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## IMMUNOHISTOCHEMICAL EXPRESSION OF MMP9 IN DIFFERENT GRADES OF ORAL SQUAMOUS CELL CARCINOMA: OUR EXPERIENCE AT TERTIARY CARE CENTRE

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MMP9, OSCC,  
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**ABSTRACT: Introduction:** Oral carcinoma is amongst the leading malignancies worldwide, with squamous cell carcinoma (SCC) as the most common type accounting for over 90% of cases. In OSCC, MMPs, (MMP9) which belongs to gelatinase group, promote tumor progression by new blood vessel formation, disturbing tissue morphology which allows tumor growth breaks the basement membrane and enables metastasis and it's overexpression in OSCC have prognostic value. **Aims and Objectives:** To evaluate the expression of MMP 9 in different grades of OSCC. **Material and Methods:** In this prospective study, a total number of 30 blocks specimens; 10 of each well, moderately, poorly differentiated were stained with MMP9 antibodies and compared with normal oral mucosa. All the data were documented and analyzed with SPSS 20 version software. **Results:** Normal 10 tissues with a mean age of 31.30 years and 30 OSCC patients of 51.93 years mean age were studied. In OSCC, 18 male (60%) and 12 female (40%) were participated. Among them, 8% smoking, 15% chewing and 7% both habits were revealed. With the X<sup>2</sup> test, it was revealed that MMP9 in different grades of OSCC was found statistically significant. (P<0.05) in accordance with increasing order of dysplasia. **Conclusion:** Evaluation of MMP9 expression could be a prognostic marker in OSCC and can be used for cancer biotherapeutics. Which may improve the treatment protocol and prognosis of the patient.

**INTRODUCTION:** Oral squamous cell carcinoma (OSCC) is one of the most common malignancies, and it is observed over 90% of cases<sup>1, 2</sup>. In recent advances, tumor microenvironment (TME) had been accepted for the treatment of cancer.

The tumor cells contain both cancer cell and surrounding microenvironment which is considered as TME<sup>3</sup>. Various types of cells like CAF, smooth muscle cells, endothelial cells, neutrophils, lymphocytes, and macrophages consists of the tumor microenvironment.

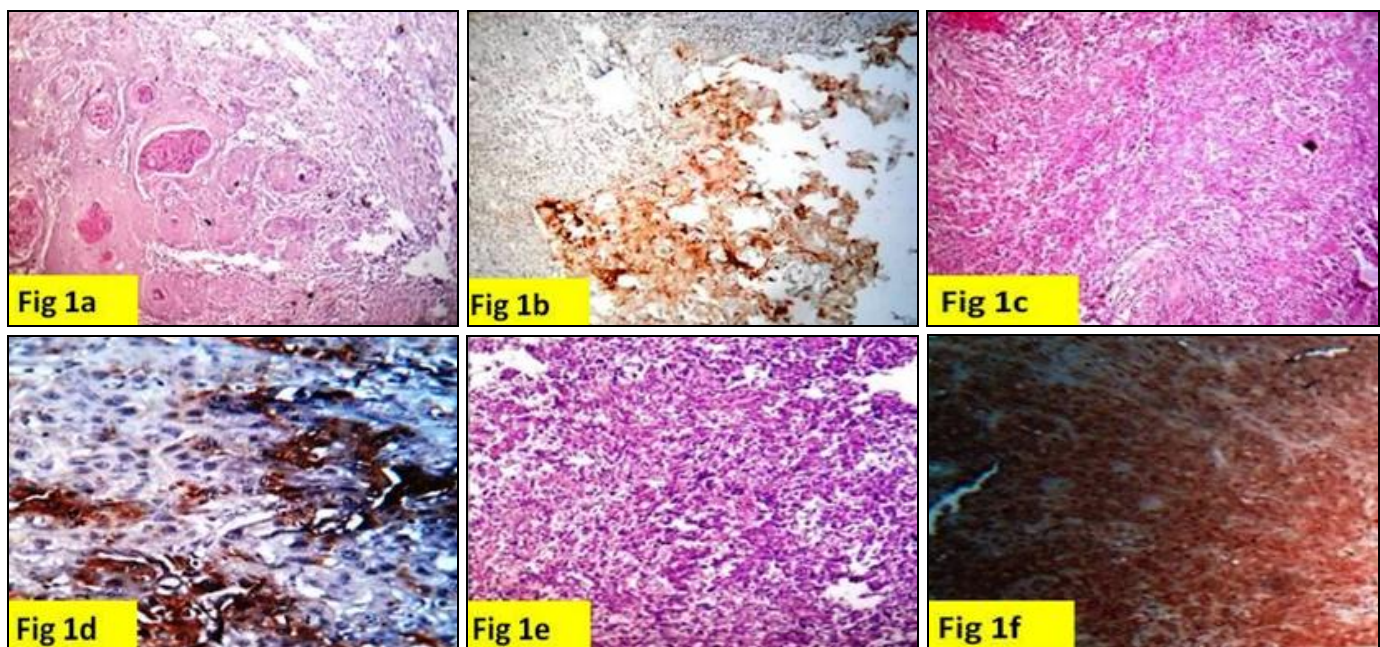
MMPs (matrix metalloproteinase) is one of the main enzymes which are calcium-dependent and zinc containing endopeptidases. These factors modulate cell behavior and enhance cancer progression by regulating angiogenesis, migration, proliferation, and invasion.

