Original Article

Expression of matrix metalloproteinase-9 in histological grades of oral squamous cell carcinoma: An immunohistochemical study

Rekha Patil[1], Aarti Mahajan[1], G L Pradeep[1], Nilima Prakash[1], Swapnil Patil[2], Sabiha Mohiddin Khan[3]

Departments of [1]Oral and Maxillofacial Pathology and [2]Pedodontics and Preventive Dentistry, MGV'S KBH Dental College and Hospital, Nashik, [3]Indian Cancer Society, Mumbai, Maharashtra, India

Abstract

Context: Oral squamous cell carcinoma (OSCC) is characterized by a high degree of local invasiveness and metastasis to cervical lymph nodes and distant sites. Degradation of extracellular matrix (ECM) requires the concerted action of several extracellular enzymes, the most prominent of which are matrix metalloproteinases (MMPs). Proteolytic degradation of ECM components by (MMP-9) facilitates carcinoma cell invasion, enhances angiogenesis and tumor progression.

Objective: To assess and correlate the immunohistochemical expression of MMP-9 with clinicopathological parameters and histological grades of OSCC.

Settings and Design: Thirty histopathologically diagnosed cases of OSCC including 12 cases of well-differentiated squamous cell carcinoma, 12 cases of moderately differentiated squamous cell carcinoma and 6 cases of poorly differentiated squamous cell carcinoma were included in the study group.

Materials and Methods: The samples were subjected to staining using monoclonal antibodies against MMP-9 and visualized using the polymer-HRP detection system. Expression of MMP-9 was assessed in tumor epithelium/parenchyma and connective tissue stroma separately, and the mean of both was considered as average MMP-9 expression.

Statistical Analysis: The parametric independent samples "*t*" test, one-way ANOVA test and Pearson's correlation test were used for the statistical analysis.

Results: Immunoexpression of MMP-9 increased with advancing stage and histological grade of OSCC with statistically significant results.

Conclusion: MMP-9 plays an important role in invasion and metastasis and can serve as an independent prognostic marker.

Keywords: Extracellular matrix, immunohistochemistry, matrix metalloproteinase-9, oral squamous cell carcinoma

Access this article online

Quick Response Code:

Website:
www.jomfp.in

DOI:
10.4103/0973-029X.325121

How to cite this article: Patil R, Mahajan A, Pradeep GL, Prakash N, Patil S, Khan SM. Expression of matrix metalloproteinase-9 in histological grades of oral squamous cell carcinoma: An immunohistochemical study. J Oral Maxillofac Pathol 2021;25:239-46.

INTRODUCTION

Globally, oral cancer is a major health hazard, with an incidence rate of about 5% of all malignant tumors. The global annual incidence rate has been reported as 8.2/100,000 for males and 2.8/100,000 for females.[1,2] More than 90% of all oral cancers are oral squamous cell carcinomas (OSCCs).[3] The highest prevalence and incidence of OSCC is found in the Indian subcontinent, where it ranks among the top three types of cancer in the country.[4]

Cancer incidence and mortality are rapidly growing worldwide. The reasons are complex but reflect changes in the prevalence and distribution of the main risk factors for cancer, several of which are associated with socioeconomic development.[5,6] The 5-year survival rate for patients with head and neck squamous cell carcinoma (HNSCC) is approximately 50% and has not improved significantly over the past five decades, despite advances in treatment techniques and modalities.[7]

Oral cancer is characterized by a high degree of local invasiveness and metastasis to cervical lymph nodes. Metastasis is a complex process that promotes the dissemination of cancer cells from the primary tumor to distant sites. Cervical lymph node metastases (LNMs) is an essential malignancy criterion in oral cancer, and nearly 40% of patients with oral cancer suffered from lymph node metastatic tumors.[8]

Tissue invasion and metastasis require extensive remodeling and degradation of extracellular matrix (ECM) which requires the concerted action of several extracellular enzymes, the most prominent of which are matrix metalloproteinases (MMPs).[9] MMP belongs to a family of zinc-dependent endopeptidases which can degrade several types of collagen in the ECM. Hence, they play an important role in tissue repair and ECM remodeling and turn to promote cancer invasion.[10]

