ISSN: 0048-2706 (Print), ISSN: 2227-9199 (Online) PakHeart J2023;56(03)

Assessment Of ImmunohistochemicalexpressionOfmt1mmp/MMP14 In Oral Submucous Fibrosis And Oral Submucous Fibrosis With Concomitant Oral Squamous Cell Carcinoma

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Abstract:

Background : Recently recognized as "collagen metabolic disorder" also a well known oral potentially malignant disorder with about 7-13% malignant transformation rate, "Oral submucous fibrosis" (OSF) is incessant, unabating, ineradicable disease. From last few years researches have been conducted to study different molecular aspects of carcinogenesis in OSF through various appurtenant and supplemental methods which is important from clinical as well as the histological point of view. Ultimately targeting these molecules through tailored treatment modalities and finally improving overall survival of high risk cases of OSF patients.
Material and Methods: We have taken 25 cases each of "OSF ." and OSF with concomitant Oral Squamous Cell Carcinoma along with 25 cases of normal mucosa. Immunohistochemical staining of 4 µm thick sections of formalin fixed paraffin embedded blocks with Polyclonal rabbit MT-1 MMP antibody was done in each case All the IHC slides were blinded for analysis by two independent experienced oral pathologists for scoring of these antibodies
Results:one way ANOVA. chi square test and Mann Whitney U test. Highly statistically significant difference when comparison was made between OSF, OSF with OSCC and control groups with (p = 0.000) A statistically non-significant differences was found in the expression of stromal cells between OSF and OSF with OSCC cases. (with p= 0.090)
Conclusion The semiquantitative expression of MT1MMP could assess early invasion in high risk cases of OSF even before its clinical appearance.

Keywords: MT-1 MMP,Oral submucous fibrosis, malignant potential in OSF

Introduction

Oral submucous fibrosis(OSF)"isincessant, unabating, ineradicable "debilitating and irremediable disease with global acceptance as an Indian disease "OSF" is an "oral potentially malignant disorder"which is now considered as "collagen metabolic disorder" with one of the highest probability to carcinogenesis . This dreaded disease affects many parts of oral cavity like the mucosa, tongue, and even oropharynx and esophagus if the disease progresses..The characteristics of this disease are paleness of the mucosa along with fiery , scorching pain of the mucosa followed by inability to eat spicy food and progressive, irreversible fibrosis with restriction in mouth opening and limited movement of tongue leading to difficulty in speech and swallowing. Atrophic epithelium, juxtraepithelial hyalinization and finally fibrosis involving the lamina propria , submucosa and often extending into the underlying musculature are hallmark histopathological features of this disorder. The pathophysiology of "OSF" is complex with mainly involves chewing of" betel quid" alone or in combination with "tobacco", "lime" along with other factors like the deficiencies of iron and multinutrients, , hereditary aberrations, "Herpes simplex virus "(HSV)", "Human papilloma virus (HPV)", "autoimmunity" etcaffectingdirectly byinducing "OSF" or indirectly by arbitrating the immune system in "OSF". This disease involves fibrosis caused due to surge in interlinking of "collagen" due to over

