Pathology & Oncology Research (2020) 26:1489–1497
https://doi.org/10.1007/s12253-019-00724-y

ORIGINAL ARTICLE

Identification of Differentially Expressed Proteins from Smokeless Tobacco Addicted Patients Suffering from Oral Squamous Cell Carcinoma

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Received: 25 February 2019 / Accepted: 14 August 2019 / Published online: 24 August 2019

Abstract

Oral squamous cell carcinoma (OSCC) is the eight most common malignancy worldwide with an incidence rate of 40% in south-east Asia. Lack of effective diagnostic tools at early stage and disease recurrence despite extensive treatments are main reasons for high mortality and low survival rates. The aim of current study was to identify differentially expressed proteins to explore potential candidate biomarkers having diagnostic significance. We performed comparative proteomic analysis of paired protein samples (cancerous buccal mucosa and adjacent normal tissue) from OSCC patients using a combination of two dimensional gel electrophoresis and Mass spectrometric analysis. On the basis of spot intensity, seventeen proteins were found to be consistently differentially expressed among most of the samples which were identified through mass spectrometry. For validation of identified proteins, expression level of stratifin was determined using immuno-histochemistry and Western blot analysis. All identified proteins were analyzed by STRING to explore their interaction. Among uniquely identified proteins in this study, at least two candidate markers (Ig Kappa chain C region and Isoform 2 of fructose bisphosphate aldolase A) were found to be novel with respect to OSCC which can be explored further. Results presented in current study are likely to contribute in understanding the involvement of these molecules in carcinogenesis apart from their plausible role as diagnostic/prognostic markers.

Keywords Oral squamous cell carcinoma · Tissue proteomics · Differential expression · Biomarker · Smokeless tobacco

Introduction

Oral squamous cell carcinoma (OSCC) is the eighth common cancer in men with an incidence rate around 300,000 to 500,000 per year [1]. It is the most frequent malignancy affecting head and neck region resulting in severe complications [2]. Although many therapeutic modalities such as surgery, chemotherapy and radiotherapy are in practice, mean five-year survival rate for OSCC is around 50% and tends to regress more in metastatic stages [3].

Epidemiological studies have indicated tobacco and alcohol to be the most critical risk factors for oral cancer in Western countries [3]. Both may act independently or synergistically to increase the risk [4]. Other social carcinogens include smokeless tobacco (SLTs), betel quid, areca nut, naswar and gutka, which are frequently used in Asian countries [5, 6]. The higher prevalence rate of OSCC in Pakistan and India has been associated with widespread use of SLTs [7]. Locally, use of betel quid, areca nut, and gutka is more common in southern Pakistan whereas naswar is frequently used in northern Pakistan [6]. Use of SLTs is linked with the development of oral premalignant lesions (OPLs) such as leukoplakia, and oral submucous fibrosis that ultimately lead to primary tumor and advanced oral cancer [8]. Most common sub-site for OSCC among Western population is tongue and floor of mouth [9] while developing countries show a mixed distribution of various sites in the oral cavity [5]. Among OSCC patients from Karachi, Pakistan, the most common sub-site is reported to be buccal mucosa [9].

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Early stage diagnosis of OSCC is likely to reduce mortality rates since delayed diagnosis increases the probability of tumor spread and malignancy. Current diagnostic methods include histopathological examination followed by biopsy. Early diagnosis, however, heavily relies on the appearance of OPLs and their treatment before progression to malignancy. Histopathology remains unable to diagnose the disease at early stages subsequently leading to an unsuccessful treatment and poor prognosis [4]. Proteomic analysis has been widely accepted as a useful method for identification of candidate biomarkers and therapeutic targets [10]. Various studies have been conducted on OSCC samples obtained from different populations having exposed to variety of risk factors. Owing to the fact that sub-site and risk factors are unique in our region, we designed current study to examine differentially expressed proteins from OSCC and normal tissue samples using proteomics approach.

