Decreased Mitochondrial DNA Content in Posttreatment Salivary Rinses from Head and Neck Cancer Patients

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Abstract **Purpose and Experimental Design:** Alterations in mitochondrial DNA (mtDNA) sequence and content have been described in human tissues and tumors in association with smoking exposure. We did quantitative PCR analysis of *cytochrome c oxidase* (*Cox*) *I* and *Cox II* genes to measure changes in mtDNA content in pretreatment and posttreatment salivary rinses obtained from 76 patients undergoing surgical resection for primary head and neck squamous cell carcinoma. We also examined the relationship between changes in mtDNA content and postoperative radiation therapy, smoking exposure, alcohol intake, and other clinical characteristics.

Results: Overall, mtDNA content in posttreatment saliva was significantly decreased. The mean change for *Cox I* was −0.21 [95% confidence interval (95% CI), −0.44 to 0.01, $P = 0.06$] and for *Cox II* was −0.31 (95% CI, −0.55 to −0.08, $P = 0.01$). Patients in the radiation therapy group exhibited a significant decrease compared with the nonradiated group ($P = 0.03$ for *Cox I*; $P = 0.05$ for *Cox II*). In addition, significant decreases in *Cox I* (−0.71; 95% CI, −1.17 to −0.25, $P = 0.005$) and *Cox II* (−0.65; 95% CI, −1.17 to −0.13, $P = 0.02$) were found in never-smoking patients but not in former or current smokers.

Conclusion: Our data suggest that salivary mtDNA content is decreased in never smokers and in response to radiation therapy after primary surgical resection.

In 2004, 28,260 new cases of oral cavity and pharynx cancer and 20,260 new cases of larynx cancer were diagnosed in the United States, resulting in 7,230 and 3,830 deaths, respectively (1, 2). Recently, attention has focused on mitochondria because of their central role in cellular functions, including energy production, transport, cell motility, cell proliferation, apoptosis, and intermediate metabolism (3–5). Alterations in mitochondrial function have been found in many tumor systems (6, 7). Point mutations or deletions within the displacement loop and coding regions, as well as the 4,977 deletion or short deletion of mitochondria, have been found in cancers of the colon (8, 9), breast (10), stomach (11), kidney (12), esophagus (13), thyroid (14), and head and neck (15). In addition, evidence that alterations in mitochondrial DNA (mtDNA) may influence cellular sensitivity to cancer therapeutic agents has been reported (4, 16). It has also been noted that mtDNA content is organ and tissue specific and that an increase in mtDNA content may be a compensatory response for decline in respiratory function (17).

A proportion of head and neck cancer patients display a phenotype in which fields of genetically altered epithelia exist both adjacent to and distant from the primary head and neck squamous cell carcinoma (HNSCC) in the upper aerodigestive tract (18, 19). These clonal expansions of genetically altered cells may result in the development of premalignant lesions and, in some cases, the development of a second primary HNSCC. DNA with alterations characteristic of primary HNSCC have been detected in salivary rinses from HNSCC patients and likely reflect DNA from cells that are shed from primary tumors and/or fields of altered epithelia with alterations related to carcinogenesis (15).

We have previously showed that increased mtDNA content is found in primary HNSCC and in salivary rinses from HNSCC patients (20). We wished to determine if a population of cells with altered mtDNA remained in the upper aerodigestive tract of patients after surgical resection of primary HNSCC with or without external beam radiation therapy, and to define the presence and prognostic effect of mtDNA alteration in the remaining epithelia. In this study, we did quantitative PCR of mtDNA content [*cytochrome c oxidase I* (*Cox*) *I* and *Cox II*] from paired pretreatment and posttreatment salivary rinse samples

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obtained from 76 patients diagnosed with primary HNSCC. The effect of smoking exposure and other clinical variables on mtDNA content alterations were also examined.

