Original Article

Expression of Cytokeratin 8/18 and Matrix Metalloproteinase-9 in Oral Squamous Cell Carcinoma Using Multiplex Immunohistochemistry Technique

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Abstract

Introduction: The emphasis in the field of molecular pathology is now being laid upon identifying multiple biomarkers in the tumor microenvironment involved in the carcinogenesis process of oral squamous cell carcinoma (OSCC). The embryonic phenotype of epithelium expressing cytokeratin (CK) 8/18 having increased proliferation and cell motility, coupled with the destruction of extracellular matrix caused by matrix metalloproteinase (MMP)-9, would favor the process of carcinogenesis. In this context, our study includes an assessment of the correlation of CK 8/18 and MMP-9 in OSCC tissues by utilizing the multiplex immunohistochemistry (mIHC) technique. **Materials and Methods:** The present prospective *ex vivo* study comprised 22 OSCC samples and 22 tissues from apparently healthy controls. The OSCC tissues were graded based on the degree of differentiation. The expression of CK 8/18 and MMP-9 with respect to their intensity and localization was studied using mIHC. **Results:** CK 8/18 immunostaining was positive in 77.3% (n = 17) OSCC cases, while MMP-9 was positive in 95.5% (n = 21) cases. A statistically significant difference was seen for the values between the groups ($P < 0.01$) for all the parameters – CK 8/18 intensity, CK 8/18 staining area, MMP-9 intensity, and MMP-9 staining area with higher values in OSCC as compared to control tissue. A statistically significant difference was seen for the values between the groups ($P < 0.01$) for CK 8/18 staining area with higher values in moderately and poorly differentiated OSCC. **Conclusion:** Increased expression of CK 8/18 and MMP-9 can correlate to higher OSCC grades. CK 8/18 is absent in normal tissues and thus can be utilized as an effective indicator of OSCC. The mIHC technique enabled this simultaneous topographic analysis of both the markers in an efficient and economically feasible manner.

Keywords: Cytokeratins, matrix metalloproteinase, tumor microenvironment

Introduction

Oral squamous cell carcinoma (OSCC) is the predominant type of head-and-neck tumor, attracting significant research attention in the field of oncology for its diagnosis and treatment.[1] Despite considerable advancements, the overall survival rate for OSCC patients remains a matter of concern. Timely detection of microscopic alterations during the initial phases of the disease can substantially enhance patient survival rates. Previous studies have indicated that the 5-year survival rate for Stage I OSCC is approximately 80%, whereas it drops to 20% for Stage IV cases.[2] Hence, early diagnosis of OSCC is imperative to improve patient prognosis.

The tumor microenvironment (TME) of OSCC is complex and exhibits a range of stromal modifications responsible for the lesion's aggressive characteristics, including infiltration and metastasis.[3] Molecular

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pathology now places significant emphasis on identifying multiple biomarkers within the TME that play roles in carcinogenesis. Simultaneous detection of several biomarkers on a single tissue section can enhance the visualization of the TME, consequently improving the diagnostic and prognostic capabilities of pathologists.

Squamous epithelial cells, which constitute the infiltrating cell population in OSCCs, predominantly contain cytokeratins (CKs) in their cytoskeleton.[4] Recent attention has been focused on the significance of various CKs in cancer diagnosis and prognosis. Among the 23 identified CK subtypes, the expression of CK 8/18 distinguishes adult mucosal epithelium from fetal mucosal epithelium. In adults, these CKs are only expressed in simple epithelia, while in the fetus, they are ubiquitously present in mucosal epithelium. However, their expression in adult cancerous mucosal tissues may suggest a transition toward an embryonic cell phenotype.[5]

Microinfiltration resulting from basement membrane (BM) breaches is another critical prognostic factor in OSCC. Endopeptidase enzymes, including matrix metalloproteinases (MMPs), can degrade connective tissue stroma and the BM. MMPs such as MMP-2, 9, 12, 13, and 14 have been associated with head-and-neck cancers.[6] MMP-9, a member of the gelatinase group, promotes tumor cell invasion by breaking down the BM and creating a microenvironment conducive to micrometastasis. Overexpression of MMP-9 has been observed in various malignant tumors, including breast, prostate, liver, lung, and bladder cancers, albeit limited studies have been conducted on oral cancer.[7]