MMP-9 is known as a multifunctional modulator that is involved in very complex cell-signaling cascades. Proteolytic degradation of ECM components (including types III, IV and V collagens, as well as gelatin) by MMP-9 facilitates carcinoma cell invasion and results in the discharge of growth factors such as vascular endothelial growth factor that enhance angiogenesis and tumor progression. At the same time, antiangiogenic endostatin, angiostatin and tumstatin are released. MMP 9 has a fluctuating role in cancer, which not only affects carcinoma cells but also other cell populations. MMP-9 can act as either a carcinoma protector or promoter depending on the specific situation,

which is related to patient characteristics, including the stage, grade, and location of the tumor.[11]

The independent prognostic significance of MMP-9 has been shown in carcinomas of breast,[12] pancreas,[13] bladder,[14] colorectal[15] and HNSCC.[16,17] However, there are no consistent results between MMP-9 expression, disease progression, prognosis or metastasis in OSCC. Therefore, the present study is aimed to evaluate the MMP-9 expression in different clinical stages and histological grades of OSCC.

MATERIALS AND METHODS

The study was conducted in the Department of Oral Pathology and Microbiology at the institute hospital. Thirty histopathologically diagnosed cases of OSCC were included in the study group. Ethical clearance from the institutional ethical committee and informed consent from the patients was obtained for the present study. Demographic data of the cases, habit history, duration and frequency of habit and clinical diagnosis were recorded.

Staging of OSCC was done according to the staging system by the American Joint Committee for Cancer Staging and End Result Reporting.[18] The OSCC cases were graded according to the histologic malignancy grading system given by Bryne *et al*.[19] The clinicopathological parameters of these patients are summarized in Table 1.

Immunohistochemical staining
Formalin-fixed paraffin-embedded tissues were sectioned at

Table 1: Clinicopathological characteristics of oral squamous cell carcinoma patients (*n*=30)

Characteristics	*n* (%)
Gender	
Male	25 (83.3)
Female	5 (16.7)
Age (years)	
≤50	20 (66.6)
51-60	5 (16.6)
61+	5 (16.6)
Primary sites	
Buccal mucosa	10 (33.3)
Tongue	10 (33.3)
Gingivobuccal sulcus	6 (20)
Floor of mouth	3 (10)
Retromolar trigone	1 (3.3)
Clinical stage	
I	5 (16.7)
II	4 (13.3)
III	15 (50)
IV	6 (20)
Histological grade	
Grade I	12 (40)
Grade II	12 (40)
Grade III	6 (20)

4 μm and mounted on aminopropyltriethoxysilane-coated slides. Sections were deparaffinized in xylene and ethanol. Endogenous peroxidase activity was blocked by incubating sections with PBS- Phosphate Buffered Saline (PBS +2% hydrogen peroxide) and then washed with phosphate-buffered saline (PBS). Nonspecific binding was blocked with protein block for 5 min.

Sections were incubated with primary antibody MMP9 antibody (lyophilized mouse monoclonal antibody MMP-9, Clone-15W2, Ig Class-IgG2aκ, Hybridoma Partner Mouse myeloma, Novocastra, Leica Biosystems, United Kingdom) using a Novolink™ Polymer Detection Systems for 2 h in humidifying chamber. The sections were incubated with postprimary block for 30 min. Then, the slides were incubated with the Novolink Polymer antibody for 30 min in the humidifying chamber. The slides were washed thoroughly with PBS between all stages of the procedure.

The antibody reaction was visualized by using fresh substrate/chromogen solution of 3,3-diaminobenzidine (DAB) in the provided buffer (by mixing 25 μl concentrated DAB in 500 μl of substrate buffer) for 10 min. The sections were counterstained with Hematoxylin, dehydrated and mounted using DPX- Dibutyl phthalate Polystyrene Xylene.

Breast cancer tissue was used as a positive control. For the negative control, the primary antibody for MMP-9 was replaced by a solution of bovine serum albumin in PBS solution and each set of staining always included a separate known positive control.