Materials and Methods

Subjects. One hundred fifty-two salivary rinse samples were collected from 76 patients with primary HNSCC at the Department of Otolaryngology-Head and Neck Surgery, School of Medicine, The Johns Hopkins University, after appropriate approval was obtained from the Johns Hopkins institutional review board. All of the 76 selected patients were diagnosed with primary HNSCC and had not undergone external beam radiation therapy, although one patient had radiation therapy 2 months before surgery, with pretreatment samples harvested before radiotherapy. Full-course (6 weeks) adjuvant radiotherapy (50-60 Gy) was initiated after surgery in 47 patients. Pretreatment salivary rinse samples were collected before tumor resection on the day of tumor resection. Posttreatment samples were collected after completion of therapy. Sample collection time interval was defined as the time elapsed from the day of tumor resection to the day the posttreatment saliva was collected. The distribution of overall HNSCC saliva rinse sampling time interval was shown in Fig. 1A. Smoking was defined as having undergone at least 1 continuous year of daily smoking. Former smokers were defined as patients who had undergone at least 1 year of smoking cessation. Appropriate staging, outcome, tobacco exposure, and clinical measures were obtained by retrospective chart review. For this cohort, mean follow-up time was 24.9 months (median, 22.8 months; range, from 4 to 61 months).

Collection of salivary rinse/brushing mixture samples. Salivary rinse/brushing mixture 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. DNA was isolated using standard phenol-chloroform extraction and ethanol precipitation procedures.

Quantitative PCR. A Perkin-Elmer/ABI 7900 thermocycler was used to perform quantitative PCR amplification for *β-actin* and mtDNA regions for *Cox I* and *Cox II*. *Cox I* primer sequences used previously were used in this study (11). 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′-AAGATTATTACAAATGCATGGGC. *Cox II* region amplification was done using forward primer 5′-CCCCACATTAGGCTTAAAAA-CAGAT-3′ and reverse primer 5′-TATACCCCCGGTCGTGTAGC-3′. *β-actin* amplification was done using forward primer 5′-ACCCA-CACTGTGCCCATCTAC-3′ and reverse primer 5′ -TCGGTGAGGATCTT-CATGAGGTA-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. PCR amplifications were carried out in buffer containing 16.6 mmol/L ammonium sulfate, 67 mmol/L Trizma (pH 8.8), 2.5 mmol/L $MgCl_2$, 10 mmol/L β-mercaptoethanol, 0.1% DMSO, 600 nmol/L each of forward and reverse primers, 200 nmol/L TaqMan probe, 0.6 units Platinum Taq polymerase, and 2% ROX reference dye. Five hundred picograms of DNA were used to amplify the 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 (ARPE-19) cell line DNA. Data analysis was done using Microsoft Excel software. Mitochondrial to nuclear DNA ratios were calculated by dividing the mtDNA signal for each gene by the corresponding *β-actin* signal.

