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Estimation of salivary and serum basic fibroblast growth factor in treated and untreated patients with oral squamous cell carcinoma

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ABSTRACT

Objective: The purpose of this study was to evaluate salivary and serum basic fibroblast growth factor (bFGF) level in OSCC patients to provide a reliable biomarker for the early detection, monitoring, and prognosis of OSCC patients.

Materials and methods: The study enrolled 90 subjects, equally grouped as recently diagnosed & untreated OSCC patients (Group I), treated OSCC without any recurrence (Group II) & as control (Group III). Enzyme-linked immunosorbent assay was employed to measure bFGF concentrations in saliva and serum samples of all three groups. The results were tabulated and analyzed statistically.

Results: Group I showed high-level of bFGF expression profile in saliva (8.80 ± 1.26 pg/ml) whereas the levels of bFGF in group II (2.69 ± 0.17 pg/ml) and Group III (3.17 ± 0.43 pg/ml) are significantly lower than group I. Serum bFGF levels were also high in group I (6.33 ± 0.81 pg/ml) and decreased significantly in group II (3.52 ± 0.45 pg/ml) however, the control group showed elevated range of levels (7.63 ± 0.88 pg/ml).

Conclusions: This study demonstrates the reliability of salivary bFGF marker as diagnostic as well as prognostic marker. Serum bFGF could also be used as prognostic marker only in Oral Squamous cell carcinoma patients.

1. Introduction

Squamous cell carcinoma of the oral cavity ranks as the 15th most common neoplasm in the world and the 11th most frequent cancer in males, contributing 2.1% of all malignancies.[1]More than 90% of the oral malignancies are squamous cell carcinomas (OSCCs), which arise from the epithelial lining of the oral cavity.[2] Despite significant advancement in diagnostic tools and therapeutic modalities the overall survival rate of oral cancer patients has not improved significantly from last few decades. Five year survival rates remains between 60 and 80% when it is detected during stage1 and II. Also OSCC patients specially with lymph node metastasis have very high recurrence rate therefore early detection of relapse or occult secondary's is always very challenging.[3,4]

At present, the available traditional methods lack sensitivity to detect oral cancer at an early stage and are not very informative regarding the aggressiveness of neoplasm and prognosis.[5]Therefore the need of hour is to devise a method which is non invasive, economical, easy, less time consuming and precise for its diagnosis & prognosis and one such modality is identification of biomarkers.

Oral cancerous cells are immersed in the salivary bio-fluid of oral cavity.[6]Saliva is preprogrammed to have a certain composition in response to events in oral cavity and secretions from salivary glands contain proteins which are uniquely associated.[7–9]Therefore, in comparison with serum based biomarkers, salivary proteins may be a more sensitive and specific indicator for certain oral diseases.

Several potential salivary biomarkers for early diagnosis of OSCC have been reported in the literature including bFGF.[10] In OSCCs the biomarkers are usually locally released from the tumor site and can be broadly divided into protein and RNA based biomarkers. Protein based biomarkers include a group of biomarkers such as cytokines, fibroblast growth factor, cyfra 21-1, cancer antigen-125, tissue polypeptide antigen, matrix metalloproteinases etc and RNA based biomarkers include messenger RNAs and micro RNAs.[11]

Basic fibroblast growth factor (bFGF) also known as fibroblast growth factor-2(FGF-2),a heparin-binding growth factor which occurs in several isoforms resulting from alternative initiations of translation: an 18 kD cytoplasmic isoform and four larger molecular weight nuclear

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isoforms (22, 22.5, 24 and 34 kD).[12]bFGF is synthesized by fibroblasts, endothelial cells, glial cells and smooth muscle cells and characteristically distributed in various tissue[13] and has been found to be involved in, bone development and remodeling,[14]hematopoiesis,[15] neuronal degeneration,[15,16] cardiac hypertrophy,[17] wound healing,[16,18] vascular remodeling.[19]

The detection of bFGF from saliva in the diagnosis of OSCC may emerge as a helpful tool for the community and can aid in screening and monitoring for high risk patients like tumor recurrence and improvement of the survival rate. Thus, this study was aimed to estimate and compare the salivary and serum basic fibroblast growth factor in untreated & treated patients of oral Squamous cell carcinoma.

1.1. Materials and methods

1.1.1. Subject population

A prospective comparative analytical study was conducted in which 90 subjects irrespective of age and sex were included. The study subjects were selected randomly from the Out Patient Department of Oral Medicine and Radiology and Department of Surgical Oncology of King George's Medical University, Lucknow. Subjects were divided into the following three groups of 30 participants each. Subjects were included in the study after the fulfillment of inclusion and exclusion criteria and obtaining patient's written consent. The study protocol was reviewed and approved by institutional ethical committee.

