Correlation of matrix metalloproteinase-9 expression with morphometric analysis of mucosal vasculature in oral squamous cell carcinoma, oral epithelial dysplasia, and normal oral mucosa

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ABSTRACT

Objective: Oral squamous cell carcinoma (OSCC) invades and metastasizes, by degrading the extracellular matrix (ECM) and is associated with poor prognosis. Matrix-metalloproteinase (MMP-9) can initiate ECM degradation and angiogenesis which brings a significant change in tumor microenvironment favoring tumor progression. A major thrust has been laid on understanding this key enzyme as it has significant implications for cancer therapy. Comprehending the association of vasculature with MMP-9 expression in precancerous lesions oral epithelial dysplasia (OED) and OSCC is essential since the data regarding the same are fewer. The aim is to evaluate and correlate MMP-9 expression with morphometric analysis of mucosal vasculature in the normal oral mucosa (NOM), OED, and OSCC.

Methods: A total of 60 histologically diagnosed cases of OED ($n = 30$); OSCC ($n = 30$); along with 10 NOM ($n = 10$) as control were included. Immunohistochemical staining of MMP-9 and vascular morphometric analysis was performed for all the cases. Results were analyzed using the Chi-square test, Fischer exact test, and Spearman correlation test.

Results: A statistically significant difference in MMP-9 was noted among the groups with $P = 0.011^*$ (epithelium); $P = 0.001^*$ (stroma) by the highest value in OSCC group. Morphometry also revealed a progressive increase from NOM to OED to OSCC. Spearman's correlation of MMP-9 with vascular parameters illustrated a positive relation of MMP-9 with mean vascular density (MVD) and mean vascular area percentage (MVAP).

Conclusion: Positive correlation of MMP-9 with MVD and MVAP demonstrates this markers effect on angiogenesis. Henceforth, MMP-9 can be embattled as a potential therapeutic target in combating tumor progression.

Keywords: Epithelial dysplasia, immunohistochemistry, matrix metalloproteinase, squamous cell carcinoma

Introduction

Cancers of head and neck comprise the heterogeneous group of tumors arising from mucosal lining of the oral cavity, pharynx, larynx, and account for one-sixth of the total cancer cases worldwide.[1] Head and neck cancer incidence varies globally depending on the mode of consumption of tobacco, alcohol habits, altered sexual behaviors, and other associated risk factors. Major hurdle that inhibits successful cancer treatment is the wide locoregional and metastatic spread.[2]

Oral carcinogenesis is a multistep process, which progress through distinct genotypic and phenotypic alterations, seen initially in pre-cancerous and subsequently in cancerous lesion. Recent developments in molecular medicine have led us to perceive that neoplastic cell growth is a result of disruption of normal mechanisms that regulate cellular proliferation, differentiation, and death. It is, therefore, progressively more important to understand the molecular mechanisms for implications of new therapies.[3]

The tumor infiltration and metastasis occur due to the degradation of the basement membrane (BM) between the epithelium, lamina propria, around cancer nests, and surrounding vascular structures. A direct involvement of matrix-metalloproteinases (MMPs) in the development and

progression of many epithelial cancers have been reported.[3] High levels of proteases facilitate degradation of the matrix, thus allowing tumor cells to migrate and metastasize through the vascular and lymphatic systems. Juarez *et al*. speculated that MMP-9 is the key factor in determining the invasive phenotype of at least a subpopulation of oral carcinoma cells.[4] Furthermore, MMP 9 expression has also been correlated with invasion and poor prognosis in oral squamous cell carcinoma (OSCC).

Mounting evidence suggests that angiogenic process commences in the premalignant stages of most cancers. Identification of this stage, where the shift in the balance of factors influencing angiogenesis is important as it helps in developing strategies to prevent the progression of premalignant lesions to malignant tumors.[5] MMP-9 activates angiogenesis by proteolysis of extracellular matrix and activation of proangiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor.[6] Massova *et al*. suggested that understanding the structure and function of this key enzymes has significant implications for cancer therapy.[7]

In the current scenario, improved immunohistochemical techniques and morphometric analysis of histopathological images help in providing the better appreciation of the mucosal alterations related to the progress of cancer.[8] Expression of some MMP's has been studied in the preneoplastic stages of several carcinomas including cervical, colorectal, and gastric premalignancy, while only a few studies have examined MMP-9 expression in oral dysplasia.

