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Salivary Basic Fibroblast Growth Factor in Patients with Oral Squamous Cell Carcinoma or Oral Lichen Planus

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Abstract

Objective—To gather preliminary data concerning the feasibility of using salivary basic fibroblast growth factor (bFGF) for detecting development of oral squamous cell carcinoma (OSCC) in oral lichen planus (OLP) patients, and in OSCC patients whose disease was in remission.

Study Design—Saliva samples were collected from five patient groups: newly diagnosed OSCC patients; OSCC patients in remission; OLP patients in disease-active state; OLP patients in disease-inactive state; and normal controls. Salivary bFGF levels were determined by ELISA, and data was analyzed using the Mann Whitney U test.

Results—Salivary bFGF levels were significantly elevated in newly diagnosed OSCC patients compared with OSCC remission patients, disease-active OLP patients, and normal controls. No significant difference was found between newly diagnosed OSCC patients and disease-inactive OLP patients.

Conclusion—Our results suggested that salivary bFGF might be a potential biomarker for detecting OSCC development in OSCC patients in remission, but not in OLP patients.

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Keywords

Salivary biomarkers; basic fibroblast growth factor; FGF-2; oral lichen planus; oral squamous cell carcinoma; oral cancer

INTRODUCTION

Oral squamous cell carcinoma (OSCC) accounts for the majority of oral cancers diagnosed worldwide.^{1, 2} Despite the advances in treatment modalities, the five-year survival rate has not changed much for several decades, staying at 50–60%.³ This unfavorable survival rate could be because OSCC is frequently not diagnosed until it is at an advanced stage, and also because secondary tumors often develop after treatment, due to field cancerization.^{4, 5} The annual incidence of development of secondary tumors in patients who have been treated for OSCC is reported to be 3.2–4%.^{6, 7} Monitoring for recurrence of cancer in OSCC patients who are in remission obviously is important; and a non-invasive screening tool which could detect the early development of recurring tumors would be very helpful to these patients.

Oral lichen planus (OLP) is a chronic inflammatory mucocutaneous disorder and one of the most common oral mucosal diseases seen in dental clinics. The prevalence of OLP worldwide is unknown, but has been reported to be 0.1–2.2%.^{8–11} OLP usually affects multiple sites in the oral cavity, appearing bilaterally.^{8, 11} Clinically, it can present as white reticular-type striations, small white papules, white plaques, erosions, ulcers, or blisters. The symptoms of OLP vary and often alternate between periods of exacerbation and remission. Patients may be symptomatic, with sore mucosal surfaces, sore and bleeding gums, and sensitivity to cold, hot, and spicy foods; or they may be asymptomatic, especially with the reticular-type lesions.^{9, 11} Since the etiology of OLP is as yet unknown, there is no permanent cure for OLP. Several clinical studies and case reports have suggested that patients with OLP have an increased risk for developing OSCC.^{12–15} Therefore, the currently recommended management for OLP includes an annual check-up in addition to symptomatic relief with the use of topical corticosteroids and/or other immunosuppressants.¹⁶ A non-invasive screening tool for detecting early OSCC development would also be very beneficial for the OLP patients.

In recent years, salivary biomarkers for various cancers have become a subject of strong research interest. Advantages of using saliva over serum include the fact that saliva collection is non-invasive, and that there is no possibility of needle prick injuries.^{17–20} Several potential salivary biomarkers for early diagnosis of OSCC have been reported in the literature²¹, including basic fibroblast growth factor (bFGF, FGF-2).²² bFGF is a strong mitogen that stimulates the proliferation of cells of mesodermal and neuroectodermal origin.²³ It has been found to be involved in hematopoiesis;²⁴ angiogenesis;^{25–27} vascular remodeling;^{28, 29} cardiac hypertrophy;³⁰ neuronal degeneration;^{24, 27, 31, 32} bone development and remodeling;³³ tumor progression;^{34, 35} and wound healing.^{27, 36} Vucicevic Boras et al.³⁷ found an increase in salivary and serum levels of bFGF in OSCC patients and suggested that bFGF could be a potential salivary biomarker for OSCC detection. However, whether salivary bFGF would potentially be a good biomarker for detecting the development of OSCC in OLP patients as well as in patients who previously had OSCC was still unknown.