expression of "lysyl oxidase" activity, 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 thus causing decreases in degeneration of collagen ."[1,2]For any Oral Potentially malignant disorder, the main topic of discussion and concern is always the conversion of that disease into malignancy. In case of OSF it was first described in "1956"by "Paymaster". And has been estimated to be 7- 13% with significant mortality rate.It appears to be a myriad process with many complex as well as diverse routes. The oral squamous cell carcinoma"" (OSCC)" arising in the background of "OSF" which is now considered to form a clinic-pathologically different disease[2]. Auxillary screening aids like molecular biomarkers, salivary diagnosticshave progressed remarkably in the past few years allowing us to appreciate the process involved in conversion of "OSF" into malignancy.at molecular level with appropriate tumour markers . Thesepotentially early markers would definitely help to identify high risk OSF cases that may become malignant in future. We can have improved therapeutic prime management with focus on target drug therapy, ultimately helping in improving prognosis in these cases.[2]Matrix metalloproteinases (MMPs) are "zinc dependent proteases family of 28 human "MMPs, identified according to the selectivity of substrate and resemblance of substrate structure, They are "collagenases", "gelatinases", "stromelysins", "matrilysins", and "membrane-type".. Among the 6 "MT-MMPs" known in humans."MT1-MMP" is most explored enzyme . It causes the degradation of fibrillary collagens: types I, II, and III (but not type IV) collagens. , thereby promoting cellular invasion into matrices,. It is also as a cell surface proMMP-2 activator expressed in invasive cancer cells. It promotes migration , invasion , growth , angiogenesis and metastasis of cancer cells . This invasive property is acted on multiple levels by MT1MMP. Firstly activates the cell signaling pathways by causing proteolysis of extracellular matrix (ECM) biomolecules, and invasion by ECM. Secondly a structural changes in MT!MMP is produced due to binding of ligands, ultimately effecting its interactions to cell surface partners and intracellular signaling. Thirdly, acting as a transcript or factor and causing intracellular proteolysis.This nature of MT1MMP has been astronomically researched in other carcinomas like "melanoma", "pancreatic cancer, "advanced neuroblastoma", "small cell" and "non-small cell lung cancer","mesothelioma", "tongue squamous cell carcinoma"," head and neck carcinoma", "bladder cancer", "breast cancer", "colorectal cancer", and "ovarian cancer[3,4,5,6]For Clinicohistological correlation & assessment we need to authorize, ,understand as well as identify the main molecules which will help in identification of possible mechanism involved in transformation of OSF to malignancy that is OSCC. So that these molecules might be considered as an emerging approach to individualize and facilitate treatment planning to formulate a better regime for target drug therapy for high risk cases of OSF patients who might be kept under aggressive surveillancefor better prognosis. Thus, the aim of present study is to compare the immunohistochemical expression of "MT-1 MMP" immunohistochemically in "oral submucous fibrosis" and "oral submucous fibrosis" with coexisting "Oral Squamous Cell Carcinoma"

Materials and Method:

Twenty f ive blocks of patients diagnosed with Oral submucous fibrosis and also cases of Oral submucous fibrosis with concomitant Oral Squamous Cell Carcinoma were retrieved from archives and Twenty five samples of normal tissue were included. From these blocks 4 µmthick sectionswere prepared and were immunohistochemical stained with Polyclonal rabbitMT-1 MMPantibodyScoring of antibodies were done by two independent experienced oral pathologists who were unaware of the clinical details the cases as well as histopathological diagnosis of cases

Scoring of antibodiesMT-1MMP

The staining was seen in both stromal cells and epithelial cells

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

Immunohistochemistry Staining: The immunohistochemical investigation was performed using super sensitive polymer-HRP Twenty Five micron section from each group were cut from paraffin block and mounted on super frost slides. Sections were dewaxed, washed and antigen retrieval carried out in PT Link Module with 1 mM EDTA solution (pH 9) for 20 minutes. Endogenous peroxidase was blocked by using 3% hydrogen peroxide in methanol at

room temperature for 10 min.Immunostaining was carried out on the i6000 Biogenexautostainer. Slide were washed PBS briefly and incubated with primary antibody* for 60 min Section were washed with PBS and incubated with the Super sensitive detection system (Biogenex) for 30 minutes. Section were washed with PBS. Diaminobenzidine (DAB) was used as the chromogen in hydrogen peroxide for 10 minutes.Sections were then counterstained with haematoxylin and mounted

Inclusion criteria: All the samples of patients with primary disease as "Oral submucous fibrosis" and "Oral submucous fibrosis" with coexisting" oral squamous cell carcinoma" were taken.

Exclusion criteria: Samples of patients with carcinomas without pre=existing oral submucous fibrosis or individuals suffering from collagen disorders. were excluded from the study

Results

Data was subjected to statistical analysis using Statistical package for social sciences (SPSS v 26.0, IBM). Descriptives, one way ANOVA, chi square test Kruskall Wallis ANOVA, Mann Whitney U test. Were applied For all the statistical tests, $p<0.05$ was considered to be statistically significant, keeping α error at 5% and β error at 20%, thus giving a power to the study as 80%.