Moreover, comprehending the association of mucosal vasculature with the expression of MMP-9 in oral epithelial dysplasia (OED) and SCC is essential since the data regarding the same are lacking. Hence, the present study aims to evaluate and compare the expression of MMP-9 and morphometric analysis of mucosal vasculature in normal oral mucosa (NOM), OED, and OSCC.

Materials and Methods

Study groups

Institutional Ethical Clearance and waiver of informed consent were obtained for this retrospective study. The study material included 70 formalin fixed paraffin embedded tissues blocks consisting of 10 NOM as control (Group I - NOM); 30 OED (Group II - OED); and 30 OSCC group (Group III - OSCC) as the study group. Clinicopathologic data in each case regarding age, gender, site, habit, clinical, and histologic diagnosis were collected from departmental records.

NOM was obtained from gingival and vestibular mucosa after extraction of impacted teeth. 10 cases of these tissues were thoroughly evaluated for any pathological changes, and only

those with minimal inflammation were considered as normal. Cases of OED were graded based on the WHO 2005 grading system and categorized into 10 cases each of mild, moderate, and severe dysplasia.[9] Cases of OSCC were graded based on Border's histological grading criteria and categorized into 10 cases each of well differentiated (WDSCC), moderately differentiated (MDSCC), and poorly differentiated carcinomas (PDSCC)[10]

Immunohistochemistry

Immunostaining with an antibody against MMP-9 (Monoclonal Rabbit Anti-Human MMP-9, BioGenex Lab) using Super Sensitive Polymer DAB detection kit (Biogenex Lab) was performed.

Immunohistochemical analysis

Immunostaining was evaluated for intensity and area of MMP-9 expression based on a Four-Point Scale. Intensity of expression was graded as 0 or (−), no staining, 1 or (+), weak staining (Light brown), 2 or (++), moderate staining (Golden Brown), 3 or (+++), intense intensity (Dark brown). Area of expression was graded based on the percentage of the area of expression as 0: No expression, Grade 1: >25%, Grade 2: 26–50%, and Grade 3: >50%.[11]

Morphometry

Areas showing intense expression of MMP-9 and vasculature were subjected to morphometric analysis using Q win standard (Leica TM DM2500). The images of five representative fields from each section under ×400 magnification were captured and analyzed by outlining the blood vessels [Figure 1]. Morphometry of the blood vessels was evaluated for the parameters such as mean vascular density (MVD), mean vascular area (MVA), MVA perimeter (MVAP), and mean vascular luminal diameter (MVLD).

Statistical analysis

Association between the clinical parameters and immunohistochemical results was analyzed with Chi-square test and Fischer exact test. ANOVA was used for the comparison of morphometric measurements. The correlation between MMP-9 expression and vascularity was assessed using Spearman correlation coefficient. $P \leq 0.05$ considered as statistically significant.

