Original Article

Ratio of Salivary Sialic Acid to Fucose as Tumor Markers in Potentially Malignant Disorders and Oral Cancer

Anandhi Sekar Arthisri, Asokan Sathiyamoorthy[1], Bhagavatham Meenakshi[2], Chitraa R Chandran[3]

Department of Oral Medicine and Radiology, Ragas Dental College and Hospital, Departments of [1]Oral Medicine and Radiology, [2]Biochemistry and [3]Periodontics, Tagore Dental College and Hospital, Chennai, Tamil Nadu, India

Abstract

Background: Lack of noninvasive and economically feasible laboratory investigations in the early detection of cancer is a set-back in the prognosis of oral cancer (OC) patients. **Aims:** The aim of the study was to evaluate the ratio of sialic acid to fucose in salivary samples of OC and oral potentially malignant disorder (OPMD) patients. **Materials and Methods:** A total of 60 participants were selected and divided into three groups based on clinical and histopathological diagnosis: OC patients (n = 20), OPMD patients (n = 20), and healthy patients (n = 20). Unstimulated whole saliva of 1.5 ml was collected from the selected individuals for evaluating the salivary levels of sialic acid and fucose using the biochemical assay. **Results:** The difference in mean salivary sialic acid and fucose among the study groups was statistically significant (P = 0.001); one-way analysis of variance. The mean sialic acid to fucose ratio in the control group, OPMD group, and OC group were 0.34 mg/dl, 0.88 mg/dl, and 0.89 mg/dl, respectively. OC patients had significant elevation in the levels of salivary sialic acid, fucose, and their ratios (P = 0.001, P = 0.001, P = 0.003, respectively); Tukey's *post hoc* test. **Conclusions:** The ratio of salivary sialic acid to fucose is a predictable tumor marker for the diagnosis of OC. Further investigations are required to evaluate the influence of OC grading on this diagnostic marker.

Keywords: *Fucose, oral cancer, saliva, sialic acid, tumor marker*

Introduction

Oral cancer (OC) remains as one of the major causes of death, accounting for nearly 50%–70% of total cancer mortality and is a matter of great concern as 80,000 new cases of OC are reported each year.[1] Early detection of OC is the most effective way to improve survival rates.[2] Biochemical changes have been occurring in biological fluids and tissues of different types of malignancies. Most molecules found in blood and urine are found in saliva, but their concentrations were estimated to be tenth to one thousandth of that in the blood. Salivary analysis can also be a new diagnostic tool for OC.[3] Saliva, composed of a wide variety of organic and inorganic constituents, is a complex fluid that collectively act to regulate the oral environment.[4]

Tumor markers or biochemical serum markers involves all the substances in the serum that change quantitatively during tumor growth. These markers are released in large quantities by malignant cells into the circulation.[5] Tumor markers play a major role in the secondary prevention and in the detection of malignancies.[6]

The control of a normal cell proliferation depends on the genetic changes which are expressed on the cell membrane which, in turn, is made of glycoproteins and glycolipids.[7,8] The terminal end of the glycoconjugates are attached to sialic acid which are crucial in cell-cell recognition, adhesiveness, and invasiveness. The malignant cells express an alteration in the sialic acid content of the glycoconjugates, and this seems to be an initial event in carcinogenesis.[9] The elevated glycoconjugates are shed from tumor cells and released into circulation.

Fucose is a monosaccharide that is a common component of many N and O-linked glycans and glycolipids produced by mammalian cells.[10] Similarly, fucose is a deoxyhexose sugar that the body requires for optimal functions of cell to cell communications which plays a role in several biological events which mediate

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Address for correspondence:
Dr. Anandhi Sekar Arthisri, Department of Oral Medicine and Radiology, Ragas Dental College and Hospital, Chennai - 600 119, Tamil Nadu, India.
E-mail: thillaarthisri@yahoo.com

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inflammation, embryonic development, carcinogenesis, and antigen recognition.[11] Cellular glycosylation changes are associated with different types of neoplastic transformation.[12-15] Fucose has been considered to play a significant role in cancer transformation and its spread.[16]

It has already been reported that changes of serum sialic acid and fucose levels in cancer patients relate well with decrease in tumor mass, relapse, and metastasis of the disease.[17-19] In addition to OC patients, similar changes of salivary sialic acid and fucose levels have also been observed in patients with oral potentially malignant disorder (OPMD).[20] Therefore, monitoring the salivary sialic acid and fucose levels will be a promising approach for the early diagnosis of OPMD and prognosis of OC.

