RESEARCH ARTICLE

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Quantitative Proteomic Analysis of Non-Tobacco Associated Oral Squamous Cell Carcinoma Reveals Deregulation of Cytoskeletal and Apoptotic Proteins

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Abstract

Background: The exact etiology of non-tobacco associated oral squamous cell carcinoma (NT-OSCC) is still unknown. The lack of established biomarkers for oral NT-OSCC has resulted in less effective management and poor prognosis. Here, we report for the first time a panel of potential markers identified from the quantitative proteomic analysis of NT-OSCC using two-dimensional gel-electrophoresis (2D-GE) using matrix-assisted laser desorption/ ionization - time of flight (MALDI-TOF) coupled with mass spectrometry (MS) and further analysis using protein analysis through evolutionary relationships (PANTHER) database. **Objective:** To quantitatively analyze the proteomic profile of non-tobacco associated oral squamous cell carcinoma. **Methods:** Twenty fresh tissue samples were collected from healthy controls and NT-OSCC, ten each, and were subjected to proteomic analysis. Sample quantification for the presence of protein was done using Bradford assay and bovine serum albumin was used as a standard protein to obtain the standard graph. Fractionation of protein was done using sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and they were separated based on their molecular weight. MS analysis was done and the purified peptides were analysed using MALDI-TOF. PANTHER database for functional classification and pathway analysis was done for identification of protein expression. **Results:** Our approach of combining 2D-GE with MS identified four candidate proteins including keratin, alpha-1-antitrypsin (AAT), S100 and serpin B5 with significant differential expression in NT-OSCC as compared with healthy controls. The results showed that the levels of these proteins were significantly upregulated in NT-OSCC when compared to the healthy controls that suggests that these proteins can be used as candidate targets for NT-OSCC therapeutics. **Conclusion:** The differentially expressed proteins are found to be involved in apoptotic signalling pathways, cytoskeletal dynamics and are known to play a critical role in oral tumorigenesis. Put together, the results provide available baseline information for understanding the development and progression of NT-OSCC. These identified proteins on further validation may be used as potential biomarkers in future for early detection and predicting therapeutic outcome of patients with NT-OSCC.

Keywords: Non-tobacco associated OSCC- keratin- alpha-1-antitrypsin- S100- serpin

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Introduction

Oral squamous cell carcinoma (OSCC) constitutes 45% of all cancers occurring in the Indian sub-continent thus leading as the single most commonly encountered cancer of the head and neck region. OSCC contributes to over 80% of malignant head and neck lesions and is well recognized as the most common malignant tumour of oral structures. Every year 300,000 new cases are diagnosed worldwide (Krishnan et al., 2014)

The use of the molecular approach in the last few decades has revealed the proteomic picture in the pathogenesis of OSCC. There are powerful search engines to help identify alterations in genetic expression, the role of gene instability and epigenetic modifications that bring about differential gene expression in oncogenesis (Williams, 2000; Ananthi et al., 2018).

Understanding such gene alterations and the differential expression of genetic patterns are essential to understanding the molecular pathogenesis of oral squamous cell carcinoma. Despite the achievement of significant leads, a thorough understanding of the pathogenesis of oral cancer at the molecular level and its interrelation with etiological agents might, at the current rate, need at least another decade of exhaustive research (Yost et al., 2018).

Although tobacco usage is the primary cause of OSCC, there is a subset of OSCCs that are non-habit related. As

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most of the studies assessing the etiology are focused on habit associated OSCCs, there is a void regarding the pathogenesis of non-habit associated OSCCs (NT-OSCC) and is still understudied. Hence, this study tends to address the lacuna associated with the identification of molecular biomarkers that are implicated in the pathogenesis of non-tobacco associated oral squamous cell carcinoma. This may improve the precise diagnosis of the pathology at the early stages of the disease and therefore may help in understanding the basic process linked to aggressiveness and would aid in the development of a novel therapeutic modality for improved treatment and better outcome.

