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Orginal Article

IMMUNOREACTIVITY OF MMP-9 IN TISSUE SAMPLES OF ORAL POTENTIALLY MALIGNANT DISORDERS AND NORMAL ORAL MUCOSA

Fiza Shafiq[1], Aneela Bashir[2], Alamgir[3], Mehreen Malik[2], Syeda Munazza Gillani[2], Asfand Khan[2], Abbas Saleem Khan[4]

[1]Department of Oral Pathology, Women Medical and Dental College, Abbottabad, Pakistan
[2]Department of Oral Pathology, Abbottabad International Medical College, Pakistan
[3]Department of Oral Pathology, Ayub Medical College, Abbottabad, Pakistan
[4]Department of Oral Pathology, Peshawar Dental College, Pakistan

ABSTRACT

Objectives: *This study's objective is to evaluate the immunoreactivity of MMP-9 in tissue samples from diagnosed cases of oral precancerous conditions and from normal oral mucosa.*

Materials and Methods: *This study evaluated MMP-9 immunoreactivity in normal oral mucosa and oral potentially malignant disorders (OPMDs). Tissue samples were collected from surgical procedures and departmental archives, with H&E and immunohistochemical staining performed following standard protocols. Immunoreactivity was assessed based on staining intensity and positive cell percentage, using spleen tissue as a positive control. Statistical analysis showed significant differences in age between normal mucosa and OPMD cases ($p < 0.05$).*

Results: *MMP-9 expression was high in OPMDS patients than in normal oral mucosa. While comparing the degree of MMP-9 expression among OPMDs and normal oral mucosa , a statistically significant relationship was found.*

Conclusion: *MMP-9 expression was shown to be higher in OPMDs than in normal oral mucosa. Evaluation of MMP-9 biomarker may be clinically significant in OPMDs and may be utilized as a diagnostic indicator for OPMDs.*

Key words: *Disorders, Matrix Metalloproteinase-9, Oral potentially malignant, normal oral mucosa, Immunohistochemistry*

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INTRODUCTION

Interleukin-8 (IL-8) and transforming growth factor (TGF-) stimulate neutrophils, macrophages, and fibroblasts to create matrix metalloproteinases (MMPs)[1]. MMPs contribute to the malignant transformation process through two mechanisms: suppression of NK cell activity and activation of certain growth factors. MMPs affect the bioactivity of the endothelial growth factor receptor (VEGFR), which leads to the development of angiogenesis[2,3]. The zinc metalloproteinase family of enzymes includes matrix metalloproteinase-9 (MMP), also known as 92 KDa type IV collagenase/gelatinase, which is linked to the breakdown of extracellular matrix. One of the main indicators of cancer progression is extracellular matrix (ECM) disintegration, which promotes tumour cells' invasion into surrounding tissues[4,5].

Correspondence:
Fiza Shafiq
Assistant Professor
Department of Oral Pathology, Women Medical and Dental College, Abbottabad, Pakistan
Email: fizashafiq818@yahoo.com

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Oral premalignant lesions are typically present before the OSCC. The WHO defines OPMDs as clinical manifestations that increase the risk of cancer in the oral cavity, whether in clinically normal oral mucosa or in defined precursor lesions. Oral erythroplakia and leukoplakia are examples of oral conditions that could be cancerous (proliferative verrucous, speckled etc.). Lichen planus, smokeless tobacco keratosis, oral submucosa fibrosis, and chronic candidiasis[6]. The prevalence of OPMDs varies from 1% to 5% globally. 80% of oral malignancies were preceded by OPMDs, according to the epidemiological studies[5].

Leukoplakia prevalence globally ranges from 1.49% to 2.6%, while oral submucous fibrosis (OSF) affects 1.0%–3.03% of the population[7,8]. OPMDs can occur at any intraoral site, with distribution varying by disorder, patient age, gender, and etiological factors. Erythroplakia commonly affects the floor of the mouth, buccal mucosa, and soft palate, while proliferative verrucous leukoplakia (PVL) frequently involves the alveolar mucosa, gingiva, and palate. Buccal mucosa is the most affected site, accounting for 18.4% of lesions with a malignant transformation rate of 3.35%. Tongue lesions show a higher transformation rate of 24.22%, while the combined "floor of mouth and tongue" site has a rate of 14.85%[9].

