Interleukin 6 and Interleukin 8 as Potential Biomarkers for Oral Cavity and Oropharyngeal Squamous Cell Carcinoma

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Background: Since morbidity and mortality rates due to oral cavity and oropharyngeal squamous cell carcinoma (OSCC) have improved little in the past 30 years, early detection or prevention of this disease is likely to be most effective. Using laser-capture microdissection, we have identified the expression of 2 cellular genes that are uniquely associated with OSCC: interleukin (IL) 6 and IL-8. These cytokines may contribute to the pathogenesis of this disease, and have been linked with increased tumor growth and metastasis.

Objectives: To investigate whether IL-6 and/or IL-8 could serve as informative biomarkers for OSCC in saliva and/or serum and to determine if there is a role for saliva as a diagnostic medium for OSCC.

Patients and Methods: Patients with newly diagnosed T1 or T2 oral cavity or oropharyngeal histologically confirmed squamous cell carcinoma were recruited for the study. Age and sex-matched disease-free subjects were used as controls. Using quantitative real-time polymerase chain reaction analysis and enzyme-linked immunosorbent assay, we respectively assessed the expression of IL-6 and IL-8 in serum (controls, n=32; patients with OSCC, n=19) and saliva (controls, n=32; patients with OSCC, n=32) at the messenger RNA (mRNA) and protein levels.

Main Outcome Measures: Specificity and sensitivity of these biomarkers for OSCC and their predictive value.

Results: Interleukin 8 was detected at higher concentrations in saliva ($P<.01$) and IL-6 was detected at higher concentrations in serum of patients with OSCC ($P<.01$). We confirmed these results at both the mRNA and the protein levels, and the results were concordant. The concentration of IL-8 in saliva and IL-6 in serum did not appear to be associated with sex, age, or alcohol or tobacco use ($P>.75$). Using statistical analysis, we were able to determine the threshold value, sensitivity, and specificity of each biomarker, as well as a combination of biomarkers, for detecting OSCC.

Conclusions: Our findings indicate that IL-8 in saliva and IL-6 in serum hold promise as biomarkers for OSCC. A saliva-based test could be a cost-effective adjunctive tool in the diagnosis and follow-up of patients with OSCC.

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which are uniquely associated with OSCC: interleukin (IL) 6 and IL-8. These cytokines have also been linked with increased tumor growth and metastasis, and could thus contribute to the pathogenesis of this disease. Their expression is silenced in normal keratinocytes. Others have also detected elevated concentrations of IL-6 and IL-8 in cell-line supernatants, tumor specimens, and the serum of patients with head and neck squamous cell carcinoma.

Genetic alterations can be successfully identified in bodily fluids draining the organ affected by the tumor. With this in mind, we investigated whether the ability to analyze saliva for potential biomarkers would be feasible in the diagnosis of OSCC. Specifically, we examined IL-6 and IL-8 at the messenger RNA (mRNA) and the protein levels in both the serum and saliva of OSCC patients with OSCC and age- and sex-matched controls. Furthermore, our data were subjected to statistical analysis to determine the specificity and sensitivity of these biomarkers for OSCC as well as their predictive value.

## METHODS

### PATIENT SELECTION

Patients were recruited from the Division of Head and Neck Surgery at the University of California Los Angeles Medical Center; the University of Southern California Medical Center; and the University of California San Francisco Medical Center over a 6-month period. Thirty-two patients with documented primary T1 or T2 squamous cell carcinoma of the oral cavity or oropharynx were included in this study. All patients had recently been diagnosed with primary disease, and had not received any prior treatment in the form of chemotherapy, radiotherapy, surgery, or alternative remedies. An equal number of age- and sex-matched disease-free subjects with comparable smoking histories were selected as a control comparison.