Results

Demographic data

A significant male predominance was observed in our study groups, by 86.7% of OED and 88.3% of OSCC group, respectively. Buccal mucosa showed more predilections among OED cases, and buccal mucosa (50%) and gingival buccal complex (50%) were the prime sites in OSCC. A chewing
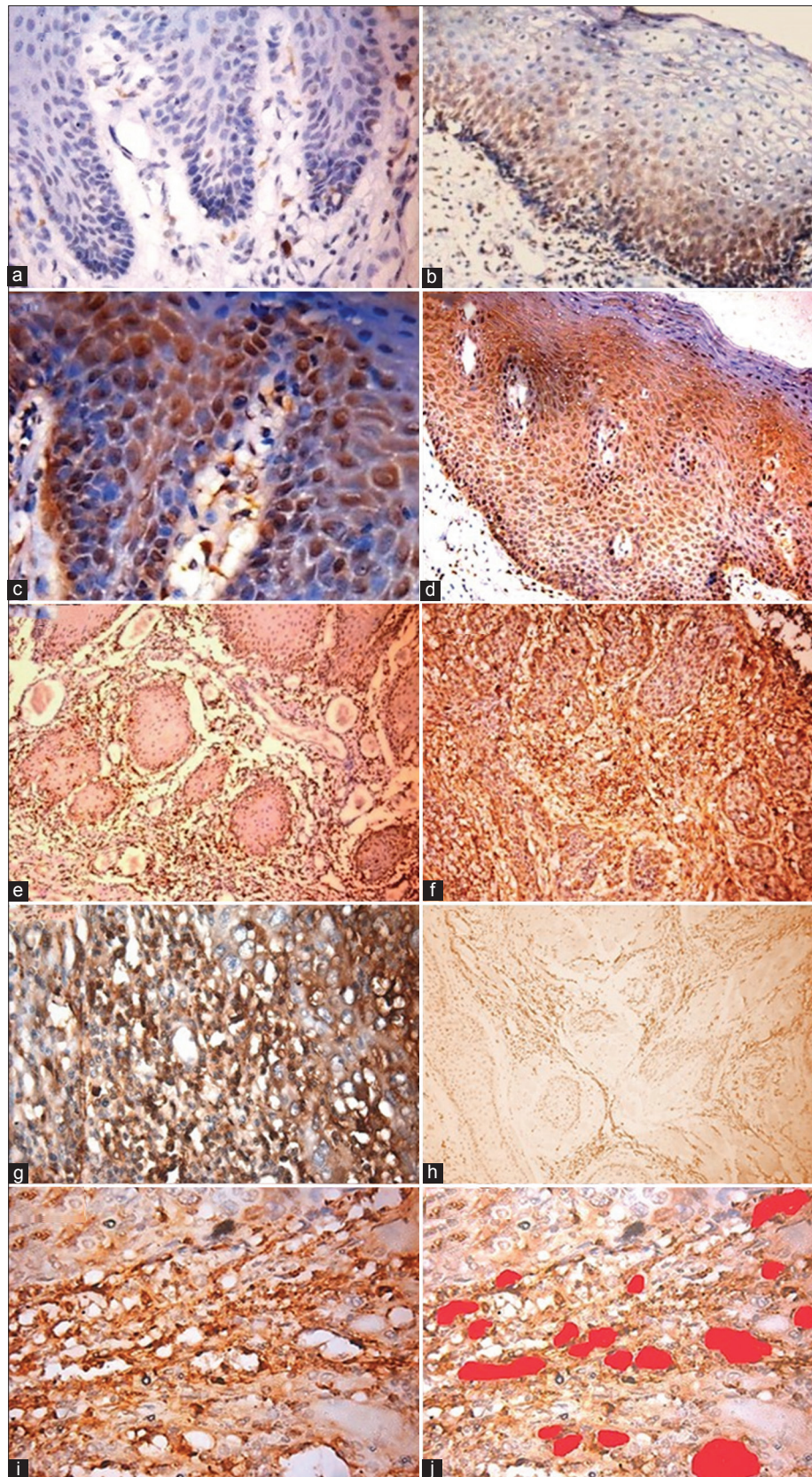
Figure 1: Photomicrograph showing (a) normal oral mucosa (IHC ×400); (b) mild oral epithelial dysplasia (OED) (IHC ×400); (c) moderate OED (IHC ×400); (d) severe OED (IHC ×400); (e) well-differentiated squamous cell carcinoma (IHC ×100); (f) moderately differentiated squamous cell carcinoma (IHC ×100); (g) poorly differentiated squamous cell carcinoma (IHC ×100); (h) matrix-metalloproteinase (MMP) 9 invading muscle; (i) MMP9 and vascularity; and (j) blood vessel outline (IHC ×100)

type of tobacco habit was noted in almost all the cases in our study group. Due to the indistinct distribution of parameters such as male predominance, common site, and type of habit, a correlation was not done between MMP-9 and clinical data.

