Original Article

Estimation of serum sialic acid in oral submucous fibrosis and oral squamous cell carcinoma

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Abstract

Background and Objectives: Sialic acid (SA) *N*-acetyl neuraminic acid is a negatively charged 9-carbon monosaccharide, commonly attached to the nonreducing residues of carbohydrate chains of glycoconjugates by glycosidic linkage. SA is widely distributed in glycoproteins (GPs) of cell membrane. The alterations in GPs start at an early stage of tumorigenesis. Hence, the aim of the present study is to evaluate the levels of serum SA in normal individuals, in patients with oral submucous fibrosis (OSMF), oral squamous cell carcinoma (OSCC), and compare the levels with respect to the clinical staging and histological grading.

Materials and Methods: A total of 90 individuals were selected for the purpose of the study. Thirty cases of clinically diagnosed and histopathologically confirmed cases of OSMF and OSCC each were included. A control group of 30 age and gender-matched individuals with no systemic diseases were selected. Serum levels of SA were measured based on the reaction between SA and ninhydrin. The absorbance was read using a spectrophotometer.

Results: Serum SA levels were significantly increased in OSMF, OSCC patients as compared with controls. When multiple comparison was done using *post hoc* Tukey test, there is a statistically significant difference between clinical staging and histopathological grading of OSMF and OSCC ($P < 0.05$).

Conclusion: The serum SA levels in OSMF and OSCC patients were increased as compared with controls suggesting that, it can be used as a reliable biomarker for prognostic evaluation, and also give a clue about the amount of tumor burden in the individual.

Keywords: Oral squamous cell carcinoma, oral submucous fibrosis, sialic acid

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INTRODUCTION

Head and neck cancers (HNCs) have emerged as a leading cause of cancer-related mortality and morbidity worldwide.[1] Oral cancer encompasses an important group of HNC with ≥90% of them being oral squamous cell carcinomas (OSCCs).[2]

Although the oral cavity is frequently examined, 60% of intraoral carcinomas are in advanced stage at the time of detection.[3] Persistent difficulties arising in oral cancer are late diagnosis, poor response of tumor to chemotherapy, lack of reliable biomarkers for early diagnosis and posttherapeutic monitoring.[4] Therefore, early detection of oral cancer is of utmost importance

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for improving the survival rate and prognosis of patients with the disease.[2]

OSCC is generally preceded by oral potentially malignant disorders (OPMDs), such as oral submucous fibrosis (OSMF).[5] The hallmark of the disease is submucosal fibrosis that affects most parts of the oral cavity, pharynx, and upper third of esophagus.[6] The rate of malignant transformation has been reported to be 7%–13%.[7]

Sialic acid (SA) which is also called as *N*-acetyl neuraminic acid is a promising cancer biomarker. It is a negatively charged 9-carbon monosaccharide and present as the terminal components of side chains of glycoproteins (GPs) and glycolipids (GLs), which are the important constituents of cell membranes.[8,9] Cell surface is transformed during carcinogenesis, and the malignant cell surface GPs and GLs have altered carbohydrate compositions that may contribute to aberrant cell–cell recognition, cell adhesion, antigenicity, and the invasiveness demonstrated by malignant cells.[10] The altered glycoconjugates are released into the circulation and body fluids through increased turnover, secretion, and/or shedding from malignant cells and are of considerable interest for their potential diagnostic and prognostic value.[9]

The significant elevations in these GP constituents in patients with OPMDs and OSCC could be indicators of early biochemical changes. Hence, the present study was undertaken to estimate the serum SA levels in OSMF and OSCC.

Objectives

1. To compare the serum SA levels in normal individuals, patients with OSMF and OSCC
2. To evaluate and compare the serum SA levels with respect to clinical staging in OSMF
3. To evaluate and compare the serum SA levels with respect to clinical staging in OSCC
4. To estimate serum SA levels with respect to histopathological grading of OSMF
5. To estimate the serum SA levels with respect to histopathological grading of OSCC.