There were no significant differences between the groups in terms of mean (SD) age: OSCC patients, 49.3 (7.5) years; controls, 48.8 (5.7) years (t test P > .80); sex (t test P > .90); or smoking history (t test P > .75). Smoking history was determined by asking patients with cancer and controls (1) how many years they had smoked, (2) how many packs per day they smoked, (3) how many years they had elapsed since they had quit smoking (if they had indeed quit), and (4) whether they only smoke cigarettes, or did they also use cigars, pipes, chewing tobacco, or marijuana. We then aimed to optimize the match between patients and controls in terms of the following: (1) similar pack-year history, (2) similar time lapse since they had quit smoking, (3) use of cigarettes exclusively. No subjects had a history of prior malignancy, immunodeficiency, autoimmune disorders, hepatitis, or human immunodeficiency virus infection. Each of the combinations in the control group underwent a physical examination by a head and neck surgeon, to ensure that no suspicious mucosal lesion was present.

### SALIVA AND SERUM PROCEDURES

Informed consent was given by all patients. Saliva and serum procurement procedures were approved by the institutional review board at each institution.

Saliva from the 32 patients with OSCC and the 32 controls were obtained for a prospective comparison of cytokine concentration.

The subjects were required to abstain from eating, drinking, smoking, or using oral hygiene products for at least 1 hour before saliva collection. Saliva collection was performed using the “draining (drooling)” method of Navazesh and Christiansen, for a total sample of 5 mL of saliva. Saliva samples were subjected to centrifugation at 3500 rpm (2600g) for 15 minutes at 4°C (Sorvall RT6000D centrifuge; DuPont, Wilmington, Del). The fluid phase was then removed, and RNase (Superase-In, RNase Inhibitor; Ambion Inc, Austin, Tex) and protease (aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate; Sigma, St Louis, Mo) inhibitors were then added promptly on ice. The conditions for the separation of the cellular and fluid phases of saliva were optimized to ensure no mechanical rupture of cellular elements that would contribute to the mRNA detected in the fluid phase. All samples were subsequently treated with DNase (DNAsel-DNA-free; Ambion Inc). The cell pellet was retained and stored at –80°C.

Serum samples from 19 patients with OSCC and 32 controls were obtained for a prospective comparison of cytokine concentration. Blood was drawn from controls and patients before treatment. Serum samples were collected by centrifuging whole blood at 3000 rpm (1000g) for 10 minutes at 15°C (Sorvall RT6000D centrifuge). Serum was then separated, and RNase (Superase-In, RNase Inhibitor) and protease (aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate) inhibitors were then added promptly on ice. All samples were subsequently treated with DNase (DNAsel-DNA-free). The aliquots were stored at –80°C until further use.

### RNA ISOLATION

A sample of 560 µL of saliva supernatant was processed using the QIAamp Viral RNA mini-kit (QIAGEN, Chatsworth, Calif). RNA was extracted according to the manufacturer's instructions. Samples were air-dried and resuspended in water treated with diethyl pyrocarbonate and were kept on ice for immediate usage or stored at –80°C. Aliquots of RNA were treated with RNase-free DNase (DNAsel-DNA-free) according to the manufacturer's instructions. Concentrations of RNA were determined spectrophotometrically, and the integrity was checked by electrophoresis in agarose gels containing formaldehyde.

### RT-PCR ANALYSIS

Reverse transcription–polymerase chain reaction analysis (RT-PCR) was performed as follows. RNA from each sample was reverse-transcribed in 40 µL of reaction mixture containing 2.5 U of Moloney murine leukemia virus reverse transcriptase (Applied Biosystems Inc [ABI], Foster City, Calif) and 50 pmol of random hexanucleotides (ABI) at 42°C for 45 minutes. Based on the published sequences, oligonucleotide primers were synthesized commercially at Fisher Scientific (Tustin, Calif) for PCR as follows:

- β-actin: 5'-AGATGCAGAGAGGACATGCTG-3' and 5'-ATACTCCTGCTGTGATCCAC-3';
- IL-8: 5'-AGGGTTGTGGAGAAGTTTTTG-3' and 5'-GAGGGTTGTGGAGAAGTTTTTG-3';
- IL-6: 5'-GAGGGTTGTGGAGAAGTTTTTG-3' and 5'-CTGGACATCTCAGTCTTGGT-3';
- IL-6: 5'-ATGATTTCACCCCTAAGGAG-3'.

