ISSN2515-8260 Volume 08,Issue 04,2021

A quantitative analysis to evaluate the role of serum total and lipid bound sialic acid level as diagnostic markers in oral potentially malignant condition and oral squamous cell carcinoma

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Abstract
Oral cancer is the sixth most common cancer throughout the world and is a crucial issue in regions where tobacco habits, in the form of chewing and/or smoking, with or without alcohol intake, are common. Squamous cell carcinoma is the cause for 95% of oral cancers and is associated with avoidable aetiological risk factors. The most successful means to enhance the survival and reduce morbidity, damage, duration of treatment and hospital cost is the detection of oral cancer at an early stage. It is essential to study marker levels in patients with oral precancer who are at a high risk of developing oral cancer.
The present study was carried out to evaluate usefulness of serum Total Sialic Acid (TSA) and serum Lipid-Bound Sialic Acid (LSA) as markers of oral submucous fibrosis and oral cancer. Study consisted of 60 patients and 30 controls. There were 3 study groups, Group I control, Group II OSMF, and Group III oral cancer consisting of 30 patients each. Serum of all the patients in the control group and study groups were collected and stored at -20 ^{o}C until analyzed. Levels of serum TSA and LSA were estimated spectrophotometrically. The results also demonstrated that the assessment of TSA and LSA by simple, in-expensive and reproducible methods can provide significant clinical information about the extent of malignant disease and can differentiate between patients with oral precancer and oral cancer.

Keyword: Oral cancer, TSA, LSA, oral submucous fibrosis

1. Introduction
India is a heavily populated country with over a billion population isunique in its extreme diversity in climate and culture. Although, innumerable cancer-related practices are included with such a varied diversity that need to be analyzed and taken into consideration while planning a prevention strategy for resident and immigrant Indian population [1]. The most common malignant disease in the oral cavity is squamous cell carcinoma [2]. Squamous cell carcinoma accounts for 95% of oral cancers and is associated with avoidable aetiological risk factors [3]. During the last decade of the 20th century there was an 18% to 30% increase in oral cancer incidence in males and females respectively. Despite being more prevalent in the elderly oral cancer is affecting younger patients. Globally, new cases of oral cancer exceed 410000 annually and accounts for between 1% and 5% of all cancers worldwide, but in India

may be as high as 15-40% of all cancers registered [4]. Stage at diagnosis is the most important prognostic indicator for oral and oropharyngeal squamous cell carcinoma. If lesions are detected when they are small, localized and treated expeditiously, survival rates of 70–90% can be achieved [5].

The majority of oral squamous cell carcinomas (OSCC) are preceded by visible changes of the oral mucosa. the terms pre-cancer', precursor lesions', pre-malignant', intra epithelial neoplasia' and potentially malignant' have been used in the international literature [6]. Earlier workers have reported elevated serum levels of total sialic acid (TSA), lipid-bound sialic acid (LSA) and TSA to total protein ratio, in various malignancies [7, 8].

So, the objective of the present study is to estimate serum levels of sialic acid, in the oral pre-cancerous conditions (OPC) along with the oral cancer patients and in healthy control group to evaluate their role in diagnosis and prognosis of oral cancer.

2. Materials and Methods

The present Study was conducted in the Department of Oral Pathology and Microbiology, in collaboration with Regional cancer hospital and Department of Biochemistry. After obtaining an informed consent, all patients were subjected to routine clinical examination and detail case history was recorded. Sample size with exclusion criteria:

Group I: 30 healthy individuals without any systemic disease.

Group II: 30 Subjects with clinically & histopathologically diagnosed oral submucous fibrosis without any other underlying systemic disease.

Group III: 30 Subjects with clinically & histopathologically diagnosed oral Squamous cell carcinoma without any other underlying systemic disease.

Blood sample collection: Fasting Blood samples were collected and allowed to clot at room temperature followed by centrifugation at 3000 rpm for 10 minutes. To avoid possible diurnal variation the samples were drawn between 8.45 a.m. and 9.30 a.m. The serum samples were separated and stored at -20 ^{o}C until assayed.

