Increased Mitochondrial DNA Content in Saliva Associated with Head and Neck Cancer

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Abstract Alterations of the mitochondrial DNA (mtDNA) have been described in human tumors and in other tissues in association with smoking exposure. We did quantitative PCR of *cytochrome c oxidase I* (*Cox I*) and *cytochrome c oxidase II* (*Cox II*) genes on oral rinse samples obtained from 94 patients with primary head and neck squamous cell carcinoma (HNSC) and a control group of 656 subjects. Mitochondrial DNA/nuclear DNA in saliva from HNSC patients and controls in relationship to smoking exposure, ethanol intake, and tumor stage were examined. Mean levels of *Cox I* and *Cox II* in saliva samples were significantly higher in HNSC patients: *Cox I*, 0.076 [95% confidence interval (95% CI), 0.06-0.09] and *Cox II*, 0.055 (95% CI, 0.04-0.07) in comparison with controls *Cox I*, 0.054 (95% CI, 0.05-0.06), $P < 0.0001$ and *Cox II*, 0.046 (95% CI, 0.04-0.05), $P = 0.003$ (*t* test). MtDNA levels were elevated in primary tumors when compared with matched, pretreatment saliva and significant correlation was noted (*Cox I*, $r = 0.30$, $P = 0.005$ and Cox *II*, $r = 0.33$, $P = 0.002$, respectively, Pearson's correlation). On univariate analysis, smoking, age, HNSC diagnosis, and advanced stage of HNSC were associated with higher level of mtDNA content in saliva. Multivariate analysis showed a significant and independent association of HNSC diagnosis, age, and smoking with increasing mtDNA/nuclear DNA for *Cox I* and *Cox II*. mtDNA content alteration is associated with HNSC independently of age and smoking exposure, can be detected in saliva, and may be due to elevation in mtDNA content in primary HNSC.

Mitochondria are an important biological source and target of reactive oxygen species and free radicals and are sensitive to environmental mutagens, including tobacco smoke. Normal assembly and operation of the respiratory chain requires an intact and functional mitochondrial genome. The functional complement of mitochondrial DNA (mtDNA) in a cell depends on both the copy number of mitochondrial genomes as well as the integrity of each mtDNA molecule (1). The number of mitochondria per cell type is highly variable depending on the cell's energetic demand (2). Any event invalidating the respiratory chain, either issuing from genetic damage or from the cell environment (3) could provide a proliferating advantage to a transformed or tumor cell.

During the past few years, point mutation or length instability of displacement loop and Coding region, 4,977 deletion or short deletion of mitochondria have been found involved in the colorectal (4, 5), breast (6), gastric (7), renal (8), esophageal (9), thyroid cancer (10), and head and neck cancer (11). Furthermore, The increase of mtDNA content with age has been hypothesized to be a compensatory response for the decline in respiratory function (12).

On the other hand, there are over 3,800 compounds in cigarette smoke, including a large amount of mutagenic and oxygen radical-forming substances and over 60 documented carcinogens. These organic radicals and genotoxic agents may cause extensive damage to DNA, proteins, and lipids (13). Evidence concerning a connection between smoking and head and neck cancer has been extensively documented (14). Recently, mtDNA content was identified to be altered in aging and smoking-associated lung tissue (15). We hypothesized that elevation in salivary mtDNA associated with development of head and neck squamous cell carcinoma (HNSC) is independent of other factors that may contribute to mtDNA increase and determined associations with other clinicopathologic variables.

To test these hypotheses, we did quantitative PCR of mtDNA content (*Cox I* and *Cox II*) on oral rinse samples obtained from 94 of primary HNSC subjects and a control group of 656 participants without HNSC, examining known risk factors for HNSC development, mtDNA content alteration, and clinical variables associated with HNSC.

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Materials and Methods

Subjects. Ninety-four oral rinse samples were collected from patients with primary HNSC at Department of Otolaryngology-Head and Neck Surgery, School of Medicine, Johns Hopkins University, after appropriate approval was obtained from the Johns Hopkins institutional review board. We also obtained control oral rinse samples from 656 non-HNSC subjects with a variety of ages and risk factors, including smoking and alcohol intake, were recruited for a head and neck screening study. All control subjects were given a confidential survey of risk factors for head and neck cancer (alcohol, tobacco use, etc.) as well as the presence of comorbid illness. Smoking was defined as at least one continuous year of daily smoking. Data regarding tobacco exposure and ethanol intake from HNSC subjects was abstracted from hospital records.