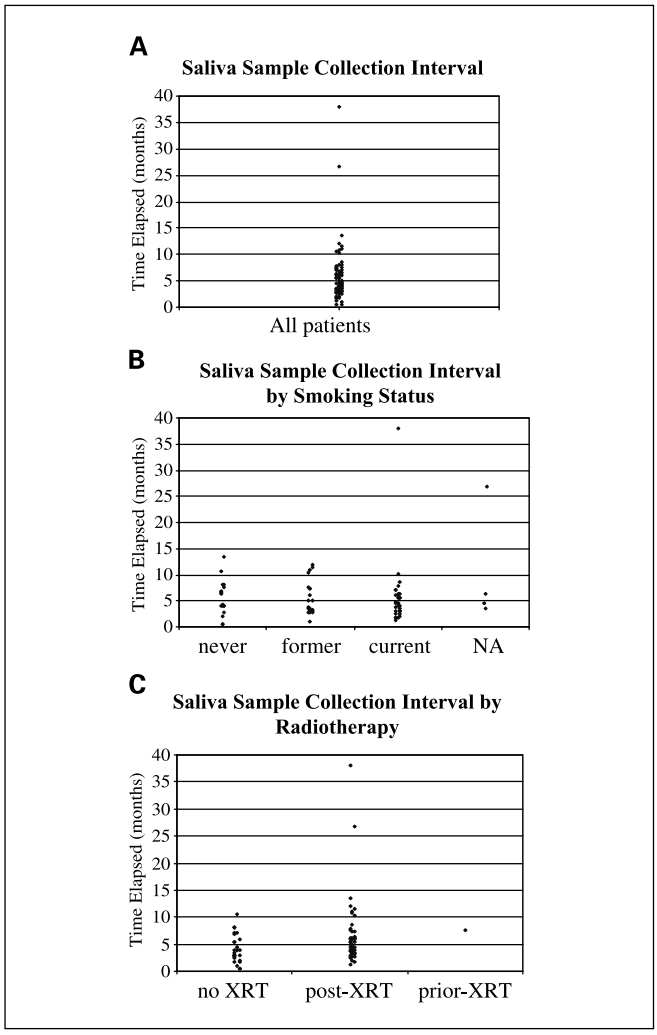

Fig. 1. Elapsed time distribution of HNSC saliva rinse sampling. *A,* all patients. *B,* subgroups by smoking status: never, never-smoker; former, former smoker; current, current smoker; NA, smoking status not available. *C,* subgroups by radiotherapy; of note, one patient underwent radiotherapy before resection of a second primary HNSC (*prior-XRT*): XRT, radiation therapy.

Statistical analysis. The major statistical end point in this study was the change in saliva mtDNA content following surgery and postoperative radiotherapy. Distributions of mtDNA in pretreatment and posttreatment saliva were examined graphically using scatterplots on the natural and log scales. Log transformation was used for these values and the difference (post − pre) was taken for statistical analyses. Paired *t* tests were used to determine if these changes were significantly different from zero. Changes in mtDNA content were also evaluated with univariate and multivariate linear regression models to determine the effects of radiation therapy, smoking, alcohol intake, age, gender, stage, and site. The correlation between *Cox I* and *Cox II* mtDNA content changes were also assessed. Scatter plots and Pearson product-moment correlation coefficients were computed between *Cox I* and *Cox II* mtDNA content change values. All statistical computations were done using the SAS system and all *P* values reported are two-sided.

Results

Characteristics of pretreatment and posttreatment HNSCC saliva rinse samples. We did quantitative PCR analysis of

normalized mitochondrial *Cox I* and *Cox II* genes in the paired pretreatment and posttreatment saliva of 76 primary HNSCC patients. Table 1 shows the baseline characteristics of the study participants. As expected, the two major causes of head and neck cancer, i.e., smoking and alcohol drinking, were highly prevalent in these patients. To eliminate the possible confounding effect of time interval between surgery and the collection of the second saliva sample in the subgroups, the collection interval was compared in the surgery alone and surgery plus radiation groups. For the surgery alone group, the mean was 4.1 months compared with 7.0 months in the surgery plus radiation group ($P = 0.026$). The Kaplan-Meier plot of the time to postsurgery sample for these two groups showed that the surgery plus radiation group had longer intervals to collection of the second sample (data not shown). The median time to collection of the second sample was 3.6 months in the surgery group and 5.3 months in the surgery plus radiation group (log-rank $P = 0.009$). However, postsurgery duration was not associated with mtDNA content changes when considered in univariate regression analysis. Figure 1 shows the sampling time interval distribution of overall and various subgroups of HNSCC saliva rinses (Fig. 1A-C). In multivariate regressions where stage and smoking were considered as well as radiation, postsurgery duration was also not significant and did not alter regression coefficients of other factors in the models.