Common risk factors include tobacco use (smoked and smokeless) (10), alcohol consumption chronic viral infections (e.g., HPV, HSV)[10,11],, alcohol-containing mouthwash[12], poor oral hygiene[13], socioeconomic conditions[14], dietary deficiencies, and genetic predisposition[15]. Most OPMD patients are aged 50-69 years, though 5% of cases are now seen in individuals under 30. Leukoplakia primarily affects middle-aged men, while erythroplakia is more common in men aged 50-70. PVL predominantly affects women (male-to-female ratio 1:4) around 60 years[9], and OSF-related oral squamous cell carcinoma (OSCC) is more common in men, with a mean age of 46 years[16].

Malignant transformation rates vary: lichen planus (1.4%), OSF (5.2%), erythroplakia (33.1%), and PVL (49.5%). Overall, OPMDs exhibit a malignant transformation rate of 7.9%, emphasizing the need for early detection and management[17,18].

The present descriptive cross sectional study is design to assess the Immunoreactivity of MMP-9 in formalin fixed Paraffin embedded block among oral potentially malignant disorders. This would help in exploring it's biological behavior and it's predictive role in probable malignant potential of oral potentially malignant disorders. We evaluate the expression of MMP-9 between oral normal mucosa and conditions that have a chance of being malignant in the oral cavity.

MATERIALS AND METHODS

For normal oral mucosa: Individuals with histologically diagnosed normal mucosa. The mucosa was acquired after getting informed consent from healthy individuals for already planned minor Surgical procedure e.g impacted third molar extraction, alveloplasty, implant placement. In which normal oral tissue is cut and remove as part of treatment plan. Slides were prepared for MMP-9 monoclonal antibody by IHC staining. Antigen retrieval was done by placing in the citrate buffer and than heat in the microwave oven. Slides were stained with MMP9 monoclonal antibody. Immunoreactivity of MMP9 was evaluated by marking a case negative and positive (on basis of staining intensity and percentage of positive cells). One positive control was processed with each batch of IHC staining. Spleen tissue was taken as positive control.

MMP-9 Immunohistochemistry Method

Paraffin embedded tissue 3-5 micrometer thick section were cut through microtome and picked the tissue section on poly –L- lysine coated slide or AKO IHC microscope slide. The slides were fixed in oven at 56-60 C for 20-25 min. Then deparaffinized the formalin fixed embedded tissue 2-5min (dewax in xylene). Then rehydration through descending alcohol series, first 100%, 90%, 70%, 50%, followed by a through wash in running tap water for 30min. Couplin jar or other suitable containerwere filled with sufficient quantity of target retrieval solution). The antigen retrieval endogenous peroxidase activity was carried out by soaking of slides in blocking solution peroxidase for 5 min (Annexure-VB). Then primary antibody (MMP 9) were applied on tissue ction for 30 min Then washed with buffer (TBST), (Tris buffer saline), (Annexure-VD) for 30 min at room temperature. Then secondary antibody (HRP) were applied. Then washed with buffer 2-3 min.). Then the slides were wiped and then DAB substrate chromogen solution were applied to cover section). Incubated

for 30 second to 2 mints. Then washed with distilled water. Then Counter stain in hematoxylin 1 to 2 dips or incubate it on strength of hematoxylin used. Rinse gently in running tap water. Dehydrated in ascending part of alcohol (clear in xylene and mount in DPX.

The cases came from the department's archives. Patients of all ages with cases of OPMDs that have been histopathologically diagnosed are included in the study. Following a tissue sectioning using a microtome, the MMP-9 immunohistochemical staining and H&E staining were carried out in accordance with standard histopathology laboratory protocol. The study comprised 10 cases of healthy people and a total of 25 cases of OPMDs with histological diagnoses (n=25).

Inclusion Criteria

Cases of OPMDs that were categorized by histopathology as either oral epithelial hyperplasia or oral epithelial dysplasia (Mild, moderate, Severe dysplasia, Carcinoma in situ).

Exclusion Criteria

Patients who's OPMDs (such as leukoplakia, erythroplakia, or oral submucous fibrosis) are already associated with or present alongside oral squamous cell carcinoma (OSCC) were not included in the study. This exclusion ensures that the study focuses only on OPMDs without any concurrent cancerous lesions, allowing a clearer analysis of their behavior and malignant transformation potential.

Statistical analysis

Data was analyzed. SPSS version 20 was used to further analyze the data for statistical significance. Single sample't' test showed the difference in age of cases of normal oral mucosa and OPMDs were found to be statistically significant (<0.05)

RESULT

10 healthy control cases and 25 cases with OPMD were included in the study.

