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Evaluation of Salivary Sialic Acid in Patients With Different Clinico-Pathological Stages of Oral Leukoplakia and Oral Squamous Cell Carcinoma - A Cross Sectional Study

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Early detection of oral cancer is significantly potential with noninvasive methods like salivary analysis which is of utmost importance to reduce the risk rate of advanced stage.As there is paucity in studies performing clinicopathological staging and grading of precancerous and cancerous conditions, this study prompted us to evaluate salivary sialic acid in patients with different clinico pathological stages of oral leukoplakia and oral squamous cell carcinoma in order to develop cost effective,simple diagnostic method. Unstimulated salivary sample for the present study comprised a total of 20 healthy controls (Group I), 20 Oral leukoplakia patients(Group II) and 20 oral squamous cell carcinoma patients(Group III). Estimation of salivary sialic acid levels (free and protein bound) were performed by using Ultra violet visible Spectrophotometer. This was correlated with the clinicopathological staging and grading of oral leukoplakia and oral squamous cell carcinoma. The stastical comparisons were performed by analysis of variance (ANOVA) followed by student's paired t-test and f test. Sialic acid (free & protein bound) levels were significantly increased in oral squamous cell carcinoma patients and oral leukoplakia patients compared to controls . Protein bound Salivary sialic acid was significantly increased in OSCC when compared with oral leukoplakia patients. Significant higher levels of free sialic acid and protein bound sialic acid was found in well-differentiated OSCC cases when compared to other two grades. Salivary parameters offer scope for detailed future research on their applications in screening, diagnosis, and management of cancer. Sialic acid is a sensitive marker of oral cancer. Elevated levels of free and protein-bound salivary sialic acid in leukoplakia and oral cancer patients indicate its importance as a tumor marker.

Key words: Oral cancer, Sialic Acid (Free and Protein bound), OSCC (Oral squamous cell carcinoma), Oral leukoplakia, histopathological differentiation.

Oral cancer is the most common cancer and constitutes a major health problem in developing countries, representing the leading cause of death. A key factor in the lack of improvement in prognosis over the years is the fact that a significant proportion of oral squamous cell carcinoma (OSCC) are not diagnosed or treated until they reach an advanced stage.[1] It is believed that identification and monitoring of these potentially malignant lesions and conditions allows

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clinicians to detect and treat early intraepithelial stages of oral carcinogenesis, for example mild, moderate or severe dysplasia and carcinoma in situ, all of which generally precede the development of invasive OSCC[2].

The International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) have recently stressed that we can reduce a third of a predicted 15 million cancer cases in the future and more effectively manage another third by planning effective cancer control and screening strategies.[3] This paved our interest to detect the role of saliva in early detection of oral cancer.

Saliva is a complex fluid composed of a wide variety of organic and inorganic constituents which collectively act to modulate the oral environment[4-5]. It is a unique fluid and interest in it as a diagnostic tool has advanced exponentially in recent years. Biological markers can be used to monitor cancer, predict the therapeutic response and prognosis of cancer, and in certain situations even diagnose cancer. These markers, referred to as tumour markers, are naturally occurring or modified molecules that can be measured in serum, plasma, or other body fluids and their concentration becomes changed in the presence of cancer .[6]

Aberrant glycosylation is a universal phenomenon, such glycosylations including sialylation in cell membranes are the important events in malignancy. Terminal sugars of glycoproteins and glycolipids play significant role in cell to cell interactions and in development of cell adhesion which is significant in malignant transformations[7]. N-acetylneuraminic acid (referred to as sialic acid, SA) is a negatively charged nine-carbon monosaccharide commonly attached by an glycosidic linkage to the non-reducing residues of the carbohydrate chains of glycoproteins and glycolipids[8].

The knowledge gained from various studies have reported elevated levels of total sialic acid (TSA), lipid-bound sialic acid (LSA) and TSA to total protein ratio,in various malignancies[9-11]. They were limited to focus on saliva and to give importance in performing clinicopathological staging and grading of oral cancer patients and precancerous conditions.This prompted us to estimate salivary levels of sialic acid (free & protein bound) in oral cancer patients and in leukoplakia in order to develop cost effective, simple diagnostic method.

