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ORIGINAL ARTICLE

Identification of GIMAP7 and Rabl3 as Putative Biomarkers for Oral Squamous Cell Carcinoma Through Comparative Proteomic Approach

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Abstract

Oral squamous cell carcinoma (OSCC) accounts for more than 90% of all oral cancers and has been listed as sixth most common human cancer. Due to late diagnosis and insufficient therapeutic response among patients, the survival rate remains very low accentuating the importance of early diagnostic markers. The study aimed to identify differentially expressed proteins in search for putative serum biomarkers and drug targets. Serum samples (n = 45) were depleted and resolved on two dimensional gel electrophoresis. Among differentially expressed proteins, two were identified using MALDI-TOF mass spectrometry. Gene expression levels of identified proteins were quantified in malignant and normal tissue using RT-qPCR. To validate serum Rabl3 expression, sandwich ELISA was performed. Proteomics analysis revealed two proteins which were found to be associated with oral cancer. The expression of GIMAP7 was found to be down regulated in serum of patients suffering from oral cancer while the expression of Rabl3 was found to be up-regulated. Gene expression analysis in malignant tissue and adjacent normal tissue revealed the same pattern. Quantitative ELISA was used to validate expression of Rabl3 in serum from oral cancer patients and healthy subjects which demonstrated significant up-regulation in cancer patients. Findings in current study demonstrate differential expression of novel putative biomarkers GIMAP7 and Rabl3 in oral cancer which suggests their potential role in oral cancer pathology and can be considered as predictive biomarkers.

Keywords Serum biomarkers · OSCC · GIMAP7 · Rabl3 · Proteomics

Introduction

Oral squamous cell carcinoma (OSCC) is a major sub-type of malignancy [1] which is considered as sixth most common cancer in the world [2]. Five year survival rate among patients suffering from OSCC is nearly 50% which is decreased drastically in advanced stage [3]. OSCC is reported to be a major cause of morbidity and mortality among males in south East Asia and especially in Pakistan [4]. Highest incidence in males has been linked with exposure to risk factors [5]. In developed countries, major risk factors of oral cancer are alcohol and tobacco use [6] while in developing countries like Pakistan and India, smokeless tobacco products (gutka, naswar) and betel quid with or without tobacco are considered major risk factors [7]. In most of the cases, diagnosis of oral cancer takes place at an advanced stage of diesase [8] hence it is difficult to treat OSCC even with the advancements in standard treatments procedures such as surgery and radiotherapy. The detection of the disease at an earlier stage, therefore, is likely to decrease the mortality rate.

Proteomic techniques have been utilized in identification of biomarker using diseased tissues and body fluids [9] including OSCC [10]. Most of the work has been focused on tissue proteomics and various putative biomarkers have been suggested till date [11, 12]. None of them, however, is being used in clinical practice as diagnostic/prognostic marker, maybe due to restriction of being tissue markers. Serum/plasma proteomics offers an attractive tool due to use of minimal invasive method and safety in handling [13]. Differentially expressed proteins identified using plasma /serum samples have been reported. Almost 45 different bio-molecules are suggested to be potential biomarkers on the basis of analysis from different geographical regions [12, 14]. Data from local Pakistani population, however, is not

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available and is worth examining since main risk factor for local population is use of smokeless tobacco products (SLTs) such as niswar, gutka, manipuri etc.

In order to search for unique biomarkers related to SLTs, we performed comparative proteomics analysis on serum samples from oral cancer patients. Among differentially expressed spots, several differentially expressed proteins were identified; two unique proteins namely Rabl3 and GIMAP7 are reported here. We verified the expression levels of both proteins in malignant and normal tissue through RT-qPCR analysis. Current study focused on expression of Rabl3 and GIMAP7 at protein and mRNA levels from OSCC serum and OSCC tissue samples, respectively. In this study, we report differential expression of GIMAP7 and Rabl3 in serum from oral cancer patients along with oral (malignant and healthy) tissue.

Materials & Methods

Sample Collection

The study was approved from Institutional Ethical Committee. Diagnosis and tumor grade categorization was conducted by expert surgeons through physical examination and histopathological confirmation. Selected candidates were interviewed and data was recorded regarding their exposure to risk factor, duration of exposure, overall life style, socio-economic status, occupational activity educational background, age and gender. Majority of samples were obtained from smokeless tobacco users. Blood samples from OSCC patients (n = 45) were collected before surgery while for controls, samples were collected from age and sex matched healthy volunteers (n = 30). For collection of serum, blood was left to form clot for 20 minutes at room temperature then centrifugation was carried out at 3500 rpm for 10 minutes. Supernatant was collected and stored at -80 °C.