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They are responsible for tissue remodeling, degradation of ECM, which includes type IV collagen, gelatine, fibronectin important part of the basement membrane<sup>4</sup>. Till now 24 MMPs are identified, out of which 23 are found in humans. In OSCC, MMPs, especially MMP9 which belongs to gelatinase group, promote tumor progression by new blood vessel formation, disturbing tissue morphology that allows tumor growth which breaks the basement membrane and enables metastasis and its overexpression in OSCC have prognostic value<sup>5</sup>. Therefore, through this study, we attempt to understand the importance of MMP9 in the tumor microenvironment further. By correlating it with

clinicopathologic parameters and evaluating its expression in different grades of OSCC.

**METHODOLOGY:** The present study was done in the Department of Oral & Maxillofacial Pathology & Microbiology, SOA Deemed to be University. The Institutional Ethical Committee of "Siksha O Anusandhan University" has approved to carry out this dissertation work (IMS SH/IEC/2013/193/8). The detail information on each case including age, sex, was collected from departmental archives. The study includes patient of all age group with histo-pathological diagnosed primary carcinoma case.



**FIG. 1: a) SHOWS WELL DIFFERENTIATED SQUAMOUS CELL CARCINOMA IN H & E SECTION 10 X VIEW b) SHOWS MODERATE EXPRESSION OF MMP9 IN WELL DIFFERENTIATED SQUAMOUS CELL CARCINOMA c) SHOWS MODERATELY DIFFERENTIATED SQUAMOUS CELL CARCINOMA IN H & E SECTION 10 X VIEW d) SHOWS MILD EXPRESSION OF MMP9 IN MODERATE DIFFERENTIATED SQUAMOUS CELL CARCINOMA e) SHOWS POORLY DIFFERENTIATED SQUAMOUS CELL CARCINOMA IN H & E SECTION 10 X VIEW f) SHOWS SEVERLY EXPRESSION OF MMP9 IN POORLY DIFFERENTIATED SQUAMOUS CELL CARCINOMA**

Patients are having carcinoma with any secondary infections and immunocompromised diseases, secondarily the tumors which are under treatment of chemotherapy or radiotherapy were excluded from the study. 30 blocks of various histopathological grades *i.e.* 10 well differentiated, 10 moderately differentiated, 10 poorly differentiated and 10 normal tissue blocks were taken as control group. Immunohistochemistry was used for the study to evaluate different grades of OSCC using IHC marker MMP9. Immunohistochemical staining was performed on formalin

fixed paraffin-embedded biopsy specimens that were obtained in the pre-treatment period and preserved in the departmental archives **Fig. 1a-f**. Identical sections were taken on a slide coated with 3-aminopropyl triethoxy silane (Sigma-Aldrich Co., St. Louis, MO, USA), deparaffinized, hydrated through descending grades of alcohol and placed in the tris-EDTA buffer (pH of 9.0) for 5 min in the microwave to retrieve antigenicity<sup>8</sup>. Endogenous peroxidase activity and the non-specific antigen binding sites were blocked using 3% hydrogen peroxide and protein block for 5 min each.