Collection of oral rinse samples. Oral rinse samples were obtained by swishing and gargling for 15 seconds with 25 mL of sterile 0.9% NaCl and brushing oral and oropharyngeal mucosal surfaces with an exfoliative brush and rinsing in the salt solution. The 25-mL swish and gargle samples were subjected to centrifugation at 2,500 rpm for 15 minutes. The supernatant was discarded, and the cell pellet was retained and placed in 1% SDS/proteinase K (0.5 mg/mL) at 48°C for 72 hours. Digested product was then subjected to phenol-chloroform extraction and ethanol precipitation.

Quantitative PCR. A Perkin-Elmer/ABI 7900 thermocycler was used to perform quantitative PCR amplification for *β-actin*, mtDNA regions for *cytochrome c oxidase I* (*Cox I*) and cytochrome *c* oxidase II (*Cox II*). Primers were custom made and obtained from Invitrogen (Carlsbad, CA). The *Cox I* region was amplified using forward primer 5′-TTCGCCGACCGTTGACTATTCTCT-3′ and reverse primer 5′-AAGATTAT-TACAAATGCATGGGC. *Cox II* region amplification was done using forward primer 5′-CCCCACATTAGGCTTAAAAACAGAT-3′ and reverse primer 5′-TATACCCCCGGTCGTGTAGC-3′. *β-Actin* amplification was done using

Table 1. Characteristics of HNSC subjects and controls

	HNSC (%)	Control (%)	*P*
Sex			
Male	71 (75.5)	413 (63.3)	0.0197
Female	23 (24.5)	240 (36.8)	
Age (y)			
Mean	56.8	61.8	0.0008
Median	57	63	
Range	31-85	18-94	
Race			
White	70 (75.3)	510 (78.5)	0.4865
Other	23 (24.7)	140 (21.5)	
Smoking			
Never	19 (20.9)	248 (38.0)	<0.0001
Former	24 (26.4)	326 (49.9)	
Current	48 (52.8)	79 (12.1)	
Ethanol			
Never	30 (39.0)	124 (20.6)	0.0012
Former	13 (16.9)	115 (19.1)	
Current	34 (44.2)	364 (60.4)	
mtDNA (natural scale)			
Cox I	0.076	0.054	<0.0001
Cox II	0.055	0.046	0.003

NOTE: Race distribution is similar in two groups. Gender, age, smoking, and ethanol intake have different distribution between two groups.

