DETECTION OF HOST-SPECIFIC IMMUNOGENIC PROTEINS IN THE SALIVA OF PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA

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DETECTION OF HOST-SPECIFIC IMMUNOGENIC PROTEINS IN THE SALIVA OF PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA

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The main purpose of this article is to develop a new and reliable saliva-based clinical diagnostic method for the early detection of oral squamous cell carcinoma (OSCC). This study used an immunoproteomic approach which allowed the detection of immunogenic host proteins in patients’ samples using pooled human antibodies. In an attempt to investigate potential biomarkers of OSCC, two-dimensional electrophoresis (2-DE) followed by immunoblotting of saliva from patients and controls were compared. The protein spots of interest were analyzed using 2-DE image analyzer and subsequently subjected to MALDI-TOF/TOF and then matched against NCBI database. The result showed that four protein clusters, namely Human Pancreatic Alpha-amylase (HPA), Human Salivary Amylase (sAA), keratin-10 (K-10), and Ga Module Complexed with Human Serum Albumin (GA-HSA), had exhibited immunoreactivity in western blot. The results are suggestive of the potential use of the differentially expressed saliva protein as tumor biomarkers for the detection of OSCC. However, further studies are recommended to validate this finding.

Keywords: immunoblotting, oral squamous cell carcinoma, saliva immunogenic protein, silver staining, tumor biomarker, two-dimensional electrophoresis

INTRODUCTION

Oral cancer is a type of head and neck cancer.¹ It refers to all malignant lesions that are originated from the lips, oral cavity, and
pharynx.[2] It is the sixth most common cancer in the world, which constitutes approximately 2–3% of all malignancies, with more than 300,000 cases were newly diagnosed yearly worldwide.[2–5] Of all the cancer cases reported, 90% were oral squamous cell carcinoma (OSCC) due to its high degree of local invasiveness and high rate of metastasis.[2,4]

In 2007, Cancer Facts & Figures by American Cancer Society reported that overall 5-year survival rate for oral cancer still hovers around 50%, which is essentially unchanged during the past few decades although with the advances in surgery, chemotherapy, radiotherapy, and combined modalities therapy(CMT).[4–6] One of the major impediments to improve the survival rate in OSCC patient is the failure to detect OSCC at an early stage, which leads to high mortality rate.[2,4,6] Therefore, early detection of OSCC is very important because the early stage of OSCC patients have an excellent 5-year survival rate of 80%.[2,6] Until now, biopsy of suspicious lesion remains the gold standard method to confirm the diagnosis. However, this method becomes less common for screening purposes due to its invasiveness and non uniform appearance of (pre)cancerous lesions which may impede determination of site for biopsy and histopathological verification of oral cancer. Therefore, new screening tumor markers are essential to improve identification of OSCC at an early stage, particularly in at-risk populations.[4–6]

Saliva is a good diagnostic medium in the biomarker discovery for OSCC compared to other diagnostic bio-fluids, such as blood, urine, tears, and cerebral spinal fluid. It has been increasingly evaluated as a diagnostic fluid for detecting periodontitis, caries risk, oral cancer, breast cancer, salivary gland disease, and systemic disease, such as hepatitis.[6] Besides, compared with blood sampling or biopsy, advantages of using saliva for oral cancer screening include saliva collection is cost effective, safer, easier, less invasive and does not clot like blood samples.[2,6,7] Up until now, there have been a small number of studies that search for salivary protein markers as potential diagnostic marker for OSCC.[5] For example, defensin-1, interleukin-6 and the soluble form of CD44 were found to be elevated in OSCC patients compared with healthy individuals.[2,6,8]

This study applied the use of human immune response to capture certain unique immunogenic proteins in the saliva for early detection of OSCC. The human immune system, including IgM, definitely could respond to even low levels of antigenic or immunogenic proteins.[9] This natural IgM antibodies not only involve the early recognition of external invaders, such as bacteria and viruses, but also involve identification and eradication of precancerous and cancerous lesions.[10,11] Therefore, in this study, the immunoproteomic approach using 2-DE and immunoblotting method were chosen and developed to detect those immunogenic host proteins in patient’s saliva.
MATERIALS AND METHODS

Clinical Samples

For samples collection, the saliva and serum samples of oral squamous cell carcinoma (OSCC) were obtained from Malaysian Oral Cancer Database & Tissue Bank System (MOCDTBS) at Oral Cancer Research & Coordinating Center (OCRCC), Kuala Lumpur, Malaysia with consent and approval granted by the Ethical Committee of the University Malaya. A total of four saliva samples and four serum samples were collected in this study which included two control saliva samples, two control serum samples, two OSCC saliva samples, and two OSCC serum samples. Newly diagnosed OSCC patients who have never received any form of treatment were included in this study. No subject had a history of prior cancer or other systemic diseases.

