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ORIGINAL ARTICLE

Differential expression of salivary S100A7 in oral submucous fibrosis

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KEYWORDS

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Abstract *Aim:* To investigate the expression of salivary S100A7 levels among patients with oral submucous fibrosis (OSF) and healthy controls.

Method: A total number of 60 participants were included in the study (30 OSF cases and 30 healthy controls). Demographic data was collected using a structured baseline questionnaire. Salivary S100A7 levels were quantified using enzyme-linked immunosorbent assay. Data was analyzed using Student *t*-test. Pearson correlation test was used to evaluate correlation between S100A7 levels and independent variables such as frequency and duration of areca nut use, *gutka* use, and mouth opening.

Results: The mean value of salivary S100A7 for OSF group was 0.275 ng/ml, whereas mean value of salivary S100A7 for healthy controls was 0.195 ng/ml. Student *t*-test indicated that there was statistically significantly higher levels of S100A7 in OSF group as compared to healthy controls ($p < .001$). When the clinical variables of individual groups were analysed, a significant negative correlation was found between salivary S100A7 and duration of areca nut ($p = .009$) and *gutka* chewing ($p = .03$), whereas a significant positive correlation was found for mouth opening ($p = .04$).

Abbreviations: HRP, horseradish peroxidase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OSCC, oral squamous cell carcinoma; OSF, oral submucous fibrosis; ROS, reactive oxygen species; RAGEs, receptors of advanced glycated end products; Psoriasin, S100A7; TMB, tetramethylbenzidine; UWS, unstimulated whole saliva; SPSS, Statistical Package for the Social Sciences.

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Conclusion: OSF presented higher levels of salivary S100A7 levels as compared with healthy individuals and may be used as surrogate measure to identify subjects at risk for OSF.

1. Introduction

Oral submucous fibrosis (OSF) is a high risk precancerous condition characterized by excessive fibrotic changes in the lamina propria and the associated connective tissue of the oral mucosa (Mehrotra et al., 2013). Approximately 2.5 million people worldwide suffer from OSF, most being diagnosed in Southern India (Mehrotra et al., 2013). The rate of malignant transformation of OSF is about 7.6%, calculated over a period of 17 years, with a prevalence of about 0.2% to 0.5% (Bansal et al., 2013; G. Arakeri et al., 2014). Areca nut (in the form of different preparations) has been established as the major etiological agent causing OSF through epidemiological and in-vitro experimental studies (Hazarey et al., 2007). Pakistan is one of the foremost countries in South Asia where high amounts of areca nut and smokeless chewable tobacco are consumed due to easy availability, thus contributing to high incidence of OSF in this region (Maher et al., 1994; Shah et al., 2009). One of the studies conducted in a rural area of Pakistan reported 99% of OSF patients with history of areca nut use (Memon et al., 2015). Studies carried out on areca nut using school children in Pakistan reported a high frequency of OSF ranging from 50% to 79.6% (Ali et al., 2011; Maqsood et al., 2013).

Family of S100 proteins are a small, acidic, multigene, calcium-binding family containing approximately 25 members, each coded by a separate gene and found exclusively in vertebrates (Donato et al., 2013; Wang et al., 2017). These proteins play an significant role in regulation of cytoskeletal components, protein phosphorylation, enzyme activity, calcium homeostasis and transcription (Donato et al., 2013). S1007, a member of the S100 family was first isolated from squamous epithelial cells of skin affected by psoriasis, hence it is also known as "Psoriasin" (Madsen et al., 1991). High expression of S100A7 is seen in hyperplasia associated with inflammatory lesions and dysplasia associated with neoplastic progression (Zhou et al., 2008; Kaur et al., 2014). Similarly, elevated levels have also been found in oral premalignant lesions with risk of progression to oral squamous cell carcinoma (OSCC) (Zhou et al., 2008; Kaur et al., 2014). This could be attributed to the primary cells including epithelial cells and collagen producing fibroblasts where increment of salivary S100A7 are expressed in oral lesions such as OSF. Previous studies have also reported S100A7 levels in OSCC patients using saliva to be a non-invasive and convenient medium for detection (Jou et al., 2014; Dey et al., 2015). In this context, S100A7 may also be a potential biomarker in OSF patients, a premalignant condition prevalent in areca nut users among Pakistani population (Memon et al., 2015). Moreover, to the authors' knowledge, there has been no study that has evaluated the levels of S100A7 in OSF. So its exclusive role in this condition remains unclear. The aim of this case control study was to investigate the expression of salivary S100A7 levels between patients with OSF and healthy controls.

