ORIGINAL RESEARCH PAPER
Volume-8 | Issue-9 | September - 2019 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

2D ELECTROPHORESIS AND MALDI-TOF-MS OF SALIVA FROM PATIENTS WITH ORAL SUBMUCOUS FIBROSIS: A CASE CONTROL STUDY

Dental Science

Srivastava Siddharth M.D.S. (Oral Medicine and Radiology), Reader, Department of Oral Medicine & Radiology, I.T.S Dental College & Hospital, Greater Noida, Uttar Pradesh, India

Gundareddy N Suma M.D.S.(Oral Medicine & Radiology), Professor and Head, Department of Oral Medicine & Radiology S.G.T. Dental College, Hospital & Research Institute, Chandu-Budhera Gurgaon, Haryana, India

Krishnamoorthy Bhuvana M.D.S. (Oral Medicine & Radiology), Private Practitioner, Bangalore, Karnataka, India.

Mittal Sajili M.D.S (Periodontics), Private Practitioner, City Life Dental, A-85, Yojana Vihar, Delhi, India.

Rathi Chhabra Vidhi* M.D.S (Oral & Maxillofacial Surgery), Reader, I.T.S. Centre for Dental Studies and Research, Muradangar, Ghaziabad, U.P. *Corresponding Author

Sareen Chanchal M.D.S (Oral & Maxillofacial Pathology), Reader, Department of Oral & Maxillofacial Pathology, I.T.S Dental College & Hospital, Greater Noida, Uttar Pradesh, India.

ABSTRACT

BACKGROUND: Salivary proteomics technology can be used to evaluate the disease progression of Oral Submucous Fibrosis (OSMF), a morbid, crippling and premalignant condition of the oral mucosa, associated with the use of areca nut in various forms.
AIMS: The objective of this preliminary study was to compare the proteomic profile of whole unstimulated saliva of subjects with OSMF with that of healthy volunteers to identify proteins, the levels of which were significantly altered between the two groups.
SETTINGS AND DESIGN: Whole unstimulated saliva was obtained from 40 subjects with OSMF and age-sex matched healthy subjects
METHODS AND MATERIALS: salivary proteins were separated using two-dimensional gel electrophoresis. Proteins, the levels of which were significantly different between the two groups, were identified by computer image analyses and subsequent MALDI-TOF-Mass Spectrometry.
RESULTS: 184 protein spots were detected over control gel, while 169 on experimental gel. Significant no of protein spots were altered in both the groups, proteins which were found to be significantly different were immunoglobulin heavy chain variable region and methyl transferase.
CONCLUSION: Comparison of the proteomic profile of whole unstimulated saliva of OSMF subjects with that of healthy control subjects revealed differential protein expression. The approach applied herein might be helpful in molecular insights of OSMF.

KEYWORDS

2D Electrophoresis, MALDI-TOF-Mass Spectrometry, Oral Submucous Fibrosis, Saliva, Proteomics

INTRODUCTION

Betel nut and tobacco in various forms have left the field of healing sciences with challenging diseases like oral cancer and other potentially malignant disorders [1]. Oral submucous fibrosis (OSMF), a premalignant condition has been linked with various predisposing factors primarily, the consumption of commercial preparations of betel nut, while HLA-Human leucocyte antigen, auto antibodies, hypersensitivity to capsaicin, chronic iron or vitamin B complex deficiency (malnutrition) are other reported factors. It is considered as a slow progressive disease of the oral cavity characterized by rigidity of the oral mucosa and development of palpable fibrous bands with a malignant potential rate of 3-7.3% [2-3]. It predominantly affects the people from South-East Asia in between the age group of 20 to 40 years with varying sex predilection [4]. Burning sensation on consuming hot and spicy foods with progressive reduction in mouth opening are major complaints with which patients usually report to the clinicians. Severity of disease can be staged clinically based on relative mouth opening and histopathology [5-6]. A definitive diagnosis of oral submucous fibrosis is basically confined to its histopathological examination [4].

There is a growing interest in exploring whole salivary proteins using advanced proteomic technology (2D Electrophoresis, MALDI-TOF-MS) and their expression by which one can estimate or analyze the change at genetic level, which is the central dogma of molecular biology [7-8].

