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Clinical significance of total and lipid bound sialic acid levels in oral pre-cancerous conditions and oral cancer

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BACKGROUND: Altered glycosylation of glycoconjugates is among the important molecular changes that accompany malignant transformation. The purpose of our study was to investigate clinical usefulness of circulatory levels of total and lipid bound sialic acid for early diagnosis and management of oral cavity cancer patients.
METHODS: Blood samples were collected from 41 untreated oral cancer patients, 20 patients with oral pre-cancerous conditions (OPC) and 20 healthy subjects. Serum sialic acid (total and lipid bound) levels were measured spectrophotometrically.
RESULTS: Serum levels of total and lipid bound sialic acid were significantly elevated ($P < 0.001$) in untreated oral cancer patients as compared to healthy individuals as well as patients with OPC. Multivariate analysis documented that the progressive rise in total and lipid bound sialic acid was significantly associated ($P = 0.0001$ and 0.039, respectively) with stage of malignant disease.
CONCLUSION: The data revealed significant elevations in sialic acid levels in oral cancer patients and suggested potential utility of these parameters in diagnosis as well as determining clinical stage of the malignant disease.
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Keywords: glycoproteins; oral cavity cancer; oral pre-cancerous conditions; serum; sialic acid; tumours

Introduction

Both, the incidence as well as mortality rate of cancer have shown a sharp acceleration since last two decades. Therefore, more intense efforts are required for fight against this life threatening disease. Oral cavity cancer is currently the most frequent cause of cancer-related deaths among Indian men, which is usually preceded by oral pre-cancerous conditions (OPC) like leucoplakia and/or oral submucous fibrosis (1). The idea of screening and following patients with malignancy by blood-based tests is appealing from several points of view including its ease, economic advantage, non-invasiveness and possibility of repeated sampling.

Over the past several decades, experience gained from the studies of tumours has led to the recognition of the significant role of glycoconjugates in malignant transformation (2–4). Terminal epitopes of carbohydrates have been proposed to play a significant role in cell–cell interactions, in the development of cell adhesion and in malignant transformation (5). One of the most common changes in glycoconjugates during malignant transformation is the increase in size of oligosaccharides resulting in branching sites for incorporation of sialic acid (6). The occupancy of sialic acid at the terminal or near to terminal position underlies its vital role in determining surface characteristics of cells and secreted glycoproteins. Being non-reducing termini, sialic acid has gained outstanding importance in cancer research (7).

Total sialic acid (TSA) consists of glycoprotein and glycolipid bound sialic acid whereas lipid bound sialic acid (LSA) comprises of glycolipid bound sialic acid (8). These glycocongugates are released into circulation through increased turnover, secretion and/or shedding from malignant cells. (9). Significant elevations in serum levels of TSA and LSA in sera of patients with OPC and oral cancer compared with controls are reported (3, 4, 10). Elevated serum sialic acid levels are reported to be correlated with the clinical staging, prognosis and recurrence of malignancies. (9, 4, 11, 12).

However, the precise relevance of the alterations in different forms of sialic acid with extent of disease, its impact on survival and response to therapy in cancer patients have not been systematically studied. Therefore, the current investigation was aimed at determining role of alterations in TSA and LSA in diagnosis and staging of oral cancer (OC). Further, previous studies have been carried out in a small number of subjects with comparisons between controls and cancer patients. To assess the diagnostic specificity of the

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markers, individuals at risk of cancer should be distinguished from those having OPC. Hence, patients with OPC were also included in the study, to serve as pathological controls.

Materials and methods

Subjects

The present study included three populations: (i) controls: the study included 20 age-matched healthy males who had no major illness in recent past, (ii) pathological controls: twenty patients with OPC (age range: 17–67 years, median age: 37.8 years) were also enrolled for the study. Among the 20 patients, with OPC 12 had oral submucous fibrosis and eight had oral leucoplakia and (iii) cancer patients: the study also included 41 histopathologically proven untreated OC patients; all were males. Due consent to participate in the study was obtained from all the subjects. Diagnosis of OPC and oral cancer was based on their clinical, radiological and histopathological reports. Clinical stage of the disease was determined as per American Joint Committee of Cancer (AJCC) norms (13). As detailed in Table 1, stage-I, II, III and IV disease was found in nine, 10, eight and 14 patients, respectively. The age range of oral cancer patients was 14–80 years with median age of 43.8 years. Histopathological reports showed that all the patients had squamous cell carcinoma with grade I ($n = 11$), II ($n = 25$) and III ($n = 14$). Histopathological grades were stamped according to Shafer et al. (14). Majority of the patients were tobacco consumers with more than 5 years.