2. Materials and methods

2.1. Ethical guidelines

The present study was approved by the Ethics Review Committee of Ziauddin University, Karachi, Pakistan (Reference code: 00190716MAOP). The entire procedure was explained to the study participants who were requested to read and sign the consent form before commencement. All participants were informed that the participation was voluntary and they had the right to terminate their participation without any consequences.

2.2. Sample calculation

Sample size was calculated using prevalence of Oral Submucous Fibrosis through the website (www.openepi.com). The absolute precision was set on 2% and the confidence level was set at 95%. The prevalence for OSF was taken as 0.5%. Total of 60 participants were inducted into the study by using (non-probability) quota sampling with 30 cases of OSF and 30 subjects as controls.

2.3. Study participants and eligibility criteria

The study comprised of 60 non-smoking participants split into two groups: 30 cases clinically diagnosed with OSF and 30 clinically healthy participants. Both cases (clinically diagnosed OSF) and healthy participants (with no clinical signs of OSF) of both gender, with history of areca nut and smokeless chewable tobacco consumption were included in this study. OSF patients previously treated with steroids or suffering from any chronic oral and systemic conditions were excluded.

2.4. Research questionnaire

A detailed and structured questionnaire was provided to all the participants by one trained examiner. Items within the questionnaire for study groups had details regarding name, age, gender, occupation, ethnicity, habits (i.e., areca nut, betel quid, *gutka* use), duration and frequency of habit use, clinical examination.

2.5. Clinical assessment of OSF patients

All cases of OSF were diagnosed following intraoral evaluations of: (a) Inter-incisal mouth opening of the patient; (b) Presence of fibrous bands in the buccal mucosae; (c) Appearance of the buccal and palatal mucosae; (d) Alteration in tongue movement or appearance and; (e) Degree of fibrosis of soft palate.

2.6. Saliva sampling

Unstimulated whole saliva (UWS) samples were collected with the technique described elsewhere (Akram et al., 2017). After interview and clinical examination, UWS from the 60 participants were collected using passive drool technique. Participants were seated comfortably with head inclined forward. Patients were requested to pool saliva inside the oral cavity without any stimulation or muscular movement for the duration of 5 min (min). Patients were then instructed to expectorate the saliva into a funnel connected to a falcon tube under ice. Samples were transferred to the Research Laboratory, Ziauddin University, where they were placed in a centrifuge machine at 1792*g* for 15 min at 4 °C. Supernatant was collected and all the samples were stored at −80 °C until further analysis.

2.7. ELISA for estimation of S100A7 in saliva

Salivary S100A7 was quantified using sandwich enzyme-linked immune-sorbent assay technique (ELISA) (Human Protein S100A7 ELISA Kit - Abbexa Ltd, Cambridge Science Park, Cambridge, UK). ELISA was performed once for all the participants with their samples triplicated. Test samples and antibody (conjugated biotin) were combined in the wells. After washing with wash buffer, horseradish peroxidase (HRP) Streptavidin was added to the wells. Unbound conjugates were then removed the help of wash buffer again. For the purpose of visualization of HRP enzymatic reaction 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) substrate was used. Catalysed product initially gave a blue colour that soon changed into yellow after adding stop solution provided in the ELISA kit. The density of yellow product corresponded to the amount of S100A7 in the sample. Absorbance of optical density was measured by Stat Fax 2100 microplate reader at 450 nm and concentration of S100A7 calculated.

2.8. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) (version 23 for Windows, IBM, Chicago, IL). Age, duration and frequency of habit use, mouth opening and concentration of salivary S100A7 were expressed as means and standard deviations. Normality of distribution of the variables was tested with Kolmogorov-Smirnov and Shapiro-Wilk test. Percentage and frequencies were computed using the descriptive analysis. Student *t*-test was applied to compare the levels of S100A7 among cases and controls. The Pearson correlation test was used to evaluate correlation between salivary S100A7 levels and independent variables such as frequency and duration of areca nut use, *gutka* use, and mouth opening.

