Original Article

Usefulness of salivary sialic acid as a tumor marker in tobacco chewers with oral cancer

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ABSTRACT

Aim: This study aims to assess the usefulness of salivary sialic acid (SA) as a tumor marker in the detection of oral squamous cell carcinoma (OSCC) among tobacco chewers.

Materials and Methods: After the approval of study protocol by the Institutional Ethics Committee and informed voluntary consent, salivary samples were collected from 96 participants in each group of tobacco chewers with OSCC, tobacco chewers without precancerous or cancerous lesion, and healthy controls. Salivary protein-bound SA (PBSA) and salivary-free SA (FSA) were measured by Yao *et al.*'s method of acid ninhydrin reaction, and the data were subjected to appropriate statistical analysis.

Results: The salivary PBSA and FSA levels in the Groups 1, 2, and 3 participants were 31.17 ± 7.6 mg/dL and 63.45 ± 9.8 mg/dL, 25.45 ± 16.61 mg/dL and 33.18 ± 11.38 mg/dL, and 22.73 ± 3.01 mg/dL and 21.62 ± 8.86 mg/dL, respectively. Salivary FSA levels were significantly increased among the tobacco chewers with OSCC patients (Group 1) and tobacco chewers with no premalignant lesions of the oral cavity (Group 2) compared to the healthy controls (Group 3) with $P < 0.05$ being statistically significant. Salivary FSA levels were significantly increased in Group 1 as compared with Group 2. The salivary PBSA was high among Group 1 as compared to the control Group 3; there was however no significant difference in the levels of salivary PBSA between Group 1 and Group 2. There was no significant difference in the PBSA levels between OSCC patients of Group 1 and the tobacco chewers without precancerous or cancerous lesion in the oral cavity of Group 2.

Conclusion: Salivary PBSA and FSA are significantly raised in both tobacco chewers with OSCC and in tobacco chewers with no precancerous or cancerous lesions in the oral cavity. SA should therefore be used cautiously while considering it as a marker for the early detection of oral cancer. Tobacco can be a crucial confounding factor when SA is used as a biomarker in OSCC since their levels are elevated to some extent even in tobacco chewers without any clinically obvious precancerous or cancerous lesions in the oral cavity.

KEY WORDS: Oral cancer, saliva, sialic acid, tobacco chewers

INTRODUCTION

Head and neck cancers are the 6th most common cancers globally.[1] In India, head and neck cancers constitute 30%–35% of all cancers, and the majority of them are oral cancers. It is reported that there is an annual increase in the incidence of these cancers worldwide.[2-4]

Mortality due to oral cancer is high because patients generally present in the late stages of cancer when the symptoms of pain, bleeding, or an oral or neck mass appear; the survival rate in these patients with advanced disease has been reported to be as low as 40%–50%. Recently, studies have shown the 5-year survival rate to be 85% if the oral cancer is detected in the early stages.[5] Although the gold standard for the diagnosis of oral cancer is the histopathological examination of a biopsy specimen from the suspicious lesion, the procedure, however, is difficult and time-consuming and requires an expert to conduct and opine. Further, the histopathology report will be reliable only when the specimen is representative, due to field cancerization and condemned mucosa. The procedure is quite unpleasant for the patient

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and unsuitable for a community screening of the high-risk population, for early diagnosis during the asymptomatic phase of the disease.[6]

The various factors that are implicated in the etiology of oral cancer are tobacco abuse, genetic predisposition, alcohol, immunodeficiency, diet, and viral infections such as human papillomavirus and human herpes virus.[3,7] In India, tobacco consumption accounts for the majority of oral cancer cases.

