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Tissue and serum α2-3- and α2-6-linkage specific sialylation changes in oral carcinogenesis

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Abstract Increased sialylation of cell surface glycoconjugates is among the key molecular changes associated with malignant transformation and cancer progression. We investigated significance of linkage-specific sialylation changes in oral carcinogenesis. Tissue and serum levels of total sialic acid (TSA), linkage-specific sialyltransferases (ST) and sialoproteins were analyzed from patients with oral precancerous conditions (OPC) and oral cancer as well as the post-treatment follow-up blood samples of oral cancer patients. TSA levels were measured using a spectrophotometric method. The linkage-specific lectins, *Sambusus nigra* (SNA) and *Maackia amurensis* (MAM) detects α2-6- and α2-3-linked sialic acid, respectively, were used to analyze ST activity and sialoproteins. Malignant tissues showed significantly higher levels of TSA, reactivity of SNA and MAM, and α2,3-ST activity compared to the adjacent normal tissues. α2,6-ST was also higher in malignant tissues. Similarly, the marker levels were higher in precancerous tissues than their adjacent normal tissues. Serum levels of TSA, TSA/ total proteins, α2-6-sialoproteins and α2,6-ST were markedly increased in untreated oral cancer patients compared to the controls and OPC as well as responder (CR) patients. Serum levels of the markers were higher or comparable between untreated oral cancer patients and non-responders (NR). Serum levels of α2-3-sialylation were elevated in non-responders compared with the responders. Further, the observed sialylation changes in tissue and serum were found to be associated with various clinicopathological features and disease progression. Thus, the data suggest potential utility of sialylation markers in early detection, prognostication and treatment monitoring of oral cancer.

Keywords Oral cancer · Oral precancerous conditions · Glycoproteins · Sialylation · Treatment monitors

Abbreviations

CR	complete responders
ELISA	enzyme-linked immunosorbent assay
MAM	*Maackia amurensis* agglutinin
Neu5Ac (NANA)	*N*-acetylneuraminic acid
NR	non-responders
OPC	oral precancerous conditions
SNA	lectin *Sambusus nigra* agglutinin
ST	Sialyltransferase
TBA	thiobarbituric acid
TP	total proteins
TSA	total sialic acid

Introduction

Oral cancer is the sixth major cause of cancer-related morbidity and mortality globally [1] with incidence reaching high proportions in India, where there is 4.5 times more prevalence in males (Hospital-based cancer registry, Gujarat Cancer and Research Institute, 2001). Clinical, epidemiological and laboratory studies suggest direct etiological relationship with prolonged tobacco use [2]. In India, high

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risk of oral cancer and oral precancerous conditions (OPC) such as sub-mucous fibrosis and leukoplakia in young population is attributed to different types of tobacco chewing [2]. Oral cancer has emerged as a good model to study the molecular changes of multistep carcinogenesis preceded by precancerous changes. The transformation rates from premalignant to malignant varies from 0.6 to 36% [3].

The neoplastic transformation is often associated with profound alterations in cell-membrane glycosylation. It represents one of the most frequently occurring post-translational modifications. During oncogenic transformation and metastasis, increased expression of complex oligosaccharides and branching sites on tumor cells has been detected which facilitate the incorporation of terminal epitopes [4]. Sialic acids (*N*-acetylneuraminic acid, NANA) frequently occupy the terminal, non-reducing position on membrane glycoproteins. The negative charge due to presence of a carboxyl group enables sialic acid to mediate a wide array of cellular functions such as transport of positively charged molecules, cell–cell and cell–matrix interactions, influence the conformation of membrane glycoproteins and masking antigenic sites of receptors [5]. The presence of sialic acid at the terminal or near the terminal position underlies its importance in determining chemical and biological diversity, and characteristics of cell-surface and secreted glycoproteins [6]. Human and experimental model based studies have documented clinical significance of this sugar moiety [7, 8]. Numerous investigators have reported possible relation of increased sialic acid levels with various malignancies [9–11]. A recent study suggests that selective inhibition of linkage specific sialic acid incorporation significantly suppresses metastatic potentials of tumor cells [12].

Altered expression of sialylated glycoproteins during differentiation and oncogenic transformation has been previously reported [13]. The different sialyl linkages can be determined by linkage-specific lectins. The extent of sialylation of glycoconjugates can be determined by examining sialyltransferase (ST) activity (EC 2.4.99.1–11); a family of about 20 enzymes catalyzing the incorporation of sialic acid from donor substrate CMP-sialic acid to the oligosaccharide side chains of glycoconjugates. STs differ in their substrate specificities, biochemical parameters, and cell and tissue expression [14]. They are classified according to the commonly found sialyl linkages they catalyze. For instance, α2,3-ST transfer sialic acid to galactopyranosil residue, α2,6-ST transfers to subterminal GalNAc or GlcNAc and α2,8-ST adds to another sialic acid forming polysialic acid [14]. Expressions of STs are more often deregulated in cancer and its up-regulation might indicate activation of a specific signaling pathway [15, 16]. Abnormally high levels of ST mRNA expression and total activities were found in tumor-bearing cells and sera of cancer patients [17–19]. ST6Gal.I is one of the most frequently up-regulated glycosyltransferases in human cancers [20]. Linkage specific STs are involved in synthesis of cell surface carbohydrate antigens in epithelial cancers [15].

Given the importance of altered sialylation in cancer, the present study was undertaken to gain further insights into detailed analysis of TSA, α2,6- and α2,3-sialylated proteins, and α2,6-ST, α2,3-ST activities in tissue and serum and their importance in diagnosis, prognostication and treatment monitoring of oral cancer. We enrolled the patients with OPC to detect the sequential precancerous changes during neoplastic transformation. Healthy individuals were also included as controls to obtain baseline levels of the serum markers. The marker levels in cancerous and OPC tissues were compared with their adjacent normal tissues. Serum levels of the markers in patients with oral cancer and OPC were compared with the controls. In an attempt to elucidate clinical significance, the correlations of the marker levels in tissue and serum with various clinicopathological features were also evaluated.