3. Results

3.1. General characteristics of the study participants

A total number of 60 participants were included in the study (30 OSF cases and 30 healthy controls). The included percentage of males were 56.3% and 47.7% in test and control groups, respectively. The mean age for test and healthy controls was 28.2 years and 26.9 years, respectively. Out of 30 OSF patients, 16 patients used areca nut (≈10.4 packets per day) and 14 patients used *gutka* (≈10.5 packets per day). Out of 30 healthy controls, 20 patients used areca nut (≈10.4 packets per day) and 10 patients used *gutka* (≈10.5 packets per day). Mean duration of areca nut and *gutka* use among OSF and healthy controls was 8.9 and 9.7 years and 5.2 and 6.9 years, respectively. The mean mouth opening for patients with OSF and healthy controls was 1.72 ± 0.68 cm and 3.9 ± 0.45 cm, respectively.

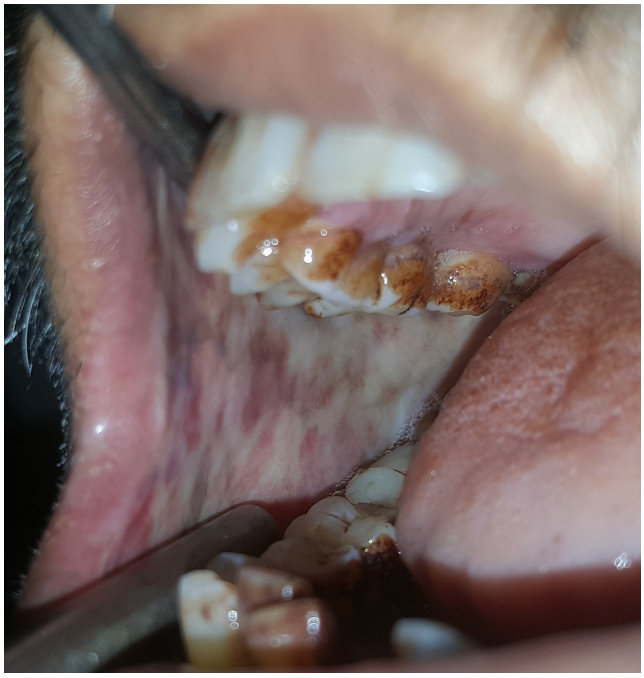

Fig. 1 Clinical changes affecting the buccal mucosa.

Twenty-four patients had bilateral fibrosis of buccal mucosa, while only 6 patients had unilateral fibrotic bands on the buccal mucosa (Fig. 1). Twenty five patients had restricted tongue movement. Twenty three patients had fibrosis of the soft palate (Fig. 2). Sixteen patients reported burning

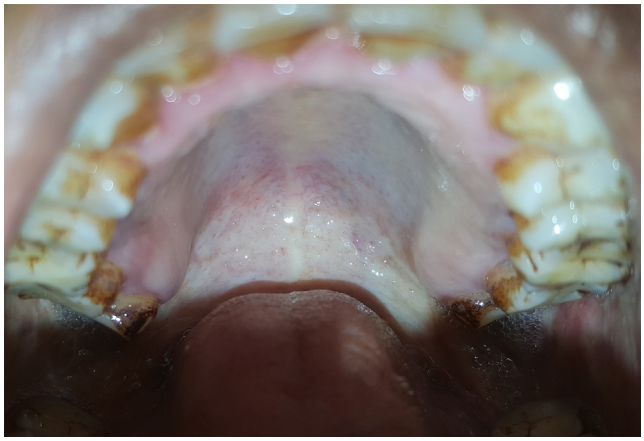

Fig. 2 Clinical changes affecting the palate, pale marble appearance.

Table 1 Mean salivary S100A7 levels between OSF and healthy control.

Variable	Groups (n)	Mean ± SD (ng/ml)	Std error of mean	95% CI	
				Lower bound	Upper bound
Salivary S100A7	OSF Cases (30)	.28 ± .90*	.016	0.205	0.562
	Healthy Controls (30)	.19 ± .03	.005	0.075	0.316

* *P*-value denotes significant difference with healthy control at < .001 by Independent *t* test.

sensation of the mouth while 14 patients had not experienced any burning sensation.