The embryonic phenotype characterized by the expression of CK 8/18 is associated with inherent qualities such as increased proliferation and cell motility. When combined with the extracellular matrix (ECM) destruction caused by MMP-9 in the TME, such an epithelium would support the process of carcinogenesis. Their heightened expression in seemingly normal oral mucosa has also been predictive of field cancerization changes.[8] To our knowledge, no prior studies have concurrently assessed CK 8/18 and MMP-9 expression in OSCC using multiplex immunohistochemistry (mIHC). In this study, we investigate the correlation between CK 8/18 and MMP-9 in OSCC by employing the mIHC technique.

Materials and Methods

The present prospective *ex vivo* study was conducted over the course of 3 years in the period of May 2019–May 2023. This study complies with the Declaration of Helsinki and the protocol was approved by the Institutional Ethical Review Board (Reference Number: GDCH/SS/EC/6693; Dated 16/09/2019). A total sample size of 21 patients per group was determined adequate to complete the present study. Patients with clinical presentation suspicious of primary OSCC (n = 22) were included in the study, and an incisional biopsy was obtained from the lesional area. Cases of recurrent OSCC were excluded from the study, as the focus of the present study was to identify biomarkers in primary OSCC. The chemotherapy or radiotherapy provided to such patients could alter the level of biomarkers. Patients in which biopsy was contraindicated, such as those with uncontrolled diabetes or cardiovascular disorders, were also excluded from the study.

Normal oral mucosal tissues (n = 22), obtained from patients opting for routine minor surgical procedures such as crown-lengthening procedures or surgical extraction of teeth in the institution, were used as controls. It was ensured that controls did not have tobacco-related habits or systemic diseases and had a similar age distribution pattern as the individuals in the study group.

Eligible patients were then explained the purpose of the study, involved procedures, and possible complications, following which informed consent was obtained. After recording the demographic and clinical details of the lesion, an incisional biopsy was performed.

Histopathological procedures

The biopsy specimens were fixed in 10% neutral-buffered formalin for 24 h, after which they were subjected to routine histopathological processing and embedded in paraffin wax. Sections of 4 μm thickness were then obtained from the paraffin-embedded tissue blocks using a semi-automated rotary microtome (Leica RM 2145, Leica Biosystems, Germany). The slides were stained with hematoxylin and eosin and then microscopically observed for histopathological confirmation of OSCC. Once confirmed, the OSCC was then graded according to the grading system recommended by the WHO, which is based on Broder's grading system.[9]

Multiplex immunohistochemistry

The sections were standardized by maintaining the thickness at 4 μm and obtained on silane-coated slides. The slides were fixed overnight at 60°C in the incubator. The sections were cleared in xylene, followed by rehydration to water through decreasing grades of alcohol. To block the endogenous peroxidase activity, the sections were covered with peroxidase block for 10 min, followed by washing with buffer twice.

Antigen retrieval was carried out in Tris-EDTA buffer using a microwave-based antigen retriever system (BioGenex EZ-Retriever® System). The slides were subjected to a temperature of 90°C for 10 min followed by 97°C for 5 min. The slides were incubated for 1 h in a cocktail solution comprising CK 8/18 rabbit monoclonal (Clone NCH-38, M3612, DAKO; Agilent Technologies, Inc., Santa Clara, CA, USA) and MMP-9 mouse

monoclonal antibody (Clone 8.1.1 BioLegend, Inc., San Diego, CA, USA) at room temperature in a humidifying chamber.

The slides were then incubated with detection reagent for 20 min (Anti Rabbit polymer AP and Anti-mouse Super Sensitive™ polymer-HRP immunohistochemistry (IHC) detection system). The diaminobenzidine and red chromogen substrates were then applied for 20 min followed by washing in deionized water. Mayer's hematoxylin was used for subsequent nuclear counterstaining for 1 min, followed by rinsing in water, dehydration in increasing grades of alcohol, clearing in xylene, and mounting with Dibutylphthalate polystyrene xylene.

Normal duodenal tissue was used as positive control, while brain tissue was used for negative control. Tris-buffered saline instead of an antibody cocktail was used as negative batch immunostaining control.

