Comparative evaluation of Immunohistochemical expression of MT-1 MMP, TIMP-1, TGF-β1, α-SMA in oral submucous fibrosis and oral submucous fibrosis with coexisting Oral Squamous cell carcinoma

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Abstract: Background : 'Oral submucous fibrosis" (OSF) is chronic, progressive, and irreversible disease but also as oral potentially malignant disorder with high probability of malignant transformation. From a clinical as well as the histological point of view it appears to be of major importance to establish and understand the molecular nature of the mechanism of malignant transformation in OSF .So that these molecules could be targeted to formulate a standard treatment for high risk cases of OSF patients in need for better prognosis. Material and Methods: 25 Cases of "oral submucous fibrosis ." and 25 Cases of oral Submucous Fibrosis with coexisting oral squamous cell carcinoma will be analyzed.4 μm thick sections of formalin fixed paraffin embedded blocks were immunohistochemical stained with Polyclonal rabbit MT-1 MMP antibody , Monoclonal mouse TIMP-1 antibody ,Monoclonal mouse TGF-β1antibody , Monoclonal mouse α-SMA antibody in each case

Statistical Analysis: Sample size formula based on difference between two proportions:

This study uses the following formula for the sample size n: $n = (Z_{\alpha/2}+Z_\beta)^2 * (p_1(1-p_1)+p_2(1-p_2)) / (p_1-p_2)^2$,

Discussion: The Incidence of malignant potential in OSF has been estimated to be 7-13%[2]. After years of persistent efforts and research, satisfactory and definitive treatment modality still eludes us. These markers could be used on regular basis to assess malignant potential of OSMF. This will help to formulate and decide the standard treatment plan with a better regime of target drug therapy.

Keywords: MT-1 MMP, TIMP-1, TGF-β1, α-SMA, Oral submucous fibrosis, malignant potential in OSF

Introduction:

"Oral submucous fibrosis(OSF)" , seen in "India" as well as in many western countries ,is not only considered as chronic, progressive, and irreversible disease but also as oral potentially malignant disorder with high probability of malignant transformation. The

main sites involving this disease is oral mucosa and if it progresses may involve the "pharynx" and "upper third of the oesophagus".[1]Depending on the various stages of this diseases, its clinical features manifests as blanching of the mucosa followed by inability to eat spicy food and rigidity of mucosa of "lip", "tongue" and "palate" resulting in restriction in mouth opening and limited movement of tongue...The main histopathological features include atropic epithelium, juxtraepithelial hyalinization and finally fibrosis.[2] The pathogenesis of this includes increase in production of collagen and decrease in breakdown of collagen leading to fibrosis. The main causative factor responsible for this disease is proven to be alkaloid (arecoline) which abnormally increases production of collagen and flavonoid component (tannins and catechins) both present in arecanut which directly influences on metabolism of collagen.

. The malignant potential of "oral submucous fibrosis" was first described by "Paymaster" in 1956,. It has been estimated to be 7- 13% with significant mortality rate.[2]. For understanding the pathogenesis and the possible mechanisms of malignant transformation of this disease numerous researches have been carried out across the world and in these researches auxillary screening aids like molecular biomarkers, salivary diagnostics can give more improved accuracy After years of persistent efforts and research, satisfactory and definitive treatment modality still eludes us.. These researches can help us to detect malignancy at molecular level with appropriate tumor markers . This knowledge would definitely help to find appropriate treatment modalties to either delay or prevent malignant transformation in high risk cases resulting in better prognosis.

Extracellular matrix (ECM) and its components are degraded by a family of zinc dependent proteases called as Matrix metalloproteinases (MMPs) which are produced by a both physiologic as well as pathological processes. Among the family of 28 human MMPs MT1MMP have been proven to have role in tumor invasion, tumor growth progression , and angiogenesis in other carcinomas like gastric carcinomas, bladder carcinomas , genitor-urinary carcinomas, gliomas etc. So it may prove to be significant independent marker to detect early invasion in high risk cases of OSF[3,4]

There is a family of four protease endogenous tissue inhibitors which inhibit MMPs are known as "tissue inhibitors of metalloproteinases (TIMPs)",These have a role in maintaining balance between synthesis and physiological degradation of the extracellular matrix. Whenever there is a disturbance in balance between MMPs and TIMPs the pathological conditions like OSF arrives..Normally it regulates proliferation and moity of cell, apoptosis, angiogenesis etc.Hence it may prove to be significant marker to detect early invasion in high risk cases of OSF..[5]