Evaluation of immunoexpression of matrix metalloproteinase-9

For quantitative analysis, MMP-9 positive cells were counted in 10 high-power fields (magnification: ×400) of a light microscope (Olympus CH 20*i*). Expression of MMP-9 was assessed in tumor epithelium/parenchyma and connective tissue stroma separately, and the mean of both was considered as average MMP-9 expression. The slides were analyzed by the observer blinded to clinical data. Expression was analyzed semi-quantitatively and scored according to the method proposed by Franchi *et al*.[20] Scores were interpreted as 0 – no stained cells, 1 – ≤25% stained cells, 2 – >25% and ≤50% stained cells, 3 – >50% and ≤75% stained cells and 4 – >75% stained cells.

Statistical analysis

All statistical analyses were performed using the SPSS software system version 19 (IBM Inc, Chicago, Illinois, USA). Descriptive statistics were used for demographic data and summarized as mean with standard deviation and as a number with percentage for discrete variables.

The parametric independent samples "*t*" test and one-way ANOVA test were applied to evaluate significant differences among the mean values in different groups. Pearson's correlation test was applied to study the correlation between MMP-9 scores with clinicopathological parameters. $P < 0.05$ was considered to indicate statistical significance at 95% of the confidence interval.

RESULTS

On comparison of MMP-9 expression with demographic data, we found that the mean MMP-9 score increased with advancing age. The results were statistically significant ($P = 0.05$). Females exhibited higher mean MMP-9 scores compared to males. The mean MMP-9 score was significantly higher in OSCC of the tongue followed by the floor of the mouth, buccal mucosa and other sites [Table 2]. However, the comparison in the expression of MMP-9 with gender ($P = 0.188$) and site ($P = 0.259$) of OSCC was not found to be statistically significant.

On comparing MMP-9 expression with tumor size, the mean MMP-9 score increased as the tumor size increased ($P = 0.002$). Pairwise intragroup comparison showed a statistically significant difference for T1 versus T2 ($P = 0.003$) and T1 versus T3 group ($P = 0.004$) [Table 3].

We observed the expression of MMP-9 with the nodal status of OSCC and found that the mean MMP-9 score increased with the regional lymph node involvement and was also highly statistically significant ($P = 0.00$) [Table 4].

A comparison of MMP-9 expression with the stage of OSCC showed that the Mean MMP-9 score was higher in advanced stages of OSCCs ($P < 0.05$). Pairwise comparison showed that mean MMP-9 score was significantly lower in Stage I as compared to Stage III ($P = 0.01$) and Stage IV ($P = 0.02$) OSCC and also significantly lower in Stage III compared to Stage IV.($P = .001$) [Graph 1 and Table 5].

On comparison of MMP-9 expression with histological grades of OSCC, we found a higher mean MMP-9 score in poorly differentiated carcinomas squamous

Table 2: Correlation of matrix metalloproteinase-9 expression scores with site

Pairwise comparisons by Mann-Whitney *U*-test			
	P	*P*	*P*
BM versus GBS	0.572	0.149	0.164
BM versus tongue	0.370	0.016*	0.055
GBS versus tongue	0.787	0.587	0.829
BM versus FOM	0.391	0.058	0.026*
BM versus RMT	00.811	0.568	0.830

*$P < 0.05$. BM: Buccal mucosa, GBS: Gingivobuccal sulcus, FOM: Floor of mouth, RMT: Retromolar trigone

Table 3: Comparison of matrix metalloproteinase-9 expression scores with tumor size by Kruskal-Wallis ANOVA

Tumor size	Parenchyma			Stroma			Average MMP 9 expression		
	Mean	SD	Mean rank	Mean	SD	Mean rank	Mean	SD	Mean rank
T1	2	0.894	7.17	0.67	0.516	4.17	1.50	0.837	5.67
T2	3.06	0.659	15.94	2.18	0.636	16.24	2.94	0.659	16.50
T3	3.57	0.535	21.57	2.86	0.378	23.43	3.43	0.535	21.50
Total	2.97	0.850		2.03	0.928		2.77	0.935	
H		10.268			17.605			12.727	
P		0.006			0.000			0.002	
Pairwise comparisons by Mann-Whitney *U*-test									
		P			P			P	
T1 versus T2		0.015*			0.000*			0.003*	
T1 versus T3		0.007*			0.001*			0.004*	
T2 versus T3		0.083			0.052			00.099	

