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DOI: 10.46903/gjms/18.01.2120

ORIGINAL ARTICLE

EXPRESSION OF MATRIX METALLOPROTEINASE-9 IN ORAL SQUAMOUS CELL CARCINOMA AND ORAL PSEUDOEPITHELIOMATOUS HYPERPLASIA

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ABSTRACT

Background: Oral pseudoepitheliomatous hyperplasia (PEH) appears histologically similar to oral squamous cell carcinoma (OSCC) in small oral biopsies, thus posing diagnostic dilemma. The objective of this study was to compare the expression of matrix metalloproteinase-9 (MMP-9) in differential diagnosis of OSSC and oral PEH.

Materials & Methods: This comparative cross-sectional study was conducted in the Department of Histopathology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan from January 2013 to March 2016. It included sixty archival cases, 30 each of OSCC and PEH. Paraffin embedded blocks were prepared, hematoxylin and eosin stained sections taken and immunostained with MMP-9. The expression of MMP-9 was evaluated in OSCC and PEH.

Results: The OSCC group included 16 (53.33%) men and 14 (46.67%) women, whereas PEH group included 18 (60%) men and 12 (40%) women. The mean age of OSCC group was 60.1±17.3 and that of PEH group was 52.7±16.6. In OSCC group, site of lesion was buccal mucosa in 12 (40%), gingiva 10 (33.33%), tongue 7 (23.33%) and floor of mouth 1 (3.34%) case. In PEH group, site of lesion was buccal mucosa in 12 (40%), tongue 11 (36.66%), gingiva 6 (20%) and palate 1 (3.34%) case. The expression of MMP-9 was positive in all the 30 cases of OSCC and negative in all 30 cases of PEH.

Conclusion: Compared to pseudoepitheliomatous hyperplasia (PEH), MMP-9 revealed a higher expression in oral squamous cell carcinoma (OSCC). This finding has become mainstream strategy in distinguishing OSCC from PEH in oral mucosal biopsies in cases difficult to diagnose.

KEY WORDS: Pseudoepitheliomatous Hyperplasia; Squamous Cell Carcinoma; Matrix metalloproteinase-9; Immunohistochemistry; Buccal Mucosa; Gingiva; Tongue; Floor of Mouth; Palate.

Cite as: Awan AM, Naz I, Mahmood MK, Uddin H. Expression of matrix metalloproteinase-9 in oral squamous cell carcinoma and oral pseudoepitheliomatous hyperplasia. Gomal J Med Sci 2020 Jan-Mar; 18 (1): 24-9. https://doi.org/10.46903/gjms/18.01.2120

INTRODUCTION

Pseudoepitheliomatous hyperplasia (PEH) is an unusual benign reactive phenomenon, which occurs in the mucosal epithelium secondary to infections, inflammation or malignant lesions. Clinically and histologically it can be easily confused with other malignancies especially with squamous cell carcinoma.[1] It appears microscopically as a benign epithelium with broadened, elongated rete pegs and epithelial extensions into the stroma. The epithelium on the surface of the PEH demonstrates acanthosis with loss of its normal architecture. There are also few mitotic figures present along with the keratin which may be arranged in the form of pearls in surface epithelium but there is absence of any cellular or nuclear atypia. The stroma demonstrates the presence of inflammatory cells and lacks perineural, vascular or lymphatic invasion.[2] Oral squamous cell carcinoma makes up 90% of all oral malignant tumours having a poor survival rate.[3] It is the second most common malignancy in both genders in Pakistan.[4] In our Asian part of the world, OSCC is caused mainly due to high-risk habits of

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Date Submitted: 23-06-2019
Date Revised: 17-11-2019
Date Accepted: 27-12-2019

tobacco and betel quid chewing and also considered as a major cause of deaths.[5] The development of cancer is a complex and multi-step process which includes degradation of the basement membrane and extracellular matrix, loss of cell adhesiveness, tumour cell motility and formation of new blood vessels.[6-7] Although PEH shows no cytological evidence of malignancy microscopically, still it can be mistaken for an invasive OSCC.[8] In essence it is significantly important to differentiate between the two pathologies as the therapeutic management is entirely different. OSCC being a malignancy involves radical surgery whereas wait and see policy throughout life is essential to keep an eye on behavior for PEH.[2] Accurate diagnosis of these lesions is of paramount importance and immunohistochemical evaluation of biomarkers can be helpful in their differentiation. Keeping this in view, we investigated the role of MMP-9 in differential diagnosis of OSCC and PEH.

