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Research paper

Secreted Down Regulated Protein Markers in Oral Squamous Cell Carcinoma

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is the key reason for illness and death by oral cancer. The discovery of candidate markers to differentiate normal from malignant cells can result in an accurate and early clinical diagnosis of OSCC. We aimed to identify candidate biomarkers for OSCC diagnosis and to enhance understanding of their role in disease progression and pathogenesis. Of particular interest are proteins that are secreted in saliva to allow non-invasive detection. We employed a high throughput protein profiling approach involving single shot peptide fractionation coupled to mass spectrometry analysis of the fourteen paired human malignant OSCC and normal adjacent mucous samples to identify protein markers. We found a total of 5,123 protein fractions in the proteome dataset of OSCC and normal samples, including 94 proteins with strong differential expression (p-value <0.01 and the fold change <-2). Functional data analysis revealed that these 94 down-regulated proteins were significantly associated with multiple biological processes, like myogenesis, fatty acid metabolism, and KRAS Signaling. To further highlight candidate markers for non-invasive OSCC detection, we combined our data with public salivary datasets, identifying 3 candidate markers for further study. Our investigation identified proteins and TF involved in key pathways regulating them is likely to provide insight into the mechanism of OSCC progression which may lead to developing novel OSCC diagnostics and therapeutics strategies.

KEYWORDS: Down-regulation, protein markers, oral squamous cell carcinoma, proteomics

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is among the major causes of oral cancer. The prevalence of oral cancer is diverse in different regions, like South Asia, it is the most diagnosed carcinoma in male patients however it is responsible for 1–4% of all cancers in the Western world. Lip oropharynx and oral cancer are accountable for 4,47,751 new cancer cases with approximately 2,28,389 deaths in 2018 (i.e. 2.4 percent of all cancer deaths). Head and neck cancer is 13th in mortality and 14th in terms of incidence among other cancers (1). As per 2012 and 2018 worldwide data, in the HNSCC group, the prevalence of oral squamous cell carcinoma is the 11th and 18th most frequent cancer, respectively. Pakistani and Bangladeshi males in Asia ranked first in this cancer (2).

The frequency of oral cancer is on the rise due to heavy smoking, unhealthy nutrition (irregular intake of strong alcohols, spices, etc.), and longer lifespans. Among the main causes for the increased incidence is the late diagnosis, usually revealed once complications start. Oral cancer is an “Epithelial Neoplasia,” although the basement membrane begins as clonal increased growth of changing stem cells enlarged upward and in lateral position and progress to invasive and in situ carcinoma by

changing normal epithelium mucosa from hyperplasia to dysplasia. The tumor-Node-Metastases System used for staging OSCC samples was developed by American Joint Committee on Cancer (3). International Histological Classification of Tumors (WHO) and tumor invasion front (TIF) were recently two systems used for tumor lesions histological classification. The spread of the tumor and the growth rate of the tumor are necessary to be assessed. Degree of tumor differentiation (either well, moderately, or undifferentiated) used initially for classification (4). It is necessary to develop diagnostic tools for OSCC early detection and its progression monitoring and therapeutic drugs targets identification.

For cell signaling pathways and protein expression elucidation, the study of Proteomics becomes increasingly useful (5). Proteomic research was carried out in our study, by gel electrophoresis and mass spectrometry to examine the differential expression of proteins for the identification of novel markers. Identification of altered protein expression in cancer will help in identifying key biological pathways, leading to a better understanding of the underlying molecular mechanism of the disease, and can be used in precise therapy selection in OSCC management. Therefore, in the present study normal adjacent tissue and tumor tissue were taken as samples for comparison.

MATERIALS AND METHODS

Collection of tissue specimens of OSCC patients

This study includes fourteen patients; Tissue samples of male and female subjects diagnosed with oral squamous cell carcinoma (OSCC) were collected from a local hospital in Lahore, Pakistan according to the approved guidelines of the Ethical Review Committee of the King Edward Medical University [Ref. No. 306/RC/KEMU] with the consent of the patients. Patients with either Hyperplasia, Papilloma, Pleomorphic Adenoma, Soft Tissue Tumor, Odontogenic tumors, HIV infection, hepatitis C, B, and/or with a history of current or earlier chemotherapy or radiotherapy were excluded from the study. All tumors of OSCC patients included in the study were HPV-negative. Tumors were TNM staged according to AJCC recommendations. After surgery tissue specimens (tumor and adjacent non-tumor tissue) were immediately frozen in liquid nitrogen and stored at -80°C for future use. Non-tumor tissues were cancer cell-free and around 80% of cancer cells were present in stored tumor tissues as revealed by pathological evaluation. The histological findings showed cases of moderately differentiated SCC and well-differentiated. Clinical information of patients was collected from the hospital files including medical history and demographic data (Table 1).

