Salivary Expression of Sclerotium Rolfsii Lectin – Binding Mucin-Type Glycoprotein in Human Oral Cancer.

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ABSTRACT

Aim: Oral cancer is a major health problem in India associated with significant morbidity and mortality. Due to the absence of truly specific symptoms, the early diagnosis of oral cancer is quite challenging. Aberrant glycosylation of cell surface glycoconjugates is an important feature of malignant changes. Abnormal glycosylation in cancer leads to formation of altered glycoconjugates such as Thomsen – Friedenreich (TF) antigen. In this study we have analyzed saliva for various constituents and used a novel, TF antigen binding fungal lectin (SRL) from Sclerotium rolfsii, to detect glycosylation changes in oral squamous cell carcinoma (OSCC).

Materials and Methods: Unstimulated whole 30 saliva samples each collected from OSCC patients and normal healthy individuals and were analyzed for biochemical constituents and subjected to lectin precipitation assay using SRL. The results were compared between healthy and OSCC subjects.

Results: Our results showed that levels of salivary constituents like proteins, sugars, hexosamines and sialic acids were significantly high in OSCC patients compared to healthy controls. Lectin precipitation assay with SRL revealed the presence of altered mucin-type glycoproteins in saliva of OSCC patients in significantly higher quantity as compared to healthy individuals.

Conclusion: A non invasive method like salivary analysis can be developed for the early diagnosis of OSCC and SRL can be developed as a probe to detect and quantify salivary TF antigen in OSCC.

Key words: OSCC, TF antigen, SRL, saliva, salivary glycoprotein

1. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy that is the sixth most common neoplasm in the world [1]. Around 95% of the HNSCC arise most commonly in the oral cavity. Oral cancer is a major problem in India too, which accounts for 50 to 70% of all cancers diagnosed [2]. This high incidence of oral cancer is attributed to chronic abuse of tobacco. The early diagnosis of oral carcinoma tends to be a problem because there are no truly specific early symptoms. It is probable that, even with different therapeutic strategies tumor cells remain in patients resulting in local and / or distant relapse. Therefore, current methods have some limitations in respect of their sensitivity, suggesting that new strategies should be developed. In this context, the identification of tumor markers is crucial. Many glycoproteins and glycolipids have been described as tumor-associated antigens since they may be over expressed or be different to normal as a consequence in alteration in their synthesis.

Cell surface carbohydrates are modified upon malignant transformation, tumor cell differentiation and metastasis [3, 4, 5]. Altered glycosylation of cell surface glycoconjugates may result from any of these events, such as increased sialylation or desialylation, increased branching, increased fucosylation or altered sugar chain sequence. These glycoforms are released into the circulation through increased turn over, secretion, and / or shedding from malignant cells. Such aberrant glycosylation, a universal feature of cancer cells, results in the formation of mucin-type antigens such as Thomsen-Friedenreich (TF) antigen, Tn and sialyl Tn antigens in a variety of cancers.
Among these mucin-type antigens, the extensively studied is the TF antigen, a blood group antigen which is characterized by class 1 core sequence Galβ1 – 3GalNAc-α-O-ser/thr in oligosaccharide chains [9, 10]. TF antigen is considered as pan carcinoma antigen as it is expressed in varieties of cancers [11]. Cancer associated antigens occurring in mucins and mucin - type glycoproteins have been of special interest in cancer biology for studying novel cancer cell surface molecules [12, 13] and lectins binding to such marker antigens are widely employed for detection of cancer - associated glycosylation changes [13, 14]. The exquisite carbohydrate specificity of lectins makes them particularly useful for detection and quantification of mucins [15].

Lectins are a class of carbohydrate binding proteins, commonly detected by their ability to precipitate glycoconjugates in solution or to agglutinate cells. Lectins form cross-links between polysaccharides or glycoproteins in solution and induce their precipitation, which can be inhibited by the sugar ligands for which the lectins are specific. Analogy of lectin-saccharide interactions with those of antibody-antigen reactions prompts the application of lectins in the detection, quantification and purification of glycoproteins and glycopeptides in solution [16].