Various studies have been performed the evaluation of serum and salivary sialic acid levels among OPMD and OC patients.[21-24] Few studies have evaluated the salivary fucose levels in normal and OC patients.[1,20] Chinnannavar *et al*. in 2016 have evaluated the serum sialic acid and fucose levels among patients with OPMD and OC. They concluded that the ratio of sialic acid to fucose can be used as a diagnostic marker in OC patients.[20] However, similar ratio has not been analyzed in the salivary samples of OC patients.

Therefore, the aim of this preliminary study is to perform a comparative assessment of the salivary sialic acid, fucose, and their ratios in patients with OPMD and OC.

Materials and Methods

This study was approved by the Institutional Ethical Committee (Ref no: IEC/TDCH/084/2016). A total of 60 participants were selected from the patients visiting the oral medicine and radiology department in the college. People with known history of systemic abnormalities such as hypertension, diabetes, pregnancy, renal problems, cystic fibrosis, TB, viral diseases such as HIV and salivary gland disorders, and individuals below 20 years and above 60 years were excluded from the study. Patients were divided into three groups based on the clinical and histopathological diagnosis: OC group (n = 20), OPMD group (n = 20), and healthy group (n = 20). Patients with histopathologically diagnosed OC and OPMD (leukoplakia, erythroplakia, lichen planus, and oral submucous fibrosis) were included in Group B and C, respectively.

Collection of saliva

Informed consents were obtained from the patients for carrying out biopsy and salivary analysis. Unstimulated whole saliva of 1.5 ml was collected from the selected individuals using draining method during morning hours to minimize the changes in salivary composition as a result of diurnal variation. The patients were advised to restrain intake of food, beverages, or chewing gum an hour before sample collection. They were instructed to rinse the mouth with distilled water to remove any food debris. Saliva samples were centrifuged at 3000 rpm and the supernatant were separated and stored at −80°C till further analysis. The estimation of salivary levels of sialic acid and fucose in healthy, OPMD, and OC patients was performed.

Estimation of sialic acid

It was performed by Ninhydrin method[25] using spectrophotometer (Deep Vision, Cordoba, Argentina). Acidic Ninhydrin Reagent was prepared by dissolving 250 mg ninhydrin (SLR Diagnostics, Gurugram, India) in 6 ml of glacial acetic acid and 4 ml of concentrated Sulphuric acid, by vortexing for 30 min. Later 0.1 ml saliva was added to 0.9 ml of normal saline, to this 4 ml ethyl alcohol was mixed and centrifuged at 3000 rpm for 30 min. The supernatant was kept separately. The precipitate obtained was dissolved in 1 ml of distilled water and to this 1 ml of glacial acetic acid and 1 ml of acid ninhydrin agent (freshly prepared) was added. This was labeled as protein-bound sialic acid. 1 ml of glacial acetic acid and 1 ml of acid ninhydrin reagent was added to 1 ml of supernatant collected earlier. This was labelled as free sialic acid. Protein bound sialic acid tubes and free sialic acid tubes were kept in the boiling water bath for 10 min, cooled under tap water, and absorbance was read at 470 nm using spectrophotometer.

Standards (N acetyl Neuraminic acid [NANA]) were prepared by dissolving 10 mg NANA (SLR Diagnostics) in 100 ml distilled water. NANA standards ranging in concentration from 20 to 100 ug/ml (0.2–1 ml) were run simultaneously. The optical density value of sample was compared with that of standards at various concentrations S1, S2, S3, S4 and S5, and calculated accordingly. The concentration of sialic acid in each sample was expressed in mg/dl.

Estimation of fucose

It was done based on the method of Shettles and Shettles[26] and Winzler.[27] Fucose can be analyzed by dissolving ethanol precipitated proteins of serum/saliva in alkali, heating with sulfuric acid, and determining the color after the addition of cysteine reagent.

Initially sulfuric acid mixture was prepared by dissolving 60 ml of concentrated sulfuric acid in 10 ml of water and kept refrigerated until use. Cysteine reagent (3%) was prepared by adding 3 g in 100 ml of water. Next 400 mg of NaOH was dissolved in water and made up to 100 ml. Stock Standard was prepared by dissolving 200 mg of fucose (SLR diagnostics) in 100 ml of water and working standard was prepared by diluting 0.1 ml of stock solution in 10 ml of water.