Tobacco consumption is highly prevalent among the adult population, both as smoking and as smokeless forms. The main form of smokeless tobacco in India is tobacco chewing as betel quid, which is the betel leaf, areca nut, slaked lime, and tobacco.[8,9] Tobacco chewing and smoking causes oxidant/antioxidant imbalance, which elevates oxidative stress.[10] Due to which, there is increased lipid peroxidation, oxidative DNA damage, damage to macro- and micro-molecules of cells, and disturbances of antioxidant defense, which can induce a malignant process.[10,11]

With regard to this fact, there is a search for biomarkers that can be measured in serum, plasma, or other body fluids. The concentration of these biomarkers changes in the presence of cancer and facilitates early detection of oral cancer to provide less aggressive treatment option and better prognosis.[11,12]

Many biochemical parameters in blood and saliva have been proposed as biomarkers of oral cancer, such as lactate dehydrogenase, metalloproteinase 9, 8 oxoguanine, and p16 protein.[11]

Studies on saliva of oral squamous cell carcinoma (OSCC) patients have also shown the increased expression of salivary cytokines, especially interleukin 6 (IL6), IL8, and tumour necrosis factor-α to play an important role in cancer progression and angiogenesis and hence can serve as potentiotial biomarkers.[13] The pro-inflamatory cytokine IL6 was also found to be elevated in type 2 diabetes mellitus patients with periodonditis.[14]

Recently, estimation of sialic acid (SA) has attracted great interest as a tumor marker in oral cancer, SA is the generic term given to a family of acetylated derivatives of neuraminic acid which occur mainly at terminal positions of glycoprotein and glycolipids oligosaccharide side chains.[15,16]

Salivary SA is increased in oxidative stress conditions like chronic alcoholism and in diseases like diabetes mellitus.[17] Current studies have indicated that SA can be considered as a potential biomarker for the early detection and histopathological correlation in OSCC as the aberrant glycosylation and sialylation of the cell membrane glycoconjugates is an important event in the malignant transformation of cells.[18] SA is a component of the oligosaccharide side chains of these glycoconjugates and plays an important role in cell to cell interaction and malignant transformation.[12,15,19] Studies done on tobacco chewers without any clinically obvious premalignant or malignant lesions of the oral cavity have also found the levels of SA to be elevated significantly.[15,17,19]

Despite the vast number of research studies done on the usage of SA as a biomarker for various cancer, there is a paucity of studies on salivary SA in India, particularly in comparing the potential use of SA as a tumor marker for oral cancer screening, among the general population consuming tobacco in the form of tobacco chewing. There is a paucity of studies in India that have compared the potential role of SA as tumor marker of oral cancer among tobacco chewers, a risk factor for oral cancer present even among the general population in these regions. Therefore, the present study was planned to assess if tobacco chewing confounds the use of salivary SA as a tumor marker for oral cancer detection among tobacco chewers.

MATERIALS AND METHODS

Source of data

This study was a hospital-based cross-sectional analytical study done from February 2017 to September 2017 approved by the institutional ethics committee. The study group consisted of 96 participants from the outpatient department of otorhinolaryngology and head and neck surgery, in a tertiary care hospital. Individuals aged between 31 and 60 years were included in the study. Group 1 consisting of tobacco chewers with OSCC, clinically obvious and histopathologically confirmed cases ($n = 32$). Group 2 consisting of tobacco chewers without any precancerous or cancerous lesion in the oral cavity ($n = 32$). Group 3 consisting of healthy controls without any tobacco habits as control group ($n = 32$).

A detailed clinical history, local, and systemic examination findings of the participants were recorded in a semistructured pro forma.

The study excluded patients with recurrent or chronic ulcerative lesions of the oral cavity like pemphigus/Behcet's syndrome, patients who have undergone radiotherapy, oncosurgery or neoadjuvant chemotherapy or with immunodeficiency, with mixed habits such as alcohol abusers and tobacco smokers, and on regular medication that can affect the salivary flow.

Method of collection of data

Sample collection

After obtaining informed consent from the participants, an unstimulated whole saliva sample was collected according to the method of Navazesh.[20] The sample was collected between 9 am and 12 noon. The individuals were asked to rinse their mouth thoroughly to remove any food debris and then after 10 min were asked to spit into sterile plastic containers, avoiding forcible spitting. The collected samples were centrifuged at 3000 rpm for 15 min, and supernatants were collected. Protein-bound SA (PBSA) and free SA (FSA) levels were estimated.

