Original Article

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Quick Response Code:

Website: www.carcinogenesis.com

DOI: 10.4103/jcar.JCar_8_20

Prediction of metastasis in oral squamous cell carcinoma through phenotypic evaluation and gene expression of E-cadherin, β-catenin, matrix metalloproteinase-2, and matrix metalloproteinase-9 biomarkers with clinical correlation

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Submitted: 30-Mar-2020
Revised: 06-May-2020
Accepted: 17-May-2020
Published: 06-Aug-2020

Abstract:

CONTEXT: Controversies prevail regarding the true predictive role of epithelial–mesenchymal transition (EMT) biomarkers in metastasis of oral squamous cell carcinoma (OSCC). There is also limited research carried on till date wherein the protein and gene expression of EMT biomarkers have been investigated simultaneously in the Indian population.

AIM: The aim of this study was to assess the gene expression and quantitative protein expression of EMT biomarkers using conventional method and MATLAB software and to determine if there is any difference in the expression between metastatic and nonmetastatic OSCCs with clinicopathologic correlation.

SETTINGS AND DESIGN: Twenty metastatic and nonmetastatic OSCC tissue sections each were obtained from department archives. Gene expression and quantified protein expression of EMT markers were done and correlated with clinical parameters.

SUBJECTS AND METHODS: Sections immunostained for EMT biomarkers were evaluated using semi-quantitative and quantitative (MATLAB) methods. Gene expression using semi-quantitative reverse transcriptase–polymerase chain reaction was done. These findings were correlated with clinical parameters.

STATISTICAL ANALYSIS USED: Pearson's Chi-square test, Student's *t*-test, and univariate logistic regression analysis were performed using SPSS software.

RESULTS: The low immunoexpression of E-cadherin and β-catenin and the high expression of matrix metalloproteinase (MMP)-2 and MMP-9 correlate with Stages III and IV showing high metastatic risk. Furthermore, the upregulated MMP-2 and MMP-9 gene expressions in advanced clinical stages of OSCC have high metastatic potential.

CONCLUSIONS: This study is the first of its kind to employ texture and color segmentation in MATLAB to objectively assess the protein expression of EMT biomarkers. This research is instrumental in studying the protein and gene expressions of EMT markers with clinical correlation.

Keywords:

Epithelial–mesenchymal transition, immunohistochemistry, MATLAB, metastasis, oral squamous cell carcinoma, reverse transcriptase–polymerase chain reaction

How to cite this article: Sowmya SV, Rao RS, Prasad K. Prediction of metastasis in oral squamous cell carcinoma through phenotypic evaluation and gene expression of E-cadherin, β-catenin, matrix metalloproteinase-2, and matrix metalloproteinase-9 biomarkers with clinical correlation. J Carcinog 2020;19:8.

Introduction

Cancer risk prediction models have been formulated so far to assess the cost of population prevention strategies, genetic counseling, planning trials, etc.[1] Literature search has revealed the use of a variety of statistical models to predict metastasis and survival with the analysis of various clinicopathological variables and individual biomarkers. However, due to its multifactorial etiology, it is difficult to recognize a single etiological factor for oral cancer metastasis.[2]

Early prediction of metastasis to regional lymph nodes is a significant oncological factor for the prognosis of oral squamous cell carcinoma (OSCC). At present, various molecular mechanisms have been identified for metastasis, and numerous biomarkers have been investigated for specific mechanisms. It becomes really challenging for a pathologist to predict the metastatic potential of a particular patient as it can have direct implications on treatment. The expression of many biomarkers may correlate with the histopathological features, clinical staging, and behavior of the malignancy.[3] Literature search has revealed that for providing effective treatment for OSCC, tumor differentiation and regional metastasis to the lymph nodes act as reliable predictors. Therefore, research in predicting the metastatic risk of OSCC preoperatively has a significant scope.

Although major progress has been achieved due to technical advancements in the surgical procedures, radiotherapy and chemotherapy, OSCC continues to have poor survival and prognosis. The challenges in providing treatment amplify with the complex anatomy of the head and neck region and late presentation of the patients to health care.[4] The unaffordable advanced treatment procedures add to the increased mortality rate.[5] Several biomarkers have been employed to identify the processes leading to EMT.[6-8] However, controversies prevail regarding the true predictive role of EMT biomarkers in metastasis of OSCC.[9] There is also limited research carried on till date wherein the protein and gene expression of EMT biomarkers with clinical correlation have been investigated simultaneously in the Indian population. Therefore, an attempt has been made to assess the phenotypic protein expression and gene expression of EMT biomarkers using conventional method and MATLAB software and to determine if there is any difference in their expression between metastatic and non-metastatic OSCCs with clinicopathologic correlation. This may facilitate the clinician in appropriate decision-making in OSCC treatment.

Subjects and Methods

The workflow of the present study is summarized in Figure 1.