Biomarker detection has been made possible by the latest technological advancements of mass spectrometry (MS) instrumentation and the several bioinformatics aids available in this current era of science. Together, they help in being the swiftest and at the same time, being the simplest method of profound, all-inclusive, and high output proteomic biomarker analysis (Cation and Ramos, 2022)

The aim of this study was to identify the proteins that participate in functional alterations such as metastasis and apoptosis and assessing their expression levels, which on further validation can serve as potential sites for target therapy in future.

Materials and Methods

Patient selection: The study was approved by the Institutional Review Board of SRM Institute of Science and Technology, Ramapuram, Chennai (IRB Approval no: SRMDC/IRB/2017/MDS/No.604). Informed consent was obtained from all patients recruited for the study. A total of 20 samples were used in the present study and all samples were obtained from patients attending SRM Dental College, Ramapuram between 2019 to 2021. Tumor and normal tissue samples were snap frozen and stored at liquid nitrogen until used for protein and ribonucleic acid (RNA) extraction. All methods were performed in accordance with the guidelines approved by Institutional Ethics Committee of SRM Dental College, Chennai, India.

Protein extraction: Tissue extracts were made by grinding the samples in chilled mortar and pestle with liquid nitrogen and dissolved in lysis buffer (7 M Urea, 2 M Thiourea). Samples were further fragmented by sonication for 10 minutes (min) and centrifuged at 12,000 revolutions per minute (rpm) for 15 min at 4 degree Celsius (°C).

Sample quantification for the presence of protein using Bradford assay: To determine if the quantity of protein in the sample was adequate for further analysis, sample quantitation was done using Bradford assay. The unknown protein sample was diluted to obtain 5-100 microgram (ug) protein per 30 millilitres (ml). Standards were prepared with a range of 5-100 microgram of protein. 1.5 ml of Bradford reagent was added to both standards and sample and incubated at room temperature for 5 min. Absorbance was measured at room temperature using a spectrophotometer at 595 nanometre (nm). Bovine serum albumin (BSA) was used as a standard protein to obtain the standard graph (Ananthi et al., 2018)

Fractionation of protein using SDS-PAGE

After protein estimation, the samples were analysed in sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were separated based on their molecular weight.

Mass spectrometry analysis: Total tissue proteins (250 ug) were analyzed by two-dimensional separation, first with 18 centimetre (cm) immobilized pH gradient (IPG) strips of pH 3–11 and second by PAGE analysis. Separated proteins were subjected to colloidal Coomassie blue G-250 staining and in-gel trypsin digestion. Destained gel pieces were then dehydrated in 100% acetonitrile (ACN) for 10 min and dried under vacuum for 30 min and then rehydrated/trypsinized with 5 µl of trypsin buffer (10 mM ammonium bicarbonate-NH_4HCO_3 in 10% ACN) containing 400 ng of trypsin (Sigma Aldrich, USA) on ice for 30 min and overlayed with 25 µl of buffer (40 mM NH_4HCO_3 in 10% ACN) and incubated at 37°C for 16 hours (h). Then, peptides, extracted twice with 25 µl of 0.1% trifluoroacetic acid (TFA) in 60% ACN by sonication (10 min) followed by 20µl of 100% ACN, were dried under vacuum for 90 min, dissolved in peptide resuspension solution (0.1% TFA in 5% ACN) and desalted using C18 zip tips (Merck Millipore, USA) according to the manufacturer's instructions. Zip tip purified peptides were analyzed using matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF) (Ananthi et al., 2018).

Functional classification of proteins and pathway analysis: The gene symbols of the differentially expressed proteins were given as input to protein analysis through evolutionary relationships (PANTHER) database for functional classification and pathway analysis. STRING (www.string.db.org) was used for protein network construction.

Results

Protein extraction and quantification: Protein extraction was done and quantified using Bradford assay. The assay revealed presence of protein that was assessed through measurement of absorbance difference from standard BSA using a spectrophotometer at 595 nm. The degree of change in absorbance between the standard and the test solutions indicated the presence of protein in adequate quantities for subsequent assays.