We hypothesized that when there is no sign of OSCC in their mouths, salivary bFGF levels in both the OSCC patients in remission and the OLP patients would be within the range of levels found in the normal controls. Furthermore, since salivary bFGF levels had also been found to be elevated in OLP patients,³⁸ and because bFGF has been shown to be involved in

wound healing,^{27, 36} we hypothesized that its levels in saliva in OLP patients with multiple symptomatic lesions (the disease-active state) would be significantly different from the OLP patients with no lesions, or with asymptomatic reticular lesions in the mouth (the disease-inactive state). Therefore, the purposes of this study were to: 1) assess the feasibility of using salivary bFGF as a biomarker for detecting OSCC recurrence in OSCC patients in remission, as well as detecting OSCC in OLP patients; and 2) investigate whether the salivary bFGF levels were significantly different in OLP patients when the disease was active compared to when it was inactive.

MATERIALS AND METHODS

The study protocol was reviewed and approved by the Institutional Review Board at the Texas A&M Health Science Center - Baylor College of Dentistry, Dallas, Texas, USA. Signed informed consent forms were obtained from all the participants.

Subject Population

Study subjects were recruited from the Stomatology Center, Baylor College of Dentistry, and were also referred by local ear-nose-throat doctors and head-and-neck surgeons. Our Stomatology Center at Baylor College of Dentistry is one of the largest oral medicine specialty clinics in the Southwest US. There were more than 800 OLP patients in our database and we had seen an average of 29 new OLP cases annually since 2004. The OSCC patients were recruited from our Stomatology Center, and by referral from the local surgeons, ear-nose-throat doctors and dentists.

Study information was provided to the patients at the Stomatology Center who fit the inclusion criteria, and information was sent out to the local doctors who see or manage OSCC patients. The doctors gave the study information to those patients who fit the inclusion criteria. If the patient was interested, he/she would contact us and we would arrange an appointment for saliva collection. All patients were from the Dallas/Fort Worth, Texas area and the nearby towns.

The subjects were classified into the following five groups: Group A: Patients with newly diagnosed OSCC; Group B: OSCC patients who had completed treatment at least two years prior to saliva collection and had had no recurrence; Group C: OLP patients in the disease-active state (with multiple symptomatic lesions); Group D: OLP patients in the disease-inactive state (with no lesions, or with asymptomatic reticular lesions in the mouth); Group E: Normal controls who had no oral mucosal disease, such as lichen planus, aphthous stomatitis, geographic tongue or candidiasis. The inclusion and exclusion criteria for all the groups of participants are listed in Table 1. Convenient samples were used. Any OSCC or OLP patients who were interested in participating and fit the criteria were included in the study. Normal controls were volunteers who fit the criteria, and were recruited from our institution or from the community.

Sample Size Determination

There had been only two previous studies investigating salivary bFGF levels in OSCC or in OLP patients, and only one of them provided information necessary for calculating effect size and sample size. Therefore, we used that study (Magnusson et al.³⁸) as a reference, to estimate the sample size for our study. The effect size was measured for the Student's t test as the standardized difference between the means by G power,³⁹ and it was 2.7 (The means and standard deviations in the OLP and the normal controls were 5.9 ± 2.9 pg/ml and 0.3 ± 0.3 pg/ml, respectively). According to Cohen,⁴⁰ an effect size (d) larger than 0.8 for a Student's t test is defined as large. Therefore, the effect size for salivary bFGF level (for this study)

was considered to be large. To identify a large difference ($d=0.8$), if present, while maintaining an α (Type I) error rate of 0.05 and a β (Type II) error rate of 0.20 (with a power of at least 0.80), a sample size of at least 21 for each group is indicated.

Saliva Sample Collection

Saliva samples were collected based on the method described by Navazesh et al.⁴³ Saliva samples from the subjects of the above-named five groups were collected in the morning between 6 am and 12 pm. Subjects were asked to refrain from eating, drinking and performing any kind of oral hygiene procedures, such as brushing, rinsing with water or mouth rinse products prior to saliva collection. At time of collection, a cup of water was first given to the participant for rinsing. Five minutes after rinsing, the participant was asked to spit into a 50ml sterile plastic tube kept in ice. Approximately 5ml of saliva was collected within 30 minutes from each subject/participant.

Saliva Sample Processing

Saliva samples were processed based on methods previously described in the research literature.^{44,45} Immediately after the saliva sample was collected, it was centrifuged at 2600 g for 20 minutes at 4°C. After centrifugation, the supernatant was separated from the pellet. Three proteinase inhibitors were added to 1ml of the supernatant: 1 μ l of Aprotinin (10mg/ml) (Sigma, St. Louis, MO, USA); 3 μ l of sodium orthovanadate (Na_3VO_4 , 400 mM) (Sigma, St. Louis, MO, USA); and 10 μ l of phenyl methyl sulfonyl fluoride (PMSF, 10 mg/ml) (Sigma, St. Louis, MO, USA). The samples were then stored at -80 °C until future use.