3.2. Mean concentration of salivary S100A7 among OSF and controls

Mean salivary S100A7 levels between OSF and healthy control is presented in Table 1. The mean salivary flow rate among OSF and healthy controls was 0.41 ± 0.07 and 0.54 ± 0.3 ml/min. The mean value of salivary S100A7 for OSF group was 0.275 ± 0.089 ng/ml, whereas, mean value of salivary S100A7 for healthy controls was 0.195 ± 0.026 ng/ml. Student *t*-test indicated that there were statistically significant higher levels of S100A7 in OSF group as compared to healthy controls ($p < .001$) (Fig. 3).

3.3. Pearson correlation analysis among S100A7 levels and clinical variables

Pearson correlation coefficient was calculated to analyse for any correlations among salivary S100A7 levels and the explanatory variables assessed in OSF cases. When the individual groups were analysed, a significant negative correlation was found between salivary S100A7 and duration of areca nut ($r = -.45$, $p = .009$) and *gutka* chewing ($r = -.20$, $p = .03$), respectively, whereas a significant positive correlation was found for mouth opening ($r = .03$, $p = .04$) (Table 2).

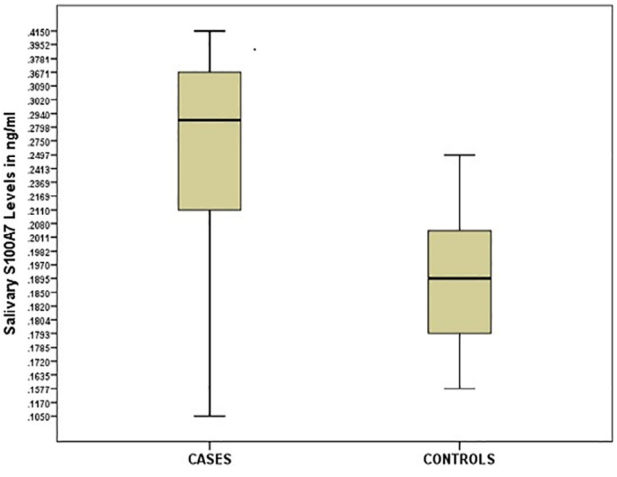

Fig. 3 Box and whisker plot representing mean concentrations of salivary S100A7 among OSF and healthy controls.

Table 2 Pearson correlation analysis among salivary S100A7, habits and mouth opening.

	Correlation coefficient	*P* value
Frequency of areca nut use		
OSF cases	−.60	.90
Healthy controls	.36	.22
Duration of areca nut use		
OSF cases	−.45	**.009**
Healthy controls	.01	.92
Frequency of gutka use		
OSF cases	−.30	.30
Healthy controls	−.25	.39
Duration of gutka use		
OSF cases	−.20	**.03**
Healthy controls	.32	.25
Mouth opening		
OSF cases	.03	**.04**
Healthy controls	.05	.85

Bold indicates statistically significant p value at <0.05.

4. Discussion

In the present study, we hypothesized that salivary S100A7 levels are higher in patients with OSF as compared to the healthy control group and that there was a correlation between salivary S100A7 levels and clinical parameters assessed. The findings of the present study showed statistically significantly higher expression of S100A7 in OSF cases as compared to healthy controls. Moreover, there was a significant positive association between salivary S100A7 levels and duration of *gutka* use and mouth opening.

Oral submucous fibrosis is characterized by epithelial atrophy, juxta-epithelial inflammation, avascularization of connective tissues and altered collagen activity. These effects result in increased inflammatory response in OSF (Pitiyage et al., 2011). In addition, overexpression of S100A7 is reported in numerous cancers including OSCC (Zhou et al., 2008; Kesting et al., 2009; Raffat et al., 2018). Research indicates that S100A7 directly binds with receptors of advanced glycated end products (RAGEs) which are involved in inflammatory processes. RAGEs in turn stimulates and activates NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) protein complex that results in proinflammatory cytokine production (Wolf et al., 2011). Furthermore, S100A7 increases the intracellular levels of reactive oxygen species (ROS) in keratinocytes, suggesting a feedback loop where S100A7 is both stimulated by ROS and intensifies hypoxia (Vegfors et al., 2016). The results of the present study showed that the salivary

levels of S100A7 were increased in patients with OSF as compared to healthy controls. Therefore, our findings raised the possibility that S100A7 may be one of the main biomarkers for inflammatory changes in OSF.

