Review Article

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Significance of Connective Tissue and Immunological Markers in Oral Submucous Fibrosis

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ABSTRACT

OSF has high malignant transformation rate. It mainly affects the South-Asian population, majorly because of areca nut consumption habit.

Many biochemical markers are studied for OSF, ranging from serum iron, trace metal elements, antioxidants and many genetic markers. The ones focused in this review are immunological markers (immunoglobulins, cytokines, complement derivatives) connective tissue markers (β- Fibroblast Growth Factor (FGF), myofibroblast, α-Smooth Muscle Actin (SMA), Transforming Growth Factor-β (TGF)) and genetic predisposition (like higher expression of Sister Chromatid Exchange (SCE) and Human Leukocyte Antigen (HLA), genetic involvement of Suppressor of Mothers against Decapentaplegic (SMAD)-2, SMAD-3, SMAD-7, matrix metalloproteinase (MMP1), MMP2, MMP9, etc).

Biomarkers are very useful in predicting the disease, its progression, rate of malignant transformation and prognosis. For this reason, identifying and exploring the biochemical markers of OSF is of utmost importance to reduce the mortality and morbidity associated with the disease.

Keywords: Biomarkers; connective tissue; genetic predisposition to disease, immunology; oral submucous fibrosis.

INTRODUCTION

Oral submucous fibrosis (OSF) is slowly progressive South-Asian disease with highest rates of malignant transformation amongst oral potentially malignant disorders.[1] It is always associated with a juxta-epithelial inflammatory reaction followed by fibro-elastic change of lamina propria, with epithelial atrophy leading to stiffness of mucosa and causing trismus and inability to eat.[2] Clinical pathognomonic features include burning sensation of oral mucosa accompanied by pallor and progressive, irreversible fibrosis leading to difficulty in mouth opening, speech, and swallowing.[1,3]

Although mechanism is not fully understood, it is considered "potentially malignant disorder" due to high rate of malignant transformation (7-12%).[4] Betel nut (*areca catechu*) is recognised as cause.[5] While arecoline and arecadine are thought to be main causative factors, several co-factors such

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as malnutrition, immunological alterations, and genetic predisposition are also implicated.[6]

Trace elements such as copper, iron, selenium, and molybdenum have been studied to identify any modifying effects on etiology.[7] Extensive data for molecular markers, like p53, proliferation associated antigens, cytokeratins, Bcl2 proteins, exist for other premalignant lesions, data for OSF is limited. Immunological and biochemical studies can help in early diagnosis, appropriate treatment, and indicate prognosis.[8] Hence, this review highlights implication of connective tissue and immunological markers in OSF.

IMMUNOLOGICAL CELLS IMPLICATED IN THE PATHOGENESIS OF OSF

Macrophage: Macrophages are important cells for the innate immunity. Circulating monocytes are attracted to tissues by chemotactic factors and become macrophages under the influence of their microenvironment. Pereira et al. in their study showed that the mean macrophage cell density in OSF study group epithelium was significantly higher as compared to control group. Also, the mean cell density of macrophages in subepithelial connective tissue in the OSF group was significantly higher than the corresponding mean cell density in control group. These macrophages were studied using CD68. Thus, this study had highlighted the utility of macrophage estimation in OSF using CD68 as an immunohistochemical marker.[9] Another study by Wang et al. suggests that arecoline promotes CD147 expression via the TGF-β1 signaling pathway in human oral keratinocytes, whereas overexpression of CD147 may promote OSF progression.[10]

Langerhans cells: Langerhans cells are dendritic, non-keratinolytic clear cells of the oral epithelium present in the suprabasal layer, derived from myeloid stem cells of bone marrow. They are Antigen Presenting Cells (APCs) that aid to provoke a specific T cell reaction by the interaction of the Major Histocompatibility Compatibility (MHC) Class II with the CD4+ cells. A study by Narayanan and Narasimhan showed increase in the Langerhans cells in the OSF epithelium when compared to normal epithelium with active dendritic morphology in OSF tissues. There was a significant difference in the number of LCs between normal and OSF tissues (p value <0.001). This emphasized the role of T cell immunity in OSF.[11]

BIOMARKERS FOR IMMUNOLOGICAL STATUS

Immunological mediation is a well-established factor in the pathogenesis of human disorders. Predictably, assessment of immunological parameters has been one of the earliest investigations in OSF. Evaluation of immunoglobulins (Ig), autoantibodies, cytokines, complement derivatives, and circulating immune complexes have been carried out in OSF.