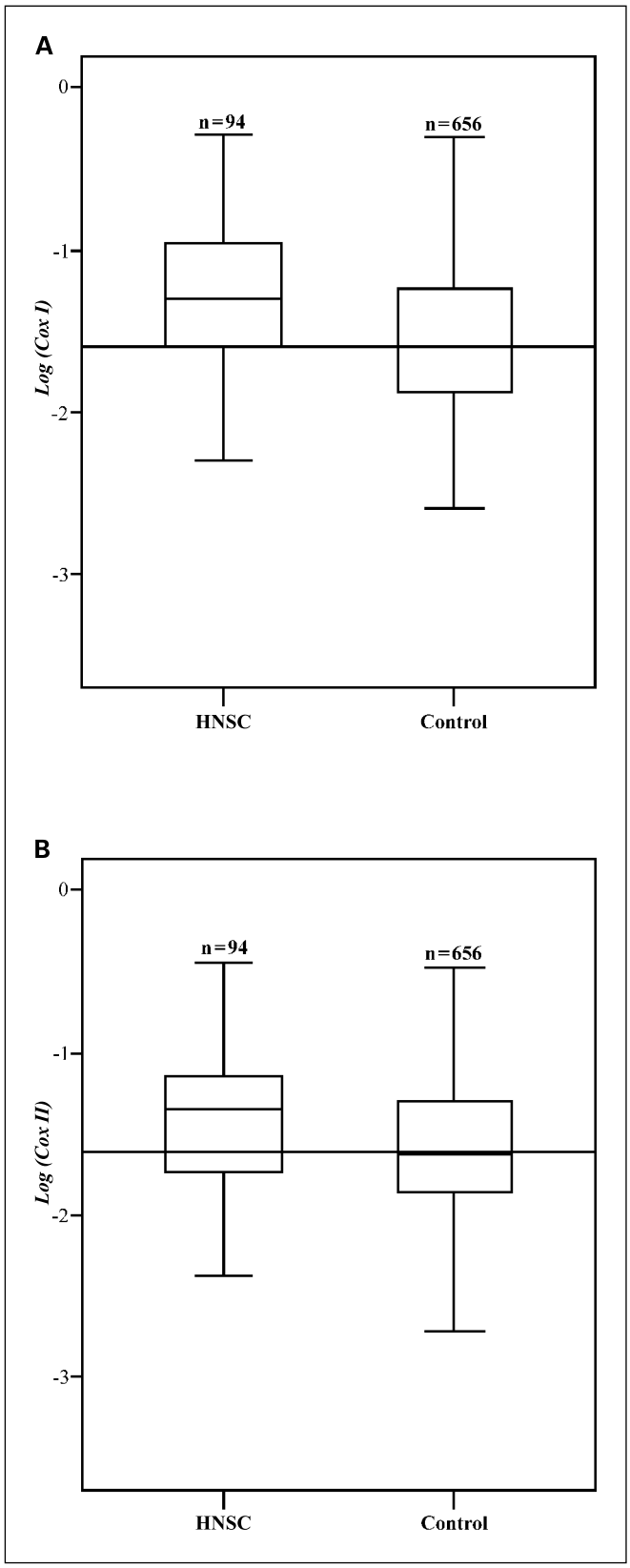

Fig. 1. Distributions of mtDNA are graphed with box plots on log scales. *A,* in the saliva of HNSC group, the mean level of *Cox I*, 0.076 (95% CI, 0.06-0.09) was significantly elevated compared with controls, *Cox I*, 0.054 (95% CI, 0.05-0.06; $P < 0.0001$). *B,* in the saliva of HNSC group, the mean level of *Cox II*, 0.055 (95% CI, 0.04-0.07) was significantly elevated compared with controls, *Cox II*, 0.046 (95% CI, 0.04-0.05; $P = 0.003$). Bars, ±SD. Boxes, 95% CI. Line in Box, mean value.

Table 2. mtDNA content (natural scale) in saliva samples with smoking status

Subjects	mtDNA content	Smoking	*n*	Mean	95% CI
Control	*Cox I*	Never	248	0.043	0.03-0.05
		Former	325	0.059	0.05-0.07
		Current	79	0.066	0.05-0.09
	Cox II	Never	248	0.037	0.03-0.04
		Former	326	0.051	0.04-0.06
		Current	79	0.051	0.04-0.06
HNSC	*Cox I*	Never	19	0.074	0.04-0.10
		Former	24	0.076	0.05-0.10
		Current	48	0.075	0.05-0.10
	Cox II	Never	19	0.058	0.04-0.08
		Former	24	0.049	0.03-0.07
		Current	48	0.06	0.04-0.08

forward primer 5′-ACCCACACTGTGCCCATCTAC-3′ and reverse primer 5′-TCGGTGAGGATCTTCATGAGGTA-3′. All Taqman probes (Applied Biosystems, Foster City, CA) were 5′-FAM and 3′-TAMRA labeled. *Cox I* probe 6-FAM-AACGACCACATCTACAACGTTATCGTCAC-TAMRA, *Cox II* probe 6-FAM-CAATTCCCGGACGTCTAAACCAAACCACTTTC-TAMRA, and *β-actin* probe 6-FAM-ATGCCCTCCCCCATGCCATCC-TAMRA were used. Five hundred picograms of DNA were used to amplify mitochondrial regions, whereas 10 ng were used to amplify *β-actin*. The real-time PCR reactions were done in triplicate for each gene. Standard curves where obtained using adult retinal pigmented epithelia 19 cell line DNA from untreated control cells. mtDNA/nuclear DNA (nDNA) ratios were calculated by dividing the mtDNA signal for each gene by the corresponding *β-actin* signal. Of note, primers were chosen to avoid amplification of genomic pseudogenes that are homologous to mitochondrial genes, this was confirmed by BLAST results for all primers (http://www.ncbi.nlm.nih.gov/BLAST/). In addition, to exclude the possibility that random nicking due to tobacco use may lead to an apparent increase in amplification of these shorter targets, we treated 100 matched samples from subjects with varied smoking exposures with and without *Pvu*II digestion to linearize mtDNA before amplification and noted no difference in the smoking-related increase in mtDNA/nDNA. Finally, mtDNA content was compared with mitochondrial mass as measured by amount of *Cox II* protein within samples by performing Western blot analysis on samples with a variety of mtDNA/nDNA ratios, demonstrating excellent agreement between mtDNA content and *Cox II* protein normalized to β-actin controls.

