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RESEARCH ARTICLE

MMP-9 EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA USING IMMUNOHISTOCHEMISTRY, WESTERN BLOTTING, AND REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION TECHNIQUES

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Abstract

Research into matrix-metalloproteinases (MMP) and their inhibition in certain cancers has moved significantly, studies on MMP expression in oral squamous cell carcinoma (OSCC) remains scarce. MMP-9, a gelatinase-degrading enzyme, destroys matrix proteins in gingivitis and adult periodontitis. MMP-9 genetic (mRNA) and protein profiles were examined to determine MMP-9 expression in oral cancer in this study. Our gender findings indicate that the majority of patients were males and consumers of various tobacco forms. The buccal mucosa was involved in 52% of OSCCs, followed by the tongue (33%), and the gingivobuccal sulcus (9%).The levels of MMP-9 detected in OSCC grades were higher than controls in the order of well-, followed by moderately- and lastly poorly-differentiated grades. Immunohistochemistry seemed to be a reliable approach for measuring MMP-9 expression in OSCC samples when compared to RT-qPCR and western blotting. The association of high MMP-9 levels with well-differentiated OSCC grade implies its potential as a biomarker for early detection of OSCC which could lead to the development of early OSCC treatments. More studies with larger samples might aid in the evaluation of the techniques used.

Introduction: -

One of the top ten most prevalent malignancies and a severe life-threatening condition is oral cancer. GLOBACAN 2020 reports 3,77,713 cases and 1,77,757 deaths worldwide, making it India's second most common cancer[1]. Additionally, 60% of oral cancer cases in India are gingivo-buccal complex tumors (lower alveolus, buccal mucosa, and retro molar trigone)[2]. Despite new technology and therapeutic advances, oral cancers have no meaningful outcome and a 5-year survival rate of 50%[3]. Oral cancer therapy relies on the stage, as improper treatment without scanning or diagnostics can cause patient's death. Prognostic and diagnostic markers may help eradicate cancer with advances in technology and immune-therapeutics[3].

The significance of matrix-metalloproteinases (MMPs) in certain malignancies and their inhibition as a treatment have advanced rapidly, however, oral cancer studies on MMP expression have been rare. MMPs are calcium-

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dependent zinc-containing endopeptidases that degrade extracellular matrix components. MMPs remodel extracellular motion, which is essential to tissue growth and differentiation. One of the MMPs, MMP-9, is a sign of poor prognosis in human carcinogenesis, although its source is unknown. MMP-9 degrades type IV collagen, the major component of BM. It may be linked to BM dysfunction and cancer cell distant metastasis. The destruction of collagen IV and extracellular matrix by MMP-9 facilitates cancer progression, including invasion, metastasis, growth, and angiogenesis[4].Oral dysplasia can develop to malignancy with MMP-9 mRNA overexpression[5].

Given the aforementioned, this study assessedMMP-9 expression in oral squamous cell carcinoma (OSCC) at mRNA and protein levels using quantitative reverse transcription PCR (RT-qPCR) and immunohistochemistry (IHC)/western blotting (WB). Our study sheds light on MMP-9 and its mechanisms in OSCC apoptosis and carcinogenesis, and promotes MMP-9 research as an oral cancer biomarker and therapeutic target.

Materials and Methods: -

Patients and specimens

Sixty oral cancer tissue biopsies were collected from histopathologically diagnosed and well-differentiated (15), moderately differentiated (15), and poorly differentiated (15) OSCC cases and 15 control oral mucosal tissue samples. The samples were collected from patients who had not received radiation or chemotherapy before surgery at the Department of Oral and Maxillofacial Pathology, Divya Jyoti College of Dental Sciences and Research, Modi Nagar, UP, India, and the Department of Dentistry, University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi, India.

IHC and its scoring

IHC was done on histopathologically verified oral cancer paraffin tissue slices. Fixed in 10% formalin and paraffin embedded, the tissue sections (~5µm thick) were microtome-cut and adhered on poly L-lysine slides via heat fixing. Rehydration with different alcohol grades followed deparaffinization with fresh xylene. Antigen retrival was done by boiling tris-buffer (Dako-EnVision). Dark peroxidase block inhibited endogenous peroxidase activity for 10 min. After washing with wash buffer, polyclonal rabbit primary antibody against MMP-9 (Abcam) was incubated at 4°C in humidified chamber at the standardized dilution (1:50). The next day, slides were washed in wash buffer for 5 minutes and incubated with HRP-conjugated secondary antibody for 45 minutes. The sections were washed again with wash buffer, stained with chromogen and diamino benzidine for 2–5 min in dark, rinsed with tap water, counterstained with Mayer's haematoxylin, and mounted in DPX. The slides were seen under a light microscope (Radical, India). Cell Sens took images at various magnifications and quantitated them using IHC Profiler plugin Image J (NIH) USA.