Salivary proteomic studies have identified biomarkers whose expression is significantly altered in the saliva of patients with dental caries, chronic periodontitis, oral lichen planus, oral leukoplakia and head and neck squamous cell carcinoma (HNSCC). Varying levels of differentially expressed proteins in tissues, from oral leukoplakia were also established [9-15]. However, no such clinical trials have been undertaken to demonstrate clinical applicability of these techniques in the most common premalignant condition, OSMF.

Hence a preliminary study was undertaken to analyze global expression of salivary proteome in OSMF patients with the objectives of establishing the proteome profile and identification of the differential expression of salivary proteins.

METHODS

This case control study was conducted in the Department of Oral Medicine and Radiology, I.T.S Centre for Dental Studies and Research, Ghaziabad, Uttar Pradesh, India, in collaboration with Department of Biophysics, All India Institute of Medical Sciences (AIIMS), New Delhi, India between November 2010 to May 2012.

Patient selection

A total of 80 subjects (40 each in the experimental and control group) both males and females in the age group of 20-35 years were included in the study. The ethical clearance for the study was obtained from the institutional ethical committee and an informed written consent was obtained from all the participants.

Inclusion criteria

All subjects in the experimental group were gutkha chewers with characteristic clinical features of burning sensation of oral mucosa, intolerance to spicy food along with restricted mouth opening. On clinical examination mucosal blanching and fibrous bands were present. Control group included apparently healthy age and sex matched individuals with no associated deleterious habit.

Exclusion criteria

Subjects with conditions that may affect normal functioning of salivary glands example any systemic diseases (Sjogrens' syndrome,

scleroderma, autoimmune diseases) or mental illness (depression /anxiety /mood disorders), post surgical defects (surgery involving major salivary gland, post-surgical fibrosis/defect etc.), those on any medications / drugs for illness that can transiently alter salivary functioning, those suffering or have undergone treatment for a significant illness (neuromuscular disorders, psychoactive medication, radiotherapy etc.), those with symptoms of dry eyes or hyposalivation (dry mouth / xerostomia) or hypersalivation, pregnant or nursing women were excluded from the study.

Subjects unfit for biopsy procedure, compromised oral health status i.e. gingivitis, multiple caries, periodontitis, oral mucosal disease viz keratosis/ulceration were not included under the control group.

The clinical examination was performed by an oral medicine specialist under strict aseptic conditions and adequate lighting, followed by recording of findings in a customized proforma. Clinical diagnosis of OSMF was made according to Khanna et al [5]. A punch biopsy was performed under local anaesthesia and confirmed histopathologically.

Saliva Collection
Unstimulated whole saliva was obtained from all subjects in both the groups according to Navazesh guidelines [16].

Saliva processing and storage
Collection of saliva was done into ice-cooled graduated test tube containing a cocktail of protease inhibitors (A-BS380) followed by centrifugation of sample at 1400g at 4 degree C for 15 minutes to remove debris.

Supernatant was later collected and pooled for 80 subjects within separate sterile plastic containers marked as experimental pool and control pool. A pinch of sodium azide was added to it, to prevent fungal growth. Sample was then stored at -70 degree C. This procedure was performed within 2 hours from the time of collection.

Proteins were then precipitated using 90% acetone (v/v: volume / volume), 10% (v/v) TCA solution (100% w/v: weight / volume) and 0.07% 2-mercaptoethanol (v/v). After incubation at –70 degree C for 2 hours, insoluble material was pelleted at 20,000g. Pellet was then washed three times with pure acetone containing 0.07% β-mercaptoethanol (v/v), air dried and solubilized in lysis buffer (containing 8M (moles) urea , 4% CHAPS (w/v) , 0.05% Triton-X-100 (v/v) and 65mM DTT). Protein amount was estimated by Bradford's method.

Two dimensional (2D) gel electrophoresis
Rehydration of IPG strips
Precast IPG strips, PROTEAN (Bio-Rad) with nonlinear immobilized pH 3-10 gradient were rehydrated overnight with 1000 µg (microgram) of protein sample complemented with 0.0025% v/v bromophenol blue and 1 % v/v IPG buffer.