Amplification of the complementary DNA (cDNA) was carried out using 50 cycles at 95°C for 20 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension cycle of 72°C for 7 minutes. Specificity of the PCR products was verified by the predicted size and by restriction digestion. To establish the specificity of the responses, negative controls were used in which input RNA was omitted or in which RNA was used but reverse transcriptase omitted. As a positive control, mRNA was extracted from total salivary gland RNA (Human Salivary Gland Total RNA, Clontech, Palo Alto, Calif).
To ensure RNA quality, all preparations were subjected to analysis of expression.

REAL-TIME PCR FOR QUANTIFICATION OF IL-6 AND IL-8 mRNA

To analyze quantitatively the result of RT-PCR, we used quantitative real-time PCR (qRT-PCR) (Bio-Rad iCycler, Hercules, Calif). Each sample was tested in triplicate. The amplification reactions were carried out in a 20-µL mixture, using iQ SYBR Green Supermix (Bio-Rad Laboratories). After initial denaturation at 95°C for 3 minutes, 50 PCR cycles were performed at 60°C for 20 seconds, then 20 seconds at 72°C, then 20 seconds at 83°C, followed by 1 minute at 95°C and by a final 1-minute extension at 55°C. Aliquots were taken from each well and checked by electrophoresis in agarose gels to ensure the specificity of the products.

ELISA FOR QUANTIFICATION OF IL-6 AND IL-8 PROTEIN CONCENTRATIONS

Enzyme-linked immunosorbent assay (ELISA) kits for IL-6 and IL-8 were used (Pierce Endogen, Rockford, Ill) according to the manufacturer’s protocol. Each sample was tested in duplicate in each of 2 replicate experiments. After development of the colorimetric reaction, the absorbance at 450 nm was quantitated by an 8-channel spectrophotometer (EL800 Universal Microplate Reader, BIO-TEK Instruments Inc, Winooski, Vt), and the absorbance readings were converted to picograms per milliliter based on standard curves obtained with recombinant cytokine in each assay. If the absorbance readings exceeded the linear range of the standard curves, ELISA was repeated after serial dilution of the supernatants. Each sample was tested in at least 2 ELISA experiments, and the data were calculated from the mean of tests for each sample.

STATISTICAL ANALYSIS

The distributions of patient demographics were calculated overall and separately for OSCC cases and controls, and were compared between the 2 arms with either the t test for continuous measures or 2 × 2 χ² tables for categorical measures. The distributions of IL-6 and IL-8 levels in saliva and serum were computed and compared between the OSCC cases and controls using 2 independent group t tests. Differences were considered significant for P values less than .01. Because of the range of the IL-6 and IL-8 levels, log transformations of these measures were also used in the analyses. Data were expressed as the mean ± SD. Age, sex, and smoking history were controlled at the group level in the experimental design; these patient factors were also adjusted in the analyses when comparing IL-6 and IL-8 through regression modeling.

Using the binary outcome of the disease (OSCC cases) and nondisease (controls) as dependent variables, logistic regression models were fitted to estimate the probability of developing OSCC as a function of each of the potential biomarkers (IL-6 or IL-8), controlling for patient age, sex, and smoking history. Using the fitted logistic models, receiver operating characteristic (ROC) curve analyses were conducted to evaluate the predictive power of each of the biomarkers. Through the ROC analyses, we calculated sensitivities and specificities by varying the criterion of positivity from the least (cut at probability of 0) to the most stringent (cut at probability of 1). The optimal sensitivity and specificity were determined for each of the biomarkers, and the corresponding cutoff/threshold value of each of the biomarkers was identified. The biomarker that has the largest area under the ROC curve was identified as having the strongest predictive power for detecting OSCC.

RESULTS

mRNA ISOLATION FROM THE FLUID PHASE OF SALIVA

By using RT-PCR, we tested the hypothesis that IL-6 and IL-8 mRNA transcripts are present in the fluid phase in saliva. The RT-PCR studies showed that saliva and serum contained mRNA encoding for IL-6 and IL-8. The PCR products had the sizes (95 and 88 base pairs, respectively) that were expected from the selected primers (Figure 1). The same-sized products were expressed in the positive control.

To ensure that the RNA and protein analyzed were from the fluid phase of saliva only (and to ensure the lack of contamination by intracellular components), we optimized the centrifugation speed for the saliva and serum samples. We utilized genomic DNA as a marker of cell lysis and spillage of intracellular components. We performed PCR for the housekeeping genes β-actin and ubiquitin on whole saliva and serum samples, and samples that had been centrifuged at various speeds. We were able to determine the optimal centrifugation speeds at which there was no spillage of intracellular contents (Figure 2).