Materials & Methods

Tissue Samples

The study was conducted after approval from Institutional review board (IRB). Informed consents were obtained from all participants and all procedures adapted were as per Helsinki guidelines [11]. Tissue samples and normal adjacent tissues (2–4 cm away) were collected from patients after confirmation of diagnosis by histopathology. OSCC samples were graded using Tumor node metastasis (TNM) staging system [12]. The samples were snap frozen and transported in liquid nitrogen followed by the storage at −70 °C till further analysis.

Protein Extraction & Estimation

Fifty mg of each normal and cancerous tissue was rinsed using Phosphate buffer saline (PBS) followed by homogenization in 1 ml of lysis buffer containing 2 M Urea, 0.25 M EDTA, 0.5 M DTT, 2% Ampholyte (pH 3 to 10) and protease inhibitor cocktail. The homogenate was centrifuged at 12,000×g for ten minutes and supernatant was collected. Total protein was estimated using BCA assay as per manufacturer's instructions (Pierce BCA protein assay kit).

Two-Dimensional Gel Electrophoresis (2DE)

Equal quantities of protein (80 μg each) from cancerous and adjacent normal tissue samples were applied on 7 cm NL-IPG strips pH 3 to 10 (Bio-Rad). After overnight rehydration, strips were subjected to isoelectric focusing using Multiphore II system (GE healthcare, Amersham Biosciences). Focusing was performed for approximately 10,000 V/h at 20 °C. After IEF, strips were equilibrated with 2DE-equilibration buffer. For second dimension, SDS-PAGE was performed using 12% polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue G-250.

Gel Visualization & Spot Selection

For comparison of protein expression pattern, gels were analyzed using PDQuest software (Bio-Rad, USA). Intensities of all spots in both groups were calculated and differentially expressed protein spots were excised using ExQuest Spot cutter (Bio-Rad USA).

LC-MS/MS Analysis Using Q-Exactive Orbitrap Mass Spectrometer

In-gel Digestion

Excised spots were in-gel digested using an earlier protocol [13]. Briefly, 20 μg of reagent grade trypsin was dissolved in buffer (10 mM ammonium bicarbonate into 10% *v*/v acetonitrile) to get a final concentration of 13 ng/μL trypsin. Gel spots were reduced by adding 10 mM DTT in 100 mM ammonium bicarbonate and alkylated by 50 mM iodoacetamide in 100 mM ammonium bicarbonate. After washing, 50 μl trypsin buffer was added and kept at 37 °C for overnight digestion. Peptides were extracted using extraction buffer followed by drying in speed vac (Eppendorf).

Desalting & Extraction of Peptides

Extracted peptides were re-suspended in 1% TFA and loaded on C18 stage tips for desalting previously activated by loading buffer (50% ACN in 0.1% TFA). Tips were then centrifuged and column was again washed with 1% TFA. Peptides were eluted by adding 50% acetonitrile in 0.1% TFA and collected into a separate eppendorf tube. Tips were washed and the residual volume was also collected. Peptides were dried down using speed vac and a little volume was allowed to remain in the tube.

LC-MS/MS Analysis

The extracted peptides from each sample were run on a Q-Exactive mass spectrometer (Thermo Fisher Scientific) attached with a Dionex Ultimate 3000 (RSLCnano) chromatography system. The peptides were re-suspended in 0.1% formic acid and then loaded onto Biobasic Picotip Emitter (120 mm length, 75 μm ID) filled with Reprocil Pur C18 (1.9 μm) reverse phase media. The peptides were eluted by a rising acetonitrile gradient over 19.5 min at a flow rate of 250 nl/min. The mass spectrometer was in positive ion mode running with a capillary temperature of 220 °C, along with a potential of 2100 V applied to the frit. The data was acquired by using

automatic data dependent switching mode of the mass spectrometer. A high resolution (70,000) scan (300–1600 m/z) was conducted for the selection of the 12 most intense ions in order to perform MS/MS analysis using higher-energy collision dissociation (HCD).