Table 1. Characteristics of HNSC patients

Sex	
Male	61 (80.3%)
Female	15 (19.7%)
Age (y)	
Mean	57
Median	56
Range	25-89
Race	
Caucasian	63 (82.9%)
African-American	11 (14.5%)
Other	2 (2.6%)
Smoking	
Never	17 (23.6%)
Former	20 (27.8%)
Current	35 (48.6%)
Alcohol	
Never	25 (37.9%)
Former	8 (12.1%)
Current	33 (50%)
Radiation	
No radiation	28 (37.3%)
Radiation	47 (62.7%)
Pathologic stage	
I	13 (18.1%)
II	8 (11.1%)
III	12 (16.7%)
IV	39 (54.2%)
Tumor site	
OC	41 (54.7%)
OP	14 (18.7%)
L	17 (22.7%)
HP	3 (4%)
Disease status	
NED	51 (67.1%)
AWD	5 (6.6%)
DOD	10 (13.2%)
DUC	4 (5.3%)
LFU	6 (7.9%)
Mean mtDNA content	
Cox I	
Pretreatment saliva	0.0813
Posttreatment saliva	0.0588
Cox II	
Pretreatment saliva	0.0602
Posttreatment saliva	0.0408

Abbreviations: OC, oral cavity; OP, oral pharyngeal; L, laryngeal; HP, hypopharyngeal; NED, no evidence of disease; AWD, alive with disease; DOD, dead of disease; DUC, dead of unknown cause; LFU, lost follow-up.

Table 2. Overall saliva mtDNA change: log (post) − log (pre)

	n	Mean (SD)	95% CI	Min	Max	*P*
Cox I	76	−0.21 (0.97)	−0.44 to 0.01	−2.76	2.21	0.06
Cox II	76	−0.31 (1.03)	−0.55 to −0.08	−3.04	2.17	0.01

NOTE: *P* values from paired *t* tests.

mtDNA content is decreased in posttreatment HNSCC salivary rinses. To evaluate whether changes in salivary rinse of mtDNA content occurred after HNSCC patients underwent resection with or without radiotherapy, the mean changes for mtDNA content were analyzed. In posttreatment compared with pretreatment saliva, overall mean changes in *Cox II* content was significantly decreased and *Cox I* was borderline statistically significantly decreased (Table 2). In addition, the changes of *Cox I* DNA content showed significant correlation with *Cox II* DNA content changes ($R^2 = 0.79$, $P < 0.001$, Pearson product-moment correlation coefficiency test). The reduction of mtDNA content was reflected in the dosage of both mitochondrial subunit genes (*Cox I* and *Cox II*) in HNSCC posttreatment saliva in a consistent manner.

Postoperative radiation therapy and smoking status are associated with mtDNA content alterations in posttreatment HNSCC salivary rinses. To determine which factors are strongly associated with mtDNA content decrease in the posttreatment saliva, we used a univariate regression model to analyze possible factors that may affect mtDNA, including use of postoperative radiation therapy, smoking, alcohol, age, tumor site, stage, and gender. Recruited HNSCC patients were first grouped into two categories: (*a*) surgery—primary treatment based on surgery without radiation; (*b*) combined treatment—surgery plus postoperative radiation therapy. When comparing these groups, for both *Cox I* and *Cox II* (Table 3), the combined treatment group had a decrease of mtDNA in posttreatment saliva, whereas the surgery-only group had little or no change in mtDNA content. For both measures of mtDNA, these means were significantly different from each other when comparing the combined therapy group with the surgery-only group,

Table 3. Radiation therapy and saliva mtDNA change: log (post) − log (pre)

mtDNA	Radiation	*n*	Mean (SD)	95% CI	Min	Max	*P*
Cox I	No	28	0.1 (1.17)	−0.35 to 0.56	−2.76	2.21	
	Yes	47	−0.41 (0.8)	−0.64 to −0.17	−2.62	1.17	0.03
Cox II	No	28	−0.01 (1.15)	−0.46 to 0.43	−2.99	2.17	
	Yes	47	−0.51 (0.93)	−0.78 to −0.24	−3.04	2.04	0.05

NOTE: *P* values from regression analysis comparing means.

$P = 0.03$ for *Cox I* and $P = 0.05$ for *Cox II*. Within the combined treatment group, measures of difference before and after therapy were significantly different for individual patients as well (paired *t* tests), $P = 0.001$ for *Cox I* and $P = 0.001$ for *Cox II*. Postoperative sampling interval was not associated with *Cox* changes when considered in univariate regressions. Using a regression model to adjust the simple *Cox I* and *Cox II* *t*-test comparisons between radiation groups for postsurgery sampling interval, the difference between the surgery and surgery plus radiation groups remained significant ($P = 0.05$ for both). These unadjusted and adjusted results are shown in Tables 3 and 4.

