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Comparative Analysis of Differentially Expressed Genes in Oral Leukoplakia and Oral Submucous Fibrosis Using Bioinformatics Approach

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims and Objectives: There is lack of understanding of distinct molecular pathways and associated gene mediators of the Potentially malignant disorders of the oral cavity (OPMD), such as Leukoplakia and Oral sub mucous fibrosis. This microarray data analysis aims to highlight common and distinct gene signatures by identifying the differentially expressed genes and the pathways in which they participate to help researchers & clinicians to distinctly characterize the Oral Leukoplakia and Oral Submucous Fibrosis disease conditions for developing appropriate treatment strategies

Methods: Gene Expression Omnibus datasets of Oral leukoplakia GSE85195 (OL) and Oral submucous fibrosis (OSMF) GSE64216 were analyzed using GEO2R package (using Log 2 fold change; false discovery rate < 0.05). The lists of differentially expressed genes of both datasets were analyzed for common and distinct members. These were then submitted to Database for

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Annotation, Visualization and Integrated Discovery (DAVID) for gene set enrichment analysis and functional annotation, to identify enriched Gene Ontology terms and KEGG pathways (Fisher's Exact Test for P-value < 0.05).
Results: In total 1107 in OL, 607 in OSMF and 143 common differentially expressed genes in both OL and OSMF were identified. The gene set enrichment analysis by DAVID revealed 26% integral component of membrane, 20% extracellular region and 14% nucleus as cellular components and molecular functions. The distinct gene signatures and altered biological pathways reported here clearly distinguish molecular profiles of both OL and OSMF, which may help to identify potential drug targets for developing and designing new treatment strategies in future.

Keywords: Oral leukoplakia; oral submucous fibrosis; Homo sapiens; gene ontology.

ABBREVIATIONS

OPMD: Oral Potentially Malignant Disorders
GEO : Gene Expression Omnibus
OL : Oral Leukoplakia
OSMF: Oral Submucous Fibrosis
ECM : Extracellular Matrix

1. INTRODUCTION

Oral cancer mainly occurs in areas of precursor lesions in the oral cavity, which has been known for over a century. These lesions were characterised to in the literature as "pre-cancer," "precancerous/premalignant lesions," and "intra epithelial neoplasia." The WHO Collaborating Centre selected a more accurate phrase - "potentially malignant illnesses" - because there is no guarantee that all precancerous lesions would develop into mouth cancer [1].

The most prevalent of these lesions include Oral Leukoplakia (OL), Oral Submucous Fibrosis (OSMF), Lichen Planus (LP), Oral Lichenoid Lesions (OLL), Oral Erythroplakia (OE) and Proliferative VerrucousLeukoplakia (PVL). Oral dysplasia is a mucosal disorder characterised by cellular and architectural abnormalities that may or may not be linked to OPMDs [1].

The overall global prevalence of OPMD is believed to be around 4.5 percent, with large variations depending on the geographic regions studied[2].

OPMDsis linked to a variety of etiologic variables, both genetic and environmental. Tobacco, alcohol, and betel chewing have all been linked to the development of particular lesions in the past. Other predictors of OPMDs and its malignant transformation are ultraviolet radiation which leads to lip cancer, nutritional intake deficiencies. Along with all the risk factors various mutations and genetic mechanisms have been documented in past which plays an important role in progression of these disorders.

Oral squamous cell carcinoma (OSCC) constitutes 92-95% of all oral cancer.Oral cancer is the 12th most common cancer among women and the sixth most common cancer among men. OPMDs are a heterogeneous group of lesions with different probabilities of malignant transition (MT) into invasive cancer[2].

Till date various treatment modalities have been approached for the OPMDs and Oral cancer starting from habit cessation to the radiation therapy, surgery or combination therapies. But ability to treat has been constrained due to lack of understanding of the specific key genes that may underlie the growth of the cancer[3,4].