Inter group comparison of mean age of the subjects

	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum	F value	p value of one way ANOVA
1	25	47.16	7.581	1.516	35	66		
2	26	47.88	6.364	1.248	37	64	.490	.615#
3	26	48.92	5.067	.994	42	60		

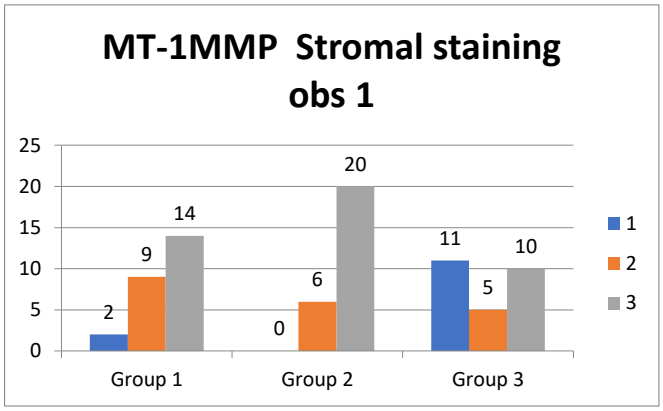
Inter group comparison of sex * group

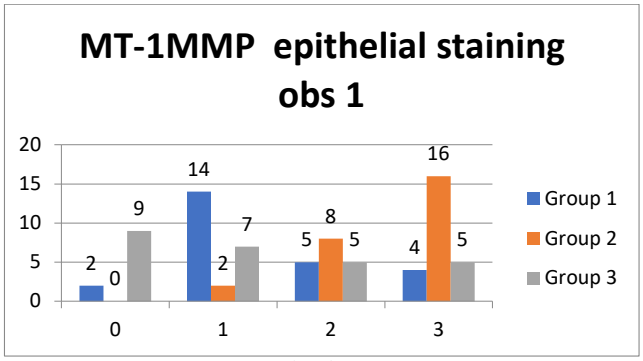
		Group					
		1	2	3	Total	Chi-Square value	p value of Chi-Square test
Sex	F	4	3	5	12		
	M	21	23	21	65	0.590	0.745#
	Total	25	26	26	77		

There was a statistically non significant difference seen for the frequencies between the groups ($p>0.05$)
Inter group comparison of demographic data was done to know whether the groups were similar at baseline after randomization; Matched groups (similar groups) can be ideally be compared

This also means that these independent demographic variables are less likely to have an effect on the outcome

Results for Observer 1MTIMMP : When the intensity of staining of stromal cells and epithelial cells were compared between OSF and OSF with coexisting OSCC cases, a statistically highly significant difference was seen for frequencies between groups. The stromal and epithelial staining were more in OSF with coexisting OSCC than in OSF. In Group 1 (OSF) in case of stromal staining, mostly score 2 (>50% of positive cells) staining was seen. In Group 2(OSF with coexisting OSCC). Score 3(extensive staining)was seen . In Group,3 (control group) score 1 &3 (<50% of positive cells and extensive staining) both were evident. In Group 1 in case of epithelial staining, score 1(<50% of positive cells) was seen. Group 2 showed mainly score 3 (extensive staining while Group 3 showed score 1(<50% of positive cells)