We further observed that the mean change for *Cox I* in the never-smoker group was significantly different than in current smokers and marginally different for *Cox II* (Table 5). Mean changes significantly different from zero in Table 5 were in the never-smoker group, paired *t* tests $P = 0.005$ for *Cox I* and $P = 0.02$ for *Cox II*. Scatterplot analyses also showed significant changes between pretreatment and posttreatment saliva in the subcategory of never smokers with postradiation therapy (Fig. 2).

Patients with stage III-IV HNSCC exhibited significantly decreased *Cox I* mtDNA (−0.36; 95% CI, −0.62 to −0.10; $P = 0.03$) following treatment compared with stage I-II patients (0.18; 95% CI, −0.29 to 0.65). *Cox II* mtDNA was also decreased in stage III-IV patients (−0.41; 95% CI, −0.70 to −0.12; $P = 0.17$) more so than in stage I-II patients (−0.04; 95% CI, −0.52 to 0.44), but these mean changes were not significantly different from each other. Paired analyses to test if mean changes in mtDNA were significantly different from zero indicated that both *Cox I* and *Cox II* mtDNA were significantly decreased in stage III-IV patients before and after therapy ($P = 0.008$ and $P = 0.006$) but not stage in I-II patients ($P = 0.43$ and $P = 0.87$).

Table 4. mtDNA change adjusted for postsurgery interval

mtDNA	Variable	Estimate (95% CI)	*P*
Cox I	Intercept	0.172 (−0.22 to 0.56)	
	Radiation	−0.462 (−0.92 to 0.001)	0.05
	Postsurgery duration (mo)	−0.017 (−0.06 to 0.02)	0.43
Cox II	Intercept	−0.027 (−0.45 to 0.39)	
	Radiation	−0.504 (−1.00 to −0.008)	0.05
	Postsurgery duration (mo)	0.003 (−0.04 to 0.05)	0.88

Factors not associated with *Cox I* or *Cox II* mtDNA changes included age, gender, alcohol, and tumor site.

Multivariate regression model analysis of mtDNA content change in pretreatment and posttreatment salivary rinses. We used a multivariate regression model to adjust for more than one factor at a time, which included significant factors found by univariate analysis: postoperative radiation therapy, smoking, and tumor stage (Table 6). Postoperative radiation therapy and smoking remained significant factors influencing mtDNA content when analyzed in a multivariate fashion. Stage was not significant in the multivariate analysis when adjusted for radiation and smoking. However, radiation and stage were significantly correlated. Stage III-IV patients were more likely to be treated with radiation following surgery. Among 21 stage I-II HNSCC patients, 8 (38.1%) were treated with postoperative radiation therapy, whereas 70.1% stage III-IV HNSCC patients received postoperative radiation therapy (odds ratio, 4.18; 95% CI, 1.43 to 12.25, $P = 0.009$). This raised a question as to whether stage was significant if postoperative radiation therapy was not included in the multivariate analysis. A multivariate regression model was analyzed again by only adjusting for smoking and stage categories. Using this model, stage was marginally significant for *Cox I* but not for *Cox II* (Table 7).

Discussion

Mitochondria are involved in multiple cellular processes, such as energy metabolism, apoptosis, and generation of reactive oxygen species. Alterations in these processes are proposed to play a causative a role in tumor formation or manifestation of clinical phenotype and malignant potential of the tumor (21). The increase in mtDNA content may be a compensatory response for a decline in respiratory function (17). In this study, we showed that *Cox I* and *Cox II* DNA content were significantly and concomitantly decreased in HNSCC postoperative salivary rinses, and that never-smoker status and postoperative radiation therapy were associated with a reduction in mtDNA content after therapy. It is intuitive that mtDNA content in a salivary rinse reflects the mtDNA content of the entire cell population in this rinse, including both tumor cells and surrounding nonmalignant epithelia. Excision of a population of cells with increased mtDNA would be expected to result in a net decrease in mtDNA content. Conversely, persistent elevation of mtDNA content after excision would imply that cells with elevated mtDNA content are continuing to be harvested in postoperative salivary rinses and are still present in the remaining epithelium.