Immunohistochemical evaluation

The expression of CK 8/18 and MMP-9 was determined independently. The grading system was adopted from a similar previous study carried out by Kale *et al*.[8] Each tissue section was scored according to the staining intensity and staining area [Table 1].

A total score of ≥3 was regarded as positive for each marker. Five high-power fields having the most abundant expression of markers were selected, and an average of all the fields was taken as the final score. Grading was performed by two pathologists; any discrepancy was resolved by mutual agreement.

Results

Out of the total 44 subjects in the study, 59.1% (n = 26) were males and 40.9% (n = 18) were females. The overall male:female ratio was 1.44:1; however, the ratio was much higher in the OSCC patients (3.4:1) wherein 77.3% were males. The patient's ages ranged from 22 to 71 years, with a mean of 49.20 years (standard deviation [SD] = ±11.22). Most of the OSCC patients (n = 17) belonged to the age group of 40–70 years.

All the OSCC patients (n = 22, 100%) had ≥5 years of tobacco and/or betel-quid chewing habits. The duration of habit ranged from 6 to 30 years, with a mean of 14.36 years (SD = ±6.63). On grading the H and E-stained slides according to the WHO grading system, 54.5% were graded as well-differentiated OSCC (n = 12), 27.3% were graded as moderately differentiated OSCC (MDOSCC) (n = 6), and 18.2% were graded as poorly differentiated OSCC (PDOSCC) (n = 4). None of the cases were of anaplastic OSCC.

CK immunostaining was absent in all the control tissues (n = 22), whereas MMP-9 was found to be positive in 27.27% (n = 6) of control tissues [Figure 1]. In the study group, CK 8/18 immunostaining was found to be positive in 77.3% (n = 17) and MMP-9 in 95.5% (n = 21) OSCC tissues. An increase in the intensity of immunoexpression of CK 8/18 was noted across WDOSSCC, MDOSCC and PDOSCC respectively [Figure 2].

On analysis by ANOVA test, a statistically highly significant difference was found between the groups (P < 0.01) for all the IHC parameters – CK 8/18 and MMP-9, with staining intensity and area, being higher in the OSCC tissues as compared to the controls [Table 2]. Furthermore, a statistically highly significant difference was observed for the values between the grades of OSCC (P < 0.01) for CK 8/18 staining area with higher values in PDOSCC and MDOSCC [Figure 3]. Assessment of the correlation between CK 8/18 and MMP-9 by Spearman's rho value revealed that there was a statistically nonsignificant correlation between MMP and CK (P > 0.05).

Table 1: Histopathological grading system for hematoxylin and eosin and scoring system used for evaluation of immunohistochemical expression

Grading of OSCC	Evaluative scoring of immunohistochemical markers			
	CK 8/18		MMP-9	
	Staining intensity	Staining area	Staining intensity	Staining area
WDOSCC → <25% of undifferentiated cells MDOSCC → 25%–50% of undifferentiated cells PDOSCC → 50%–75% of undifferentiated cells Anaplastic/pleomorphic OSCC → >75% of undifferentiated cells	0 - No staining 1 - Light pink 2 - Pink to light red 3 - Dark red	0 - No staining 1 - Positive staining in less than one-third of the epithelial cells 2 - Positive staining in one-third to two-third of the epithelial cells 3 - Positive staining in more than two-third of epithelial cells	0 - No staining 1 - Light yellow 2 - Yellow to brown 3 - Dark brown	0 - No staining 1 - Positive staining for less than one-third of connective tissue stroma 2 - Positive staining area ranged from one-third to two-third of connective tissue stroma 3 - Positive staining for more than two-third of connective tissue stroma

CK: Cytokeratin, MMP: Matrix metalloproteinase, OSCC: Oral squamous cell carcinoma, WDOSCC: Well differentiated OSCC, MDOSCC: Moderately differentiated OSCC, PDOSCC: Poorly differentiated OSCC