Iso-electric focusing (1st dimension)
Iso-electric focusing was carried out using the IPGphor ™ iso -electric focusing system (Amersham Biosciences) at 100 Volts (1hour), 300 Volts (1hour), 500 Volts (1 hour), 1000 Volts (2 hours), linear gradient from 1000 Volts to 8000 Volts, and a final phase at 8,000 Volts for 5 hours, resulting in a total of 100,000 Volts/ hours.

Equilibration
Strips were then equilibrated for 15 min in 8M urea, 50 mM (millimoles) Tris HCl buffer at pH 8.8, 30 % v/v glycerol, 2 % SDS, 0.001 w/v bromophenol blue and 65 mM DTT, and finally for 15 min in the same solution excluding DTT, where it was replaced by 13.5 mM iodo- acetamide.

SDS- PAGE (2nd dimension)
Proteins were separated on 10 % SDS-poly acryl amide gels (customized) at a constant voltage of 50 Volts for 2 hours followed by 150 Volts for 6 hours at 25 °C, using an Iso-DALT electrophoresis unit (Amersham Biosciences). The procedure was repeated for three to four times subsequently and the best out of all gels were taken for further analysis .

Staining of gels
Gels were stained with colloidal Coomassie blue (G-250), overnight and later destained for further analysis.

Identification of protein spots on gels using image master 2D platinum software 7.0
Gel images were analysed with the above software, to detect protein spots. A spot delineates a small region in the gel where protein is present. This shape is automatically differentiated by a spot detection algorithm and quantified by calculating its intensity, area, volume and volume%.

In-gel digestion
Protein spots of interest were excised from colloidal Coomassie (G–250) blue stained gels, and washed successively with water, 25 mM ammonium bicarbonate, acetonitrile / 25 mM ammonium bicarbonate (1:1, v/v) and acetonitrile.

Gel fragments were dried at 37 °C. The digestion was carried out at 37°C for 5 hours after addition of 10µL of 0.0125 µg.µL-1 trypsin in 25 mM ammonium bicarbonate (pH 7.8). The resulting tryptic fragments were extracted twice with 50 µL of acetonitrile / water (1:1, v/v) containing 0.1 % trifluoroacetic acid for 15 min. The pooled supernatants were concentrated to a final volume of 20 µl by heating at 37°C. The tryptic peptides were desalted and concentrated to a final volume of 3 µL with C18 Zip-Tip.

MALDI-TOF-MS
(Matrix-assisted laser desorption/ ionization- Time of Flight- Mass spectrometry)
The α-cyano-4-hydroxycinnamic acid matrix was prepared at half saturation in acetonitrile / water (1:1, v/v), acidified with 0.1 % trifluoroacetic acid.

0.8 µL of each sample was mixed with 0.8 µL of the matrix prepared and the mixture was immediately spotted on the MALDI-TOF-Mass analyser. (Bruker Daltonics, Flex Series (microflex),Germany).

Analysis of peptide fragments from protein spots
PMF (peptide mass fingerprinting) spectra were obtained for the peptide fragments and molecular masses of the peptides were measured and searched against human protein sequence database NCBInr through the application of the search program, MASCOT (matrix science).

RESULTS
Of the 80 subjects, 74 (92.5%) were males and 6 (7.5%) were females, with age ranging between 20-35 years.

After running the gel three to four times it was analysed using image master 2D platinum 7.0 software.

184 protein spots were detected over control gel, while 169 on experimental gel. Out of this 167 were found to be matched while 19 were unmatched. To be precise 17 additional spots were present over control gel which were not present over experimental gel and 2 additional spots were present over experimental gel which were not found anywhere over the control gel [Fig.1].

With the software volume% of protein spots were calculated, proteins with volume% less than 0.5% were considered underexpressed, those with more than 1.5% as overexpressed. Among the matched protein spots, spots with marked under and over expression (protein spot 1 & 2) were selected for identification using MALDI-TOF-MS [Table 1].

Volume % of protein spot 1 over experimental (OSMF) gel was less when compared to control gel, showing its underexpression over experimental gel. Similarly volume % of protein spot 2 over experimental (OSMF) gel was more when compared to control gel, showing its overexpression over experimental gel.