Group I: Histologically diagnosed oral squamous cell carcinoma patients.

Group II: Oral squamous cell carcinoma patients who have completed treatment at least 2 years before sample collection and had no recurrence.

Group III: Healthy controls.

2. Selection criteria

2.1. Inclusion criteria

1. Histologically proven OSCC patients and had not started any treatment.
2. Patients who have had completed their treatment for OSCC at least two years back and had no history of relapse since then.

2.2. Exclusion criteria

1. Patients who had been previously diagnosed with any systemic disorder.
2. Patients who had been previously diagnosed with inflammatory condition of oral cavity.
3. Patients who had been previously diagnosed with autoimmune disorders.

Saliva Sample Collection: Unstimulated whole saliva samples were collected by draining method as described by **Navazesh *et al.***[20]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 collection of saliva. The participants were asked to rinse to remove any food debris and then instructed to spit passively (not forcibly) to collect 5 ml of saliva within 30 min. After collection of saliva, the sample was placed in ice carrier box and transferred to the biochemistry laboratory.

Serum Sample Collection: Five milliliter of whole blood was withdrawn from cubital vein of antecubital fossa through veni-puncture from each subject at the same time after saliva collection.

Saliva Sample Processing: Following collection, the saliva was immediately centrifuged at 3000 rpm for 20 min to remove solid constituents (desquamated oral epithelial cells, keratin debris, blood cells, bacteria and food residuals, if any). The resulting supernatant was separated and 1 ml of it was transferred into the 2 ml sterile plastic eppendorf tube. Proteinase inhibitors were then added to 1 ml of supernatant. The samples were then stored at −80 °C freezer till further analysis.

Serum Sample Processing: Blood samples were collected without anticoagulant into 10 ml centrifuge tubes and allowed to clot for at least 20 min at room temperature. Sera were separated by centrifugation 3000 rpm for 15 min.1 ml of serum was pipetted into the 2 ml sterile eppendorf tube. Proteinase inhibitors were added into 1 ml of serum. The serum samples were then stored at −80 °C freezer till further analysis.

Saliva and Serum analysis: Saliva and serum sample were analyzed within 6 month from the time of collection. Concentrations of bFGF in saliva and serum samples were measured by a commercially available enzyme linked immunosorbent assay (ELISA) (GenAsia), according to the manufacturer's instructions. The absorbance of saliva sample at 450 nm wavelengths was measured by thermofischer microplate reader. The concentration of bFGF in serum and saliva samples was calculated based on standard curve. Each saliva and serum sample was tested in triplate.

2.3. Statistical analysis

Data were summarized as Mean ± SE (standard error of the mean) and analyzed using SPSS software version 17.0. Groups were compared by independent Student's t-test. Groups were also compared by one way analysis of variance (ANOVA) and the significance of mean difference between (inter) the groups was done by Tukey's HSD (honestly significant difference) post hoc test. Discrete (categorical) groups were compared by chi-square (χ^2) test. A two-tailed ($\alpha = 2$) p value less than 0.05 ($p < 0.05$) was considered statistically significant.\

2.4. Observations & results

Present study was performed in 90 subjects with mean age of 46.60 ± 2.07 years, 57.27 ± 1.86 years and 45.73 ± 1.67 years in group I, II, III respectively (Fig. 1).

The mean saliva level of bFGF was 8.80 ± 1.26 pg/ml, 2.69 ± 0.17 pg/ml, 3.17 ± 0.43 pg/ml were in Group I, II and III respectively (Fig. 2). The mean salivary bFGF level of Group I was comparatively higher than both the other groups. Significantly different mean salivary bFGF level in all three groups was found by ANOVA.

Tukey's test showed that the difference in the levels was found to be highly significant and lower in both Group II & III as compared to group I (Table 1). However, there was no significant difference between Group II & III though it was higher (15.2%) in control as compared to treated OSCC.

Similarly, serum bFGF values were recorded. The mean serum bFGF level of group I (6.33 ± 0.81 pg/ml) and group II (3.52 ± 0.45 pg/ml) was found comparatively lower than the control (7.63 ± 0.88 pg/ml)

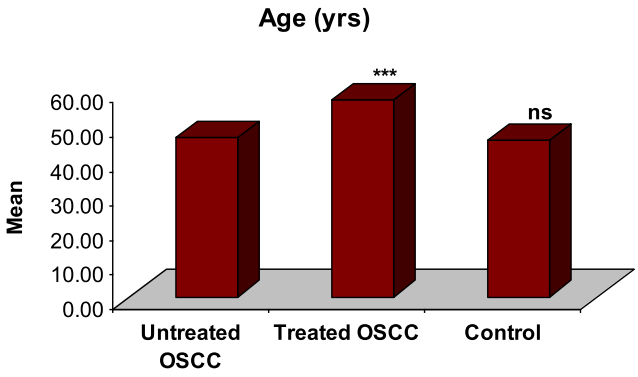

Fig. 1. Showing mean age of three groups.