With the advancement of genomic technology, bioinformatics has become popular for analysing gene profiles, which aids in explaining the molecular process as well as disease-specific biomarkers. Being the powerful analysis differential gene expression provides a method for studying molecular pathogenesis which is the basis of understanding the altered pathways and processes at the cellular level[5]. Many Microarrays and transcriptome analyses have acted as prominent tools in oral cancer research, allowing gene expression monitoring in tumour cells as well as gathering important information[6,7].

The aim of the study was to use High-throughput microarrays datasets for transcriptome analyses to decipher altered expression profiles of genes and simultaneously to characterize the biological behaviours of cellular components in oral leukoplakia and oral submucous fibrosis. The objective here is to elucidate the probable components, processes, functions and molecular pathways underlying pathological situations through bioinformatics approach, all of which depend on altered gene expression patterns.

To the best of our knowledge, this is the first study to compare and investigate differentially expressed genes in Oral leukoplakia and Oral submucous fibrosis for transcriptome analysis using microarray data, in the hope that the findings may help identify potential biomarkers/ targets for the diagnosis and clinical treatment of Oral premalignant disorders (OPMDs), namely, Oral leukoplakia and Oral submucous fibrosis. Due to the paucity of OL and OSMF microarraydata, only two datasets were discovered relevant to the field of study, in order to decipher their distinctness and similarity in process of progression towards malignant transformations.

2. MATERIALS AND METHODS

2.1 Obtaining Microarray Gene Expression Data from NCBI

The high throughput microarray gene expression datasets analyzed in the present study were obtained from the Gene Expression Omnibus (GEO), a public repository for data storage in National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov/geo). Two GEO datasets of oral leukoplakia and oral sub mucous fibrosis, GSE85195 and GSE64216, were included in the present study. The dataset GSE85195 based on GPL6480 Agilent-014850 Whole Human Genome Microarray 4×44K G4112F, includes data from 15 individual Gingivobuccal complex study RNA samples, while the dataset GSE64216 using GPL10558 platform Illumina Human HT-12 V4.0 expression beadchip contains data from 6 individual oral buccal mucosa RNA samples.

The RNA profile of 15 OPL and 34 OSCC samples was compared with 1 independent controls Gingivobuccal complex tissue from healthy donors.

2.2 Mining of Differentially Expressed Genes

Both the datasets were analysed using GEO2R online analysis tool (www.ncbi.nlm.nih.gov/geo/geo2r) which is a web-based tool that allows users to compare two or more groups of Samples in a GEO datasets to detect the differentially expressed genes (DEGs) under different experimental settings. The adjusted $P<0.05$ and Log fold change or $|LogFC|$ were set as DEGs cutoff criterion was applied using the Benjamini and Hochberg 1995 false discovery rate (FDR) method[8]. Statistical analysis was carried out for each datasets.

2.3 Functional Annotation and Statistical Analysis of DEGs

Database for Annotation, Visualization and Integrated Discovery (DAVID) (david.abcc.ncifcrf.gov) is an online application that provides a complete collection of functional annotation tools for researchers to comprehend the biological significance underlying multiple genes. Gene Ontology (GO) enrichment analysis and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis were used in this study, which was conducted using the DAVID software analysis. The GO (http://www.geneontology.org) database is integrated in it and classifies gene list data into biological processes (BP), cellular components (CC) and molecular function (MF).

2.4 Statistical Analysis of DEGs

The above mentioned DAVID database was also used to statistically analyse gene list using Fisher's exact test and Gene Set Enrichment Analysis. Fisher's exact test is a statistical significance test for contingency tables analysis in DAVID database. It is used to test two types of classification for categorical data that results from classifying objects in two separate ways and Gene Set Enrichment analysis (GSEA) also known as functional enrichment analysis is a method for identifying gene or protein classes that are over-represented in a large set of genes or proteins that may be linked to disease characteristics. The strategy use statistical techniques to discover gene groupings that are significantly enriched or deficient. Common genes between both datasets were found with intersect function followed by the combined P-value <0.05 computation based on Fisher's $\chi 2$-based method.