Salivary mucins being the major constituents of saliva, are characterized by high sugar to protein ratio, a seemingly subtle change in sugar sequence of these mucous glycoproteins may indicate the onset of disease, such as is observed in cancer [7]. The immunotherapeutic and / or diagnostic, metastatic potential of some of these mucin structures such as Galβ1-3GalNAc (TF antigen), sialosyl-Tn and Tn have been demonstrated in human saliva [17].

The paucity of reports on compositional changes associated with salivary glycoproteins and the salivary expression of cancer associated mucin-type glycoproteins in OSCC, which utilized lectins to recognize and to quantify these glycoproteins in saliva, prompted us to use a lectin from phytopathogenic fungus *Sclerotium rolfsii* (SRL), a novel TF antigen - binding lectin, which was identified and purified in our laboratory [18, 19].

2. MATERIALS AND METHODS
2.1. Study population and data collection
The study group consisted of 30 OSCC patients. OSCC was diagnosed clinically and histopathologically during their visit to the Oral and Maxillofacial Surgery Unit, SDM College of Dental Sciences and Hospital, Dharwad, India. The control group consisted of 30 normal, apparently healthy, age and sex matched subjects. The methodology was reviewed and permission was granted for the study by the institutional ethical committee. The study was carried out with the consent of study subjects.

2.2. Collection of Saliva samples:
The saliva samples from OSCC patients were collected prior to any therapeutic intervention. Unstimulated whole saliva samples were collected 2 hours after the subject’s breakfast to minimize diurnal variations in salivary flow and composition. The subject was asked to rinse the mouth with sterile distilled water thoroughly to remove any food debris. After 10 minutes the subject was directed to spit into a chilled wide-mouth sterile container for a period of 5 minutes [20]. Saliva samples were centrifuged (Kubota refrigerated centrifuge RA 300 F, angular rotor) at 4°C at a speed of 10,000 rpm for 30 minutes. The supernatant was diluted and was filtered through a membrane filter (Filtech Pharmalab Pvt. Ltd. Mumbai, India). The filtrate was
subjected to extensive dialysis against distilled water. Aliquots of the dialyzed samples were lyophilized and stored at –20 °C.

2.3. Analysis of Salivary Constituents:

Lyophilized saliva samples were used for analysis of biochemical constituents. Levels of salivary total proteins, total sugars, hexosamines and total sialic acids were determined and were compared between the study group and the test group.

Total proteins were estimated by using Bovine Serum Albumin (BSA) as standard [21]. Salivary total sugars were estimated by Phenol-sulphuric acid method with some modifications using D-Glucose as standard [22, 23].

Further, salivary hexosamines were estimated using N-acetyl D-glucosamine as standard [24]. Salivary total Sialic acids were estimated by Ferric-Orcinol method using N- acetyl Neuraminic Acid (NANA) as standard [25].

2.4. Purification of SRL:

*Sclerotium rolfsii* lectin (SRL) was purified from the sclerotial bodies. In brief, *Sclerotium rolfsii* was grown in corn-sand culture medium as it promotes larger and higher yield of sclerotial bodies. These sclerotial bodies were harvested and ground to fine powder which was used for preparing the crude extract of lectin. Crude extract of the lectin was prepared in 50mM acetate buffer containing 150mM NaCl, pH 4.3 and this was subjected to methanol precipitation (0 – 30%). This precipitate was used for further purification of lectin using cation exchange chromatography on CM Cellulose. These chromatography fractions were used for further purification of lectin by gel filtration Chromatography on Bio Gel P-60 and finally the lyophilized Bio Gel P-60 fractions of lectin were subjected to Gel filtration Chromatography on Sephadex G–75. The purified lectin fractions were lyophilized and used in the study [18, 19]

2.5. Lectin - precipitation assay:

Quantitative precipitation assays were carried out to detect and quantify the SRL-reacting salivary glycoproteins in the saliva of cancer patients and normal individuals. Precipitation assay was performed in duplicate in 1.5ml micro centrifuge tubes [26].