Immunoexpression of MMP-9

MMP-9 expression was noted as brownish granules in the proliferating epithelial cells and stromal cells such as fibroblasts, endothelial cells, and inflammatory cells.

Group I: NOM (control group)

A weak expression of MMP-9 was noted in the basal layer of epithelium in 60% of cases, and a negative expression was seen in connective tissue [Figure 1a].

Group II: OED [Table 1]

Intensity and area of MMP-9 expression in different strata of the epithelium of OED increasedprogressively with the grades.

Intensity and area of epithelial expression

Intensity of expression
Mild and moderate OED samples predominantly expressed light brown staining (+), whereas 80% of severe OED showed intense staining (++). We also observed that few cases of mild dysplasia did not express MMP-9 (30%). A statistically significant difference in intensity of expression between the grades of dysplasia was found (P = 0.001*) [Table 2].

Area of expression
The extent of staining area was limited to the basal/parabasal layer in mild OED (Grade I - 60% cases), increased progressively to the spinous layer in moderate OED (Grade II - 80% cases), and to the corneal layer in cases of severe OED (Grade III - 60% cases). [Figure 1b-d]. This difference in MMP-9 expressions between the grades of OED was statistically significant (P = 0.001*).

Intensity and area of stromal expression

Very minimal expression of MMP-9 was noted in the stroma of all grades of OED.

Group III: OSCC [Table 3]

MMP-9 showed diffuse expression pattern in tumor cells, stromal fibroblasts, inflammatory cells, endothelial cells, at invasive front and around the tumor islands. [Figure 1e-g]. An enhanced MMP 9 expression was noted in all grades of OSCC.

Intensity and area of epithelial expression

In the tumor cells a predominant expression in the range of golden brown intensity (++) were seen in 50% cases of WDSCC, 60% cases of MDSCC, and 50% cases of PDSCC, and Grade II area of expression, i.e., 25–50% of tumor cells

were seen in WDSCC (60%), MDSCC (60%), and PDSCC (50%) cases.

Intensity and area of stromal expression

In the stroma surrounding the tumor cells, we observed mainly a dark brown intense (+++) MMP-9 expression spreading >50% stromal component. The percentage for intensity and area of expression being almost same between the grades, i.e., WDSCC (30%), MDSCC (60%), and PDSCC (50%) and a statistically significant variation was not present [Table 3].

Comparison of the three groups by Fischer exact test [Table 4]

On comparing the intensity and area of MMP-9 between the groups, we found a significant difference of immunoexpression in tumor cells by P = 0.011* and 0.007*, respectively, with

Table 1: Demographic data of patients included in the study

Group	Male	Female	Total	Age	Predominant site
NOM	60%	40%	10	38.1±13.34 yrs	Gingiva (100%)
OED	86.7%	13.3%	30	44.1±13.42 yrs	BM (90%)
OSCC	83.3%	16.7%	30	53.3±11.98 yrs	GBS (50%)

Table 2: Immunoexpression of MMP-9 in Epithelium & Stroma of OED group

Intensity of MMP-9 in Epithelium (FE, P=0.001+)				Area of MMP-9 in Epithelium (FE P=0.001 +)			
Grade	-	+	++	+++	0	1	2	3
Mild	30%	70%	0	0	30%	60%	0	10%
Moderate	0	60%	40%	0	0	20%	80%	0
Severe	0	10%	80%	10%	0	0	40%	60%
Intensity of MMP-9in Stroma				**Area of MMP-9 in Stroma**			
Grade	-	+	++	+++	0	1	2	3
Mild	100%	0	0	0	100%	0	0	0
Moderate	90%	1	0	0	100%	0	0	0
Severe	100%	0	0	0	100%	0	0	0