Assays done

The assays were done by the standard spectrophotometric method using Perkin Elmer ultraviolet/visible spectrophotometer Lambda 35. PBSA and FSA levels in saliva were estimated by the method of Yao *et al*.[21] Proteins were precipitated with ethanol; SA contents of precipitate and supernatant were assayed, which gave the values of PBSA and FSA, respectively. To the saliva sample, 0.8% NaCl and 4 mL ethanol were added, vortex mixed, and centrifuged for 30 mins at 3000 rpm. The supernatant was removed. The precipitate obtained was dissolved in distilled water, and then, glacial acetic acid and acidic ninhydrin reagent vortex were mixed for 30 mins and analyzed for PBSA. To 1 mL supernatant, 1 mL acetic acid and 1 mL acidic ninhydrin reagent were added and mixed and analyzed for FSA. Both PBSA and FSA tubes were kept in boiling water bath for 10 min, cooled, and the absorbance of PBSA and FSA were read at 470 nm against reagent blank.

N-acetyl neuraminic acid standards ranging in concentration from 20 to 100 μg/mL were also run simultaneously; concentration of PBSA and FSA in saliva was calculated from the standard graph and expressed as mg/dL.

Statistical analysis

The data collected were presented by mean, standard deviation, and confidence interval for the three groups.

Comparison of data between the groups was made by Student's *t*-test to test for significance. ANOVA test, Tukey-B for *post hoc* pairwise comparison was done, Tukey-B for *post hoc* pairwise comparison used as the test for association between the groups. $P < 0.05$ was considered as statistically significant.

RESULTS

In the present study, all the participants were from a lower socioeconomic status and with poor nutritional status. The number of tobacco chewers with oral cancer according to age group was 22 patients in 51–60 years age group, eight patients in 41–50 years of age group, and two patients between 31 and 40 years [Figure 1]. The number of tobacco chewers without oral malignant or premalignant lesions according to age groups was nine individuals in 31–40 years, nine individuals in 41–50 years, and 14 individuals in 51–60 years [Figure 2]. The number of healthy controls without tobacco consumption according to age groups was 18 in 31–40 years age group, 9 in 41–50 years age group, and 5 in 51–60 years age group [Figure 3]. The number of male individuals was 12 and female individuals were 20 in Group 1; in Group 2, there were 28 female and four male individuals. In Group 3, there were 21 females and 11 males [Figure 4]. Majority of the oral cancer patients had advanced disease. As per the American Joint Committee on cancer staging manual, tumor node metastasis staging of OSCC patients in the study, three patients were T1N0, three patients were T2N0, four patients were T2N1, one patient were T2N2b, two patients were T3N0, five patients were T3N1, six patients were T4N1, three patients were T4N2a, and five patients were T4N2b disease [Table 1]. All patients with oral cancer had Grade 1 well-differentiated squamous cell carcinoma.

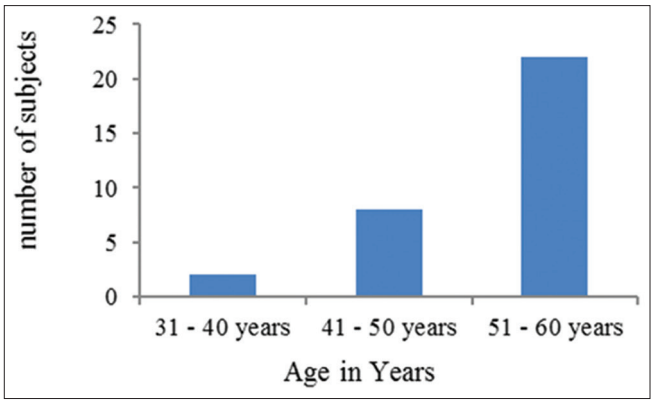

Figure 1: The number of tobacco chewers with oral cancer accordiong to age groups, most of the patients belonged to the age group between 51 and 60 years