Samples Collection and Preparation

The saliva samples were collected and centrifuged at 1585×g for 15 min at 4°C by using microcentrifuge 5415 R (Eppendorf, Hamburg, Germany) to remove debris and cells based on a modified method by Dowling et al.\textsuperscript{12} and Arellana-Garcia et al.\textsuperscript{13} The resulting supernatants were concentrated using Suprema 21 high-speed refrigerated centrifuge (TOMY, Tokyo, Japan) by centrifugation at 2683×g for 15 min at 4°C for further analysis. On the other hand, the blood samples were collected and spin down at 5000×g for 15 min at 4°C by using microcentrifuge 5415 R (Eppendorf).

2-D Electrophoresis

The concentrated supernatant saliva samples were subjected and rehydrated in 11 cm rehydrated precast Immobiline Drystrips pH 4–7 (GE Healthcare Bioscience, Uppsala, Sweden) overnight.\textsuperscript{12,14,15} Isoelectric focusing was carried out by using a Protean Isoelectric Focusing Cell (BioRad Inc., Berkeley, CA, USA). Focused sample in the gel strips were subjected to second-dimension electrophoresis separation by using the 10% linear polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS).\textsuperscript{12,14,15} All samples were analyzed in triplicate.

Silver Staining

The 2-DE gels were developed using the silver staining method described by Heukeshoven and Dernick.\textsuperscript{15,16} A modified silver staining method by Shevchenko et al. was used for mass spectrometry analysis.\textsuperscript{15,17}
Image Analysis

The silver-stained 2-DE gels were scanned using ImageQuant™ LAS500 (GE Healthcare Bioscience, Uppsala, Sweden). The Image Master 7.0 platinum was utilized to detect, match, and quantify protein spots. To compensate for non-expression related variations in protein spot intensity, the proteins that were differentially expressed in saliva were evaluated through normalization. The percentages of volume contributions (vol %) were used to calculate the proteins that were differentially expressed in saliva which referred as the percentage of a protein taken against the total spot volume of all proteins including the unresolved peptides in each gel.[14,15]

Immunoblotting

The ready ran 2-DE pooled saliva were divided into four categories (Table 1). Immunoblotting was performed using Trans-blot® Turbo™ Transfer Starter System (Bio-Rad). The separated proteins were transferred on a nitrocellulose membrane (0.45 μm) (Bio-Rad). Electroblotted membrane was then blocked with Superblock (Pierce, Rockford, IL, USA) for 1 hr and incubated overnight at 4°C with pooled serum of control individuals or pooled serum of OSCC patients as primary antibodies. Primary antibodies were removed and the membranes were washed with pH 7.5 Tris-buffered saline Tween-20 (TBS-T). The membranes were then incubated with monoclonal anti-human IgM-HRP (Invitrogen, Carlsbad, CA, USA) for 1 hr at room temperature. The membranes were then washed with TBS-T and developed using chemiluminescence substrate (Pierce). The signal of the membranes were visualized using chemiluminescent blotting reagent (Pierce) and the ImageQuant™ LAS500 (GE Healthcare).[14,15]

<table>
<thead>
<tr>
<th>Category</th>
<th>Sample on the Blotted Membrane</th>
<th>Primary Antibody</th>
<th>Immunogenic Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal pooled saliva</td>
<td>Normal pooled sera</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Normal pooled saliva</td>
<td>OSCC pooled sera</td>
<td>B</td>
</tr>
<tr>
<td>3</td>
<td>OSCC pooled saliva</td>
<td>Normal pooled sera</td>
<td>C</td>
</tr>
<tr>
<td>4</td>
<td>OSCC pooled saliva</td>
<td>OSCC pooled sera</td>
<td>D</td>
</tr>
</tbody>
</table>

/ detected; – undetected.