Immunoglobulins (Igs): Elevated levels of major Igs were noted in patients with OSF by Gupta et al.[6] A similar increase in salivary and serum IgG and IgA have been reported in other studies.[12] Correlating the Ig levels to total serum protein, the authors found a significant drop in the latter in all patients with OSF. Based on the findings a nutritional basis for the disease was suggested. Concurrent studies report similar increases in IgG, IgA, and IgM.[13] Anil *et al*. evaluated the tumor marker serum beta-2 microglobulin in oral cancer, oral lichen planus and OSF patients. A significant increase in the marker in OSF and oral cancer patients was noted with little or no increase in the oral lichen planus group. With this finding, the authors postulated the theory of increased production or impaired excretion of the protein. There is similar correlation of the levels of protein with oral cancer, this similarity between OSF and oral cancer also suggests high malignant potential of OSF.[5]

Cytokines: Cytokines are products of cellular reactions involved in defense. They are the mediators through which tissue reactions occur and symptoms are reflected. Haque *et al*. evaluated the levels of several cytokines in OSF patients. The cytokines studied included: IL-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). A significant increase of all cytokines with a decrease of IFN-γ was noted in patients. Mediation by pro-inflammatory cytokines and modulation by IFN-γ were postulated as possible pathways for the disease.[14] Hsu *et al*. evaluated the effects of arecoline on the levels of IL-2, TGF-β, TNF-α and IFN-γ in patients with OSF, mucosal disorders, and oral cancers. They found decreased levels of all the cytokines in OSF patients as

compared with subjects that indulged in the habit of betel nut consumption, but without oral lesions. The decrease in IFN-γ was consistent with the above study.[15] In the study by Illeperuma et al., it was found that GRO-a, IL-6 and IL-8 were more highly expressed in the fibroblasts of OSF tissue samples, as compared with those of normal oral mucosa.[16] Cytokines have also been evaluated as indicators to therapy in various potentially malignant disorders. In a study on Chinese patients, Sun et al. used IL-6 levels as indicators of therapy with levamisole and traditional Chinese herbs. IL-6 levels were higher in OSF as compared with controls, but much lower than other mucosal disorders.[17] The other marker, which has been studied for OSF, is TGF-β. This is a key mediator of tissue fibrosis resulting from accumulation of extra cellular matrix (ECM). A study by Kale et al. showed that early cases of OSF showed more intense TGF-β staining of epithelium, fibroblast, macrophages and inflammatory cells than the advanced cases. The study thus concluded that TGF-β plays a key role in OSF and is secreted more during early course of the disease than in advanced stage.[18] Mediation by cytokines as a possible pathway in OSF seems to be the impression from the above studies. Yet, cytokine production and its effects on the oral tissues are a normal phenomenon in all reactions ranging from inflammations to oral cancer.

Autoantibodies: A high incidence of autoantibodies including, antinuclear, anti-smooth muscle, anti-gastric parietal cell, antithyroid microsomal has been demonstrated in a Taiwanese study. The authors opined that altered auto-antigens released are coline ingredients. It was also highlighted that damaged cells may induce autoantibody production. The trauma caused to the oral and gastric mucosa from ingestion of the betel nut may increase absorption and help in the process. The authors further stressed the role of human leukocyte antigen (HLA) in speeding the process.[19]

GENETIC PREDISPOSITION IN OSF

The deposition of collagen fibers has long been related to genetic mechanisms. There are mainly two events modulated by TGF-β, which decreases the collagen degradation:[20]

1. Activation of tissue inhibitor of matrix metalloproteinase gene (TIMPs).
2. Activation of plasminogen activator inhibitor gene (PAI).

Initial investigations centered around genetic parameters evaluated from blood and serum of patients with OSF. These include sister chromatid exchanges (SCEs) and Human Leucocyte Antigen (HLA) genotypes.

Sister Chromatid Exchange (SCE): SCE is the exchange of genetic material between two identical sister chromatids. During the S-phase of the cell cycle, DNA is replicated and each chromosome is present in a duplicated state with the two genetically identical chromatids joined together at the centromere. These two sister chromatids are readily apparent in late prophase or early metaphase of mitosis. SCE is the process wherein the two sister chromatids break and re-join with one another, physically switching positions on the chromosome. Because the exchanges occur with tremendous precision with respect to the DNA sequence, and the sister chromatids are genetically identical, no information is altered during the exchange. Such exchanges are natural events during cell replication with each cell typically undergoing three to four SCEs during each replication cycle.