Determination of Salivary bFGF Concentrations

Saliva samples were thawed and typically analyzed within 6 months from time of collection. Salivary bFGF concentrations were determined by a commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Each sample was only thawed once, at the time for ELISA. There was no repeated freezing and thawing for any of the saliva samples in this study. The absorbance of the saliva samples at 450 nm wavelengths was measured by FLUOstar OPTIMA microplate reader (BMG Labtech, Cary, NC, USA,) and corrected by the absorbance at 650 nm. The concentrations of bFGF in the saliva samples were calculated based on the standard curve. The minimal level of detection was 0.313 pg/ml. Each saliva sample was tested in triplicate.

Method of statistical analysis

The Mann-Whitney U test was used to compare differences in salivary bFGF concentrations between members of each pair of groups, using the SPSS IBM, Version 17.0, software. $P<0.05$ was considered significant.

RESULTS

A total of 139 saliva samples were collected from September 1, 2009 to March 31, 2011. Three samples showed salivary bFGF levels below the minimum detection level and were excluded from analysis. The demographic and clinical information of the other 136 participants are listed in Table 2 by group. The ages of the participant were similar amongst the different study groups ($p=0.642$). Gender has never been reported to be a confounding factor for salivary bFGF. But in this preliminary study, we also compared the bFGF levels between male and female participants in each study group. We found no significant difference in salivary bFGF levels between males and females in each group (Group A: $p=0.291$; Group B: $p=0.559$; Group C: $p=0.976$; Group D: $p=0.122$; Group E: $p=0.252$).

Most of the newly diagnosed OSCC patients (Group A) were in Stages I and IV. Most patients who were in remission (Group B) had had OSCC Stage I lesions at the time of diagnosis. In both Groups A and B, most of the patients had OSCC lesions on the tongue. There were three patients each in Group A and in Group B who had OSCC affecting more than one location. For OLP, most of the patients in both Groups C and D had combined reticular and erosive types of lesions at the time of diagnosis. Common sites for OLP lesions for both these two groups of patients were buccal mucosa and gingiva/alveolar ridge area.

The mean, standard deviation, range, median and standard error of salivary bFGF concentrations (pg/ml) with coefficient of variance in each of the study groups are shown in Table 3. Group F was created by combining our Groups C and D (OLP disease-active and disease-inactive groups) in order to facilitate a comparison between our results and the results previously published in the literature, because all previously-published salivary biomarker studies regarding OLP did not separate disease-inactive patients from disease-active patients.^{38,46,47}

A significantly higher salivary bFGF levels were found in newly diagnosed OSCC patients compared to the levels found in OSCC remission patients ($p=0.002$), OLP disease-active patients ($p=0.038$) and normal controls ($p=0.018$). The salivary bFGF levels in Group F (combined OLP disease-active and disease-inactive groups) were significantly higher when compared to the levels found in OSCC remission patients ($p=0.001$) and normal controls ($p=0.011$). The salivary bFGF levels in OLP disease-inactive patients were significantly higher compared to the levels found in OSCC remission patients ($p<0.001$), OLP disease-active patients ($p=0.001$) and normal controls ($p<0.001$). There was no significant difference between newly diagnosed OSCC patients and OLP disease-inactive patients ($p=0.789$); newly diagnosed OSCC patients and combined OLP patients ($p=0.303$); OSCC remission patients and OLP disease-active patients ($p=0.073$); OSCC remission patients and normal controls ($p=0.051$); or OLP disease-active patients and normal controls ($p=0.703$).

DISCUSSION

Our study showed that the salivary bFGF levels in newly diagnosed OSCC patients was significantly elevated compared to the levels found in the normal controls, a result consistent with the findings reported previously by Vucicevic Boras et al.³⁷ These findings suggest that salivary bFGF could be a potential biomarker for detection of OSCC in patients who had never been diagnosed with it. We also found that the salivary bFGF level in the newly diagnosed OSCC patients was significantly higher than the levels found in the OSCC patients who had completed treatment and had shown no recurrence for at least two years. This result suggests that salivary bFGF might be a potential biomarker for detecting OSCC recurrence in such patients. Because there was no significant difference in the salivary bFGF levels between the groups of OSCC patients in remission and the normal controls, the evidence is further strengthened for the potential efficacy of salivary bFGF as a biomarker for detecting recurrence in post-treatment OSCC patients. However, further studies investigating salivary bFGF in a group of OSCC patients with recurrence will be required for confirmation.