Statistical analysis. The major statistical end point in this study was the comparison of mean mtDNA content in normal versus HNSC saliva specimens and the effects of age, gender, smoking, and ethanol intake on these means. Within HNSC patients, mtDNA content by tumor stage and site was also compared. Distribution of mtDNA/nDNA values were examined graphically with box plots and scatter plots, on the natural and log scales. To obtain normal distribution, the log transformation was used for all statistical analyses, whereas means and SDs are reported on the natural scales. Two or more group means were compared with an analysis of variance and the relationship between the logarithm of mtDNA and multiple factors was evaluated by regression analyses. The correlation between saliva and tissue mtDNA was also of interest. Scatter plots and Pearson product-moment correlation coefficients were computed between age and mtDNA content and between saliva and tissue mtDNA values. All statistical computations were done using the SAS system and all *P*s are two sided.

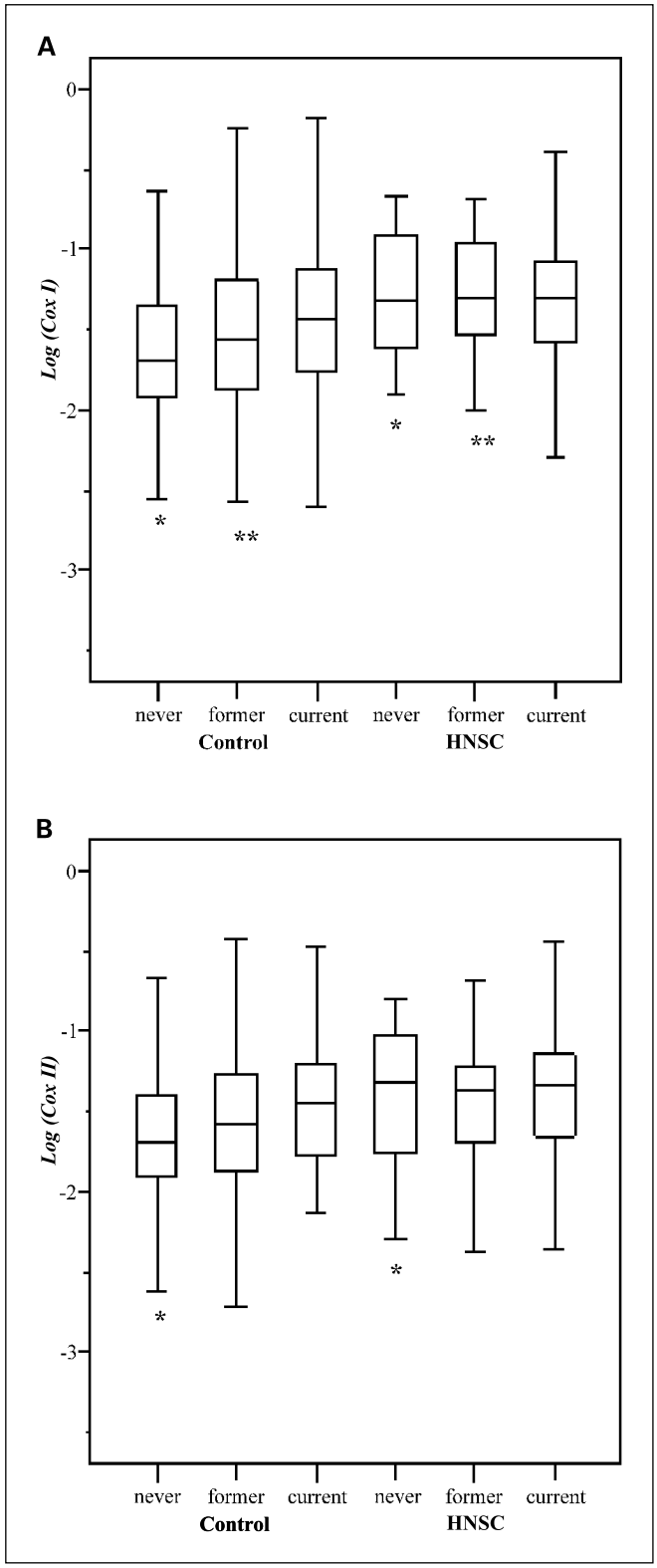

Fig. 2. Box plots of mtDNA content stratified by cancer diagnosis and smoking. *Cox I* (*A*) and *Cox II* (*B*) show that the smoking related increase in mtDNA are limited to the noncancer controls. *, $P_{CoxI} = 0.002$ and $P_{CoxII} = 0.011$ in the never-smoker saliva between HNSC *Cox I*, 0.074 (95% CI, 0.044-0.104) and *Cox II*, 0.058 (95% CI, 0.037-0.080) and controls *Cox I*, 0.043 (95% CI, 0.034-0.051) and *Cox II*, 0.037 (95% CI: 0.030, 0.043). **, $P = 0.016$ of the former-smoker saliva between HNSC *Cox I*, 0.076 (95% CI, 0.050-0.103) and control *Cox I*, 0.059 (95% CI, 0.050-0.069). No significant difference in salivary mtDNA content was found for current smokers between HNSC and control.

Results

Mitochondrial DNA content in saliva of head and neck squamous cell carcinoma and control group. We did quantitative PCR of normalized *Cox I* and *Cox II* genes in the saliva of 94 HNSC patients and 656 controls. Table 1 shows the baseline characteristics of the study participants. Race distribution was similar in two groups. Seventy-five percent of the HNSC cohort was male, 63.3% in controls. Current drinkers comprised 44.2% (34 of 77) of the HNSC, a significantly lower proportion than that 60.4% (364 of 603) of the control. HNSC patients had more current smokers (52.8%) than the control (12.1%). The mean age in the HNSC group, 57 years (95% CI), 54-59 was significantly younger that the mean age in the control group of 62 years (95% CI, 61-63; $P = 0.0008$). Mean content levels of *Cox I* and *Cox II* in saliva samples were significantly higher in HNSC patients compared with normal controls (Fig. 1).