MATERIALS AND METHODS

Thirty cases each of clinically proven and histopathologically confirmed OSMF and OSCC attending the outpatient department of our college were included in the study group. A group of 30 healthy individuals were taken as controls. The research protocol was reviewed and approved by the Ethical Committee of our institution.

Inclusion criteria

This include patients with OSMF and OSCC.

Exclusion criteria

1. Patients with a history of systemic diseases such as diabetes mellitus, cardiovascular diseases and infectious diseases
2. Patients treated previously for oral cancer and potentially malignant disorders.

These patients were subjected to a detailed history and a thorough clinical examination.

The OSMF cases were staged clinically [Figure 1a] based on the interincisal distance according to Lai *et al*.,[11] and graded histopathologically [Figure 2a-c] according to Utsunomiya *et al*.[12]

The OSCC cases were staged clinically [Figure 1b] based on tumor-node-metastasis staging[13] and histopathologically [Figure 2d-f] into three grades based on the modified Broder's[14] system of classification.

After histological confirmation, the patients were recalled for the collection of blood. A volume of 5 ml of venous blood was collected and serum was immediately separated by centrifugation for 5 min at 3000 rpm. The supernatant was separated and stored at −20°C until analyzed.

Biochemical analysis of the serum collected was analyzed based on the reaction between SA and ninhydrin in the presence of acidic medium (according to Yao *et al*.).[15] This leads to the formation of a colored product which can be measured by using spectrophotometer at 470 nm.

Acid ninhydrin reagent was freshly prepared. About 250 mg ninhydrin was dissolved in 6 ml glacial acetic acid and 4 ml concentrated HCL, by thorough vortexing for 30 min.

Procedure

A volume of 0.1 ml of serum is mixed with 0.9 ml of saline. To this solution, 4 ml of ethanol is added and the precipitate is obtained, followed by centrifugation. To the precipitate,

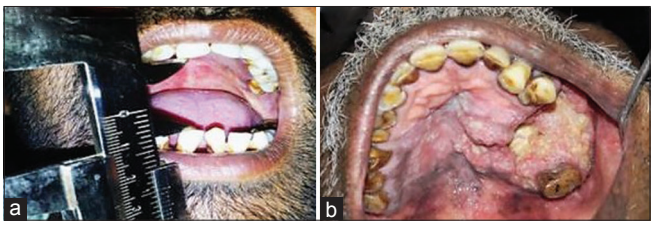

Figure 1: Clinical photograph depicting (a) Oral submucous fibrosis. (b) Oral squamous cell carcinoma

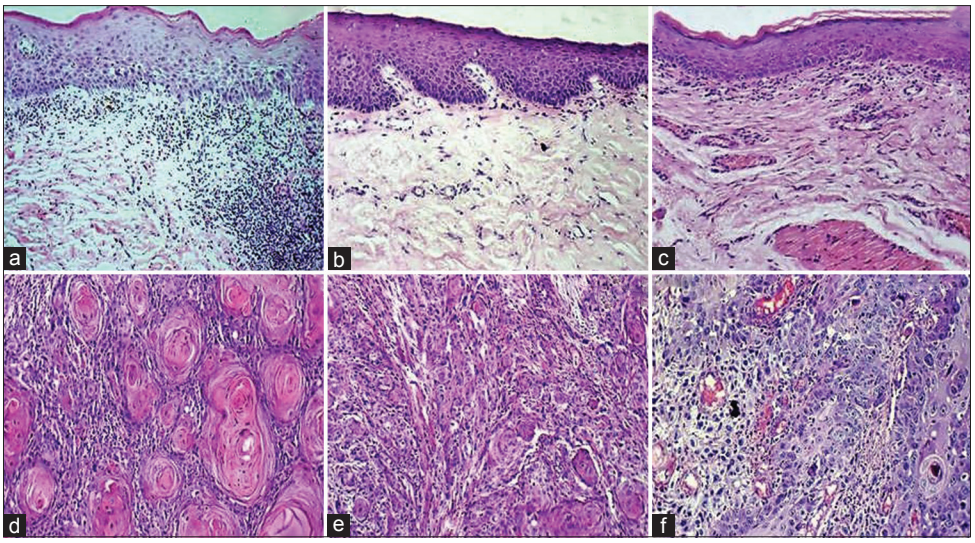

Figure 2: Photomicrograph illustrating histopathological grading of oral submucous fibrosis cases (a) Early (b) Intermediate (c) Advanced and oral squamous cell carcinoma cases (d) Well-differentiated (e) Moderately differentiated (f) Poorly differentiated, respectively in low-power magnification (H and E, ×10)