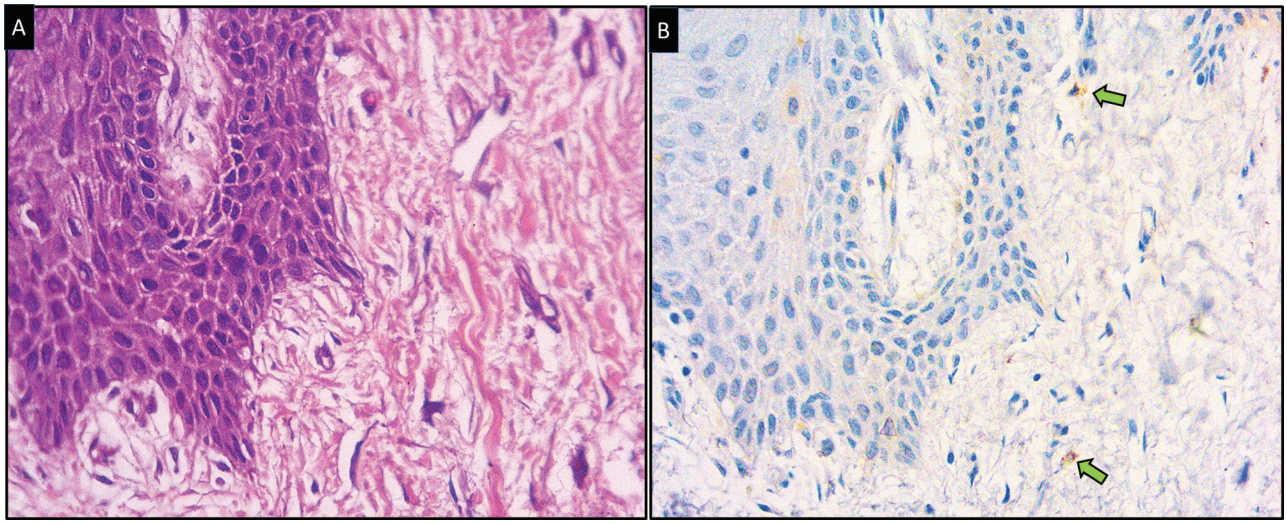

Figure 1: Normal gingival tissue (a) H and E, ×400, (b) Negative expression of cytokeratin 8/18 and weak expression of matrix metalloproteinase-9 (green arrows)