Image J's IHC Profiler plugin was used to measure MMP-9 staining intensity[6]. The photos were taken using a 40X light microscope in full light. At least 10 random photos were picked from each slide. IHC profiler scored staining intensity pixels as high positive (score3), positive (score 2), low positive (score 1), and negative (score 0). The average scores of ten random photos were used to classify MMP-9 expression as no expression (0-0.9), low expression (1+), moderate expression (2+), and high expression (3+) (**Table 1**).

Table 1:- Scoring criterion for immunohistochemistry (IHC) for analyzing MMP-9 expression.

Score	Staining Intensity	Interpretation	MMP-9 Expression (Total 45 patients)
0-0.9	Negative	No expression	0
1+	Low positive	Low expression	13
2+	Positive	Moderate expression	14
3+	High positive	High expression	18

Preparation of protein extracts and WB

Frozen oral tissue cancer samples and controls (50mg dry weight) were homogenized in 1X cold PBS and centrifuged at 4000rpm for 5 minutes. Supernatant was discarded and pellet washed again with 1X PBS. 0.3-0.5 ml RIPA lysis buffer (1M Tris-HCl, 5M NaCl, 0.1M PMSF, 0.5M EDTA, 10% Triton-X-100, 10% Sod.). Deoxycholate, 10% SDS, 0.1mg PI, and dH20 were added to the pellet and iced for 45 minutes, tapping every 10 minutes. The extraction mixture was centrifuged at 14,000 rpm for 20 min at 4°C. The whole cell lysatesupernatant

was transferred to fresh tubes. Qubit 3.0 (Thermo Scientific) estimated protein in the extracts as per manufacturer's instructions. Lysates were stored at -80°C until use.

50µg of whole cell lysatewas loaded per lane in 10% polyacrylamide gel (**Table 2**) and electro-transferred to Immobilon-PSQ membranes using a Bio-rad semi-dry trans-blotter (Millipore Corporation, Bedford, MA). Polyclonal rabbit primary antibody against MMP-9 (ab38898, Abcam, UK) was incubated overnight in 1X PBS with 5% skimmed milk powder, 0.05% Tween 20 (Sigma-Aldrich, CHEMIE GmbH, Germany) at 1:1000. Bands were visualized using a 1:5000 dilution of goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase and a chemilumniscentAmersham ECL™ detection kit (GE Healthcare) on X-rayed blots at various time intervals. Developer and fixer (Carestream, India) created and corrected exposure blots. The membrane was stripped and re-probed with β-actin to ensure equivalent loading and normalization. Densitometric analysis was performed using image J Software (NIH) USA to measure MMP-9 expression in different oral cancer grades.

Table 2:- Preparation of 10% SDS polyacrylamide gel.

Resolving gel 10%		**Stacking gel 5%**	
30% Acrylamide (29:1)	6.65 ml	30% Acrylamide (29:1)	1.7 ml
1.5mM Tris-HCl (pH.8.8)	6.25 ml	1.5mM Tris-HCl (pH.6.8)	1.25 ml
10% SDS	0.25 ml	10% SDS	0.1 ml
10% APS	0.25 ml	10% APS	0.1 ml
TEMED	0.025 ml	TEMED	0.01 ml
Distilled water	6.58 ml	Distilled water	6.85 ml
Total volume	20 ml	Total volume	10 ml

RNA extraction, cDNA synthesis, and RT-qPCR

From oral cancer tissue biopsies and controls, TRIzol® Reagent (Sigma-Aldrich, USA) was used to extract total cellular RNA. Cells were removed from 35 mm plate and rinsed with 1X PBS. Cell pellet was dissolved in trizol and incubated at room temperature for 2–5 minutes. 0.2ml chloroform was added to trizol reagent and carefully stirred for 2-3 minutes. This was followed by 15 minutes of 10,000 rpm centrifugation. Upper aqueous layer was separated in a new tube and 500□l isopropanol was added to precipitate total RNA at -20□C for 30 minutes. After centrifuging at 10,000 rpm for 15 minutes at 4□C, the supernatant was removed and the RNA pellet was found at the bottom. Pellet impurities were cleaned with 80% ethanol and air-dried. The pellet was dissolved in nuclease-free water and kept at -80□C for experiments. Electrophoresis on an ethidium bromide stained 2% agarose gel evaluated RNA quality and purity by detecting at least two bands of 28S and 18S RNA in a 2:1 ratio (**Figure 1**). Qubit 3.0 Fluorometer (Thermo Fisher, USA) quantified total RNA.iScriptTM cDNA Synthesis Kit (BIO-RAD) was used to synthesize cDNA from 2□g of RNA.

