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SALIVARY PROTEIN EXPRESSION ANALYSIS IN ORAL SUBMUCOUS FIBROSIS & ORAL SQUAMOUS CELL CARCINOMA: A PROTEOMIC STUDY

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ABSTRACT: OSMF is a pre-cancerous condition of the oral cavity.[1]The oral precancerous condition as defined by WHO is a generalized pathological state of the oral mucosa associated with a significantly increased risk of cancer, which accords well with OSMF characteristics.[2] OSCC is a common human malignant tumour with an increasing incidence. It is a high-effect local disease in the oral cavity affecting over 300,000 people worldwide annually.[3] The coexistence of the oral squamous cell carcinoma (OSCC) and oral sub mucous fibrosis (OSMF) is well documented in the literature and various studies have shown it to be 5% to 42%. The aim of the study was to identify proteins that are differentially expressed in Oral Sub Mucous Fibrosis and Oral Squamous Cell Carcinoma Patients.

METHODOLOGY: Tissue samples of 9 patients (3 oral sub mucous fibrosis+ 3 oral squamous cell carcinoma + 3 healthy volunteers with habit of gutka chewing) were collected. The proteins in the samples were analysed using mass spectrometry to identify differentially expressed proteins.

RESULTS & OBSERVATION: The total number of proteins identified was 172 in OSMF saliva samples and 114 in OSCC saliva samples. In OSMF,22 proteins were up regulated and 5 proteins were down regulated. In OSCC, 11 proteins were up regulated and 1 protein was down regulated. The common protein which is differentially expressed in OSMF and OSCC saliva samples was identified.

CONCLUSION: These differentially expressed proteins could serve as potential biomarkers/ biomarker in early detection and diagnosis of malignant transformation in OSMF.

KEYWORDS: Oral sub mucous fibrosis, Oral squamous cell carcinoma, Proteomics, Mass spectrometry.

I. INTRODUCTION

OSMF is a pre-cancerous condition of the oral cavity.[1]The oral precancerous condition as defined by WHO is a generalized pathological state of the oral mucosa associated with a significantly increased risk of cancer, which accords well with OSMF characteristics.[2] OSCC is a common human malignant tumour with an increasing incidence. It is a high-effect local disease in the oral cavity affecting over 300,000 people worldwide annually.[3] The coexistence of the oral squamous cell carcinoma (OSCC) and oral sub mucous fibrosis (OSMF) is well documented in the literature and various studies have shown it to be 5% to 42%.

When OSCC is originating from OSMF, clinically it will be more invasive and shows metastasis and increased recurrence rate than OSCC which is not originating from OSMF. Therefore, investigating biomarkers is very important for the prevention and early detection of carcinomatous transformation.[4]

There are many factors in the pathogenesis of OSMF and OSCC. The Gold Standard in the diagnosis is biopsy. Tissue diagnosis is usually confirmed by a wedge or punch biopsy obtained from the periphery of the lesion or from the centre of the lesion with an adequate volume of viable tissue retrieved from the specimen.

A biomarker is an objectively measured and evaluated indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to therapeutic intervention. Alterations in their concentration, structure, function, or action are associated with the onset, progression, or even regression of a particular disorder/condition or the result of the body's response to biomarkers. Thus, biomarkers serve as a valuable tool in the detection, risk assessment, diagnosis, prognosis, and monitoring of disease.[5]

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Therefore, sensitive and specific biomarkers for OSMF & OSCC may be helpful to screening high-risk patients. Scientists are searching for biomarkers in the saliva, an easy-to-obtain body fluid, for non-invasive detection of oral cancer.

Human saliva is especially attractive for disease diagnosis because i) its collection is totally non-invasive as compared to blood for serum/plasma analyses; and ii) many, if not all blood components, are reflected in the oral fluid. This bio-fluid has been proven to be very valuable for the diagnosis of HIV, periodontal diseases and hepatitis.[6]

Proteomics is a powerful approach for the global study of the structure and function of all proteins expressed in a biological system. Different proteomics studies have been successfully engaged in the discovery of biomarkers in human saliva. Interest in rapid and less invasive diagnostic tests has grown exponentially in the past decade, leading to extensive research on saliva as a biological fluid for clinical diagnosis.[7]

There exists a dilemma for clinicians in cases where OSMF transforms to OSCC, but there is lack of clinical evidence on the matter. Such situations demand the need for an early diagnostic tool, which is less invasive and accepted by the patient, to build a primary diagnosis to the condition.