Total sialic acid (TSA): Determination was carried out using the Thiobarbituric acid method [12].

Lipid bound sialic acid (LSA): Determination was carried out using method described by Katopodis and coworkers [13].

Estimation of Serum Total sialic acid (TSA) [12]: Serum Total sialic acid level were determined as suggested by Amnioff (1961), duly modified by Skoza and Mohos (1976).

Principle: After oxidation with Periodic acid followed by heating with Thiobarbituric acid, sialic acid develops non-fading chromophore with dimethyl sulfoxide. The colour intensity of the chromophore is in direct proportion to sialic acid contents.

Estimation of Serum lipid bound sialic acid level: (LSA) (Katopodis and coworkers) [13].

Principle: Serum gangliosides are treated with chloroform: methanol mixture and extracted in aqueous phase followed by precipitation with phosphotungstic acid. The precipitates, when boiled with resorcinol reagent, give blue colour which is directly proportional to the amount of lipid bound sialic acid present in serum.

3. Observations and Results

The Present study included total 90 subjects with age range from 20-70 years, which were divided into three groups. Group I-Control, Group II-Oral submucous fibrosis and Group III-oral Squamous cell carcinoma of 30 subjects each. All subjects were analyzed for Serum level of total sialic acid (TSA) and lipid bound sialic acid (LSA). The data was collected, tabulated and analyzed by SPSS 15.0 © (statistical package for social science) software. Various statistical tests were applied to determine if a statistically significant difference (level of 0.05) existed between the studied groups.

Age profile of subjects in three groups

The mean age of subjects from group I was 31.27(±8.448) years with an age range from 20 to 52 years. While Group II patient's shows mean age of 30.57(±8.349) years with an age range from 20 to 58 years. In Group III mean age of 56.33(±9.679) years with an age range from 38 to 70 years was noted.

Distribution of study population according to gender

In group I out of 30 cases, 20 were males (66.66%) and 10 were females (33.34%). In Group II out of 30 cases, 27 were males (90%) and 3 were females (10%). Out of 30 cases of Group III 24 were males (80%) and 6 were females (20%). The total sample size was 90 out of which males were 71 (78.9%) and female were 19(21.1%).

Distribution of study population according to habits

Out of all 90 subjects 54(60%) patients were with habit of Smokeless tobacco and Betal nuts. 8(8.9%) patients were with habit of Smokeless tobacco and Betal nuts with Smoking. 4(4.4%) were with habit of Smoking. 7(7.8%) patients were with habit of Smokeless tobacco betal nuts and alcohol and 6(6.7%) patients were with habit of chewing betal nuts. These values show definite relationship of habits in oral precancer and cancer.

Distribution of oral squamous cell carcinoma patients according to site

Table 1 shows site wise distribution of oral Squamous cell carcinoma patient. In our study the most common site involved was Buccal mucosa 53.3% followed by Alveolus 20%, Tongue 10%, Palate and Floor of mouth 6.7% each and lips 3.3%.

Table 1: Distribution of oral squamous cell carcinoma patients according to site

Sr. No.	Site of Cancer	Total No of Patients	Percentage
1	Buccal mucosa	16	53.3%
2	Tongue	3	10%
3	Alveolus	6	20%
4	Floor	2	6.7%
5	Palate	2	6.7%
6	Lip	1	3.3%
Total		30	100%

Distribution if oral squamous cell carcinoma patients according to TNM staging

In table 2 the oral cancer group was divided clinically into four subgroups according to TNM staging by American Joint Committee of cancer. Out of 30 patients 5 patients were with TNM stage I, 8 patients with TNM stage II, 15 patients with TNM stage III while 2 patients with TNM stage IV.