Table 5. Smoking and saliva mtDNA change: log (post) − log (pre)

mtDNA	Smoking	*n*	Mean (SD)	95% CI	Min	Max	*P*
Cox I	Never	17	−0.71 (0.89)	−1.17 to −0.25	−2.62	0.38	
	Former	20	−0.27 (0.8)	−0.65 to 0.10	−2.51	1.17	0.17
	Current	35	0.1 (1.04)	−0.26 to 0.46	−2.76	2.21	0.01
Cox II	Never	17	−0.65 (1.02)	−1.17 to −0.13	−3.04	0.99	
	Former	20	−0.38 (1.11)	−0.90 to 0.14	−3.04	2.04	0.44
	Current	35	−0.09 (1.02)	−0.45 to 0.26	−2.99	2.17	0.08

NOTE: *P* values from regression analysis comparing means.

The lack of decrease in current smokers implies that there is a significant population of residual epithelial cells with elevated mtDNA related to smoking exposure in these patients. These data are consistent with mtDNA content alteration as an indicator or result of mitochondrial derangement early on the pathway to smoking-related carcinogenesis or as a direct result of smoking toxicity (20, 22).

Interestingly, postoperative radiotherapy resulted in reduction of mtDNA content. Similarly, recent genetic and epigenetic studies have shown the presence of alterations in morphologically normal tumor surroundings or nonneoplastic tissue (23, 24). Our data may indicate that radiotherapy ablates a population of cells with elevated mtDNA. These could be nonneoplastic cells or populations of premalignant cells that have not yet undergone malignant transformation (19). This is consistent with the ability of radiation therapy to reduce local recurrence rates. Alternatively, this decrease of mtDNA after radiotherapy may reflect an effect of external beam radiation that influences mtDNA content or mitochondrial number in cells, effectively reducing mtDNA.

A more significant decrease in mtDNA in posttreatment salivary rinses from patients with advanced HNSCC was noted; however, the majority of advanced HNSCC also received postoperative radiation therapy. The greater magnitude of decrease in mtDNA content associated with advanced-stage HNSCC may simply reflect an initial, larger primary tumor burden in advanced stage cases, resulting in a larger relative difference between preoperative and postoperative burden of cells with altered mtDNA content or may be related to a higher incidence of postoperative radiotherapy. In the current study, we were unable to draw a clear conclusion because of limitations in subgroup size.

Besides being interested in comparing mtDNA content between the various subgroups of preoperative and postoperative saliva, we were concerned about the comparability of the time interval between surgery and the collection of the second saliva sample in the compared groups. As presented above, the time interval between sampling was significantly different (P = 0.026) between surgery alone and surgery with radiation groups. However, given this is a retrospective, observational study, there is no way to conclusively correct for this in the analysis. We can, at best, adjust our t-test results for this factor and report the adjusted results. This does not, however, rule out the time difference as a confounding factor. Univariate regression analysis indicated that postsurgery duration was not associated with mtDNA changes. Both *Cox I* and *Cox II* changes remained significant when multivariate regression was adjusted for time duration and this adjustment did not alter regression coefficients of other factors in the models. This indicates that postsurgery duration may not be confounding our previous

