured human cells (1).

Interestingly, mtDNA copy number appears to increase with age in various human tissues (10) as well as in cells affected by the exogenous application of an oxidative agent such as hydrogen peroxide (11). This increase in mitochondrial copy number has been viewed as a potential compensatory effect for the generalized decline in mitochondrial respiratory function (12). Thus, mtDNA content is directly proportional to oxidative DNA damage yet inversely proportional to function.

Oxidative DNA damage has been implicated in the development of many human cancers through the mutation and dysregulation of critical genes. Molecular damage created in part by oxidative insults can drive the progression of normal tissues to cancer. First proposed in colorectal cancer, the tumor progression model states that normal tissues progress to cancer through a series of premalignant intermediates (13). The histopathological appearance of these stages of tumorigenicity is caused by a stepwise accumulation of critical genetic alterations. Specifically, alterations of mitochondria have also been discovered in a variety of primary human cancers including gastric (14), esophageal (15), thyroid (16), breast (17), and non–small-cell lung cancer (18). In head and neck premalignant lesions, alterations in the mtDNA poly C-tract, an important area located in the mitochondrial D-loop, are proportional to degrees of premalignancy (19).
It is established that reactive oxygen species damage DNA, increase mitochondrial content, and increase the potential for cancer development. In addition, it has been demonstrated that cancer develops through a series of premalignant intermediates in keeping with a tumor progression model. Therefore, our goal was to establish a link between a spectrum of head and neck premalignant lesions and mitochondrial content in the context of normal mucosal cells and tumors. If such a link can be established, quantification of mtDNA content may serve as a molecular marker that may have use in evaluating the tumorigenic potential of head and neck lesions. Our study explored the relationship between mtDNA content and a spectrum of head and neck tissues of differing histopathological grades.

MATERIALS AND METHODS

Sample Procurement. Institutional review board approval was obtained to perform a retrospective analysis of premalignant upper aerodigestive lesions at Johns Hopkins Hospital. The surgical pathology records of the Johns Hopkins Hospital were searched for consecutive cases of premalignant and head and neck mucosal lesions of the upper aerodigestive tract without a previous or concurrent head and neck malignancy over a 28-year period. Ninety-one patients with available tissue were identified and stratified on the basis of the corresponding premalignant lesion’s histologic grade and classified as mild, moderate, or severe. Hematoxylin and eosin-stained sections of each premalignant case were reviewed by a head and neck pathologist with extensive experience in grading dysplasias of the upper aerodigestive tract (W. H. W.). The degree of dysplasia was determined according to established World Health Organization guidelines (20). From each case, tissue blocks were selected for histologic sectioning and DNA extraction.

Ten-micron sections were cut from paraffin blocks and microdissected using hematoxylin and eosin-stained slides as a guide. The samples were placed in xylene for 12 hours with subsequent centrifugation at 13,500 rpm. The tissue pellets were then digested in 1% SDS/proteinase K over 48 hours at 48°C. Subsequent phenol-chloroform extraction and EtOH precipitation were performed, and samples were stored at −20°C. Sixteen paraffin-embedded head and neck squamous cell carcinoma (HNSCC) tumors were processed in the same manner.

Local community organizations with a significant proportion of members with tobacco exposure were contacted for participation in a screening study for upper aerodigestive tract malignancy. Informed consent was obtained by an institutional review board-approved protocol, and 655 participants with a variety of ages and risk factors were recruited. At the screenings, subjects filled out a detailed health/demographic questionnaire, provided blood and saliva samples, and received a physical examination by a head and neck surgeon. To procure mucosal cells from the upper aerodigestive tract, subjects were given 25 mL of saline to swish, gargle, and brush. The patients were instructed to brush their buccal mucosa, rinse, and in keeping with a tumor progression model. Therefore, our goal was to establish a link between a spectrum of head and neck premalignant lesions and mitochondrial content in the context of normal mucosal cells and tumors. If such a link can be established, quantification of mtDNA content may serve as a molecular marker that may have use in evaluating the tumorigenic potential of head and neck lesions. Our study explored the relationship between mtDNA content and a spectrum of head and neck tissues of differing histopathological grades.

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Although these saliva samples were from a mixed population with and without smoking histories, their oral mucosal cells were considered normal because their physical exams were devoid of suspicious lesions. Each wash sample underwent centrifugation at 2,500 rpm for 15 minutes. The supernatant was discarded, and the cell pellet was retained. One percent SDS/proteinase K (0.5 mg/mL) was added, and the resultant mixture was placed at 48°C for 72 hours. Subsequent DNA extraction was performed through phenol-chloroform and EtOH precipitation.