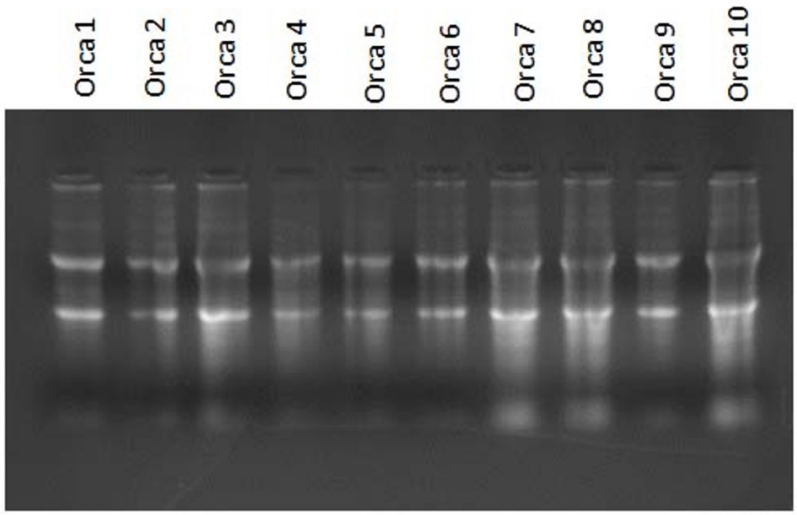

Figure 1:- Gel picture showing the quality of RNA on 2% EtBr-stained agarose-formaldehyde gel.

RT-qPCR was performed on a Bio-Rad CFX-96 using SYBR Green chemistry. Different oligonucleotide primer sequences were utilized to measure MMP-9 expression (**Table 3**). Amplification settings for MMP-9 genes were initial denaturation at 95ºC for 5 minutes, cycling steps of denaturation at 95ºC for 20s, annealing at 60ºC for 20s, and extension at 72ºC for 20s, repeated 40 times. It was followed by melting reaction at 95ºC and 65ºC for 10s and 60s, respectively, and data collection at 95ºC for 1 second. Normalization was done using β-actin as a reference gene, and gene expression change was assessed using $2^{-\Delta\Delta CT}$.

Table 3:- Real time primers used in the study.

MMP-9	5′-CTGGAGACCTGAGAACCAA-3′	5′-ACTGCTCAAAGCCTCCACAAGA-3′
β –actin	5′-GGATGCAGAAGGAGATCACTG-3′	5′-CGATCCACACGGAGTACTTG-3′

Statistical analyses

GraphPad Prism software (version 5) was used for statistical analysis, which included one-way ANOVA with Bonferroni's Multiple Comparison Test. A statistically significant p-value of less than 0.05 was evaluated.

Results: -

Clinical details of OSCC patients

The age range of patient in our study was 25 to 71 years. There were 87% male patients and 13% female patients in our study. We noted that nearly 80% of the patients in poorly-differentiated OSCC grade were males and only 20% were females; in moderately-differentiated OSCC grade nearly 87% were males and 13% were females; and in well-differentiated OSCC grade nearly 93% were males and 7% were females (**Table 4**).

About 52% of OSCCs were involving buccal mucosa, followed by tongue (nearly 33%) and gingivobuccal sulcus (9%) (**Table 5 and 6**).

Table 4:- Distribution of OSCC grades among patient genders.

Grading	Age range (in years)	Female (%age)	Male (%age)
Poorly-differentiated	28 - 71	3 (20%)	12 (80%)
Moderately-differentiated	31 - 70	2 (13.34%)	13 (86.67%)
Well-differentiated	25 - 71	1 (6.67%)	14 (93.34%)
Total	25 - 71	6 (13.33%)	39 (86.67%)

Table 5:- Distribution of cancer location among genders of OSCC patients.

Cancer location	Total (%age)	Female (%age)	Male (%age)
Buccal Mucosa	24 (52.17%)	2 (8.34%)	22 (91.67%)
Gingivobuccal sulcus	4 (8.70%)	0 (0%)	4 (100%)
Lower lip	2 (4.35%)	0 (0%)	2 (100%)
Tongue	15 (32.61%)	4 (26.67%)	11 (73.34%)
Soft palate	1 (2.17%)	0 (0%)	1 (100%)
Total	46* (100%)	6 (13.04%)	40 (86.96%)

*One patient has involvement of both Lower lip and Gingivobuccal sulcus (GBS).

Table 6:- Distribution of cancer location among different OSCC grades.