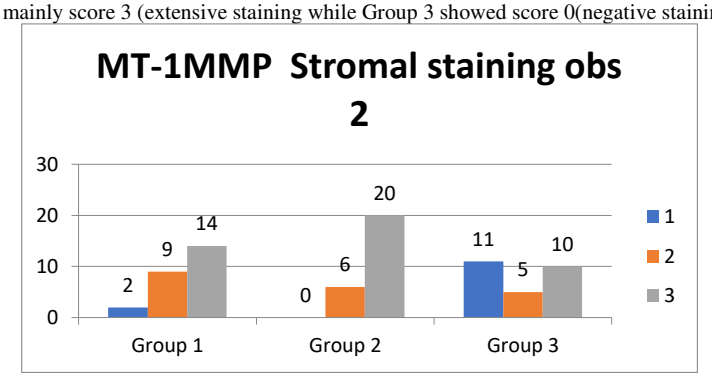


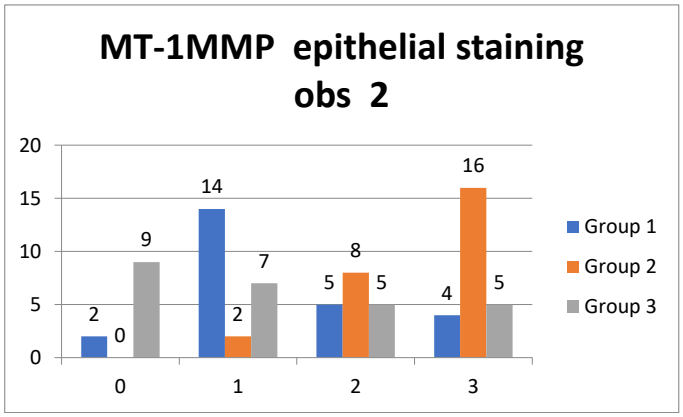


Results for Observer 2

MTIMMP

When the intensity of staining of stromal cells and epithelial cells were compared between OSF and OSF with coexisting OSCC cases, a statistically highly significant difference was seen for frequencies between groups.The stromal and epithelial staining were more in OSF with coexisting OSCC than in OSF. In Group 1 (OSF) in case of stromal staining, mostly score 3(extensive cells) staining was seen. In Group 2 (OSF with coexisting OSCC). Score 3(extensive staining)was seen . In Group,3 (control group) score 1 &3 (<50% of positive cells and extensive staining) both were evident.In Group 1 in case of epithelial staining, score 1(<50% of positive cells) was seen. Group 2 showed mainly score 3 (extensive staining while Group 3 showed score 0(negative staining)





When MT1MMP stromal staining obs 1 in group 1 (2.08±0.759) was compared with group 2 (2.77±0.514) and group 3 (2.01±0.916) they showed a highly statistically significant difference between them with 'p' value of 0 .001. When MT1MMPstromal staining stromal obs 2 in group 1 (2.48±0.653) was compared with group 2 (2.77±0.430) and group 3 (1.96±0.916) they showed a statistically nonsignificant difference between them with 'p' value of 0 .090.

When MT1MMP epithelial staining obs 1 in group 1 (1.08±0.640) was compared with group 2 (2.35±0.797) and group 3 (-73±0.667) they showed a highly statistically significant difference between them with 'p' value of 0 .000. When MT1MMP epithelial staining stromal obs 2 in group 1 (1.44±0.870) was compared with group 2 (2.54±0.647) and group 3 (1.23±1,142) they showed a highly statistically significant difference between them with 'p' value of 0 .000.

		N	Mean	Std. Deviation	Median	Mean rank	Chi square value	p value of Kruskal-Wallis ANOVA
MT-1MMP Stromal staining obs 1	1	25	2.08	.759		32.66	13.629	0.001**
	2	26	2.77	.514		51.02		
	3	26	2.04	.916		33.08		
MT-1MMP Stromal staining obs 2	1	25	2.48	.653		40.10	12.327	0.002**
	2	26	2.77	.430		48.12		
	3	26	1.96	.916		28.83		
MT-1MMP epithelial staining obs 1	1	25	1.08	.640		33.04	36.913	0.000**
	2	26	2.35	.797		58.83		
	3	26	.73	.667		24.90		
MT-1MMP epithelial staining obs 2	1	25	1.44	.870		32.46	22.465	0.000**
	2	26	2.54	.647		55.15		
	3	26	1.23	1.142		29.13		

Inter group pair wise comparison between groups 1 vs 2

	Mann-Whitney U value	Z value	p value of Mann-Whitney U test
MT-1MMP Stromal staining obs 1	160.000	-3.501	0.000**
MT-1MMP Stromal staining obs 2	251.000	-1.693	0.090#
MT-1MMP epithelial staining obs 1	89.500	-4.743	0.000**
MT-1MMP epithelial staining obs 2	116.000	-4.168	0.000**

There was a statistically highly significant / significant difference seen for the values between the groups ($p<0.01$, 0.05) for almost all the variables except for MT-1MMP Stromal staining obs 2

Inter group pair wise comparison between groups 1 vs 3

	Mann-Whitney U value	Z value	p value of Mann-Whitney U test
MT-1MMP Stromal staining obs 1	318.500	-0.130	0.897#
MT-1MMP Stromal staining obs 2	223.500	-2.064	0.039#
MT-1MMP epithelial staining obs 1	238.500	-1.873	0.061#
MT-1MMP epithelial staining obs 2	279.500	-0.900	0.368#