Data Analysis

MS/MS data was analyzed by PEAKS studio 6.0 and protein identification search was performed against IPI human database. Parent mass error tolerance was set to 10 ppm and fragment mass error tolerance was set to 0.03 Da. Three missed cleavages and three variable PTMs [Carbamidomethyaltion, 4-hydroxynonenal (HNE), Acetylation] per peptide were allowed.

Immunohistochemistry and Western Blotting

Immunohistochemical staining of normal and OSCC tissues was performed as described earlier [14]. Briefly, tissues fixed in phosphate-buffered formalin. Cryosections (3–5 μm thickness) were immunostained using mouse monoclonal primary antibody for 14–3-3σ (Abcam, Cambridge, UK) at a dilution of 1:50 and visualized with Alexa Fluor-546 conjugated goat anti-mouse secondary antibody (1:1000). For Western blotting, equal amounts of protein from both cancerous and normal tissue were applied to 12% SDS–PAGE. The gel bands were transferred to PVDF membrane at a constant current of 250 mA. After blocking the membrane with 3% BSA, the blot was incubated with human 14–3-3σ/SFN monoclonal antibody (ab14123) overnight (1:50) followed by incubation with horseradish peroxidase- conjugated goat anti-mouse IgG. Protein bands were visualized using Diaminobenzidine (1 mg/ml in 30% of hydrogen peroxide).

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

To confirm the expression of identified proteins, respective gene expression analysis for *cytokeratin (KRT), fructose-bisphosphate aldolase A (ALDOA) and 14–3-3 σ/stratifin (SFN)* was performed using reverse transcriptase quantitative PCR. Malignant tissue samples and adjacent healthy tissue from six oral cancer patients were examined in triplicate. Total RNA was extracted using TRI reagent and 500 ng RNA was reverse transcribed. cDNA synthesis was performed in a thermal cycler under the following conditions: 5 min at 25 °C, 60 min at 24 °C and 5 min at 70 °C. RT products were used for qPCR SYBR green detection assay (Real time ABI 7300). qPCR temperature conditions were 15 min at 94 °C, followed by 40 cycles of 14 s at 94 °C, 1 min at 60 °C and 1 min at 72 °C. The data was normalized by the *GAPDH* gene and analyzed by 2-ddCt relative quantification method. Dissociation curve analysis was also carried out. All the samples were amplified in triplicate.

Network Analysis and Functional Classification

For Network Analysis, Uniprot accession numbers of the identified proteins were uploaded in the STRING database and analyzed using multiple protein search option. Online functional annotation tool PANTHER was used for the functional classification.

Results

Demographic Details of OSCC Subjects

Among the eight sets of cancerous and normal adjacent mucosa, six were obtained from male subjects whereas two were from female subjects. All subjects were SLT users among which five were using tobacco, betel quid, and betel nut for twelve years or more. The rest of the three subjects were using naswar for twenty years or more. Three subjects were diagnosed with stage I cancer and five were diagnosed with stage II cancer.

Two-Dimensional Gel Electrophoresis and MS/MS Analysis

All gels were analyzed using PDQuest software. On average, 62 ± 17 and 67 ± 21 spots were observed in Coomassie stained gels for cancer and normal tissue, respectively. Seventeen spots were found to be consistently differentially expressed in most of the gels with at least more than 1.5 fold change. Thirteen spots were found to be up-regulated whereas four were down-regulated. Figure 1 shows two dimensional gel electrophoretic pattern of cancer tissue and normal mucosa depicting differentially expressed spots. Labeled spots were subjected to LC-MS/MS analysis and identified against IPI human database using PEAKS proteomics data software.

The list of identified proteins is given in Table 1. Over-expressed proteins included different Cytokeratins, Calreticulin, Heat shock protein 70 and Stratifin. The four down-regulated proteins were Actin alpha skeletal muscle, Tropomyosin beta chain, Heat shock protein beta 1, and Phosphoglycerate kinase 1.

