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Comparison of Salivary Sialic Acid Levels between Patients with Potentially Malignant Disorders of Oral Cavity and Healthy Subjects

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Abstract: ***Background & Objectives: Many oral squamous cell carcinomas develop from potentially malignant disorders of the oral cavity[1] and are an important target for cancer prevention. Studies shows reduction in tumor mass; recurrence and metastasis correlate well with changes of serum sialic acid levels in cancer patients and has been considered as a valuable tumor marker in monitoring the clinical status of the carcinoma patient. Sialic acid a constituent of salivary glycoproteins are released into the saliva through increased turn over, secretion, and / or shedding from malignant cells.[2] This study aims to evaluate and compare the levels of salivary sialic acid (bound and non-bound) in patients with potentially malignant disorders of oral cavity and healthy subjects. Methods: Unstimulated whole saliva of 1-2 ml was collected between 10 AM- 12PM; according to the method of Navazesh.[7] Saliva sample was centrifuged at 3000 rpm for 15 minutes and estimation of sialic acid was done. Results: The mean value of bound salivary sialic acid of oral potentially malignant diosrder group was 2.848 mg/dl with standard deviation of 0.289 while that of control group was 3.364 mg/dl with standard deviation of 0.376. The mean value of free salivary sialic acid of oral potentially malignant disorder group was 3.859 mg/dl with standard deviation of 0.554 and that of control group was 3.364 mg/dl with standard deviation of 0.376. The difference in salivary sialic acid in both groups was found to be significant (P<0.05). Conclusion: Saliva as a diagnostic medium is less expensive, less invasive and has less risk both to patient and clinician. Although the amount of salivary sialic acid in saliva was low when compared to serum, detectable amount can be appreciated in subjects with potentially malignant disorders of oral cavity and oral cancer when compared to normal subjects. Thus salivary sialic acid appears to be a potential biomarker for screening purposes.***

Key words: Oral potentially malignant disorders, salivary sialic acid, bound salivary sialic acid, free salivary sialic acid

1. Introduction

Oral potentially malignant disorders[1] (OPMD) are relatively common, occurring in about 2.5% of the general population and are an important target for cancer prevention. Many oral SCCs develop from potentially malignant disorders of the oral cavity.[2,3] A wide array of OPMD's have been implicated in the development of oral cancer, including leukoplakia, erythroplakia, palatal lesion of reverse cigar smoking, oral lichen planus, oral submucous fibrosis, discoid lupus erythematosus, and hereditary disorders such as dyskeratosis congenita and epidermolysis bullosa.[4] Detection of oral premalignant lesions at an early stage is of utmost importance to prevent further morbidity as they have shown a rate of progression and cancer transformation of up to 17% within a mean period of 7 years after diagnosis.[5]

Aberrant glycosylations are the universal features of cancer. Glycoproteins and glycolipids are important constituents of cell membrane; hence, they play an important role in malignancy. These glycoconjugates are released into the circulation through increased turn over, secretion, and / or shedding from malignant cells. Usefulness of assay of serum glycoconjugates in early detection of cancer and in monitoring the progress of treatment, have been evaluated in previous studies. [6] Sialic acid is a constituent of many salivary glycoproteins. N-acetylneuraminic acid is a negatively charged nine carbon monosaccharide commonly attached by a glycosidic linkage to the non-reducing residues of the carbohydrate chains of glycoproteins and glycolipids. Studies show that there is a significant difference in both serum and salivary sialic acid level (total and bound sialic acid) in pan chewers, leukoplakia, oral squamous cell carcinoma, endocrine gland cancer and showed positive correlation between stages of cancer and levels of sialic acid.[7,8,9] Determination of salivary sialic acid levels is an easily reproducible, non-invasive and inexpensive tool and so is used to evaluate its significance as a potential biomarker by a comparison between patients with various common OPMD and healthy subjects.

2. Methodology

The study was conducted over a period of 18 months at Dept. of Oral Pathology & Microbiology, Govt Dental College, Kottayam on 100 samples after obtaining ethical clearance. The sample population were divided into 2 groups:

Group 1 consisted of 50 previously untreated clinically and histopathologically diagnosed patients with oral potentially malignant disorders satisfying the inclusion and exclusion criteria. OPMD included leukoplakia, oral submucous fibrosis, erythroplakia, oral lichen planus and proliferative verrucous leukoplakia.

Review of the same patients was done after 6 months of initiation of treatment.

Group 2 consisted of 50 healthy volunteers attending OPD of Govt Dental College, Kottayam.

All stastistical calculations were done using computer program Microsoft Excel version 7 (Microsoft Corporation, NY, USA), SPSS version 16.0 (Statistical Package for Social Science: Chicago, IL, USA).The statistical comparison are performed by chi-square test and paired t

test. A p value of less than 0.05 was considered as statistically significant.