Two test tubes, one named blank and the other named test were taken. To these 0.1 ml of saliva, 5 ml of 95% ethyl alcohol were added and triturated in a Vortex mixer.

The tubes were centrifuged for 15 min (1500 rpm). The precipitate was suspended in 5 ml of 95% ethyl alcohol, recentrifuged for 15 min, and then the supernatant was decanted. The precipitate was dissolved in 1 ml of 0.1N NaOH. Reagent blank and standard tubes were prepared by adding 1 ml of distilled water and 1 ml of working standard to approximately marked 15 mm × 150 mm test tubes. All the test tubes (both standard, test and blank) were placed in a stand in ice water bath. To the above 4.5 ml of cold sulfuric acid-water mixture was added to each tube and triturated. The solution was transferred to already boiling water bath, heated for 3 min and cooled in tap water. Cysteine reagent (0.1 ml) was added to reagent blank, standard tube and test tube. After 60 min, the solutions were transferred to appropriate cuvettes, and absorbance was read at 400 nm and 430 nm in spectrophotometer set at zero with the reagent blank.

Results

The collected data were analyzed with the SPSS statistics software 23.0 Version (SPSS Inc. Chicago, IL, USA). The biochemical parameters were expressed as mean and standard deviation. One-way analysis of variance (ANOVA) followed by Tukey HSD *post hoc* test for evaluating the significance of mean difference ($P < 0.05$). The mean salivary sialic acid levels and fucose levels in the healthy patient group were 1.35 mg/dl and 3.91 mg/dl respectively. In OPMD group, the mean salivary sialic acid levels and fucose level were 4.27 mg/dl and 4.83 mg/dl respectively. In OC group, the mean sialic acid levels and fucose levels were 5.30 mg/dl and 6.14 mg/dl. The difference in salivary sialic acid and fucose levels in all the study groups was highly significant ($P = 0.004$) [Table 1].

The ratio of sialic acid to fucose was relatively higher in the OC group (0.89 ± 0.67 mg/dl) when compared with OPMD group (0.88 ± 0.75 mg/dl) [Table 1].

On comparison of salivary sialic acid and fucose between the study groups using one-way ANOVA with Tukey's *post hoc* test, the results were statistically significant. The ratio of salivary sialic acid to fucose was highly significant between the healthy group and OC group ($P = 0.003$) [Table 2].

Discussion

Glycoproteins and glycolipids are the major components of the cell membrane.[7] Sialic acid-rich glycoproteins (sialo glycoproteins) binds the selectin in humans. High density of sialic acid rich glycoprotein is expressed by cancer cells. Thus, increased expression of sialic acid on surfaces produces a negative charge on the cell membranes, which creates cell opposition and helps these late-stage cancer cells to enter the blood stream.[28] Fucose a natural deoxyhexose sugar is incorporated onto the glycoproteins during the synthesis of N-and O-linked glycans in mammalian cells. Fucosylated glycans play an important role in signal transduction, cell growth, transcription, and adhesion.[29] Cancer is characterized by the deregulation of normal cellular and molecular processes which results in increased fucosylation.[30]

Previous studies have proved that out of several glycoproteins the sialic acid and fucose levels are increased in the circulation in both serum and saliva.[22,31] The main challenge of using saliva as a diagnostic tool is that the release of glycoprotein levels is comparatively less in saliva than serum. To scrutinize this Pradeep *et al*. compared the serum and salivary sialic acid and fucose levels in OC patients. The results of the study revealed detectable amounts of both sialic acids, fucose are present and increased in the saliva of OC patients.[1]

Salivary diagnostics is a dynamic field that is being incorporated as part of disease diagnosis in the recent years.[32] Hence, we have evaluated the salivary sialic acid, fucose levels and their ratios in normal individual, patients with OPMD and OC.