Materials and methods

Patients and controls

The study included three groups of the subjects: (a) Controls: 100 healthy individuals who had no major illness in the recent past. (b) Patients with OPC (pathological controls): 75; among which 50 patients had oral submucous fibrosis and 25 patients had oral leukoplakia. (c) Cancer patients: 130 histopathologically proven untreated oral cavity cancer patients. Due consent was obtained from all the subjects to participate in the study. Ninety-seven percent of the patients with OPC and oral cancer were tobacco users and majority of them were males (Table 1). Clinical TNM staging of malignant disease was determined as per AJCC norms [21]. Distribution of oral cancer patients with regard to various clinicopathological characteristics including stage, tumor differentiation, lymph node metastasis and nuclear grade of the tumors were recorded (Table 2). Cancer patients were followed up at various intervals after initiation of anticancer treatment. The study also included post-treatment follow-up serum samples (N=75) of cancer patients. Patients' response to anticancer treatment was assessed on the basis of their clinical and radiological findings during follow-up. Clinical status of the patients during and/or after anticancer treatment was evaluated as suggested by Therasse *et al.* [22] and were grouped into complete responders (CR, N=52) and non-responders (NR, N=23). The patients with partial response, stable disease and progressive disease were termed as NR.

Table 1 Details of controls and patients

Variable	Controls (*N*=100)	OPC (*N*=75)	Cancer patients (*N*=130)
Age			
Median (years)	33	28.5	42
Range (years)	20–71	14–80	18–77
Gender			
Males (%)	88	92	94
Females (%)	12	08	06
Male:Female	7.6:1	11.7:1	15.7:1
Tobacco habits			
Yes (%)	61	97.4	97.7
No (%)	39	02.6	02.3
Type of habits			
Chewing (%)	62	67.5	40
Smoking (%)	19	13.5	24
Chewing + smoking (%)	15.5	19	33
Snuff (%)	03.5	–	03

Samples collection and processing

Malignant and uninvolved adjacent normal tissues (*N*=75) from oral cancer patients were obtained on ice immediately after surgical intervention. A portion of malignant tissue and adjacent normal mucosa (free margins at least 1–2 cm away from the tumor) were dissected after examination by the pathologist and were also investigated for histopathological analysis. Precancerous and adjacent normal tissues (*N*=10) from the patients with OPC were also collected at the time of biopsy. Tissues specimens were washed with cold phosphate buffer saline (PBS, pH 7.4) and immediately stored at −70°C for biochemical analysis. Blood samples were drawn from all the subjects by venipuncture between 9 and 11 AM, to avoid any possible diurnal variations. Sera were separated, divided into multiple equal fractions and were stored at −70°C until analyzed. Each sample was analyzed in duplicate.

Methods

Estimation of tissue and serum total proteins

Total proteins from tissues and serum were determined using the Lowry and Biuret methods, respectively.

Estimation of tissue and serum total sialic acid (TSA)

Sialic acid levels in tissue and serum were determined using thiobarbituric acid (TBA) method described by Warren [23] and modified by Crook [24, 25]. Briefly, malignant and adjacent normal tissues were thoroughly homogenized in ten volumes of 0.05 M sulfuric acid (H_2SO_4) using glass homogenizers. Homogenates were then incubated at 80°C for 1 h followed by centrifugation at 5,000 g. The supernatants obtained were used for sialic acid and protein estimation. 100 μl of supernatant was used for TSA determination. In order to eliminate 2-deoxy-ribose interference the absorbance of the sample was measured at 549 nm and 532 nm. Each sample was analyzed in duplicate and for each determination, spectrophotometric absorbance of the pink chromophore against the sample blank were measured at 549 nm. The calibration curve was prepared using concentration range of 0–20 μg of *N*-acetylneuraminic acid (Sigma). Tissue TSA levels were expressed as microgram (μg)/mg proteins and serum TSA values were expressed as milligram/deciliter (mg/dl). Concentration of serum TSA was normalized with total proteins (TP) and the ratio TSA/TP was expressed as (μg/mg proteins).

Processing of tissue specimens for sialyltransferases and sialoproteins analysis

Malignant, precancerous and adjacent normal tissues were homogenized in PBS (pH 7.4) (100 mg tissue/300 μl PBS). The whole process was carried out on ice. The homogenates were spun at 5,000 g at 4°C and the resulting

Table 2 Clinical details of oral cancer patients

Oral cancer patients	*N*=130
Sites	
Buccal mucosa (%)	45.4
Oral tongue (%)	20.8
Alveolus (%)	10.7
Retromolar trigon (RMT; %)	07.7
Lip (%)	04.7
Others (Gum, gingival sulcus, Hard palate; %)	10.7
Histopathology	
Squamous cell carcinoma (%)	100
Lymphnode involvement	
No (%)	49.4
Yes (%)	50.6
Stage	
Stage I (%)	10.6
Stage II (%)	17.0
Early stage (I+II; %)	(27.6)
Stage III (%)	17.9
Stage IV (%)	54.5
Advanced stage (III+IV; %)	(72.4)
Tumor differentiation	
Well (%)	30.8
Moderate (%)	57.7
Poor (%)	11.5
Nuclear grade	
I (%)	27.0
II (%)	61.5
III (%)	11.5

supernatants were used for protein estimation, enzyme assays and dot blot analysis.

Biotinylation of lectins for enzyme activity assay and dot blot analysis

The α2-6- and α2-3-sialyl-linkage specific lectins, *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAM) probes were used for the detection of α2-6- and α2-3-linked sialic acid, respectively. Biotinylation of SNA and MAM (Sigma) was performed as instructed in Sulfo-NHS-Biotinylation Kit (Pierce, IL). Biotin conjugated lectins were used for detecting linkage specific sialoproteins and sialyltransferases activity assay from tissue homogenates and serum samples.

