Published in final edited form as:

Oral Oncol. 2011 December ; 47(12): 1122–1126. doi:10.1016/j.oraloncology.2011.07.032.

Salivary Endothelin-1 Potential for Detecting Oral Cancer in Patients with Oral Lichen Planus or Oral Cancer in Remission

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Abstract

Objectives—Endothelin-1 (ET-1) is a potent vasoconstrictor involved not only in vascular biology but also in carcinogenesis. Results of a study in 2007 suggested salivary ET-1 as a potential biomarker for oral squamous cell carcinoma (OSCC), but a later study showed conflicting results. The purpose of our pilot study was to investigate feasibility of using salivary ET-1 as a biomarker for OSCC in two groups: oral lichen planus (OLP) patients and patients with OSCC in remission.

Materials and Methods—Saliva samples were collected from five groups of subjects: patients with newly diagnosed, active OSCC (Group A); patients with OSCC in remission (Group B); patients with active OLP lesions (Group C); patients with OLP in remission (Group D); and normal controls (Group E). Salivary ET-1 levels were determined by enzyme-linked immunosorbent assay, and the results were analyzed by the Mann Whitney U test.

Results—The mean salivary ET-1 level in Group A was significantly higher than that found in Group C ($p=0.001$), Group D ($p=0.015$) or Group E ($p=0.004$). There were no significant differences ($p>0.05$) in the mean salivary ET-1 levels between Groups A and B; Groups B and C; Groups B and D; Groups B and E; Groups C and D; Groups C and E; or Groups D and E.

Conclusion—Salivary ET-1 could be a good biomarker for OSCC development in OLP patients regardless of the degree of OLP disease activity. However, it appeared not to be a good biomarker for detecting recurrence of OSCC in patients in remission.

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Conflict of Interest Statement

None declared.

Keywords

Oral cancer; oral squamous cell carcinoma; saliva; endothelin; ET-1; lichen planus; biomarker

Introduction

Using salivary biomarkers for early cancer detection is a promising line of research, especially because saliva collection is easy and non-invasive. More than 20 potential salivary biomarkers for oral squamous cell carcinoma (OSCC) have been reported in the literature in recent years.¹ The application of salivary biomarkers in early detection of OSCC would be especially important for high-risk groups, such as patients with oral lichen planus (OLP) and patients with previous history of OSCC. However, because the levels of most of the potential salivary biomarkers for OSCC had not been previously investigated specifically in those two groups of patients, the feasibility of using these salivary biomarkers for detecting OSCC development in the groups was unknown.

Endothelin-1 (ET-1) is one of the reported potential salivary biomarkers for OSCC detection.² The ET-1 belongs to the endothelin family of potent vasoconstrictors recently found to be involved not only in vascular biology but also in various pathological conditions, including inflammation, wound healing, and carcinogenesis.³⁻⁴ Increased levels of ET-1 mRNA and ET-1 protein have been reported in OSCC tissue.² However, results from the only two previous studies of its potential as a salivary biomarker for OSCC were inconsistent.^{2,5} Additional study was needed in order to conclusively determine whether ET-1 should be considered a useful OSCC biomarker.

Therefore, in this pilot study we sought to investigate the salivary ET-1 levels in our OSCC patients in north Texas, USA. It would add to the research data of the biomarker potential of ET-1 for OSCC, since the two previous studies were done with patients in different geographic areas (San Francisco, USA, and Brazil, respectively).^{2,5} We also investigated the feasibility of ET-1 as a salivary biomarker for OSCC development in OLP patients and for detection of recurrence in patients whose OSCC had been in remission. In addition, because ET-1 is known to be involved in inflammation and wound healing, we thought it likely that levels of ET-1 in OLP patients might be influenced by whether the patient was showing active inflammation, with multiple symptomatic lesions in the mouth, or whether the patient was in a disease-inactive state, with no lesions or with the asymptomatic reticular type of lesion. Therefore we designed our study to gather data regarding that research question as well.