In the present study, the salivary sialic acid levels were highly significant among the study groups. The mean salivary sialic acid level in the control group, OPMD group and OC group was 1.354 mg/dl, 4.277 mg/dl, and 5.300 mg/dl, respectively, which is in complete agreement with other studies performed earlier.[21-23]

Sanjay *et al*. has evaluated the role of sialic acid as a marker of oral squamous cell carcinoma and suggested the correlation of elevated salivary sialic acid levels to the progression of oral squamous cell carcinoma.[21] Chaudhari *et al*. estimated the salivary sialic acid levels in patients with OPMD and squamous cell carcinoma and concluded that salivary sialic acid can be used as a cost effective, noninvasive diagnostic parameter for OC.[23]

In our study, the salivary fucose levels were significantly high in the patients with OPMD and OC. The mean

Table 1: Salivary sialic acid level, fucose level, and their ratios in the study groups

Groups	*n*	Sialic acid		Fucose		Sialic acid to fucose ratio	
		Mean±SD (mg/dl)	*P*	Mean±SD (mg/dl)	*P*	Mean±SD (mg/dl)	*P*
OC	20	5.30±1.45	0.001*	6.14±2.16	0.016*	0.89±0.67	0.004*
OPMD	20	4.27±2.19		4.83±2.90		0.88±0.75	
Healthy	20	1.35±1.53		3.91±1.94		0.34±0.78	

One-way ANOVA test, *Statistically significant at ($P<0.05$). OC: Oral cancer; OPMD: Oral potential malignant disorder; SD: Standard deviation; ANOVA: Analysis of variance

Table 2: Comparison of salivary sialic acid, fucose levels, and their ratios between the study groups

Dependent variable	Groups	Comparative group	Mean difference (mg/dl)	*P*
Sialic acid	Healthy	OPMD	−2.92	0.001*
		OC	−3.94	0.001*
	OC	OPMD	−1.02	0.001*
Fucose	Healthy	OPMD	−2.19	0.001*
		OC	−2.23	0.001*
	OC	OPMD	−3.92	0.016
Sialic acid to fucose ratio	Healthy	OPMD	−0.538	0.016
		OC	−0.516	0.003*
	OC	OPMD	−0.021	0.107

Tukey HSD, *Statistically significant (P<0.016). OC: Oral cancer, OPMD: Oral potential malignant disorder, SD: Standard deviation; HSD: Honestly significant difference

salivary fucose level in the control group, OPMD group and OC group was 3.192 mg/dl, 4.837 mg/dl, and 6.145 mg/dl, respectively. On reviewing the literature, only one study has been conducted to estimate the fucose levels in the saliva of OC patients, the result of which was consistent with our study.[1]

In one study, the author[1] has estimated the serum sialic acid, fucose levels and their ratio in OSCC patients and deduced that sialic acid to fucose ratio would be a more specific diagnostic biomarker for OC if further studies are intended in this aspect.

In another study, the author has used the serum samples to estimate the sialic acid, fucose levels, and their ratio in OC patients and subsequently appraised the potential of sialic acid to fucose ratio as a highly specific diagnostic biomarker in such patients.[20]

Few of the factors affecting the biochemical composition of salivary components including sialic acid and fucose are salivary flow rate, viscosity and pathophysiology of the oral cavity.[33] The calculation of sialic acid to fucose ratio remains unaffected by any of these factors mentioned above, thereby making it as a reliable diagnostic parameter. To the best of our insight, no studies have focused on evaluating the ratio of sialic acid to fucose in saliva of OC patients. In the present study, salivary sialic acid to fucose ratio was significantly elevated in OC group, suggestive of its use as a diagnostic tumor marker.

According to Chittemsetti *et al*.[34] and Chaudhari *et al*.,[23] the levels of sialic acid increase progressively from histopathological Grades 1–4 in oral squamous cell carcinoma patients and patients with OPMD based on Bryne's Grading system. Further research should be focused on evaluating the ratio of salivary sialic acid to fucose based on histopathological grading of OC with larger sample size and better techniques for the estimation of salivary sialic acid and fucose.

Conclusions

Many studies have spotlighted the use of salivary sialic acid as a biomarker for the diagnosis of OC. Merely a sprinkling number of studies have implicated the use of salivary fucose as a diagnostic marker. We have evaluated the ratio of salivary sialic acid to fucose, thereby implicating it to be a more distinctive diagnostic tool for OC. This study would be an insinuate for conducting numerous studies in this facet for early diagnosis of OC with cost effective and noninvasive diagnostic tool like saliva.

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Conflicts of interest

There are no conflicts of interest.

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