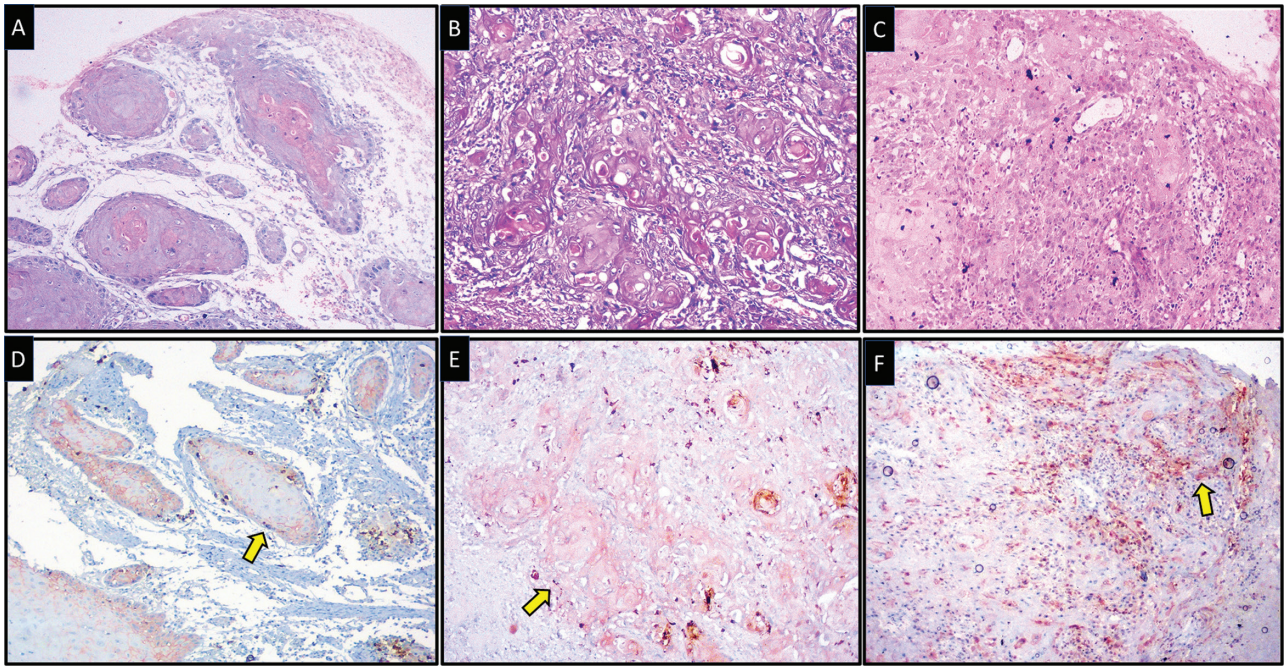

Figure 2: H and E-stained sections of (a) Well-differentiated oral squamous cell carcinoma (OSCC), (b) Moderately differentiated OSCC, (c) Poorly differentiated OSCC with corresponding multiplex immunohistochemistry showing an increase in the intensity of immunoexpression of cytokeratin 8/18 from light red to dark red (yellow arrows) through d-f (Original magnification x100)

Discussion

The conventional IHC technique can identify only a single biomarker, although the pathogenesis of OSCC involves numerous biomarkers.[3] This study aimed to investigate the changes occurring during the carcinogenesis process and the spatial relationship between CK 8/18 and MMP-9. Therefore, the simultaneous visualization of both markers within a single field was achieved using mIHC.

CK 8/18 is typically observed in fetal buccal mucosal and lingual epithelium for up to 27 weeks postgestation. In adults, these markers are associated with simple epithelia, normal glandular epithelia, transitional epithelium, and hepatocytes but not with squamous stratified epithelium.[8,10] Thus, the absence of CK 8/18 in stratified squamous epithelial cells from normal adult oral mucosal tissues explains its negative expression in controls. The presence of CK 8/18 in pathological oral mucosal epithelium indicates dedifferentiation or an embryonic cell phenotype.

In our study, CK 8/18 immunoexpression was positive in 77.3% of OSCC cases, which was similar to that reported

Table 2: Comparison of immunostaining parameters between grades of oral squamous cell carcinoma by one-way ANOVA test

Immunostaining parameter	*n*	Mean	SD	SE	95% CI for mean		Minimum	Maximum	*F*	*P*
					Lower bound	Upper bound				
CK 8/18 intensity										
Poorly differentiated	4	2.25	0.957	0.479	0.73	3.77	1	3	1.617	0.225#
Moderately differentiated	6	1.67	0.816	0.333	0.81	2.52	1	3		
Well differentiated	12	1.25	1.055	0.305	0.58	1.92	0	3		
CK 8/18 staining area										
Poorly differentiated	4	2.50	0.577	0.289	1.58	3.42	2	3	7.751	0.003**
Moderately differentiated	6	2.50	0.548	0.224	1.93	3.07	2	3		
Well differentiated	12	1.08	0.996	0.288	0.45	1.72	0	3		
MMP-9 intensity										
Poorly differentiated	4	2.50	0.577	0.289	1.58	3.42	2	3	1.234	0.313#
Moderately differentiated	6	2.17	0.408	0.167	1.74	2.60	2	3		
Well differentiated	12	2.00	0.603	0.174	1.62	2.38	1	3		
MMP-9 staining area										
Poorly differentiated	4	1.50	0.577	0.289	0.58	2.42	1	2	0.864	0.438#
Moderately differentiated	6	1.33	0.516	0.211	0.79	1.88	1	2		
Well differentiated	12	1.17	0.389	0.112	0.92	1.41	1	2		

CK: Cytokeratin, MMP: Matrix metalloproteinase, SD: Standard deviation, SE: Standard error, CI: Confidence interval

*statistically significant difference (p<0.05); **statistically highly significant difference (p<0.01); #non-significant difference (p>0.05)