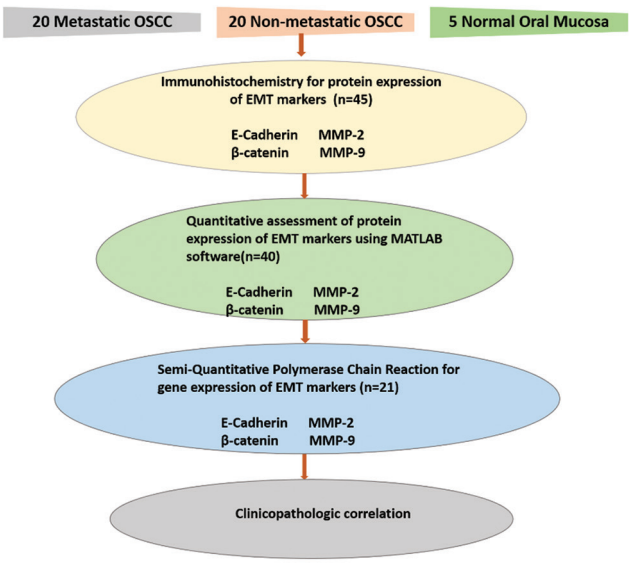

Figure 1: Workflow of the study

Patient selection and tissue specimens

Archival tissue samples of surgically excised primary OSCC with and without lymph node metastasis confirmed by histopathology formed the inclusion criteria. The control group included histopathologically confirmed normal oral mucosa. Recurrent OSCC tissue samples were excluded from the study. On screening 117 formalin-fixed paraffin-embedded (FFPE) OSCC specimens, a total of 40 cases (20 – metastatic and 20 – nonmetastatic OSCC) from patients diagnosed between 2014 and 2017 were obtained from archival material at the Department of Oral Pathology. Five cases of normal oral mucosa were selected as control specimens to assess the normal immunoexpression of the epithelial–mesenchymal transition (EMT) biomarkers considered in the research. The details of clinical parameters for each specimen were obtained from the patient database.

Immunohistochemistry

All the 45 study specimens were subjected to immunohistochemistry (IHC) using standard protocols. Four-micrometer thick tissue sections were obtained using semi-automated microtome, deparaffinized in xylene, and rehydrated through graded alcohols. Using microwave method, antigen was retrieved and endogenous peroxidases were blocked using 0.3% hydrogen peroxide for 30 min. Primary monoclonal antibodies were used to incubate the sections overnight at 4°C in a moist chamber for anti-E-cadherin (rabbit/mouse, 1:50 dilution; Dako, Denmark), anti-β-catenin (MIB-1, 1:50; Dako, Denmark), anti-matrix metalloproteinase (MMP)-2 (mouse, 1:100, Dako, Denmark), and anti-MMP-9 (mouse, Dako, Denmark) diluted in 1% bovine serum albumin and

phosphate-buffered saline (PBS). Polymer detection was performed using Envision system (horseradish peroxidase [HRP]-based two-step IHC-staining method). Anti-rabbit immunoglobulin G peroxidase-linked secondary antibody at 1:100 dilutions was used for 60 min at room temperature after PBS wash. The sections were subjected to streptavidin-conjugated HRP for 30 min at normal room temperature. Chromogen staining was developed with diaminobenzidine tetrahydrochloride for 4 min with Tris buffer, counterstained with Mayer's hematoxylin, dehydrated with graded alcohols, and mounted in Dibutylphthalate Polystyrene Xylene.[10]

Evaluation of immunoreactivity

Immunoreactivity was assessed independently by three examiners, and the mean score was considered for further analysis. Breast cancer tissue was used as a positive control for E-cadherin, β-catenin, MMP-2, and MMP-9. Omission of primary antibodies in each slide run constituted negative controls which gave appropriate results. Five most representative tumor areas were selected for scoring the immunostaining pattern. The degree of membranous positive staining for E-cadherin and membranous/cytoplasmic β-catenin antibodies was evaluated by a well-established semi-quantitative scoring on a scale of 0–3 for intensity (I) such as none, mild, moderate, and strong and for distribution (D) such as negative = 0, <10% = 1, 10%–50% = 2, 50%–80% = 3, and >80% = 4. Tissues with I × D less than or equal to four were considered as low and those with I × D greater than four were designated as high immunoreactive score.

For the interpretation of MMP-2 and MMP-9, five representative fields at the invasive tumor front were considered. The degree of cytoplasmic staining of both tumor and stromal cells was evaluated using semi-quantitative scoring on a scale of 0–3 for intensity (I) such as none, mild, moderate, and strong and for distribution (D) such as negative = 0, <10% = 1, 10%–50% = 2, and >50% = 3. Tissues with scores of 0 and 1 were considered as low and those graded as 2 and 3 were designated as having a high immunoreactive score.[11]

Quantitative assessment of protein expression of immunostained sections using image processing in MATLAB

Image acquisition

Ten representative fields of immunohistochemically stained sections of metastatic and non-metastatic OSCC cases (n = 20 each) were photographed using a charge-coupled device color video camera – Jenoptik Progres Gryphax Arktur USB 3.0 microscope camera, Jena, Germany, attached to the Olympus research microscope (BX53F2, Tokyo) and were saved as 24-bit color 2080 × 1542 bitmap image (bmp) file format in a computer. The images were subjected to image processing using MATLAB 2016 Ra 9.0 software (The MathWorks, Inc., Natick, Massachusetts, USA) with integrated Image Processing Toolbox.[12]

Image processing

Image processing was employed to quantify the phenotypic protein expression of the EMT biomarkers. Texture segmentation was employed to identify regions based on their texture. An algorithm was developed [Figure 2a] to perform segmentation based on texture and involved image preprocessing, feature extraction, clustering, and post processing. Image segmentation on the basis of color was performed using an algorithm [Figure 2b] for CIE L*a*b*/International Commission on Illumination (CIE) or CIELAB. There are three colors in an immunostained photomicrograph of OSCC sections – light blue, dark blue, and brown – that has been clustered. The positively stained brown-colored cells were segregated from the remaining structures and saved as an image. The number of brown-colored positive cells was counted using an algorithm shown in Figure 2c and displayed on the processed image. The mean intensity of the brown staining was further assessed using an algorithm displayed in Figure 2d. The code snippets corresponding to these algorithms are depicted in Figures 2a-d.