Two selected protein spots were identified using MALDI-TOF-MS. PMF spectra was obtained for the peptide fragments and molecular masses of the peptides were measured. Mowse score was assigned to two selected protein spots (71: protein spot 1) and (86: protein spot 2) based on their molecular weight and number of peptide sequence matched. These scores were searched against human protein sequence database NCBInr through the application of the search program, MASCOT (matrix science). The two selected proteins were immunoglobulin heavy chain variable region and methyl transferase [Table 2].

DISCUSSION

Oral Submucous fibrosis is a morbid, crippling and a premalignant condition of the oral mucosa, associated with the use of areca nut in various forms. Definitive diagnosis of OSMF is histopathology, which at times is not possible secondary to reduced mouth opening or is not representative of the entire condition and doesn't indicate the changes at molecular level. In such a background salivary diagnostics is proving to be a useful diagnostic adjunct.

Applicability of salivary markers among patients with leukoplakia, oral lichen planus and head and neck squammous cell carcinoma is well known phenomenon. Studies reported overexpression of urinary prokallikrein (12), CD44 (18), IL-4 (Interleukin-4) (19) and underexpression of short palate, lung and nasal epithelium associated protein (PLUNC) (12), IFN-gamma (19) in salivary samples of patients with oral lichen planus while transthyretin, transferrin, S100 calcium binding protein A9, immunoglobulin heavy chain constant region gamma 2, fibrin beta, cofilin 1, keratin (ck) 6, 13, alpha-2-actin, heat shock protein (hsp) 70 and 90, beta globin, thioredoxin were significantly overexpressed in salivary samples of patients presenting with oral squamous cell carcinoma (13, 20). Present study observed the differential expression of salivary protein among subjects with oral submucous fibrosis with that of healthy controls. A total of 169 proteins in osmf group and 184 proteins in control were detected using 2D gel electrophoresis. Further analysis for their differential expression was done using image master 2D Platinum 7.0 software and MALDI-TOF-MS. The results showed under-expression of immunoglobulin heavy chain variable region and over-expression of methyl transferase among subjects with OSMF as that of healthy control.

Methyl Transferase

Methyl transferase is a type of transferase enzyme that transfer methyl group from a donor to an acceptor. Methylation often occurs on nucleic bases in DNA or amino acid in protein structure. Literature review revealed no previous studies on salivary proteome analysis of OSMF, a premalignant condition, however recently Sartini et al concluded increased expression of salivary enzyme nicotinamide N-methyltransferase (NNMT) among patients with OSCC. While Huang SH et al observed absence of O(6)-methylguanine-DNA methyltransferase MGMT expression and promoter hypermethylation among betel quid chewers presenting oral squammous cell carcinoma (OSCC). Sartini et al found significant upregulation of nicotinamide N-methyltransferase (NNMT) among favourable cases of OSCC, while no marked expression was seen among unfavourable cases of OSCC, making it a potential biomarker and therapeutic target for OSCC. Zhang J et al found that NNMT is over-expressed in a large proportion in renal cell cancers (20-23).

Immunoglobulin heavy chain variable region

Immunoglobulin heavy chain is the large polypeptide subunit of an antibody, each heavy chain has two regions, one is a constant region and the other one is a variable region. Gao LW et al found significant under expression of immunoglobulin heavy chain variable region among human large cell lung cancer strains with high metastasis potential. Zhang YT et al found expression of immunoglobulin heavy chain variable region in human colorectal carcinoma cell lines. These findings are found consistent with our results, which could be explained on basis of OSMF as a premalignant condition (24-25).

Finding of the present study revealed applicability of these salivary markers as a potential candidate in depicting the nature of the disease process and could be used as diagnostic indicator. However, applicability of these protein expressions as prognostic or therapeutic indicators is still an area of further research. Moreover, it important to remark that this number may not be a presentation of the complete protein population, due to the nature of inherent limitations in the use of this technique. As only those spots exhibiting a good repeatability were used for further identification, it is likely that some proteins were omitted.

In addition , in traditional 2-DE, the pH of the immobilized pH gradient strips ranges between 3 and 10; hence it is likely that proteins that appeared in the low or high pH range were not separated and detected. Furthermore it cannot be excluded that some proteins may have been degraded during sample preparation.

Financial constraints limited the study for the identification of only two significantly altered protein spots. , and others were not analyzed.

Hence it is suggested that future studies using large sample size, subject population divided according to disease severity combined with financial support and alternative approaches to analyze all the proteins, which are altered in saliva of various stages of OSMF subjects are needed.