We speculated that the elevated salivary bFGF levels found in the newly diagnosed OSCC patients could come from several possible sources, including salivary glands, neoplastic cells and tumor-associated angiogenesis. BFGF is present in many tissues and body fluids including salivary glands, saliva and tears.^{48–50} In salivary glands, bFGF is present in the parasympathetic nerve⁵¹ and in ductal epithelial cells,^{52–53} from where it can be secreted into saliva upon stimulation. BFGF has also been found to be involved in endothelial cell proliferation²³, and is expressed in the basal and suprabasal keratinocytes of normal oral

mucosa and in OSCC cells.⁵⁴ Increased bFGF levels have been found to be associated with progression, invasion and angiogenesis associated with OSCC.^{54–56} Breakdown of cell-cell and cell-matrix adhesions, proteolysis of extracellular matrix, and induction of angiogenesis have all been postulated to play important roles in tumor progression and invasion.^{57,58} Reduced cell-cell and cell-matrix adhesions might not only make it easier for the cancer cells to escape from their site of origin and metastasize, but would also let locally increased levels of bFGF and other cytokines in the extracellular matrix get into saliva during the saliva sample collection. Some or all of these factors might contribute to the elevated salivary bFGF levels found in the newly diagnosed OSCC patients.

In our study, we found a significant increase in salivary bFGF levels in the combined OLP group (Group F) compared to the levels found in normal controls, a result that is consistent with the finding reported in the previously published study.³⁸ However, we found no significant difference in the salivary bFGF levels between the newly diagnosed OSCC group (Group A) and the combined OLP group (Group F). This result suggested that salivary bFGF would not be a good biomarker for detecting OSCC development in OLP patients, as it appeared to be increased in OLP patients without OSCC to a level that was not significantly different from the OSCC patients.

No study had been done previously to investigate whether fluctuations in the OLP disease activity would influence the salivary bFGF levels in OLP patients. In our study, we found a significantly higher level of salivary bFGF in the OLP disease-inactive group compared to the level found in the OLP disease-active group. As a result, it appears that OLP disease fluctuations do have a significant effect on the salivary bFGF levels. We also found a significantly higher level of salivary bFGF in the OLP disease-inactive group when compared to the levels found in the normal controls. Although the reason(s) for both of these findings is still unknown, we speculate that it might be partly related to the changes in bFGF levels contributed by salivary glands and granulation tissues during the process of wound healing.

There has been evidence reported in previous studies suggesting that bFGF, along with other growth factors secreted by salivary glands, might play an important role in wound healing in the upper gastrointestinal mucosa.^{51,53,59} In addition, bFGF plays an important role in granulation tissue formation and in proliferation of fibroblasts and endothelial cells, which are major events in the proliferative phase of wound healing, but not in the inflammatory phase.^{60,61} Our OLP disease-inactive group consisted of patients who either had no lesions or had asymptomatic reticular lesions. Although the patients had a history of OLP, there was no sign of active inflammation at the time of saliva sample collection. It could be possible that these patients were in a post-inflammatory phase (or the proliferative phase) at the time of saliva collection. In the OLP disease-active group, the patients showed signs of active inflammation (OLP lesions and soreness) which would represent the inflammatory phase of the wound healing. As bFGF was more active in the proliferative but not the inflammatory phase of wound healing^{58,59}, this might explain the elevated levels of bFGF found in the saliva of OLP patients in the disease-inactive state. From this perspective, the finding that the OLP disease-inactive group showed increased salivary bFGF levels compared to the levels found in the normal controls may also indicate that the wound-healing process continues even when the OLP patients show no lesion or have asymptomatic reticular lesions.

CONCLUSIONS

Our results suggested that salivary bFGF might be a potential biomarker for detecting OSCC recurrence in OSCC patients in remission, and that prospective well-controlled larger

population studies are needed in patients with OSCC to validate these preliminary findings. However, since salivary bFGF appeared to be significantly higher in OLP patients and reached a level that was not significantly different from the level found the OSCC patients, it would appear not to be a potential biomarker for detecting OSCC development in OLP patients. Furthermore, fluctuations in OLP disease activity appeared to significantly influence the salivary bFGF levels in OLP patients.

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Table 1

Inclusion and Exclusion Criteria for the Study Groups.