MMP-9 is gelatinase B with molecular weight of 92 kDa and is the largest member of matrix metalloproteinases family. It cleaves several extracellular matrix proteins including type IV collagen, gelatin and fibronectin.[9] It also stimulates release of vascular endothelial growth factor from extracellular matrix.[10] Their ability to degrade the basement membrane collagen appears to be very crucial in tumour cell invasion and spread of tumour.[11] Novel biomarkers involved in the development of cancers would be beneficial not only in the early diagnosis of the oral malignant lesions but also helpful in their differential diagnosis.[12] However, the literature on certain biomarkers in differentiating between OSCC and PEH is scant.

The objective of this study was to compare the expression of matrix metalloproteinase-9 (MMP-9) in differential diagnosis of oral squamous cell carcinoma (OSSC) and oral pseudoepitheliomatous hyperplasia (PEH).

MATERIAL AND METHODS

Design, Settings & Duration

This comparative cross-sectional study was conducted from January 2013 to March 2016 in the Department of Histopathology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan. Prior to commencement, the project was approved by the Institutional Ethical Review Committee.

Sampling

It included sixty formalin fixed paraffin embedded blocks of diagnosed cases 30 each of OSCC and PEH retrieved from archives of the AFIP. The blocks were carefully inspected to ensure they have adequate quantities while scanty and autolysed tissues were excluded. Samples were cut into 4-μm sections, stained with hematoxylin and eosin and studied by the same experienced pathologist and graded according to Anneroth's classification.[13]

Conduct of Procedure

PEH was defined histologically as characteristic down-growing, tongue-like proliferation of rete pegs into the underlying connective tissue in an irregular manner and heavily infiltrated by inflammatory cells. Hematoxylin and eosin stained slides of OSCC were reviewed and were graded according to Anneroth's classification. Invasive epithelial cells in OSCC demonstrate cytological atypia, including nuclear pleomorphism, maturational atypia and mitosis. The tissue sections were immunostained with polyclonal antibodies against MMP-9 (1:100, code A0150; Dako, Denmark). Immunostaining was carried out by fixing sections in 10% buffered formalin, casted in wax blocks cut in 4 μm sections and placed on glass slides which were dried overnight at 37 °C. The slides were deparaffinized and sections were mounted on HistoGrip coated slides. They were then washed thrice with distilled water. The Pyrex glass beaker containing 500 ml of 0.01 M citrate buffer was placed on a hot plate and the solution was heated till it boiled. Slides were put in a slide rack and placed in the beaker with boiling solution and kept boiling for 15 minutes. They were then allowed to cool at room temperature for at least 20 minutes. Later these were rinsed with Phosphate Buffered Saline (PBS) and submerged in peroxidase quenching solution and rinsed again with PBS. They were then applied with serum blocking solution followed with primary antibody and incubated for 30-60 minutes at room temperature. These were again rinsed with PBS and applied with secondary antibody, incubated for 10 minutes at room temperature and rinsed with PBS. Enzyme conjugate was applied and incubated for 10 minutes at room temperature and then rinsed with PBS. Chromogen was finally applied, incubated for 5-10 minutes at room temperature and rinsed with PBS.

Scoring criteria

MMP-9-stained slides were evaluated according to cytoplasmic positivity of epithelial cells using light microscope at $\times 4 \times 10$ and $\times 40$. Invasive ductal carcinoma was used as a positive control to ensure homogenous accurate and reproducible staining of tissue sections. Arbitrary counting was done and the sections were scanned at low magnification to identify positively stained areas (staining intensity). A maximum of 10 hotspots were selected in one slide and evaluated at 40x to determine the colour/shade of cytoplasmic particles. These were scored as weak (1- light yellow), moderate (2- yellow brown) and Strong (3- brown). Percentage of MMP-9 positive cells was calculated by dividing the number of stained cells with total number of cells multiplied by 100. The staining pattern of MMP-9 was categorized into four groups; negative as 0, $<10\%$ of positive

stained cells as 1, 10-50% of positive as 2 and slides with more than 50% of positive stained cells as 3. The immunoreactivity of MMP-9 was evaluated by multiplying the staining intensity (I) with staining pattern (P). On the said basis was categorized into two groups and given a minimum-to-maximum score of 0-9. A score from 0-3 was defined as negative while a score of 4 or greater as positive immunoreactivity.[14] (Figure 1, 2 & 3)