MATERIALS AND METHODS

The study was planned & conducted during the period of September 2009 to November 2010 in the Department of Oral Medicine and Radiology,Rajah Muthiah Dental College & Hospital,Annamalai University to evaluate the salivary sialic acid in patients with different clinico pathological stages of oral leukoplakia and oral squamous cell carcinoma. A detailed case history was recorded for all patients with special reference to their habits(chewing of betal nut,pan pareg etc),its nature,duration and frequency of use after establishing the clinical diagnosis of leukoplakia and oral cancer.

A cross-sectional study design was undertaken with a sample size of 60 which is divided into 3 groups:-

- Group A comprises of 20 subjects with age, sex and habit matched normal healthy individuals
- Group B comprises of 20 newly diagnosed cases of oral Leukoplakia
- Group C comprises of 20 newly diagnosed cases of oral Squamous Cell Carcinoma.

All the patients were subjected to routine haemogram (HB%,TC,DC,CT,BT,RBS) to rule out any systemic ailments. This was followed by a histopathological confirmation of diagnosis by performing an incisional biopsy of the lesion based on Broder's classification. Staging for the patients with leukoplakia were done by van der waal et al (2000).Patients with oral cancer were grouped clinically according to tumor,node,metastasis (TNM) system of cancer classification given by American Joint Committee on Cancer (AJCC)1997.The subjects with recurrence, the lesions undergoing treatment, patient's with systemic problems and patient's with no history of adverse habits are excluded from the study.

A formal ethical clearance to conduct this study was obtained by the ethical committee of the college. All the subjects were informed about the procedure and the study was carried out after their consent.

Method for collection of saliva sample

The unstimulated whole saliva was

analyzed in this study. Saliva samples were collected during the hours 10Am-12Pm, according to the method of Navazesh (1993) 2 hours after the subjects' usual break fast time. This was to ensure the variability in salivary flow and compositions be minimized due to diurinal variation. The subject was asked to rinse the mouth with distilled water thoroughly to remove any food-debris and then after 10 minutes directed to spit into a sterile plastic container. The subjects were instructed not to spit forcibly so as to avoid blood contamination. Once 2ml of saliva was collected, the container was placed in an ice carrier box and transferred to the laboratory for the biochemical analysis .

Method for biochemical analysis of saliva

Saliva sample was centrifuged at 3000rpm for 15 minutes. Supernatant was taken for analysis of sialic acid, proteins and sugars.

Estimation of sialic acid was estimated by the method of (Yao *et al* 1989). Saliva was treated with ethanol to precipitate proteins. Sialic acid contents of both the precipitate and supernatant were estimated on the basis of reaction of sialic acid with ninhydrin reagent. N-acetyl neuraminic acid standards ranging in concentration from 20-100µgms/ml were used. Sialic acid concentration obtained from the precipitate was "protein-bound sialic acid", and sialic acid concentration in the supernatant was "free sialic acid". The absorbance of blue colored complex was measured at 470 nm.

Method of stastical analysis

Stastical package for the social science (SPSS for windows,version 10.0)was used for the analysis. A p value of less than 0.01 was used to establish stastical significance. The stastical comparisons were performed by analysis of variance (ANOVA) followed by student's paired t-test and f test.

RESULTS

Correlation of salivary biochemical parameters among the oral leukoplakia and oral squamous cell carcinoma patients are compared with the controls as well as with different clinic-pathological staging and grading are listed in table 1.

The following observations were drawn from the study

1) Salivary sialic acid (free and protein bound) was significantly increased in OSCC patients and oral leukoplakia when compared with normal healthy controls.
2) Protein bound Salivary sialic acid was

Table 1.