Subsequently, the sections were incubated with primary antibody MMP9 (mouse monoclonal anti-MMP9, clone 15W2, Biogenex, diluted at 1:80) at 37 °C for 1 h in a moist chamber. They were incubated with a post-primary block followed by NovoLink polymer anti-mouse IgG-poly-HRP (8 lg/mL) at room temperature for 30 min each in a moist chamber. The sections were thoroughly rinsed with tris-buffered saline after every step. The reaction was visualized using DAB chromogen, counterstained with Mayer’s hematoxylin and mounted.

Intensity staining for MMP 9 was divided into 3 criteria: <sup>4,6</sup>

- Lower score - 1
- Medium score - 2
- High score - 3

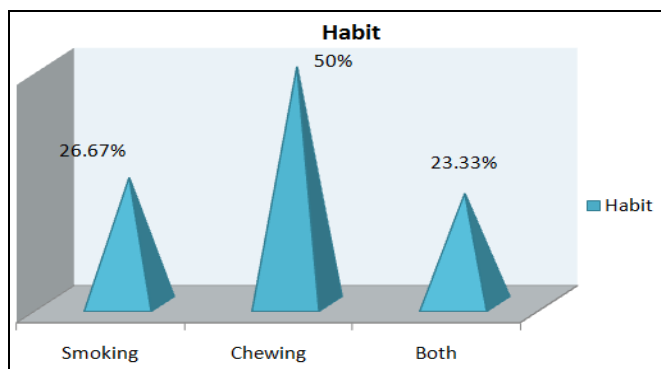
Cell population was considered as:

1. 10- 30%
2. 30-50%
3. >50%

**Counting Method:** We evaluated the slides histopathologically by our both inter/intraobserver and selected the high-density area of infiltration of MMP9 cells in the histopathological section.

**TABLE 3: HABIT WISE DISTRIBUTION IN OSCC GROUP**

		Frequency	Percent	Valid percent	Cumulative percent
Valid	Smoking	8	26.7	26.7	26.7
	Chewing	15	50.0	50.0	76.7
	Both	7	23.3	23.3	100.0
	Total	30	100.0	100.0	



**FIG. 2: SHOWS MAXIMUM NUMBER OF CASES 50% HAD LESION about HABIT**

Graph depicts 26.6% in smoking, 50% of chewing, in both (smoking and chewing) 23.33% but higher percentage shows in chewing habit in OSCC group described in **Fig. 2** in comparison to both the habits

**RESULTS:** A total number of 40 populations were studied using SPSS for analysis. Among them 10 cases from the normal population and 30 cases from OSCC. The mean age from the normal population was 31.30 years whereas in OSCC it was 51.93 years were described in **Table 1**. In the present study gender-wise distribution of patients in the OSCC group was done whereas in male 60% and females 40% their standard deviation is 51.93 ± 9.519 described in **Table 2**. Habit wise distribution in OSCC group was done taking two types of habit smoking and chewing, whereas in smoking 26.7% and chewing 76.7% seen in **Table 3**.

**TABLE 1: AGE WISE DISTRIBUTION OF PATIENTS IN NORMAL AND OSCC GROUP**

		Frequency	Percent	Mean Age
Normal	<60 yrs	9	90	31.30
	>60 yrs	1	10	
	Total	10	100	
OSCC	<60 yrs	24	80.0	51.93
	>60 yrs	6	20.0	
	Total	30	100.0	

**TABLE 2: GENDER WISE DISTRIBUTION OF PATIENTS IN OSCC GROUP**

		Frequency	Percent
OSCC	Male	18	60.0
	Female	12	40.0
	Total	30	100.0

the mean value of smoking habit is 1.88 and mean value of chewing habit is 1.80, including both the habit, mean value is 2.71, whereas the P-value is 0.098 which is statistically not significant described in **Table 3A**.