"Transforming growth factor-beta (TGF-β)" is a well known pro-fibrotic growth factor causing more of extracellular matrix assembly rather than its remodelling. Whenever there is excess production of . TGF-β , it results in conditions like scar tissue formation and fibrosis. Its isoform TGF-β1 is mostly correlated with fibrosis with playing a main role in extracellular matrix assembly and remodelling. Hence it may prove to be significant marker to detect early invasion in high risk cases of OSF.[6]

A complex molecular process of "Epithelial Mesenchymal Transition (EMT)" is seen in malignant transformation which results in total loss of epithelial cell morphology and gaining phenotype of mesenchymal cells.[7] The constant chewing of arecanut in OSF causes chemical trauma due to release of arecoline as well as mechanical irritation to mucosa of oral cavity .As a result there is release of TGF-β1 which converts fibroblasts to myofibroblasts. These have "alpha smooth muscle actin (α-SMA)" containing stress fibres. Physiologically their main function is tissue or organ's growth ,development of tissue or organ and repair and wound healing. During pathological conditions , they secrete excessive extracellular

matrix protein, in diseases like scleroderma, hypertrophic scars, kidney, and lung and heart fibrosis and OSF.[8]

From a clinical as well as the histological point of view it appears to be of major importance to establish and understand the molecular nature of the mechanism of malignant transformation in OSF .So that these molecules could be targeted to formulate a standard treatment for high risk cases of OSF patients in need for better prognosis. This leads to our research question "Does the semiquantitative expression of MT-1MMP, TIMP-1, TGF-β1, α-SMA using IHC , be effective in assessing malignant potential of "oral submucous fibrosis" and its association with coexisting "oral squamous cell carcinoma?" '.

Material and methods

Study design – Retrospective study

2 groups as follows

- Group A: 25 Cases of "oral submucous fibrosis ."

- Group B: : 25 Cases of "oral Submucous Fibrosis" with coexisting "oral squamous cell carcinoma+ (OSCC)

Inclusion criteria

- o Samples of patients with primary disease as "Oral submucous fibrosis"
- • Samples of patients with "Oral submucous fibrosis" with coexisting" oral squamous cell carcinoma"

Exclusion criteria

- o Samples of patients with carcinomas without pre=existing oral submucous fibrosis
- o Samples of individuals suffering from collagen disorders.

Data sources/ measurements –

1. **Sociodemographic details** – age, sex, habit history and other details will be obtained from departmental records.

2. **Oral Submucous Fibrosis**– Histopathologically diagnosed cases of OSF of various grades from the departmental archives .
3. **"Oral Submucous Fibrosis" with coexisting "oral squamous cell carcinoma"**– Those cases which are diagnosed histopathologically cases of "Oral Submucous Fibrosis" with coexisting" oral squamus cell carcinoma" from the departmental archives .

Methodology:

4 μm thick sections of formalin fixed paraffin embedded blocks were immunohistochemical stained . Polyclonal rabbit MT-1 MMP antibody , Monoclonal mouse TIMP-1 antibody ,Monoclonal mouse TGF-β1antibody , Monoclonal mouse α-SMA antibody.

In both groups : all 4 markers will be analysed in each case

MT-1MMP Cellular localization : membranous and cytoplasmic

The staining will be seen in both stromal cells and epithelial cells.
The degree of staining for **MT1MMP** will be scored as follows:

DEGREE F STAINING	TUMOR CELLS	STROMAL CELLS
3+	extensive staining	strong staining
2+	>50% positive staining	moderate staining
1+	<50% positive staining	weak staining
0	negative staining	negative staining

TIMP-1 Cellular localization : cytoplasmic

The staining will be seen in both stromal cells and epithelial cells.