4.1 Participants in the Research Are Described Based on Their Age and Gender. Ages of OPMD sufferers ranged from 40 to 75 years old. The majority of OPMD cases (n=20) manifested in people over 50 years old. The observed m: f ratios were 11:14=1:1.2 for OPMD cases and 6:4=1.5:1 for healthy controls, respectively. When comparing the study participants' ages, a statistically significant relationship (p=0.04) was found (Table 2).

Among the cases of OPMD were clinically viewed as oral lichen planus (40%) followed by oral leukoplakia (32%), speckled leukoplakia (20%) and oral erythroplakia represented 2%. The most commonly affected site by the OPMD were buccal mucosa (48%) followed by tongue (24%)

The commonly occurring precancerous lesion was Squamous cell hyperplasia (44%) followed by moderate dysplasia (24%), mild dysplasia (12%), severe dysplasia (8%) and CIS (4%). Among cases of OPMD's the subepithelial inflammatory infiltrate mostly comprised of acute on chronic inflammation (60%) (Table 3).

Among cases of OPMD's, 15 cases (60%) did

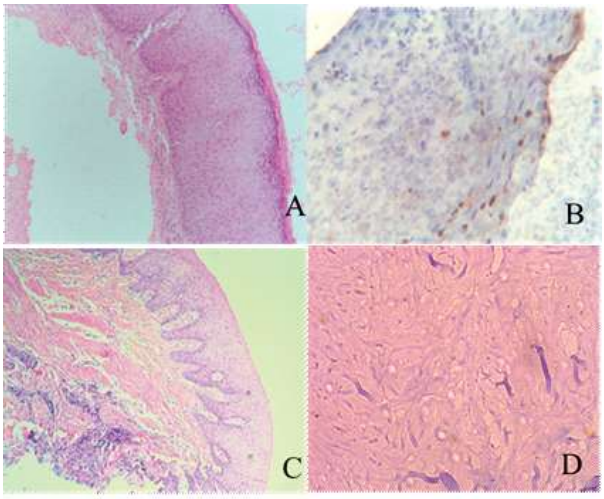

Fig 3: A.Photomicrograph of OPMD buccal mucosa showed that it was lined by stratified squamous epithelium (Magnification X 4).B. Photomicrograph show the presence of immunoreactivity of MMP-9 in OPMD cases (Magnification X40). C. Photomicrograph of H&E slide of Normal oral Mucosa (Magnification X10). D. Photomicrograph of Normal oral mucosa showed there was no staining (Magnification X 40)

Table 1: SD and Mean of Age group

Group	Minimum	Maximum	Mean	SD	p-value
A	40	75	75.5	10.7	P=0.00001
B	30	74	67.5	13.46	P=0.021

not expressed MMP–9 expression while 10 cases (40%) of OPMDs showed MMP–9 expression. There was Statistically significant relation among the MMP–9 expression and grade of staining intensity (Table 4).

Among NOM (normal oral mucosa) MMP-9 was not expressed.

Statistically significant relation was observed for MMP–9 expression among the cases OPMD's Healthy individual (Table 5). Most cases of OPMD show no expression of MMP-9 were above age of 50yrs while 6/24% cases show MMP-9 expression were above age of 50yrs. MMP-9 expression was found to be correlated statistically with OPMD patients (Table 6).

There was statistically insignificant relation observed among Binary grading system of OED and MMP–9 tissue immunoreactivity. According to Binary grading system, OPMDs diagnosed as OED, there was raised frequency of high risk lesions (n=4;33.3%) who were MMP-9 immunoreactive, compared to negative MMP–9 immunostained lesions categorized as low risk lesions

A Statistically insignificant relation was noted among MMP-9 tissue immunoreactivity, sites of OPMD and binary grading system (p=0.259) and subepithelial inflammatory changes (p=0.683). Among the cases of OPMDs MMP-9 expression

Table 2: Age, Gender of the Study Group

Study variables	Group A (OPMD's) N=25 (%)	Group B (Healthy individuals) N=10 (%)	P- value
Age			
30-39	-	2(20%)	0.04
40-50	5(20%)	2(20%)	
51-60	6(24%)	3(30%)	
>60	14(56%)	3(30%)	
Gender			
Male	11(44%)	6(60%)	0.55
Female	14(56%)	4(40%)	