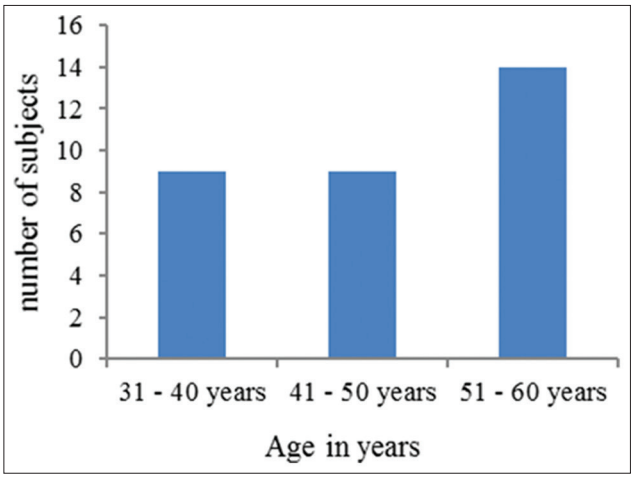

Figure 2: Number of tocacco chewers without oral malignant or premalignant lesions

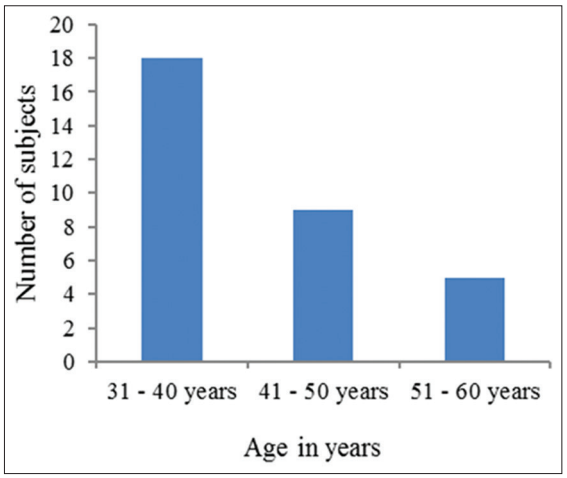

Figure 3: Number of healthy controls without tobacco use

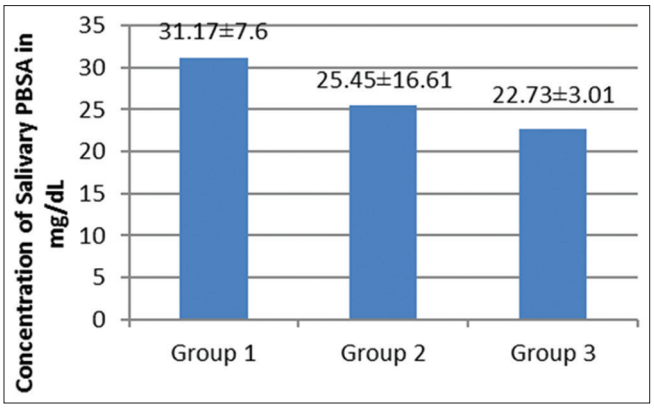

Figure 4: The levels of salivary protein-bound sialic acid in mg/dL among Group 1, Group 2, and Group 3 individuals expressed in mean ± standard deviation

Table 2 shows a significant difference in the mean values of PBSA between Group 1 and 3 (8.44 mg/dL), and mean values of FSA between Group 1 and 2 (30.27 mg/dL), 1 and 3 (41.83 mg/dL), 2 and 3 (11.56 mg/dL) are statistically significant; however, the mean difference of PBSA in Group 1 and 2 (5.72 mg/dL) was not statistically significant.

Tables 3 and 4 show the Tukey B *post hoc* pairwise comparison to test for association of tobacco use on the alteration in the levels of salivary PBSA and salivary FSA between the groups, respectively.