Parameters	Group A (Control)		Group B (Leukoplakia)		Group C (OSCC)	
	Mean	SD	Mean	SD	Mean	SD
Age	37.25	6.74	47.40	9.54	56.35	10.95
Gender	1.70	0.47	1.20	0.41	1.20	0.41
Free sialic acid	4.22	0.48	6.73	0.71	2.65	0.67
Protein bound sialic acid	2.10	0.52	2.60	0.34	2.97	0.33
Clinical Staging Vs Free sialic acid	-	-	6.73	0.71	7.16	0.69
Clinical Staging Vs Protein bound	-	-	2.60	0.34	2.97	0.33
Histo pathological Grading Vs Free sialic acid	-	-	6.73	0.71	7.16	0.69
Histo pathological Grading Vs Protein bound sialic acid	-	-	2.60	0.34	2.97	0.33

Table 2. t-test for free sialic acid (mmol/L) between Leukoplakia and OSCC group

Groups	N	Mean	SD	t-value	P value
Leukoplakia group	20	6.73	0.71	1.827	0.083
OSCC group	20	7.15	0.68		(P>0.01)

P>0.01 NS- not significant SD – Standard Deviation

significantly increased in OSCC when compared with oral leukoplakia patients (Table III).

3) Free Salivary sialic acid was not significantly increased in OSCC when compared with oral leukoplakia patients (Table II).

4) We found Significant higher levels of free sialic acid and protein bound sialic acid in well-differentiated OSCC cases when compared to other two grades (Table IV & V) (Graph I & II).

5) No statistical significant levels of free and protein bound sialic acid for different stages of OSCC. The study also revealed there is no statistical significant values for different grading and staging of leukoplakia group.

Table 3. t-test for protein bound sialic acid (mmol/L) between Leukoplakia and OSCC group

Groups	N	Mean	SD	t-value	P value
Leukoplakia group	20	2.60	0.34	3.270	0.004
OSCC group	20	2.97	0.33		(P<0.01)

P>0.01 NS- not significant SD – Standard Deviation

Table 4. F-test for free sialic acid (mmol/l) OSCC group Vs Histo pathological grading

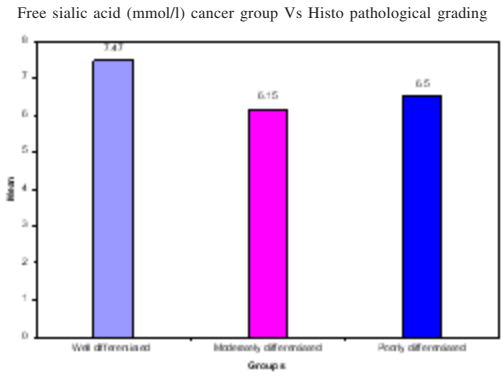
Groups	N	Mean	SD	t-value	P value
Well differentiated	15	7.47	0.37	16.44	0.000
Moderately differentiated	4	6.15	0.62		(P<0.01)
Poorly differentiated	1	6.50	0		
Total	20	7.16	0.69		

P>0.01 NS- not significant SD – Standard Deviation

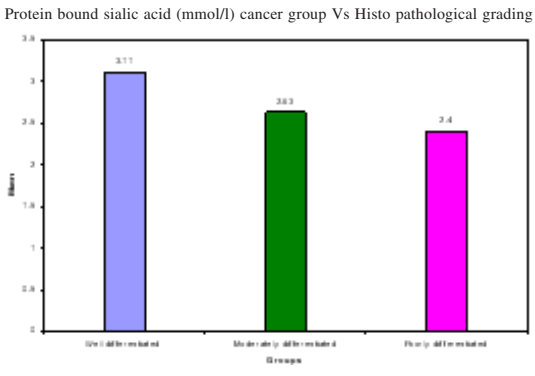
Table 5. F-test for Protein bound sialic acid (mmol/l) OSCC group Vs Histopathological grading

Groups	N	Mean	SD	t-value	P value
Well differentiated	15	3.11	0.25	9.404	0.002 (P<0.01)
Moderately differentiated	4	2.63	0.21		
Poorly differentiated	1	2.40	0		
Total	20	2.97	0.33		

P>0.01 NS- not significant SD – Standard Deviation



Graph 1.



Graph 2.

DISCUSSION

Oral cancer is largely related to lifestyle, with major risk factors being tobacco and alcohol misuse. In addition to smoking, the use of smokeless tobacco has been strongly linked to oral cancer.[2] The term leukoplakia describes a white patch or plaque that cannot be characterized clinically or pathologically as any other disease. They constitute the most precursor of oral cancer (85% of all precancerous lesions)**.** [12] More than 2/ 3rds of cancer patients are already in an advanced incurable stage at the time of diagnosis. Early detection is the key in oral squamous cell carcinoma.