Semi-quantitative reverse transcriptase–polymerase chain reaction

RNA from metastatic and non-metastatic OSCC FFPE tissues was isolated using RecoverAll™ Total Nucleic Acid Isolation Kit as per manufacturer's guidelines. The mRNA levels of E-cadherin, β-catenin, MMP-2, MMP-9, and β-actin (endogenous control) genes were determined using Techno Prime system for semi-quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). The cDNA was synthesized from 2 μg of RNA using the Verso cDNA synthesis kit (Thermo Fischer Scientific) with oligo dT primer according to the manufacturer's instructions. The reaction volume was set to 20 μl, and cDNA synthesis was performed at 42°C for 60 min, followed by RT inactivation at 85°C for 5 min. The PCR mixture (final volume of 20 μL) contained 1 μL of cDNA, 10 μL of Red Taq Master Mix ×2 (amplicon), and 1 μM of each complementary primer specific for E-cadherin, β-catenin, MMP-2, MMP-9, and β-actin (endogenous control). The forward and reverse primer sequences for each gene were synthesized at Eurofins Genomics, India. β-actin, forward TCCTCCTGAGCGCAAGTACTCT, reverse GCTCAGTAACAGTCCGCCTAGAA; E-cadherin, forward CAGCACGTACACAGCCCTAA, reverse GTCCCTGTTCCAGTAGCAACT; β-catenin, forward GAAACGGCTTTCAGTTGAGC, reverse CTGGCCATATCCACCAGAGT; MMP-2, forward

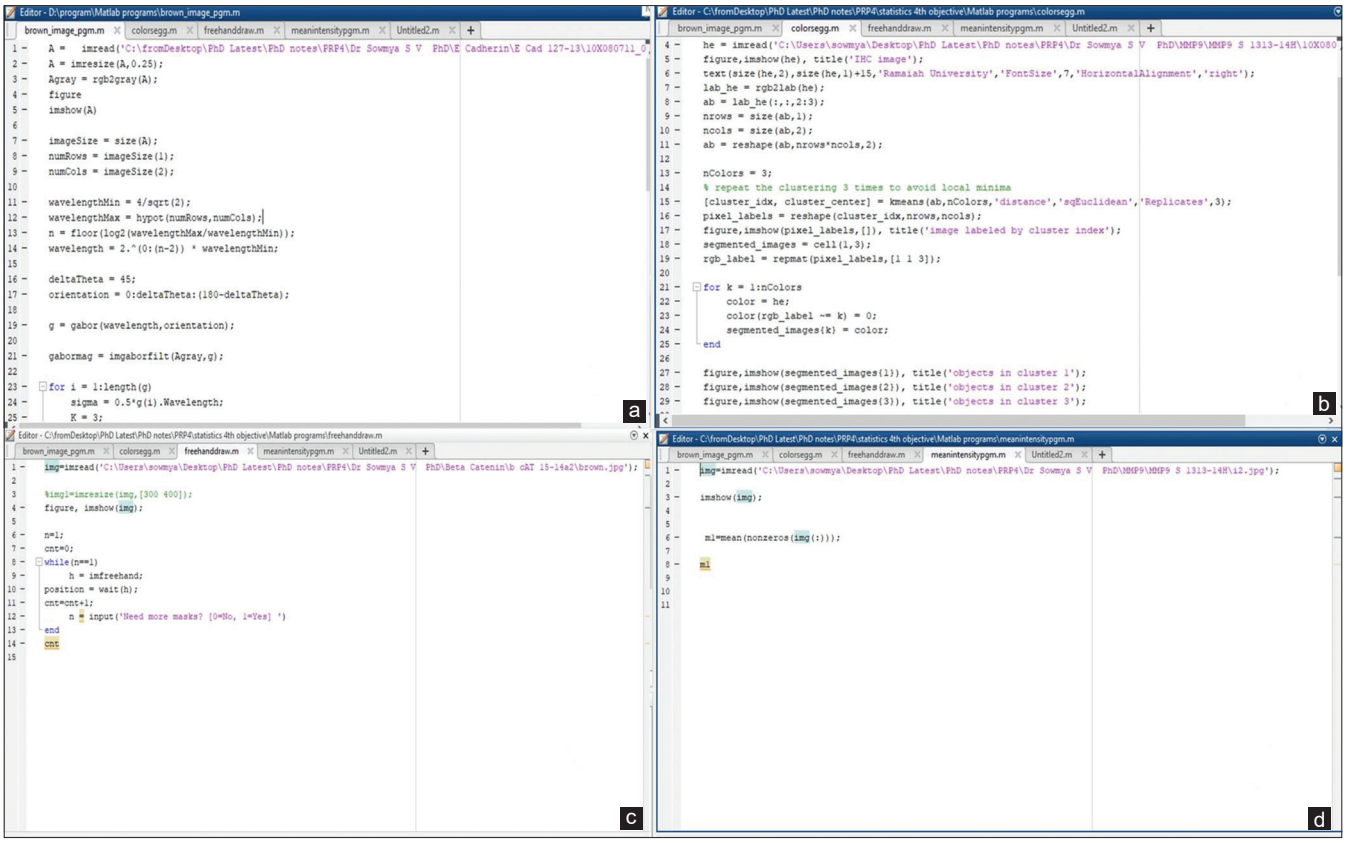

Figure 2: Code Snippets used for a- texture segmentation, b-color segmentation, c-draggable free hand regions and d-intensity of immunostaining