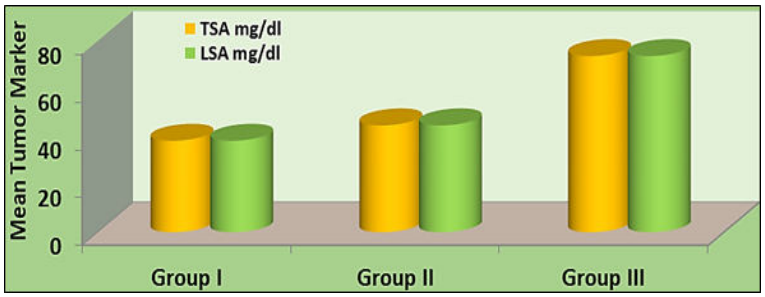
ISSN2515-8260 Volume 08,Issue 04,2021

Table 3: Distribution of oral squamous cell carcinoma patients according to TNM staging

TNM Staging	Frequency	Percentage
Stage I	5	16.3%
Stage II	8	26.6%
Stage III	15	50%
Stage IV	2	6.6%
Total	30	100%

Comparison of mean serum TSA and LSA level in controls, oral submucous fibrosis and oral squamous cell carcinoma patients

Graph 1 shows comparison of total TSA and LSA values between controls (Group I) patients with OSMF (Group II) and OSCC patients (Group III). In Group I the mean serum TSA level was 38.28 mg/dl and mean serum LSA level was 19.49 mg/dl. In Group II the mean serum TSA level was 44.72 mg/dl and mean serum LSA level was 23.11mg/dl. While in Group III patients the mean serum TSA level was 73.76 mg/dl and mean serum LSA level was 33.79mg/dl. The increase in TSA and LSA in OSCC (Group III) patients were statistically significant compared with control as well as with the patients with (Group II) OSF ($p<0.001$). The increase in TSA and LSA were not significant in OSMF when compared with controls.



Graph 1: Comparison of mean of serum TSA and LSA level in controls, oral submucous fibrosis and oral squamous cell carcinoma patients

Comparison of mean serum TSA and LSA with TNM STAGING among the oral squamous cell carcinoma patients.

Table 2 shows comparison of TSA and LSA with clinical (TNM) stages of OSCC patients. The mean serum TSA level in stage I was 63.12 mg/dl and mean serum LSA level was 30.54mg/dl. In Stage II the mean serum TSA level was 63.83 mg/dl and mean serum LSA level was 28.04mg/dl. In Stage III the mean serum TSA level was 81.31 mg/dl and mean serum LSA level was 36.87mg/dl. While in Stage IV patients the mean serum TSA level was 87.50 mg/dl and mean serum LSA level was 41.91mg/dl. Serum TSA and LSA level progressively increased with the stage of malignancy. The statistical analysis data revealed that the progressive rise in both serum TSA as well as LSA were statistically significant with stage of disease. (P value 0.000 and 0.003) When TSA and LSA were compared with in Stage I & II no statistically significant difference was observed ($p<0.001$). When TSA and LSA were compared with in Stage II & III and Stage II & IV statistically significant difference was observed ($p<0.001$). The serum TSA level in stage III was increased as compared stage I which was statistically significant. But no statistically significant difference was observed in case of LSA when comparison of LSA was made between Stage I & III.

Table 2: Comparison of mean serum TSA and LSA with TNM staging among the oral squamous cell carcinoma patients

Tumor marker	TNM Staging	N	Mean	Std. Deviation	P value (ANOVA)	Stage compared (Post Hoc Test)	P value
TSA	Stage I	5	63.12	11.61	0.000	Stage I & II	1.000

mg/dl	Stage II	8	63.83	8.83		Stage II & III	0.002*
	Stage III	15	81.31	11.01		Stage I & III	0.013*
	Stage IV	2	87.50	1.30		Stage II & IV	0.034*
LSA mg/dl	Stage I	5	30.54	6.48	0.003	Stage I & II	1.000
	Stage II	8	28.04	5.44		Stage II & III	0.011*
	Stage III	15	36.87	5.95		Stage I & III	0.265
	Stage IV	2	41.91	0.58		Stage II & IV	0.033*

*P< 0.05 (ANOVA and Post HOC (Bonferroni Test)

Comparison of mean serum TSA and LSA with his to-pathological grading among the oral squamous cell carcinoma patients.