*$P<0.05$. MMP 9: Matrix metalloproteinase-9, SD: Standard deviation

Table 4: Comparison of matrix metalloproteinase-9 expression scores with nodal status by Kruskal-Wallis ANOVA

Nodal status	Parenchyma			Stroma			Average MMP 9 expression		
	Mean	SD	Mean rank	Mean	SD	Mean rank	Mean	SD	Mean rank
N0	2.10	0.738	7.30	1.00	0.667	6.40	1.80	0.789	7.20
N1	3.40	0.507	19.60	2.40	0.507	18.40	3.13	0.516	18.43
N2	3.40	0.548	19.60	3.00	0.000	25.00	3.60	0.548	23.30
Total	2.97	0.850		2.03	0.928		2.77	0.935	
F		15.274			20.283			`16.818	
P		0.000			0.000			0.000	
Pairwise comparisons by Mann-Whitney *U*-test									
		P			P			P	
N0 versus N1		0.000*			0.000*			0.000*	
N0 versus N2		0.008*			0.001*			0.004*	
N1 versus N2		1			0.023			0.095	

*$P<0.05$. MMP 9: Matrix metalloproteinase-9, SD: Standard deviation

Table 5: Comparison of matrix metalloproteinase-9 expression scores with tumor nodes metastasis stages by Kruskal-Wallis ANOVA

Stages	Parenchyma			Stroma			Average MMP 9 expression		
	Mean	SD	Mean rank	Mean	SD	Mean rank	Mean	SD	Mean rank
Stage I	2.00	0.894	7.17	0.67	0.516	4.17	1.50	0.837	5.67
Stage II	2.25	0.500	7.50	1.50	0.577	9.75	2.25	0.500	9.50
Stage III	3.40	0.507	19.60	2.40	0.507	18.40	3.13	0.516	18.43
Stage IV	3.40	0.548	19.60	3.00	0.000	25.00	3.60	0.548	23.30
Total	2.97	0.850		2.03	0.928		2.77	0.935	
H		15.278			21.363			17.347	
P		0.002			0.000			0.001	
Pairwise comparisons by Mann-Whitney *U*-test									
		P			P			P	
I versus II		0.643			0.053			0.110	
I versus III		0.002*			0.000*			0.001*	
I versus IV		0.003*			0.000*			0.002*	
II versus III		10.00			0.023*			0.095	
II versus IV		0.001*			0.002*			0.001*	
III versus IV		0.232			0.143			0.132	

*$P<0.05$. MMP 9: Matrix metalloproteinase-9, SD: Standard deviation

cell carcinoma followed by moderately differentiated squamous cell carcinoma and well-differentiated squamous cell carcinoma [Figures 1-3]. The difference was statistically significant. Comparisons of mean MMP-9 score within grades showed statistically significant difference ($P < 0.05$) between Grade I and II ($P = 0.018$), Grade I and III ($P = 0.001$), Grade II and III ($P = 0.003$) [Graph 2 and Table 6].

DISCUSSION

Oral cancer is one of the most common cancers in the world. An estimated 378,500 new cases of intraoral cancer are diagnosed annually worldwide. In parts of India, oral cancer represents more than 50% of all cancers and is the most common cancer among males and the third most common cancer among females.[21] Indian statistics of

Table 6: Comparison of matrix metalloproteinase-9 expression scores with histological grades by Kruskal-Wallis ANOVA