Table 3: Immunoexpression of MMP-9 in Tumor cells & Stroma of OSCC group

Intensity MMP-9 in Tumor cells FE P=0.560				Area MMP-9 in Tumor cells FE P=0.531			
Grade	-	+	++	+++	0	1	2	3
WDSCC	0	30%	50%	20%	0	40%	60%	0
MDSCC	0	40%	60%	0	0	30%	60%	10%
PDSCC	0	20%	50%	30%	0	20%	50%	30%
Intensity of MMP-9 in Stroma FE P=0.43				**Area of MMP-9 in Stroma FE P=0.906**			
Grade	-	+	++	+++	0	1	2	3
WDSCC	0	30%	40%	30%	0	20%	40%	40%
MDSCC	0	0	40%	60%	0	10%	30%	60%
PDSCC	0	20%	30%	50%	0	20%	30%	50%

maximum value being in OSCC group. Similarly, on observing the intensity and area of MMP-9 expression in the stroma, a significant difference with $P = 0.001*$ by maximum value in OSCC group [Table 4].

Morphometry

OED [Table 5]
The highest mean vascular parameters were noted in severe dysplasia such as MVD 5.6 ± 3.26 μm, MVA 28979 ± 9630, MVAP 4.8 ± 2.64, and MVLD 178.7 ± 27.39 compared to lower grades of dysplasia. On the computing Fisher exact test, we observed that only MVAP found to have a significant difference between the grades of dysplasia with $P = 0.087*$ [Table 5].

OSCC [Table 6]
On noting down the vascular morphometric measurements in grades of OSCC, highest mean measurements were noted in WDSCC such as MVA of 33507 ± 18254, MVAP being 11.1 ± 2.96, and MVLD was 161.6 ± 33.39, respectively [Table 6]. We observed the highest MVD of 14.7 ± 8.33 in MDSCC cases.

Statistical analysis revealed a progressive increase in MVD from NOM to OED to OSCC. MVAP was significantly high in OSCC as compared to other groups. ANOVA revealed a significant difference in values and highest mean values in OSCC as compared OED and NOM [Table 7].

Correlation of MMP 9 expression with vascular parameters by Spearman's test

On correlating MMP-9 expression and morphometric measurements of the vasculature of the groups individually using Spearman's correlation test, NOM and MMP-9 expression did not show significant relation in any of the vascular parameters. In OED group, intensity and area of epithelial expression displayed significant relation with MVAP and MVD, respectively. In OSCC group, intensity and area of epithelial expression displayed significant relationship with MVAP and MVLD whereas stromal expression with only MVA parameter.

Discussion

Cancer is one of the most common causes of morbidity and mortality today, with >10 million new cases, and >6 million deaths each year worldwide.[12] Demographic data in the present research revealed a significant male predominance in OED and OSCC group. [Table 1] Even in most countries globally, OSCC is more common in men than in women. The reported gender differences are attributable to heavier indulgence in risk habits such as the use of tobacco, alcohol and betel nut use preferably by men.[2,13] It has been suggested that 4–6% of OSCC now occur at ages younger than 40 years.[14] An alarming increase in the incidence of oral cancers among younger people has been reported from many parts of the world, a trend that appears to be continuing. Therefore, screening for a premalignant or early stage of oral cancers is worthy of consideration and public education is imperatively needed.

Mehanna et al. in a systematic review and meta-analysis of oral dysplasia observed an overall transformation rate of 12%.[15] Considering this to be a global statistic, the scenario in a tropical country like India can be expected to be grave

Table 4: Fischer exact test for comparison of MMP- 9 in epithelium & stroma of the study groups

Intensity of MMP-9 in Epithelium (FE, P=0.011+)				Area of MMP-9 (FE P=0.007 +)			
Grade	-	+	++	+++	0	1	2	3
NOM	20%	70%	10%	0	30%	60%	10%	0
OED	10%	46.7%	40%	3.3%	10%	26.7%	40%	23%
SCC	-	30%	53.3%	16.7%	-	9 30%	56.7%	13.3%
Intensity of MMP-9 in Stroma (FE P<0.001+)				Area of MMP-9 in Stroma (FE P<0.001 +)			
Grade	-	+	++	+++	0	1	2	3
NOM	100%	0	0	0	100%	0	0	0
OED	96.7%	3.3%	0	0	100%	0	0	0
OSCC	0	16.7%	36.7%	46.7%	0	16.7%	36.7%	46.7%