**TABLE 3A: MEAN AND SD MMP 9 INHABIT**

MMP9	N	Mean ± SD		P-value
Smoking	8	1.88	.991	0.098
Chewing	15	1.80	1.014	
Both	7	2.71	.488	
Total	30	2.03	.964	

Using Chi-square test comparison of expression of MMP9 between normal and OSCC was done and it was found 7 cases are negative in OSCC, 23 cases are positive and their P-value is 0.001 which is statistically significant as described in **Table 4**.



**TABLE 4: COMPARISON OF EXPRESSION OF MMP 9 BETWEEN NORMAL AND OSCC GROUP (POSITIVE AND NEGATIVE CASES)**

			MMP9_rec1		Total	X <sup>2</sup>	P-value
			Negative	Positive			
Type of tumour	Normal	Count	10	0	10		
		% within type of tumour	100.0%	0.0%	100.0%		
	OSCC	Count	7	23	30		
		% within type of tumour	23.3%	76.7%	100.0%	18.039	0.001
Total		Count	17	23	40		
		% within type of tumour	42.5%	57.5%	100.0%		

**TABLE 5: SIGNIFICANT RELATION OF MMP 9 IN DIFFERENT GRADES OF OSCC GROUP**

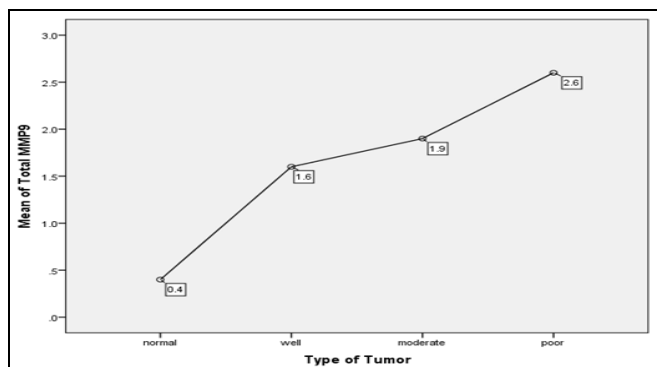
		N	Mean	Std. Deviation	P-value
Total MMP9	Normal	10	.40	.516	0.0001
	Well	10	1.60	1.174	
	Moderate	10	1.90	.738	
	Poor	10	2.60	.699	
	Total	40	1.63	1.125	

MMP 9 in different grades of OSCC group is statistically significant P-value 0.001 as shown in **Table 5**. In the above graph comparing the type of tumor mean value ranges from normal to OSCC cases whereas higher value seen in poor 2.6 depicts in **Fig 3**. By using Bonferroni test we found out the

relation between all groups of OSCC MMP9, it was found statistically significant in relation of normal to poor, but not found statistically significant with normal to well and moderately differentiated squamous cell carcinoma described in **Table 6**.

**TABLE 6: COMPARISON OF MMP9 WITH DIFFERENT GRADES OF OSCC AND NORMAL**

Dependent variable	(I) Type of tumor	(J) Type of tumor	Mean Difference (I-J)	Sig.	
Total MMP9	Bonferroni	Normal	Well	-1.200*	.014
		Moderate	-1.500*	.001	
		Poor	-2.200*	.000	
		Well	Normal	1.200*	.014
		Moderate	-0.300	1.000	
		Poor	-1.000	.058	
	Moderate	Normal	1.500*	.001	
	Poor	Well	.300	1.000	
	Poor	Poor	-.700	.382	
	Poor	Normal	2.200*	.000	
	Poor	Well	1.000	.058	
	Poor	moderate	.700	.382	



**FIG. 3: COMPARISON OF MMP 9 WITH DIFFERENT GRADES OF OSCC & NORMAL**

**DISCUSSION:** Squamous cell carcinoma (SCC) is an emerging problem in the world, due to its extremely high prevalence (90%) among oral cancers<sup>1, 7</sup>. The high rate of relapse in OSCC