Stages	Parenchyma			Stroma			Average MMP 9 expression		
	Mean	SD	Mean rank	Mean	SD	Mean rank	Mean	SD	Mean rank
Grade I (WDSCC)	2.83	1.193	15.04	1.58	0.900	11.63	2.08	0.900	9.67
Grade II (MDSCC)	3.00	0.603	15.25	2.17	0.515	16.46	2.92	0.515	16.21
Grade III (PDSCC)	3.17	0.408	16.92	2.67	0.408	21.33	3.83	0.408	25.75
Total	2.97	0.850		2.03	0.935		2.77	0.935	
H		0.232			5.706			15.657	
P		0.054			0.053			0.000	
Pairwise comparisons by Mann-Whitney U test									
		P			P			P	
Grade I versus Grade II		0.903			0.154			0.018*	
Grade I versus Grade III		0.730			0.023*			0.001*	
Grade II versus Grade III		0.552			0.219			0.003*	

*$P<0.05$. MMP 9: Matrix metalloproteinase-9, SD: Standard deviation, SCC: Squamous cell carcinoma, WDSCC: Well-differentiated SCC, MDSCC: Moderately differentiated SCC, PDSCC: Poorly differentiated SCC
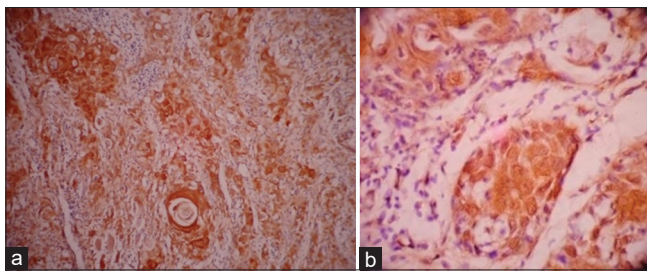

Figure 1: (a) Photomicrograph of well-differentiated squamous cell carcinoma showing matrix metalloproteinase-9 expression under ×100. (b) Photomicrograph of well-differentiated squamous cell carcinoma showing matrix metalloproteinase-9 expression under ×400
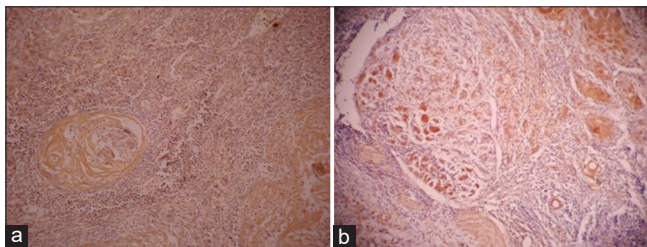

Figure 2: (a) Photomicrograph of moderately differentiated squamous cell carcinoma showing matrix metalloproteinase-9 expression under ×100. (b) Photomicrograph of moderately differentiated squamous cell carcinoma showing matrix metalloproteinase-9 expression under ×400
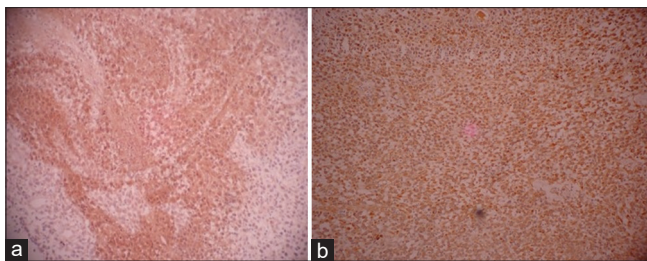

Figure 3: (a) Photomicrograph of poorly differentiated squamous cell carcinoma showing matrix metalloproteinase-9 expression under ×100. (b) Photomicrograph of poorly differentiated squamous cell carcinoma showing matrix metalloproteinase-9 expression under ×400

cancer mortality was estimated to a frequency of 71% in people aged 30–69 years for whom oral cancer was the most prevalent a fatal form of malignancy which accounted for 22·9% deaths.[22]

Cervical lymph node metastasis or distant organ metastasis, while being a potential prognostic indicator, is responsible for the poor survival rates in patients suffering from oral cancer. Epidemiological data indicated that the 5-year survival rates of oral cancer patients were 80%, 70%, 56.9% and 36.8% with Stages I, II, III and IV, respectively.[8]