Table 3 shows the variation in the salivary PBSA levels between control group (Group 3) with Group 1 and 2; there is a significant change in beta coefficients indicating the confounding effect of tobacco chewing among the Group 1 and 2 compared with control group with $P < 0.001$, and it was highly statistically significant.

Table 4 shows the variation in the salivary FSA levels between the groups, FSA levels of Group 1 and Group 2 was highly variable compared to Group 3, and there is a significant change in beta coefficients indicating the confounding effect of tobacco chewing on salivary FSA levels among the groups $P < 0.001$, which is highly statistically significant.

DISCUSSION

The present study attempted to assess the effect of tobacco consumption on the potential use of salivary SA as a tumour marker in tobacco chewers with oral cancer.

In this study, the majority of the individuals with oral cancer were female patients with a male to female ratio of 3:5, and most of the patients were in the age group of 51–60 years. This can be explained by the fact that the majority of the women in this region are addicted to tobacco quid while the male patients are more addicted to smoking. The addiction to tobacco in this region starts at a young age, and many of these people tend to develop oral cancer much later. The poor economic condition and illiteracy also contribute to chewable tobacco addiction in this region.[1,2,4,5]

Table 1: The tumor node metastasis staging of oral cancer patients

Tumor staging	Number of oral cancer patients
$T_1 N_0$	3
$T_2 N_0$	3
$T_2 N_1$	4
$T_2 N_{2b}$	1
$T_3 N_0$	2
$T_3 N_1$	5
$T_4 N_1$	6
$T_4 N_{2a}$	3
$T_4 N_{2b}$	5

Table 2: The mean difference and standard error between the salivary protein-bound sialic acid and salivary-free sialic acid levels among Group 1, 2, and 3

Variables	Group	Group	Mean difference	SE	*P*
PBSA (mg/dl)	1	2	5.72	2.67	0.107
		3	8.44*	2.67	0.009
	2	1	−5.72	2.67	0.107
		3	2.72	2.67	0.597
	3	1	−8.44*	2.67	0.009
		2	−2.72	2.67	0.597
FSA (mg/dl)	1	2	30.27*	2.52	0.000
		3	41.83*	2.52	0.000
	2	1	−30.27*	2.52	0.000
		3	11.56*	2.52	0.000
	3	1	−41.83*	2.52	0.000
		2	−11.56*	2.52	0.000

*The mean difference with $P<0.05$ is considered statistically significant.
PBSA=Protein-bound sialic acid, FSA=Free sialic acid, SE=Standard error

Among the tobacco users with oral cancer, the majority of the patients had advanced disease, with 11 patients in Stage 3 and 14 patients in Stage 4 of 32 patients. This may be attributed to the lack of awareness among the high-risk groups regarding the early signs of premalignant and malignant lesions in the oral cavity, they usually ignore the asymptomatic early lesion in the oral cavity, and commonly present with advanced disease when the symptoms of the lesions tend to appear.[4,5]

Saliva is an ultrafiltrate of plasma, and it does reflect the changes taking place in the blood.

In the present study, there was a significant elevation in the mean salivary PBSA and mean salivary FSA in tobacco chewers with oral cancer as compared to the healthy controls. The findings of this study is in accordance with the observations of previous studies of Vishakha Chaudhari *et al*., 2016,[22] in which the authors observed the mean salivary FSA and PBSA levels to be significantly elevated in malignant as compared to premalignant and healthy controls. Sanjay *et al*., 2008[23] in their study reported an increased levels of salivary FSA and PBSA in malignant group compared to the control group subjects.