ELEVATED IL-8 LEVELS IN SALIVA FROM PATIENTS WITH OSCC

On demonstrating that IL-6 and IL-8 mRNA transcripts were present in the fluid phase in saliva, we prospectively examined and compared the levels of IL-6 and IL-8 in the saliva of controls and patients with OSCC using qRT-PCR and ELISA. Saliva samples from 32 patients with OSCC and 32 control subjects were obtained. Figure 3 shows that IL-8 at both the mRNA and protein levels was detected in higher concentrations in the saliva of patients with OSCC compared with controls (t test, P < .01). There was a significant difference in the amount of IL-8 mRNA expression between saliva from OSCC patients.
Controls (shown). There was a significant difference by replicate samples in saliva in patients with cancer and control subjects. The mean±SEM concentration of IL-8 detected in reverse transcription–polymerase chain reaction (A), and enzyme-linked immunosorbent assay (B). The mean±SEM concentration of IL-8 detected in saliva of OSCC patients was significantly higher (720 pg/mL) than those in the saliva of the control group (250 pg/mL) (P<.001). To ensure that the elevated levels of IL-8 protein in saliva were not due to an elevation of total protein levels in the saliva of patients, we compared the total protein concentrations in saliva between the 2 groups. No significant differences were found (P>.05). When we compared the IL-6 levels between the 2 groups, we did not find significant differences in the salivary concentration at either the mRNA or the protein level. Within the sample size studies, we were unable to detect differences between smoking and nonsmoking subjects (data not shown).

ELEVATED IL-6 LEVELS IN SERUM FROM PATIENTS WITH OSCC

We also examined and compared the levels of IL-6 and IL-8 in the serum of controls and patients with OSCC using qRT-PCR and ELISA. Serum samples from 19 patients with OSCC and 32 control subjects were prospectively obtained. Figure 4 shows that IL-6 at both the mRNA and protein levels was detected in higher concentrations in the serum of patients with OSCC compared with controls (t test, P<.001). We noted a significant difference in the amount of IL-6 mRNA expression between serum from OSCC patients and controls. The mean copy number was 5.2×10^7 for the OSCC group and 3.3×10^7 for the control group. The difference between the 2 groups was statistically significant (P<.001). Our ELISA findings are illustrated in Figure 4B. The mean levels of IL-6 in the serum of patients with OSCC were significantly higher (87 pg/mL) than those in the serum of the control group (0 pg/mL) (P<.001). When we compared the IL-8 levels between the 2 groups, we did not find significant differences in the serum concentration at either the mRNA or the protein level. Within the sample size studies, we were unable to detect differences between smoking and nonsmoking subjects (data not shown).

ROC AND SENSITIVITY/SPECIFICITY ANALYSIS

The ROC curves and plots of sensitivity vs 1–specificity were generated for each of the potential biomarkers. Age, sex, and smoking history were controlled as described above. Based on the ELISA data, the areas under the ROC curve were calculated, as measures of the utility of each biomarker for detecting OSCC (Table). The calculated ROC values (for predicting OSCC) were 0.978 for IL-8 in saliva, 0.824 for IL-6 in serum, and 0.994 for a combination of IL-8 in saliva and IL-6 in serum. Based on the distribution of sensitivities and specificities, thresholds of biomarkers were chosen for detecting OSCC. Based on our data, for IL-8 in saliva, a threshold value of 600 pg/mL yields a sensitivity of 86% and a specificity of 97%. Similarly, for IL-6 in serum, a threshold value of greater than 0 pg/mL yields a sensitivity of 57% and a specificity of 100%. For a combination of IL-8 protein in saliva
and IL-6 protein in serum, a threshold value of greater than 600 pg/mL. IL-8 in saliva and greater than 0 pg/mL. IL-6 in serum yields a sensitivity of 99% and a specificity of 90%. Figure 5 shows the ROC curves for IL-8 in saliva, IL-6 in serum, and a combination of IL-8 in saliva and IL-6 in serum. The detailed statistics of the areas under the ROC curve, the threshold values, and the corresponding sensitivities and specificities for each of the potential biomarkers in saliva and in serum are listed in the Table. The sensitivity and specificity as well as the predictive value of the PCR measurements of IL-8 transcripts in saliva and IL-6 transcripts were also determined (data not shown) and were not as strong as the sensitivity and specificity as well as the predictive value of the measurements of these cytokines using ELISA.