Tissue lysis and protein extraction

As mentioned previously (6), 200 mg of the tumor tissue and control tissue samples were homogenized with liquid nitrogen in a pestle with mortar, suspended in modified chilled lysis buffer, vortexed for 1 hour, and centrifuged for 90 min at 1400 rpm at 4 °C (Eppendorf Centrifuge, 5417R). The resultant supernatant was aliquoted and stored at -80°C. Bradford assay (7) with bovine serum albumin (BSA) as a reference was used for estimating protein concentration in tissue lysates.

Gel electrophoresis and in-gel digestion

Tissue lysates (25µl) were separated on a 12% SDS-PAGE followed by Coomassie staining. The protein bands were cut and processed for trypsin digestion (8). Peptides were concentrated before LC-MS/MS (9).

Nano-MS/MS analysis, Database searching, and protein identification

Nano-MS/MS measurement was performed as described previously (10). MS/MS spectra

were analyzed with the help of the SwissProt human reference proteome file [downloaded February 2019 (42417 entries)] using MaxQuant (11).

Protein quantitation and differential analysis

Spectral counting quantification of proteins was used, that is the sum of all MS/MS spectra for every detected protein (12). Differential analysis of proteins between samples was done using dedicated statistics by the beta-binominal test (13,14) using the R package.

Data mining, visualization & network analysis

The "protein–protein interaction network" analysis of down-regulated proteins in OSCC was carried out by the STRING tool (15), Cytoscape (16), and WebGestalt tool to do the GSEA (17). TFs were identified using iRegulon (18).

Table 1: Clinical data of the study subjects (Histological diagnosis. *1. Poorly Differentiated SCC, 2. Moderately Differentiated SCC, 3. Well Differentiated SCC).

No.	OSCC Patients (Gel Labels)	Sex	Age	Family History	Clinical Presentation	Tumor Localization	Histological Diagnosis*	Staging TNM
1	ST1	M	70	No	Lesions with raised exophytic margin	Buccal Mucosa	3	Stage IVA (T4aN1M0)
2	MT1	M	62	No	Ulcerative type lesion	Tongue	3	Stage IVA (T4aN1M0)
3	ET	M	60	No	Ulcerative type lesion	Tongue	1	Stage II (T2N0M0)
4	ST2	M	55	No	Lump	Buccal Mucosa	2	Stage III (T2N1M0)
5	MT2	F	40	No	Ulcerative type lesion	Molar area (Mandibular Retromolar Area)	3	Stage IVA (T4aN0M0)
6	NT	M	51	No	Lichen planus	Buccal Mucosa	2	Stage IVA (T4aN0M0)
7	BT	M	38	No	Ulcerative type lesion	Tongue	3	Stage IVA (T4aN0M0)
8	AT	M	45	No	Lichen planus	Buccal Mucosa	3	Stage IVA (T4aN1M0)
9	MT3	F	65	No	Ulcerative type lesion	Hard Palate	3	Stage III (T3N0MX)
10	GT	M	60	No	Ulcerative type lesion	Soft Palate	3	Stage III (T2N1M0)
11	ANT	M	36	Yes (Father)	Ulcerative type lesion	Molar area (Mandibular Retromolar Area)	2	Stage IVA (T4aN2M0)
12	JT	M	35	No	Mixed ulcerative & exophytic lesions	Buccal Mucosa	3	Stage IVA (T2N2M0)
13	GT	F	30	Yes (Mother)	Lesions with raised exophytic margin	Tongue	2	Stage III (T3N0M0)
14	NOT	F	55	No	Verrucous type lesion	Lip	3	Stage IVA (T4aN0M0)

RESULTS

Protein profiling of OSCC tissue vs NAMs lysates

We carried out proteomics of cancer and adjacent normal tissues of 14 subjects with OSCC. The obtained dataset consisted of 5,123 proteins. On average, 3104 proteins were identified per sample. We used a paired beta-binomial test to identify the differentially expressed proteins. First, we performed GSEA on the proteins identified to investigate the most prominent biological processes. This analysis revealed that the myogenesis pathway was mostly reduced in tumor samples (or enriched in mucosa samples) (Figure 1).