Fractionation of protein using SDS-PAGE

After protein estimation, the samples were analysed in SDS-PAGE where proteins were separated based on their molecular weight. The samples along with a standard marker were loaded into the gel that was segregated into bands based on molecular weight.

Two Dimensional-Gel Electrophoresis (2D-GE)

The proteins were further segregated based on the net charge using 2D-GE. The results of this experiment showed a prominent spike in the bands of four proteins (Figure 1). These band spikes were later identified using MALDI-TOF.

Table 1. Regulation Status of the Proteins Identified Using Matrix-Assisted Laser Desorption/Ionization - Time of Flight (Maldi-Tof) Analysis.

Spot ID	Gene ID	Accession	Description	Score	Coverage	MW [kDa]	calc. pI	Regulation status in NT-OSCC
1	KRT14	P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	32.99	50.85	51.5	5.16	Up regulated
2	SERPINA1	P01009	Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3 - [A1AT_HUMAN]	50.65	31.82	46.7	5.59	Up regulated
3	S100A6	P06703	Protein S100-A6 OS=Homo sapiens GN=S100A6 PE=1 SV=1 - [S10A6_HUMAN]	159.6	75.56	10.2	5.48	Up regulated
4	SERPINB5	P36952	Serpin B5 OS=Homo sapiens GN=SERPINB5 PE=1 SV=2 - [SPB5_HUMAN]	15.44	27.47	42.1	6.05	Up regulated

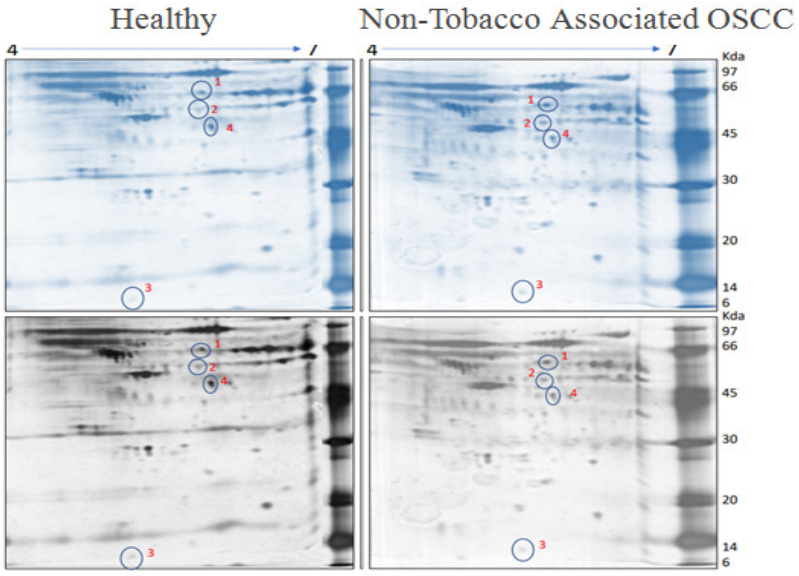

Figure 1. Representative Protein Profile of Two-Dimensional-Gel Electrophoresis (2D-GE) Containing Tissue Samples (1-Keratin, 2- Alpha-1-antiTrypsin, 3- S100, 4- Serpin B5

MALDI-TOF to identify the candidate proteins

The protein spikes were subjected to MALDI-TOF to identify the key candidate proteins. The results indicated that there was an upregulation in the level of the following proteins in NT-OSCC in comparison to healthy volunteers (Table 1).

Functional classification of identified proteins and biological network analysis

The functions of all the differentially expressed proteins were better comprehended by PANTHER (Mi H et al., 2017). The proteins were sorted into three groups based on their cellular localization, molecular function, and biological process as shown in Table 2.

Discussion

OSCC contributes to ninety percent of malignant oral lesions and is commonly known as the most habitually developing tumour of the oral cavity (Torre et al., 2012). While utilizing tobacco is the chief cause of OSCC, there is a subclass of OSCCs that are non-habit (tobacco) related.

Numerous studies have been done with regard to the genetic biomarkers in oral squamous cell carcinoma. One such study shows a positive expression of CEA mRNA marker and CK19 mRNA marker in OSCC cases. The difference was significant between the patients and normal groups (Rashid et al., 2018).