It can be concluded that Hence in conclusion, Immunoglobulin heavy chain variable region was found to be underexpresed while methy-transferase was found to be over-expressed in saliva obtained from of OSMF subjects. This preliminary study demonstrates that the use of proteome analysis may contributed to the understanding of molecular aspects of OSMF, which is not yet well understood. Potential use of salivary proteomics in early detection and staging of the disease to replace the traditional biopsy procedures must be explored further.

OSMF is a premalignant condition, with high rate of morbidity. Salivary proteomics can serve as biomarkers for its early detection and severity of the disease and can replace the traditional biopsy procedures, which induce further fibrosis.

This preliminary study demonstrates that the use of proteome analysis may contribute to our understanding of molecular aspects of OSMF, which is not yet well understood.

ACKNOWLEDGEMENTS

Sincere Thanks to Professor Dr. Hari Parkash, Former Director General, I.T.S- Centre for Dental Studies and Research, Muradnagar, Ghaziabad, Uttar Pradesh. Dr Sunil Chaudhary, Professor, Department of Oral Medicine and Radiology, ESIC Dental College, Rohini, New Delhi for their constant support in the completion of the work.

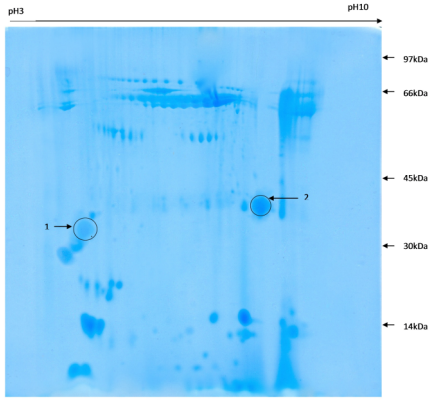

Fig. 1 (A) Two dimensional gel map of whole saliva from healthy patients, obtained in the pH range 3-10; 10%SDS-PAGE, Coomasie blue stained, showing overexpression of protein spot 1 and underexpression of protein spot 2

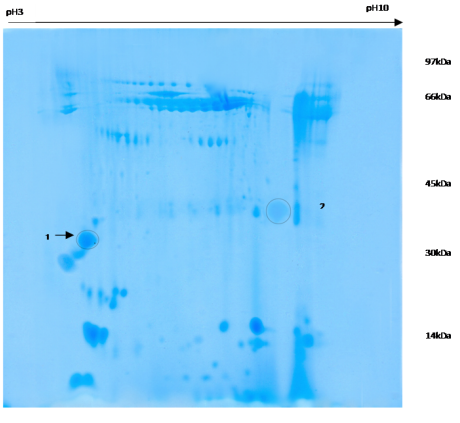

Fig. 1 (B) Two dimensional gel map of whole saliva from oral submucous fibrosis patients, obtained in the pH range 3-10; 10%SDS-PAGE, Coomasie blue stained, showing underexpression of protein spot 1 and overexpression of protein spot 2

Table 1: Volume % of Protein spots (1 and 2) over control and experimental gels

PROTEIN SPOT	% VOLUME IN CONTROL GEL	% VOLUME IN EXPERIMENTALGEL
1	3.343	1.896
2	1.682	4.833

Table 2: Two Protein spots with significant alterations identified using MALDI-TOF-MS

S. No	Protein	Experimental (Mol wt / pI)	Theoretical (Mol wt / pI)	Sequence Coverage	Mowse Score	No of peptide matched	Peptide Sequence
1.	Immunoglobulin heavy chain variable region (Protein spot 1)	39kDa / 5.6	11kDa / 5.67	45%	71	2	**1.GLEWVSGISGSGGTTDYADSVKGR 2.STLYLQMHSLRGEDTGIYYCV**
2.	Methyl-transferase (Protein spot 2)	43kDa / 8	43 kDa / 5.19	31%	86	4	**1.MICVQAGTEQRLAPTDRDAAWDPLEW KAAATLPSSMADTLTKLAYQALQQGKSLV GVAHKEVSTKLMELVAPQGAPK 2.YPMVWLDMPSTWNRR 3.VIKPLVQGLR 4.AALPQAQLVGIDLSASYLR**

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