1.0 ml of distilled water and 1.0 ml glacial acetic acid was added, followed by 1.0 ml of acid ninhydrin reagent. The reaction mixture was vortexed and then heated for 10 min in a boiling water bath. After cooling, the mixture under tap water, absorbance was measured at 470 nm using spectrophotometer.

Statistical analysis

All the variables of the study were tabulated and statistically analyzed for the mean values, standard deviation (SD) and *P* value using the statistical package software system SPSS Version 20 (SPSS Statistics for Windows, IBM Corp., Armonk, NY, USA). The statistical comparison of biochemical parameter was performed by *post hoc* Tukey's test.

RESULTS

Distribution of patients

Based on clinical staging, OSMF patients were categorized into Group A, B, C, and D which included 10 cases (33.33%), 7 cases (23.33%), 8 cases (26.66%) and 5 cases (16.66%), respectively. Similarly, based on the histopathological grading, they were categorized into early, intermediate, advanced grades which included 13 cases (43.33%), 8 cases (26.66%), and 9 cases (30%), respectively.

OSCC patients were clinically subdivided into T1, T2, T3 and T4 stages which included 13 cases (43.33%), 12 cases (40%), and 5 cases (16.66%), respectively. No cases of T4 stage were recorded during the study.

According to histopathological grading, OSCC cases were graded as well-differentiated, moderately differentiated and poorly differentiated which included 10 cases (33.33%), 16 cases (53.33%) and 4 cases (13.33%), respectively.

Serum SA levels were evaluated in both the study group and in the control group. The mean serum SA levels with SD were calculated for control group (3.78 ± 1.06), OSMF (19.99 ± 3.83) and OSCC (35.14 ± 7.87) [Table 1 and Figure 3].

In pair-wise comparison of clinical stages [Table 2 and Figure 4] and histological grading [Table 3 and Figure 5] of OSMF done by Tukey's multiple *post hoc* procedure, the increase in mean serum SA levels between any two groups was found to be statistically significant ($P < 0.05$).

Furthermore, in pair-wise comparison of clinical stages [Table 4 and Figure 6] and histological grading [Table 5 and Figure 7] of OSCC done by Tukey's multiple *post hoc* procedure, the increase in mean serum SA levels between any two groups was found to be statistically significant ($P < 0.05$).

DISCUSSION

OSCC is the sixth-most common cancer worldwide[9] with a 5-year mortality rate of almost 50%, which has not changed significantly in the last 5 decades despite the advances in the multimodality treatment.[16] OSCC is generally preceded by OPMDs such as OSMF. Detection of dysplastic changes in OPMDs is also very essential which can significantly decrease the mortality rate.

During the cancer growth, certain substances are quantitatively changed in the serum known as tumor markers

Table 1: Pair-wise comparison of mean serum sialic acid levels among controls, oral submucous fibrosis and oral squamous cell carcinoma using Tukey's multiple *post hoc* procedures

Groups	Normal group	OSMF group	OSCC group
Mean±SD	3.78±1.06	19.99±3.83	35.14±7.87
Normal group	-		
OSMF group (*P*)	0.0001*	-	
Carcinoma group (*P*)	0.0001*	0.0001*	-

**P*<0.05. OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma, SD: Standard deviation

Table 2: Pair-wise comparison of clinical stages of oral submucous fibrosis group with respect to the serum sialic acid levels by Tukey's multiple *post hoc* procedures