Immunohistochemistry and Western Blotting

Validation of differential expression of SFN in OSCC tissue and adjacent normal mucosa was performed using Immunohisotchemistry and Western blotting. Significantly increased fluorescent intensity was observed for cancer tissue in comparison with normal (Fig. 2: Top Panel). Western blot

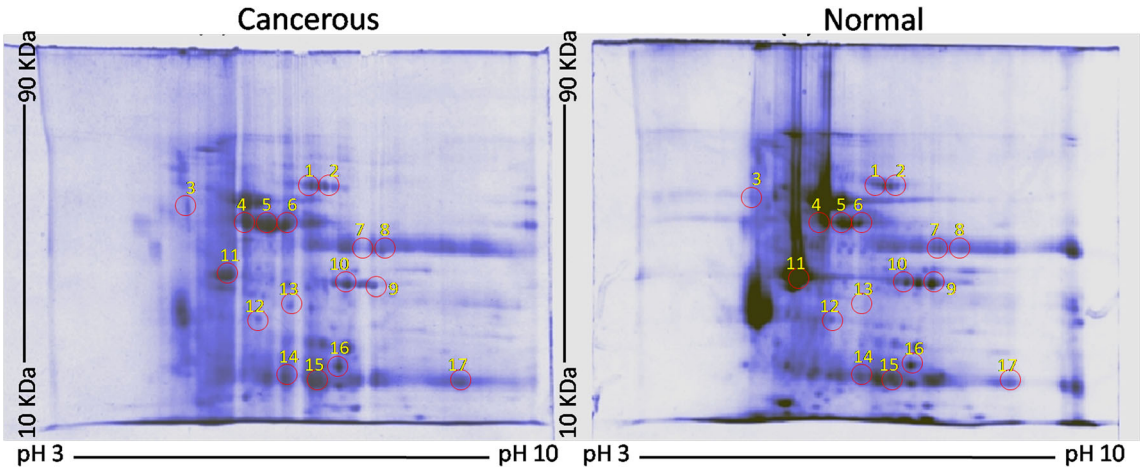

Fig. 1 2DE pattern of OSCC and normal tissue. Differentially expressed identified proteins are marked in circles and spot numbers are mentioned

analysis showed significant over-expression of SFN in cancerous tissue (Fig. 2: Bottom Panel).

RT-qPCR

To compliment proteomics studies, gene expression studies for few identified proteins was conducted using quantitative PCR. Primers for *cytokeratin (KRT), fructose-bisphosphate aldolase A (ALDOA) and 14–3-3 σ/stratifin (SFN)* genes were used. Fig. 3 shows the gene expression pattern of cancer and adjacent normal control tissue by RT–qPCR analysis. *ALDOA*, *KRT* and *SFN* were found to be up-regulated in malignant tissue while *HSP* showed down-regulated expression. Differential gene expression was found to be significant ($p < 0.05$).

Network Analysis

Network analysis of all identified proteins was performed using STRING excluding Ig kappa chain C region as STRING found no match for this protein. Some proteins including Stratifin, Cytokeratins 17, 16, 8 and Calreticulin showed direct interaction.

Table 1 Differentially expressed proteins from OSCC using LC-MS/MS

Spot No.	Uniprot accession number	Name of Protein	Name of Gene	Mol. wt. (Da)	pI	Protein coverage (%)	No. of Unique Peptides	Fold change
1	P11142	Heat shock protein 70	HSPA8	70,898	5.37	61	1	+2.3
2	P02768	Serum Albumin	ALBU	69,367	5.92	85	29	+1.7
3	P27797	Calreticulin	CALR	48,142	4.29	51	44	+2.9
4	Q04695	Cytokeratin-17	KRT17	48,106	4.97	80	2	+2.5
5	P08779	Cytokeratin-16	KRT16	51,268	4.98	86	61	+3.2
6	P02533	Cytokeratin-14	KRT14	51,562	5.09	83	18	+2.3
7	P00558	Phosphoglycerate kinase-1	PGK1	44,615	8.3	59	21	−1.9
8	P04075–2	Isoform 2 of fructose-bisphosphate aldolase A	ALDOA	45,261	8.48	67	50	+1.7
9	P04406	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	36,053	8.57	91	95	+2.8
10	P68133	Actin alpha – skeletal muscle	ACTA1	36,639	5.71	51	4	−1.7
11	P07951	Tropomyosin beta chain	TPM2	32,851	4.66	94	5	−3.2
12	P31947	14–3-3 σ/Stratifin	SFN	27,774	4.68	79	4	+1.8
13	F8VUG2	Cytokeratin-8	KRT8	30,907	5.19	12	1	+2.4
14	P08590	Myosin light chain	MYL3	21,932	5.03	81	23	+2.2
15	P01834	Ig kappa chain C region	IGKC	11,609	5.58	97	44	+1.9
16	P04792	Heat shock protein beta 1	HSB1	22,783	5.98	70	23	−1.6
17	Q99877	Histone H2B type 1	H2B1N	13,922	10.31	49	3	+2.1