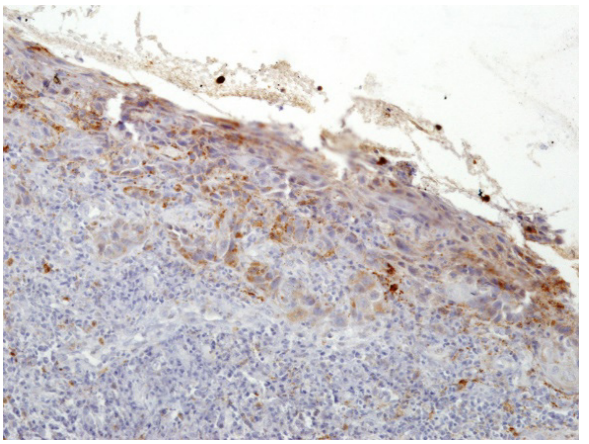

Figure 1: Moderate expression of MMP-9 in oral squamous cell carcinoma (OSCC) (x10)

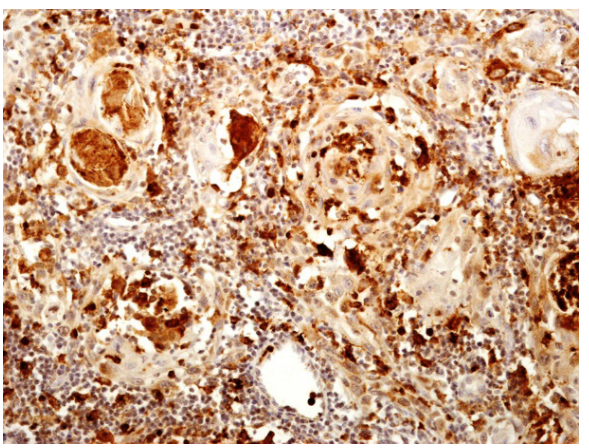

Figure 2: Strong expression of MMP-9 in oral squamous cell carcinoma (OSCC) (x10)

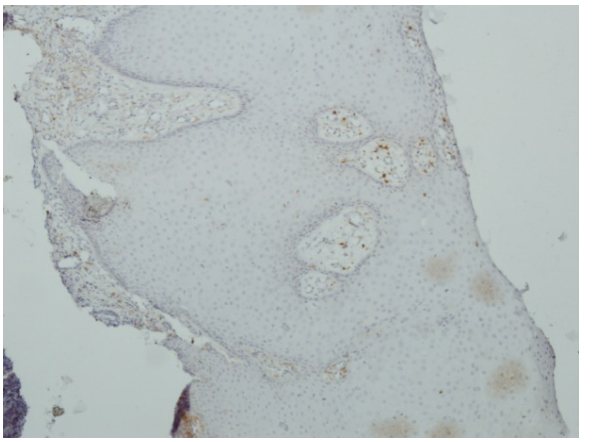

Figure 3: Negative expression of MMP-9 in pseudoepitheliomatous hyperplasia (PEH) (x10)

Data Collection & Analysis Plan

Our study included two matching variables; sex and age in years, and two research variables; site of the lesion and MMP-9 expression as positive or negative. Age was measured on numeric scale (ratio) and was described as mean, SD, minimum, maximum and range. The other variables were nominal and were described by frequency and percentage.

RESULTS

The OSCC group included 16 (53.33%) men and 14 (46.67%) women, whereas PEH group included 18 (60%) men and 12 (40%) women. The mean age of the OSCC group was 60.1±17.3 and that of PEH group was 52.7±16.6.

In OSCC group, the site of lesion was buccal mucosa in 12 (40%), gingiva 10 (33.33%), tongue 7 (23.33%) and floor of mouth 1 (3.34%) case. In PEH group, site of lesion was buccal mucosa in 12 (40%), tongue 11 (36.66%), gingiva 6 (20%) and palate 1 (3.34%) case.

We analyzed the pattern of MMP-9 expression in sixty biopsies, 30 each of OSCC and PEH. MMP-9 was seen in the cytoplasm of the epithelial cells of all the thirty (100%) cases of OSCC, with weak staining intensity in 0 (0%), moderate staining intensity in 24 (80%) and strong expression in 6 (20%) cases. (Table 1)

Table 1: Staining intensity of MMP-9 in oral squamous cell carcinoma (OSCC) and pseudoepitheliomatous hyperplasia (PEH) (n=60)

Type of the Lesion	Staining Intensity					
	Weak (1)		Moderate (2)		Strong (3)	
	Count	%age	Count	%age	Count	%age
OSCC (n1=30)	0	0%	24	80%	6	20%
PEH (n2=30)	28	93.33%	2	6.67%	0	0%

Table 2: Stained area of MMP-9 in in oral squamous cell carcinoma (OSCC) and pseudoepitheliomatous hyperplasia (PEH) (n=60)