SNA and MAM dot blot analysis for α2,6-sialoproteins and α2,3-sialoproteins

Tissue (10 μg) and serum (100 μg) proteins were spotted onto the hybond nitrocellulose membrane (Amersham Pharmacia) and air dried for 5 min at room temperature (RT) in order to immobilize proteins onto the membrane. Membranes were then blocked by incubation with 2% BSA in PBS/0.1% Tween-20 (PBS-T) for 1 h at RT. After three washes with PBS-T, blots were incubated with biotin-conjugated SNA (5 μg/ml) or MAM (20 μg/ml) prepared in antibody diluent (PBS-T/ 1% BSA), overnight at RT with gentle shaking. On the following day, membranes were washed with PBS-T then incubated for 2 h with alkaline phosphatase (ALP) conjugated streptavidin (Serotec) prepared in diluent (1:1000 dilution). The reaction was developed with CSPD, a chemiluminescent substrate for ALP (Boehringer) and detected by autoradiography. Membranes were scanned and quantitated on a Gel-Documentation system software (Bio-Rad) and the intensity of sialoproteins was expressed as OD/mm^2.

ELISA-based 96-well plate solid phase assay for α2,6-ST and α2,3-ST activity from tissue homogenates and serum

α2,6-ST and α2,3-ST activity from tissue homogenates and serum were estimated as described previously [26, 27]. Briefly, the microtiter plates (Axygen) were coated with 100 μl of desialylated acceptor asialofetuin (20 μg/ml) diluted in coating buffer (bicarbonate, pH 9.6) by overnight incubation at RT. The wells were then washed three times with wash buffer [PBS/ 0.2% sodium azide/0.1% Tween-20] and then blocked for 2 h using 2% BSA in wash buffer. After washing the wells, the standard assay conditions were provided in a final volume of 100 μl which contained 50 mM cacodylate buffer (pH 6.5), 50 μM CMP-*N*-acetylneuraminic acid (CMP-NeuAC), 10 mM $MnCl_2$ and tissue homogenates/serum as the enzyme source. The reaction mixtures were incubated at 37 °C for 2 h followed by three washes. The products of the α2,6-ST and α2,3-ST were detected by adding 100 μl of biotinylated lectins SNA (2 μg/ml) or MAM (10 μg/ml), prepared in wash buffer/ 0.1% BSA followed by 1 h incubation at RT. Unbound lectin was removed and washed three times with buffer. The bound biotinylated lectins were then incubated with 100 μl of streptavidin-ALP conjugate (1:500 dilution) for 1 h at RT. The wells were washed with wash buffer followed by distilled water and the assay was developed by incubation with freshly prepared streptavidin-ALP substrate *p*-nitrophenyl phosphate (1 mg/ml) [prepared in bicarbonate buffer/1 mM $MgCl_2$] at 37 °C for 2 h. The absorption was read at 405 nm using an automated microplate reader (Labsystem Multiscan Spectrum). Reaction mixtures with no enzyme source or substrate were used as sample blanks. Each assay was performed in duplicate. The enzyme activity (specific activity) was expressed as μmoles of PNP liberated/min/mg proteins.

Statistical analysis

Data were analyzed using a SPSS statistical software (Version 10). All values were expressed as mean±S.E.M. Student's paired *t*-test was used to compare the levels of markers between malignant and adjacent normal tissues as well as pretreatment and post-treatment serum levels of markers in oral cancer patients. Unpaired *t*-test was performed to compare the marker levels between controls, patients with OPC and oral cancer patients. Multivariate analysis was carried out to analyze the correlation of markers with various clinicopathological parameters including tumor differentiation, nuclear grade, lymph node involvement and stage of the disease. *P* values less than or equal to 0.05 were considered as statistically significant.

Results

Tissue levels of TSA, expression of α2,6- and α2,3-sialylated glycoproteins and enzyme activity of α2,6-ST, α2,3-ST

As demonstrated in Fig. 1, malignant tissues showed significantly higher concentration of TSA (μg sialic acid/mg proteins) than that of adjacent normal tissues ($p<0.001$). The mean ratio of malignant to normal tissues for TSA was 2.12 which ranged from 1.3 to 7.2. SNA and MAM lectins are broadly used probes for the detection of α2-6- and α2-3-sialoproteins, respectively, which are the products of α2,6-ST

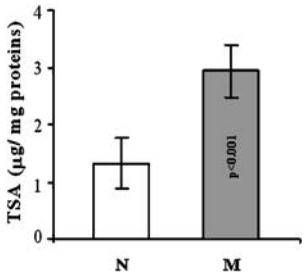

Fig. 1 Total sialic acid (TSA) levels in tissues were measured by TBA method. The graph shows comparison of TSA levels between malignant (M) and adjacent normal (N) tissues. Values (mean±SEM, *N*=75) are expressed as µg sialic acid/mg proteins

and α2,3-ST. The representative blots (Figs. 2a and 3a) revealed markedly high SNA and MAM reactivity in the majority of malignant tissues compared to their adjacent normal tissues. However, in few cases similar SNA reactivity was observed in both malignant and adjacent normal tissues. The densitometric analysis of sialoproteins is shown in Figs. 2b and 3b. As illustrated, the α2-6-SP and α2-3-SP were significantly increased in malignant tissues compared to their adjacent normal tissues (p=0.01 and p<0.001, respectively). The calculated ratio for malignant to adjacent normal tissues for α2-6- and α2-3-SP were 1.6 (range—0.9 to 6.5) and 1.9 (range—1.1 to 7.0), respectively. To evaluate whether the increased levels of TSA, α2-6- and α2-3-SP have association with α2,6- and α 2,3-ST, the enzyme activities were analysed from tissue homogenates. The comparisons of α2,6- and α2,3-ST activities in tissues are shown in Fig. 4.