Sample collection

Blood samples were collected by vein puncture in vacuette between 9.00 and 11.00 hours on every occasion to avoid any possible diurnal variations. Sera were separated and stored at −80°C until analysed.

Table 1 Clinical details of oral cancer patients

Clinical details	*n*
Number of patients	41
TNM stage	41
Stage I	9
Stage II	10
Stage III	8
Stage IV	14
Histology: squamous cell carcinoma	41
Grade I	11
Grade II	25
Grade II	5
Age range (years)	14.0–80.0
Median age (years)	43.8
Tobacco habituates	38
Chewing	11
Smoking	8
Chewing + Smoking	19
< 5 years	3
5–10 years	14
> 10 years	21

Assays

Estimation of total sialic acid

Serum TSA contents of serum were determined using a periodiate–thiobarbituric acid method (15). Briefly, to release bound sialic acid, serum samples (100 µl) were hydrolyzed with equal volume of 1 N H_2SO_4, at −80°C for 1 h. After hydrolysis, proteins were precipitated by 1.0 ml of 10% trichloroacetic acid. The supernatant was incubated with 0.025 N periodic acid at 37°C for 30 min. The reaction was terminated by addition of 2% sodium arsenite. Then 6% thiobarbituric acid was added and the mixture was kept in boiling water bath for 7.5 min; 1.5 ml of dimethyl sulphoxide was added to increase the stability of the chromophore. For each determination, spectrophotometric readings against blank were made at 549 and 532 nm to overcome any interference from 2-deoxy-D-ribose. Inter-assay and intra-assay coefficients of variations for TSA estimations were 4.5 and 5.3%, respectively.

Estimation of lipid bound sialic acid

Serum LSA levels were measured as suggested by Katopodis et al. (16). Briefly, 50 µl of serum was extracted with chloroform–methanol (2:1 v/v) maintained at 4°C. The lipid extract was separated with 0.5 ml of distilled water. The aqueous layer was precipitated with phosphotungastic acid. The precipitates were resusupended in 1 ml of distilled water and LSA in suspension was determined with resorcinol reagent. Inter- and intra-assay coefficients of variations for LSA estimations were 4.8 and 6.5%, respectively.

The TSA and LSA contents were calculated using standard curves (Fig. 1) obtained for various concentration of N-acetyl neuraminic acid (Sigma, St. Louis, MO, USA). To calculate the concentration of TSA and LSA following formula was used:

$$\text{TSA/LSA (mg/dl)} = \frac{\text{optical density of sample}}{\text{optical density of standard}} \times \frac{\text{Concentration of standard}}{\text{Volume of test}} \times 100.$$

Statistical methods

Data were analysed using the SPSS version 10 statistical software (SPSS Inc., Chicago, IL, USA). The values were expressed as mean ± SD. Student's *t*-test was carried out for comparison between the three groups of subjects. Multivariate analysis was performed to find

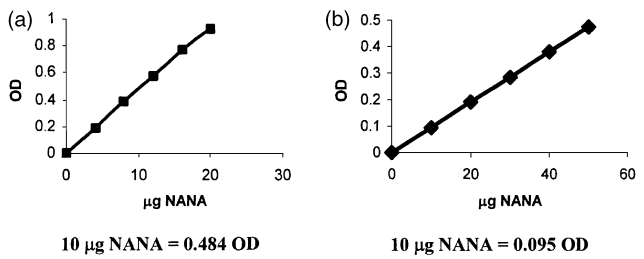


Figure 1 Standard curves for (a) total sialic acid (TSA) and (b) lipid bound sialic acid (LSA).

association of alterations in TSA and LSA levels with clinical stage and histopathological grade of oral cancer.