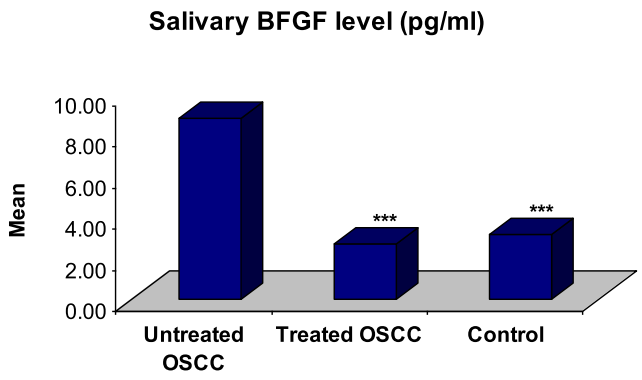

Fig. 2. Showing mean salivary bFGF level of three groups.

Table 1
Comparison (p value) of mean difference in salivary bFGF level between the groups by Tukey test.

arison	Mean difference	q value	p value	95% CI of difference
Untreated OSCC vs. Treated OSCC	−6.11	7.90	< 0.001	−8.728 to −3.496
Untreated OSCC vs. Control	−5.63	7.28	< 0.001	−8.246 to −3.015
Treated OSCC vs. Control	0.48	0.62	0.899	−2.134 to 3.097

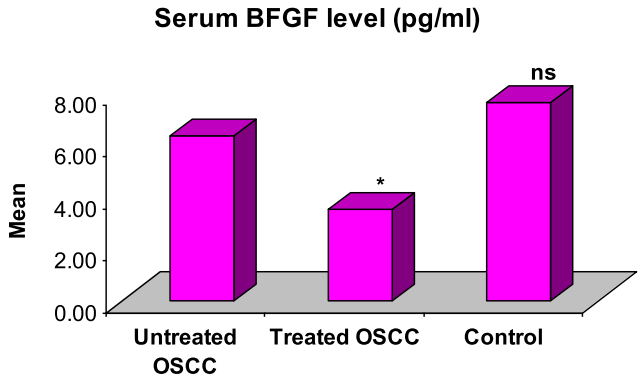

Fig. 3. Showing mean serum bFGF level of three groups.

Table 2
Comparison (p value) of mean difference in serum bFGF level between the groups by Tukey test.

Comparison	Mean difference	q value	p value	95% CI of difference
Untreated OSCC vs. Treated OSCC	−2.82	3.80	0.024	−5.321 to −0.309
Untreated OSCC vs. Control	1.30	1.75	0.434	−1.208 to 3.804
Treated OSCC vs. Control	4.11	5.55	0.001	1.607 to 6.619

(Fig. 3). Mean serum bFGF level in all three groups was found significantly different by ANOVA. Further, Tukey's test showed significantly lower serum bFGF level in group II as compared to group I but no significance was observed between Group I and group III though serum bFGF level was 17.0% higher in control as compared to untreated OSCC. However, it was significantly lower in treated OSCC as compared to control (Table 2).

3. Discussion

Oral cancer is public health problem, carries significant morbidity and mortality.[21] Oral cancer arises from sustained, stepwise accumulation of mutations resulting in transition of normal mucosa to dysplasia to invasive carcinoma.[22,23] For early detection of OSCC several diagnostic aids have been developed or are in development, including the use of salivary biomarker. Many potential OSCC salivary biomarkers are salivary proteins and are expressed in a very low quantity in saliva.[24,25] Due to presence of exfoliated cells in oral cavity, saliva may be the first choice for screening and identification for the potential biomarkers of OSCC.[26]The early detection of oral cancer offers the promise to not only detect disease at early stages but also improve the monitoring of disease progression or regression during treatment.[27]

In 1995 bFGF was detected in human saliva by van Setten[28] and in the same year Ohta T. studied that pleiotropic activity of bFGF may affect both tumor vasculature and tumor parenchyma and may contribute in carcinoma progression by inducing neovascularization or by directly acting on tumor cells.[29]

Heparin binding basic fibroblast growth factor (bFGF) protein has potent mitogenic and angiogenic properties.[30] In cancer, FGF receptors have been found to be over activated by several mechanisms, including gene amplification, chromosomal translocation and mutations. Jorgen Wesche evidence indicates that FGFs and FGFRs can act in an oncogenic fashion to promote multiple steps of cancer progression by inducing mitogenic and survival signals, as well as promoting epithelial mesenchymal transition, invasion and tumor angiogenesis.[31]