Proteomic analysis of tobacco-associated OSCC showed deregulation of collagen formation and antigen

Table 2. Functional Contribution of the Identified Proteins

Gene Ontology of Differentially expressed proteins	Keratin (In percentage)	Alpha-1-Antitrypsin (In percentage)	S100 (In percentage)	Serpin B5 (In percentage)
Cellular Component	44	54	42	40
Biological Process	7	7	6	33
Molecular Function	49	39	52	27

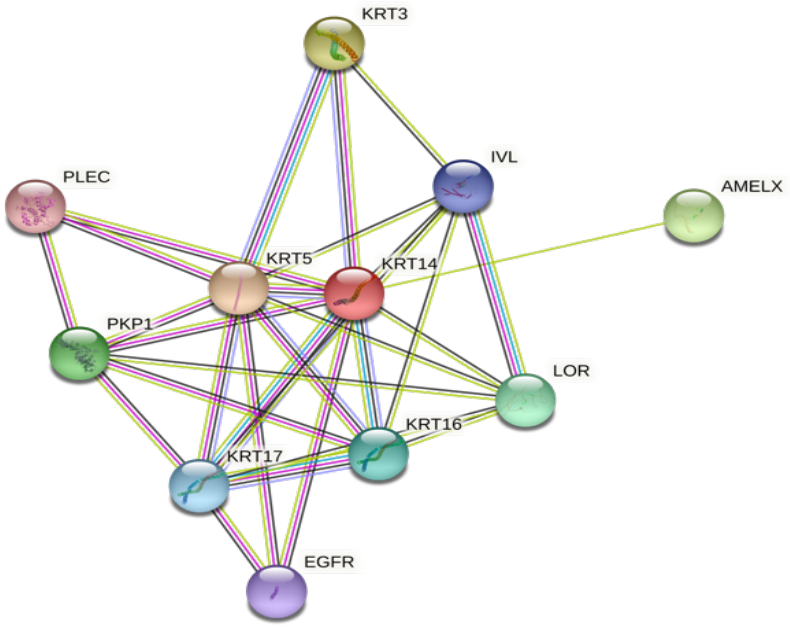

Figure 2. Keratin, Type I Cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]

processing/presentation pathway while proteins related to the mechanism of keratinization indicated upregulation of their expression. Increased expression of proteins associated with immune pathways and downregulation of muscle contraction-mediated signaling events was also seen in it. (Bhat et al., 2021)

As most studies evaluating the etiology are focused on habit-linked OSCCs, there is a vacuum regarding the pathogenesis as the non-habit stimulated carcinomas are still understudied. Therefore, detection of molecular markers may improve precision diagnosis of NT-OSCC during initial stages and may help in clear understanding the biological process associated with aggressiveness of oral cancer aiding targeted therapy for better results. Identification and assessment of expression levels of proteins that participate in functional alterations such as metastasis and apoptosis will enable prevention of disease progression. This on further validation can serve as potential sites for target therapy. Our study, therefore, aimed at the identification of proteins that are novel to NT-OSCC, thereby contributing to a better understanding of carcinogenesis.

In this study, the proteomic profile of NT-OSCC tumor tissue samples was compared with matched normal samples and observed 4 proteins were significantly deregulated. Evaluation of reported cellular functions for the 4 differentially regulated proteins indicated that cell survival and cytoskeletal remodelling pathways are

Table 3. Keratin, Type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]

Name	Go Term	Qualifier	Percentage	Count
protein binding	Molecular Function	Enables	45.21	33
cytosol	Cellular Component	Part of	21.92	16
nucleus	Cellular Component	Part of	5.48	4
cytoplasm	Cellular Component	Part of	5.48	4
intermediate filament	Cellular Component	Part of	4.11	3
keratin filament	Cellular Component	Part of	2.74	2
structural molecule activity	Molecular Function	Enables	1.37	1
structural constituent of cytoskeleton	Molecular Function	Enables	1.37	1
aging	Biological Process	Involved in	1.37	1
epidermis development	Biological Process	Involved in	1.37	1
epithelial cell differentiation	Biological Process	Involved in	1.37	1
hair cycle	Biological Process	Involved in	1.37	1
intermediate filament bundle assembly	Biological Process	Involved in	1.37	1
basal part of cell	Cellular Component	Part of	1.37	1
extracellularexosome	Cellular Component	Part of	1.37	1
cell periphery	Cellular Component	Part of	1.37	1
keratin filament binding	Molecular Function	Enables	1.37	1