Collection of saliva sample

After obtaining informed consent, unstimulated whole saliva of 1-2 ml was collected between 10 AM- 12PM; 2 hours after the subjects usual breakfast time.[10] 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. Once 2 ml of saliva was collected, the container was placed in an ice carrier box and transferred to laboratory for biochemical analysis.Review of group 1 subjects were done after 6 months of initiation of treatment and saliva samples were collected by the same technique.

Biochemical analysis of saliva

Saliva sample was centrifuged at 3000 rpm for 15 minutes and estimation of sialic acid was done.[11]

Procedure

1) Add 0.1 ml saliva to 0.9 ml normal saline to make it 1ml and add 4ml ethyl alcohol, mix and centrifuge at 3000 rpm for 30 minutes.
2) Dissolve the precipitate obtained in 1ml distilled water, add 1ml of glacial acetic acid and 1 ml of acid ninhydrin reagent. Label as "protein-bound sialic acid"(PSA).
3) Add 1 ml glacial acetic acid and 1ml acidic ninhydrin to 1 ml supernatant and label as "free sialic acid"(FSA).
4) Keep both the tubes in boiling water for 10 minutes, cool under tap water and read absorbance at 470 nm. NANA standards ranging in concentrations from 20-1000µg/ml are run simultaneously.
5) Compare the optical density of sample with that of standards at various concentrations and calculate accordingly.
6) The concentration of sialic acid in each sample is expressed in mg/dl.

3. Results

Salivary sialic acid (SSA); protein bound and free sialic acid levels were analyzed in 50 OPMD and 50 normal controls.

SSA was analyzed during the first visit to the department and reviewed after 6 months of initiation of treatment. Both the values were compared with one another.

The age of subjects in OPMD group ranged from 21 to 77 years with a mean of 55 years, while the age of subjects in control or normal group ranged from 14 to 57 years with a mean of 29.74 years. Figure 1 shows frequency distribution within OPMD group and age group.

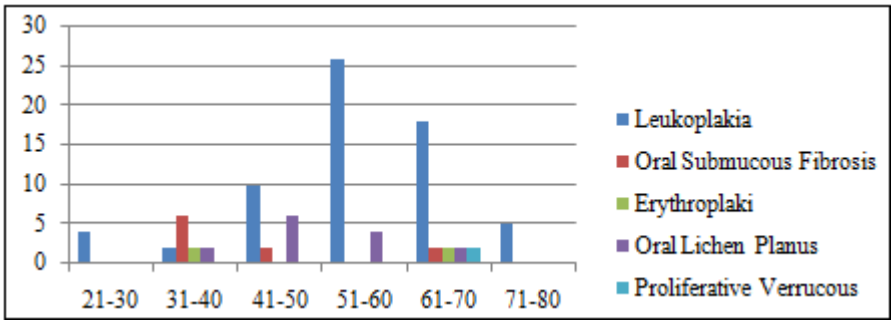

Figure 1: Age group with frequency distribution of OPMD group

Out of the 100 samples, 61 (61%) were males and 39 (39%) were females. Of the 50 samples with OPMD, 31 (62%) were males and 19 (38%) were females. In the normal or control group, 30 (60%) were males while 20 (40%) were females.

In the OPMD group, 35 (70%) subjects presented with leukoplakia, 5 (10%) with oral submucous fibrosis, 2 (4%) with erythroplakia, 7 (14%) with oral lichen planus and 1 (2%) with proliferative verrucous leukoplakia. (Figure 2)

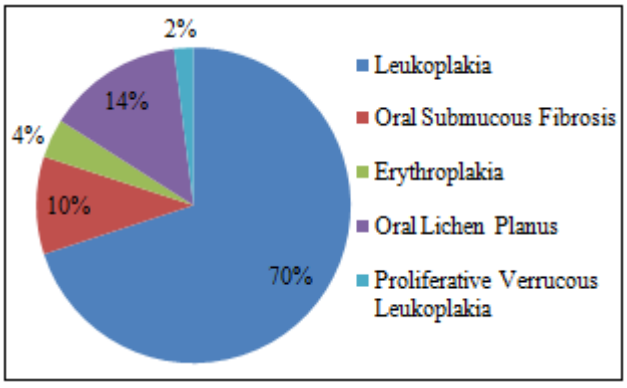

Figure 2: Frequency distribution of OPMD group

Biochemical analysis of salivary sialic acid was carried out using UV spectrophotometric method[8] with some modifications. SSA levels were recorded for both OPMD group and control group as bound SSA and free SSA. SSA levels of OPMD group was taken at 2 point of time, first during the initial diagnosis (named Baseline SSA) and secondly 6 months after initiation of treatment (named 6M SSA).