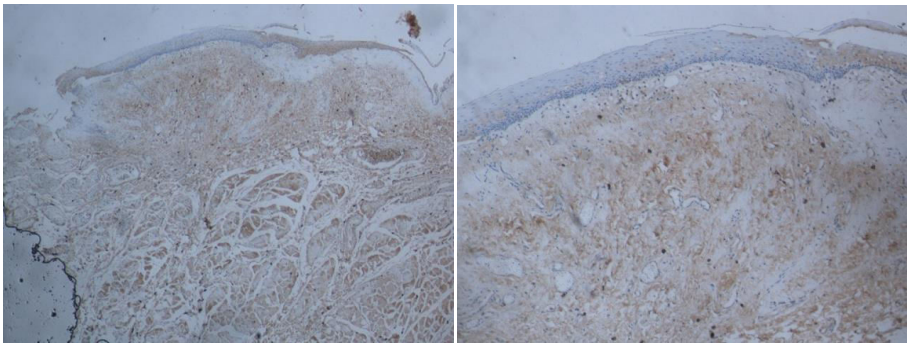
There was a statistically non significant difference seen for the values between the groups ($p>0.05$) for MT-1MMP Stromal staining obs 1,MT-1MMP Stromal staining obs 2,MT-1MMP epithelial staining obs 1,MT-1MMP epithelial staining obs 2

Inter group pair wise comparison between groups 2 vs 3

	Mann-Whitney U value	Z value	p value of Mann-Whitney U test
MT-1MMP Stromal staining obs 1	190.500	-3.112	0.002**
MT-1MMP Stromal staining obs 2	175.000	-3.358	0.001**
MT-1MMP epithelial staining obs 1	58.000	-5.328	0.000**
MT-1MMP epithelial staining obs 2	127.000	-4.051	0.000**

There was a statistically highly significant / significant difference seen for the values between the groups ($p<0.01$, 0.The mean MT1MMP for stromal staining, (for observer 1) in case of OSF, OSF with OSCC and control group were (2.08±0.759), (2.77±0.514), and (2.01±0.916 respectively while the mean MT1MMP for stromal staining, (for observer 2) for of OSF, OSF with OSCC and control group were (2.48±0.653), (2.77±0.430) and (1.96±0.916) respectively.The mean MT1MMP for epithelial staining , (for observer 1) in case of OSF, OSF with OSCC and control group were (1.08±0.640),(2.35±0.797), (-73±0.667) respectively while the mean MT1MMP for epithelial staining , (for observer 2) in case of OSF, OSF with OSCC and control group were (1.44±0.870),(2.54±0.647) ,(1.23±1,142) respectively.Highly statistically significant difference when comparison was made between OSF, OSF with OSCC and control group with MT1MMP expression seen more in OSF with OSCC followed by OSF and least in control group .A statistically non significant difference was foundwhen comparison was made between OSF, OSF with OSCC in case of stromal staining (for observer 2)

MT1MMP IN OSF(FIGURE 1)



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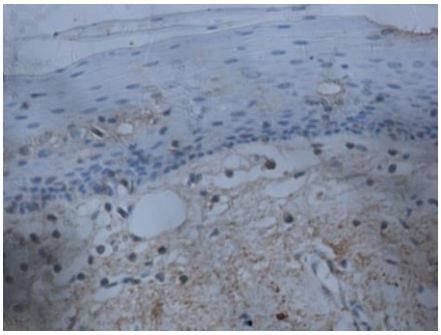

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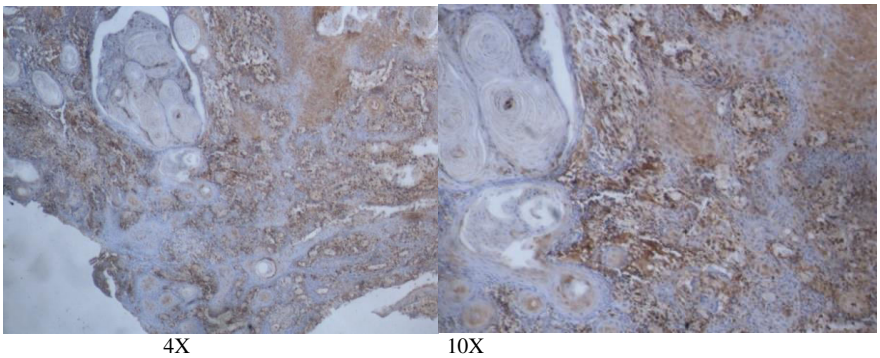
IMMUNOEXPRESSION OF MT1MMP/MMP14 IN OSF

MT1MMP IN OSCC WITH OSF (FiGURE 2)



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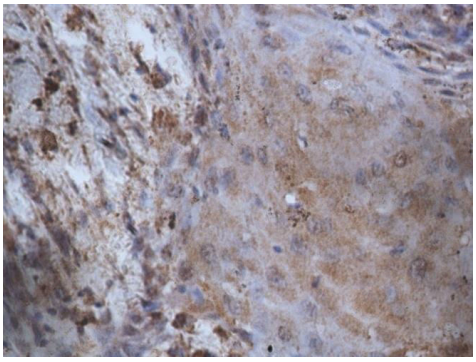