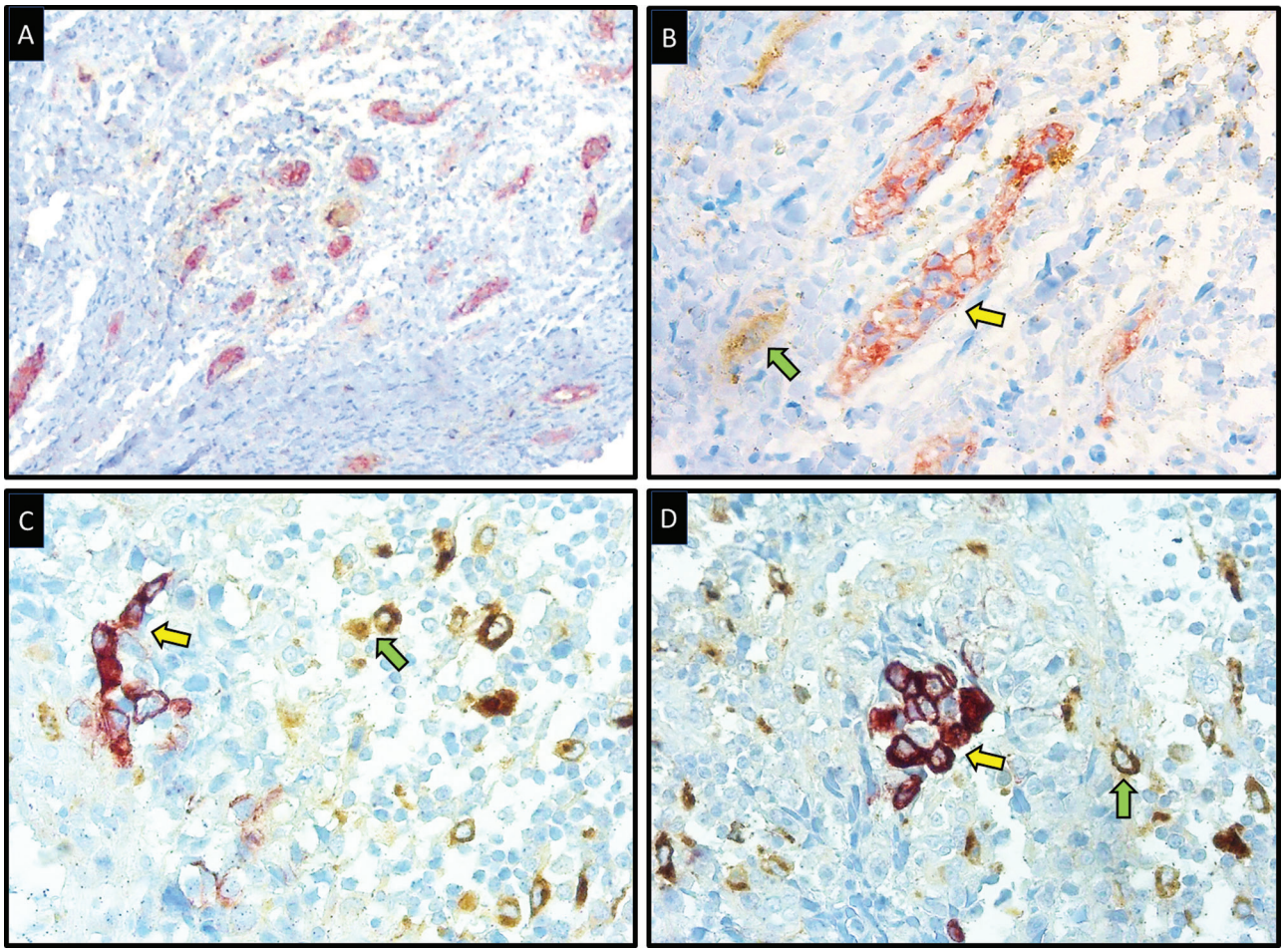

Figure 3: Tumor islands in moderately differentiated oral squamous cell carcinoma showing moderately intense positive expression of cytokeratin 8/18 (light red stain indicated by yellow arrow) and stromal fibroblasts expressing matrix metalloproteinase-9 (light brown stain indicated by green arrow) of moderate intensity (a) ×100, (b) ×400, and intense immunoexpression of both the markers (c and d) PDOSCC under high power (×400)

by Makino *et al.* (82.4%).[11] However, Nanda *et al*. (50%), Mitra *et al*. (57%), and Jaiswal *et al*. (58%) found relatively lower percentages of OSCC cases expressing CK 8/18.[12-14] CK 8/18 expression was observed throughout the tumor cells in some epithelial islands, while in other cases, only a few cells showed positive staining. The intensity and number of cells expressing CK 8/18 were significantly higher in OSCC compared to normal tissues, where it was absent. Our findings corroborate those of several authors who found that increased CK 8/18 expression is positively correlated with higher grades of OSCCs, suggesting its potential role in OSCC progression.[15]