In this study, mass spectrometry analysis will be employed to identify proteins that are differentially expressed in Oral Sub Mucous Fibrosis and Oral Squamous Cell Carcinoma Patients when compared to healthy controls that could serve as potential biomarkers.

Aim

The aim of the study was to identify proteins that are differentially expressed in Oral Sub Mucous Fibrosis and Oral Squamous Cell Carcinoma Patients when compared to healthy controls.

Objectives Of The Study

- To assess the presence of specific protein biomarkers in saliva in conditions such as Oral Sub Mucous Fibrosis and Oral Cancer.
- To compare differentially expressed proteins in patients with OSMF and OSCC with the healthy individuals in control group.

II. MATERIALS & METHODS

The patients who reported to the department of Oral and Maxillofacial of Yenepoya Dental College Hospital, Mangalore were taken up for obtaining samples for the study. Written Informed consent was obtained from the patient after explaining the aim, objective and procedure of the study in the vernacular language.

Inclusion criteria:

1. Age Group: 20- 60years
2. Both Male and Female Patients are considered.
3. Patients with Oral Sub Mucous Fibrosis confirmed through biopsy.
4. Patients with Oral Squamous Cell Carcinoma confirmed through biopsy.
5. Healthy Individuals are selected for control.

Exclusion criteria:

1. The individuals under treatment at the time of sample collection.
2. Patients above the age of 60 years and below the age of 20 years.
3. Patients without laboratory confirmation of Oral Sub Mucous Fibrosis or Oral Squamous Cell Carcinoma.

There were two study groups and one control group. Each group will contain 30 samples (n=30).

Saliva samples were obtained from the patients after diagnosis based on clinical and histopathological criteria. Control saliva samples were obtained from age and gender matched healthy donors. Samples were sent to research lab (YU IOB CSBMM).

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Depletion And Fractionation Method:

The saliva samples were centrifuged at 3,000 rpm, for 30 minutes at room temperature and the supernatant was used for further analysis. Next step is the depletion of salivary amylase. Salivary amylase is an abundant protein in the saliva hence; it was depleted using starch based affinity chromatography. Tryptic digestion of depleted serum samples was carried out. The digestion efficiency was checked by resolving the pre- and post-digested samples using SDS-PAGE and then stained. Once the digestion was confirmed, the samples were dried in speed vac and stored in -20 °C till further processing.TMT labelling of the samples was carried out with TMT mass tags 10-plex kit, Thermo Scientific. The 10-plex kit has 10 different isobaric mass tags that can be used for labelling and quantification of proteins. Strong cation-exchange (SCX) Fractionation was carried out to resolve the peptides based on their ionic property. Desalted peptides were vacuum dried and stored in -80^{o}C deep freezer until LC-MS/MS analysis.

LC-MS/MS analysis of peptides from SCX fractionation were carried out in the Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) which is connected to Easy-nLC-1200 nano flow liquid chromatography system (Thermo Scientific). Mass spectrometry derived data were analysed with Proteome Discoverer software, version 2.1(Thermo Scientific, Germany) with the SEQUEST and Mascot (version 2.5.0, Matrix Science, London, UK) search algorithms. It was searched against Human Ref Seq 81 protein database. The data was searched against decoy database and false discovery rate (FDR) score cut-off of 1 % was used for the analysis. Relative protein quantisation was carried out using Reporter Ions Quantifier node of Proteome Discoverer.

Statistical Analysis

The statistical analysis was done using software SPSS version 22. Oneway anova was used to compare between the three groups. Tukey post hoc test is used to find significance difference between the three groups.

III. RESULTS

There were a total of 172 proteins identified from the saliva samples of the histologically confirmed oral submucous fibrosis samples and a total of 114 proteins identified from the saliva samples of histologically confirmed oral squamous cell carcinoma samples by using mass spectrometry (Protein discoverer software, version 2.1(Thermo Scientific, Bremen, Germany) searched against Human Ref Seq 81 protein database using the Sequest and Mascot (version 2.2.0, Matrix Science, London, UK) search algorithms.