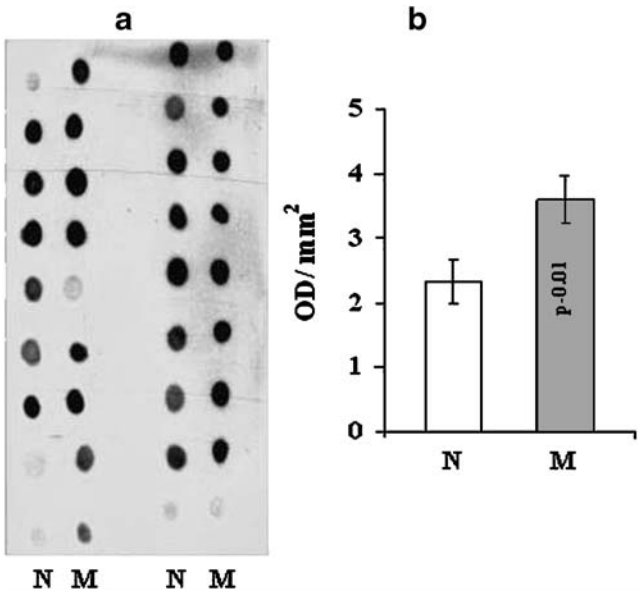

Fig. 2 Detection of α2-6-sialoproteins in tissue homogenates was done by dot-blot analysis. Ten micrograms of total proteins from tissues were spotted on a nitrocellulose membrane and probed with SNA. **a** Representative pattern of α2-6-sialoproteins blot in malignant (M) and adjacent normal (N) tissues (*N*=75) and **b** the intensity of the dot of each specimen was quantified and compared between M and N tissues. Values (mean±SEM) are expressed as OD/mm^2 in 10 µg of proteins

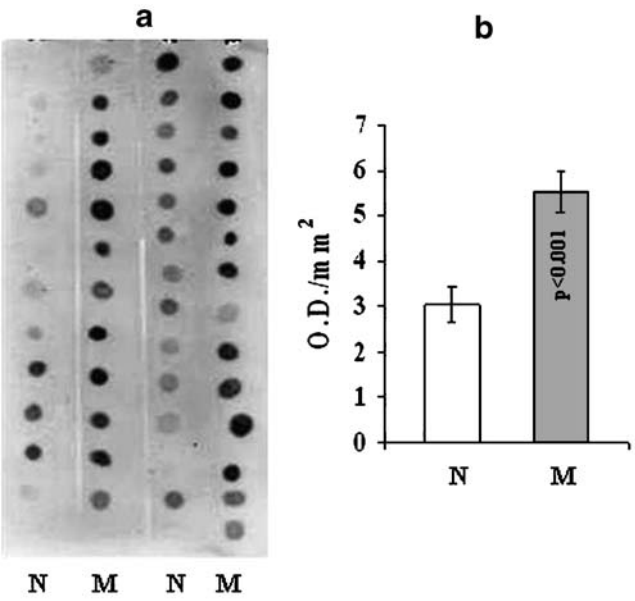

Fig. 3 Detection of α2-3-sialoproteins in tissue homogenates was done by dot-blot analysis. Ten micrograms of total proteins from tissues were spotted on a nitrocellulose membrane and probed with MAM. **a** Representative pattern of α2-3-sialoproteins blot in malignant (M) and adjacent normal (N) tissues (*N*=75) and **b** the intensity of the dot of each specimen was quantified and compared between M and N tissues. Values (mean±SEM) are expressed as OD/mm^2 in 10 µg of proteins

Statistical analysis illustrates increased α2,6-ST (p=0.08) and α2,3-ST (p=0.001) activities in malignant tissues compared to adjacent normal tissues (Fig. 4a and b). The mean ratio of malignant to normal tissues and the range for α2,6-ST and α2,3-ST were 1.54 (0.28 to 7.0) and 2.2 (0.9 to 5.2), respectively. Thus, our data suggest that higher TSA levels and strong SNA, MAM reactivity in malignant tissues may have association with increased α2,6-ST and α2,3-ST activities.

The sialylation changes were analyzed from ten OPC and their adjacent normal tissues. The higher TSA levels, SNA and MAM reactivity (data not shown) and increased α2,6-ST and α2,3-ST activities were observed in OPC tissues compared to their matched pairs. However, the relevant statistical analysis could not be performed due to less number of tissue pairs.

Serum levels of TSA and TSA/TP, reactivity of SNA and MAM lectins and the enzyme activity of α2,6-ST and α2,3-ST in the subjects

To investigate the effect of sialylation changes during neoplastic transformation in circulation, the glycoprotein constituents were evaluated from serum samples of 130 oral cancer patients, 75 patients with OPC and 100 healthy controls. The comparison of mean TSA and ratio of

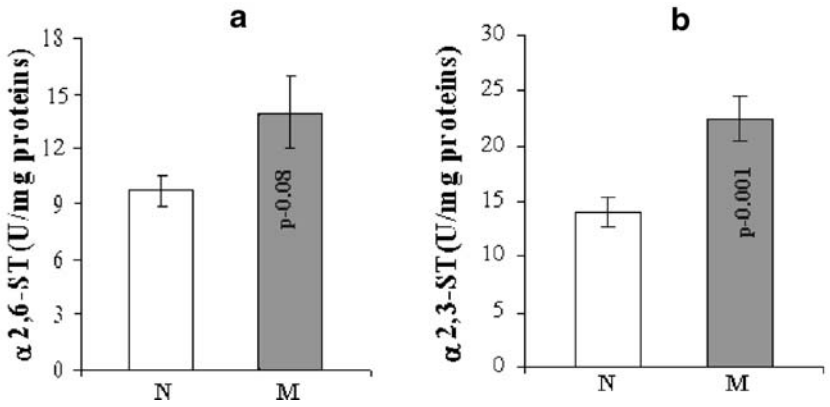

Fig. 4 Linkage specific sialyltransferase activities in tissue homogenates were measured by ELISA-based 96-well plate solid phase assay using desialylated acceptor asialofetuin. Comparison of **a** α2,6-ST and **b** α2,3-ST activities between malignant (M) and adjacent normal (N) tissues. Values (mean±SEM, *N*=75) are expressed as U/mg proteins

TSA/total proteins (TP) is represented in Fig. 5a and b. TSA and TSA/TP were significantly higher in untreated oral cancer patients compared to the OPC ($p<0.001$ and $p<0.001$, respectively) and controls ($p<0.001$ and $p<0.001$, respectively). Also, the elevations in serum TSA and TSA/TP were highly significant in patients with OPC than the controls ($p<0.001$ and $p<0.001$, respectively).