Cancer location	Poorly-differentiated (%age)	Moderately-differentiated (%age)	Well-differentiated (%age)	Total (%age)
Buccal Mucosa	11 (45.84%)	5 (20.84%)	8 (33.34%)	24 (52.17%)
Gingivobuccal sulcus	0 (0%)	3 (75%)	1 (25%)	4 (8.70%)
Lower lip	0 (0%)	1 (50%)	1 (50%)	2 (4.35%)
Tongue	4 (26.67%)	7 (46.67%)	4 (26.67%)	15 (32.61%)
Soft palate	0 (0%)	0 (0%)	1 (100%)	1 (2.17%)
Total	15 (32.61%)	16 (34.78%)	15 (32.61%)	46* (100%)

*One patient has involvement of both Lower lip and Gingivobuccal sulcus (GBS).

MMP-9 expression profile by IHC

The IHC was performed on the paraffin-embedded tissue sections of histo-pathologically confirmed sections collected previously. IHC results on oral cancer biopsies revealed higher expression, both nuclear and cytoplasmic, of MMP-9 in 40%, moderate expression pattern in 31% and low or nil expression in 29%of oral cancer tissues (**Table 7**). Furthermore, a higher expression in MMP-9 staining was found in well-differentiated in comparison topoorly-differentiated cases (**Figure 2**).

Table 7:- MMP-9 expression in oral cancer biopsies by IHC.

	Higher (n=18)	**Moderate (n=14)**	**Low/nil (n=13)**
Poorly-differentiated (N=15)	2	4	9
Moderately differentiated (N=15)	7	6	2
Well-differentiated (N=15)	9	4	2