The proteins identified were functionally classified based on their sub cellular localization, biological process and molecular functions using Panther Reference Database (www.pantherdb.org) that contains gene ontology-based information on human proteins.

Out of the 172 identified proteins from the saliva samples of the histologically confirmed oral submucous fibrosis samples, 27 proteins were found to be differentially expressed. There were 22 up regulated proteins and 5 down regulated proteins.

Among the 114 identified proteins from the saliva samples of the histologically confirmed Oral squamous cell carcinoma samples, 12 proteins were found to be differentially expressed with 11 up regulated proteins and 1 down regulated protein.

Fold change of more than 1.5 indicates the up regulated proteins and a fold change of less than 0.6 indicate the proteins which are down regulated.

Table 1 shows the list of the 22 and Table 2 shows 11 differentially expressed proteins that were found to be up regulated in the OSMF saliva samples and OSCC samples respectively. These up regulated proteins have a fold change of more than 1.5

Table 3 shows the list of 5 and Table 4 shows 1 differentially expressed protein/ proteins that were found to be down regulated in the saliva samples. These down regulated proteins have a fold change of less than 0.6

Proline Rich Protein is identified as the common protein among the three groups. The statistical analysis was done using software SPSS version 22. Oneway anova was used to compare between the three groups. Tukey post hoc test is used to find significance difference between the three groups. The expression of

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Proline rich protein has shown an average increase in expression of 2.2 fold times in OSMF than the control samples and 3.2 fold times in OSCC. The increase in expression was statistically evaluated to a p value of p=0.023, which shows the statistical significance.

Table 5 shows the comparison among the groups. The group of OSCC samples show an increase in expression of Proline rich protein of 1.33 times than the group of OSMF samples and 3.333 times than the control group.
A Tukey Post Hoc test was done to compare the expression of Proline rich proteins among the three groups. Table 6 shows the results with the group of OSCC patients having the highest value in the expression of Proline rich protein followed by the group of OSMF patients and the control group with the least.

IV. DISCUSSION

Mass spectrometry-based proteomic studies have significantly increased the identification and coverage of human salivary proteins. Some of the key proteomic studies in saliva were reported during the period of 2005–2010 by several research groups.[8]

Lazaro Alessandro Soares Nunes et al has studied the latest trends in salivary research and its applications in health and disease. The diverse physiological and pathological conditions affect the composition of saliva. It is a feasible diagnostic or prognostic utility because of the non invasiveness, ease and cost effectively of Saliva collection methods.[9] Secreted proteins have a greater likelihood of being detected in blood and other body fluids and could potentially reflect the microenvironment.[10] The major factors that facilitate the detection of these proteins in saliva is its proximity to the tumour which is developing. This will lead to the establishment of a panel of biomarkers which can be used for the early detection and therapeutic monitoring.

Proline Rich Protein is identified as the common protein among the three groups. The increase in expression was statistically evaluated to a p value of p=0.023, which shows the statistical significance. When compared among the groups, the group of OSCC samples show an increase in expression of Proline rich protein of 1.33 times than the group of OSMF samples and 3.333 times than the control group. (Table 5)

Proline-rich proteins are major components of parotid and submandibular saliva in humans as well as other animals. The acidic Proline-rich proteins will bind calcium with a strength which indicates that they may be important in maintaining the concentration of ionic calcium in saliva.[11]Dorothy L. Kauffman et al Purified Proline rich protein from the parotid saliva of a single individual.[12]

Ying Lua et al. say that salivary Proline-rich proteins (PRPs) prevent their interaction with other biological compounds and absorption from the intestinal canal by acting as a defence against tannins by forming complexes with them.[13] The increase can be of Proline rich proteins in the salivary samples of Oral Sub Mucous Fibrosis salivary samples can be attributed to the ability and function of Proline rich proteins to form complexes. Tannin is a major compound which is precipitated from areca nut which is believed to be the etiological factor in OSMF pathology. Tannin is also present in tobacco in huge quantities. The individuals with the habits of tobacco chewing and areca nut chewing will have huge amounts of tannin present in the environment of oral cavity. This abundance of tannins in turn stimulates an increased production of Proline rich proteins from the salivary glands. Thus it produces an increase in the expression of Proline rich proteins in the saliva of OSMF patients.