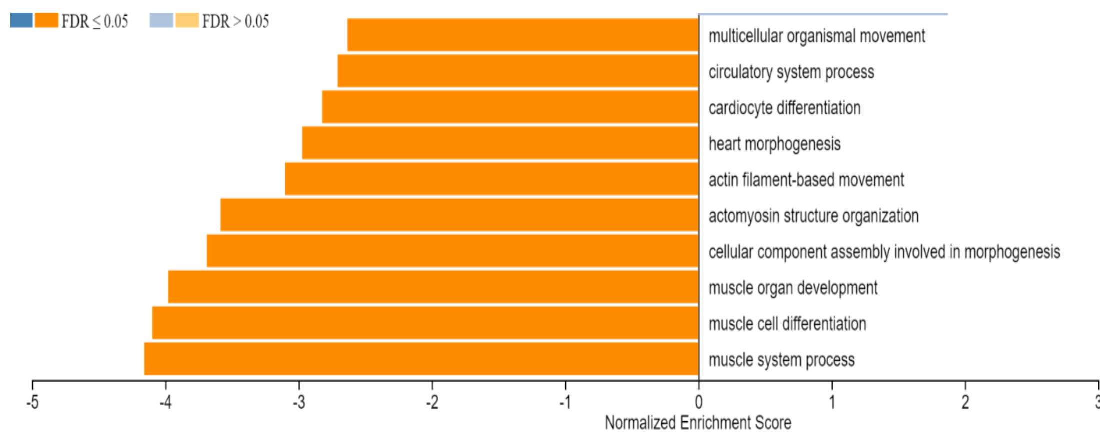

Figure 1: Down-regulated biological processes and pathways identified by GSEA.

Down Regulated protein networks associated with OSCC

To study with proteins and pathways were deregulated in OSCC tissues, we performed differential analysis. A total of 94 down regulated proteins were differentially expressed (2-fold down, $p<0.01$). We focused our analysis on the 94 downregulated proteins separately. The protein-protein interaction of the downregulated proteins revealed clusters linked to muscle system processes and actin filament organization (Fig. 2, cluster 1), regulation of sequestered Ca^{+2} into the cytosol by sarcoplasmic reticulum (cluster 2). GO analysis of these selected clusters found that these proteins were involved in different biological pathways showed in Table 2. SRF central transcription factors were identified in down regulated muscle involvement pathway cluster 1 (FC: <-2, P 0.01, average count >10) on iRegulon (Fig. 3). Drugs that induce actin stabilization or polymerization could potentially be a novel therapeutic plan to treat tumors (19).

Candidate biomarker identification

We compared salivary proteome data of normal mucosa (https://salivaryproteome.nidcr.nih.gov/) vs. OSCC datasets (20,21). Importantly, 27 out of 94 candidate proteins we identified as potential OSCC biofluid markers, with 3 top candidates detected in all 3 salivary datasets (Table 3). Thus, there is a possibility that these proteins could be utilized for the development of a non-invasive biomarker test aimed at detecting or predicting the prognosis of OSCC.