3. RESULTS

Molecular Pathways operating under the conditions of Oral Leukoplakia (OL) and Oral Submucous Fibrosis (OSMF) are still under investigation. Here, in this study we identify differentially expressed genes. Datasets of oral leukoplakia and oral sub mucous fibrosis, GSE85195 and GSE64216, were analyzed in the study.

In total, it was found that 1107 genes associated with the oral leukoplakia and 611 with the oral sub mucous fibrosis were identified using the Gene Expression Omnibus (GEO), a public repository for data storage. The GEO2R online analysis tool using DAVID was used to detect the DEGs between gingivobuccal, oral buccal mucosa and normal samples which provides one of the most extensive sets of associations of human genetics diseases and is a valuable module for the study of molecular mechanism underlying genetic diseases. After analysing sets using GEO2R by the false discovery rate (FDR)< 0.05 method of Benjamini and Hochberg, the log FC values were set as less than or equal to │LogFC│value - 2 (to identify downregulated genes) or greater than or equal to │LogFC│value 2 (to identify upregulated genes) were set as DEGs cut off criterion. Out of 1557 genes 599 were found to be upregulated genes and 958 were down regulated genes, oral leukoplakia, on the other hand, out of 826 genes, 540 were found to be upregulated genes and 286 down regulated genes in oral sub mucous fibrosis.

To identify common genes in both the datasets, we then analyzed the overlapping DEGs in both the datasets containing Set A - Oral leukoplakia with total 1107 genes and Set B - Oral sub mucous fibrosis with total of 611 gene and found 143 overlapping DEGs using Interacti Venn [9] as shown in Fig. 1.

In order to explore the biological properties of the genes and gene sets of oral lesion and conditions, the gene enrichment analysis were performed for the GSE85195 & GSE64216 datasets, with the cut-off criterion of false discovery rate (FDR)<0.05. GO annotation that contains the three sub-ontologies-biological process (BP), cellular component (CC) and molecular function (MF) were determined.

On comparing the both datasets the common DEGs i.e. top significantly enriched gene ontology terms were complied in a Fig. 2 which depicts the common expressed differentially expressed genes with the maximum percentage to negative regulation of transcription from integral component of membrane (25%), extra region (20%), nucleus as cellular components (14%), and least with the transcription factor binding, calcium ion transport etc.

In order to explore the biological properties of the genes and gene sets of oral lesion and conditions, the gene enrichment analysis were performed for the GSE85195 & GSE64216 datasets, with the cut-off criterion of false discovery rate (FDR)<0.05. GO annotation that contains the three sub-ontologies-biological process (BP), cellular component (CC) and molecular function (MF) were determined. In Fig. 3, collective gene ontology (GO) terms were explored from both the number of DEG set discussed above. These were as follows: chemotaxis (21 genes in OL & 11 genes in OSMF), chemokine-mediated signalling pathway (18 genes in OL & 7 genes in OSMF), inflammatory response (44 genes in OL & 7 genes in OSMF), extracellular region (182 genes in OL & 59 genes in OSMF), chemokine activity (14 genes in OL& 6 genes in OSMF), chemokine receptor binding (10 genes in OL & 4 genes in OSMF) and iron ion binding (14 genes in OL & 9 genes in OSMF), respectively.