Saliva and tissue mitochondrial DNA content are correlated. For 86 HNSC patients, paired primary tumor and pretreatment saliva were evaluated. A significant correlation was noted between mtDNA content in saliva and that in matching primary tumor (*Cox I* $r = 0.30$, $P = 0.005$ and *Cox II* $r = 0.33$, $P = 0.002$; Pearson's correlation). The mtDNA ratio was elevated in primary tumors in comparison with levels in matching saliva.

Head and neck squamous cell carcinoma is an independent predictor of elevated mitochondrial DNA in saliva. Using a univariate regression model, we analyzed possible factors associated with increased mtDNA content, including HNSC diagnosis, smoking, ethanol, age, and gender. We found that age, HNSC diagnosis, and smoking were significantly associated with increased mtDNA. Overall, increasing age was significantly associated with higher levels of mtDNA (*Cox I*: slope = 0.01; 95% CI, 0.007-0.019; $P < 0.0001$) and (*Cox II*: slope = 0.007; 95% CI, 0.001-0.013; $P = 0.01$). Of note, this age-related mtDNA content increase was not significant in the saliva of HNSC group. Table 2 shows mtDNA content with different smoking status in HNSC and control. Comparing with never smoker, average *Cox I* ($P_{current} < 0.0001$, $P_{former} = 0.01$) and *Cox II* ($P_{current} < 0.0001$, $P_{former} = 0.02$) content significantly increased with smoking category in the overall saliva samples. To determine whether each smoking category affects the mtDNA content in the saliva of HNSC differently from that does in the control, we compared *Cox I* and *Cox II* DNA content of never smokers, former smokers, and current smokers in the HNSC and control. Box

Table 3. Multivariate regression model for log (Cox)

Variable	mtDNA content	95% CI	*P*
Intercept	*Cox I*	−5.03 to −4.29	
Age		0.01-0.02	<0.0001
HNSC		0.28-0.78	<0.0001
Former smoker		0.19-0.66	0.05
Current smoker		0.001-0.34	0.0004
Intercept	*Cox II*	−4.65 to −3.96	
Age		0.003-0.01	0.022
HNSC		0.09-0.55	0.01
Former smoker		0.16-0.60	0.05
Current smoker		−0.003 to 0.31	0.001

NOTE: Multivariate regression model was used to adjust for more than one factor at a time, including significant factors found on univariate analysis: HNSC diagnosis, smoking, and age.