TTTCCATTCCGCTTCCAGGGCAC, reverse TCGCACACCACATCTTTCCGTCACT; and MMP-9, forward GATGCGTGGAGAGTCGAAAT, reverse CACCAAACTGGATGACGATG were employed. The samples were subjected to denaturation at 94°C for 5 min and amplified using 40 cycles of 94°C for 30 s, 51°C –61°C for 30 s, and 72°C for 30 min. Further final elongation was done at 72°C for 10 min for E-cadherin, β-catenin, MMP-2, and MMP-9 genes. For β-actin, the renaturation was set to 55°C for 30 s, followed by a final elongation at 72°C for 10 min. However, the annealing temperatures for E-Cadherin, β-catenin, MMP-2 and MMP-9 were 52°C, 54°C, 57°C and 51°C respectively.The suitable numbers of cycles were selected for amplification of these four genes so that their amplifications were exponentially ranged but did not reach a plateau. Ten microliters of the final product was run on a 2% ethidium-stained agarose gel and then photographed. The optical densities of the bands were measured, and the results were quantified using the computerized ImageJ program. The values were normalized to β-actin intensity levels.[13]

Statistical analysis

Statistical analysis using the Statistical Package for the Social Sciences (SPSS) software (IBM SPSS Statistics for Windows, version 20.0 [IBM Corp., Armonk, NY, USA]) was done. The mean score of three examiners for protein expression using IHC was analyzed using Pearson's Chi-square test. The mean score of protein expression (EMT biomarkers) from ten representative fields observed in immunostained photomicrographs was analyzed using Pearson's Chi-square test.

The gene expression of the four biomarkers E-cadherin, β-catenin, MMP-2, and MMP-9 were analyzed using Student's *t*-test. A series of univariate binary logistic regression analyses were performed to recognize the important predictors from clinical, immunohistochemical, and molecular variables, wherein the odds ratio with 95% confidence interval (CI) was calculated for each of the variables. $P < 0.05$ was considered to be statistically significant for all the analyses.

Results and Discussion

The present study was conducted to assess the combined gene expression and quantitative protein expression of the EMT markers, E-cadherin, β-catenin, MMP-2, and MMP-9, to differentiate metastatic from non-metastatic OSCC, and to identify the most predictive biomarkers for metastasis in OSCC.

The highest clinical significance of the EMT process is linked to its role in tumor cell invasion and metastasis. In the present study, there was a decreased expression of E-cadherin in the tumor cells of the metastatic

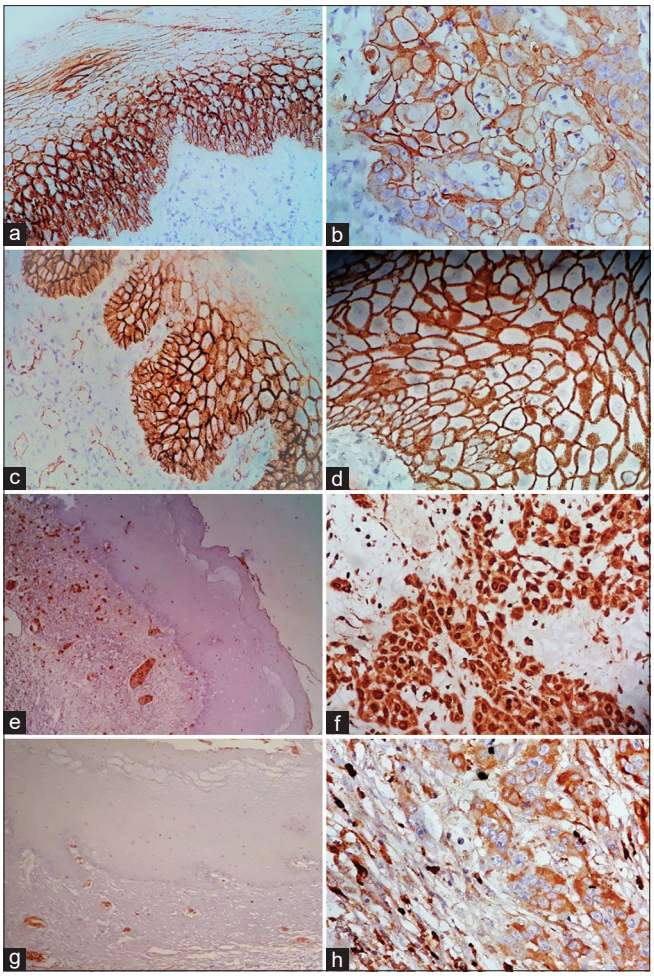

Figure 3: Photomicrographs of immunostained sections by epithelial–mesenchymal transition biomarkers; E-cadherin staining of the normal oral mucosa (a), oral squamous cell carcinoma (b); β-catenin immunostaining of normal oral mucosa (c), oral squamous cell carcinoma (d); immunostained matrix metalloproteinase-2 in normal oral mucosa (e), oral squamous cell carcinoma (f); and matrix metalloproteinase-9 immunostaining of normal oral mucosa (g), oral squamous cell carcinoma (h)