Clinical stages	Group A	Group B	Group C	Group D
Mean±SD	16.08±1.82	18.93±1.53	22.19±1.52	25.75±0.44
Group A	-			
Group B (*P*)	0.004*	-		
Group C (*P*)	0.0001*	0.002*	-	
Group D (*P*)	0.0001*	0.0001*	0.0027*	-

**P*<0.05. SD: Standard deviation

Table 3: Pair-wise comparison of histological grading of oral submucous fibrosis group with respect to the serum sialic acid levels by Tukey's multiple *post hoc* procedures

Histological grading	Early	Intermediate	Advanced
Mean±SD	16.83±2.15	20.68±3.03	23.92±2.05
Early	-		
Intermediate (*P*)	0.0037*	-	
Advanced (*P*)	0.0001*	0.0246*	-

**P*<0.05. SD: Standard deviation

Table 4: Pair-wise comparison of oral squamous cell carcinoma clinical stages with respect to the serum sialic acid levels by Tukey's multiple *post hoc* procedures

Clinical stages	T1	T2	T3
Mean±SD	28.91±6.05	37.42±4.08	43.88±2.69
T1	-		
T2 (*P*)	0.001*		
T3 (*P*)	0.0001*	0.009*	-

**P*<0.05 this indicates statistical significance. SD: Standard deviation

Table 5: Pair-wise comparison of oral squamous cell carcinoma histological grading with respect to the serum sialic acid levels by Tukey's multiple *post hoc* procedures

Histological grading	Well-differentiated OSCC	Moderately differentiated OSCC	Poor differentiated OSCC
Mean±SD	26.29±3.60	38.36±4.56	44.43±4.88
Well differentiated	-		
Moderately differentiated (*P*)	0.0001*		
Poorly differentiated (*P*)	0.0001*	0.0455*	-

**P*<0.05. OSCC: Oral squamous cell carcinoma, SD: Standard deviation

or biochemical serum markers which are receiving more attention in early diagnosis as well as predicting prognosis of the lesion.[17]

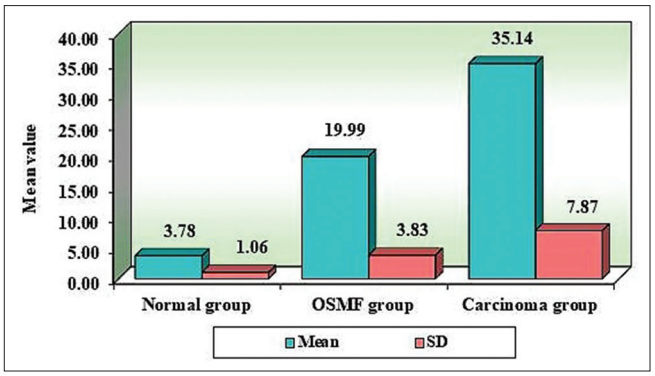

Figure 3: Graph illustrating comparison of normal, oral submucous fibrosis and carcinoma groups with respect to the serum sialic acid levels

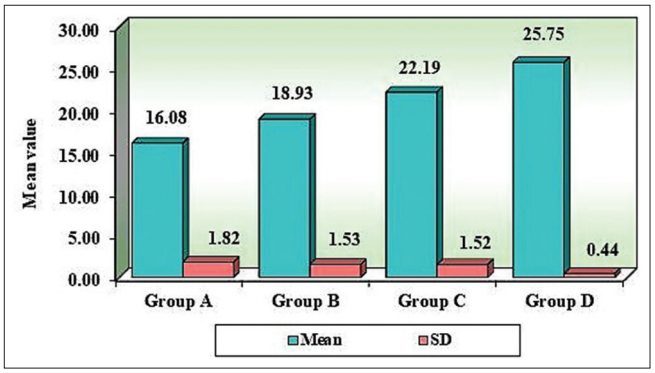

Figure 4: Graph illustrating the comparison of clinical stages of oral submucous fibrosis group with respect to the serum sialic acid levels