SNA and MAM reactivity in serum and the densitometric analysis of sialoproteins in patients and controls are provided in Figs. 6 and 7, respectively. The representative SNA blots (Fig. 6a) and the densitometry (Fig. 6b) showed markedly increased intensity of α2-6-sialoproteins in oral cancer patients compared to controls ($p<0.001$) and patients with OPC ($p=0.007$). Also, the OPC demonstrates elevated α2-6-sialoproteins than controls, whereas, no significant difference in MAM reactivity was observed amongst the groups (Fig. 7a and b).

As shown in Fig. 8a, serum α2,6-ST activity was significantly increased in oral cancer patients compared to the patients with OPC ($p<0.001$) and controls ($p=0.014$). Figure 8 provides the comparison of serum α2,3-ST amongst the groups and no significant changes in the enzyme activity were observed.

Comparison between pre-treatment and post-treatment serum levels of the biomarkers in oral cancer patients

Serum levels of sialylation markers at diagnosis were compared with their post-treatment follow-up values to examine their significance in treatment monitoring of oral cancer patients. The post-treatment follow-ups were categorized into CR and NR as described in the "Materials and methods".

The comparison of serum sialylation changes between untreated/ pre-treatment oral cancer patients with CR and NR is shown in Fig. 9. The analysis revealed a significant decline in serum TSA, TSA/TP, α2,6-ST and α2,3-ST levels in CR compared to their pre-treatment levels ($p<0.001$, $p=0.001$, $p=0.002$ and $p=0.05$, respectively). TSA and α2,6-ST were significantly higher ($p=0.035$ and $p=0.033$, respectively), while TSA/TP and α2,3-ST remained comparable between NR and pre-treatment groups. The marker levels were also found to be elevated in NR than CR. To evaluate the relationship between variations in marker levels and the effectiveness of anticancer treatment, each follow-up value was paired with the patient's corresponding pre-treatment marker value and paired *t*-test

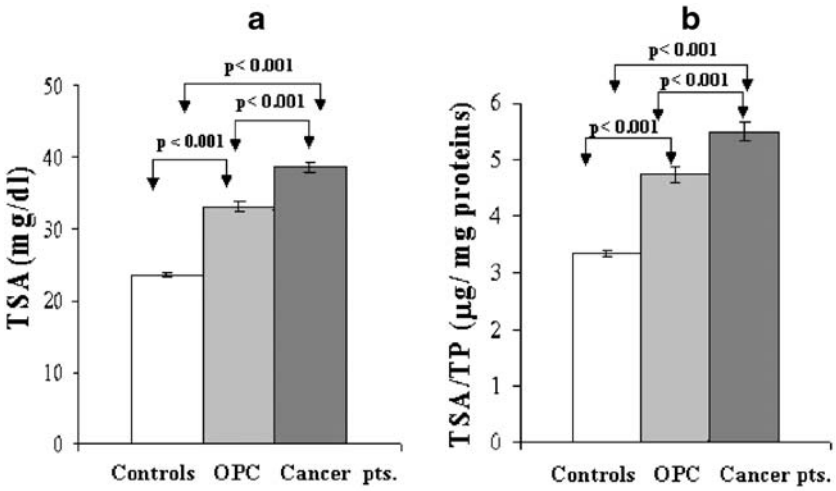

Fig. 5 TSA levels in serum samples were measured by TBA method. The levels of TSA were normalized by serum total protein levels. Comparison of serum **a** TSA and **b** TSA/TP amongst the controls (*N*=100), patients with OPC (*N*=75) and untreated oral cancer (*N*=130). Values (mean±SEM) are expressed as mg/dl for TSA and µg/mg proteins for TSA/TP

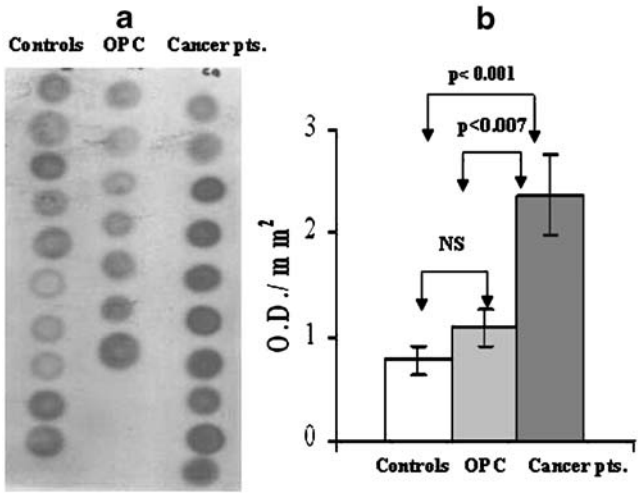

Fig. 6 SNA dot-blot analysis of serum samples. **a** Representative dot-blot for serum α2-6-sialoproteins in the controls (N=100), patients with OPC (N=75) and untreated oral cancer (N=130), **b** the intensity of α2-6-sialoproteins dot from each serum sample was quantitated and compared between the controls, patients with OPC and untreated oral cancer. Values (mean±SEM) are expressed as OD/mm^2 in 100 μg of proteins

was performed. The statistical significance of varying marker levels during favorable or poor treatment response was confirmed for each patient individually using paired t-test, which revealed similar results to those shown above. Serial estimations of the markers were made after initiation of anticancer treatment to evaluate the efficacy of the sialylation changes in treatment monitoring. The patterns of sialylation changes were studied at the time of diagnosis and during and/or after anticancer treatment in patients with different treatment outcomes. The markers showed a sharp fall in serum levels during post-treatment follow-ups and remained lower than pre-treatment levels throughout the follow-up duration in CR, whereas the markers rose progressively during follow-up period in NR. Also, serum markers declined when patients were clinically in remission. It was remarkable that the marker levels increased prior to clinical detection of the recurrence. Thus, it is evident from the results that serum sialylation changes showed significant correlation with anticancer treatment response.