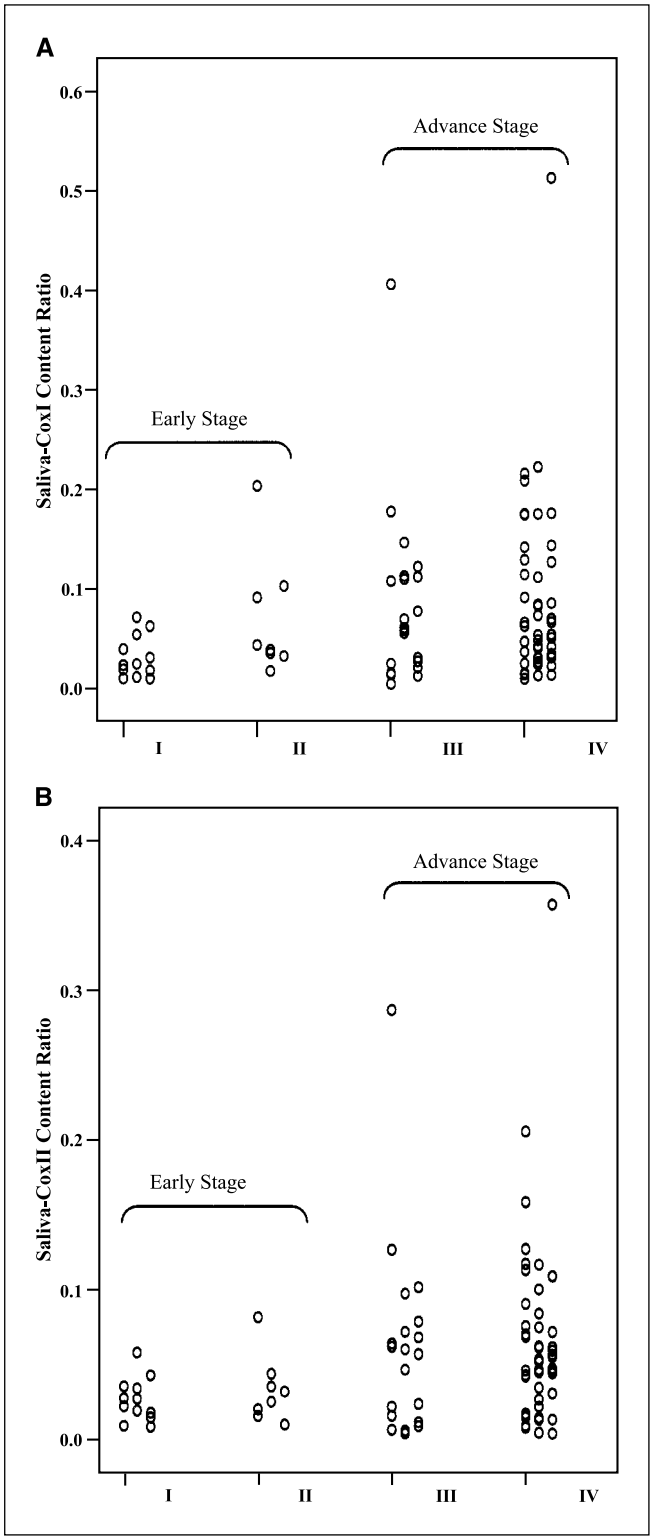

Fig. 3. Scatter plots of mtDNA content (*Cox I* and *II*) in saliva by tumor pathologic stage shows that advance stage (III and IV) patients have significantly higher mtDNA content, compared with early stage (I and II) patients. *A*, *Cox I* in HNSC saliva. *B*, *Cox II* in HNSC saliva.

plots (Fig. 2) of mtDNA content levels by HNSC diagnosis and smoking categories show that the increases by smoking category are limited to normal controls. We also found mtDNA content greatly increased in the saliva of never-smoker HNSC patients when compared with that of normal control but found no significant difference in the current-smokers saliva between saliva from current smoker HNSC patients and current smokers in the control group. Furthermore, we used a multivariate regression model to adjust for more than one factor at a time, including significant factors found on univariate analysis (HNSC diagnosis, smoking, and age) remained significant factors influencing mtDNA content. Neither ethanol intake nor gender was independently associated with mtDNA content (Table 3).