Table 5: MMP–9 Immunoreactivity in Tissue Samples of OPMD and Normal Oral Mucosa

MMP–9 Immunoreactivity	Normal oral Mucosa	OPML	P=value
Positive	-	10(40%)	0.0001
Negative	10	15(60%)	
Total	10	25(100%)	

Table 3: acute on chronic inflammation

Clinico-pathological Features	N	%
Clinical diagnosis of OPMD's		
Oral Leukoplakia	8	32%
Oral Erythroplakia	2	8%
Oral Lichen planus	10	40%
Speckled leukoplakia	5	20%
Site of OPMDS		
Lip (External and inner aspects of lip, commissure of lip)	2	8%
Tongue	6	24%
Gum (Upper and lower gum)	2	8%
Palate (Hard and soft palate, uvula)	2	8%
buccal mucosa	12	48%
Histological description of OPMDs		
Squamous cell hyperplasia	11	44%
Mild dysplasia	3	12%
Moderate dysplasia	6	24%
Severe dysplasia	4	16%
CIS	1	4%
Description of subepithelial Inflammatory infiltrate		
Chronic inflammatory infiltrate	6	24%
Acute on chronic inflammatory infiltrate	15	60%
None	4	16%

Table 4: Immunohistochemical Expression and Staining Intensity of MMP-9 among OPMDs cases

Grades of Staining Intensity	MMP–9 Expression					P-value
	Absence of staining or no staining	Grade 1 = >25%	Grade 2= 26-50%	Grade 3= >50%	Total	
No stain	15(60%)	-	-	-	15(60%)	≤0.01
Weak staining	-	-	-	-	-	
Moderate staining	-	3(12%)	-	-	5(20%)	
Intense staining	-	2(8%)	3(12%)		5(20%)	
Total	15(60%)	5(20%)	5(20%)		25(100%)	

Table 6: Description of MMP–9 Immunoreactivity status among OPMDs and healthy individuals with age and gender

OPML cases					
Age	Below 50 years	1(4%)	4(16%)	5(20%)	0.041
	Above 50 years	14(56%)	6(24%)	20(80%)	
	Total	5(60%)	10(40%)	25(100%)	
Gender	Male	5(20%)	6(24%)	11(44%)	0.188
	Female	10(40%)	4(16%)	14(56%)	
Healthy individuals	Below 50 years	6(60%)	-	6(60%)	Not computed by SPSS
	Above 50 years	4(40%)	-	4(40%)	
Gender	Male	6(60%)	-	6(60%)	
	Female	4(40%)	-	4(40%)	

Table 7: MMP–9 Immunoreactivity among cases of OPMDs with dysplastic alterations and grades of Binary system

Study Variables	MMP-9 immunoreactivity		Total	p-value
	Negative	Positive		
Low risk lesion	3(25%)	-	3	0.157
High risk lesion	5(41.6%)	4(33.3%)	9	
Total	8(66.6%)	4(33.3%)	12	

Table 8: MMP-9 Immunoreactivity and clinico-pathological parameters among OPMDs

Clinico-pathological features	MMP-9 immunoreactivity		Statistics	
Site of development of OPMLs	**Negative**	**Positive**	**Total**	**p-value**
Oral Leukoplakia	4(16%)	4(16%)	8(32%)	0.180
Oral Erythroplakia	-	2(8%)	2(8%)	
Lichen planus	6(24%)	4(16%)	10(40%)	
Speckled leukoplakia	2(8%)	3(12%)	5(20%)	
Total	12	13	25(100%)	
Site of OPMLs				
Lip (External and inner aspects of lip, commissure of lip)	2(8%)	-	2(8%)	0.433
Tongue	5(20%)	1(4%)	6(24%)	
Gum (Upper and lower gum)	1(4%)	1(4%)	2(8%)	
Palate (Hard and soft palate, uvula)	1(4)	1(4%)	2(8%)	
Buccal mucosa	6(24%)	6(24%)	12(48%)	
Histological description of OPMDs				
Squamous cell hyperplasia	7(28%)	6(24%)	13(52%)	0.259
Mild dysplasia	2(8%)	1(4%)	3(12%)	
Moderate dysplasia	5(20%)	1(4%)	6(24%)	
Severe dysplasia	2(8%)	2(8%)	4(12%)	
CIS	1(4%)	-	1(4%)	
Description of subepithelial Inflammatory infiltrate				
Chronic inflammatory infiltrate	4(16%)	2(8%)	6(24%)	0.683
Acute on chronic inflammatory infiltrate	8(32%)	7(28%)	15(40%)	
None	3(12%)	1(4%)	4(16%)	

was observed on buccal mucosa. Among the cases of OPMDs MMP-9 expression was most commonly observed in squamous hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia. (Table 8).