Malignant tissue along with adjacent normal tissue (n = 45) were also collected at the time of surgery. Tissue samples were snap-frozen in liquid nitrogen immediately after collection and kept at -80 ^{0}C until further use.

Proteomics Analysis

Albumin/Immunoglobulin G (IgG) Removal

To remove high abundant proteins Albumin and Immunolgobulin G (IgG), serum samples were depleted using ProteoExtract Albumin/IgG removal kit (Merck- Millipore, Darmstadt, Germany) in accordance with manufacturer's instructions.

Two Dimensional gel Electrophoresis

Protein concentration was estimated using BCA assay in accordance with manufacturer's guidelines (Cat # 23225, Pierce BCA protein assay kit). Equal amount of protein from OSCC and healthy control serum were suspended in rehydration buffer which contains 8M Urea, 0.5% ampholyte solution, 0.2% DTT, 0.5% CHAPS, and traces of Bromophenol blue. IPG strips 3–10 NL (Bio-Rad) were rehydrated with sample. IEF was done on Multiphor II (GE Healthcare) system at 20^{o}C with voltage rating of 10,000 V/h. After IEF, each IPG strip was equilibrated for 30 minutes each using equilibration buffer 1 (0.5M Tris-HCl pH 6.8, 6M urea, 2% SDS, 30% glycerol, 2% DTT) and 2 (0.5M Tris-HCl pH 6.8, 6M urea, 2% SDS, 30% glycerol, 2.5% w/v iodoacetamide). After equilibration, strips were located on 12% polyacrylamide gel to perform second dimension SDS PAGE. Voltage was kept constant at 65 volts. Electrohphoresis assembly mini protean electrophoresis system (BioRad) was used for second dimension. Coomassie brilliant blue G-250 was used to stain gels.

Image Analysis

Gel images were captured through ExQuest spot cutter and analysis was performed using PDQuest software (BioRad). From each matched gel set, one was selected as reference or master gel having maximum number of spots with best resolution. Comparative analysis of gels was performed to detect spot intensities and shown as a mean ± SD. Those having P values less than 0.05 were considered statistically significant.

Protein Identification Using MALDI-TOF MS

Exclusively differentially expressed spots were excised from the stained gels and digested according to reported protocols [15]. Briefly, protein spots were destained with 50% ACN and left for 15 minutes. ACN was added to the gel pieces and then desiccated using vacuum centrifuge. Samples were rehydrated in 10 mM DTT and incubated at 60 °C for 45 min to catalyze reduction of cystein residues followed by alkylation through incubation for 30 min with fresh 55 mM iodoacetamide in the absence of light at room temperature. Proteins were digested using 2 ng/L trypsin (Promega) and left at 37° C overnight. The peptides were extracted with 25 mM ammonium bicarbonate: acetonitrile (1:1) and 10% (v/v) formic acid: acetonitrile (1:1). Extracted peptides were dried using Vacufuge® vacuum concentrator (Eppendorf®) and reconstituted with 0.1% formic acid. Digested peptides were sent to core facility of ICCBS for Mass spectrometry analysis using MALDI-TOF-TOF MS (Ultraflex III, Bruker Daltonics Germany). Samples were analyzed using reported protocol [16]. Briefly, the α-cyano-4-hydroxycinnamic acid was mixed with equal amounts of samples. Reflector positive ion mode was

selected. To obtain mass spectra high voltages of 25 KV was used to accelerate ions and 6 KV for lens potential. Spectra were obtained in the mass range of (500–3000 Da).

Data Analysis Using Mascot

Protein identification was carried out through MASCOT database search – Matrix Science, using peptide mass fingerprinting from Swiss-Prot database. Search parameters were as follows, Carbamidomethylation of cysteins was selected as fixed modification of peptides and oxidation of methionine was selected as a variable modification. Missed cleavage option was selected for 1 while peptide tolerance of 100 ppm/Da. $p < 0.05$ was selected.