Table 3 shows comparison of serum TSA and LSA with histopathological grade of OSCC patients. The mean serum TSA level in Grade I, II and III were 67.01, 80.82 and 93.08 mg/dl respectively. The values for serum LSA in histopathological grade I, II and III were 31.01, 36.83 and 41.41 respectively. The above finding suggested that the levels of serum TSA and LSA were progressively increased from Grade I to Grade III. The statistical analysis data revealed that the progressive rise in serum TSA was statistically significant when compared with in Grade I & II and Grade I & III ($p<0.001$). And not significant when compared with in Grade II & III. No statistically significant difference was observed in case of LSA when comparison of LSA was made between Grade I & II and Grade II & III. However, mean serum LSA level is increased in Grade III as compared to Grade I which was statistically significant.

Table 3: Comparison of mean serum TSA and LSA with histopathological grading among the oral squamous cell carcinoma patients

Tumor marker	Histopathological grading	Mean	Std. Deviation	P value (ANOVA)	Grade compared	P value
TSA mg/dl	Grade I	67.01	12.25	0.000	Grade I & II	0.013*
	Grade II	80.82	8.64		Grade II & III	0.300
	Grade III	93.08	1.37		Grade I & III	0.002*
LSA mg/dl	Grade I	31.01	7.00	0.014	Grade I & II	0.095
	Grade II	36.83	5.26		Grade II & III	0.853
	Grade III	41.41	2.50		Grade I & III	0.040*

*P< 0.05 (ANOVA and Post HOC (Bonferroni) Test)

4. Discussion

In an epidemiological study on oral cancer and precancerous lesions in rural Indian population, the malignant transformation rate of OSMF was 7.6% over a 17- year period [14].
If lesions are detected when they are small, localized, and treated expeditiously, survival rates of 70-90% can be achieved [15]. A total of 90 subjects were selected and grouped into three equal groups of 30 subjects each in group I (control), group II (OSMF) and group III (OSCC).

Group I: control

30 healthy individuals selected for this group comprised of 66.66% males and 33.34% females. The mean TSA and LSA levels for this group were 38.94 and 19.49 mg/dl respectively. The values obtained were used as standard for comparison with Group II and Group III. The mean TSA and LSA levels obtained for control group were in accordance with previous studies conducted by Mark c. Plucinsky *et al*. (1986) [16] Baxi B R (1991) [17], G N Raval *et al*. (2003) [18] Rajpura K B *et al*. (2005) [19], Manjula Shantaram *et al*. (2009) [20].In our study, mild variation of sialic acid levels in the control group could be attributed to the different populations studied as there is wide scatter in the normal range of sialic acid for local population groups, as stated by Hangloo *et al*. (1990) [21].

Group II: OSMF

30 subjects consisting of 90% males and 10% females were included in this group. All 30 subjects were having varied clinical grades of OSMF with confirmed histopathological diagnosis. Maximum subjects in group were indulged in more than one deleterious habit. Mean values obtained for TSA and LSA in this group was 44.72 and 23.11 mg/dl respectively.

Statistically significant elevation of serum TSA and LSA was noted when compared with control group. These findings were consistent with previous studies of Baxi *et al*. (1991) [17], Rao *et al*. (1998) [23], Raval *et al*. (2003) [18] and Rajpura *et al*. (2005) [19].

The TSA and LSA levels increased significantly in OSMF group. Our findings were in concomitant with the previous study of Rajpura *et al*. (2005) [19], where they found an increase of LSA levels in OSMF patients. Baxi *et al*. (1991) [17] suggested that elevation in serum LSA can give an early indication of premalignant change.

Group III: Oral squamous cell carcinoma

In the present study we have correlated serum TSA and LSA levels in oral Squamous cell carcinoma patients with that of controls and patients with OSMF.