Table 5: Morphometric measurements of vasculature in OED

	Mild (μm)	Moderate (μm)	Severe(μm)	F2
MVD	3.6±0.9	4.5±0.65	5.6±3.26	2.444, p=0.106
MVA	26536±9650	21206±6367	28979±9630	2.093, p=0.143
MVAP	3.3±1.96	2.9±9.5	4.8±2.64	2.681, p=0.087*
MVLD	171.1±33.54	153.6±19.26	178.7±27.39	2.206, p=0.130

Table 6: Morphometric measurements of vasculature in OSCC group

	WDSCC	MDSCC	PDSCC	F2,27
MVD	10.5±7.28	14.7±8.33	9.1±4.04	1.857, p=0.176
MVA	33507±18254	19993±7976	24044±8174	3.112, p=0.061
MVAP	11.1±2.96	8.1±3.69	6.1±2.13	7.488, p=0.003+
MVLD	161.6±33.39	136±23.35	159±24.04	2.744, p=0.082

Table 7: Mean and standard deviation of various parameters in NOM, OED & OSCC

Study Group	MVD	MVA	MVAP	MVLD
NOM	3.4±0.76	15385±4499	3.7±0.84	150.4±25.15
OED	4.6±2.08	25574±9009	3.7±2.09	167.8±28.48
OSCC	11.4±6.99	25848±13306	8.4±3.52	152.6±27.21
F value	19.08	3.996	25.688	2.816
P value	<0.001 +	0.023	<0.001 +	0.067

where tobacco with various additives is consumed in a chewed form as opposed to the smoked form. Even in our study majority of the patients had a history of tobacco chewing and site distribution revealed buccal mucosa as the predominant site for OED group and gingivobuccal complex for OSCC [Table 1]. This observation is in concordance with the Prabhu et al. where they have also stressed that gingival and buccal vestibule cancers generally originate in the area of placement of tobacco quid.[16]

According to Ha et al.'s transcriptional progression model for head and neck cancer, the majority of alterations occur before the development of malignancy, i.e., during progression from a normal to a precancerous state.[17] A major thrust has been laid on the determination of molecular events in OSCCs; however, molecular alterations in the oral precancerous lesion are meager.

In our study expression profile of MMP-9 was reliably different from each other in NOM, OED, and OSCC. A weak expression of MMP-9 was noted at the basal layer of NOM, and a statistically significant increase in MMP-9 expression was noted with the grades of OED [Figure 1a-d]. Our result was in accordance with that of Fan et al. where they also detected raised expressions in dysplastic mucosa compared to NOM.[18] Jordan et al. in their polymerase chain reaction (PCR) study as well found higher levels of MMP-9 mRNA in oral dysplasia's that progress to cancer compared with those that did not.[19] The expression of MMP-9 in cases of dysplastic lesions may suggest its association with progression of phenotypic alterations acquired early during the malignant transformation pathway of oral epithelium.

Besides degradation of the BM, cancer expansion requires the induction of angiogenesis in the malignant tissue to provide nourishment for the proliferating cancer cells.[2] In the present study, morphometry shows maximum values of MVD, MVAP, and MVLD in severe dysplasia compared to other grades. Moreover, Spearman's correlation displays a significant association between MMP 9 expression and MVD in OED [Table 4]. Riedel et al. in his research have validated that MMP-9 negative tumors showed a significantly lower mean MVD per microscopic field than MMP-9 positive tumors.