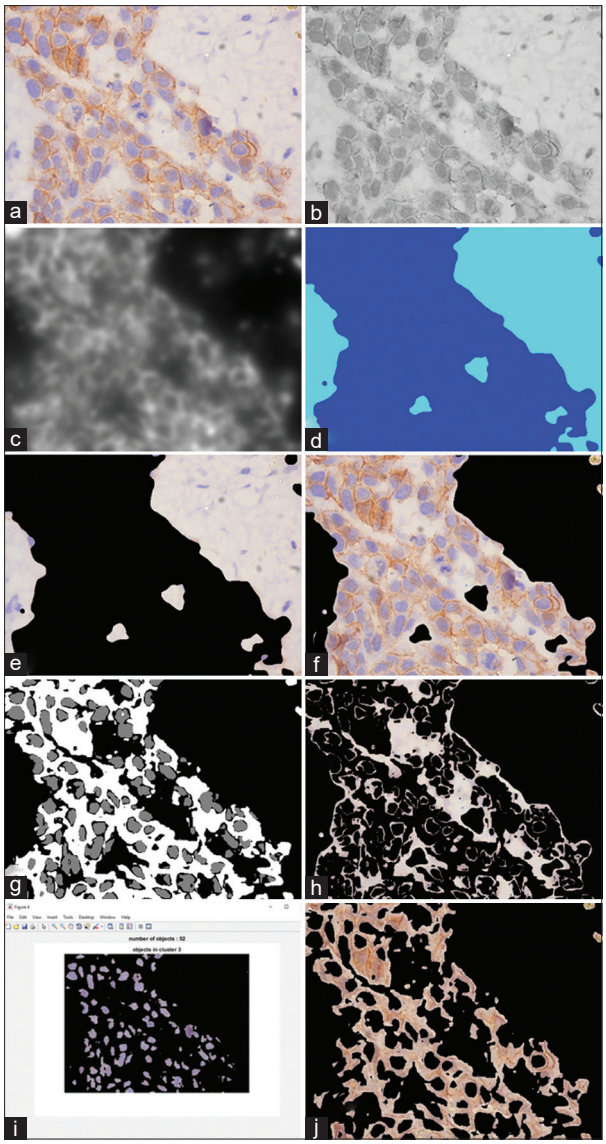

Figure 4: Photomicrographs of E-cadherin-immunostained oral squamous cell carcinoma showing series of images obtained by texture segmentation (a-f) (immunohistochemistry; ×400) and color segmentation (g-j) (immunohistochemistry; ×400)

OSCC group [Figure 3b] with all 20 (100%) cases exhibiting low expression in contrast to only 4 (20%) cases in non-metastatic OSCC group [Table 1]. The photomicrographs of immunostained sections were then subjected to texture segmentation, followed by color segmentation for obtaining the count of immunopositive cells objectively [Figure 4a-j]. It was found that the mean proportion of immunopositive cells in metastatic was significantly lower (13.95) in comparison to non-metastatic OSCC (29.20), with significant $P = 0.000$. The intensity of E-cadherin immunopositive cells was estimated using color segmentation method of image processing with the algorithm shown in Figure 2a. The mean intensity of staining obtained using color segmentation tool of MATLAB was 151.60 for metastatic and 163.80 for non-metastatic OSCC, which was statistically significant, with $P = 0.000$ [Table 2]. E-cadherin is the most important calcium-dependent cell surface protein of adherens type that anchors oral epithelial cells to each other.[5] E-cadherin displays long cytoplasmic and extracellular domains that facilitate adhesion between adjacent cells by homophilic interactions. There is a reduction in E-cadherin expression in normal embryonic development, fibrosis and during spread of cancer. E-cadherin plays a major role in transduction of signals that control cellular events such as polarity, growth, differentiation, and migration of cells. In normal epithelium, it is generally expressed in the basal and spinous layers and is absent in the superficial layers as it represents a process of continuous renewal of cells [Figure 3a]. There is an increased mobility of epithelial cells by the process of EMT leading to local invasion as tumor progresses.

This may be responsible for decreased expression of E-cadherin in these areas.[4,7]

In the present study, 18 (90%) of the metastatic OSCC cases showed a low expression of membranous β-catenin [Figure 3d] which differed significantly (P = 0.000) from the non-metastatic group (2 cases [10%]). The cytoplasmic expression of β-catenin did not reveal a significant difference between the study groups with 50% (10 cases) of metastatic and 75% (15 cases) of non-metastatic OSCC cases displaying low expression (P = 0.102) [Table 1]. Further, image processing in MATLAB software was used to assess the proportion and intensity of membranous and cytoplasmic β-catenin immunoexpression of tumor cells in the study groups [Figures 5a-f and 6a, b]. Texture segmentation of the photomicrographs revealed a reduced mean proportion of membranous β-catenin-immunopositive cells in metastatic (13.95) as compared to non-metastatic (29.20), which was statistically significant (P = 0.000) [Table 2]. The intensity of membranous and cytoplasmic immunopositive cells was then assessed on the color-segmented image [Figure 6c]. The mean intensity of immunostaining was assessed, and there was significant difference observed between the metastatic and non-metastatic OSCC groups, with P = 0.000 [Table 2]. The proportion of cytoplasmic β-catenin-immunopositive cells was determined by the draggable freehand regions [Figure 6a and b]. There was no difference in the mean proportion of cytoplasmic β-catenin-positive tumor cells between metastatic (6.80) and non-metastatic (6.05) OSCCs, with P = 0.343 [Table 2].

β-catenin is a 92-kDa protein that has a dual role in signal transduction and cell adhesion. In normal epithelium, it shows membranous expression in the basal and spinous cell layers and diminishes or remains absent in the superficial layers [Figure 3c]. It does not show cytoplasmic or nuclear expression. The E-cadherin/β-catenin complex plays a role in intercellular adhesion and may be involved in Wnt signaling pathway. In invasive lesions, E-cadherin is endocytosed and β-catenin is released leading to loss of cell adhesion. In normal and non-invasive cells, β-catenin is usually localized to cell membranes. However, it gets localized in the cytoplasm and later translocates to the nucleus leading to gene transcription and induction of EMT.[14]

The results of the present study are similar to the research done by Tanaka *et al*., who found a reduction in the immunoexpression of E-cadherin, α-catenin, and β-catenin in metastatic as compared to non-metastatic OSCC and have suggested that these markers are useful in the diagnosis and prediction of metastasis and invasion.[15] Albasri *et al*., 2015, have examined the accumulation of β-catenin in the nuclei and cytoplasm of