There is a paucity of studies / data using saliva as a diagnostic tool for oral squamous cell carcinoma and oral leukoplakia. Biochemical analysis of saliva among the malignant and pre-malignant lesions are not evaluated in detail and only a few studies have been reported in the Indian population.

Altered glycosylation is a universal feature exhibited by malignant cells. Substances like glycoproteins and glycolipids are major constituents of cell membrane and hence, they play an important role in malignancy. These glycoconjugates are released into the circulation through increased turnover, secretion, and/or shedding from malignant cells[13-14]. Usefulness of cell surface glyco-conjugates in early detection of oral cancer and in monitoring progress during treatment has been evaluated by previous studies[15-16].

Studies have reported elevated serum levels of total sialic acid (TSA), lipid bound sialic acid (LSA) and total sialic acid to total protein ratio (TSA/TP) in patients with various types of carcinoma, including oral squamous cell carcinoma. Bathi RJ *et al*., 2001. Stoyloff J and Ivanov SX (2005) in their study**,** evaluated the significance of sialic acid as tumor markers in thyroid, head and neck and lung tumors in which they found out there is significant rise in serum sialic acid levels[17-18].

Rajpura K.B. *et al.,* (2005) estimated on total and lipid bound serum sialic acid levels in oral pre-cancerous conditions, oral cancer and healthy subjects, and they found that serum levels of total and lipid bound sialic acid were significantly elevated in oral cancer patients as compared to healthy individuals as well as patients with oral precancer[19]. The significance of serum sialic acid as a sensitive tumor marker has been established in previous studies. However, its specificity is under question. Serum sialic acid levels are also reported to be elevated in non-cancerous conditions like pregnancy, inflammation and cardiovascular diseases[20].

Salivary constituents arrive from the various major and minor salivary glands and also from the gingival cervices. No effort was made to collect saliva from a particular gland, as these require sophisticated methods. Alteration in total and individual salivary contents is also influenced by the duration and intensity of the stimulus. To minimize the effects of mechanical stimulation, unstimulated salivary samples were used[21].

Very few studies have been carried out to estimate salivary sialic acid in oral diseases[4]. Salivary proteins, in particular the glycoproteins are mainly responsible for the protective qualities of saliva. These glycoproteins include mucins, proline-rich glycoproteins, á-amylases, lactoferin, salivary peroxidase and secretory IgA. A study revealed significant elevation in the salivary total protein and total sugar level in OSCC patients[8].

Koc L. *et al.,* (1996) did a comparative study on the levels of salivary sialic acid between the cancer patients and healthy controls and they found that the sialic acid levels were higher in the cancer group as compared with healthy controls.Many investigators have demonstrated that the concentration of the glycoproteins in human serum is abnormally high in a number of physiological and pathological statuses. Signfiicant increase in the glycoprotein content of serum has been associated with neoplastic diseases, tuberculosis, diabetes mellitus, and rheumatoid arthritis[22].

The present study has revealed significantly elevated free and protein bound sialic acid in the saliva of oral cancer patients as compared with healthy controls**.** We have also obtained significant higher levels of free sialic acid and protein bound sialic acid in well-differentiated OSCC cases when compared to other two grades and there was no significant results obtained in relation with different staging of OSCC. This suggests correlation of elevated salivary sialic acid levels to the progression of precancerous and

cancerous condition (i.e.,) Leukoplakia and OSCC. In the present study, we have made an attempt to establish the importance of saliva as a diagnostic fluid in Oral Leukoplakia & oral squamous cell carcinoma. As there are advantages in utilizing saliva for diagnosis of oral cancer, more studies need to be carried out to evolve sensitive and specific methods to detect salivary tumor markers

CONCLUSION

The use of saliva as a diagnostic tool is widely expanding. Due to non-invasiveness and easy method of its collection, saliva is a convenient biological fluid for diagnosis of diseases. Salivary parameters offer scope for detailed future research on their applications in screening, diagnosis, and management of cancer. Sialic acid is a sensitive marker of oral cancer. Elevated levels of free and protein-bound salivary sialic acid in leukoplakia and oral cancer patients indicate its importance as a tumor marker.

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