Figure 3. Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3 - [A1AT_HUMAN]

significantly altered in NT-OSCC. Amongst proteins regulating cell survival cascade, serpin B5 negatively regulate the apoptotic pathway and S100 encroach on cell proliferative pathways while keratin and AAT play an important role in invasion and metastasis, thereby contributing to poor prognosis in NT-OSCC.

Keratins sustain the epithelium's structure, govern metabolic activities, and drive intracellular signalling pathways that control epithelial development. Keratin, like keratin 17 (KRT17) operates as a pathogenic keratin in OSCC, facilitating tumour development by stimulating numerous signalling pathways, emphasising the role of keratin as a multifunctional tumorigenesis promoter (Khanom et al., 2016). Keratin plays the main role in

Table 4. Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3 - [A1AT_HUMAN]

Name	Go Term	Qualifier	Percentage	Count
protein binding	Molecular Function	Enables	24.32	18
extracellular space	Cellular Component	Part of	9.46	7
endoplasmic reticulum lumen	Cellular Component	Part of	9.46	7
COPII-coated ER to Golgi transport vesicle	Cellular Component	Part of	8.11	6
serine-type endopeptidase inhibitor activity	Molecular Function	Enables	6.76	5
extracellular region	Cellular Component	Part of	6.76	5
endoplasmic reticulum	Cellular Component	Part of	5.41	4
identical protein binding	Molecular Function	Enables	5.41	4
collagen-containing extracellular matrix	Cellular Component	Part of	5.41	4
extracellularexosome	Cellular Component	Part of	2.7	2
protease binding	Molecular Function	Enables	1.35	1
Golgi apparatus	Cellular Component	Part of	1.35	1
acute-phase response	Biological Process	Involved in	1.35	1
blood coagulation	Biological Process	Involved in	1.35	1
hemostasis	Biological Process	Involved in	1.35	1
negative regulation of peptidase activity	Biological Process	Involved in	1.35	1
negative regulation of endopeptidase activity	Biological Process	Involved in	1.35	1
peptidase inhibitor activity	Molecular Function	Enables	1.35	1
platelet alpha granule lumen	Cellular Component	Part of	1.35	1
endoplasmic reticulum-Golgi intermediate compartment membrane	Cellular Component	Part of	1.35	1
intracellular membrane-bounded organelle	Cellular Component	Part of	1.35	1
ficolin-1-rich granule lumen	Cellular Component	Part of	1.35	1

Figure 4. Protein S100-A6 OS=Homo sapiens GN=S100A6 PE=1 SV=1 - [S10A6_HUMAN]

cancer pathogenesis by being parts of cellular components followed by enabling certain molecular functions. There is evidence for active keratin participation in cancer cell invasion and metastasis, as well as response to therapy, reinforcing the role of keratins as multimodal regulators of epithelial tumorigenesis. Keratins are also required for chaperone-mediated intracellular signalling, which could contribute to epithelial cancer. Specifically, expression

Table 5. Protein S100-A6 OS=Homo sapiens GN=S100A6 PE=1 SV=1 - [S10A6_HUMAN]