The mean value of bound SSA of OPMD group was 2.848 mg/dl with SD of 0.289 while that of control group was 3.364 mg/dl and SD of 0.376 (Table 1). A statistically significant difference between bound SSA of OPMD and control groups was observed ($p<0.05$). The mean value of free SSA of OPMD group was 3.859 mg/dl and SD of 0.554 and that of control group was 3.364 mg/dl with SD of 0.376. The difference in salivary sialic acid in both groups was found to be significant ($P<0.05$). (Table 2)

Table 1: Comparing means of bound SSA of two groups(OPMD and control) using T test

	Mean	Standard Deviation	t value	P value
Malignancy	3.648	0.289	8.86	0.000
Normal	1.649	0.376		

Table 2: Comparing means of free SSA of two groups (OPMD and control) using T test

	Mean	Standard Deviation	t value	P value
Malignancy	3.559	0.554	9.207	0.000
Normal	2.460	0.376		

Comparison of mean of SSA in leukoplakia (OPMD 1), oral submucous fibrosis (OPMD 2), erythroplakia (OPMD 3), oral lichen planus (OPMD 4), proliferative verrucous leukoplakia (OPMD 5) with controls (Group 6) were done individually using paired t test and differences in SSA levels was found to be significant for OPMD 1 ($p<0.05$) (Table 3), OPMD 2 ($p<0.05$) (Table 4) and OPMD 4 ($p<0.05$) (Table 6).

Table 3: Comparing mean of leukoplakia subjects and controls separately using T test

	Group Means	N	Mean	Std. Deviation	P
Baseline Bound SSA	1	35	2.8116	0.262311	0
	6	50	1.64906	0.91218	0
Baseline Free SSA	1	35	3.60937	0.413342	0
	6	50	2.4608	0.920247	0
Bound SSA 6 Month	1	35	2.26823	0.350968	0
	6	50	1.64906	0.91218	0
Free SSA 6 Month	1	35	3.14009	0.414822	0
	6	50	2.4608	0.920247	0

Table 4: Comparing mean of oral submucous fibrosis subjects and controls separately using T test

	Group Means	N	Mean	Std. Deviation	P
Baseline Bound SSA	2	5	3.1672	0.220493	0.001
	6	50	1.64906	0.91218	0
Baseline Free SSA	2	5	4.3706	0.575649	0
	6	50	2.4608	0.920247	0
Bound SSA 6 Month	2	5	2.7204	0.340626	0.012
	6	50	1.64906	0.91218	0
Free SSA 6 Month	2	5	4.1406	0.306264	0
	6	50	2.4608	0.920247	0

Table 5: Comparing mean of erythroplakia subjects and controls separately using T test

	Group Means	N	Mean	Std. Deviation	P
Baseline Bound SSA	3	2	2.3895	0.210011	0.261
	6	50	1.64906	0.91218	0.032
Baseline Free SSA	3	2	3.0235	0.187383	0.396
	6	50	2.4608	0.920247	0.042
Bound SSA 6 Month	3	2	2.831	0.537401	0.077
	6	50	1.64906	0.91218	0.167
Free SSA 6 Month	3	2	3.784	0.159806	0.049
	6	50	2.4608	0.920247	0

Table 6: Comparing mean of oral lichen planus subjects and controls separately using T test

	Group Means	N	Mean	Std. Deviation	P
Baseline Bound SSA	4	7	2.84557	0.163342	0.001
	6	50	1.64906	0.91218	0
Baseline Free SSA	4	7	4.29329	0.310539	0
	6	50	2.4608	0.920247	0
Bound SSA 6 Month	4	7	2.44	0.290803	0.022
	6	50	1.64906	0.91218	0
Free SSA 6 Month	4	7	3.91586	0.278148	0.001
	6	50	2.4608	0.920247	0

Table 7: Comparing mean of proliferative verrucous leukoplakia subjects and controls separately using T test

	Group Means	N	Mean	Std. Deviation	P
Baseline Bound SSA	5	1	3.46	.	0.055
	6	50	1.64906	0.91218	.
Baseline Free SSA	5	1	5.32	.	0.003
	6	50	2.4608	0.920247	.
Bound SSA 6 Month	5	1	2.37	.	0.503
	6	50	1.64906	0.91218	.
Free SSA 6 Month	5	1	4.55	.	0.029
	6	50	2.4608	0.920247	.