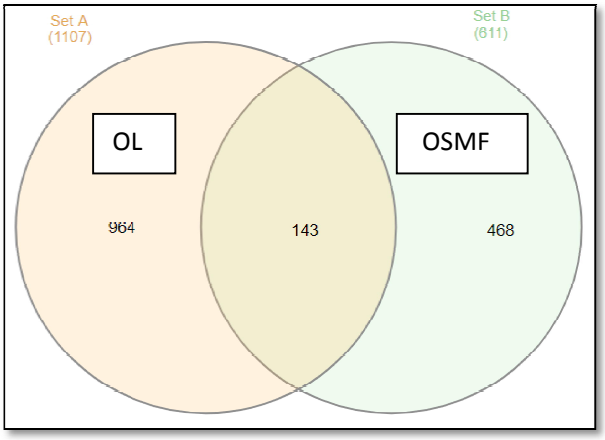

Fig. 1. Venn-Diagram depicting 143 overlapping differentially Expressed Genes (DEGs) in the both Set A – OL and Set B – OSMF datasets

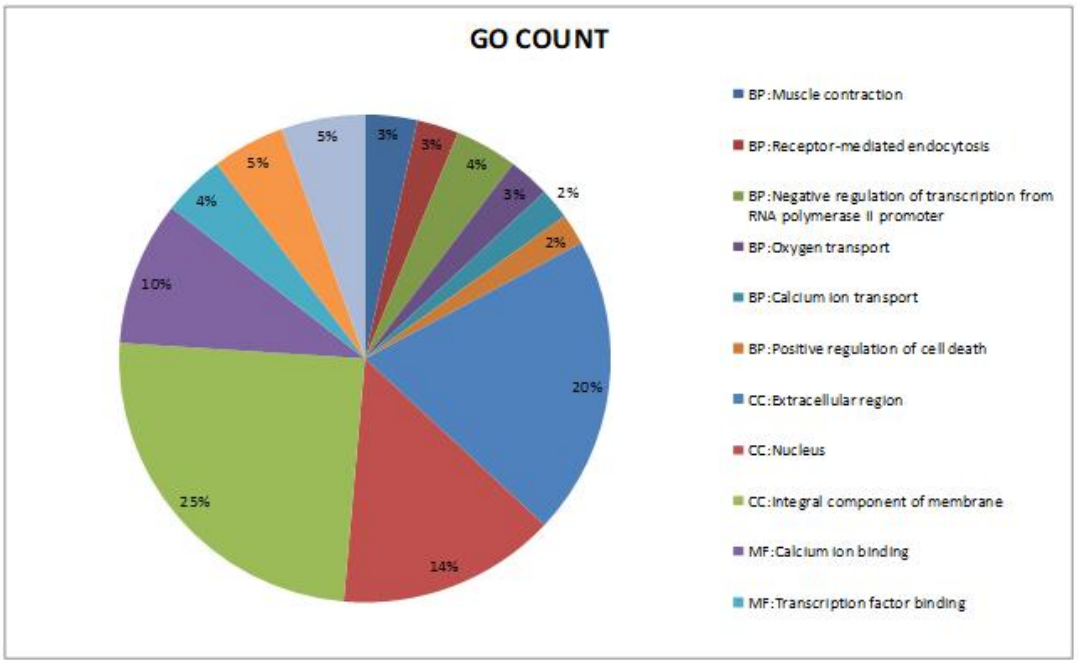

Fig. 2. Pie chart for representation of significantly enriched Gene ontology terms [biological processes (BP), cellular components (CC), and molecular function (MF)] using DAVID