Name	Go Term	Qualifier	Percentage	Count
protein binding	Molecular Function	Enables	26.56	17
calcium ion binding	Molecular Function	Enables	9.38	6
cytoplasm	Cellular Component	Part of	7.81	5
nuclear envelope	Cellular Component	Part of	6.25	4
nucleus	Cellular Component	Part of	4.69	3
plasma membrane	Cellular Component	Part of	4.69	3
cytosol	Cellular Component	Part of	3.13	2
proteinhomodimerization activity	Molecular Function	Enables	3.13	2
S100 protein binding	Molecular Function	Enables	3.13	2
calcium-dependent protein binding	Molecular Function	Enables	3.13	2
perinuclear region of cytoplasm	Cellular Component	Part of	3.13	2
collagen-containing extracellular matrix	Cellular Component	Part of	3.13	2
extracellularexosome	Cellular Component	Part of	3.13	2
ruffle	Cellular Component	Part of	1.56	1
tropomyosin binding	Molecular Function	Enables	1.56	1
extracellular region	Cellular Component	Part of	1.56	1
signal transduction	Biological Process	Involved in	1.56	1
axonogenesis	Biological Process	Involved in	1.56	1
zinc ion binding	Molecular Function	Enables	1.56	1
ion transmembrane transporter activity	Molecular Function	Enables	1.56	1
membrane	Cellular Component	Part of	1.56	1
extrinsic component of cytoplasmic side of plasma membrane	Cellular Component	Part of	1.56	1
ion transmembrane transport	Biological Process	Involved in	1.56	1
metal ion binding	Molecular Function	Enables	1.56	1
positive regulation of fibroblast proliferation	Biological Process	Involved in	1.56	1

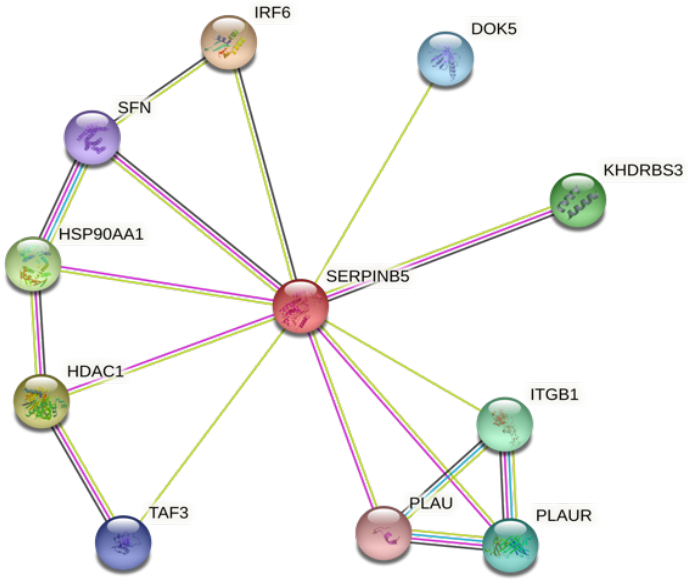

Figure 5. Serpin B5 OS=Homo sapiens GN=SERPINB5 PE=1 SV=2 - [SPB5_HUMAN]

Table 6. Serpin B5 OS=Homo sapiens GN=SERPINB5 PE=1 SV=2 - [SPB5_HUMAN]

Name	Go Term	Qualifier	Percentage	Count
extracellular space	Cellular Component	Part of	20	3
serine-type endopeptidase inhibitor activity	Molecular Function	Enables	13.33	2
protein binding	Molecular Function	Enables	13.33	2
cytoplasm	Cellular Component	Part of	13.33	2
morphogenesis of an epithelium	Biological Process	Involved In	6.67	1
extracellular region	Cellular Component	Part of	6.67	1
negative regulation of endopeptidase activity	Biological Process	Involved In	6.67	1
extracellular matrix organization	Biological Process	Involved In	6.67	1
regulation of epithelial cell proliferation	Biological Process	Involved In	6.67	1
prostate gland morphogenesis	Biological Process	Involved In	6.67	1

of keratin 14 (KRT14) was significantly correlated with well-differentiated OSCC (Kengkarn et al., 2020).