Correlation of the tissue and serum sialylation changes with clinicopathological features

The comparison of mean α2,6-ST and α2,3-ST activities in malignant tissues at different stages of the disease is shown in Fig. 10. As the disease progresses from early (stage I+II) to advanced (stage III+IV), α2,6-ST (p=0.015) and α2,3-ST (p=0.048) were gradually increased in tumors. Other markers did not show correlation with the stage of disease. A significantly higher α2,3-ST activity and α2-3-SP and α2-6-SP levels were observed in poorly differentiated tumors when compared to well/moderately differentiated tumors (p=0.055, p=0.048 and p=0.039, respectively) (data not shown).

Multivariate analysis was performed in order to determine the correlation of serum markers with clinicopathological parameters in cancer patients. As documented in Table 3, positive correlation was observed between serum TSA and tumor differentiation (p=0.03). Enhanced serum α2-6-SP levels were significantly associated with poor differentiation and advanced stage of the disease (p=0.04 and p=0.055, respectively). Increased activity of α2,6-ST in serum was also correlated with the degree of tumor differentiation, lymph node metastasis and advanced stage of the disease (p=0.03, p=0.045 and p=0.05, respectively). None of the serum markers showed association with age and gender of the patients. Further, serum α2,6-ST was significantly higher in patients with advanced stages of the disease compared to early stages of the disease (p=0.05; Fig. 11).

Discussion

A number of studies have shown altered tissue or serum sialylation profiles in various cancers, however, there is a dearth of reports on systematic evaluation of sialic acid, α2-6- and α2-3-linkage specific sialoproteins and activities of α2,6-ST and α2,3-ST in both tissue and serum of oral cancer patients. The present study, sialylation changes in patients with OPC, who are at a higher risk of developing

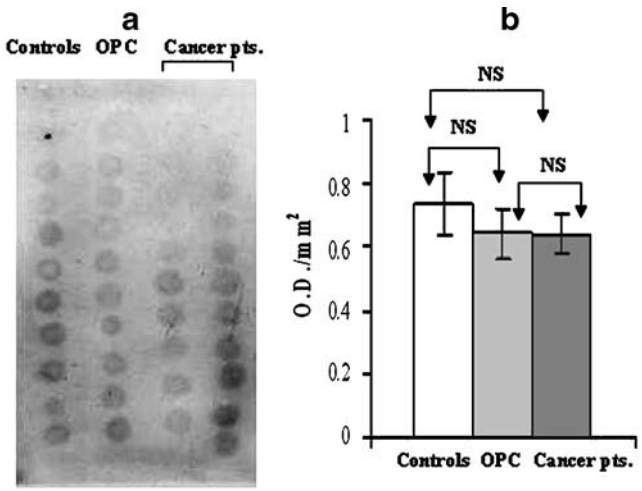

Fig. 7 MAM dot-blot analysis of serum samples. **a** Representative dot-blot for serum α2-3-sialoproteins in the controls (N=100), patients with OPC (N=75) and untreated oral cancer (N=130), **b** the intensity of α2-6-sialoproteins dot from each serum sample was quantitated and compared between the controls, patients with OPC and untreated oral cancer. Values (mean±SEM) are expressed as OD/mm^2 in 100 μg of proteins

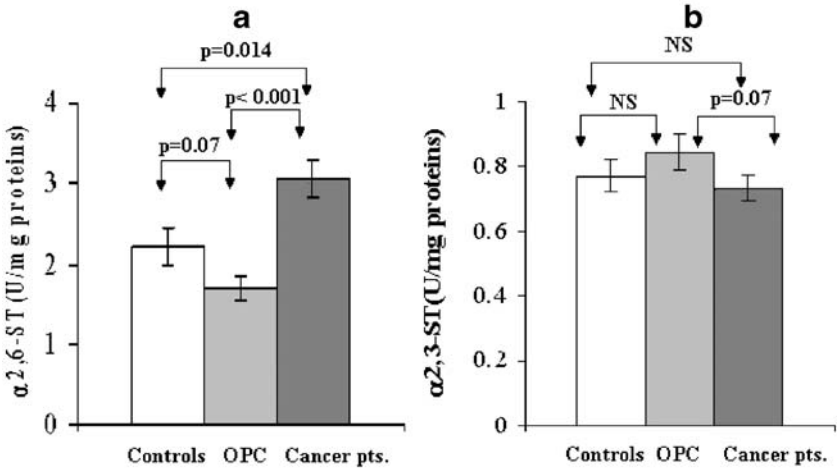

Fig. 8 Linkage specific sialyltransferase enzyme activities in serum samples. Comparison of serum **a** α2,6-ST and **b** α2,3-ST activities between controls (N=75), patients with OPC (N=75) and untreated oral cancer(N=130). Values (mean±SEM) are expressed as U/mg proteins

oral cancer, suggest that malignant transformation initiates early changes in cell surface sialylation. However, all parameters could not be analyzed from precancerous tissues due to small quantity of specimen availability at the time of biopsy and hardly obtained adjacent normal tissues for comparison. Further, the current data demonstrate importance of sialylation changes in disease progression and treatment monitoring of the oral cancer patients. The analysis of markers from tissue specimens suggest precise changes occurring during carcinogenesis, whereby, these glycoproteins are released into the circulation through increased turnover, secretion and/or shedding and are of considerable interest for their potential diagnostic and prognostic value. The screening and follow-up of patients by minimally invasive serum tests is appealing because of the accessibility to repeated sampling.