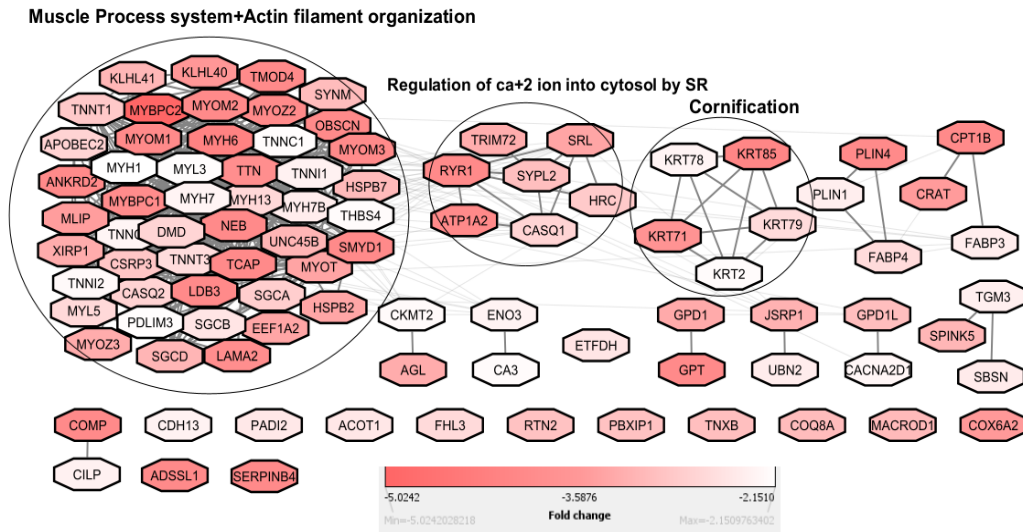

Figure 2. Down regulated biological processes associated with OSCC.

Table 2. Down regulated biological processes associated with OSCC. Biological processes associated with the most populated protein clusters analyzed using Clue Go.

PROCESS	GO TERMS	TERM P VALUE	CORRECTED P VALUE	NUMBER OF GENES	CLUSTERS
Muscle system process		3.24E-44	1.78E-42	[ANKRD2, ATP1A2, CACNA2D1, CASQ1, CASQ2, CKMT2, COMP, CSRP3, DMD, GPD1L, HRC, JSRP1, KLHL41, MLIP, MYBPC1, MYBPC2, MYH1, MYH13, MYH6, MYH7, MYH7B, MYL3, MYL5, MYOM1, MYOM2, MYOM3, MYOT, MYOZ2, NEB, RYR1, SGCA, SGCD, SYNM, TCAP, TMOD4, TNNC1, TNNC2, TNNI1, TNNI2, TNNT1, TNNT3, TRIM72, TTN]	1
Actin filament organization	Actomyosin structure organization	1.62E-17	6.64E-16	[CASQ1, CASQ2, CSRP3, KLHL41, LDB3, MYH6, MYH7B, MYOM1, MYOM2, MYOM3, MYOZ2, NEB, OBSCN, TCAP, TMOD4, TNNT1, TNNT3, TTN]	
	Actin filament-based movement	5.71E-23	2.68E-21	[ATP1A2, CACNA2D1, DMD, GPD1L, MYBPC1, MYBPC2, MYH6, MYH7, MYH7B, MYL3, NEB, SGCD, TCAP, TNNC1, TNNC2, TNNI1, TNNI2, TNNT1, TNNT3, TTN]	
Regulation of ca+2 ion into cytosol by SR	Release of sequestered calcium ion into cytosol by sarcoplasmic reticulum	3.28E-10	3.28E-10	[ATP1A2, CASQ1, HRC, RYR1]	2

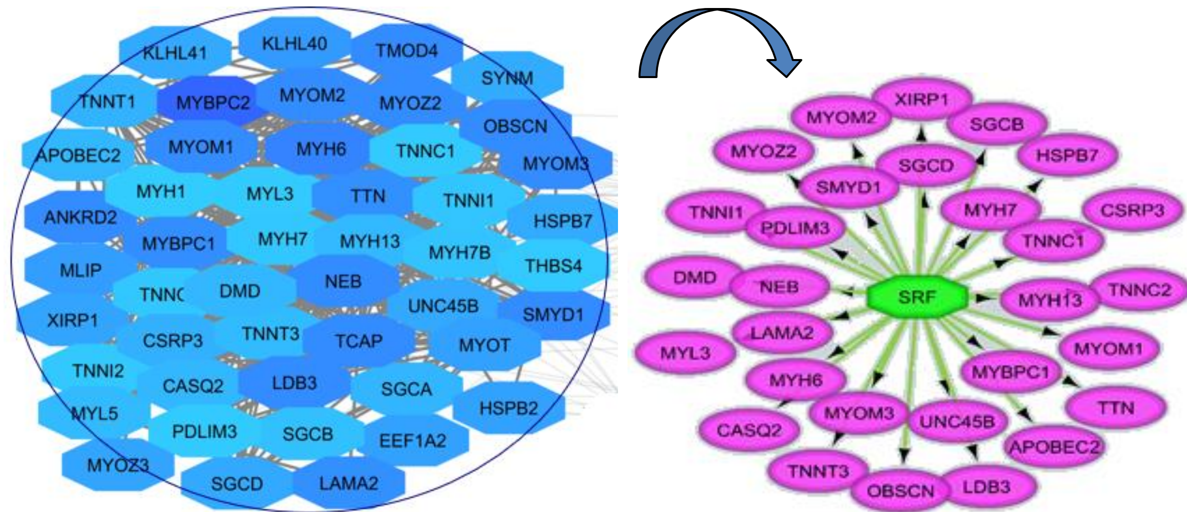

Figure 3. Down regulated biological processes associated with OSCC. Down regulated muscle involvement pathway cluster (FC: <-2, P 0.01, average count >10) iRegulon identified SRF central transcription factor.