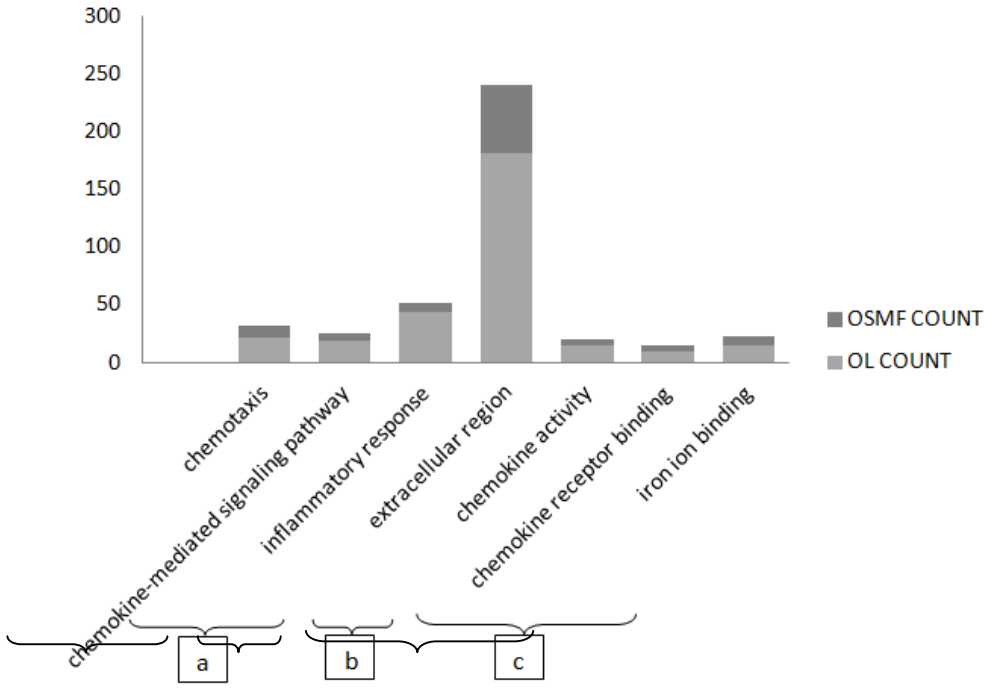

Fig. 3. represents common GO terms associated with list of differentially expressed genes (DEGs) compared between OL & OSMF (FDR < 0.05); y-axis shows percentage count of genes associated with GO terms; x-axis represents GO terms as (a) biological processes, (b) cellular components and (c) molecular function

To gain further insight into the distinct expressed gene of Oral leukoplakia and Oral sub mucous fibrosis, gene enrichment analysis was performed where the three sub – ontologies were expressed in different annotation clusters. In Oral leukoplakia the gene ontology i.e. Biological process which was highly expressed was the immune response with the gene count of 54 followed by proteolysis gene count 45, inflammatory response gene count 44 and extracellular matrix gene count 31 etc, similarly, in oral sub mucous fibrosis the highly observed gene ontology was positive regulation of transcription from RNA polymerase II promoter with gene count of 27, DNA template with gene count of 23, oxidation reduction process with gene count of 18 and inflammatory response with gene count of 17 etc as shown in Table-1.

Whereas in Oral leukoplakia and Oral sub mucous fibrosis the Cellular component, which was highly recognized with the gene count of 250 & 125, respectively, is the integral component of membrane followed by extracellular region (Table-1). Relevant Molecular function in Oral leukoplakia included ATP binding, serine-type endopeptidase activity, metalloendopeptidase activity etc. and similarly in Oral sub mucous fibrosis, zinc ion binding, ATP binding etc were found significant.

4. DISCUSSION

Oral potentially malignant disorders (OPMDs) are conditions that occur before invasive oral malignancies develop. The term encompasses precancerous lesions and diseases that were previously defined by the World Health Organization (WHO). It is proposed to introduce a new term “potentially premalignant oral epithelial lesions [PPOELs]”.The underlying concept is that these lesions have the potential to become malignant, so in their current state, that is, before malignant transformation, they are still (potentially) premalignant. Oral cancer is more likely to develop in those with OPMDs. Oral leukoplakia and oral sub mucous fibrosis are some of the most common OPMD with the higher chances of malignant transformations[2].

Being multifactorial in its aetiology and wide range of pathogenesis these disorders are still lacking a targeted approach towards its interventions. In the present analysis using bioinformatics, differences and common differential gene expression profiles under Oral Leukoplakia and Oral Sub Mucous Fibrosis pre-cancerous disease conditions were obtained and functionally annotated to find the pathways regulated by them. The differentially expressed genes were discovered in different enriched annotation clusters in all three sub groups of gene ontology i.e. biological process, cellular component and molecular function.