The study by Shivashankara and Prabhu, 2011,[24] similarly, showed a significant increase in the levels of FSA and PBSA in

Table 3: Turkey B *post hoc* pairwise comparison and linear regression model with dependent variable salivary protein-bound sialic acid among the study groups

Salivary PBSA	Unstandardized coefficients		Standardized coefficients (β)	*t*	*P*	95.0% CI for *B*	
	B	SE				Lower bound	Upper bound
Model 1	34.892	2.879	−0.310*	12.119	0.000	29.175	40.608
	−4.219*	1.333		−3.165	0.002	−6.865	−1.573
Model 2	39.881	8.241	−0.420*	4.839	0.000	23.516	56.246
	−5.716*	2.674		−2.138	0.035	−11.025	−0.406
Tobacco chewing yes/no	−2.994	4.631	−0.127	−0.646	0.520	−12.190	6.203

Considering the dependent variable salivary PBSA (mg/dl), the linear regression model 1 shows the significant variation in PBSA levels between the Groups 1, 2, and 3. In model 2 with dependent variable as salivary PBSA and considering tobacco chewing as a confounder. There is a *significant change in beta coefficients indicating the confounding effect of tobacco chewing to be significant among Groups 1, 2, and 3. CI=Confidence interval, SE=Standard error, PBSA=Protein-bound sialic acid

Table 4: Turkey B *post hoc* pairwise comparison and linear regression model with dependent variable salivary free sialic acid levels among the study groups

Salivary FSA	Unstandardized coefficients		Standardized coefficients (β)	*t*	*P*	95.0% CI or *B*	
	B	SE				Lower bound	Upper bound
Model 1	81.241	2.961	−0.844*	27.433	0.000	75.361	87.121
	−20.912*	1.371		−15.255	0.000	−23.634	−18.191
Model 2	112.425	7.763	−1.222*	14.482	0.000	97.010	127.840
	−30.268*	2.519		−12.018	0.000	−35.269	−25.266
Tobacco chewing yes/no	−18.710	4.362	−0.436*	−4.289	0.000	−27.373	−10.048

Considering the dependent variable as salivary FSA (mg/dl), The linear regression model 1 shows the significant variation in FSA levels between the Groups 1, 2, and 3. In model 2 with dependent variable as salivary FSA and considering tobacco chewing as a confounder. There is a* significant change in beta coefficients indicating the confounding effect of tobacco chewing on salivary FSA to be significant among Groups 1, 2, and 3. CI=Confidence interval, SE=Standard error, FSA=Free sialic acid

patients with malignant oral cancer as compared to the control and premalignant individuals.

Yet another study by Bose *et al*., 2013[19] observed no difference in serum glycoconjugates levels between individuals using tobacco and nontobacco users as controls.

Studies conducted by various authors using saliva samples to estimate SA levels found that the alteration in the salivary SA levels to be similar to the changes observed with SA levels in the plasma and concluded that SA could be a reliable biomarker in oral cancer.[11,12,15] SAs include a family of acetylated derivatives of neuraminic acid at terminal positions of glycoprotein and glycolipids side chains in oligosaccharides; they can affect the cell membrane permeability and develop ligands or even block the permeability. Therefore, the elevated level of SA in cancer and premalignant conditions is expected.[22,25] This was reflected in our study also.

Saliva is in constant contact with oral mucosa. Therefore, the changes in oral mucosa can be reflected in the saliva, particularly when the cells lose their cohesiveness. Saliva provides a "window" into the oral and systemic health of an individual, and like other bodily fluids, saliva can be analyzed and studied to diagnose diseases.[26] In our study, although there was an increase in the mean salivary PBSA in oral cancer patients as compared to tobacco chewers without any cancerous or precancerous lesions in the oral cavity, it was still not statistically significant, which means a mean range to differentiate between high-risk group and cancer patients with salivary PBSA may be difficult to derive; however, this statement by the authors will need to be substantiated with study taking a larger group of individuals. The mean salivary FSA was significantly higher in oral cancer patients as compared to tobacco chewers without any premalignant oral lesions; salivary FSA levels were also found to be significantly higher in tobacco chewers without any precancerous oral lesions compared to healthy controls.