Expression of MMP-9 is upregulated in a stepwise fashion, the first one when a dysplastic lesion evolves and the next one when the dysplasia progresses to invasive carcinoma.[20] As the tumor progresses, stromal cells also secrete MMPs that result in invasion and metastasis; therefore, the role of stromal cells in progression is of equal importance to that of tumor cells.[18] In our study, an enhanced MMP-9 expression was observed in the epithelial cells and stroma in all the grades of OSCC [Figure 1e-g]. However, Fischer exact analysis did not reveal any significant difference. Our result contradicts the finding of Vincent et al. where they found a significant association with an increase in tumor grade differentiation.[2]

The MMPs role is crucial when the tumor is being established, and to control the microenvironment but it is not the only one which plays an indispensable role in the maintenance of the tumor. Franchi et al. documented the existence of a correlation between MMP-9, activity of nitric oxide synthase pathway, p53 status, and angiogenesis in OSCC.[21] As the tumor progresses, there are more signals serving redundant functions, and any one of these becomes less critical for maintaining overall tumor survival.[19] This could be the probable explanation for not finding any difference in MMP-9 expression between the grades of OSCC.

Another interesting finding was the overexpression of MMP-9 around the tumor islands and into the invasion of tissues such as salivary gland, muscle, and fat [Figure h and i]. BM around the cancer nests can restrict tumor invasion and metastasis, whereas fragmented BM may accentuate the same. According to Fan et al., massive dissolution of collagen fibers accelerates the malignant progression of tumors by providing channels for cancer cells to invade the lamina propria.[18]

In our study, individual inflammatory cells were also found to express MMP-9 in OED and OSCC. A literature search revealed that leukocytic infiltration could antagonize tumor formation and growth as they secrete interleukins, chemokines, reactive oxygen species, as well as MMPs that modulate angiogenesis, cell proliferation, tumor growth, and invasion.[22] Inflammatory cells express high levels of pro-angiogenic factors such as MMP-9, VEGF, and COX-2. These mediators generate a pronounced inflammatory regenerative response with exaggerated tissue remodeling and lymphangiogenesis promoting cell motility, cancer cell invasion, and metastasis.[23]

On the morphometric assessment of mucosal vasculature in carcinoma group, maximum value in MVD was noted in MDSCC followed by WDSCC and then in PDSCC. Spearman's correlation of MMP-9 expression with vascular parameters of grades of OSCC revealed a significant association between MDSCC stromal MMP-9 expression with MVD ($P = 0.047*$) and MVLD ($P = 0.038*$). However, PDSCC did not show a statistical relationship with any of the vascular parameters. In a study done by Astekar et al. both VEGF expression and MVD did not show a uniform increase with ascending stages of oral SCC.[24] They explained that the nutrition is necessary for the initial establishment and growth of tumor mass, but

eventually, tumor houses a number of various modulating factors such as oncogenes and tumor suppressor genes.[22] This very well explains our result of morphometric values with the grades of OSCC.

In the present analysis, a progressive increase of vascular parameters from NOM to OED to OSCC is noted. [Table 3] As the tumor grows, increased proliferative activity of tissue results in more vascularity and neoangiogenesis which causes high MVAP and MVD. Moreover, adaptive response to ischemia results in vascular dilatation, which, in turn, causes high MVLD.[25] Pazouki *et al.* showed an increase in vascularization during the transformation from NOM, through dysplasia, carcinoma *in situ,* and infiltrating carcinoma supporting the pivotal role of angiogenesis in carcinogenesis.[24] A positive correlation of MMP-9 with vascular parameters supports the theory that MMP-9 functions as a regulator of tumor angiogenesis supporting endothelial cell invasion. The role of MMP-9 in carcinoma thus appears to facilitate the accessibility of angiogenic molecules to endothelial cells, an activity that represents "seed and soil" interactions.[26]

Conclusion

MMP-9 expression and vascularity have shown a progressive increase from NOM to OED and OSCC cases. The expression of MMP-9 in OED suggests its association with progression of phenotypic alterations acquired early during the malignant transformation pathway of oral epithelium. Our result of the positive correlation between MMP-9 and MVD and MVAP indicates the MMP-9 effect on angiogenesis and its role in tumor genesis. The future scope of this research is to validate the present observations with advanced molecular techniques such as PCR, cell culture, and gelatin zymography. Hence, further research is required to validate the MMP-9 role in angiogenesis and as a predictor of malignant transformation.

Acknowledgment

Conflicts of Interest

None.

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