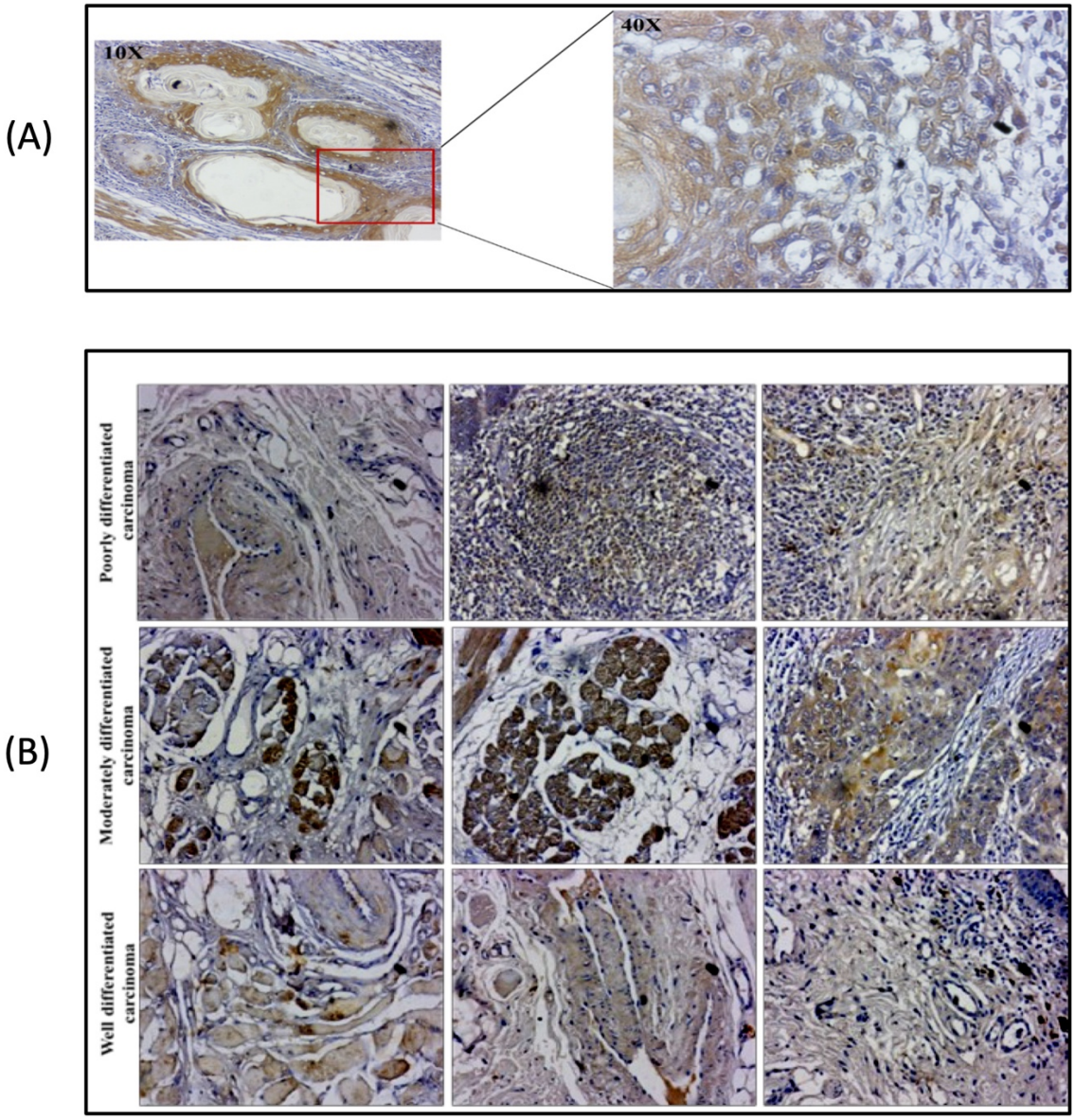

Figure 2:- (A) Immunohistochemistry (IHC) image (10X magnification) on the left showing the selection of a panel viewed under 40X magnifications on the right. **(B)**Immunohistochemistry (IHC) image showing differential MMP-9 expression in oral cancer patients who are poorly-differentiated, moderately differentiated and well-differentiated along with controls. The oral cancer tissue biopsies are at the original magnification of 20X.

Expression profile of MMP-9 by WB

A differential expression of MMP-9 in oral cancer patient samples (**Figure 3A**), with higher expression in well-differentiated when compared to moderate and poorly-differentiated cases of oral cancer was observed (**Figure 3B**).

mRNA expression profiles of MMP-9 by RT-qPCR

MMP-9 mRNA showed higher expression in OSCC tissue samples(**Figure 4A**). Comparison of the expression of MMP-9 mRNA among OSCC tissue samples andnormal tissue revealed statistically significant differences, with ap-value ≤0.05. Further, amongst the three groups, higher expressionof MMP-9 was observed in well-differentiated oral cancer patients (**Figure 4B**).

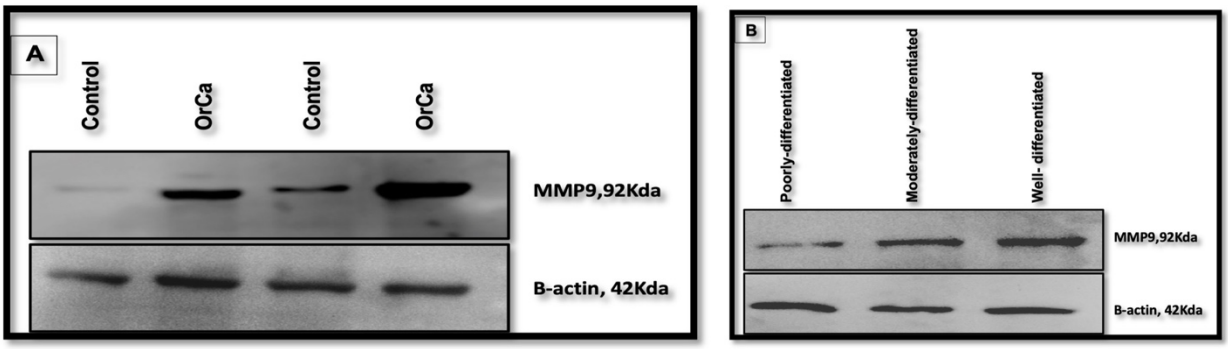

Figure 3:- MMP-9 protein expression by western blotting (WB) analysis. Protein extracts from oral tumor biopsies as well as control samples were separated in 10% SDS-PAGE and detected by specific MMP-9 antibody **(A)** expression control vs oral cancer patients. **(B)** differential expression of MMP-9 expression in control and oral cancer patients - poorly-differentiated, moderately differentiated and well-differentiated. β-actin was used as a housekeeping gene. (*OrCa: Oral cancer patients).

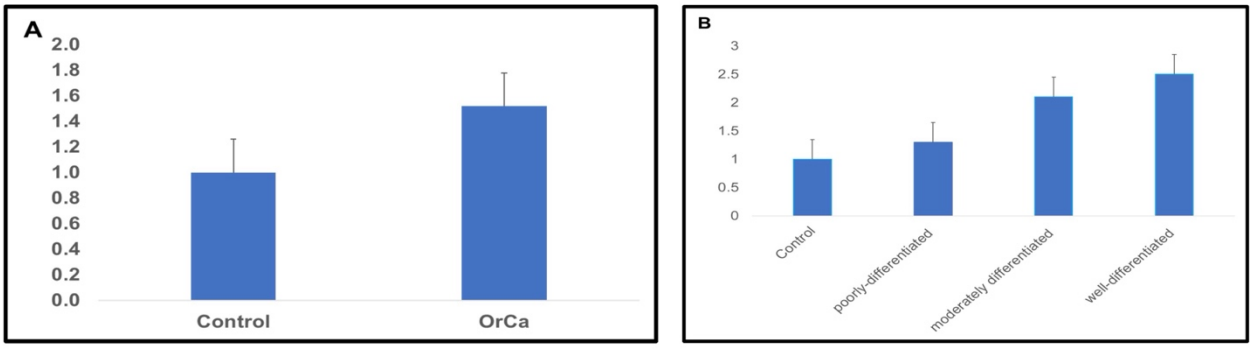

Figure 4:- mRNA expression pattern of MMP-9 in oral cancer. **(A)** MMP-9 expression in control and oral cancer patients, **(B)** differential expression of MMP-9 expression in control and oral cancer patients - poorly-differentiated, moderately differentiated and well- differentiated. β-actin was used as housekeeping gene. (*OrCa: Oral cancer patients).