Purified SRL (80 μg) in PBS was incubated with varying concentration of salivary glycoproteins (0 – 140 μg) in micro centrifuge tubes in a total volume of 300μl for 4 days at 4°C. The precipitate formed was dissolved in 500 μl of 50mM phosphate buffer (1.0M NaCl, pH 7.2). After centrifugation, the clear supernatant was used for determining protein [21]. Protein precipitated was plotted against the salivary glycoprotein concentrations added.

2.6. Statistical analysis:

Levels of salivary constituents were compared between two groups. Ratios of total sugar to total protein, hexosamines to total sugar and sialic acids to total sugar was determined to know any relationship between these variations. Student’s ‘t’ test was employed to analyze these data. As the precipitation assay was carried out in duplicate, the values were reported as mean of duplicates. The average of protein precipitated was plotted against each concentration of salivary glycoprotein added, for both the groups. The results were compared between two groups.
3. RESULTS:
3.1. Analysis of Salivary Constituents:

Salivary levels of total proteins, total sugars, hexosamines and bound sialic acids were significantly higher in OSCC patients as compared to those of normal healthy controls (Table 1). We observed a two-fold increase in protein and sugar content in the saliva of OSCC patients as compared to that of healthy subjects. Interestingly, a profound difference in the hexosamines level within the members of OSCC group ranging from a minimum of 0.5 mg/dl to maximum of 5.5 mg/dl was observed. Whereas in the normal group slight variation was seen ranging between 1.0 mg/dl and 1.4 mg/dl (data not shown). Sugar to protein ratio was determined to know whether the enhanced expression of proteins is also augmented with glycosylation of these proteins. However, we observed that there is no appreciable difference in sugar: protein ratio between two groups (data not shown).

Further, in order to understand whether the increased levels of hexosamines and sialic acids are linearly related to increase in total sugars, the ratio of concentration of hexosamines and sialic acids to total sugars were calculated, but no significant correlation could be formed. (data not shown).

3.2. Lectin precipitation assay:

The average of protein precipitated was calculated for each concentration of salivary glycoprotein added. The average of protein precipitated was plotted against the salivary glycoprotein concentration (Graph 1).

We observed that the protein precipitated against each concentration of salivary glycoprotein was nil or negligibly low with all the control samples ranging between 0.43 to 4.48 µg (average 2.97 µg), indicating the absence of any SRL-binding glycoprotein in these samples. On the contrary, the protein precipitated against each concentration of salivary glycoproteins of cancer patients was high, ranging between 28.57 µg and 69.12 µg, average being 52.31 µg (Table 2). This indicated the occurrence of the SRL-interacting glycoprotein/s in all OSCC patients in a very high concentration.

An interesting observation was that in case of control group, the rise in mean precipitation was negligible with the increase in glycoprotein concentration in contrast to test group where the mean precipitation rose up to 120µg of salivary glycoprotein concentration and then flattened as the glycoprotein concentration increased to 140 µg.

4. Discussion:

Specific cancer associated markers, expressed in saliva in OSCC, such as the tumor suppressor protein p53 [27], defensins [28], well understood tumor marker such as c-erbB-2, cancer antigens; CA15-3 and CA 125 [29, 30] are extensively studied. Tumor markers that can be identified in saliva may be potentially useful for screening malignant diseases in general and oral cancer in specific. Since such markers would require invariably expensive monoclonal antibodies, sophisticated resources and further identification, our efforts were aimed to identify simple markers and to develop cost effective methods to investigate saliva.