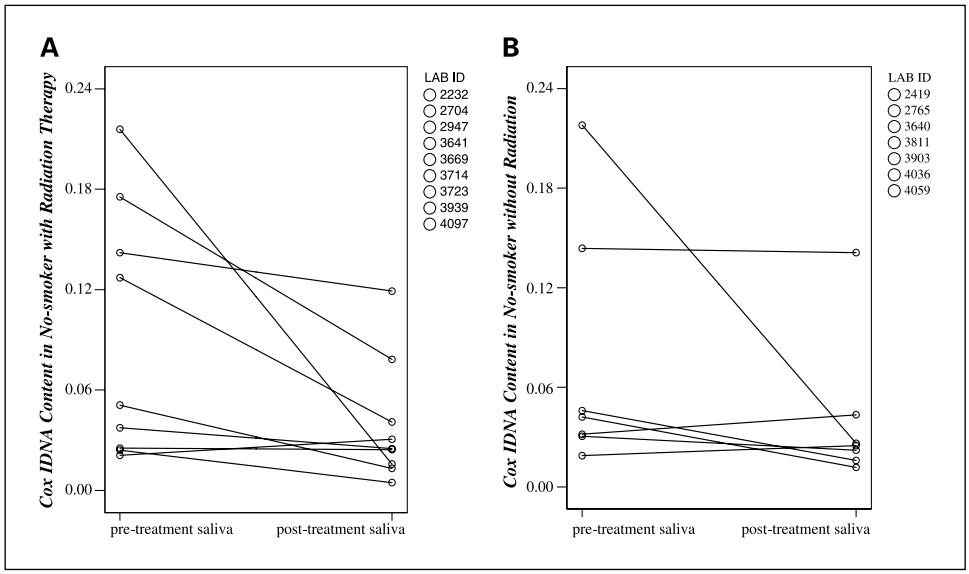

Fig. 2. Representative scatterplot analyses for *Cox I* DNA alterations in the subcategories of never-smokers with or without radiation therapy between pretreatment and posttreatment saliva rinse sampling. The mean *Cox I* in never-smokers with radiation therapy (*A*) is 0.091 for pretreatment saliva and 0.063 for posttreatment saliva. In contrast, the mean *Cox I* in never-smokers without radiation therapy (*B*) is 0.076 for pretreatment saliva and 0.067 for posttreatment saliva.

Table 6. Overall multivariate regression model for change in mtDNA

mtDNA	Variable	Estimate (95% CI)	*P*
Cox I	Intercept	−0.96 (−1.45 to −0.46)	0.0003
	No radiation	0.47 (0.02 to 0.93)	0.05
	Former smoker	0.59 (−0.03 to 1.12)	0.07
	Current smoker	0.83 (0.28 to 1.38)	0.004
Cox II	Intercept	−0.92 (−1.47 to −0.38)	0.002
	No radiation	0.49 (−0.02 to 0.99)	0.06
	Former smoker	0.45 (−0.24 to 1.13)	0.21
	Current smoker	0.59 (−0.01 to 1.20)	0.06

NOTE: The variable "no radiation" was compared with radiation group. Former and current smokers were compared with never smokers.

Table 7. Multivariate regression model (no radiation category) for mtDNA change

mtDNA	Variable	Estimate (95% CI)	*P*
Cox I	Intercept	−0.369 (−0.93 to 0.19)	
	Stage III-IV	−0.479 (−0.97 to 0.01)	0.06
	Former smoker	0.500 (−0.12 to 1.12)	0.12
	Current smoker	0.793 (0.24 to 1.34)	0.006
Cox II	Intercept	−0.412 (−1.05 to 0.22)	
	Stage III-IV	−0.334 (−0.88 to 0.21)	0.24
	Former smoker	0.333 (−0.37 to 1.03)	0.35
	Current smoker	0.541 (−0.08 to 1.16)	0.09

results. In multivariate regressions where stage and smoking were considered as well as radiation, sampling interval was also not significant.

Finally, some differences in the significance of the association between *Cox I* and *Cox II* and various clinical variables are noted. Possible reasons for this difference may include a variability in amplification efficiency of these primer and probe sets used to amplify these genes, a difference in degradation of these two genes in exfoliated aerodigestive cells, or simple variability in assay technique.

In summary, our results showed a significant decrease in *Cox I* and *Cox II* mtDNA content in postoperative salivary rinses from patients with HNSCC. This association was most pronounced in never smokers and in patients who underwent postoperative radiation therapy. Alterations in mtDNA content may reflect a role for mitochondrial dysregulation in the development of HNSCC.

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