OSCC shows a geographical variation with respect to the age, site, sex and habits of the population.[32] Majority of male and female patients in newly diagnosed and untreated OSCC group belonged to 4th decade with mean age of 46 years. Halboub,[33] Saraswathi[34] and Sherin,[35] Singh MP[36] found that Oral SCC is common in middle age group patients. Present study potentiates the existing data on age. Our study confirmed that patients with OSCC are in mean age of 46.60 years whereas treated OSCC patients are in 5th decade of life. Similarly Gangane,[37] Ravi Mehrotra[38] also reported that prevalence of oral cancer is common in 50–59 years age group peoples. However, recent trend shows the surge in prevalence of Oral cancer among younger population in USA[39] and in India as well.

OSCC is more prevalent in male than female. Male to female distribution was (7.5:1). Iype,[40] Mehrotra,[38] Singh MP[36] reported male to female ratio of predominance 2.4:1, 2.3:1, 3:1 respectively. Male predominance could be attributed to more exposure to higher risk habits. However many recent studies in the Indian subcontinent as well in western world reported an increase in tongue cancer in young females without any contributing factors.[40–42] A study conducted in a rural population of Andhra Pradesh, it was seen that females were more affected than males, but this was attributed to the more common habit of reverse smoking in female population of that region.[43]

Our study revealed that the bFGF level in saliva of the healthy individuals was in the range of 1.06–8.73 pg/dl with a mean of 3.17 pg/dl. Mitreyi[44] found slightly higher bFGF level range of 1.04–19.10 pg/dl with mean of 4.602 pg/dl in saliva of controls patients. Our study showed significant higher bFGF level in saliva of newly diagnosed untreated oral SCC patient in comparison to healthy controls, observation was consistent with previous findings of Boras[45] and Mitreyi.[44] They suggested that salivary bFGF could be a potential biomarker for detection of OSCC in patients who had no oral mucosal disease.

Basic FGF level in saliva of patients in which treatment had been completed was significantly lower than level of recently diagnosed untreated OSCC patients. This finding is similar to previous studies of Boras[45] and Mitreyi.[44] It is hypothesized that various stimuli such as tumor angiogenesis, inflammation had been decreased after treatment of oral squamous cell carcinoma and are responsible for lower bFGF level after the successful therapy of oral cancer patient.

The salivary bFGF levels in the Group II and Group III was almost

similar as found in earlier studies however in control individual bFGF level was slightly elevated (15.2%) than OSCC patient in remission. This unexpectedly high value might be appeared due to undisclosed medical conditions or habits such as heavy tobacco smoking which has caused the elevated readings for entire group. Ishizaki H observed that smoking affect the saliva concentrations of bFGF. In their study they found smoker had significantly elevated bFGF concentration than non-smokers.[46]

As regards to serum bFGF, similar trend was noticed in group I and group II however the serum levels of bFGF in group I and group III was not very different. Boras also observed that serum bFGF level was not significantly different in untreated OSCC patients and healthy control group.[45]Similar findings were also observed in carcinoma other than oral cancer. This increase in the bFGF levels in control group indicates presence of other inflammatory processes systemically.

The basic FGF is also present in physiological conditions in blood plasma and an increased concentration appears in the course of carcinoma.[47]Serum bFGF level was found significantly decreased after treatment of oral SCC patients than the healthy control group. This finding suggested that serum bFGF can be used as biomarker for oral squamous cell carcinoma patients.

This study is different to other previous studies in that both serum and saliva level of bFGF was evaluated in newly diagnosed as well as already treated oral squamous cell carcinoma patients. The rationale of the present study was to detect the reliability of salivary basic fibroblast growth factor as a biomarker in oral squamous cell carcinoma.

Present study supports salivary bFGF over serum bFGF as a reliable biomarker for early detection of oral squamous cell carcinoma as salivary fluid bathes the oral squamous cell cancer and sways away with more information on oral squamous cell carcinoma than serum. The findings of present study provide a promising basis for the development of a non-invasive effective approach for the screening of large population for OSCC detection.

In our study follow up of recently diagnosed oral squamous cell carcinoma patients were not done after completion of their treatment which can be required to further assess the salivary and serum bFGF level to monitor any recurrence in these patients if happen. Further more studies with larger sample size in different demographic area should be needed in order to establish the sensitive and specific biomarker status of Basic fibroblast growth factor.

Conflict of interest statement- None declared

We, hereby, state that there is no personal or financial conflict of interest.

Role of funding source

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jobcr.2018.08.005.

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