Materials and Methods

1. Patient groups and recruitment

All patients were recruited during the period from September 1, 2009 to November 1, 2010, from the Stomatology Center, Texas A&M Health Science Center-Baylor College of Dentistry, and from referrals by local dentists and surgeons who had diagnosed or treated OSCC and/or OLP patients. The protocol used was approved by the Institutional Review Board of Baylor College of Dentistry and informed consent was obtained from each patient prior to saliva collection. Saliva samples and clinical information were collected from a total of five groups of human subjects. The groups are defined and their inclusion and exclusion criteria are listed in Table 1.

In order to exclude confounding factors and achieve a homogeneous group population, we sought to exclude subjects who had systemic diseases or conditions that might affect the

data or the analysis. We excluded subjects who used any corticosteroids or immunosuppressants prior to saliva collection because corticosteroids had been reported to affect the levels of some potential salivary OSCC biomarkers involved in inflammation.⁶ Since ET-1 was also known to be involved in inflammation and wound healing, it seemed logical that its level would be influenced by anti-inflammatory drugs or immunosuppressants, even though no studies of this issue had been published. To exclude the possibility of including patients who had oral lichenoid lesions due to graft versus host disease, lupus erythematosus, or association with hepatitis C, we excluded patients who had any history of these systemic diseases. Smoking and excessive alcohol consumption are known risk factors for OSCC, and salivary ET-1 level has been reported to be elevated in smokers compared to non-smokers.⁷ In order to exclude these two confounding factors, for our OLP groups we only recruited non-smokers and subjects who reported drinking fewer than 14 alcoholic drinks per week. The definitions of non-smokers, ex-smokers and non-drinkers were based on the definitions used in previously published studies.^{8–12} Although no study investigated salivary ET-1 levels in ex-smokers, we included ex-smokers in our OLP groups because the risk for OSCC in ex-smokers was reported to be the same as for non-smokers.¹³ We excluded patients with Sjögren's syndrome or previous radiation therapy to the head and neck area (except for Group B) because of expected difficulties in collecting saliva from these individuals. We did not exclude post-radiation therapy patients in Group B because we found that many post-treatment OSCC patients during the past few years had been given intensity-modulated radiation therapy (IMRT) or received amfostine (Ethiol) during radiation treatment, and thus experienced a less severe degree of xerostomia as a side effect, compared with radiation patients during earlier times. Some of these patients were able to produce an adequate amount of saliva for the study.

2. Saliva collection

Whole saliva was collected from the participants between 6am and 12pm, using a previously published protocol.^{14–15} Briefly, participants were asked to refrain from eating, drinking or using oral hygiene procedures on the day of saliva collection. A water mouthrinse was administered prior to saliva sample collection. Five minutes after the oral rinse, the participant was asked to sit upright and spit into a 50ml Falcon tube kept on ice. Approximately 8 ml of saliva were collected within 30 minutes.

3. Sample processing

Saliva samples were processed immediately after collection according to a previously published method.¹⁶ Briefly, the saliva samples were centrifuged at 2,600g for 15 minutes, at 4 °C. Proteinase inhibitors including 1 ul of aprotinin (10mg/ml, Sigma, St. Louis, MO), 10 ul of phenylmethylsulfonyl fluoride (10mg/ml, Sigma, St. Louis, MO) and 3 ul of sodium orthovanadate (Na₃VO₄, 400mM, Sigma, St. Louis, MO) were added to each milliliter of the supernatant to prevent protein degradation. All samples were stored at –80 °C in aliquots until further use.

4. ET-1 level determination and data analysis

The salivary ET-1 levels were determined with an enzyme-linked immunosorbent assay kit (Assay Designs, Ann Arbor, MI) following the manufacturer's instructions. The optical density at 450 nm wavelength for each sample was recorded by FLUOstar OPTIMA microplate reader (BMG Labtech, Cary, NC). The ET-1 concentration in each saliva sample was calculated based on the standard curve. The results were analyzed with SPSS for Windows, version 18.0. The frequency, mean and standard deviation were used to describe the study variables for each group. Mann Whitney U test was used to determine if there was a significant difference in the levels of ET-1 between each pair of groups being compared.