In the present study we have attempted to detect glycosylation changes in saliva during OSCC and explore the possibility of developing salivary biochemical constituents as cancer markers in OSCC. As a prelude we analyzed salivary components like total proteins, total sugars, hexosamines and sialic acids.
Table 1

Comparison of salivary constituents in test group and control group

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Group (n=30)</th>
<th>Test Group (n=30)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total proteins</td>
<td>79.8 ± 1.15</td>
<td>168.5 ±4.18</td>
<td>S*</td>
</tr>
<tr>
<td>Total sugars</td>
<td>14.7 ± 0.29</td>
<td>28 ± 0.56</td>
<td>S*</td>
</tr>
<tr>
<td>Total Hexosamines</td>
<td>1.2 ± 0.01</td>
<td>2.7 ± 0.18</td>
<td>S*</td>
</tr>
<tr>
<td>Protein-bound sialic acid</td>
<td>2.1 ± 0.05</td>
<td>5.3 ± 0.28</td>
<td>S*</td>
</tr>
</tbody>
</table>

a) Values expressed as mean ± Standard Deviation; S*= Significant

Table 2

Lectin - precipitation assay – Protein precipitated in Control and Test group

<table>
<thead>
<tr>
<th>Salivary glycoproteins (µg)</th>
<th>Protein precipitated (Average)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control group</td>
</tr>
<tr>
<td>20</td>
<td>0.43</td>
</tr>
<tr>
<td>40</td>
<td>1.45</td>
</tr>
<tr>
<td>60</td>
<td>2.49</td>
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<td>80</td>
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<tr>
<td>120</td>
<td>4.41</td>
</tr>
<tr>
<td>140</td>
<td>4.47</td>
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</tbody>
</table>

Graph 1

Lectin precipitation assay - Protein precipitated in normal and cancer saliva
There was a significant increase in these salivary components of cancer group as compared to healthy group. Similar observations were made by other workers also (31). Though we observed significantly high level of salivary sialic acids in the present study, over expression of sialic acids in saliva and serum were observed in noncancerous clinical conditions [32]. Interestingly analysis of salivary hexosamines showed profound differences within OSCC group which was not observed in case of healthy group. Hitherto hexosamines levels in saliva could be an exciting aspect and could have diagnostic value and needs further exploration as very few studies have reported changes in hexosamines in oral cancer [33]. This preliminary analysis also showed that there is overall increase in the secretion of glycoproteins in SCC of oral cavity.

Glycoconjugates form vital constituent of cell membrane. The malignant transformation is frequently associated with altered glycosylation of cell surface glycoconjugates. Aberrant glycosylation leads to the formation of mucin-type antigens in a variety of cancers [6, 8, 34]. Synthesis and secretion of mucin are the general features of glandular epithelial tissues and the expression of mucin antigens have been investigated extensively in adenocarcinomas [8, 34]. Though normal squamous cells do not secrete mucins, these antigens were observed in the serum of esophageal squamous cell carcinoma [35] and cervical squamous cell carcinoma patients [36]. Altered expression of mucin-type glycoproteins has also been reported from OSCC [7].

In this study we attempted to detect and quantify salivary mucin-type antigen in OSCC using a novel lectin, SRL. The results of lectin precipitation assay indicate that specific glycoproteins, which interact with SRL, occur in the saliva of OSCC patients but not in the saliva of healthy individuals. The assay also implies that the quantity of interacting glycoprotein component could be occurring in high concentration in a mixture in saliva in OSCC. Such observations were shown earlier by precipitation assays demonstrating the formation of Glycoprotein-lectin cross-linked complexes in mixed precipitation systems (37). From these results it was evident that specific glycoproteins, which interact with SRL, occur in the saliva of OSCC patients but not in the saliva of normal healthy individuals. Since SRL is known to bind specifically to TF antigen, it may be concluded that TF antigen is expressed in the salivary mucins. However, these salivary glycoproteins being heterogeneous complex mixture, in the present study, lectin glycoproteins cannot identify whether it is a specific component or multiple components that occur in saliva during OSCC that are recognized by SRL.

In conclusion, we would like to say that this study could be of greater significance for diagnostic purpose and also could open up new perspectives for the investigation of novel cancer-associated antigens in saliva. And SRL with its unique TF antigen binding property would be promising tool for probing aberrant glycosylation and the changes associated with cancer.

5. ACKNOWLEDGEMENTS:

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6. REFERENCES