This suggests that the presence of oral cancer lesion tends to elevate the FSA levels over and above the levels elevated among the individuals chewing tobacco but without oral lesions and the controls. Based on our findings, salivary FSA and not PBSA may be used in the early detection of oral cancer lesions, although requiring further confirmation from larger cohort study.

The study findings are similar to the study done by Chaudhari *et al*., 2016[22] who showed that salivary SA showed a positive correlation with oral cancer. Kurtul and Gokpinar, 2012[17] also showed a positive correlation of total SA levels in saliva with both smokers and smokeless tobacco users. Zhang *et al*., 2009[27] also showed that SA levels could be used as a tumor marker even in early oral malignancy. Joshi and Patil, 2010[28] reported no positive correlation between total SA levels in serum and tobacco use. However, the levels in smokeless tobacco users were high than those in smokers. The results of our study were in accordance with the results of Kurtul and Gokpinar, 2012 study and contrary to Joshi and Patil, 2010 study with regard to the use of smokeless tobacco.[17,28]

Salivary proteomics for the identification of Zika virus and its vertical transmission from mothers to their fetus suggests cell surface SA to play an important role as one of the mechanisms in Zika virus internalization and infection.[29,30]

Various studies by Chaudhari *et al*., 2016 and Sanjay *et al*., 2008 have shown that both FSA and PBSA levels increased in oral cancer. However, these studies do not attempt to correlate the levels of FSA with PBSA.[22,23]

The correlation done with salivary PBSA and salivary FSA among the groups shows that there is a positive correlation of salivary PBSA and FSA in oral cancer patients with PBSA and FSA in tobacco chewers. This strongly suggests that the presence of tobacco use might be the cause for the increase in PBSA and FSA in these two groups. However, the significance of this correlation might be established when a larger sample size is considered for the study. The direction of alteration in the salivary PBSA and FSA levels is similar; when the salivary PBSA and FSA levels in the Group 1 and Group 2 individuals were correlated with the Group 3 controls, the correlation findings prompt us to believe that the direction of alteration in SA levels is dictated by the presence of tobacco constantly in contact with buccal mucosa and may not be due to the presence of oral lesions *per se*.

A study done in Nasik in India showed that SA levels in saliva increased progressively as the grade of cancer increased, and even in premalignant conditions, SA levels increased as the severity of dysplasia increased.[22] A study done in Orissa showed that the level of serum SA raised progressively as the clinical stage or grade of oral cancer increased. All patients of oral cancer in our study had well-differentiated squamous carcinoma.[31,32] Hence, the correlation between SA levels and grade of tumor could not be assessed. However, the fact that both FSA and PBSA levels in saliva were much higher in oral cancers compared to tobacco users without oral malignant, or premalignant lesions indicate that SA levels increase with progression of dysplasia.

Further with the coefficients arrived using Tukey B *post hoc* pairwise comparison, shows that tobacco can act as a strong confounding factor while evaluating SA as a tumor marker in oral cancer patients.

CONCLUSION

There is a high prevalence of oral cancer in rural India, particularly among women due to tobacco-chewing habits. The patients usually present with clinically advanced disease.

Salivary SA levels are significantly raised in oral cancer. The FSA levels in saliva can be used as a biomarker in the detection of oral cancer. However, tobacco chewing can be a confounding factor when SA levels are used to detect oral cancers as these levels are elevated to some extent in tobacco chewers without any malignant or premalignant lesions in the oral cavity.

Future studies need to take into account the duration of tobacco addiction or use and correlate the biochemical findings with the levels of PBSA and FSA. Changes in salivary flow rate, which may influence the concentrations of biochemical parameters in saliva, need consideration in future studies. The present study is a preliminary step in the direction of establishing biomarkers in general, salivary analysis of SA in oral cancer in particular.

The authors would like to conclude that tobacco-chewing habit can be a confounding factor in the use of salivary SA levels as a biomarker for OSCC, especially when undertaken as a screening tool among the high-risk population.

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Conflicts of interest

There are no conflicts of interest.

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