Results

A total of 111 participants were recruited. However, five of the saliva samples in Group A (OSCC patients before treatment) were discarded due to blood contamination in the samples. The distribution of the remaining 106 participants among the five groups, along with their demographic and clinical information, is shown in Table 2.

Most patients in Group A (55.6%) had been diagnosed in the advanced stages (stage III and IV)(Table 2). Of the 15 patients in our Group B, the duration of their disease-free period ranged from 2 years 1 month to 8 years 10 months. The number of patients in disease-free period of time for 2–3 years, 3–5 years, and more than 5 years were 7, 5, and 3, respectively. The most commonly affected site in our OSCC patients (Group A and B) was the tongue (Table 2). We classified the clinical types of OLP as reticular, erosive, atrophic, plaque, papular, and bullous types. None of the patients showed papular or bullous types. The most common initial clinical presentation of OLP in our patients (Group C and D) was a combination of reticular, erosive and atrophic types, and the most commonly affected sites were buccal mucosa and gingiva (Table 2).

The mean salivary ET-1 concentrations for each group are shown in Table 3. There were no significant differences among the mean ages of the participants in the five groups ($p>0.05$). The mean ET-1 level in Group A was significantly higher than that of either Group C ($p=0.001$), Group D ($p=0.015$) or Group E ($p=0.004$). There was no significant difference in the mean salivary ET-1 levels between Group A and B; Group B and C; Group B and D; Group B and E; Group C and D; Group C and E; or Group D and E ($p>0.05$). We also performed analysis comparing the OLP patients as a whole (Group F, composed of Group C and D combined), to Groups A, B and E, respectively. The mean salivary ET-1 level in Group A was significantly higher than the level in Group F ($p=0.002$), but there was no significant difference between Group F and Group B or between Group F and Group E ($p>0.05$). Among the 15 Group B patients we recruited, there were four participants who had had previous radiation therapy. The salivary ET-1 levels in these four patients ranged from 2.888 to 19.108 pg/ml, and the mean was 8.702 pg/ml. There was no significant difference ($p=0.43$) in the mean salivary ET-1 levels between the patients in Group B who had had previous radiation (8.702 ± 7.3337 pg/ml) and those who had not (6.3155 ± 4.5864 pg/ml), although admittedly the number of patients was too small to make any meaningful conclusion about the effects of radiation on salivary ET-1 levels.

Because salivary ET-1 levels have been reported to be elevated in patients with chronic heart failure¹⁷ or upper gastrointestinal diseases such as gastric ulcer, duodenal ulcer and gastritis,⁷ the systemic diseases of the participants in each group were analyzed. None of our participants had a history of the above-mentioned diseases. The major systemic diseases found in our study groups could be categorized as follows: cardiovascular diseases (including hypertension and arrhythmia); bone and joint diseases (including osteoarthritis, osteoporosis and osteopenia); diabetes mellitus; gastrointestinal diseases (including acid reflux and inflammatory bowel diseases); hypothyroidism; and other diseases (pulmonary diseases, psychological diseases, kidney diseases, anemia and fibromyalgia). The numbers of subjects within each of these categories of systemic diseases for each group are listed in Table 4. The frequency of the five most common categories of diseases found in our patient population, in descending order, is as follows: cardiovascular diseases, bone and joint diseases, gastrointestinal diseases, hypothyroidism and diabetes. Due to the small sample size of each group, we did not analyze the statistical correlation between the salivary ET-1 level and the systemic diseases of participants in this study.