Type of the Lesion	Stained Area							
	Negative (0)		<10% of cells (1)		10-50% of cell (2)		>50% of cell (3)	
	Count	%age	Count	%age	Count	%age	Count	%age
OSCC (n1=30)	0	0%	0	0%	21	70%	9	30%
PEH (n2=30)	25	83.33%	4	13.33	1	3.34	0	0%

Table 3: Immunoreactivity of MMP-9 in in oral squamous cell carcinoma (OSCC) and pseudoepitheliomatous hyperplasia (PEH) (n=60)

Type of the Lesion	Score									
	0	1	2	3	4	5	6	7	8	9
OSCC (n1=30)	0	0	0	0	20	0	5	0	0	5
PEH (n2=30)	26	2	2	0	0	0	0	0	0	0

Twenty one cases were scored as two (10-50% of cells) and 9 as three (>50% of cells) as per stained area of MMP-9. (Table 2)

Immunoreactivity to MMP-9 was designated a score of four in 20 (66.66%) cases, score of six in 5 (16.67%) cases and score of nine in 5 (16.67%) cases of OSCC. (Table 3)

All the thirty (100%) cases of PEH showed negative expression for MMP-9, with 28 (93.33%) showed weak, 2 (6.67%) showed moderate and 0 (0%) showed strong staining intensity. (Table 1)

Twenty five (83.33) of these were scored as 0 (negative), 4 (13.33%) as 1 (<10% of cells) and 1 (3.34%) was scored as 2 (10-50% of cells) according to stained areas of MMP-9 in PEH. (Table 2)

Immunoreactivity to MMP-9 was designated a score of 0 in 26 (86.66%) cases, score of one in 2 (6.67%) cases and score of two in 2 (6.67%) cases. (Table 3)

DISCUSSION

The carcinogenesis is marked by a complex interplay between tumor cells and their microenvironment. Matrix metalloproteinases (MMPs) play a chief role in the extracellular matrix remodeling.[15] They are involved in tissue regeneration and are of great importance in virtually any process that involves remodeling; ranging from angiogenesis and cell proliferation to wound healing and degradation of dysfunctional tissue. They are also involved in pathological processes such as inflammation, tumour invasion and metastasis.[16] MMP-9 is an important member of MMP family associated with degradation of type IV collagen; the main component of basement membrane responsible for the tumour invasion and spread.[17]

We observed a significant expression of MMP-9 in the epithelial cells of all 30 cases of OSCC; all were positive with majority (80%) showing moderate and 20% showing strong staining intensity. (Table 1)

A similar study by Majeed and Khalil also reported a prominent expression in all 31 cases of OSCC of their study.[10] Another study by Chandolia, et al. mentioned similar findings; 6% mild, 14% moderate and 80% strong MMP-9 expression in well differentiated OSCC.[17] We suggest that the significant higher expression of MMP-9 observed in the OSCC may be related to intense proliferative activity of this lesion. Similarly another study by Kale, et al. reported immunoreactivity to MMP-9 in all cases of OSCC.[18] Dai, et al. noted a very high expression of MMP-9 in OSCC, mainly in the cytoplasm of the tumour cells.[19] A similar trend was seen by Henriques, et al. in their study in which MMP-9 was expressed in all 35 cases of OSCC.[20] All the studies are in close accordance to our finding.[10,17-20]

All the 30 cases of PEH showed reduced expression for MMP-9 in our study; 28 (93.33%) cases showed weak and 2 (6.67%) cases showed moderate staining intensity. (Table 1)

Immunoreactivity to MMP-9 was designated a score of 0 in 26 (86.66%) cases, score of one in 2 (6.67%) cases and score of two in 2 (6.67%) cases. (Table 3)

A study by You, et al. reported similar finding of MMP-9 expression in 29 cases of PEH.[15] The assessment of positive staining has inherent subjectivity. Based

on single criteria either intensity itself or pattern alone can skew the results. To avoid this bias, we took its cumulative score.

CONCLUSION

Compared to pseudoepitheliomatous hyperplasia (PEH), MMP-9 revealed a higher expression in oral squamous cell carcinoma (OSCC). This finding has become mainstream strategy in distinguishing OSCC from PEH in oral mucosal biopsies in cases difficult to diagnose.

Acknowledgement: We are thankful to the staff of Department of Histopathology, AFIP, Rawalpindi for all their technical assistance provided during the entire research work.

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CONFLICT OF INTEREST
Authors declare no conflict of interest.
GRANT SUPPORT AND FINANCIAL DISCLOSURE
None declared.

AUTHORS' CONTRIBUTION

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

Conception or Design:	AMA, IN
Acquisition, Analysis or Interpretation of Data:	AMA, IN, MKM, HU
Manuscript Writing & Approval:	AMA, IN, MKM, HU

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.