Gene Expression Analysis

Reverse Transcriptase-Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA from OSCC tissue and adjacent healthy tissue were extracted and reverse transcribed using RevertAid First-Strand cDNA synthesis kit (ThermoFisher Scientific, Massachusetts, USA). qPCR reaction mixture containing cDNA, primer pairs, nuclease free H_2O and SYBR green was prepared and run on quantitative real time 7300 PCR system (Applied Biosystems Corp., California, USA). First cycle comprised of 5 min run at 50 °C followed by 10 min run at 95 °C. After first cycle, next 40 cycles utilized following temperatures; 95 °C for 14 s (denaturation), 58 °C for 1 min (annealing) and 72 °C for 45 s (extension). Human *glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene* was used for data normalization and the relative expressions of the Human *GIMAP7*and *Rabl3* were measured with respect to adjacent healthy tissue using the ddCt relative quantification method (Table 1). At the end, dissociation curve of PCR product was analysed.

Validation of Rabl3 Expression Through ELISA

Serum level of Rabl3 was measured using commercially available ELISA kit (SET514Hu, Cloud-Clone, USA) in accordance with the manufacturer's guidelines. Briefly, 100 µl sera samples and standards were transferred to 96 well plates coated with human Rabl3 monoclonal antibody. After incubation at 37 °C for an hour, liquid was removed and 100 µl detection reagent A was added followed by another incubation at 37 °C. 100 µl detection reagent B was added and incubated at 37 °C for 30 minutes. Careful aspiration followed by addition of 90 µl substrate solution and incubation for 15 minutes at 37 °C was then done. 50 µl stop solution was added at the end and absorbance was taken at 450 nm using a microplate reader system. Standard curve was made through serial dilutions of standard; samples were run in triplicate their mean absorbance was used to find Rabl3 concentration.

Statistical Analysis

Data was analyzed using the statistical software of SPSS 20.0 for Windows (SPSS Inc., USA). Student t-test was employed to calculate statistical significance, whenever required.

Results

Protein Identification

Sixteen spots are obtained on 2-D gels showing differential expression in serum of OSCC patients in comparison to healthy control serum. Two statistically significant differential protein spots were identified as GIMAP7 and Rabl3 using MALDI-TOF mass spectrometry. Representative gels of both OSCC serum and healthy control serum have been shown in Fig. 1. Relative spot intensities of GIMAP7 and Rabl3 are given in Figs. 2 and 3, respectively.

Gene Expression Analysis in Tissue

In order to confirm protein expression pattern of identified proteins in tissue samples, real time quantification was performed using RT-qPCR. mRNA level of *GIMAP7* was found to be down regulated in cancer tissue on the other hand, *Rabl3* showed over expression in malignant tissue as shown in Fig. 4.

Table 1 Primer sequences used in the study

S.no	Gene	Primer sequence from 5′-3′
1	*Rab-like protein 3 (Rabl3)*	F = AAACAAAGCGCCATGAAGTT R = TACCGTGGATTTGTGCAGTC
2	*GTPase IMAP family member 7(GIMAP7)*	F = ACACCACCTGCAAGGAAATC R = CCAGCAGCAGAACTAGGACA
3	*Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*	F = TGACAAAGTGGTCGTTGAGG R = ACCTGCCGTCTAGAAAAACC

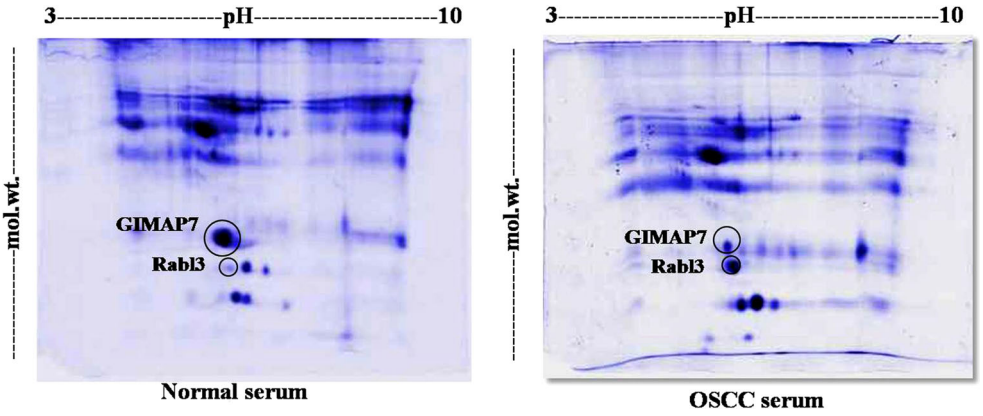

Fig. 1 Representative 2-D gel showing differential protein expression in OSCC serum in comparison with healthy control serum. Identified proteins are marked on the gel

Validation of Rabl3 Expression Through ELISA

Rabl3 concentration was found to be significantly ($P < 0.001$) higher in OSCC serum (11.6 ± 1.5 ng/mL) in comparison to control (8.113 ± 1.0 ng/mL). Figure 5 represents relative values of Rabl3 sera concentration from OSCC patients and normal controls as determined through ELISA.