The degree of staining for TIMP-1 will be scored as follows:

DEGREE F STAINING	TUMOR CELLS	STROMAL CELLS
3+	extensive staining	strong staining
2+	>50% positive staining	moderate staining
1+	<50% positive staining	weak staining
0	negative staining	negative staining

TGF-β1 Cellular localization : nucleus and cytoplasmic

. The staining will be seen in both stromal cells and epithelial cells. The intensity of staining of the epithelium (basal and superficial) and stroma was assessed as:

DEGREE F STAINING	STROMAL CELLS(tissue section)	EPITHELIAL CELLS(tissue section)
– negative	no staining;	no staining;
+ mild	Positive staining<$1/3^{rd}$	Positive staining<1/
++ moderate	Positive staining $1/3^{rd}$- $2/3^{rd}$	Positive staining $1/3^{rd}$- $2/3^{rd}$
+++ intense	Positive staining>$2/3^{rd}$	Positive staining>$2/3^{rd}$

α-SMA Cellular localization: membrane/cytoplasmic

Immunostaining will be assessed **according to the method proposed by "Tuxhorn et al."[and applied by" Etemad‑Moghadam et al."** [9] (**percentage of α‑SMA‑positive cells,** between and around the neoplastic islands) **s**o as those not inflammatory and not present around blood vessels stromal cells present in the subepithelial connective tissues

% POSITIVE CELLS	POSITIVE CELLS	INTERPRETATION
0	None/ negative	none/ negative
1	1%–33%	Mild
2	34%–66%	Moderate
3	67%–100%	Intense

.

According to "Vered et al"., myofibroblasts distribution pattern, and positive stained cells arrangements, will be classified into 3 groups:

DISTRIBUTIN PATTERN OF "MYOFIBROBLASTS"	INTERPRETATION
Focal	focal arrangement
Network(	multiple rows forming interconnected cytoplasmic extensions of myofibroblasts
Spindle	arranged in one to three concentric layers

The results of the expression of MT-1 MMP, TIMP-1 ,TGF-β1 ,α-SMA in Group A and Group B with be compared

Statistical Analysis

Sample size formula based on difference between two proportions:

This study uses the following formula for the sample size n:

$$n = (Z_{\alpha/2}+Z_{\beta})^2 * (p_1(1-p_1)+p_2(1-p_2)) / (p_1-p_2)^2,$$

where $Z_{\alpha/2}$ is the critical value of the Normal distribution at $\alpha/2$ (e.g. for a confidence level of 95%,

α is 0.05 and the critical value is 1.96),

Z_{β} is the critical value of the Normal distribution at β (e.g. for a power of 80%, β is 0.2 and the critical value is 0.84) and

p_1 and p_2 are the expected sample proportions of the two groups.

P1=Proportion of OSF showed over 70% of TGF-ß1 expression =0.70

P2= Proportion of Non OSF showed over 30% of TGF-ß1 expression =0.30

$$N = \frac{(1.96+0.84)^2 *[0.70*(1-0.70)+0.30*(1-0.30)]}{(0.70-0.30)^2}$$

 = 25 patients needed in each groups

25 individuals in Group A ,25individuals in Group B . 2groups as follows

- Group A: 25 Cases of OSF .

- Group B: : 25 Cases of OSF with coexisting OSCC.

Scope

- The complex process of malignant transformation follows many pathways. Further research is to be done for identification of main molecules which will help in identification of possible mechanism involved in transformation of OSF to malignancy that is OSCC.

Limitations

- -Gene profiling could not be done.

Implications

These markers could be used on regular basis to assess malignant potential of OSMF. This will help to formulate and decide the standard treatment plan with a better

regime of target drug therapy. This will be more effective in predicting and improving prognosis of high risk cases of OSF

Expected Outcome

This study might help in improving the quality of life in high risk OSF cases and these patients could be treated appropriately for better prognosis.

Discussion

"Oral submucous fibrosis(OSF)",is not only considered as chronic debilitating, oral disease but also as oral potentially malignant disorder with high probability of malignant transformation. OSF is a disease in which rigidity of mucosa of oral cavity, lips, tongue, palate is affected. And may progress to pharynx and upper third of oesophagus in later stages. The hallmark histopathological features include atropic epithelium, juxtraepithelial hyalinization and finally fibrosis Clinically it includes inability to eat spicy foods, whitening and stiffness of oral mucosa leading to trismus. The main causative factor responsible for this disease is proven to be alkaloid (arecoline) and flavonoid component (tannins and catechins) in arecanut.[1]