Table 3. Three OSCC tumor prediction biomarker candidate proteins (p < .01, FC -2), and their detection in public saliva datasets.

Accession number	Gene Symbol	Razor+ unique peptides count	Protein Name	p-value	OSCC Tumor vs NAT FCs	Detection frequency of the 3 saliva datasets	Subcellular localization
P35749	MYH1	151	Myosin-11	0.003	-2	3	Cytoskeleton
Q08188	TGM3	34	Protein-glutamine gamma-glutamyl transferase	0.005	-2	3	Plasma Membrane
Q6UWP8-2	SBSN	4	Suprabasin	0.000	-2	3	Extracellular

DISCUSSION

Previously, to uncover the biological roles of the differentially expressed genes in tumors on gene ontology (GO) enrichment analysis, genes related to muscle contraction have been reported to be altered in oral squamous cell carcinoma (OSCC) and suggests the presence of myofibroblasts in tumor stoma of patients with lymph node involvement. Pathway enrichment analysis identified pathways related to an extracellular matrix organization, muscle contraction, calcium signaling, and salivary secretion to be the most significantly enriched pathways in both categories. Muscle contraction pathways were reported to be deregulated in lymph node metastasis OSCC (22) and a promising pathway for therapeutics strategy designing (23). The prominent nodes with the lowest p-values signify the presence of muscle contraction pathways and associated altered expression of genes such as TTN, TNNT3,

TNN2, MYH6, and MYL2 in tumors. This finding aligns with a previous study that used microarray-based gene expression profiling and identified a distinctive signature pattern associated with the muscle contraction pathway (22). In addition, various studies have demonstrated the existence of myofibroblasts in the tumor stroma of lymph nodes through gene expression analysis, establishing their involvement in metastatic migration, invasion, and correlation with a lower survival rate among patients with OSCC (23). We also found significantly decreased levels of many proteins in tumor tissue compared to NAMs and few of them were found to be secreted in normal and tumor saliva as well. The regulation of serum response factor (SRF) and its target genes/proteins provide a classic example of the diversity of genes controlled by a single DNA-binding protein and the importance of cofactor interactions in the control of gene expression (24). SRF thereby serves as a platform to interpret cell identity and signaling by engaging various partners. The recent discovery and mechanistic dissection of the myocardia family of transcriptional coactivators (25, 26), which regulate SRF activity during cell growth, migration, and myogenesis, have provided new insights into the mechanism of action of SRF, as well as the roles of coactivators in the control of gene expression in general. We suggest more studies be focused to illuminate the role of muscle-related proteins in "OSCC" carcinogenesis.

CONCLUSIONS

By analyzing the proteomics data of OSCC, we identified proteins that are down-regulated and associated with biochemical pathways and biological networks, potentially contributing to the development of cancer. The proteins that were found to be differentially expressed and associated with multiple biochemical pathways could enhance our comprehension of the molecular mechanisms involved in OSCC tumors. Nevertheless, additional research is necessary to comprehend the impact of etiological factors on the pathogenesis of this oral cancer.

List of Abbreviations

Normal Adjacent Tissue (NAT)

Oral Squamous Cell Carcinomas (OSCC)

Head and Neck Squamous Cell Carcinoma (HNSCC)

Liquid chromatography-mass spectrometry (LC-MS/MS)

Gene Set Enrichment Analysis (GSEA)

Gene ontology (GO), Differentially expressed Proteins (DEPs)

Fold Changes (FCs), Transcription Factors (TFs)

Ethics approval and patient consent

All patients or legal guardians consented to participation in the study in accordance with the Institutional Ethical Review Board, King Edward Medical University [Ref. No. 306/RC/KEMU].

Consent for publication

All authors consent to the publication of this manuscript.

Availability of data and materials

The datasets generated or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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