An increase of proline rich protein +2.35 times was observed by Paul Dowling in the saliva of Oral Squamous cell carcinoma patients compared to a control group of non-malignant conditions.[14]

Peter M Steinert et al said that barrier function to squamous epithelial cells is a contribution of cornified cell envelope (CE) which is a specialized structure. Proline rich (PR) protein is one set of the structural proteins present in CEs. Differential expression patterns of PRs apparently reflect specific barrier requirements of different epithelia. They are ubiquitous cross-bridging proteins [15]

Yoshitaka et al proposed that P*roline-rich protein has* shown to be over expressed in $ALDH1^{br}$ cells by a cDNA microarray and RT-PCR. Proline rich Protein was shown to have a role in cell growth and maintenance of $ALDH1^{br}$ cells by SPRR1B over expression and knockdown experiments.

There are many studies showing increase in calcium levels in the saliva of OSCC patients. Hidayatullah G. Munshi has studied about the increase in calcium in the saliva of patients with OSCC. Calcium is an important

regulator of keratinocyte function, and in oral squamous cell carcinoma.[16] Edan et al has studies on the increase in calcium level in saliva of smokers who developed OSCC.[15]

The increase in expression of Proline rich protein in OSCC patients could be mainly due to two reasons. Firstly, Proline rich protein has an integral part as one of the major structural proteins by cross linking itself to other structural proteins in facilitating the multiplication of squamous epithelial cells. It also supports as a structural protein in the expression of ALDH 1 cells that complement cell multiplication and tumour progression in squamous epithelial cells. Proline rich protein expression in the saliva of OSCC patients is attributed to the increased abundance of the proteins that are involved in tumour progression. The major factor leading to the detection of these differentially expressed proteins in saliva is its proximity to the developing tumour. The identification of these as a biomarker will be useful for therapeutic monitoring and early detection of OSCC. Secondly, another major function of basic Proline rich protein is binding to calcium to form stable complexes. Matrix metalloproteinase activation plays a significant role in the behaviour of tumour cells. The activation of MMP requires calcium. Therefore the level of calcium increases in the environment of actively dividing cells in OSCC which will cause an increase the level of calcium in saliva. Proline rich proteins are released more for forming stable complexes with calcium, thus increasing their expression in saliva.

Previous literature on studies conducted by Sreelatha S Hosthor et al and many others are suggestive of an increase in Calcium in the saliva of OSMF and OSCC patients than the control group along with a significant difference of increase in saliva of OSCC patients than OSMF patients.[18]A comparison of the levels of Calcium in the human saliva of patients with OSCC and OSMF shows a significant increase in level in the saliva of OSCC patients than the OSMF patients. Both these conditions have a higher calcium level than the healthy controls.

Further studies should be conducted keeping the current study as a base to confirm the expression of Proline rich protein as a definitive biomarker to diagnose the transformation of OSMF to OSCC and use it as a prognostic tool.

V. CONCLUSION

In the present study, there was a significant increase in the expression of Proline rich proteins among the three groups. The group of OSCC patients has the highest value in the expression of Proline rich protein followed by the group of OSMF patients and the control group with the least.

To prove the useful identification of potential biomarkers for the early detection of transformation of oral submucous fibrosis into oral squamous cell carcinoma, the differentially expressed proteins should be validated in a large cohort of clinical samples and appropriate clinical conditions. Dentists' knowledge and education in detecting oral cancer at its precancerous phase is the key to prevent its progression to later stages. In order to improve early detection, it is imperative to increase the health-care providers' depth of knowledge about oral cancer, their risk factors and the most common oral precancerous conditions.

ETHICAL CLEARANCE

Ethical Clearance was obtained from the Institutional Ethical Committee.