Results

Comparison of serum TSA and LSA levels between controls, patients with OPC and oral cancer patients

For simultaneous assessment of sensitivity as well as specificity of the alterations in TSA and LSA, the marker levels were compared not only with healthy individuals but also with patient with OPC (Table 2). The mean values of both TSA and LSA showed more than twofold and 1.4-fold rise in oral cancer patients as compared with controls and patients with OPC, respectively. The increase in TSA and LSA in oral cancer patients were statistically significant compared with controls as well as with the patients with OPC ($P < 0.001$). The increase in the levels of TSA and LSA were also significant in patients with OPC compared with controls ($P < 0.001$).

Comparison of serum TSA and LSA levels with histopathological grade of oral cancer

To find out association of glycoprotein changes with histolopathological grade of oral cancer, TSA and LSA levels were compared between oral cancer patients with various histopathological grades. As documented in Table 3, mean ± SD values for serum TSA in histopathological grade I, II, and III were 56.84 ± 17.86, 64.79 ± 20.57 and 73.28 ± 19.41, respectively. The values for serum LSA in histopathological grade I, II and III were 32.94 ± 3.35, 32.54 ± 6.08 and 33.94 ± 2.80, respectively. Thus, TSA levels were progressively increased from grade I to III. However, alterations in mean serum LSA values did not show any correlation with grade of oral cancer. Multivariate analysis revealed that the alteration neither in TSA nor in LSA showed any significant association with histopathological grade of the disease ($P = 0.265$ and 0.155, respectively).

Comparison of serum TSA and LSA levels with clinical Tumor, Node, Metastasis (TNM) stage of oral cancer

Recently, serum category for serum concentrations of various tumour markers is incorporated in clinical staging of malignancies because of their independent clinical significance (13). Therefore, in the present study, the association of the biochemical changes with the disease activity was compared between various stages of oral cancer, which is illustrated in Fig. 2. Serum TSA and LSA levels rose progressively with the stage of malignancy. As clear from the figure as well as the table positive increase in mean values of TSA with stage of the malignancy was more prominent than LSA. Multivariate analysis was carried out to evaluate statistical significance of the alterations. The multivariate analysis data revealed that the progressive rise in both serum TSA as well as LSA were statistically significant with stage of the of the disease ($P = 0.0001$ and 0.039, respectively).

Discussion

Numerous workers have compared alterations in different biomarkers between controls and oral cancer patients. However, earlier reports have not compared

Table 2 Comparison of total sialic acid (TSA) and lipid bound sialic acid (LSA) values between controls, patients with oral pre-cancerous conditions and oral cancer patients

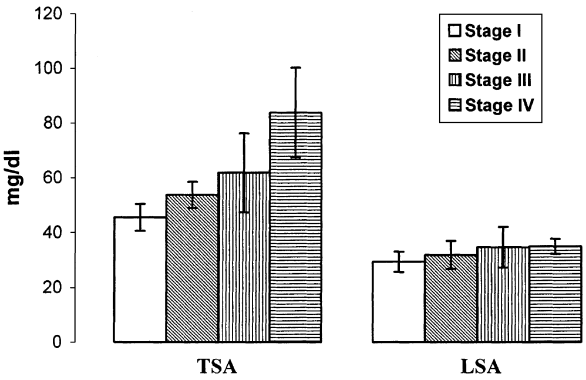
	TSA (mg/dl)	*LSA (mg/dl)*
Controls ($n = 20$)	30.25 ± 2.49	16.32 ± 2.97
Leucoplakia ($n = 08$)	44.40 ± 7.53	22.31 ± 4.73
OSMF ($n = 12$)	39.69 ± 4.72	28.72 ± 3.47
Oral cancer ($n = 41$)	63.70 ± 19.40	32.81 ± 5.10
Statistical significance:		
Control vs. leucoplakia/OSMF	$P < 0.001$	$P < 0.001$
Control vs. OC	$P < 0.001$	$P < 0.001$
Leucoplakia/OSMF vs. OC	$P < 0.001$	$P < 0.001$

Values are expressed as mean ± SD.
OSMF, Oral submucous fibrosis; OC, Oral cancer.