Discussion: -

Paucity of early detection marker(s) in OSCCimpelled us to examine MMP-9 expression in oral cancer patients in this study. Our study population consisted of 87% male and 13%female patients, with an age range of 25 to 71 years and a mean age of 45.42 years. 40% of OSCC patients were under 40 years of age in our study. Due to riskier lifestyles, OSCC is growing in North Indian youth. According to our gender findings, the majority of patients were male and tobacco consumers, which was similar to other studies from South East Asia[7,8]and international studies[9], suggesting that males in South East Asian countries are at an increased risk of oral cancer.

52% of OSCCs in our study involved buccal mucosa, followed by tongue (33%), and gingivobuccal sulcus (9%). Nearly 46% of poorly-differentiated cases involved buccal mucosa, while 47% of moderately-differentiated cases involved tongue. Most well-differentiated cases in our investigation included buccal mucosa (33%), followed by

tongue (27%). Studies have reported that tobacco and alcohol use made the border of the tongue the most common site for OSCC in America and Europe[10]. Buccal mucosa has been found to be the most prevalent location for OSCC in southeastern Asia because to areca nut- and tobacco-chewing[11]. Brazilian and other occidental studies have found that OSCC most often affects the tongue border and floor of mouth[12]. They thought tobacco, alcohol, and poor oral hygiene contributed to OSCC in their community. Evaluation of mortality data from 20,647 SEER 9 patients found that OSCC was most usually identified in the floor of the mouth and tongue[13]. They also discovered that tongue OSCC had higher cause-specific mortality than other subsites. This contradicts our findings, possibly due to cultural and nutritional differences. Varying subsites of OSCC have changing survival rates. Investigation in Taiwanese population found that hard palate OSCC patients had the worst 5-year survival rate, followed by gingival and floor of mouth OSCC patients, and were most likely to be identified at an advanced stage[14].

Since betel quid (BQ) chewing is a major risk factor for chewer's mucosa, leukoedema, oral leukoplakia, ulcer, and oral carcinogenesis[15], nearly 18% patients were betel nut chewers, suggesting that its components may stimulate oral mucosal cell MMP-9 expression and secretion. BQ chewing may cause chewer's mucosa, leukoedema, ulcer, oral carcinogenesis, and cancer progression by inducing MMP-9 and promoting tumor invasion and metastasis.

IHC results on oral cancer biopsies showed increased nuclear and cytoplasmic MMP-9 expression in 40%, moderate expression in 31%, and low or no expression in 29%. In addition, well-differentiated cases showed increased MMP-9 staining compared to poorly-differentiated instances. A significant difference in MMP-9 expression on IHC was seen between moderately and poorly differentiated oral cancer cases. However, same was not the case between moderate and well-differentiated grades. The IHC grade-wise analysis eliminated spurious findings because two independent observers and the IHC profiler scored similarly. Previous studies have found that IHC increases MMP-9 protein expression in OSCC tissues[16]. All these data imply MMP-9 contributes to oral carcinogenesis and tumor invasion.

MMP-9 expression in dysplastic lesions may indicate phenotypic changes acquired early in oral epithelium's malignant transformation process. Stromal cells generate MMPs that cause invasion and metastasis, hence their involvement in progression is equal to that of tumor cells[17]. We observed MMP-9 expression increase from poorly differentiated to well-differentiated OSCC grades. MMP-9 increases tumor grade differentiation[18], supporting our findings.

Higher MMP-9 expression in well-differentiated oral cancer patients compared to moderate and poorly-differentiated cases was also confirmed by WB. Studies linking MMP-9 expression to OSCC clinical and pathological characteristics are scarce. MMP-9 is involved in cancer formation and metastasis in several forms of cancer[19,20]. Many genetically engineered animal models of cancers[21,22] show that MMP-9 gene deletion delays tumor start or suppresses tumor progression. We found strong MMP-9 expression in most well-differentiated tumors, suggesting its significance in OSCC onset. Early tumor invasion relies on MMP-9 to degrade and modify ECM homeostasis[23]. ECM breakdown is crucial to tumor metastasis. However, inconsistent data has also been reported depicting that MMP-9 was not related with all OSCC clinical and pathological factors[24,25].