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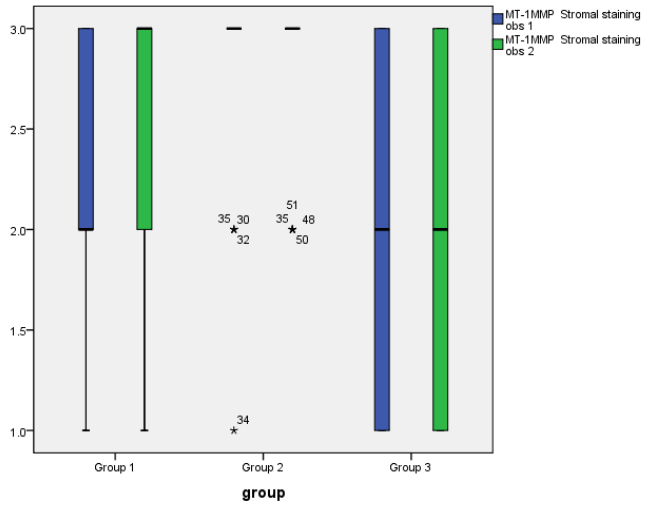
IMMUNOEXPRESSION OF MT1MMP/MMP14 IN OSF WITH OSCC

Intra class correlations & Cronbach's Alpha (Inter and Intra rater)

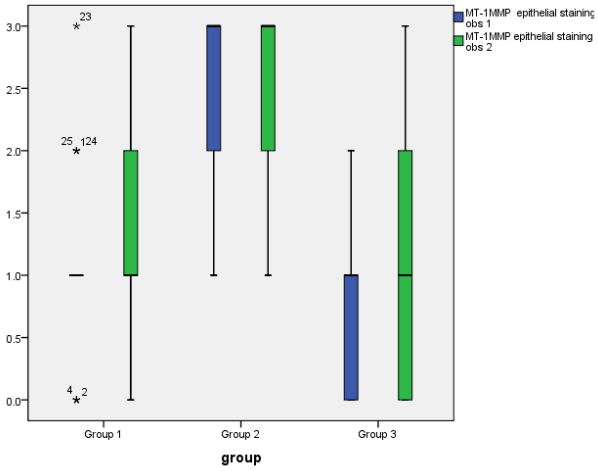
Variable	Cronbach's Alpha	Intraclass Correlation		Lower Bound	Upper Bound	Value	p value
MT-1MMP Stromal staining	.986	Single Measures	.959	.917	.982	71.579	.000**
		Average Measures	.986	.971	.994	71.579	.000**
MT-1MMP epithelial staining	.968	Single Measures	.910	.822	.960	31.316	.000**
		Average Measures	.968	.933	.986	31.316	.000**

There was an overall **excellent internal consistency** (alpha value >0.9) There was an **overall almost perfect agreement** (Single measures value >0.8) with a statistically highly significant p value (p<0.01)