The list includes respective spot no. from 2D image analysis, Uniprot accession number of protein, name of protein, gene name, molecular weight, pI, protein coverage, no. of unique peptides, and fold change

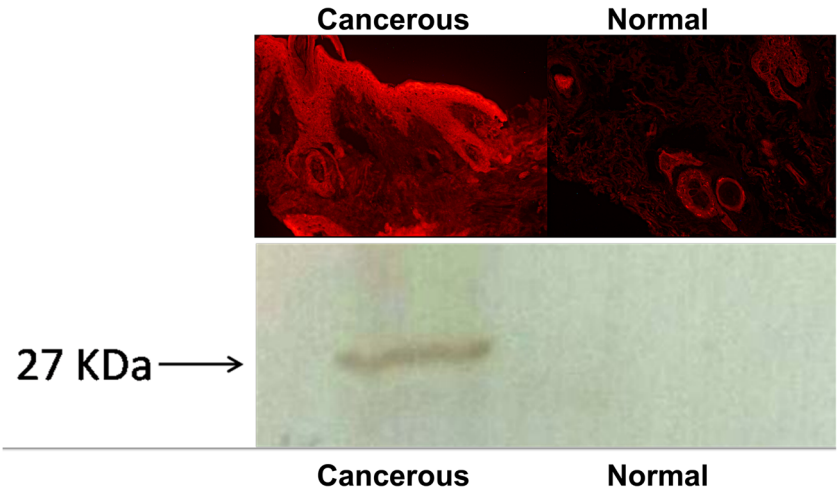

Fig. 2 Immunohistochemical (top) and Western blot (bottom) analysis of SFN in cancer and normal tissue

Histone H2B type 1 did not show interaction to any other identified protein. Figure 4a shows network obtained by STRING representing protein-protein interactions.

Ontological Classification

PANTHER analysis categorized identified proteins on the basis of ontologies (Fig. 4b). They were divided into nine different classes including biological regulation, cellular component organization, cellular process, developmental process, Immune response, localization, metabolic process, multicellular organismal process, and stimuli response.

Discussion

Identification of potential candidates as clinical biomarker and therapeutic target may offer possibilities for early diagnosis and prognosis [15]. Biomarker identification is also significant for studying the related cellular pathways and their role in carcinogenesis [16]. Current study deals with comparative analysis of normal and cancerous tissue samples from smokeless tobacco associated oral carcinoma subjects in order to identify potential biomarker. This study is likely to reflect distinct protein profile since apart from variation in risk factors and physiological/environmental stimulants [5, 9], tumor subsite in local samples is also unique. Most common sub-site of OSCC worldwide is tongue while among Pakistani population, tumors of buccal mucosa are most common [5].

In total, 103 spots were differentially expressed across all gels and seventeen consistent spots were subjected to LC-MS/MS. Our analysis revealed three unique proteins including Ig Kappa chain C region, Isoform 2 of fructose-bisphophate aldolase A and serum albumin. We found higher expression of Ig kappa chain C region expression (IGKC) in OSCC tissue. Ig kappa chain C region is a small immunoglobulin involved in immune response, phagocytosis, receptor-mediated endocytosis, and B receptor signaling pathway regulation [17, 18]. IGKC has been identified as