Quantitative Polymerase Chain Reaction. A Perkin-Elmer/ABI 7900 thermocycler was used to perform real-time polymerase chain reaction (PCR) amplification for β-actin and mitochondrial DNA regions for cytochrome c oxidase I (CoxI). CoxI primer sequences used previously were used in this study (21). Primers were custom made and obtained from Invitrogen (Carlsbad, CA). The CoxI region was amplified using forward primer 5′-TTGCCGACCGTGTAATCTCTC-3′ and reverse primer 5′-AAGATTATACAAATGCTATGGGC. β-Actin amplification was done using forward primer 5′-ACCCACGTGTGCCCATCTAC-3′ and reverse primer 5′-TCGGTGAGCATCTTCATGAGGTA-3′. All TaqMan probes (Applied Biosystems, Foster City, CA) were 5′-FAM and 3′-TAMRA labeled. CoxI probe6-FAM-ACCGACCATCTACAAACGTATCGTCACTAMRA and β-actin probe 6-FAM-ATGCCCTCCCCCATGTCATCC-TAMRA were used. Probe and primer combinations were designed to avoid amplification of pseudogenes, by performing BLAST analysis of a primers and probes. PCR amplifications were carried out in buffer containing 16.6 mmol/L ammonium sulfate, 67 mmol/L Trizma, 2.5 mmol/L MgCl2, 10 mmol/L β-mercaptoethanol, 0.1% dimethyl sulfoxide, 600 mmol/L each of forward and reverse primers, 200 mmol/L TaqMan probe, 0.6 unit of platinum Taq polymerase, and 2% Rox reference dye. Five hundred picograms of DNA were used to amplify mitochondrial regions. Whereas 10 ng were used to amplify β-actin, a single copy gene. The real-time PCR reactions were performed in triplicate for each gene. Standard amplification curves for CoxI and β-actin genes were obtained using JHU-O11 cell line DNA.

Statistical Analysis. The major statistical end point in this study was the comparison of the mean mtDNA content of specimens taken from five different types of head and neck lesions. A regression model was fit to test if the means from the mild, moderate, severe, and tumor groups were different from the normal group. Mitochondrial DNA values were transformed with the log transformation for this analysis. Means and SDs were determined on the natural scale. All statistical computations were performed using the SAS system (SAS Institute Inc., Cary, NC), and a two-sided P value is reported.

RESULTS

Using quantitative PCR techniques, we determined the relative content of mtDNA with respect to the β-actin gene in 91 premalignant lesions of different grades, 14 malignant lesions, and in the normal mucosal cells in 655 individuals without disease (Fig. 1). Eighteen, 32, and 41 patients exhibited mild, moderate, and severe dysplasias, respectively. In the population with premalignant disease, mean age was 67.1 years, and median age was 70 years. Eighteen, 32, and 41 patients exhibited mild, moderate, and severe dysplasias, respectively. The mean and median age of patients with tumors was 38.8 and 40 years, respectively. In the population without lesions, mean age was 62.4 years, and median age was 63 years.

Mean CoxI/β-actin DNA ratios for mild, moderate, and severe premalignant lesions were 0.0529, 0.0607, and 0.1021, respectively.
respectively (Table 1). A combined mean ratio for mild and moderate dysplasias was 0.0578, and a combined ratio for all dysplasias was 0.0780. The mean ratio for the normal mucosal cells contained in saliva was 0.0537, whereas the mean ratio for tumors was 0.1667 (Fig. 2).

This showed a clear increase in mtDNA/nuclear DNA (nDNA) ratios from normal tissue to invasive tumors. The greatest increase in mtDNA content was seen in the transition from moderate dysplasia to invasive tumor because mild dysplasia had only a minimal increase in the mtDNA/nDNA ratio when compared with saliva (normal cells). We performed an analysis of variance to examine the significance of our findings as described (see Materials and Methods). As a whole, our model demonstrated significance \( P = 0.0358 \). Comparisons between individual categories showed borderline significance when compared with the normal (saliva) group, with \( P \) values of 0.0673, 0.0747, and 0.0824 for moderate dysplasia, severe dysplasia, and invasive tumor, respectively.

**DISCUSSION**

The vast majority of upper aerodigestive tract cancers are related to smoking (22), and like most cancers, HNSCCs are thought to arise through premalignant intermediates that are the phenotypic expression of accumulated genetic alterations. These genetic alterations are a result of exposure to oxidative agents conferred through tobacco consumption. Mitochondrial DNA is more susceptible to oxidative damage and possesses a mutation rate that may be as much as 2 orders of magnitude greater than that of nDNA (2). Therefore, it is not surprising that exposure to extrinsic oxidative and mutagenic agents, such as cigarette smoke, augments mutagenicity, resulting in the generalized decline of mitochondrial respiratory chain function (23) with concomitant increases in mtDNA content (11, 24). Decreases in mitochondrial function are also a function of age because the effects of mutations and concomitant failures of mtDNA repair accumulate over time (23).