Tumor metastasis is facilitated by a highly coordinated tandem of increased migratory ability coupled with increased proteolytic activity toward ECM components. Proteolytic degradation of ECM is an essential part of this process and several enzyme systems like serine proteinases, cysteine proteinases and MMPs are involved. The first step in metastasis formation involves the degradation of the underlying basement membrane which mainly consists of type IV collagen. MMP-9 plays an important role in its degradation because of its ability to destroy this type of collagen.[23]

In this study, assessment of MMP-9 expression was done by the semi-quantitative scoring method described by Franchi *et al.*[20] Our study showed that MMP-9 expression was present in all OSCC cases ranging from weak to strong expression. We found that the intensity of MMP-9 staining in the parenchyma was stronger than in the tumor stroma. It is believed that MMP-9 produced by stromal cells potentiates the action of MMPs produced by the parenchyma. This fact supports the view of an interaction between neoplastic cells and the adjacent stroma as demonstrated in some experiments.[24] This strategic interaction permits neoplastic cells to induce stromal cells

to produce proteolytic enzymes that act in synergism with tumor enzymes, opening a tissue space for tumor invasion, migration and metastasis.

On comparison of demographic data with MMP-9 expression, we found a statistically significant difference between patient's age but not in sex and site. Our results are in accordance with the studies done by O-Charoenrat *et al.*,[17] Ruokolainen *et al.*,[25] Dunne *et al.*,[26] Zhou *et al.*[27] and Mäkinen *et al.*[28] On the contrary, Dai *et al.*[29] found higher MMP-9 expression in male OSCC patients than female OSCC patients ($P < 0.05$). Mohtasham *et al.*[30] found a positive correlation between MMP 9 and E-cadherin expression with the primary site of tumors.

In the present study, MMP-9 expression increased as the tumor size (T) increased (from T1 to T3) and was also found to be statistically significant. We found a statistically significant difference between the MMP-9 expression in the presence (N1, N2) and absence (N0) of cervical LNM with the increased intensity of staining in nodal-positive cases compared to node-negative cases [Graph 3]. Our results are in concordance with the studies done by O-Charoenrat *et al.*,[17] Franchi *et al.*,[20] de Vicente *et al.*,[24] Dunne *et al.*,[26] Zhou *et al.*,[27] Kurahara *et al.*,[31] Hong *et al.*,[32] Katayama *et al.*[33] and Ogbureke *et al.*[34] All these studies found a significant correlation of MMP-9 expression with the T stage and regional lymph node involvement. On the contrary, Ruokolainen *et al.*,[25] Ikebe *et al.*,[35] Riedel *et al.*[36] and Guttman *et al.*[37] did not find a correlation between MMP-9 expression and primary tumor size and neck node metastasis.