Various genes were found to be highlighting important roles in metabolism and pathways like immune response, inflammatory responses, muscle contraction and most were found to be part of extra cellular matrix as cellular components. Muscle contraction has high relevance with the progression events of oral premalignant lesion and disorders as its is characterized by juxta epithelial inflammatory cell infiltration followed by fibrosis in the lamina propria and sub mucosa of the oral mucosa leading to muscle contraction & stiffness[10]. Similar discovery was made by Wandhan[11]in their respective study, in which the investigators emphasised the role of muscle contraction in oral premalignant lesions and conditions.

Extracellular matrix (ECM) and the epithelial–mesenchymal transition (EMT) are documented for the progression of the disease and its malignant transformation with the likelihood of EMT in OPMDs. It is further supported by the findings that many cytokines, nucleus proteins and signalling pathways involved in EMT had been expressed and activated in oral premalignant lesions and conditions, especially in oral leukoplakia and oral submucous fibrosis[12]. Due to altered ECM composition and structures there is activation of fibroblasts which might result in differentiation of ECM fibroblasts into myofibroblasts resulting in increased levels of ECM-degrading proteases. This differentiation finally leads to the progression of disease state. Fibroblasts in intact tissue are stress shielded by a functional ECM; they do not develop contractile features and cell matrix adhesions[12].

In present study oral leukoplakia has shown high affinity towards integral component of plasma membrane and cell adhesions,It is well documented that cell membranes uses primary receptors for binding to the extracellular matrix[11]. Proteins function as transmembrane linkers between the extracellular matrixes and one of the most important components of membrane is integrins which helps in the cells attachment to the ECM and the ECM's signal transmission to the cells. In support to above results Hamidi[13]conducted a study to clarify

Table 1. Distinct gene enrichment analysis of Oral Leukoplakia and Oral Submucous Fibrosis significant genes (FDR < 0.05) based on biological processes, cellular components and molecular function

S.NO.	Gene ontology term associated with Oral Leukoplakia		Gene ontology term associated with OSMF	
	GO TERM	Gene Percentage	GO TERM	Gene Percentage
1	collagen catabolic process	2.1	Chemotaxis	2.3
2	extracellular matrix organization	3.2	inflammatory response	
3	immune response	5.1	G-protein coupled receptor signaling pathway	3.6
4	inflammatory response	4.5	oxidation-reduction process	3.8
5	proteolysis	4.	positive regulation of transcription from RNA polymerase II promoter	5.7
6	G-protein coupled receptor signaling pathway	5.	transcription, DNA-templated	4.9
7	extracellular region	18.9	extracellular region	12.6
8	plasma membrane	23.7	integral component of membrane	26.7
9	integral component of membrane	25.9	nucleus	18.4
10	cell junction	2.4	zinc ion binding	6.8
11	nucleus	18.0	transcription factor activity, sequence-specific DNA binding	4.4
12	extracellular matrix structural constituent	1.3	DNA binding	4.2
13	chemokine activity	1.4	ATP binding	4.9
14	metalloendopeptidase activity	2.2		
15	extracellular matrix structural constituent	1.3		
16	serine-type endopeptidase activity	2.4		
17	iron ion binding	1.4		
18	protein coupled receptor activity	3.0		
19	ATP binding	4.5		

whether epithelial cells and its components in oral leukoplakia expressions could be associated to malignant transformation of the lesions and concluded that integrins and its components are associated with the inflammation, malignant transformation and may have multiple roles in tumor formation.

Numerous models have been proposed for the pathogenesis of oral premalignant lesions and conditions related to its aetiology and it is found that various parameters like biochemical; Immunological mediation and genetic parameters are responsible in alteration and progression of the disease. Most frequent analysed parameters which were seen in present module were calcium ions binding, iron binding, zinc ion binding, immunoglobulin's (Ig), auto antibodies, cytokines, complement derivatives, deposition of collagen fibres and circulating immune complexes which is known to carried out the pathogenesis of various oral lesions and condition as shown in Table-1.