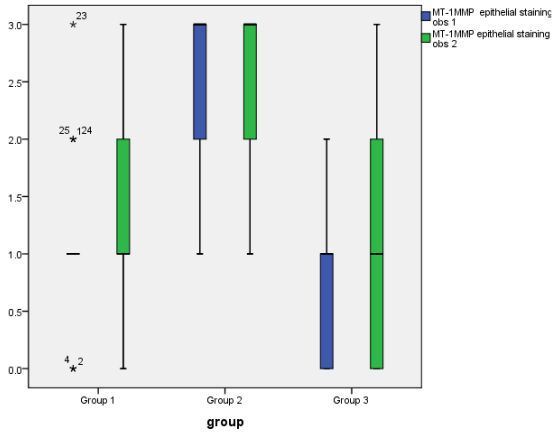
MT-1MMP Stromal staining



MT-1MMP epithelial staining



MT-1MMP epithelial staining



Discussion

Atrophiaidiopathica (tropica) mucosae oris" was reported by Schwartz in 1952 and later Joshi in 1953 named it as "oral submucous fibrosis"(OSF). Now well established as "oral potentially malignant disorder" well as complex collagen metabolic disorder with malignant transformation rate up to 7% to 14% according to Paymaster in 1956. With a high prevalence and malignant transformation rate in India along with many western countries, OSF has emerged as a challenge in terms of therapeutic interventions especially in high risk cases. Carcinogenesis in OSF that is appearance of Oral squamous cell carcinoma (OSCC) in background of OSF is now recognized as separate disease entity.Early detection of invasion can best be studied at molecular level even before the clinical changes are imperious for better prognosis of high risk cases. Copious efforts have been made by using ancillary screening aids like molecular biomarkers, salivary diagnostics to detect the early event in process of malignant transformation of OSF.Targeting these molecules will help to design and plan the standard treatment modalities for high risk OSF cases with hope for better prognosis. The present study has mainly used MMP!4/ MT1MMP marker to detect early invasion in OSF cases.MT1-MMPcauses the degradation of fibrillary collagens: types I, II, and III (but not type IV) collagens., thereby promoting cellular invasion into matrices,. It is also as a cell surface proMMP-2 activator expressed in invasive cancer cells. It promotes migration , invasion , growth , angiogenesis and metastasis of cancer

Kommentar [DN1]:

cells . This invasive property is acted on multiple levels by MT1MMP. Firstly activates the cell signaling pathways by causing proteolysis of extracellular matrix (ECM) biomolecules, and invasion by ECM. Secondly a structural changes in MT!MMP is produced due to binding of ligands, ultimately effecting its interactions to cell surface partners and intracellular signaling. Thirdly, acting as a transcript or factor and causing intracellular proteolysis. This has been proven in melanoma", "pancreatic cancer, "advanced neuroblastoma", "small cell" and "non-small cell lung cancer","mesothelioma", "tongue squamous cell carcinoma"," head and neck carcinoma", "bladder cancer", "breast cancer", "colorectal cancer", and "ovarian cancer. But no study till now has been conducted in OSF.[7,8,9,10]In our study the mean MT1MMP for stromal staining, (for observer 1) in case of OSF, OSF with OSCC and control group were (2.08±0.759), (2.77±0.514), and (2.01±0.916 respectively while the mean MT1MMP for stromal staining, (for observer 2) for of OSF, OSF with OSCC and control group were (2.48±0.653), (2.77±0.430) and (1.96±0.916) respectively.The mean MT1MMP for epithelial staining , (for observer 1) in case of OSF, OSF with OSCC and control group were (1.08±0.640),(2.35±0.797), (-73±0.667) respectively while the mean MT1MMP for epithelial staining , (for observer 2) in case of OSF, OSF with OSCC and control group were (1.44±0.870),(2.54±0.647) ,(1.23±1,142) respectivelyHighly statistically significant difference when comparison was made between OSF, OSF with OSCC and control group with MT1MMP expression seen more in OSF with OSCC followed by OSF and least in control group .A statistically non significant difference was foundwhen comparison was made between OSF, OSF with OSCC in case of stromal staining To the best of our knowledge, no studies have been conducted in the past for evaluating expressions of MT-1MMP in OSF cases. Few studies in the past had been conducted in past by Kurahara et al in OSCC,[11] Tetu et al in breast carcinoma[12] ,Yan T et al in nasopharyngealcarcinoma[13], Martins J et al in cervical carcinoma [14] In all these studies MT-1MMP expression was higher in carcinoma cases which is similar to our studies.A more relevant finding was the non-significant differences in expression of stromal cells between OSF and OSF with OSCC cases pointing to alterations in thechange in tumor microenvironment in cases of high risk cases which might be turning into malignancy at later stages even before clinical appearance. In the tumorigenesis of OMPDs, several pathways play role for the development of tumor. The primary change is incertitude in microenvironment, The factors promoting carcinogenesis are the changes caused by potentially malignant cells in microenvironment of immunosuppressive, hypoxic and acidic conditions. Along with this, the interaction between the networks formed by immune cells, metabolic, mechanical and neural microenvironments also augment these procarcinogenic effects. All is followed by most critical event being invasion of basement membrane and metastasis which happens somewhere in the process of carcinogenesis[15]

The present study found a significant relation between probability of malignant transformation and expression of Mt1mmp. The present study focus on the role of and thus points to the need of formulating and designing the microenvironment-based targeting strategies for early detection of carcinogenesis in high risk cases of OSF . A prospective study with large sample size will help to gain insight about the usefulness of Mt1mmp for assessing early invasion and better prognosis of high-risk cases of OSF.

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