Table 1: Comparison of epithelial-mesenchymal transition biomarkers immunoexpression between metastatic and non-metastatic oral squamous cell carcinoma groups using semi-quantitative assessment

Biomarker	Group of OSCC	Immunoexpression of EMT biomarkers		*P*
		Low, *n* (%)	High, *n* (%)	
E-cadherin	Metastatic	20 (100%)	0	0.000
	Non-metastatic	4 (20%)	16 (80%)	
Membranous β-catenin	Metastatic	18 (90)	2 (10)	0.000
	Non-metastatic	2 (10)	18 (90)	
Cytoplasmic β-catenin	Metastatic	10 (50)	10 (50)	0.102
	Non-metastatic	15 (75)	5 (25)	
Proportion of MMP-2 tumor cells	Metastatic	2 (10)	18 (90)	0.001
	Non-metastatic	12 (60)	8 (40)	
Staining intensity of MMP-2 tumor cells	Metastatic	2 (10)	18 (90)	0.001
	Non-metastatic	12 (60)	8 (40)	
Proportion of MMP-2 stromal cells	Metastatic	3 (15)	17 (85)	0.018
	Non-metastatic	10 (50)	10 (50)	
Staining intensity of MMP-2 stromal cells	Metastatic	2 (10)	18 (90)	0.002
	Non-metastatic	11 (55)	9 (45)	
Proportion of MMP-9 tumor cells	Metastatic	2 (10)	18 (90)	0.000
	Non-metastatic	19 (95)	1 (5)	
Staining intensity of MMP-9 tumor cells	Metastatic	3 (15)	17 (85)	0.000
	Non-metastatic	14 (70)	6 (30)	
Proportion of MMP-9 stromal cells	Metastatic	0	20 (100)	0.000
	Non-metastatic	14 (70)	6 (30)	
Staining intensity of MMP-9 stromal cells	Metastatic	1 (5)	19 (95)	0.037
	Non-metastatic	6 (30)	14 (70)	

OSCC: Oral squamous cell carcinoma, MMP: Matrix metalloproteinase

oral cancer and leukoplakia using the Iterative Method of Expectation–Maximization algorithm and concluded that it is an efficient technique to help the pathologist to evaluate the histological changes on photomicrographs of oral cancer.[16]

MMPs are a group of enzymes that cause degradation of collagen and other proteins in the extracellular matrix (ECM). Degradation of basement membrane (BM) at the epithelium–lamina propria interface and around tumor nests and vascular tissues is a vital step in invasion and metastasis. Cancer cells act as initiators of carcinogenesis, whereas the stromal cells take up the function of promoters, thereby playing a synergistic role in the microenvironment. MMP-2 and MMP-9 are gelatinases/Type IV collagenases that mainly degrade Col-IV, the main component of BM and ECM, and play a role in neovascularization. It is generally absent in the normal epithelium [Figures 3e and g] and is expressed in the cytoplasm of neoplastic tumor and stromal cells.[17,18]

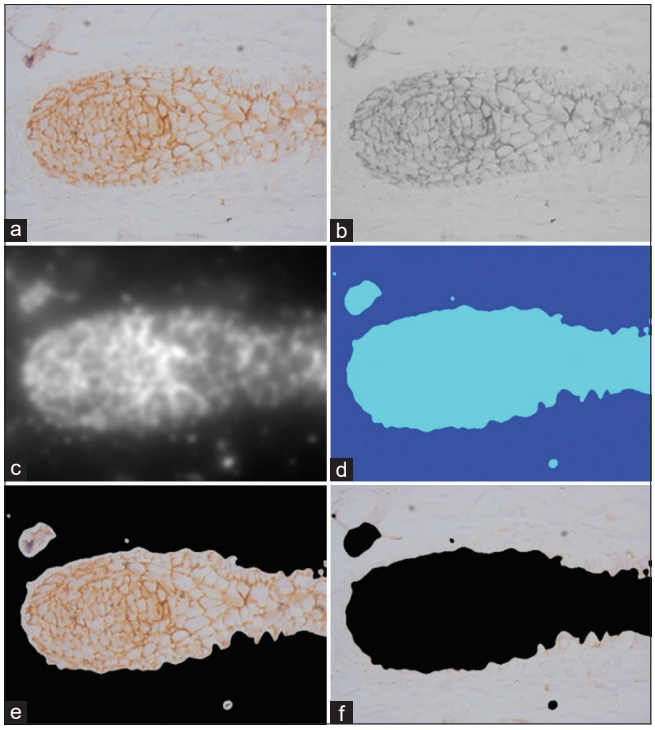

Figure 5: Photomicrographs of membranous β-catenin-immunostained oral squamous cell carcinoma showing a series of images obtained by texture segmentation (a-f) (immunohistochemistry; ×400)

The current study showed a high proportion and intensity of MMP-2-positive tumor cells in metastatic with 18 cases (90%) each displaying high expression as compared to non-metastatic OSCC, with $P = 0.001$. A similar expression was noted in the stromal cells which showed a high proportion and intensity of MMP-2-immunopositive cells in metastatic in comparison to non-metastatic, with significant $P = 0.018$ and 0.002, respectively [Figure 3f and Table 1]. Texture and color segmentation in image processing were used to assess the proportion of immunopositive tumor and stromal cells and determine their count. The mean proportion of MMP-2-immunopositive tumor cells was significantly increased in metastatic (29.10) as compared to non-metastatic (18.85) OSCC, with $P = 0.000$. The intensity of MMP-2-positive tumor cells also showed a significant difference between the study groups with the mean value of 180.20 in metastatic as compared to non-metastatic OSCC, with a mean intensity of 165.70 [Figure 7a-h and Table 2]. The proportion of immunopositive stromal cells showed mean values of 34.80 and 24.15 in metastatic and non-metastatic OSCC, respectively, with significant $P = 0.000$, as shown in Table 2 [Figure 7i-l]. However, there was no significant difference in the intensity of MMP-2 between the groups, with $P = 0.376$.