Discussion

Our results showed that salivary ET-1 levels in OSCC patients before treatment (Group A) were significantly higher than the levels found in normal controls (Group E). This finding was consistent with the finding reported by Pickering *et al.*² in a study based on patients in the San Francisco area, but was contrary to the finding reported by Hoffmann *et al.*⁵ in a study based on patients in Brazil. Comparing our data with the reported data in these two studies, some interesting factors are noteworthy. First, the salivary ET-1 concentrations in both our OSCC and normal control groups were much higher than the levels reported by Pickering *et al.*² (Table 5). This might be due to the use of proteinase inhibitors in our saliva samples, which was not done in the Pickering *et al.*² study. Without proteinase inhibitors, statistically significant reduction in the ET-1 levels has been reported in saliva samples stored at -80°C over 5 weeks.¹⁸ Second, although our findings in OSCC patients were different from those in the Hoffmann *et al.*⁵ study, the means and the ranges of salivary ET-1 concentrations for both the post-treatment OSCC patients and the normal controls were quite similar (Table 5). The reason for this is unclear. Comparing the methodology used in these two studies, Hoffmann *et al.*⁵ appeared to store the saliva samples immediately after collection, without centrifugation and separation of supernatant as we did in our study. Therefore, the results in our study (as well as in the Pickering *et al.*² study) should represent the ET-1 levels of the supernatant (cell-free) portion of the saliva samples, while the levels found in the Hoffmann *et al.*⁵ study should represent the ET-1 levels in the whole saliva samples without processing. Whether this might account for their different conclusion remains to be clarified.

Our results showed that salivary ET-1 levels in OSCC patients before treatment (Group A) were significantly higher than the levels found in OLP patients, regardless of the symptoms of disease activity (Group C and D). This result indicated that salivary ET-1 would be a potential biomarker for detecting OSCC development in OLP patients. However, further study including a group of OLP patients with presence of OSCC will be needed to confirm this inference. On the other hand, our results did not show a significant difference between the salivary ET-1 levels in OSCC patients with a current cancer presence (Group A) and OSCC patients who had shown no recurrence for at least 2 years (Group B). This suggests that salivary ET-1 would not be a good biomarker for monitoring recurrence in previously treated OSCC patients.

While we hypothesized that differences would be seen between OLP disease-active and disease-inactive states, due to the involvement of ET-1 in inflammation and wound healing, our results surprisingly showed no significant difference in salivary ET-1 levels between these two groups of OLP patients (Group C and D). Salivary ET-1 level also was not significant elevated in the OLP patients as a whole (Group F) compared to the normal controls (Group E), despite its reported involvement in inflammation and wound healing. The reason for these two interesting results is unclear, although we speculate that certain systemic diseases and oral conditions may contribute to it. ET-1 has been found to be expressed in normal striated duct cells (but not in the acinar cells or intercalated cells)¹⁹ and in normal gingival epithelial cells.²⁰ An increased ET-1 level was reported in the cell culture system when gingival epithelial cells were infected by periodontopathic bacteria.²¹ Therefore, any factor that can affect secretion of ET-1 from the striated duct cells or the gingival epithelial cells can also potentially affect the salivary ET-1 level. On the other hand, salivary ET-1 levels have been reported to be elevated in patients with chronic heart failure¹⁷ or upper gastrointestinal diseases such as gastric ulcer, duodenal ulcer and gastritis.⁷ Although none of our participants were known to have these diseases, the possibility that other systemic diseases may affect the salivary ET-1 level similarly cannot be totally excluded. Large-scale studies investigating the potential influence of systemic

diseases on salivary ET-1 levels will be needed for a meaningful answer regarding this possibility.

Based on the results of our pilot study, we concluded that salivary ET-1 could be a good biomarker for OSCC development in OLP patients regardless of the degree of OLP disease activity. However, it appeared not to be a good biomarker for detecting recurrence of OSCC in patients in remission.

Acknowledgments

This project is funded by the National Institute of Dental and Craniofacial Research (1R21DE018757-01A2).

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Table 1

The Inclusion and Exclusion Criteria of the Study Groups (OSCC= Oral Squamous Cell Carcinoma; OLP= Oral Lichen Planus).