TABLE 1 The 2-DE Blotted Nitrocellulose Membranes were Incubated Overnight (4°C) with Pool Sera of Patients or Controls (As Primary Antibody) Before Subjected To Monoclonal Anti-Human IgM-HRP. Immunogenic Proteins that Showed on the 2-DE Immunoblotted Nitrocellulose Membrane (Refers to Figures 1 and 2)
Statistical Analysis

The Student’s *t*-test was used to analyze the differences between controls and OSCC patients. A *p*-value of less than 0.05 was considered as statistically significant different.\(^{14,15}\)

Mass Spectrometry Analysis and Database Search

By using a commercial kit ProteoExtract \(^{\text{TM}}\) All-in-One Trypsin Digestion Kit (Calbiochem, Darmstadt, Germany), the selected protein spots were excised and subjected to in-gel tryptic digestion. The mass spectrometry analysis was carried out at the Proteomic Centre, Faculty of Biological Sciences, National University of Singapore. One of the representative gels was used to performed MALDI mass spectrometry to identify the proteins by using the Applied Biosystem 4800 Proteomics Analyzer. For the MS/MS analysis, the mass standard kit (Applied Biosystems/MOS Sciex, Toronto, Canada) was used as a calibrator. After that, the protein were identified by submitted the data from the MS/MS to the MASCOT search engine assessed on December 4, 2012.\(^{14,15}\)

RESULTS

2DE Saliva Protein Profiling

The present study demonstrated comparable 2-DE saliva protein profiles of OSCC patients and controls were obtained. Figure 1 shows a representative 2-DE saliva protein profile. Four protein clusters were consistently appeared in all 2-DE profile of patients and also controls. The protein clusters were designated as protein A, B, C, and D, as indicated in Figure 1.

![Representative 2-DE saliva protein profiles of pooled normal saliva and pooled OSCC saliva. Whole saliva samples of patients and controls were subjected to 2-DE and silver staining. Protein spots were compared and analyzed using Image Master Version 7.0 platinum.](https://example.com/fig1.png)
Image Analysis of 2-DE Gels

The clusters of protein spots were analyzed using Image Master 2D Platinum Software version 7.0 (GE Healthcare Biosciences). It demonstrated that the level of proteins detected in saliva of OSCC patient were comparable to those of the control groups.

Detection of Immunogenic Protein by 2-DE Immunoblotting

The 2-DE immunoblots demonstrated that there were differences between the saliva profiles of OSCC patients compared to control groups. Protein A was identical in all immunoblotted profiles of normal saliva and

![Image](image_url)

**FIGURE 2** 2-DE immunoblot results for (a) category 1, (b) category 2, (c) category 3, and (d) category 4 (refer to Table 1). Whole saliva samples OSCC patients and controls were subjected to 2-DE and blotted onto the nitrocellulose membrane followed by probing with pooled sera and monoclonal anti-human IgM-HRP.
OSCC saliva. It appeared as a common immunogenic protein for all four categories, as shown in Figure 2. Protein B only appeared as immunogenic proteins in two categories which were control groups probed with human normal serum and OSCC serum. At the same time, Protein C and D were only detected in OSCC saliva which was probed with normal serum. Table 1 shows the immunogenic proteins that detected on the 2-DE immunoblots.

Identification of Proteins Using Mass Spectrometry

Host-specific proteins A, B, C, and D were further identified by subjecting the protein spot cluster to MALDI-TOF analysis followed by database searching. Table 2 shows the information of the identified proteins. Through database searching, Protein A, B, C, and D were identified as Chain A, 3-D structure analysis of the R337q variant of Human Pancreatic Alpha-amylase (HPA), Chain A structure solution, and refinement of the recombinant Human Salivary Amylase (sAA), Keratin-10 (K-10), and Chain A crystal structure of Ga module complexed with Human Serum Albumin (GA-HSA), respectively.

DISCUSSION

In the present article, comparable results of 2-DE saliva protein profiles of OSCC patients and controls were obtained. Interestingly, protein A, B, C, and D, namely HPA, sAA, K-10, and GA-HSA, respectively, were consistently appeared in all silver-stained 2-DE saliva protein profiles but not in the immunoblotted profiles. From the western blot profile, it seems that immunogenic property has shown only in protein C and D of OSCC patients. Besides, we also identified the potential loss of IgM immunoreactivity