Our hypothesis is that in upper aerodigestive mucosal cells, mtDNA experiences alterations and resultant dysfunction as a reflection of progression to a malignant phenotype. In response to this dysfunction, mitochondrial content increases to compensate for its functional deficiencies. Our study supports this hypothesis by showing not only an increase in mtDNA/nDNA ratios but also a consistent and proportional relationship between mitochondrial content and different degrees of histopathologic grade. This implies that a progressive increase in mtDNA content is a hallmark of tumor progression in HNSCC and occurs in a manner similar to that found by oxidative injury.

One potential limitation is our relatively small numbers of premalignant head and neck lesions, tissues that are difficult to obtain in large numbers. Although the differences in values closely approach significance, the addition of greater sample numbers may be necessary to definitively demonstrate differences between each degree of dysplasia. Another limitation to our study was the relative dearth of information with respect to smoking that accompanied our archival premalignant tissues. If tobacco consumption information were readily available, then direct conclusions could be made regarding the relationship of smoking to both mtDNA/nDNA ratio and degrees of premalignancy. Instead, our data illustrate the relationship between mtDNA/nDNA ratios and histopathology only. However, because numerous previous studies have outlined a direct relationship between dysplastic tissues and smoking, we can safely assume that this relationship exists in our study as well.

**Table 1** Relative mtDNA content \((\text{CoxI}/\beta\text{-actin ratio})\) versus category of dysplasia

<table>
<thead>
<tr>
<th>Lesion type</th>
<th>Sample size</th>
<th>Mean CoxI/β-actin ratio</th>
<th>25th, 50th, and 75th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal cells (saliva)</td>
<td>655</td>
<td>0.0537</td>
<td>0.0134, 0.0257, and 0.0584</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>18</td>
<td>0.0529</td>
<td>0.0306, 0.0396, and 0.0760</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>32</td>
<td>0.0607</td>
<td>0.0282, 0.0428, and 0.0732</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>41</td>
<td>0.1021</td>
<td>0.0233, 0.0474, and 0.0766</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>14</td>
<td>0.1667</td>
<td>0.0307, 0.0773, and 0.2693</td>
</tr>
</tbody>
</table>

![Fig. 1](http://example.com/fig1.png) **Fig. 1** Quantitative PCR of CoxI and β-actin genes (representative curve). CoxI and β-actin are ubiquitous genes found in the mitochondrial and nuclear genomes, respectively. Using quantitative PCR in samples from the same patient, the mtDNA/nDNA ratio can be assessed. Subsequently, the CoxI/β-actin ratio is calculated to detail the relative mitochondrial content of the sample.

![Fig. 2](http://example.com/fig2.png) **Fig. 2** Mean mtDNA content is directly proportional to histopathologic stage. Consistent upward trending between tissues of increasing histopathologic severity was observed. This represents a molecular validation of the tumor progression model in HNSCC.
The age disparity among the patients from whom the samples were collected was also an initial concern because of previously published associations between increased mitochondrial content and advanced age. Premalignant and saliva samples corresponded to patients in their early 60s, whereas tumor-bearing patients had a median age of 40 years. This disparity became less of a concern when we discovered relative increases in tumors despite these patients being 20 years younger. Therefore, we have likely underestimated the difference in mtDNA content between premalignant lesions.

Originally developed from colorectal cancer (13), the tumor progression model, which relates cancer development to a stepwise accumulation of genetic alterations, has been extended to HNSCCs (25). Our results support this model by revealing a progressive increase of mitochondrial content across a spectrum of benign, premalignant, and malignant lesions.

This study also complements previous research that explored the relationship between mitochondrial C-tract alterations and oral premalignancies of different histopathological grade (19). The mitochondrial C-tract region, a mononucleotide repeat of cytosine residues located in the D-loop region of the mitochondrial genome, had previously been shown to be altered in HNSCCs (26). Ha et al. (19) observed significant C-tract alteration rates of 22%, 37%, and 62% in benign hyperplastic, premalignant, and carcinoma in situ tissues, respectively. These observations and ours provide evidence that increases in both mutational alterations of mtDNA and mitochondrial content are directly proportional to histologic grade.

These dual phenomena may be explained by a positive feedback mechanism whereby the cell, to compensate for a generalized decline in respiratory chain function caused by mutagenic exposure, up-regulates the replication of mitochondria. As such, measurement of mtDNA content may reflect genetic alteration or DNA injury. However, impaired oxidative phosphorylation may also result in increased oxidative injury to both nuclear and mtDNA, resulting in additional mutagenic insult.

In summary, mtDNA alterations and content increases are common progression events in the development of HNSCC from benign lesion to premalignant intermediates and, ultimately, to tumors. Although this relationship may be simply associated with HNSCC carcinogenesis, mitochondrial derangements demonstrate characteristic behavior of progression events that are etiologic in tumor development. This gives support to investigation of the functional consequences of mitochondrial alteration in the development of HNSCC.

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