Sialic acid, an important component of cell surface and soluble glycoconjugates, undergoes substantial variations during malignant transformation. Thus, particular interest has been focused on changes in the composition and metabolism of sialic acid in transformed cells. Earlier reports on alterations in the sialic acids in cancer patients have stimulated interest in this sugar as possible marker [5, 9–11]. However, more attention has been devoted to studying serum sialic acid than the tissue levels. A significant increase in TSA levels in tumors and serum of oral cancer patients in the present study propose its implication in oral cancer. The high levels of serum TSA in patients with OPC suggest that early changes occur in cell surface glycoconjugates during malignant transformation. The ratio of TSA/TP were studied to normalize any variations caused by alterations in total protein levels and also has been reported as the most promising of those

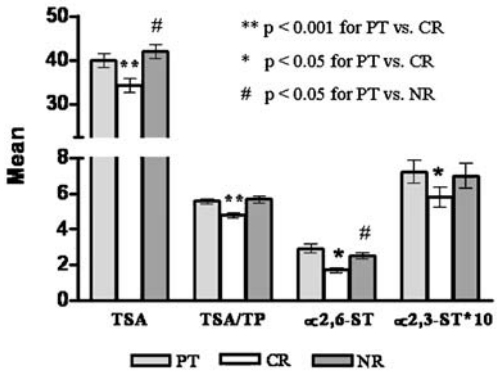

Fig. 9 A follow-up study on sialylation changes in serum samples during and/or after anticancer treatments in oral cancer patients. The post-treatment follow-ups were categorized into CR and NR as described in the "Materials and methods". The follow-up serum samples were analysed for sialylation changes using Paired t-test analysis for each follow-up of the patients. Graph showing the comparison of serum TSA, TSA/TP, α2,6-ST and α2,3-ST between untreated/ pre-treatment (PT) oral cancer patients (N=130), and CR (N=52) and NR (N=23). Values (mean±SEM) are expressed as mg/dl for TSA and mg/gm proteins for TSA/TP and U/mg proteins for enzyme activities

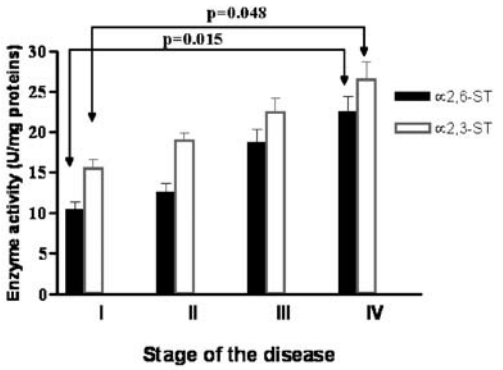

Fig. 10 Levels of sialyltransferases in oral malignant tissues depending on the stages of disease. Statistical analysis reveals linear relationship (p=0.015 for α2,6-ST and p=0.048 for α2,3-ST) of higher enzyme activities with increase in the stage of the disease (stage I to stage IV). Values (mean±SEM) are expressed as U/mg proteins

Table 3 Association of serum sialylation changes with various clinicopathological characteristics

Variable	TSA	TSA/TP	α2,6 SP	α2,3 SP	α2,6 ST	α2,3 ST
Age	0.06	0.065	0.90	0.33	0.16	1.00
Gender	0.75	0.89	0.11	0.81	0.13	0.5
Nuclear grade	0.09	0.26	0.08	0.91	0.76	0.93
Tumor differentiation	*0.03*	0.14	*0.04*	0.58	*0.03*	0.41
Lymphnode metastasis	0.25	0.38	0.17	0.77	*0.045*	*0.04*
Stages of disease	0.30	0.60	*0.055*	0.23	*0.05*	0.16
Early vs. advanced stage	0.53	0.72	*0.04*	0.18	*0.045*	0.62

markers previously tested [10, 28]. The increased TSA/TP in oral cancer patients suggests that sialic acid alterations is primary phenomena during malignancy rather than the secondary effect of the protein changes. This is in accordance to the earlier reports by us [19] and others [29] on different forms of serum sialic acid. Further, we observed positive correlations between increased serum TSA, TSA/TP and tumor grade, suggest that sialylation changes are associated with the disease progression. Previous reports support our observations that higher levels of serum or tissue sialic acid associated with disease progression [9, 10, 19] and tumor differentiation [30]. Taken together, our results suggest that sialic acid levels reflect the tumor burden and are associated with the stage of primary lesion and presence of distant metastasis. The spontaneous metastasis of murine cells was found to be associated with sialic acid content of the cell culture and the degree exposed on the tumor cell surface [31]. A recent study suggested that inhibition of cell surface sialic acid might provide an anti-metastatic therapeutic target [32].

One of the consistent alterations associated with malignant transformation and metastasis is increased β1-6-branching of oligosaccharides [33]. Most of the lactosamine antennae are substituted with sialic acid, therefore, the increased β1-6-branching may contribute to the carcinoma-associated unique sialylation pattern of glycoproteins [31]. The role of linkage specific sialylation in development of an invasive phenotype has been reported earlier [34, 35].