Groups	Inclusion Criteria	Exclusion Criteria
A: OSCC patients before treatment	1 Patients who had pathology reports of OSCC and had oral lesions of OSCC at the time of saliva collection. 2 Patients who had no previous diagnosis of OLP.	1 Subjects who had used any corticosteroids or immunosuppressants, for any reason, for at least 1 week prior to saliva collection 2 Subjects who had bone marrow transplants, hepatitis C, or lupus erythematosus or Sjögren's syndrome. 3 Subjects who had previous radiation therapy to the head and neck area (except Group B)
B: OSCC patients post-treatment for at least two years without recurrence	1 Patients who had past history and pathology reports of OSCC but no previous diagnosis of OLP. 2 Patients who had completed treatment for OSCC at least 2 years prior to saliva collection, and had had no recurrence of cancer since then.	
C: OLP patients in disease-active condition	1 Patients who had lesions with clinical features of OLP, had been biopsied with a pathology diagnosis of OLP and the treatment of OLP had not been started. 2 Patients who did not have a solitary lichenoid lesion adjacent to a dental restoration. 3 Patients who had no previous diagnosis of oral epithelial dysplasia or OSCC. 4 Patients who stated that they were non-smokers* or ex-smokers**. 5 Patients who stated that they had consumed fewer than 14 alcoholic drinks*** per week	
D: OLP patients in disease-inactive condition	1 Patients who had been diagnosed with OLP previously and had a pathology report of OLP 2 Patients who had no clinically visible lesions of OLP, or had the asymptomatic reticular type of OLP 3 Patients who had no previous diagnosis of oral epithelial dysplasia or OSCC. 4 Patients who stated that they were non-smokers* or ex-smokers**, and had consumed fewer than 14 alcoholic drinks*** per week.	
E: Normal controls	1 Subjects who had no history of OLP, epithelial dysplasia or OSCC. 2 Subjects who stated that they were non-smokers* or ex-smokers** and had consumed fewer than 14 alcoholic drinks*** per week.	

*“non-smoker” was defined as a person who had smoked fewer than 100 cigarettes in his/her lifetime and had not smoked at all within one calendar year prior to beginning participation in the study, and who had not smoked pipe or cigar or used smokeless tobacco, for any more than a total of 6 months in their lifetime, and not at all within the calendar year prior to beginning participation in the study.

**
“Ex-smoker” was defined as a person whose last smoking or other use of tobacco products was at least 20 years prior to beginning participation in the study.

***
One alcoholic drink was defined as approximately 150 ml of wine, 330 ml of beer or 30 ml of hard liquor.

Study Groups, Demographic Data, OSCC Clinical Stage and Type(s) of Treatment, OLP Clinical Types, and Locations of the Lesions in the Patients.

Table 2

Group	Number of Subjects	Mean Age (Range)	Gender		OSCC Stage*				OSCC treatment**				OLP Clinical Types***				Location of the Lesion(s)****					
			M	F	I	II	III	IV	S	S + R	S + C	S + R + C	R/P	E/A	Combination	B	G/A/R	T	V	F	P	
A	18	59.11 (26-75)	15	3	5	3	3	7	10	2	2	4	0	-		1	4	9	0	3	1	
B	15	66.67 (35-95)	8	7	9	4	2	0	11	3	0	0	1	-		0	2	11	0	2	0	
C	21	63.71 (45-83)	2	19	-						-			2	2	17	15	5	6	0	1	
D	28	62.68 (47-80)	11	17	-						-			3	8	17	18	11	4	0	0	
E	24	62.92 (42-85)	11	13	-						-			-		-						
F (C+D)	49	63.12 (45-83)	13	36	-						-			5	10	34	35	33	16	10	0	1

* The OSCC stage is based on the TNM classification.