indicates the inadequacy of current prognostic predictors in predicting metastatic potential. There are various proteolytic enzymes like MMP2, MMP9, MMP12, MMP14 which has a significant role in determining the prognosis of head and neck cancers<sup>8</sup>. In this study, we have observed the infiltration of MMP9 in different grades of OSCC. Oral squamous cell carcinoma occurs at any intraoral site and generally involve tobacco user in the middle age group. Higher incidence of OSCC seen frequently in females<sup>9, 10, 11</sup>. In our study we found 80% of OSCC cases below 60 yrs of age, 60% were males, and 40% were females; majority cases (53.33%) had a lesion in the buccal mucosa, and MMP9 infiltration was not significantly associated with a different form of tobacco habit

pattern. According to Training Dai *et al.*, MMP9 expression amongst men and women was markedly different, which indicates a high potential for OSCC metastasis in males. Our findings were in concordance with the studies done by Aparna *et al.*,<sup>7</sup> Elahi *et al.*,<sup>9</sup>. But de Vicente *et al.*,<sup>12</sup> Yorioka *et al.*,<sup>13</sup> and Sutinen *et al.*,<sup>14</sup> found a significant correlation with the patient's age, gender, the habit of MMP9 expression.

There are various scoring criteria of MMP9 used by different authors like Maryam Elahi *et al.*,<sup>9</sup> the proportion and intensity scores of the stained cells in the carcinomatous stroma were determined on 10 randomly selected fields (by counting all cells in each field. The proportion scores (PS) of the MMP9 marker comprised 0( $\leq 20\%$ ), 1(21%-50%), 2(51%-75%), and 3( $\geq 76\%$ )<sup>15</sup>. In study done by Aparna *et al.*,<sup>7</sup> were classified into four types which are: very mild- score-1 *i.e.* 0-25%; mild- score-2 *i.e.* 25.01-50%; moderate- score- 3 *i.e.* 50.01-75%; and marked score-4, *i.e.* >75. We have also used the quantitative method for counting the MMP9. For MMP9 expression we have used the total score of intensity and cell population score. Intensity staining; weak-0, lower-1, medium-2, high-3, cell population: <10% is scored 0, 10-30% is scored 1, 30-50% is scored 2, >50% is scored as 3. Total score if more than 3 then staining was considered as an IHC positive.

We have compared MMP9 expression between normal and OSCC cases. There was statistically significant infiltration of MMP9 in OSCC cases as compared to normal group. Total numbers of OSCC cases were divided into a positive and negative group according to the expression of MMP9. We have found MMP 9 expression 23 positive cases and 7 negative cases. We also found that there was increased expression of MMP9 with different histological grades of oral squamous cell carcinoma. MMP9 in Well-differentiated  $1.60 \pm 1.174$ , mod differentiated mean, S.D  $1.90 \pm .738$ , to poorly differentiated  $2.60 \pm .699$  (lower to higher mean and S.D value). This expression is increasing from well to poorly differentiated squamous cell carcinoma.

MMP9 showed over-expression in various malignant tumors of breast, prostate, liver, lung, and bladder with a poor prognosis<sup>16, 17</sup>. There are

very few studies in oral cancers. Our study results were concordance with the studies done by Yao YL *et al.*,<sup>18</sup> and confirmed that relation of MMP9 expression. Therefore, it can be assumed that extensive presence of MMP9 cause breakdown of the basement membrane of the tissue which causes invasion and metastasis of OSCC, which resulted in high metastatic rate and poor prognosis Also There are many other methods (ELISA, PCR) for MMP9 expression which are more specific<sup>19, 20</sup>. IHC has been used to avoid false positive results though it is technique sensitive. Thus, further studies should be done with a larger sample size along with the use of another specific method to confirm the expression MMP9 in OSCC cases, and their expression should be correlated with the IHC technique.

**CONCLUSION:** In recent years many studies have suggested the role of MMP9 expression in oral cancer. In our research we have found that MMP9 expression increases from well to poorly differentiated squamous cell carcinoma which concludes that MMP9 plays an essential key role in different histological grades of OSCC. It plays an essential role in the process of invasion and metastasis in OSCC and upregulates the expression of MMP9.

**Future Prospective:** Evaluation of MMP9 expression could be a prognostic marker in OSCC and can be used for future cancer biotherapeutics. These findings will improve the treatment protocol and prognosis of the patients in the future.

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**CONFLICT OF INTEREST:** Nil

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