The Incidence of malignant potential in OSF has been estimated to be 7-13%[2]. After years of persistent efforts and research, satisfactory and definitive treatment modality still eludes us. For understanding the processes involved in pathogenesis and malignant transformation of OSF many studies and researches have been carried out worldwide. If ancillary screening aids like molecular markers are included in these researches, they will further guide and add to our knowledge and help to formulate various treatment modalities for high risk cases and thus improving the quality of life of patients. There are no exact biomarkers by which the mechanism of association of OSF and its progression to OSCC can be studied Many molecular studies in the past were mainly focussed on different grades of dysplasia and degree of fibrosis. in OSF. In previous studies the intensity of expression of MMP 1, TIMP-1, TGF-β1,α-SMA have studied separately in OSF and OSCC and they have not been evaluated in combination and semiquantitatively.. In our study we have used the combination of MT-1MMP, TIMP-1, TGF-β1,α-SMA cancer markers which has not been studied earlier and also will be studied semiquantitatively. The expression of all four markers might vary from one individual to another. So that they can be used to detect early invasion in high risk cases of OSF and establish border between OSF and those OSF cases which have turned to OSCC.. This study attempts to find the best and reliable marker and its association with malignant transformation in oral submucous fibrosis cases in regard to Indian population.

Till recent date among diverse group of zinc dependent proteases, 28 types have been identified. These are known as MMPs .They influence myofibroblasts thus playing dual role that is both stimulatory and inhibitory in fibrosis.[3] Of the diverse family of MMPs MT1 MMP represent membrane bound form of MMPs which are activated intracellularly. They are the mediator of cell migration, tumor invasion, and also correlated with tumor growth progression and angiogenesis. Increased Mt1MMP expression is seen genito-urinary tumors, gastric cancers, gliomas, bladder carcinoma and has been linked to tumor invasion and metastasis[4] So it may prove to be significant independent marker to detect early invasion in high risk cases of OSF.Many studies in the past are done with MMP-2, MMP-9 in OSF and OSCC and their intensity of expression is studied. Here in our study we will using MT-1MMP(MMP-14) in OSF and OSF with coexisting OSCC. It will be analysed semiquantitatively which is not done earlier.

TIMPs are family of four protease inhibitors which inhibit MMPs. They maintain the integrity of healthy tissue by maintaining balance between extracellular matrix assembly and

degradation..Disturbed balance between two affects various cell behaviours like cell proliferation, angiogenesis, apoptosis. Increased expression of TIMP1 is identified as one of the early marker of OSF and also correlated with tumor invasion and metastasis. So can be used to detect early invasion in high risk cases of OSMF. Many studies in the past are done with TIMP1 in OSF and OSCC separately and their intensity of expression is studied. Here in our study we will using TIMP1in OSF and OSMF with coexisting OSCC. It will be analysed semiquantitatively which is not done earlier. [5]

TGF-β is a well known profibrotic growth factor and key regulator in extracellular matrix assembly as well as remodelling. Its isoform TGF-β1 is mostly related to fibrosis. TGF-β1 Elevated expression is seen in OSF cases and also correlated with tumor invasion. So can be used to detect early invasion in high risk cases of OSF Many studies in the past are done with TGF-β1 in OSF and OSCC separately and their intensity of expression is studied. Here in our study we will using TGF-β1 in OSF and OSF with coexisting OSCC. It will be analysed semiquantitatively which is not done earlier.[6]

α-SMA physiologically play a key role in tissue growth, development and repair. In response to tissue injury in any form, they secrete excessive extracellular matrix protein. Its expression in tissue indicates the presence of myofibroblasts but can show various grades of intensity in different grades of OSF and OSF with coexisting OSCC.It is correlated with tumor progression. So can be used to detect early invasion in high risk cases of OSF Many studies in the past are done with α-SMA in OSF and OSCC and their intensity of expression is studied. Here in our study we will using α-SMA in OSF and OSF with coexisting OSCC. It will be analysed semiquantitatively which is not done earlier. [7,8,9.]

A number of studies on Oral submucous fibrosis were reported[10-15]. Chole et al reported on estimation of serum malondialdehyde in oral cancer and precancer and its association with healthy individuals, gender, alcohol, and tobacco abuse[16]. Chourasia et al reflected on concomitant association of oral submucous fibrosis and oral squamous cell carcinoma[17]. Studies were also reported on Ki67, CD105 and Alpha-Smooth Muscle Actin Expression in Oral Submucous Fibrosis by Gadbail et al[18,19,20,21].

Conclusion:

Thus It is very crucial to establish and understand the molecular nature of malignant potential of OSF. So this study protocol will help to understand the molecules involved in progression of OSF to OSCC specially in high risk OSF cases by studying semiquantitative expression of biomarkers in OSF and its correlation with coexisting OSCC. Furthermore these molecules could be targeted for therapeutic interventions in OSF which can help in management of such cases resulting in better and improved prognosis and thus improving the quality of life of patients.

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