Groups	Inclusion Criteria	Exclusion Criteria
A: Patients with newly diagnosed OSCC	<ol style="list-style-type: none"> 1 Patients who had been diagnosed with OSCC based on pathology reports, and 2 Had not started any OSCC treatment 	<ol style="list-style-type: none"> 1 Patients who had used any corticosteroid or immunosuppressant, for any reason, within a week prior to saliva collection 2 Patients who had had previous diagnosis of OLP, bone marrow or kidney transplants, hepatitis C, or lupus erythematosus 3 Patients who had Sjögren's syndrome
B: Patients who had completed treatment for OSCC and were in remission	<ol style="list-style-type: none"> 1 Patients who had a history and pathology reports of OSCC, and 2 Had completed treatment for OSCC and had had no recurrence for at least two years prior to participating in the study 	
C: OLP patients, in the disease-active state	<ol style="list-style-type: none"> 1 Patients who had been diagnosed with OLP, based on pathology reports, and 2 Who, at the time of saliva collection, were experiencing sore mouth and had clinically visible erosive lesions of OLP, and 3 Had not started any treatment for OLP, and 4 Clinically did not show a solitary lichenoid lesion adjacent to a dental restoration 	<ol style="list-style-type: none"> 1 Patients who had used any corticosteroid or immunosuppressant, for any reason, within a week prior to saliva collection 2 Patients who had had OSCC; oral leukoplakia, bone marrow or kidney transplants; hepatitis C; or lupus erythematosus 3 Patients who had Sjögren's syndrome or who had had radiation therapy to the head and neck area 4 Patients who were smokers ^a 5 Patients who reported that they consumed more than 14 alcoholic drinks per week ^b
D: OLP patients, in the disease-inactive state	<ol style="list-style-type: none"> 1 Patients who had been diagnosed with OLP based on pathology reports, and 2 Who, at the time of saliva collection, had no clinically visible lesions of OLP or had asymptomatic, reticular type of OLP lesions 	
E: Normal controls	<ol style="list-style-type: none"> 1 Patients who had no oral mucosal disease such as lichen planus, aphthous ulcer, geographic tongue or candidiasis 	

^a**Smoker** is defined as a person who smoked more than 100 cigarettes in his/her lifetime, had smoked within one calendar year prior to participating in the study; or who had smoked pipe or cigar or used smokeless tobacco for any more than a total of 6 months in their lifetime and within one calendar year prior to participating in the study.³⁹

^bOne drink is defined as approximately 50 ml of wine, 330 ml of beer, or 30 ml of hard liquor.⁴⁰

Table 2
The Demographics, Mean Age (Years), Age Range (Years) and the Clinical Information of the Study Groups.

Groups	N		Mean Age (Range)	OSCC Stage				OLP Type			Location of the Lesion																				
	M	F		I	II	III	IV	Reticular/Plaque	Erosive/Atrophic	Comb ined types	Buccal Mucosa/Vestibule	Alveolar Ridge/Retromolar pad/gingiva	Tongue	Floor of Mouth	Palate	Labial mucosa/Vestibule	Tonsil														
A: OSCC, newly diagnosed	24		59.33 (33–78)	10	4	3	7	--			1	5	15	4	1	0	1														
	16	8																													
B: OSCC, treated and in remission	25		64.96 (35–95)	16	3	3	3							0	4	17	4	0	1	3											
	15	10																													
C: OLP disease-active	29		63.14 (45–83)					4	5	20				20	20	8	0	1	0	0											
	4	25																													
D: OLP disease-inactive	29		62.45 (47–80)									2	7	20	19	19	11	0	0	3	0										
	11	18																													
E: Control	29		61.97 (32–85)	--																											
	15	14																													
F: Combined OLP (C +D)	58		62.98 (45–83)					6		12	40											39		39		19	0	1	3		0
	15	43																													

Table 3

The Mean, Standard Deviation (SD), Range, Median and Standard Error of Salivary Concentrations of BFGF (pg/ml) with Coefficient of Variance in the Study Groups.

Groups	Mean $\pm$ SD	Range	Median	Standard Error	Coefficient of Variance
A: OSCC, newly diagnosed	9.876 $\pm$ 14.468	0.97–73.77	7.080	2.953	0.012–0.321
B: OSCC, treated, and no recurrence for 2 years prior	3.571 $\pm$ 3.018	0.79–10.93	1.786	0.604	0.018–0.745
C: OLP disease-active	5.062 $\pm$ 3.510	1.01–16.01	4.393	0.652	0.015–0.360
D: OLP disease-inactive	8.155 $\pm$ 5.112	2.59–28.27	7.012	0.949	0.015–0.776
E: Control	4.602 $\pm$ 3.388	1.04–19.10	3.839	0.619	0.019–0.415
F: Combined OLP (C+D)	6.609 $\pm$ 4.618	1.01–28.27	6.396	0.606	0.015–0.776