Our mRNA expression analysis revealed that OSCC tissue samples express more MMP-9 than normal tissue samples. Several studies have found reduced MMP-9 gene expression in normal oral tissues. Out of 10 cases of normal oral mucosa, 40% mild, 30% moderate, and 30% high MMP-9 expression was found in each subgroup[26]. MMP-9 expression was observed in fewer than 10% of normal epithelial cells[27]. Weak positive cytoplasmic MMP-9 expression in normal tongue mucosa epitheliumappeared as brownish granules in growing epithelial cells[28]. Normal epithelium did not express MMP-9according to another study[29].Elevated MMP-9 expression in OSCC has been linked to advanced disease[30]. Similar investigations have found that MMP-9 makes OSCCaggressive[31]. OSCC tissue samples had higher MMP-9 gene expression than controls by RT-PCR[32].

Our study found highly enhanced MMP-9 expression in well-differentiated OSCC patients compared to controls, suggesting its potential as an early OSCC diagnostic adjunct. Our findings suggest that MMP-9 expression contributes to carcinogenesis, depending on patient features such as tumor grade (well-, moderate-, or poorly-differentiated) and location. Different techniques and the low sample size likely explain the substantial variation in OSCC samples in this investigation. Thus, more research with bigger, well-documented clinical materials and sensitive, specific technologies like IHC are needed to assess MMP-9's impact on OSCC.

Conclusion: -

MMP-9 expression was higher in OSCC patients than in controls. Association of high MMP-9 with well-differentiated OSCC gradesuggests its use as a biomarker for early identification which in turn may lead to development of early OSCC therapy. Additional research with bigger sample sizes would assist in assessing the significance of our study.

Conflict of interest: -

The authors declare no conflict of interest.

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References: -

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209-249.
2. Byakodi R, Byakodi S, Hiremath S, Byakodi J, Adaki S, Marathe K, et al. Oral cancer in India: an epidemiologic and clinical review. J Community Health. 2012;37(2):316-319.
3. Kim DW, Lee S, Kwon S, Nam W, Cha IH, Kim HJ. Deep learning-based survival prediction of oral cancer patients. Sci Rep. 2019;9(1):6994.
4. Groblewska M, Siewko M, Mroczko B, Szmitkowski M. The role of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in the development of esophageal cancer. Folia Histochem Cytobiol. 2012;50(1):12-19.
5. Rosenthal EL, Matrisian LM. Matrix metalloproteases in head and neck cancer. Head Neck. 2006;28(7):639-648.
6. Varghese F, Bukhari AB, Malhotra R, De A. IHC Profiler: an open-source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. PLoS One. 2014;9(5):e96801.
7. Sameera A, Tahera A, Navid R, Abbas Z, VazirB, Qureshi NR. Clinicopathological profile of squamous cell carcinoma presenting in Tertiary Care Hospital, Karachi. Prof Med J. 2020;27(5):939-943.
8. Yasin MM, Abbas Z, Hafeez A. Correlation of histopathological patterns of OSCC patients with tumor site and habits. BMC Oral Health. 2022;22(1):305.
9. Schmidt Jensen J, Jakobsen KK, Mirian C, Christensen JT, Schneider K, Nahavandipour A, et al. The Copenhagen Oral Cavity Squamous Cell Carcinoma database: protocol and report on establishing a comprehensive oral cavity cancer database. Clin Epidemiol. 2019;11:733–741.
10. Marocchio LS, Lima J, Sperandio FF, Corrêa L, Sousa SO. Oral squamous cell carcinoma: an analysis of 1564 cases showing advances in early detection. J Oral Sci. 2010;52:267–273.
11. Johnson NW, Jayasekara P, Amarasinghe AA. Squamous cell carcinoma and precursor lesions of the oral cavity: epidemiology and etiology. Periodontol 2000. 2011;57:19–37.
12. Grimm M. Prognostic value of clinicopathological parameters and outcome in 484 patients with oral squamous cell carcinoma: microvascular invasion (V+) is an independent prognostic factor for OSCC. Clin Transl Oncol. 2012;14:870–880.
13. Farhood Z, Simpson M, Ward GM, Walker RJ, Osazuwa-Peters N. Does anatomic subsite influence oral cavity cancer mortality? A SEER database analysis. Laryngoscope. 2019;129:1400–1406.
14. Su WW, Su CW, Chang DC, Chuang SL, Chen SL, Hsu CY, et al. Impact of varying anatomic sites on advanced stage and survival of oral cancer: 9-year prospective cohort of 27 717 cases. Head Neck. 2019;41(5):1475-1483.
15. Jeng JH, Chang MC, Hahn LJ. Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. Oral Oncol. 2001; 37:477–492.
16. Tsai CH, Hsieh YS, Yang SF, Chou MY, Chang YC. Matrix metalloproteinase 2 and matrix metalloproteinase 9 expression in human oral squamous cell carcinoma and the effect of protein kinase C inhibitors: preliminary observations. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2003; 95:710–716.
17. Peschos D, Damala C, Stefanou D, Tsanou E, Assimakopoulos D, Vougiouklakis T, et al. Expression of matrix metalloproteinase-9 (gelatinase B) in benign, premalignant and malignant laryngeal lesions. Histol Histopathol. 2006;21:603–608.