Discussion

Oral cancer is the major cause of morbidity and mortality among males in Pakistan [4]. It has been predicted that oral cancer incidence in Pakistan is likely to increase with passage of time [17]. Major risk factor for oral cancer in local population is intense use of smokeless tobacco among young generation [5]. Regardless of the incredible developments in surgery, radiotherapy and chemotherapy, the survival rate has not improved primarily because of late stage diagnosis [8]. Combination of two-dimensional gel electrophoresis (2-DE) and MALDI-TOF MS has been widely used for profiling serum proteins using diseased samples.

Serum proteomics is an attractive tool to identify putative biomarker from different cancer types including OSCC. Several biomarkers from OSCC serum have already been identified such as alpha-1-B-glycoprotein (ABG), clusterin (CLU), complement C3c (C3), haptoglobin (HAP), leucine-rich α2-glycoprotein (LRG), pro-apolipo protein A1 (proapo-A1), and retinol-binding protein 4 precursor (RBP4) [18]. In current study, we have identified two differentially expressed proteins from OSCC serum in comparison to healthy serum obtained from volunteers. To check their expression pattern in malignant and healthy tissue, we quantified mRNA levels through real time RT-qPCR.

We observed down-regulation of GIMAP7 in serum samples from OSCC patients and it's concurrent down expression at mRNA level in OSCC tissue. The GIMAP (GTPase of immunity-associated proteins), has been closely linked with cell survival of lymphoid cells [19]. GIMAP is a unique GTPase family member which is conserved in vertebrates [20]. Regulation of cell viability or death is likely to be regulated through GIMAP family members in immune cells [21, 22]. Involvement of GIMAP family members in pathogenesis of cancer have also been reported in case of non small cell lung cancer (NSCLC). Microarray studies and qPCR analysis revealed down regulation of GIMAP family members in lung cancer tissue [23]. Mouse model studies have indicated that GIMAP4, a GIMAP family member, has capability to accelerate apoptosis [22]. In hepatocellular carcinoma (HCC), down-regulation of GIMAP5 and GIMAP6 has been reported

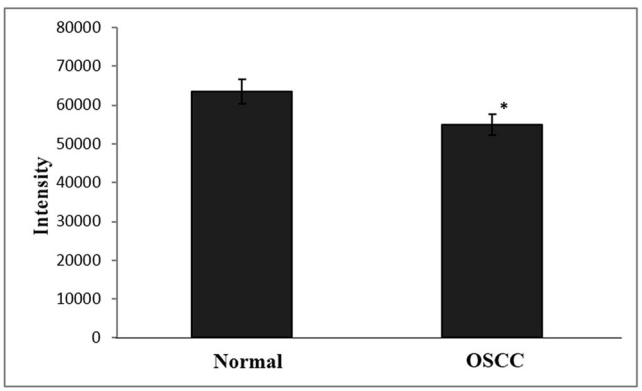

Fig. 2 Relative spot intensity of GIMAP7. Each bar represents mean ± SD whereas * represents statistical significance when $p < 0.05$

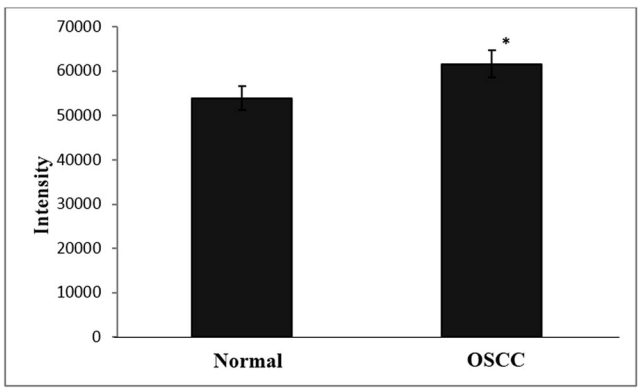

Fig. 3 Relative spot intensity of Rabl3. Each bar represents mean ± SD whereas * represents statistical significance when $p < 0.05$