Iron is hypothesised to play a role in the development of epithelium in the oral mucosa via the activity of cytochrome oxidase, as well as the maintenance of the permeability barrier. In the present study one of the enhanced gene ontology was iron-containing enzymes and it is known that they participate in many[14].

The importance of iron in collagen production is also well-known. The enzymes proline hydroxylase and peptidyl lysine hydroxylase, which hydroxylateproline and lysine, respectively, require iron for collagen formation. Peptidylproline hydroxylase uses iron, as well as molecular oxygen alpha-Ketoglutarate and ascorbic acid, as a cofactor in this process [15].

In concurrence with the results, Ramanathan[16], Lippard[14], and Anuradha[17] also stated and discussed the possible alternative hypothesis and its causes emphasising the iron binding ,genetic and an immune parameters and stated their role in mechanism of action for progression of the pathogenesis of the OPMDs.

Hambidge [18] stated that zinc plays a variety of biological roles, as it can interact with an extensive range of organic biochemicals and has a function in RNA and DNA metabolism, signalling, gene expression and apoptosis. Similarly in the present study it was observed that few proteins were also involved in zinc ion binding as a prominent molecular function term.

Immunological mediation has long been known to play a role in the development of human diseases. The evaluation of immunological parameters was, predictably, one of the first experiments in OPMDs which lead to altered immune and inflammatory responses. Similarly cytokines are the mediators which regulate defense-related and tissue reactions. Similar data is postulated in the present analysis where it was observed that maximum count and percentage of GO term in a particular annotation clusters were associated with the immune and inflammatory responses. To this concurrent studies reported by Shah[19] and Gupta[20], where they hypothesised that elevated levels of major Immunoglobulins was due to the increased protein synthesis or poor protein excretion. The link between these levels had suggested that OPMDs had a high malignant potential.

Ramanathan[16] stated in their study that the occurrence of Circulating Immune Complexes (CIC) actsas marker for tumor burden and prognosis in the sera of patients with Oral leukoplakia, oralsubmucous fibrosis and oral cancer.Similar to the above result,Maheswari[21] conducted a study to estimate the levels of Circulating Immune Complexes in patients with Oral Leukoplakia ,Oral sub mucous fibrosis and normal subjects and concluded that the CIC may be taken as a prognostic marker for disease progression of patients with Oral Leukoplakia and Oral sub mucous fibrosis.In addition, Sun[22] indicated cytokine levels as indicators to be altered increased in oral mucosal disorders. Mediation by cytokines may be considered as a possible pathway in OPMDs and yet cytokine production and its effects on the oral tissues can range from the inflammations to malignant changes in oral lesions and conditions.Thus, immune response plays a significant role in pathogenesis of OL and OSMF as observed.

Biochemical studies in oral leukoplakia and oral sub mucous fibrosis have thus produced results that can be used as indications of disease progression or as intermediate routes in pathogenesis. They are those pieces of the puzzle that reflects the disorder, and their research could help define the broad range of causes for this potentially fatal ailment.

5. CONCLUSION

A comprehensive perspective was provided by the bioinformatics analysis, via which an attempt was made to decode the differentially expressed genes and the pathways distinctly representing molecular profile of OL and OSMF. In this study, a list of total 143 common expressed genes, 1107 in OL and 607 in OSMF along with their corresponding distinct enrichment pathways, functions, biological processes, cellular component clusters were studied. A detailed functional annotation analysis identified potential targets for developing new therapeutic interventions for oral leukoplakia and oral sub mucous fibrosis. Only a limited number of microarray studies and gene expression datasets were available online on Oral Leukoplakia and Oral sub mucous fibrosis, which is the limitation of the study. Thus, further studies and experiments need to be designed on the basis of the above findings to prove the role of gene signatures and pathways identified above as potential drug targets. This will help in distinguishing and understanding the development, pathogenesis, and mechanism of action in OL and OSMF to limit their progression towards malignancy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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