On the assessment of MMP-9 expression in different clinical stages of OSCC, strong MMP-9 expression was noted in advanced stages of OSCC with statistically significant results. The pairwise intragroup comparison showed MMP-9 expression score was significantly lower in Stage I as compared with Stage III and stage IV OSCC patients. Furthermore, the MMP-9 score was significantly lower in Stage II as compared with Stage IV. Thus, MMP-9 expression adds a predictive power of the outcome of pathological stages. Our results are in concordance with the studies done by O-Charoenrat *et al.*,[17] Dunne *et al.*,[26] Dai *et al.*[29] and Riedel *et al.*[36] who found a statistically significant MMP-9 expression with advanced stages of HNSCC. Riedel *et al.*[36] concluded in their study that MMP-9 may be a useful marker for clinical monitoring of HNSCC patients. On the contrary Ruokolainen *et al.*,[25] Mäkinen *et al.*,[28] Guttman *et al.*[37] and Kato *et al.*[38] did not find a correlation between MMP-9 expression and tumor nodes metastasis staging of OSCC.
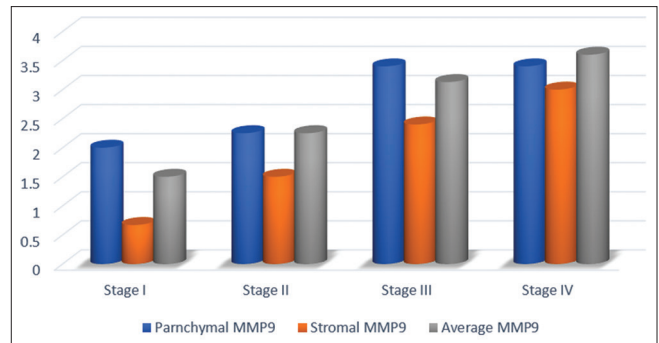
Graph 1: Comparison of mean values of matrix metalloproteinase-9 expression score with tumor-node-metastasis stages
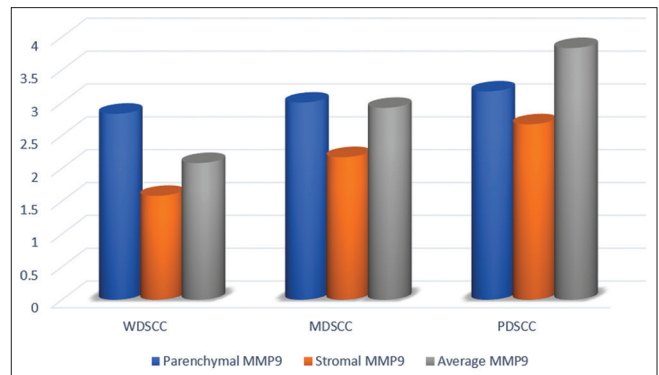
Graph 2: Comparison of mean values of matrix metalloproteinase-9 expression score with Histological grades
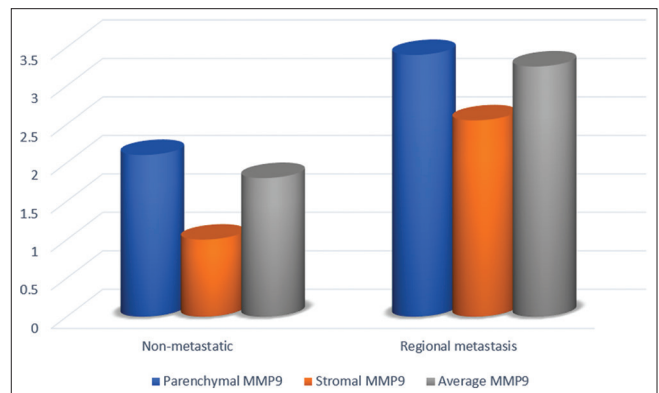
Graph 3: Correlation of mean values of matrix metalloproteinase-9 expression score in regional nonmetastatic (Stage I + II) and regional metastatic groups (Stage III + IV)

On the correlation of MMP-9 expression with histological grades of OSCC, we observed MMP-9 expression gradually increased as the tumor progressed from Grade I to Grade II to Grade III and was also found to be statistically highly significant ($P = 0.00$) [Table 6]. On intragroup assessment, we found a significant difference in MMP-9 expression score between Grade I and II, Grade I and III, Grade II and III.

We observed the expression of MMP-9 largely in tumor cells and also in the adjacent stromal cells and inflammatory cells. It is conceivable that dynamic host-tumor interactions modulate MMPs levels and influence the progression of human tumors and tumor stroma is also a determinant factor for tumor progression.

We found that overexpression of the MMP-9 was strongly associated with nodal metastasis and advanced stages of OSCC, so MMP-9 expression can be considered as a strong prognostic factor for the locoregional spread and clinical behavior of OSCC. MMP-9 overexpression in higher grades of OSCC closely correlated with carcinoma invasion and progression. Thus, MMP-9 may be useful in determining the prognosis of patients with OSCC.

CONCLUSION

Immunohistochemical analysis of MMP-9 in tumor and stromal cells at the tumor invasion front demonstrated an overall high expression of these proteins in all the cases of OSCC studied, suggesting that these molecules play an effective role in the tumor invasion and progression. This observation may be important in determining appropriate strategies to target MMP-9 in cancer which may require the use of inhibitors of its catalytic activity and also the development of new tools to inhibit its protein binding functions. Taken together, these observations suggest the importance of targeting MMP-9 and opens new perspectives for the therapeutic inhibition of protease function in cancer.

Financial support and sponsorship
Nil.

Conflicts of Interest
There is no conflict of interest.

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