** S= surgery; R= radiation; C= chemotherapy

*** R/P= reticular and/or plaque; E/A= erosive and/or atrophic; Combination= some combination of reticular, plaque, erosive and atrophic forms. The OLP clinical types of Group D listed were the presenting types when the patient was diagnosed (biopsied). At the time of saliva collection, all Group D patients showed either no OLP lesion or showed asymptomatic reticular type lesions, as specified in the inclusion criteria.

**** B= buccal mucosa; G/A/R= gingival, alveolar ridge or retromolar pad; T= tongue; V= vestibule; F= floor of mouth; P= palate.

Table 3

Salivary ET-1 Levels (pg/ml) in OSCC and OLP Patients

Group	Number of Subjects	Salivary ET-1 Concentration	
		Mean $\pm$ Standard Deviation	Range
A	18	13.5103 $\pm$ 14.1447	2.140 – 52.229
B	15	7.7227 $\pm$ 5.6890	2.476 – 19.108
C	21	4.2965 $\pm$ 3.1845	1.363 – 14.013
D	28	5.7947 $\pm$ 4.6473	0.759 – 18.471
E	24	4.5299 $\pm$ 3.7380	0.506 – 19.280
F (C+D)	49	5.1526 $\pm$ 4.1152	0.759 – 18.471

Table 4
The Number of Subjects with Various Major Systemic Diseases in Each Study Group.

Group	Cardio-vascular disease		Bone and joint disease		Gastro-intestinal disease		Diabetes mellitus		Hypo-thyroidism		Other diseases	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
A (N=18)	8	10	1	17	2	16	2	16	2	16	3	15
B (N=15)	7	8	1	14	4	11	0	15	3	12	2	13
C (N=21)	13	8	11	10	5	16	2	19	4	17	3	18
D (N=28)	11	17	10	18	7	21	6	22	5	23	7	21
E (N=24)	8	16	6	18	1	23	1	23	0	24	0	24
Total (N=106)	47	59	29	77	19	87	11	95	14	92	15	91

Table 5

Comparison of the Reported Salivary ET-1 Levels (pg/ml) in OSCC and OLP Patients.

	Pickering et al. (2007)	Hoffmann et al. (2011) ^{a,b}	Cheng et al. (2011)
OSCC, before treatment	4.37 ± 1.35 (0.97–13.21)	6.4789 (0–18.8238)	13.5103 ± 14.1447 (2.140–52.229)
OSCC, at least two years after treatment		8.4725 (0.7999–20.5632)	7.5099 ± 5.5617 (2.476–19.108)
Oral leukoplakia		6.9773 (0–23.5559)	
OLP, disease-active			4.4209 ± 3.3467 (1.262–14.013)
OLP, disease-inactive			5.7947 ± 4.6473 (0.759–18.471)
Normal controls	1.16 ± 0.29 (0.27–2.55)	5.9806 (0–23.994)	4.5299 ± 3.7380 (0.506–19.280)

^aThe means and standard deviations for each group were not provided in the text but were shown in Figure 1 in the original article. Therefore, the means and standard deviations listed in this table are an estimation based on Figure 1 in the original article. The estimated mean and standard deviation for each group used in this table are as follows: OSCC patients before treatment: 2.6 ± 0.6 fmole/ml; OSCC patients who had completed treatment at least 5 years earlier: 3.4 ± 0.65 fmole/ml; oral leukoplakia: 2.8 ± 0.95 fmole/ml; normal controls: 2.4 ± 0.6 fmole/ml.

^bThe range of salivary ET-1 concentrations for each group was provided in the text in the original article but was presented in fmole/ml. For comparison purposes, the original data was converted to pg/ml based on ET-1 molecular weight=2491.90 (C₁₀₉H₁₅₉N₂₅O₃₂S₅, Sigma-Aldrich, St. Louis, MO). The range for each group provided in the original article was as follows: OSCC patients before treatment: 0–7.554 fmole/ml; OSCC patients who had completed treatment at least 5 years earlier: 0.321–8.252 fmole/ml; oral leukoplakia: 0–9.453 fmole/ml; normal controls: 0–9.629 fmole/ml.