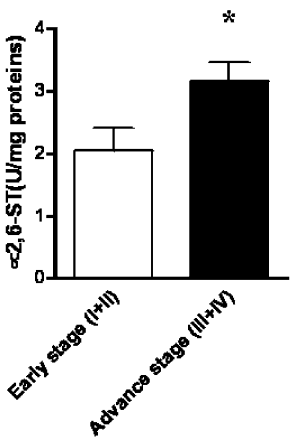

Fig. 11 Comparison of serum α2,6-ST activities in patients with early (stage I+II) and advanced (III+IV) stage of oral cancer as detailed in Table 2. Positive correlation was observed between activities and disease progression ($p<0.05$). Values (mean±SEM) are expressed as U/mg proteins

The use of lectin specific patterns has been a useful approach of analyzing the complex oligosaccharide sequences of glycoproteins. The use of lectin probes SNA and MAM for detection and discrimination of α2-6- and α2-3-sialoproteins in patients with oral cancer and OPC revealed several important observations in this study. Although few tumors showed similar or lower SNA reactivity, the overall densitometric analysis demonstrated significantly higher SNA and MAM reactivity. This suggests that both α2-6- and α2-3-sialylated glycoconjugates accumulates and associates with invasive and metastatic potentials of oral tumors. It has been previously reported that α2-6-sialylation changes associated with invasiveness of cancer cells [34, 36]. We observed that precancerous changes were associated with increased SNA and MAM reactivity. Although, the number of OPC tissues examined in this study does not allow us to make a definitive conclusion. In the current investigation, serum analysis revealed markedly higher α2-6-sialoproteins in oral cancer patients than controls and patients with OPC. No difference was seen in MAM reactivity among the groups; however, we could not devise an explanation for observed discrepancies between tissue and serum levels. Furthermore, multivariate analysis illustrated positive correlation of α2-6-sialoproteins with poor differentiation of tumors and advanced disease stage. Sata *et al.* demonstrated histochemical detection of both sialoproteins in colon carcinoma and dysplasia. Further, they reported that normal colon mucosa and mild dysplasia lacked α2-6-linked sialic acid residues but were positive for α2-3-linked sialic acid residues [35]. The colon tumors and severe dysplastic tissues were positive for α2-6-linked sialic acid which support our observations and suggest that sialoproteins may be differentially up-regulated at different stages of oral carcinogenesis. A recent study explains the association of aberrant expression of SNA- and MAM-positive sialoglycoconjugates with unfavorable pathological characteristics and worse prognosis [37]. Strong SNA reactivity has been observed in colon carcinoma [34, 35] and proposed as an indicator of a poor 5-year survival [38].

Altered sialylation in cancer is associated with specific type of sialyltransferases activity [15]. Expression of α2,6- and α2,3-ST mRNA isoforms, and total enzyme activity

and their regulation have been studied in various malignancies [20, 36, 39–41]. Traditional assays for STs using radioactivity-based methods detect total enzyme activity instead of individual isoforms. Therefore, to precisely determine the linkage specific isoform involved in sialylation in oral cancer, we employed absorbance based solid-phase non-radioactive method for the measurement of α2,6- and α2,3-ST activities as described previously [26, 27]. This technique exploits the linkage specificity of lectins SNA and MAM, which bind to the terminal sequence with high affinity. Our results showed high activities of both STs in malignant tissues, among them α2,3-ST showed significantly higher activity, while α2,6-ST were up-regulated in serum. In an attempt to find out the clinical applicability of increased STs, we carried out the correlation of ST activities in malignant tissues and serum, with clinicopathologic features. The α2,6-ST and α2,3-ST activities were significantly enhanced in tumors with advanced disease stage. Also, increase in serum α2,6-ST was found to be associated with advanced stage of the disease and poor differentiation of tumors. In this context, we state that α2,3-ST more likely associates with hypersialylation during early phase of tumor development, whereas α2,6-ST is likely to be associated with disease progression and metastasis of oral cancer. This also suggests that isoforms may have diverse roles during disease progression and increased sialylation on the cell surface may help the tumor cells to escape from the primary site. Increased α2,6-ST activity was previously reported in metastatic tissues and serum [42]. Over-expression ST6Gal-I mRNA in primary tumors is associated with poor differentiation of tumors [43], invasiveness and lymph node metastasis [40], and poor prognosis [44]. On the other hand, ST3Gal-I, III, IV expression is found to be responsible for enhanced sialylation of O-glycans and up-regulation of these isoforms correlated with poor prognosis of breast and colorectal carcinoma [43, 45, 46].

Hypersialylation due to enhanced ST is found to be associated with the presence of several cell surface antigens on tumor cells [15]. α2-3-sialylation of *O*-linked glycoproteins has been proposed to have a decisive role in biosynthesis of sialyl Lewis-x/a and mediate a critical binding of neoplastic cells to activated endothelium, which is one of the key steps in hematogenous metastasis [47]. Sialyl-α2-6-linkage associated with sTn-epitope expression on cell surface and useful predicting patients' survival [48]. In a separate study, we have found increased sialyl Lewis X in oral precancerous and cancerous tissues (unpublished data). Sialilated cell surface epitopes are promising tools for the strategies used to design carbohydrate mimetic and the structure-activity relationships of substrate-based ST inhibitors [49].

The analysis of serum markers is the most appealing idea of screening and following cancer patients during their anti-cancer treatment. Hence, the present study determined the usefulness of alterations in serum sialylation profile as treatment monitors in oral cancer patients. Serum levels of TSA, TSA/TP, α2,6-ST and α2,3-ST decreased upon successful therapeutic response in CR and remained elevated or comparable in NR than their pre-treatment levels. Response to therapy, no response and persistent disease correlated well with the levels of serum sialylation changes in the patients. Results of present study strongly suggest that serum sialylation changes occur during anticancer treatment and may serve as indicators of clinical status and help to predict treatment response, recurrence or possible therapy failure. Thus, close monitoring of serum sialylation changes may be a promising approach and being non-invasive method, may be useful to assess treatment outcome during post-treatment follow-up in oral cancer patients.

In conclusion, our findings suggest that increased sialylation of tissue and serum glycoproteins in patients with oral cancer and OPC are associated with oral cancer development and treatment monitoring of the patients. The increased levels of α2-6- and α2-3-sialoproteins and their enzyme activities are found to be associated with various stages of neoplasia. The data also suggests that malignant transformation in oral epithelium is accompanied by α2-3-sialylation, whereas, α2-6-sialylation may be associated with disease progression and metastatic potentials. These results also provide a foundation for in-depth studies into the role of sialylation-associated early changes in OPC cells.

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