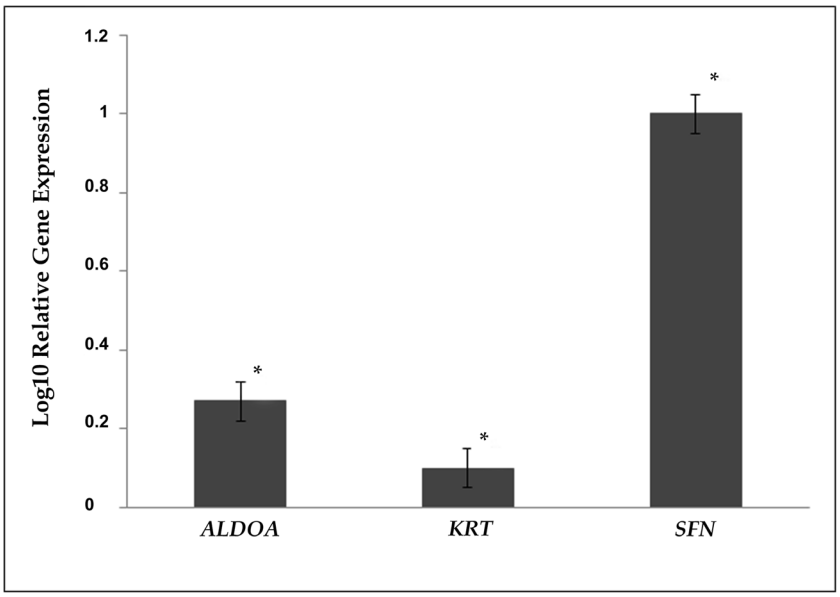

Fig. 3 Relative gene expression of *KRT, ALDOA* and *SFN* in OSCC tissue. 2-ddCt method was used to calculate relative differences. *GAPDH* was used as a reference gene and the adjacent normal tissue as calibrator. * shows statistical significance with $P < 0.05$