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession Number</th>
<th>Score</th>
<th>Mr (kDa)</th>
<th>pI</th>
<th>Sequence Coverage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Chain A, three-dimensional structure analysis of the R337q variant of Human Pancreatic Alpha-amylase</td>
<td>gi</td>
<td>18655893</td>
<td>231</td>
<td>56</td>
<td>6.16</td>
</tr>
<tr>
<td>B: Chain A, structure solution and refinement of the recombinant Human Salivary Amylase</td>
<td>gi</td>
<td>14719766</td>
<td>911</td>
<td>56</td>
<td>6.21</td>
</tr>
<tr>
<td>C: Keratin-10</td>
<td>gi</td>
<td>307086</td>
<td>184</td>
<td>46</td>
<td>5.11</td>
</tr>
<tr>
<td>D: Chain A, crystal structure of Ga module complexed with Human Serum Albumin</td>
<td>gi</td>
<td>55669910</td>
<td>842</td>
<td>67</td>
<td>5.57</td>
</tr>
</tbody>
</table>
function towards protein B in OSCC patients. Intriguingly, immunoprofiles of OSCC patients’ saliva probed with pooled OSCC serum have showed no protein spot of interest except for protein A, which may be due to the change of the epitope structure of immunoproteins for the IgM binding during the carcinogenesis process.\textsuperscript{[18]}

Protein A, HPA, is an enzyme widely found in plants, mammalian tissues, and microorganism which is responsible for catalyze hydrolysis of α-1,4-glycosidic linkages of starch component (amylose and amylopectin), glycogen and various oligosaccharides.\textsuperscript{[19]} In humans, HPA is present in the gastrointestinal tract for degradation of starch for body storage and usage.\textsuperscript{[20]} Protein A appeared in all four categories of 2-DE immunoblotting study, indicating that it is a common immunogenic protein detected by natural occurring antibody of IgM.

Protein B, sAA, is an enzyme which is secreted by salivary gland in responsible for food digestion and breakage of complex carbohydrate into maltose.\textsuperscript{[21]} It has also been identified as the first line of immune defense because of its responsibility in the prevention and clearance of bacteria from the mouth.\textsuperscript{[22]} From the study, we identified sAA as another natural occurring antigen recognized by normal human IgM but not by patients’ IgM as immunogenic protein. Loss of this IgM immunoreactivity may due to the molecular conformation changes of α-amylase in OSCC patients which triggered by pH changes and the formation of sulfhydryl groups.\textsuperscript{[23,24]} Besides, there are reports on the facts that sAA abundance and activity will significantly reduce by 25% in the OSCC patient.\textsuperscript{[25]}

K-10, a type of cytokeratin which protects epithelial cells from mechanical and non mechanical stresses results in cell death despite of playing the role in cell signaling, the stress response, apoptosis, and other tissues-specific functions.\textsuperscript{[26]} It had been used as tumor markers in histodiagnosis of certain cancers due to their abundance and tissue specificity. Cytokeratin expression pattern in the malignant cells is usually retained from the cell of origin.\textsuperscript{[27,28]} Anti-keratin antibody which was established as natural antibodies in human sera reacts strongly with K-10 in OSCC saliva due to keratinisation that occur in OSCC.\textsuperscript{[28,29,30]} In this study, K-10 was only detected in pooled OSCC saliva probed with normal human serum and thus K-10 is very specific for OSCC diagnosis.

GA module complex appears on the surface protein of various bactericide species which interact with HSA to form albumin-binding GA module complex. Typically, this protein G-related albumin binding modules promote bacterial growth, virulence, and erosion of host’s immune response system by allow infecting bacteria to coat their surfaces with host proteins in mammalian plasma.\textsuperscript{[31]} In the saliva of OSCC patients, this protein is captured by normal human IgM as immunogenic protein. The immunogenic property might derived from the albumin that bind or complex with
certain bacteria which contain high level of antigenic GA module and appear high abundant in cancer patients’ mouth. Protein peptostreptococcal albumin-binding (PAB) from anaerobic human commensal and pathogen *Finegoldia magna* (formerly *Peptostreptococcus magnus*) and Protein G of group C and G Streptococci are examples of some surface protein which have repeated albumin-binding domains known as GA module.\[32–34\] Although the current results showed that immunogenic proteins may have a potential to use as salivary tumor biomarkers for OSCC, this requires further extensive validation in a study that has to be carried out on clinically representative populations.

**CONCLUSIONS**

In conclusion, the analysis of saliva samples from controls and OSCC patients using 2-DE followed by immunoblotting has enabled the identification of immunogenic proteins in saliva which subsequently highlighted the potential of sAA, K-10, and GA-HSA to be served as salivary tumor biomarkers for OSCC.

**ACKNOWLEDGMENTS**

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