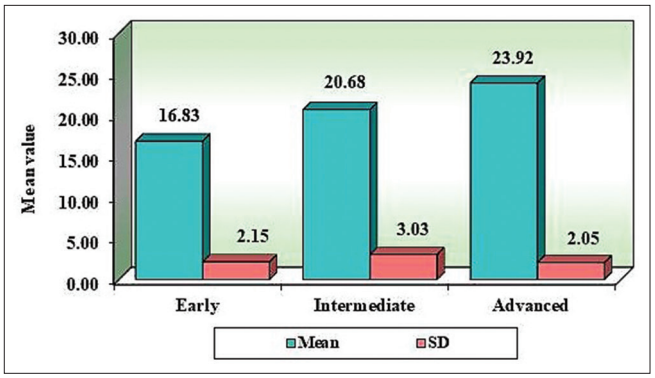

Figure 5: Graph illustrating the comparison of histological grading of oral submucous fibrosis group with respect to the serum sialic acid levels

Neoplastic transformation is associated with altered cell surface components and the identification of such changes may provide the basis for using carbohydrate antigens as tumor markers. Measurements of these entities may be valuable in establishing the diagnosis, staging of disease, detecting metastasis, identifying patients at high risk for recurrence and evaluating therapeutic response.[18]

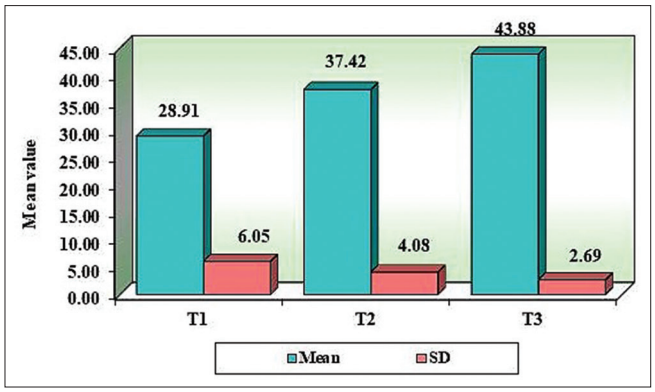

Figure 6: Graph illustrating the comparison of oral squamous cell carcinoma clinical stages with respect to the serum sialic acid levels

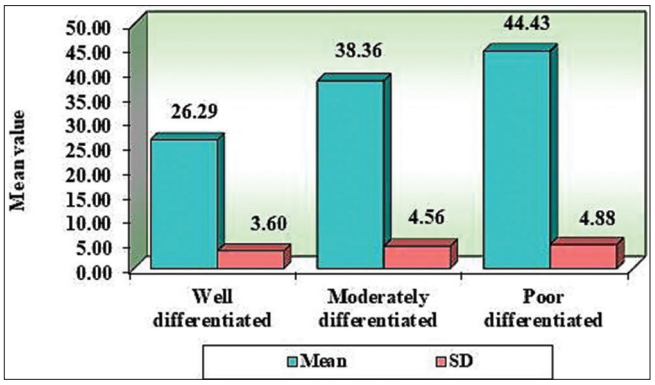

Figure 7: Graph illustrating the comparison of oral squamous cell carcinoma histological grading with respect to the serum sialic acid levels

The alterations in GPs start at an early stage of tumorigenesis. GPs and GLs are the major constituents of cell membrane. The carbohydrate portions of these glycoconjugates project from the outer surface of the membrane and form the cell coat. The cell coat is made up predominantly of SA which is attached to glycoconjugates by glycosidic linkage.[19-21] SAs have been implicated in a number of phenomena, including metastatic spread, cell contact, cell recognition, tumor antigenicity, transport process and viral receptors. Being nonreducing termini, SA has gained outstanding importance in cancer research.[22-24] One of the most common changes in glycoconjugates during malignant transformation is the increase in size of oligosaccharides resulting in branching sites for the incorporation of SA.[24]