Oral submucous fibrosis is caused by chewing betel quid and *gutka* that contains areca nut and tobacco (Niaz et al., 2017). There are numerous biologically-active alkaloids and flavanoids present in areca nut that stimulate fibroblast cells to increase collagen synthesis and concomitantly reduce collagen degradation due to increased stability of the collagen structure and reduced collagenase activity (Gururaj Arakeri et al., 2017). Research data indicates that S100A7 has antifibrotic activity and reduced fibroblast proliferation in high collagen associated diseases (Gauglitz et al., 2015). Therefore, it may be postulated that the negative correlation between salivary S100A7 and duration of areca nut and *gutka* use among OSF cases may elaborate the negative association between salivary S100A7 levels and high collagen activity by fibroblasts in the oral mucosa of OSF cases. Future studies are warranted to test this hypothesis.

Biomedical researchers routinely investigate different biomarkers in serum in order to assess the presence of malignancy (Kosaka et al., 2014; Jia et al., 2017). Research suggests that saliva can also be used to measure levels of certain biomarkers which helps in the detection of various malignancies including OSCC. Several studies have investigated the salivary levels of S100A7, A8, A12 and S100P respectively in OSCC patients (Hu et al., 2008; Jou et al., 2014; Dey et al., 2015). They suggested that S100 proteins are potential salivary biomarkers for the diagnosis of OSCC in humans and assessment of these proteins in saliva may be a promising approach for the detection of OSCC related biomarkers. This makes saliva the fluid of choice for routine assessment of different proteins in clinical studies. Our study aimed to assess the levels of S100A7 in the saliva samples of OSF and healthy cases. Detection of salivary levels of S100A7 may provide a generalized implication of oral premalignant conditions including OSF.

Like any other study, this study has certain limitations which should be taken into account. The cross-sectional nature of the data does not allow us to comprehend the molecular pathogenesis of OSF and its association with salivary S100A7 levels. Assessment of salivary S100A7 levels were performed once with the clinical examinations. For this reason, longitudinal study may help us to analyse the levels of S100A7 and correlate with the soft tissue changes in OSF. Furthermore, it is well-documented that OSF is stratified on the basis of several clinical parameters and histopathological presentations (Bose and Balan, 2007). Therefore, it is difficult to assess the severity of the disease and its malignant transformation that corresponds with the levels of S100A7 in saliva. Future studies are warranted to evaluate the levels of salivary S100A7 among different clinical stages of OSF. The authors of the present study relied for the diagnosis of OSF on clinical examination only. For this purpose, invasive techniques such as "punch biopsy" assist pathologist for more accurate diagnosis and staging of OSF. However, this notably affects compliance. Our study indicates higher expression of S100A7 in saliva among OSF patients. This indicates that the measurement of S100A7 levels in saliva is just as sensitive and reliable as in serum to detect a difference between healthy and premalignant states (Kaufman and Lamster, 2002). Findings from the present study indicate that measurement of S100A7 levels may be used as a surrogate/diagnostic salivary biomarker to identify patients at risk. It must be noted that the present study did not calculate the sensitivity/specificity of S100A7 in saliva. Therefore, the threshold for diagnostic levels of S100A7 protein cannot be translated in OSF. The present study is only a preliminary finding and further case control/cohort studies should be undertaken in order to define the threshold value of S100A7 in OSF.

5. Conclusion

In conclusion, results of the present study suggested that patients with OSF had higher levels of salivary S100A7 compared with healthy individuals and may be used as a surrogate measure to identify subjects at risk for OSF. However, further studies based on the disease severity can be carried out to further extrapolate results and provide strong findings.

Ethical statement

The authors would like to thank the Board of Advanced Studies and Research at Ziauddin University for funding the project. The authors also extend their sincere thanks to Dr. Kevin Joseph Jerome Borges and Mr. Moazzam Ali Shahid for revising the document for English language correction and assisting in the laboratory work at Ziauddin Medical University, respectively.

Conflict of interest statement

The authors declare that they have no conflict of interest and all authors have read and approved the final draft.

Acknowledgement

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