To evaluate the predictive power of salivary IL-8 and serum IL-6 for the presence of OSCC, we performed a small blinded test on 10 independent samples: 5 OSCC patient specimens and 5 control specimens. Using qRT-PCR and ELISA, we blindly assessed the expression of IL-6 in serum and IL-8 in saliva at the mRNA and protein levels. In each case, we were able to determine with 100% accuracy which samples were from control subjects and which were from patients with OSCC.

In the present study, we set out to identify whether 2 specific cytokines, IL-6 and IL-8, could be measured in the saliva and serum of patients with OSCC, and whether these cytokines could potentially be useful as biomarkers for head and neck cancers. Interleukin 8 was detected at higher concentrations in the saliva of patients with OSCC and IL-6 was detected at higher concentrations in the serum of patients with OSCC. We confirmed these results at both the mRNA and the protein levels, and the results were concordant. The concentration of IL-8 in saliva and IL-6 in serum did not appear to be associated with sex, age, or alcohol or tobacco use. We subjected our data to ROC analysis, and we were able to determine the threshold value, sensitivity, and specificity of each biomarker as well as a combination of the 2 biomarkers for detecting OSCC (Table). Furthermore, we were able to measure mRNA in salivary specimens.

Numerous studies have shown that genetic alterations can be successfully identified in bodily fluids that drain from the organ affected by the tumor. The ability to analyze saliva would therefore be beneficial in the diagnosis and treatment of OSCC. The use of saliva has been criticized as a diagnostic medium since informative analytes are generally present in lower amounts than in serum. However, with new amplification techniques and highly sensitive assays, this objection is no longer valid. We tested the hypothesis that IL-6 and IL-8 mRNA transcripts are present in the fluid phase in saliva by using RT-PCR. The RT-PCR studies demonstrated that saliva and serum indeed contained mRNA encoding for IL-6 and IL-8 (Figure 1).

The use of the fluid phase of saliva has unique advantages over the use of exfoliated cells. Depending on the location of the tumor, one may not be able to easily access and swab the tumor bed. Although salivary biomarkers could not identify the site from which the tumor originated, they could identify patients at risk. Such a saliva test could be administered by nonspecialists in remote locations as a screening tool to select patients for referral for careful evaluation of the upper aerodigestive tract. Finding early-stage, previously undetected dis-

<table>
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<th>Biomarker</th>
<th>Area Under ROC Curve</th>
<th>Threshold/Cutoff, pg/mL</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
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</thead>
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<td>600</td>
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<td>97</td>
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<td>IL-6 serum protein</td>
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<td>&gt;0</td>
<td>57</td>
<td>100</td>
</tr>
<tr>
<td>IL-8 saliva protein and IL-6 serum protein</td>
<td>0.994</td>
<td>&gt;600 and &gt;0</td>
<td>99</td>
<td>90</td>
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Abbreviations: IL, interleukin; ROC receiver operating characteristic.
Interleukin 6 and IL-8 may therefore play a role in the pathogenicity of OSCC as well as serve as useful biomarkers. Elevation of IL-6 has been shown to promote immune unresponsiveness and induction of wasting, cachexia, and hypercalcemia, all of which are observed in patients with OSCC who have a poor prognosis. Interleukin 8 plays an important role in the stimulation of angiogenesis, proliferation, and chemotaxis of granulocytes and macrophages, which are prominent constituents in the stroma of OSCCs. In our study, the IL-6 levels in serum and IL-8 levels in the saliva of patients with OSCC were all higher than the determined cutoff levels in serum and IL-8 levels in the saliva of age-matched control subjects (at P<0.05). Our IL-6 serum data confirm these results, and thereby serve as an internal control for our experiments. It has also been suggested from studies using cultured OSCC lines and tumor specimens that IL-6 and IL-8 expression may play a role in the increased pathogenicity of OSCC by providing a growth advantage. This is consistent with the study of Dong et al., in which a murine model demonstrated a higher constitutive production of cytokine and resulted in a selective advantage of in vivo tumor growth and metastasis. Preliminary results of an analysis of the effect of surgery or chemotherapy or radiotherapy on IL-6 levels indicate that serum cytokine levels decrease in posttreatment patients. Using the Affymetrix 133A high-density oligonucleotide arrays, our laboratory has independently profiled the salivary transcriptome of 10 of the 32 OSCC samples and confirmed IL-8 was significantly overexpressed (>2-fold) in all samples examined (P<0.05).