In contrast, our findings regarding MMP-9 positivity in OSCC were slightly higher than previous reports (75%–85%). MMP-9, a gelatinase, plays a physiological role in development, wound healing, and inflammation.[16] In normal tissues, MMP-9 is primarily expressed in the cytoplasm and membrane of inflammatory cells and fibroblasts. Tumor cells secrete chemokines that attract inflammatory cells, and MMPs can cleave these chemokines, enhancing tumor growth.[17] Our study found a significantly higher number of cells expressing MMP-9 and more intense immunostaining in OSCC compared to normal tissues. In the tumoral area, MMP-9 expression was present in the cytoplasm of peritumoral inflammatory cells and, to a lesser extent, in the ECM. However, in some cases, MMP-9 expression was diffuse and unrelated to the tumor area.

MMP-9 degrades components of the ECM and BM, specifically type IV collagen, a major BM constituent. Increased stromal MMP-9 expression implies increased ECM degradation, facilitating neoplastic cell invasion and OSCC progression.[18] However, our study did not find a definite correlation between MMP-9 immunoexpression and OSCC histopathological grade, in contrast to findings by Kosunen *et al*., who reported a correlation between PDOSCC grades and strong stromal MMP-9 expression.[19]

No statistically significant correlation between CK 8/18 and MMP-9 immunoexpression and clinicodemographic parameters such as age, gender, lesion size, intraoral site, or lymph node palpability was observed in our study. These findings are consistent with previous research that did not find significant correlations between marker expression and these parameters.[11,20,21] However, studies by Dai *et al*. and Silva *et al*. found significantly higher stromal MMP-9 expression in male patients with OSCC compared to female patients.[22,23]

While there was no statistical correlation between the two markers' expression, mIHC enabled visualization of their spatial distribution within the lesion tissues. CK 8/18 was observed in surface epithelium and infiltrated neoplastic cells, while MMP-9 was primarily expressed in the cytoplasm of connective tissue stromal cells in the peritumoral area. However, in some cases, there was no direct relationship between cells expressing CK 8/18 and MMP-9.

In conclusion, mIHC allowed a more precise assessment of the topographical relationship between CK 8/18 (expressed in tumor islands) and MMP-9 (expressed in peritumoral stromal cells) within lesional tissues. The technique preserved tissue specimens, eliminating the need for serial sectioning, and provided advantages despite its complexity compared to conventional IHC, which examines a single marker. Careful attention during each step of the mIHC procedure is essential as it is technique-sensitive. Preventing cross-reactivity between immunoreagents and selecting contrasting colors are crucial for mIHC success.[24,25] Cross-reactivity from primary antibodies can be avoided through techniques such as manual heating, microwaving, or antibody elution using buffers.[26] In our study, microwave-based antigen retrieval was employed to minimize antibody cross-reactivity. The use of monoclonal antibodies against both biomarkers further minimized cross-reactivity among different antibodies.[27]

Longitudinal studies with adequate follow-up would provide more clarity concerning the expression of CK 8/18 and MMP-9 in the long-term prognosis of OSCC. The correlation of expression of the biomarkers can also be compared in recurrent and primary cases of OSCC, which could provide an insight into their biological nature. Immunoexpression of both markers could also be evaluated in oral premalignant disorders such as leukoplakia. This would elucidate the alterations in biomarkers during the transition from normal mucosa to premalignant conditions to OSCC.

Conclusion

Increased expression of CK 8/18 and MMP-9 can be correlated to higher grades of OSCC. CK 8/18 is absent in normal tissues and thus can be utilized as an effective indicator of OSCC. The increased expression of CK 8/18 in tumor cells and MMP-9 in peritumoral stromal cells and ECM is indicative of crucial changes occurring in the carcinogenesis process at the epithelial-stromal interface. The mIHC technique proved efficient and economically feasible by cutting short the time and materials required for multiple IHC procedures. This, in turn, reduced the cost borne by laboratory technicians as well as the pathologist and ultimately by the patients. It also benefited the patients in terms of time, wherein early and more precise assessment of biomarkers could be provided, which is much crucial for improving the prognosis of OSCC.

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Conflicts of interest
There are no conflicts of interest.

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