18. Vincent-Chong VK, Salahshourifar I, Karen-Ng LP, Siow MY, Kallarakkal TG, Ramanathan A, et al. Overexpression of MMP13 is associated with clinical outcomes and poor prognosis in oral squamous cell carcinoma. Scientific World J. 2014;2014:897523.
19. Ok Atılgan A, Özdemir BH, Yılmaz Akçay E, Tepeoğlu M, Börcek P, Dirim A. Association between focal adhesion kinase and matrix metalloproteinase-9 expression in prostate adenocarcinoma and their influence on the progression of prostatic adenocarcinoma. Ann Diagn Pathol. 2020;45:151480.
20. Yang XZ, Cui SZ, Zeng LS, Cheng TT, Li XX, Chi J, et al. Overexpression of Rab1B and MMP9 predicts poor survival and good response to chemotherapy in patients with colorectal cancer. Aging (Albany NY). 2017;9(3):914-931.
21. George AK, Homme RP, Majumder A, Tyagi SC, Singh M. Effect of *MMP-9* gene knockout on retinal vascular form and function. Physiol Genomics. 2019;51(12):613-622.
22. Martin MD, Carter KJ, Jean-Philippe SR, Chang M, Mobashery S, Thiolloy S, et al. Effect of ablation or inhibition of stromal matrix metalloproteinase-9 on lung metastasis in a breast cancer model is dependent on genetic background. Cancer Res. 2008;68(15):6251-6259.
23. Daniele A, Abbate I, Oakley C, Casamassima P, Savino E, Casamassima A, et al. Clinical and prognostic role of matrix metalloproteinase-2, -9 and their inhibitors in breast cancer and liver diseases: A review. Int J Biochem Cell Biol. 2016;77:91–101.
24. de Vicente JC, Fresno MF, Villalain L, Vega JA, Hernandez Vallejo G. Expression and clinical significance of matrix metalloproteinase-2 and matrix metalloproteinase-9 in oral squa- mous cell carcinoma. Oral Oncol. 2005;41:283-293.
25. Tao DT, Gao ZL, Zhou JP, He LP, Shi LX, Fang Y, et al. Zinc dependent endopeptidases of matrix metalloproteinases-9 expressions is associated with tumor metastases of oral squamous cell carcinoma in Chinese popula- tion: a meta-analysis. Int J Clin Exp Med. 2014;7:1531-1536.
26. Chandolia B, Basu SK, Kumar M. Can MMP-9 be a Prognosticator Marker for Oral Squamous Cell Carcinoma? J Clin Diagn Res. 2016;10(1):ZC09-13.
27. Zhou CX, Gao Y, Johnson NW, Gao J. Immunoexpression of matrix metalloproteinase-2 and matrix metalloproteinase-9 in the metastasis of squamous cell carcinoma of the human tongue. Aust Dent J. 2010;55(4):385–389.
28. Fan HX, Li HX, Chen D, Gao ZX, Zheng JH. Changes in the expression of MMP-2, MMP-9 and ColIV in stromal cells in oral squamous tongue carcinoma: relationships and prognostic implications. J Exp Clin Cancer Res. 2012;31:1–8.
29. Tamamura R, Nagatsuka H, Siar CH, Katase N, Naito I, Sado Y, et al. Comparative analaysis of basal lamina type IV collagen α chains, matrix metalloproteinases-2 and -9 expressions in oral dysplasia and invasive carcinoma. Acta Histochemica. 2013;115(2):113–119.
30. Kato K, Hara A, Kuno T, Kitaori N, Huilan Z, Mori H, et al. Matrix metalloproteinases 2 and 9 in oral squamous cell carcinomas: Manifestation and localization of their activity. J Cancer Res Clin Oncol. 2005;131:340–346.
31. Yorioka CW, Coletta RD, Alves F, Nishimoto IN, Kowalski LP, Graner E. Matrix metalloproteinase-2 and -9 activities correlate with the disease-free survival of oral squamous cell carcinoma patients. Int J Oncol. 2002;20:189–194.
32. Umashankar K, Jayaraj G, Sherlin HJ, Don KR, Santhanam A. Evaluation of matrix metalloproteinase 9 gene expression in oral squamous cell carcinoma and its clinicopathological correlation. Natl J Maxillofac Surg. 2021;12:339-343.