MMP-9, another collagenase, was also evaluated for its expression in OSCC. It was found that the proportion and intensity of MMP-9 tumor cells was significantly high in metastatic OSCC with 18 cases (90%) and 17 cases (85%) of positivity as compared to non-metastatic OSCC, with $P = 0.000$. A similar high expression of MMP-9 was observed both in proportion and intensity of immunopositive stromal cells in metastatic OSCC compared to the non-metastatic group. All the 20 cases (100%) showed an increased proportion of positive stromal cells, and the staining intensity was high in 19 cases (95%) in metastatic OSCC which showed a statistically significant difference in the expression, with $P = 0.000$ and 0.037, respectively [Figure 3h and Table 1]. The proportion of immunopositive tumor and stromal

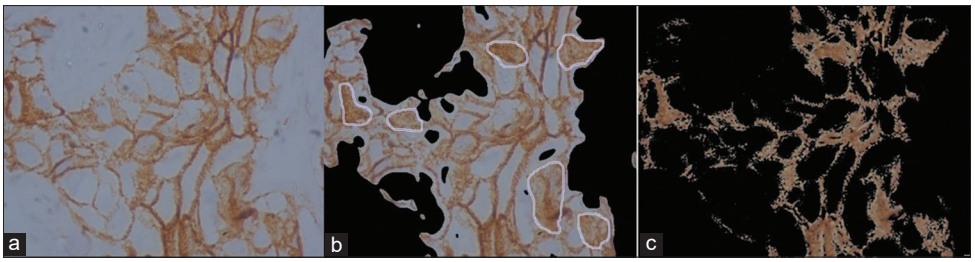

Figure 6: Photomicrographs showing the cytoplasmic β-catenin-immunopositive tumor cells of oral squamous cell carcinoma selected by draggable freehand regions (a and b); color-segmented image to estimate the intensity of immunopositive cells (c) (immunohistochemistry; ×400)

Table 2: Comparison of epithelial-mesenchymal transition biomarkers immunoexpression between metastatic and non-metastatic oral squamous cell carcinoma groups using MATLAB software

Biomarker	Group	*n*	Minimum	Maximum	Mean±SD	*P*
Proportion of E-cadherin-positive cells	Metastatic	20	10	20	13.95±2.395	0.000
	Non-metastatic	20	14	58	29.20±11.678	
Intensity of E-cadherin-positive cells	Metastatic	20	140	166	151.60±7.970	0.000
	No-nmetastatic	20	138	180	163.80±10.871	
Proportion of membranous β-catenin-positive cells	Metastatic	20	10	20	13.95±2.395	0.000
	Non-metastatic	20	14	58	29.20±11.678	
Proportion of cytoplasmic β-catenin-positive cells	Metastatic	20	3	13	6.80±2.587	0.343
	Non-metastatic	20	2	12	6.05±2.350	
Intensity of membranous and cytoplasmic β-catenin-positive cells	Metastatic	20	140	166	151.60±7.970	0.000
	Non-metastatic	20	138	180	163.80±10.871	
Proportion of MMP-2-positive tumor cells	Metastatic	20	20	34	29.10±3.553	0.000
	Non-metastatic	20	17	25	18.85±1.981	
Intensity of MMP-2-positive tumor cells	Metastatic	20	154	189	180.20±9.855	0.00
	Non-metastatic	20	163	170	165.70±1.689	
Proportion of MMP-2-positive stromal cells	Metastatic	20	26	43	34.80±4.819	0.000
	Non-metastatic	20	19	30	24.15±3.083	
Intensity of MMP-2-positive stromal cells	Metastatic	20	162	190	183.35±7.782	0.376
	Non-metastatic	20	162	198	185.55±7.756	
Proportion of MMP-9-positive tumor cells	Metastatic	20	25	29	27.35±1.226	0.000
	Non-metastatic	20	11	16	13.75±1.650	
Intensity of MMP-9-positive tumor cells	Metastatic	20	163	184	176.15±3.843	0.000
	Non--metastatic	20	158	179	167.85±5.509	
Proportion of MMP-9-positive stromal cells	Metastatic	20	26	33	29.50±1.792	0.000
	Non-metastatic	20	10	26	18.95±5.063	
Intensity of MMP-9-positive stromal cells	Metastatic	20	175	189	186.05±2.982	0.000
	Non-metastatic	20	156	180	164.50±5.155	

MMP: Matrix metalloproteinase, SD: Standard deviation

cells was assessed by texture segmentation, followed by color segmentation and then determining the cell count using an appropriate algorithm in image processing was done. There was a significant increase in the proportion and intensity of tumor cells, with mean values of 27.35 and 176.15, respectively, in metastatic as compared to non-metastatic, which was 13.75 and 167. 85, with $P = 0.000$. Similarly, stromal cells also showed a significant difference ($P = 0.000$) between the study groups in proportion and intensity with metastatic displaying mean proportion of 29.50 and intensity of 186.05. There was a reduced proportion (18.95) and intensity (164.50) of stromal cells in non-metastatic OSCC [Table 2 and Figure 8a-f].