Table 3 Comparison of serum total sialic acid (TSA) and lipid bound sialic acid (LSA) with histopathological grade of cancer patients

Histopathological grades	*TSA (mg/dl)*	*LSA (mg/dl)*
Grade I	56.84 ± 17.86	32.94 ± 3.35
Grade II	64.79 ± 20.57	32.54 ± 6.08
Grade III	73.38 ± 19.41	33.94 ± 2.80
Multivariate analysis		
F-value	1.374	0.265
Significance	$P = 0.155$ (not significant)	$P = 0.857$ (not significant)

Values are expressed as mean ± SD.



	TSA mg/dl (Mean ± SD)	LSA mg/dl (Mean ± SD)
Stage I	45.5 ± 4.92	29.3 ± 3.72
Stage II	53.7 ± 4.8	31.8 ± 5.13
Stage III	61.8 ± 14.45	34.6 ± 7.42
Stage IV	83.8 ± 16.43	35.0 ± 2.76
Statistical significance: Multivariate analysis with stage of oral cancer		
***F*-value**	22.26	3.08
Significance	$P = 0.0001$	$P = 0.039$

Figure 2 Comparison of serum total sialic acid (TSA) and lipid bound sialic acid (LSA) with clinical (TNM) stages of cancer patients.

the alterations between patients with OPC and oral cancer patients. It is therefore essential to study marker levels in patients with OPC who are at a higher risk of developing oral cancer. Hence, the current study included patients with OPC also as pathological controls.

It is reported that altered carbohydrate compositions of malignant cell surface glycoproteins and glycolipids play an important role in a normal biochemical circuit of cells (17–20). These glycoproteins and glycolipids can be released into the circulation through increased turnover, secretion and/or shedding and are of considerable interest for their potential diagnostic and prognostic value (21, 22). Sialic acids are important terminal sugars of the carbohydrate chains of cell membrane glycoproteins and glycolipids (7). Earlier reports on alterations in the TSA, LSA as well as small amount of free sialic acid in cancer patients have stimulated interest in this sugar residue as possible tumour markers. Significantly elevated levels of serum sialic acid forms including TSA, LSA, free sialic acid and TSA/total proteins in cancer patients have been compared with healthy individuals (4, 5, 23). Differences in mean sialic acid levels between benign and malignant tumours have been reported. It is also found that different forms of sialic acid are more suitable for monitoring disease extent and anticancer therapy. In the current study, we observed significantly elevated serum TSA and LSA levels in patients with OPC as compared with the healthy individuals. Further, sialic acid levels were increased in untreated oral cancer patients as compared with healthy individuals and patients with OPC. The significant elevations in these important glycoprotein constituents in patients with OPC could be indicators of early biochemical changes because of the malignant transformation of the cell. Thus, the alterations in TSA and LSA could discriminate between patients with OPC and oral cancer patients. This suggested that malignant transformation brings changes in cell surface glycoconjugates, which is in accordance with earlier reports (10). Elevations in the levels of sialic acid appeared to reflect tumour burden and correlated well with the stage of the primary lesion and presence of distant metastasis (4, 10, 17, 21). We also observed significant progressive elevations in serum TSA and LSA values in oral cancer with stage of the malignant disease. Thus, the results of the current study showed a positive correlation between serum levels of different forms of sialic acid with extent of the malignant disease. This also indicate that higher levels of these parameters may be associated with poor prognosis in patients which remains to be studied.

In conclusion, our results suggested that sialylation by increased sialic acid levels is dominant in oral cancer patients. The present study also indicated that glycoprotein metabolism is significantly altered in patients with OPC and the regulation of sialylation may be partially responsible for the changes associated with malignant transformation. The results also demonstrate that the assessment of TSA and LSA by simple, non-invasive, in-expensive and reproducible methods can provide significant clinical information about the extent of malignant disease and can differentiate between patients with OPC and OC patients.

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