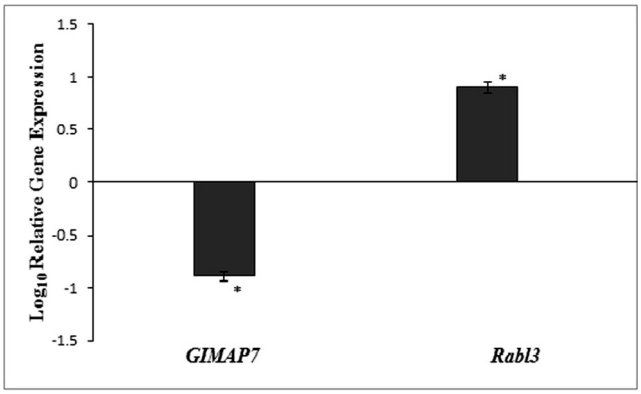

Fig. 4 Gene expression analysis in OSCC tissue sample using RT-qPCR. Relative gene expression was calculated using the 2^{-ddCt} method, with GAPDH mRNA expression as reference gene and the adjacent normal tissue as the calibrator. Each bar represents mean ± SD whereas * represents statistical significance when $p < 0.05$

both from tissue and serum samples [24]. Down regulation of GIMAP7 both at protein and mRNA level observed in serum and tissue samples from oral cancer patients might suggest tumor suppressor role for this protein.

The second protein identified in current study is Rabl3 which is suggested to be a positive regular in various malignancies. However, the relationship of Rabl3 expression with oral cancer has not been reported earlier. We found up-regulated expression of Rabl3 in OSCC serum and analogous mRNA level in OSCC tissues. Identification of Rabl3 in serum using proteomic approach was validated using ELISA which indicated significant over expression in oral cancer patients. RABL3 is a member of Rab subfamily of small GTPases [25] which plays key role in tumorigenesis. There is strong evidence indicating correlation between tumorigenesis and their elevated expression level [26]. In-vitro gene silencing experiments in breast cancer cell lines demonstrated inhibition of cell proliferation, tumor formation, metastasis and cell motility [27] suggesting it to be an oncogene. Over expression of Rabl3 in non-small cell lung cancer is linked with poor survival [28]. Up-regulation of Rabl3 in hepatocellular carcinoma cell lines and malignant tissue at protein and mRNA level has shown its association with cancer progression and poor survival suggesting possibility of using Rabl3 as a possible diagnostic and prognostic marker in hepatocellular carcinoma [29, 30]. It has been postulated that Rabl3 mediates its role through phosphorylation of focal adhesion kinase (FAK) and can stimulate cell proliferation and antiapoptotic activities through activation signaling pathway [27]. Considering our results and previous data, we speculate that Rabl3 can be considered as a putative therapeutic target for anti cancer treatment.

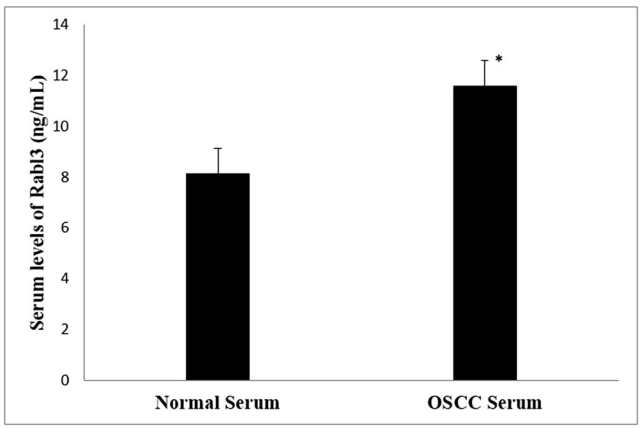

Fig. 5 Concentration of Rabl3 in OSCC serum (n = 45) with reference to healthy controls (n = 30) as measured through ELISA. Each bar represents mean ± SD whereas * represents statistical significance when $p < 0.001$

Conclusion

Taken together, our study reports differential expression of two novel putative biomarkers GIMAP7 and Rabl3 both at protein and mRNA level from oral cancer samples suggesting their possible role in tumor pathology. Our results offer new evidence to examine prospective role of these proteins as a potential anti tumor therapeutic target.

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Compliance with Ethical Standards

Conflict of Interest Authors declare that there is no conflict of interest.

Ethical Approval The study was approved from Institutional Ethical Committee (IEC), University of Karachi (#NCP-IRB-108). All procedures adapted were as per 1964 Helsinki guidelines.

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