4. Discussion

The term 'Sialic Acid', was derived from the Greek word “Sialos” meaning “Saliva” and was first introduced by Swedish biochemist, Gunnar Blix in 1952. Sialic acid is a generic term for the *N*- or *O*-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon backbone.[12] Sialic acid is a protein-bound monosaccharide which occurs in combination with other mono-saccharides like galactose, mannose, glucosamine and fucose.[13] Its level is studied for the evaluation of synthesis and secretion of glycoprotein.[14] Normally they can be found as components of both external and internal membrane areas where they are very exposed and develop important functions.[15] Serum sialic acid levels has been known to increase in patients with various malignancies and also in conditions like acute inflammation, high fever and rheumatoid arthritis.[13]

Aberrant glycosylations are the universal feature of cancer and the levels of these glycoconjugates increases as the cancer advances. The increase in sialylation in cancer may be due to overexpression of sialyltransferases.[16] Metastatic cancer cells often express a high density of sialic acid-rich glycoproteins. This overexpression of sialic acid on surfaces

creates a negative charge on cell membranes. This creates repulsion between cells (cell opposition)[17] and helps these late-stage cancer cells enter the blood stream. Studies have shown that changes of serum sialic acid levels in cancer patients correlate well with reduction in tumor mass, recurrence and metastasis and has been considered as a valuable tumor marker in monitoring the clinical status of the carcinoma patient.[17]

Oral potentially malignant disorders (OPMDs) occurring in about 2.5% of the general population include a variety of lesions and conditions characterized by an increased risk for malignant transformation to oral squamous cell carcinoma (OSCC). Correct diagnosis and timely treatment of PMDs at an early stage is of utmost importance to prevent further morbidity as they have shown a rate of progression and cancer transformation of up to 17% within a mean period of 7 years after diagnosis.[5]

In our study, the mean value of bound SSA and free SSA of OPMD group as a whole was 3.648 ± 0.289 mg/dL and 3.559 ±0.554 mg/dL respectively, the difference in salivary sialic acid in both groups was found to be significant (P<0.05). while that of control group was 1.649 ±0.376 and 2.460 ±0.376 mg/dL respectively. Results showed that there were increased levels of bound salivary sialic acid in PMD subjects when compared with normal or control group.

A study on salivary sialic acid in patients with different clinico-pathological stages of oral leukoplakia and oral squamous cell carcinoma showed results similar to our study where sialic acid (free & protein bound) levels were significantly increased in oral squamous cell carcinoma patients and oral leukoplakia patients compared to controls. Significant higher levels of free sialic acid and protein bound sialic acid were found in well-differentiated OSCC cases when compared to moderate and poorly differntiated grades.[18]

A significant rise (P < 0.005) in salivary sialic acid was observed in the study conducted by Dadhich M et al[19], in which the mean serum sialic acid levels in normal, leukoplakia and oral cancer group as 7.515, 19.620, and 55.235 mg/dL, respectively, whereas the levels of salivary sialic acid were 1.5113, 2.3302, and 9.0304 mg/dL, respectively.

The mean value of bound SSA and free SSA of OPMD group of the present study was 3.648 ± 0.289 mg/dL and 3.559 ±0.554 mg/dL respectively and was compared with a study conducted by Sanjay P R et al[20]. Results of this study were consistent with our study and gave values of bound SSA and free SSA in OSCC as 3.02±.01 and 4.3±0.01 respectively.

The sub-groups of PMD involved in our study were leukoplakia, oral submucous fibrosis, erythroplakia, oral lichen planus, proliferative verrucous leukoplakia which were the commonly reported cases in the institution. The mean of bound SSA and free SSA of leukoplakia subgroup was 2.5±0.30 and 3.3±0.41 respectively, oral submucous fibrosis subgroup was 2.92±0.28 and 4.41±0.43, erythroplakia subgroup was 2.58±0.38 and 3.45±0.16, oral lichen planus was 2.63±0.23 and 3.95±0.29 and proliferative verrucous leukoplakia subgroup was 2.86±0.00 and 4.95±0.00 respectively. The means were compared with normal or control subjects and were found to be statistically significant (P<0.05).

But there are no other studies with OPMD's involving erythroplakia, oral lichen planus, oral submucous fibrosis, proliferative verrucous leukoplakia to compare our results with and as there was a major difference in the sample size between the compared groups, statistical bias should be taken into consideration. Further studies with ample sample size in each subgroup of OPMD are required to corroborate the statistical significance of our study. However detectable amount of bound SSA and free SSA were noted in all the OPMD's studied. Correlating with previous studies on leukoplakia, it would not be hasty to conclude that other OPMD's also does show a significant difference of SSA as in oral leukoplakia.

Saliva as compared to serum is easy to collect, store and ship. Saliva as a diagnostic medium is less expensive, less invasive and has less risk both to patient and clinician. Although there is a lower amount of salivary sialic acid in saliva when compared to serum, detectable amount can be appreciated in subjects with PMD of oral cavity and oral cancer when compared to normal subjects. Results of our study also shows that there is an increase in levels of bound salivary sialic acid in PMD subjects when compared with normal or control group and this difference is statistically significant. Thus bound salivary sialic acid appears to be a potential biomarker and after validation studies on a larger sample size these parameters could be used in future as a non-invasive method of screening of OPMD's for their malignant transformation potential.

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