The major activity of keratin, as per our study, is its role of enabling protein binding constituting 45% of the individual actions of keratin (Table 3; Figure 2). Tumour cells produce proteolytic enzymes that are thought to be essential for the invasion and degradation of the extracellular matrix and patients with a high concentration of trypsin have a poor prognosis. In lieu of estimating trypsin levels, the serum concentrations of AAT can be measured as this antiprotease has been found to be elevated in numerous malignancies. The levels of AAT may provide insight into the magnitude of proteolysis caused by the malignancy. Researchers have looked into the link between high levels of AAT and various cancers and showed that when tumour burden increased, AAT levels increased dramatically, functioning as a predictive marker for the disease process. Cancer patients frequently have an immunocompromised state at the time of diagnosis, which results in an overexpression of AAT levels. The increase might be due to the host's numerous defensive systems in reaction to the tumour load, or it could be due to a normal immunoregulatory process (Greene and McElvaney, 2010) (Table 4; Figure 3).

The S100 gene family is the biggest subfamily of genes that code for EF-hand calcium-binding proteins. Downregulated expression of the S100A2, A100A4, S100A6, S100A7, and S100A8 has been observed in oral cancers, precancerous tissues, and/or various oral cancer cell lines in earlier investigations. S100 proteins have key roles in carcinogenesis, cancer metastasis, tumour microenvironment, pluripotency maintenance (Sapkota et al., 2008; Chen et al., 2014). Signalling pathways are crucial in proper cell development, growth, differentiation, and death and are affected by oscillations in intracellular zinc levels controlled by these carrier families. The development of cancer has been linked to zinc dyshomeostasis (Table 5; Figure 4).

Serpin B5 [also known as maspin (mammary serine proteinase inhibitor)] is a protein belonging to the serpin family. Serpin B5 is a tumour suppressor that adheres to extracellular matrix components directly, implying that the surface binding relationship is responsible for the prevention of tumor-induced angiogenesis, invasion, and

metastatic dissemination. Loss of Serpin B5 expression is associated with increased aggressiveness in a variety of tumours, including breast, thyroid, prostate and skin Cancers. In the complicated tumorigenesis process, Serpin B5 is a key senescence-associated biomarker and predictive tumour suppressor factor (TamazatoLonghi et al., 2016; Yang et al., 2016; Roversi, 2018) (Table 6; Figure 5).

It is clear that in our study, of the four proteins expressed, each protein was involved in different activities in tandem with all the other three proteins or in different combinations of the remaining three proteins. Although candidate proteins have been identified in NT-OSCC, which can be potential targets for future therapy, the study is not without its own limitations. The first limitation of our study is the smaller sample size. As the study is a first-of-its-kind attempt to identify the candidate proteins expressed in NT-OSCC as compared to the healthy controls, a sample size of ten in each group was taken. The second limitation is the quantification of the actual candidate proteins that are exclusive to NT-OSCC. Hence, conducting this study on a larger-scale with more subjects involved may help overcome this limitation in future.

In conclusion, Cancer is a global burden and oral cancer is the second most common cancer in the Indian subcontinent. Oral cancer has an increased tendency for metastasis and relapse that has been attributed to the expression of certain proteins which are resistant to conventional chemotherapy and radiotherapy. The advent of newer treatment modalities has channelled an increased interest in proteomic studies and the results of the study indicate that an assessment of how these protein biomarkers behave in combination with each other in NT-OSCC may provide a unique understanding of the process of tumorigenesis, thereby allowing targeted therapy and minimizing the unpleasant co-morbidities associated with this grave disease.

Author Contribution Statement

In this study, STA and MN contributed to the design of the experimental study. STA carried out the experiments related to the study. STA and MN collected the data and contributed in data analysis. MN and RK supervised the experiments and results. STA, MN, DKT, NG and PS contributed to data extraction from selected articles. MN and DKT contributed to writing the manuscript and article management. MN, RK, DKT and NG revised the manuscript. All authors reviewed and approved the final manuscript for submission.

Acknowledgments

The study was approved by the Institutional Review Board of SRM Institute of Science and Technology, Ramapuram, Chennai (IRB Approval no: SRMDC/IRB/2017/MDS/No.604).

The study was approved by the Institutional Ethics Committee of SRM Dental College, Chennai, India.

Any conflict of interest

The authors declare no potential conflicts of interest.

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