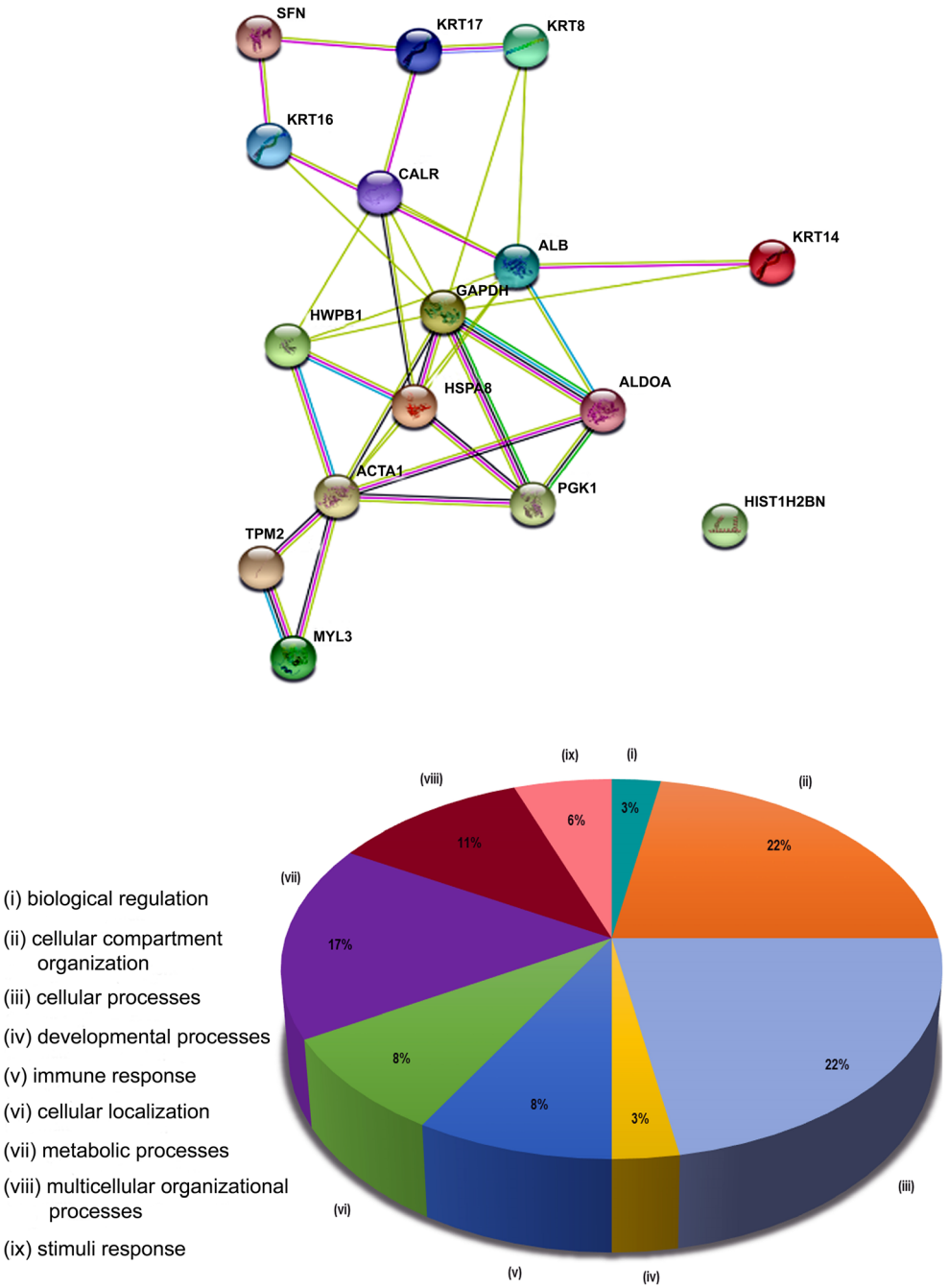

Fig. 4 **a** Network and Functional classification analysis of identified proteins by STRING (**a**) and PANTHER (**b**)

a predictive and prognostic marker in breast cancer, non-small cell lung carcinoma and colorectal cancer. It also proved to be a unique biomarker capable of predicting chemotherapeutic response in breast cancer [19] suggesting its role in clinical applications [18]. Whether IGKC would offer similar role in case of OSCC is yet to be investigated.

Second unique protein found in this study is Isoform 2 of fructose-bisphosphate aldolase A (Aldolase A) which is a metabolic enzyme involved in fructose metabolism and glycolysis cycle [20]. Glycolysis cycle and cancer progression have long been associated with each other [21]. Due to up-regulation in various cancers [22], Aldolase A is likely to act as an oncogene. The higher expression of this protein in colon cancer [23] and lung squamous carcinoma [24] has been reported. Aldolase A expression is also significantly correlated with metastasis and poor prognosis [25]. Higher expression of Aldolase A in malignant tissue was also confirmed at mRNA level in individual samples thus confirming its role as an oncogene. This is first study where we report up-regulated expression of Aldolase A in OSCC tissue.

Third uniquely identified protein is serum albumin (ALBU) that showed up-regulated expression in OSCC tissue

as compared to the adjacent normal epithelia. ALBU is considered very important both physiologically and biologically as it is involved in various critical processes in the body [26]. Albumin also works as a nutrient supply for the cells and in case of nutritional deficiency; cells increase the uptake of albumin from the blood stream. In order to fulfill nutritional demands during tumor progression, uptake of albumin from serum is increased resulting in elevated tissue albumin with a concurrent decrease in serum levels [26, 27]. Serum albumin level has been positively associated with survival rate among lung, gastrointestinal, and ovarian cancer patients [26–28]. This hypothesis, however, needs to be tested on large population.