Untreated 30 patients with oral Squamous cell carcinoma were included in this group. There were 80% males and 20% females in this group. The most common site involved was buccal mucosa followed by Alveolus Tongue, Palate and Floor of mouth and lips. Smokeless tobacco and Betal nuts chewing was the most predominant habit observed in this group. 73.76 And 33.79 mg/dl were the mean serum levels obtained for TSA and LSA respectively for this group.

In the present study, statistically significant elevation of serum TSA and LSA level was noted when compared with control group. These findings were consistent with previous studies of Baxi *et al*. (1991) [17], Shashikant *et al*. (1994) [24], Rao *et al*. (1998) [23], Raval *et al*. (2003) [18] and Rajpura *et al*. (2005) [19] who stated that significant elevation of TSA & LSA in patients with oral cancer could be due to the malignant transformation of the cell.

In the present study, serum TSA & LSA found to be significantly elevated in patients with oral cancer as compared with OSMF group. These findings were in accordance with findings of Baxi *et al*. (1991) [17], Raval *et al*. (2003) [18] and Rajpura *et al*. (2005) [19] who suggested that malignant transformation brings changes in cell surface glycoconjugates which results in increase in the level of circulating biomarkers.

Correlation of TNM clinical stages of oral cancer and serum TSA and LSA levels in our study showed progressively increased levels of these markers from Stage I to IV. Serum TSA level was significantly increased in Stage III compared to II and in Stage IV compared to II. These findings were consistent with previous studies of Baxi *et al*. (1991) [17], Rao *et al*. (1998) [23]and Rajpura *et al*. (2005) [19].

There was no significant difference of TSA & LSA levels between Stage I & II which was in accordance with the studies of Baxi *et al*. (1990)

In our study positive increase in mean values of TSA with stage of the malignancy was more prominent than LSA which was in accordance with Rajpura *et al*. (2005) [19].

The present study also includes correlation of TSA and LSA level with histopathological grading of tumor. Correlation of histopathological grades of oral cancer and serum TSA and LSA levels in this study showed that serum TSA and LSA levels were progressively increased from Grade I to III and was statistically highly significant. These findings were in accordance with the findings of Vallikanthan *et al*. (1992) [25] & Rao *et al*. (1998) [23].

There was no significant difference of TSA & LSA levels between histopathological Grades I & II and Grades II & III. The reason best discussed for this finding could be that the histopathological grades correlate with number of other clinical parameters like size of the lesion, time for which it has persisted, exogenous irritants and immune status as stated by Burkhardt *et al*. (1981) [26]. Pindborg J *et al*. (1978) [27]also stated that it is not necessary that all the particular histopathological changes will be seen in any one case and that there is

considerable subjectivity involved in their recognition and also small amount of biopsy material is not always representative of the whole lesion.

Summary and conclusion

The present study was carried out to evaluate usefulness of serum TSA and serum LSA as markers of oral submucous fibrosis and oral cancer. Study consisted of 60 patients and 30 controls. There were 3 study groups. Serum levels of TSA and LSA were statistically analyzed between the control and study groups. The present study also incorporated the correlation of serum levels of TSA and LSA with the histopathological grades and also with the clinical stages for Group III patients.

There was a significant increase in serum TSA & LSA levels in OSMF and oral cancer as compared to control group. Serum TSA and LSA levels of Group III (OSCC) also elevated significantly when compared with Group II (OSMF).

There was progressive significant rise in serum TSA and LSA levels in oral Squamous cell carcinoma group with the clinical stages from Stage I to IV and with histopathological grades from Grade I to III. However, not much comparative changes were observed in biomarkers between other clinical stages (Stage I to II) and histopathological grades. (Grade II & Grade III). Thus, it can be concluded that serum TSA and LSA can be used as biochemical markers adjuvant to other markers for oral precancerous lesions and early detection of oral cancer.

The results also demonstrated that the assessment of TSA and LSA by simple, in-expensive and reproducible methods can provide significant clinical information about the extent of malignant disease and can differentiate between patients with oral precancer and oral cancer.

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