The reason for the SCE is not known, but it is required and used as a mutagenic testing of many products. Four to five SCE per chromosome pair, per mitosis is in the normal distribution, 14-100 exchanges are not normal and presents a danger to the organism.[21,22] Studies on SCEs in OSF indicate increased frequency expression in the condition along with oral cancer as matched with controls. Ghosh *et al*. evaluated SCEs in patients with OSF and those consuming areca nut products and tobacco and found a significantly higher expression than controls. Interestingly, levels in patients with combined habits of smoking and chewing was higher than in the other groups. It is difficult to assess whether the mutagenic indicator expression was due to the effects of the habits or the disease process. In another study, patients with OSF and those chewing betel nuts alone were assessed for SCEs. Expression was higher in both the groups as compared with controls. The authors proposed that the habit of chewing betel nut is the primary mutagenic factor and the tissue change of OSF probably follows. It was also postulated that the

malignant transformation of the condition is a result of this mutagenic effect.[5]

Human Leucocyte Antigen (HLA): The HLA system is the name of the major histocompatibility complex in humans. The super locus contains a large number of genes related to the immune system function in humans. This group of genes resides on chromosome 6 and encodes cell-surface antigen-presenting proteins and many other genes. The major HLA antigens are essential elements for immune function. They are important in disease defense and organ transplant rejections. They may protect against or fail to protect (if down regulated by an infection) against cancers and may mediate autoimmune disease (e.g., Type I diabetes, coeliac disease).[23]

Impairment of the immune system has long been propagated as a mechanism in the pathogenesis of OSF. The geographical location and the almost definite association with habit of chewing betel nut have led to suggestions of an autoimmune basis for the condition. In one of the earliest studies on HLA prototypes, Caniff et al. evaluated HLA type A10 and DR3 in OSF patients. The authors found conclusive evidence of raised expression of these prototypes in OSF patients. They proposed that the results support the concept that OSF is a chronic autoimmune disease, initiated by constituents of betel nut, and occurring in genetically susceptible individuals. It was also suggested by them that genes situated in the HLA region are important determinants of genetic susceptibility in OSF. In contrast, in studies on larger samples of betel nut chewers in South African subjects of Indian origin, Vanwyk et al. could not find any significant association of HLA antigens and have discarded the hypothesis of autoimmunity for this disorder.[5]

POTENTIAL BIOCHEMICAL AND IMMUNOHISTOCHEMICAL PARAMETERS IN OSF

Lipids and Lipoproteins: Changes in lipid profile have long been associated with malignancies as lipids play a key role in maintenance of cell integrity. Studies on lipid profiles of OSF patients have consistently revealed lower levels compared to controls. Three studies on the role of lipids in OSF, analyzed a range of lipids including total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, very low-density lipoprotein cholesterol, triglycerides, Apo-A1, Apo-B and LPa in the sera of patients with OSF.[24-26] A consistent observation of lower lipid levels in OSF as compared to controls was found in all the studies.

The inference was unanimous that lower levels of plasma cholesterol and other lipid constituents in patients might be due to their increased utilization by neoplastic cells for new membrane biogenesis.

Lactate dehydrogenase: Tissue breakdown releases Lactate dehydrogenase (LDH); and therefore, LDH can be measured as a surrogate for tissue breakdown, e.g., haemolysis. Other disorders indicated by elevated LDH include cancer, meningitis, encephalitis, acute pancreatitis, and human immunodeficiency virus.[27] In the study by Sivaramakrishnan et al., significant difference was seen between OSF group and normal controls in the serum and salivary LDH levels. The level was higher in OSF group.[28] This indicates the evidence of tissue breakdown in OSF.