DISCUSSION

In the present study, cases of OPMDs had a mean age of 75.5 and a M/F ratio of 1:1.2. This result is consistent with the research by Hadzic et al. regarding the role of gender and age in the development of disease [19]. Contrary to Hosagadde et al observation 's that the young age (21–30 year) group was most frequently affected[20].

Men were more effected than women (2.5:1). In this study, the mean age of people with OPMD was shown to be statistically different from that of healthy oral mucosa (67.5) (p 0.05). (Table 1). The majority of OPMD cases, or those who appeared before the age of 50, were observed in study by Aroquiadasse et al and Balsaraf et al[21,22].

In our study, the majority of OPMD cases presented in the over-50 age range (Table 2). In terms of site distribution, buccal mucosa (48%) were the most often affected areas by OPMDs. Our study's findings (Table 3) are consistent with those of Kumar et al., Hadzic et al and Siriwardena et al[19,23].This result is in contrast to the study by Mello et al., who found that the gingiva (59%) and tongue (41%) were more frequently affected[24].

In this study squamous cell hyperplasia (48%) was the most common epithelial precancerous lesion among the cases of OPMD (mild, moderate, severe dysplasia). Contrary to Chandolia et al. findings,'s which showed that epithelial dysplasia found high from mild to severe (55%)[25].

Clinically, oral lichen planus (40%) and oral leukoplakia (32%) was observed in the majority of OPMD cases, while 20% of individuals had speckled leukoplakia and 8% had oral erythroplakia (Table 3).

The study conducted by Kumar et al. found that lichen planus (21.33%) and leukoplakia (49.33%) were the most prevalent subtypes, followed by oral submucous fibrosis (49.33%)[26]. According to the study by Mello et al., leukoplakia (88%) was the most common diagnosis, followed by erythroleukoplakia (23%) and erythroplakia (10%)[24].

The present study also showed that MMP 9 expression was not observed in 15 (60%) cases while MMP-9 showed expression in 10 cases so there was statistically significant relation in OPMDs cases regarding grades of staining intensity and MMP-9 expression (Table 4).

This study showed a statistically insignificant relation between MMP-9 tissue immunoreactivity and sites of OPMD which is inconsistent to other study reported by Jose et al., they observed statistically significant relation between MMP-9 tissue immunoreactivity and site of OPMD.Also our study showed statistically insignificant relation of MMP-9 Immunoreactivity and binary grading system

In the current study, we compared MMP-9 Immunoreactivity and expression among cases of OPMDs and healthy oral mucosa, also compare the MMP-9 tissue immunoreactivity and binary grading system of OED. The expression of MMP-9 was higher in OPMDs than in normal oral mucosa. Thus the higher expression of MMP-9 in OPMD cases compared to normal oral mucosa, suggesting its potential as a diagnostic marker for the early detection of oral precancerous lesions.

LIMITATIONS

The present study's limitations include the use of

- IHC rather than a molecular approach.
- Low number of cases of normal oral mucosa.
- Convenient sampling method.

CONCLUSION

There were statistically significant variations in mean age between OPMD cases and healthy adults. A statistically significant relationship between the cases' older age groups and healthy individuals was found. There was Statistically insignificant relation between binary grading system and MMP-9 Immunoreactivity. A statistically significant correlation between staining intensity levels and MMP-9 expression was found among OPMD cases. There was no expression of MMP-9 In normal oral mucosa of healthy individuals. MMP-9 expression was observed in OPMDs cases as compare to normal oral mucosaMMP-9 can be use as diagnostic marker in early detection of oral precancerous lesion.

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CONFLICT OF INTEREST
Authors declare no conflict of interest.
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AUTHORS' CONTRIBUTION

The following authors have made substantial contributions to the manuscript as under:

Conception or Design:	FS, AB, A, MM, SMG, AK, ASK
Acquisition, Analysis or Interpretation of Data:	FS, AB, A, MM, SMG, AK, ASK
Manuscript Writing & Approval:	FS, AB, A, MM, SMG, AK, ASK

All the authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.