Our findings indicate that IL-8 and IL-6 may hold promise as biomarkers for OSCC. According to the National Cancer Institute’s “Early Detection Research Network (EDRN),” which defines biomarker validation as a 5-phase process, this study represents the completion of the second phase. Phase 3 will be a retrospective longitudinal study including different patient groups (ie, OSCC of different stages including precancers and controls including other oral diseases). Our approach must now be refined and validated for improved detection, treatment, and prevention of head and neck cancers.

A saliva-based test could be a cost-effective adjunct diagnostic tool in the postoperative management of patients with OSCC. It could potentially be used for monitoring the efficacy of treatment or disease recurrence after therapy has concluded. The use of these biomarkers may also improve the staging of the tumor. In recent years, there has been a shift from locoregional failure to distant failure for patients treated for presumed locoregional disease. This is in part a reflection of subclinical...
distant disease present before the initiation of therapy. Testing for the presence of biomarkers may allow for the detection of small amounts of tumor cells in a background of normal tissue.

Our laboratory is continuing to enroll patients in this study and will investigate the relevance of salivary and serum levels of IL-6 and IL-8, respectively, as biomarkers for prognosis, efficacy of therapy, and disease recurrence. Furthermore, we are embarking upon another study focusing on patients with oral dysplasia. It will be interesting to discover whether there is a continuum of increasingly elevated levels of IL-6 in serum and IL-8 in saliva, as disease progresses from dysplasia to frank carcinoma, as well as to discover novel biomarkers associated with precancerous lesions.

The assessment of such biomarkers in saliva and serum must eventually be tested in a prospective, blinded fashion in clinical settings requiring actual cancer detection. The fact that no healthy control subject had a saliva or serum marker above our reported threshold is encouraging, indicating the excellent test specificity. The combination of biomarkers—IL-8 in saliva and IL-6 in serum—holds great potential for OSCC diagnostics as ROC analysis yields a sensitivity of 99% and a specificity of 90% (Figure 5C; Table). Future refinement of our approach may focus on several areas. More biomarkers could be added to the panel. Using oral fluid-based microarray technologies, our laboratory has identified other molecular biomarkers that appear specific for OSCC. We will pursue the validation of these genes, and test their efficacy as biomarkers. A comprehensive panel of markers capturing all tumors and low-cost, high-throughput technology is ideal so that early molecular detection can be applied in real-life screening.

The cumulative outcomes of the proposed studies will be used in a series of next steps toward the eventual creation of micro-/nano-electrical mechanical systems (MEMS/NEMS) for the ultrasensitive detection of molecular biomarkers in oral fluid. RNA and protein expression for the validated OSCC biomarkers will be selected as targets for cancer detection. The integration of these detection systems for the concurrent detection of mRNA and protein for multiple OSCC biomarkers will result in an efficient, automated, affordable system for oral fluid-based cancer diagnostics.

The ultimate question for the clinician and the patient is, Will this information improve the treatment of head and neck cancer? The efficacy of these biomarkers can only be determined through a well-designed, large, prospective multi-institutional trial, upon which our laboratory is now embarking. It is hoped that biomarkers, like IL-6 and IL-8, will allow for earlier detection of primary or recurrent cancers and improved staging, as well as treatment. The result will be something that has eluded clinicians for the past 30 years—improvements in survival for patients with head and neck cancer.

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Dr Wong, DMD, DMSc, had full access to all the data in this study and takes full responsibility for the integrity of the data and the accuracy of the data analysis.

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**REFERENCES**


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