Tumor stage associated with increased mitochondrial DNA content. To assess the alteration of mtDNA content on tumor stage, we examined the relative content of *Cox I* and *Cox II* in saliva from the early stage (stages I and II) versus advanced stage (stages III and IV) HNSC. The results showed a significant increased mtDNA content in the saliva with advanced stage (Fig. 3). *Cox I* of early stage in HNSC saliva was 0.048 (95% CI, 0.03-0.07) and advance stage, 0.086 (95% CI, 0.07-0.11), $P = 0.03$. *Cox II* of early stage was 0.029 (95% CI, 0.02-0.04) and advance stage, 0.063 (95% CI, 0.05-0.08), $P = 0.04$.

Within the HNSC group, tumor site was also associated with significant differences in mtDNA/nDNA, demonstrating an increase for salivary rinse *Cox I* in the oropharynx, 0.098 (95% CI, 0.07-0.13; $P = 0.006$) and hypopharyx groups. The small number of patients in the hypopharyx group ($n = 4$) limits the conclusions that can be drawn about this group, however.

Discussion

We found that mtDNA content (*Cox I* and *Cox II*) in the saliva from HNSC patients is elevated in comparison with the saliva from normal control saliva, independent of age, smoking status, and other possible confounding variables. Mitochondrial alterations have long been suspected as contributes to carcinogenesis (16). Previous studies showed that the increased mtDNA in human cells harboring 4,977 bp deleted mtDNA in response to oxidative stress (17). In addition, mitochondrial genomic mutations have been found in HNSC (11). It is possible that the increase on content of mtDNA may be a compensatory response for the decline in respiratory function, as has been described *in vitro* systems. The association of elevated salivary mtDNA content with advanced stage HNSC, as well as the correlation of increased salivary mtDNA with increased mtDNA in individual primary tumors also supports the concept that this is a reflection of mtDNA alteration in cells shed from primary tumors in the upper aerodigestive tract.

The higher mtDNA content in the current smokers, particularly in control subjects, may be explained by multiple factors. Tobacco smoke is one of the exogenous sources which may significantly increase direct mutagenic effects on mtDNA as well as the endogenous oxidant load via injury to the respiratory chain (13, 18, 19). The increase in reactive oxygen species production from a defective respiratory chain is thought to play a role in the increase of mitochondrial mass and mtDNA content (20, 21). Furthermore, both the reactive oxygen species and Ca^{2+} may act as the second messengers to trigger the expression of nuclear respiratory factors (NRF-1 and NRF-2) and mitochondrial transcription factors to induce mitochondrial biogenesis and mitochondrial proliferation (20–22).

Interestingly, the content of mtDNA in saliva from current smokers with HNSC comparing with nonsmokers with HNSC showed no significant difference. It is possible that this is due to a compensatory increase in mtDNA replication due to factors directly related to or present in primary HNSC in nonsmokers, and that elevated mtDNA in saliva of nonsmokers with HNSC is related to the increase in mtDNA from shed tumor cells or from altered fields of mucosa with mtDNA alteration.

It is worth noting that increased mitochondrial production of reactive oxygen species (23), oxidation of mitochondrial proteins (24), depressed oxidative phosphorylation activity (25), and disrupted fatty acid metabolism (26) occur in the liver following consumption of alcohol. One objective of our study was to evaluate and compare the possible role of ethanol intake on mtDNA content alteration in HNSC and control saliva. Our simple linear regression and multivariate regression model indicated that ethanol intake did not have an independent role on alterations of mtDNA content. However, we were unable to effectively segregate the smoking and ethanol exposures, and were not able to exclude whether effect of ethanol on mtDNA content in HNSC saliva independent of smoking. It is possible that underreporting of ethanol by our HNSC population as compared with our control population may have influenced (i.e. underestimated) the effect of ethanol on relative increase of mtDNA. However, we did not observe an association of ethanol intake with increased mtDNA within our control population, making this effect unlikely.

In this study, we provided novel evidence that elevated mtDNA does not only occur in the tobacco-exposed individuals without HNSC but also exists in the saliva of patients with HNSC, is independently associated with presence of HNSC, and is associated with advanced stage disease. Further investigation may elucidate the timing of mtDNA alteration in HNSC carcinogenesis and provide a mechanism for this increase in smoking and nonsmoking-related HNSC.

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