Cytokeratins were also included among other proteins identified. The over-expression of Cytokeratins (8, 14, 16 and 17) has been reported previously with the exception that Cytokeratin 14 and 16 had been studied only in tongue tissue samples of OSCC [29]. The up-regulation of these proteins in current study is the first report for buccal mucosa samples. It has been reported that dephosphorylation of Cytokeratin 8 may lead to normal cell cycle disruption and tumor progression in OSCC [30]. Over-expression of Cytokeratins 14 and 16 [29] observed previously is more likely due to involvement of both proteins in epithelial cell differentiation leading to abnormal cellular growth in OSCC. Cytokeratin 17 is suggested to be a potential biomarker candidate in OSCC since it binds to the adaptor protein Stratifin in order to regulate protein synthesis and cell growth through stimulation of Akt/mTOR pathway [31]. Gene expression analysis also indicated higher expression in malignancy. Our data is in agreement with earlier studies suggesting their possible role as a biomarker.

One of the down-regulated proteins observed in current study is Heat shock protein beta 1 which is also called heat shock protein 27. It is a small heat shock protein that has been reported to contribute in invasion and metastasis [32]. A significantly decreased expression of Heat shock protein beta 1 was earlier found in pre-malignant lesions, OSCC cells, and lymph node, highlighting the role of this protein as an indicator of poor prognosis in OSCC [33].

The increased expression of Calreticulin in OSCC tissue is one of the noteworthy findings of the current study because of its involvement in cell proliferation and previously reported in OSCC tongue tissue samples. Calreticulin is a calcium-binding chaperone involved in folding and oligomeric assembly of the proteins [34]. A clinical cohort study and knockdown-model of oral cancer cell line were used to elucidate the role of Calreticulin in oral cancer. The protein was found to be important for cell cycle progression and regulation of cell motility through FAK signaling pathway thus it was suggested to be a potential biomarker for OSCC in tissue samples [35].

Another significant protein among the identified proteins in our study is Stratifin which is also called 14–3-3σ. The protein is involved in regulation of many signaling pathways. It binds to a number of molecules including Cytokeratin 17 thus regulating the activity of binding partners [36]. A strong association between higher stratifin expression and the poor prognosis in adenocarcinoma has been observed [37]. Studies also confirmed that SFN contributes in drug resistance mechanism among patients receiving chemotherapy [38]. An elevated expression of stratifin was strongly correlated with the decreased survival rate in OSCC patients suggesting stratifin as a potential prognostic marker [39]. It has been demonstrated that it is a multifunctional unique protein induced by p53 in response to DNA damage [40]. Stratifin also positively regulates p53 by increasing its transcriptional activity and inducing the autodegradation of MDM2 thus being involved in cancer progression [41]. The p53 pathway is down-regulated in more than 70% of the cases of OSCC resulting in inhibition of the normal apoptotic cycle [42]. An over-expression of p53 and stratifin could be correlated with genetic or epigenetic mutations of either of the two molecules resulting in prevention of normal cell death cycle [41]. Up-regulation of stratifin in the current study in OSCC tissue was confirmed through immune-histochemical analysis and western blotting analysis. Results observed at protein level were complimented by mRNA expression which was also significantly higher in malignancy. Consistent up-regulation of SFN in current study is in accordance with earlier reports that indicate elevated expression in OSCC tissue, cell lines, and serum [43, 44]. As Cytokeratin 17 has also been found over-expressed in the current study, we propose that the binding of Stratifin with Cytokeratin 17 might be of importance for the cell proliferation and carcinogenesis in OSCC.

In conclusion, we identified a number of differentially expressed proteins in tissue samples from OSCC patients. Novel proteins identified in this study included Serum albumin, Ig Kappa chain C region, and Isoform 2 of fructose bisphosphate aldolase A; among which, later two are worth studying on larger dataset. Results of our study not only compliment earlier investigations but also extend the existing knowledge in the area of biomarker identification.

Acknowledgements Financial assistance was provided by Higher Education Commission, Pakistan (Project no. 20-1809). UUM was recipient of EMEA scholarship (SGA 2012-2642) funded by the Erasmus Mundus Programme of EU. Authors acknowledge the technical assistance offered by Kieran Wynne, UCD Conway Institute.

Compliance with Ethical Standards

Ethical Approval The study was approved by IRB, (NCP-108), University of Karachi and guidelines proposed by the Declaration of Helsinki were followed.

Declaration The work described has not been published previously and the authors declare no competing financial interests.

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