Aberrant glycosylation of glycoconjugates is one of the important molecular changes that accompany malignant transformation where the transformed cells increase synthesis of carbohydrates, thereby increase the levels of SA on their surfaces.[25] This altered process responsible for proliferation may be due to either the absence of normal glycosyltransferases or the activation of new tumor-related enzymes.[26]

In the present study, the serum SA levels were evaluated in controls, OSMF and OSCC individuals. When multiple comparison was done by using *post hoc* Tukey's test, there is a significant increase in the levels of serum SA in subjects with OSMF and OSCC compared to controls ($P = 0.0001$). The present study results were in accordance with the study done by Vajaria *et al*.,[4] Dadhich *et al*.,[9] Chandrabose *et al*.,[18] Taqi[25] Baxi *et al*.,[27] Joshi and Patil,[28] Kadam *et al*.,[29] Sawhney and Kumar[30] and Pradeep *et al*.,[31] where progressive increase in mean serum SA levels was noticed in patients with OSCC than OPMDs and controls. The significant elevations in these important GP constituents in patients with OPMDs could be the indicators of early biochemical changes because of the malignant transformation of the cell. Thus, the alterations in SA could discriminate between patients with OPMDs and OSCC patients.

The present study results were also in accordance with the study conducted by Chinnannavar *et al*.,[3] Rajpura *et al*.,[32] Xing *et al*.,[33] Shashikanth and Rao[34] Kimura *et al*.,[35] Wilma Delphine Silvia *et al*.[36] and Dhakar *et al*.,[37] who reported a significant increase in the mean serum SA levels in OSCC compared to the normal individuals. It has been demonstrated that SA increases at the tumor cell surface, so the increase in their serum levels may be related to their increased release through increased turnover, secretion and shedding.

In our study, serum SA levels in OSMF patients were compared with respect to clinical staging, and histopathological grading using *post hoc* Tukey's test. The results showed a statistically significant increase in the levels as the clinical stage of OSMF advances. Similarly, as the histopathological grade of OSMF increases from early to intermediate to advanced, there is a statistically significant rise in the serum SA levels ($P < 0.05$). This study was first of its kind to compare the SA levels in OSMF cases based on the clinical staging and histopathological grading.

In the present study, serum SA levels were also compared in OSCC patients with respect to clinical staging, and histopathological grading using *post hoc* Tukey's test. The results showed statistical significant increase in levels as the stage advances from I to II to III ($P < 0.05$). Stage IV cases were not recorded during the course of the study. These findings were similar to the study done by Taqi,[25] Baxi *et al*.,[27] Kadam *et al*.,[29] Sawhney and Kumar[30] and Rajpura *et al*.[32] where SA levels progressively increased as the stage advanced from I to IV suggesting SA levels were

directly proportional to the tumor burden. In contrast to the present study, the study conducted by Shashikanth and Rao[34] and Dhakar *et al*.[37] did not show any correlation of SA levels with respect to the clinical staging.

Furthermore, results revealed statistical significant increase in levels from well-differentiated to moderate to poorly differentiated squamous cell carcinoma. This may be due to the tumor differentiation and increased shedding of the malignant cells into the circulation as a result of metastasis. These findings were similar to the study done by Rajpura *et al*.[32] and Dhakar *et al*.,[37] who reported the rise in levels as the grade of OSCC progressed. A study conducted by Taqi,[25] Joshi *et al*.[28] and Shashikanth and Rao[34] did not show any significant changes in histopathological grading-wise analysis of SA which is contrary to the present study.

The present study reveals that serum SA levels may be taken as a reliable biomarker for prognostic evaluation, and also it gives a clue about the amount of tumor burden in the individual.

CONCLUSION

The present study is a simple and a cost-effective method of estimating serum SA levels and therefore can be used as a screening marker in identifying individuals with suspected OPMDs such as OSMF and assessing early malignant change. This marker also aids in increasing the accuracy of clinical diagnosis, and assessing the spread and invasiveness of the cancer of the oral cavity suggesting its use as a prognostic indicator. However, further research should be carried out in a larger sample size to support the findings of the current study.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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