Serum glycoconjugates: The changes in lipid profile have long been associated with cancer because lipids play a key role in maintenance of cell integrity. A glycoconjugate is a molecule in which one or more glycan units are covalently linked to a noncarbohydrate entity. Glycoconjugates are very important compounds in biology and consist of many different categories such as glycoproteins, glycopeptides, peptidoglycans, glycolipids and lipopolysaccharides. They are involved in cell-cell interactions, including cell-cell recognition, and cell-matrixinteractions.[29]

Evaluation of serum glycoconjugates is frequently carried out in tumors and is an indicator of the metastatic potential of the tumor. Serum glycoconjugates have been evaluated in OSF and other oral potentially malignant conditions including oral cancer. Baxi *et al*. evaluated serum glycoconjugates (serum sialic acid, lipid bound sialic acid, mucoid proteins and hexoses) in oral precancerous conditions including OSF and found elevated levels of the entities when compared to controls and chewers. There was a progressive increase of the markers with increasing grade of

malignancy. Though no specific inferences were drawn relating to the elevated levels in OSF, the authors have postulated a role for these biochemical investigations in monitoring of the lesions. Other studies showed related findings of increased levels in OSF without alluding to the probable role of the marker in the pathogenesis of the disease.[5,25,30,31]

It is feasible that increased expression of serum glycoconjugates may indicate a tendency toward malignant transformation in OSF, especially in view of the potential of the markers in cell-to cell interactions.

NOVEL BIOMARKERS IN OSF: WHAT IS THE CURRENT TREND?

There are various markers which have been identified for the detection of increased collagen deposition in patients with OSF. Apart from the ones discussed in previous section of the review, current trends of novel biomarkers in OSF focuses on few other aspects.

Recently, the expression profiles of genes in OSF and normal oral mucosa have been studied more intensively. In one study, 14,500 genes were analyzed using gene chip arrays. The study demonstrated that 716 genes were upregulated and 149 genes were downregulated in OSF. The gene expression profiles of normal controls and OSF patients were clearly distinct, in particular the genes involved in immune response, inflammatory response and TGF-β-induced epithelial-mesenchymal transition. In the same line of research, recently a study conducted by Fang et al. demonstrated that arecoline-induced myofibroblast trans differentiation was via LINC00974-mediated activation of TGF-β signalling.[32] Another study by He et al. suggested that high dickkopf-1 methylation levels in oral submucous fibrosis and oral squamous cell carcinoma tissues may decrease dickkopf-1 expression, which may induce an abnormal activation of the Wnt/β-catenin pathway and oral submucous fibrosis.[33] In a comprehensive analysis of water-soluble and ethanol soluble areca nut constituents, it was demonstrated that both alkaloid and polyphenol fractions induced TGF-β signaling in human keratinocytes. Involved genes included *TGF*-β*2*, *SMAD*-2, SMAD-3, SMAD-7, matrix metalloproteinase MMP1, MMP2 and MMP9 and others.[34-36]

Also, Anura et al. have showed that c-Myc and HIF-1 alpha have emerged as potential screening markers and VEGFRII & CD105 as prognostic markers of OSF.[37] According to other studies, proteins like Cyclophilin A expression level is found to correlate with progression of OSF, VEGF is identified for risk stratification and S100A4 (a member belonging to S100 super family of calcium- binding proteins), Hsp-70 1B, Calreticulin, and Lumican variant is significantly up-regulated in OSF patients.[38-40]

Wollina et al. demonstrated overexpression of stress protein colligin in 70% of OSF patients. Colligin, a collagen-binding protein, is a major glycoprotein in many cells. It was suggested that colligin may contribute to the increased deposition of collagen I and thereby to fibrosis development in oral submucosa.[34] CD34, a marker of mucosal vascular endothelium and basic fibroblast growth factor is both increased in OSF and demonstrate an association to the stage of fibrosis.[34]

Bag et al. validated α-enolase as a biomarker for early diagnosis of malignant potentiality of OSF. Alpha-enolase was found to be overexpressed protein in biopsies of oral submucous fibrosis with dysplasia compared with oral submucous fibrosis without dysplasia and normal oral mucosa.[41]

Another collagen marker, which is being studied recently, is the Myofibroblast or the carcinoma-associated fibroblasts (CAF). They are contractile cells expressing α-smooth muscle actin (α-SMA) and are considered primary producers of extracellular matrix after injury. Their accumulation has been established as a marker of progressive fibrosis in organs like lungs, liver, kidney and skin.[42,43] The number of α-SMA-stained myofibroblasts in OSF has been found to be significantly higher when compared to that of the normal controls. Additionally, a significant increase in the myofibroblast population has been observed from early to advanced stages of OSF to carcinoma progression.[43-45]

Conflict of interest: None.

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