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Description	AVG. Fold Change

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cystatin-SA precursor	2.332965371
N-acylneuraminate-9-phosphatase	1.389795493
PREDICTED: protein FAM193B isoform X5	1.109294701
protein S100-A12	3.122838807
sodium/potassium-transporting ATPase subunit alpha-3 isoform 3	4.761224874
actin-related protein 2/3 complex subunit 5 isoform 1 [Homo sapiens]	1.515441695
zymogen granule protein 16 homolog B precursor [Homo sapiens]	1.639885798
myelin-associated glycoprotein isoform c [Homo sapiens]	1.902249013
histone H2B type 2-E [Homo sapiens]	1.357938154
pentraxin-related protein PTX3 precursor [Homo sapiens]	1.507082621
podocalyxin isoform 2 precursor [Homo sapiens]	1.873733145
apolipoprotein A-I isoform 1 preproprotein [Homo sapiens]	1.350488554
myelin proteolipid protein isoform 2 [Homo sapiens]	0.933896289
14-3-3 protein gamma [Homo sapiens]	1.47881304
adenylyl cyclase-associated protein 1 [Homo sapiens]	1.595578353
PREDICTED: annexin A5 isoform X1 [Homo sapiens]	1.578744679
basic salivary proline-rich protein	2.226996062
PREDICTED: desmocollin-2 isoform X1 [Homo sapiens]	1.965726842
PREDICTED: 5'-nucleotidase domain-containing protein 3 isoform X1 [Homo sapiens]	1.598079262
cysteine-rich secretory protein 3 isoform 2 precursor [Homo sapiens]	1.719383196
L-lactate dehydrogenase A chain isoform 5 [Homo sapiens]	1.620371074
PREDICTED: BRCA2-interacting transcriptional repressor EMSY isoform X12 [Homo sapiens]	2.316369328

Table 1: List Of Up Regulated Proteins In Saliva Samples Of Oscc

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Tukey HSD **Multiple Comparisons**

Description	AVG. Fold Change
basic salivary proline-rich protein	2.736298279
IgGFc-binding protein precursor	2.065180311
annexin A1	2.079327113
serum albumin preproprotein	1.584517833
L-lactate dehydrogenase B chain isoform LDHBx	1.768113426
protein LEG1 homolog precursor	1.889388618
cystatin-S precursor	1.5332878
PREDICTED: F-actin-capping protein subunit alpha-1 isoform X2	1.807937132
haptoglobin-related protein precursor	2.282835477
PREDICTED: clusterin isoform X1	1.853664646
PREDICTED: FH1/FH2 domain-containing protein 1 isoform X3	1.8283376

Table 2: List Of Up Regulated Proteins In Saliva Samples Of Oscc

Description	AVG. Fold Change
calmodulin-like protein 3 [Homo sapiens]	0.435771696
small proline-rich protein 3 [Homo sapiens]	0.498285083
PREDICTED: alpha-amylase 1 isoform X1 [Homo sapiens]	0.375389742
kallikrein-6 isoform A preproprotein [Homo sapiens]	0.374392902
histone H4 [Homo sapiens]	0.422945083

Table 3: List Of Down Regulated Proteins In Saliva Samples Of Osmf

Description	AVG. Fold Change
CD44 antigen isoform 4 precursor	0.39852547

Tab`Le 4: List Of Down Regulated Proteins In Saliva Samples Of Oscc

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Dependent Variable	(I) Gps	(J) Gps	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
1	1	2	-1.333	.861	.336	-3.97	1.31
		3	2.000	.861	.128	-.64	4.64
	2	1	1.333	.861	.336	-1.31	3.97
		3	3.333*	.861	.019	.69	5.97
	3	1	-2.000	.861	.128	-4.64	.64
		2	-3.333*	.861	.019	-5.97	-.69

*. The mean difference is significant at the 0.05 level. P= 0.023

Table 5: Statistical Comparisons Between The Cases And Controls Of Proline Rich Protein

Tukey HSD

Groups	N	Subset for alpha = 0.05	
		1	2
3	3	.00	
1	3	2.00	2.00
2	3		3.33
Sig.		.128	.336

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table6:Statistical Comparison Among The Groups For The Expression Of Proline Rich Protein