Research by Akihiro Katayama *et al*., in 2004, has found significantly higher scores in the expressions of MMP-9 and TIMP-2 in metastatic OSCC than patients without metastasis using computer-assisted semi-quantitative analysis with NIH image for immunostained sections. They concluded that MMP-9 and TIMP-2 had a predictive value for metastasis and cause-specific survival which correlates well with the present study results. However, they failed to demonstrate the correlation of MMP-2 and MT1-MMP expressions with tumor metastases and prognosis.[19] Moreover, the use of image processing with texture and color segmentation used in the current study has not been employed till date for evaluating the immunostained photomicrographs of E-cadherin, β-catenin, MMP-2, and MMP-9. de Vicente *et al*., in 2005, immunohistochemically evaluated the expression of MMP-2 and MMP-9 in 68 OSCC cases and found their higher values correlated with increased invasion process.[20] In the present study, the use of MATLAB has brought in objectivity, reproducibility and reliability. The use of texture and color segmentation has saved human labor, is less time –consuming, causes less mental fatigue and provides high quality results.

The gene expression of the biomarkers under study was further analyzed using semi-quantitative RT-PCR for 10 samples each representing the metastatic and non-metastatic OSCC groups. A relative RNA value of 1 represented no overexpression in comparison with the controls, a value of <1 represented reduced expression, and >1 represented overexpression as compared with the controls. All the biomarker genes and housekeeping genes were initially assessed for their expression in SCC9 cell line, and there was a good expression of all the genes of interest. The housekeeping gene β-actin was also amplified using PCR and showed varying optical densities for the study samples [Figure 9]. The

internal control β-actin was used to normalize the gene expression. The relative gene expression (fold expression compared to control) was assessed, and Student's *t*-test statistics was employed.

The relative mean fold mRNA expression of E-cadherin in the metastatic group was found to be 0.50 folds less expressed as compared to normal control, whereas the non-metastatic group showed 0.74. However, there was a significant difference in the relative expression between the two groups, with $P = 0.04$. Similarly, β-catenin gene expression was significantly lower in metastatic OSCC showing 0.27 folds less expression compared to normal control, whereas non-metastatic was found to be 0.77 ($P = 0.002$). Kudo *et al*., 2004, have isolated invasive clones from OSCC cell lines. Using methylation-specific PCR, they have shown a significant reduction of E-cadherin and membranous β-catenin proteins with invasive capacity compared to parent cells and attributed it to methylation of E-cadherin and/or degradation of membranous β-catenin.[21]

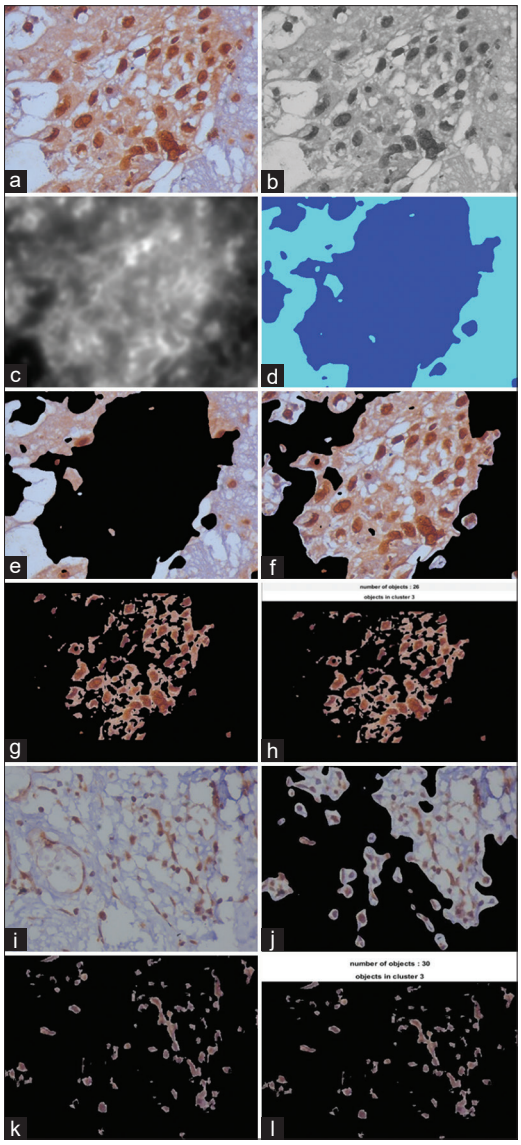

Figure 7: Photomicrographs of matrix metalloproteinase-2-immunostained oral squamous cell carcinoma showing a series of images obtained by texture and color segmentation for determining proportion and intensity of tumor cells (a-h); texture segmentation of stromal cells (i-l) (immunohistochemistry; ×400)

The relative mean fold mRNA expressions of MMP-2 was found to be 2.89- and 1.84-fold upregulated expression in the metastatic and non-metastatic groups of OSCC, respectively, as compared to normal control sample and showed a statistically significant difference, with $P = 0.07$. The relative mean fold mRNA expression of MMP-9 was also assessed in the study groups in a similar manner and was found to be 5.54 and 2.32 folds highly expressed compared to normal control in the metastatic and non-metastatic groups, respectively. The difference in the mRNA expression between the groups was significant, with $P = 0.000$ [Table 3 and Figure 9]. Pornchai O-Charoenrat *et al*., 2001, have studied the comprehensive profiles of MMPs and their inhibitors and correlate the expression values with clinicopathologic features and metastasis using semi-quantitative PCR on fresh tissue samples. They found an increased expression of MMP-9 in patients with lymph node metastasis and have suggested that it can be used as an early predictor for metastasis and permit appropriate therapy.[22]

Clinicopathologic correlation

All the 8 and 12 cases of the age groups of 31–45 years and >45 years, respectively, demonstrated low E-cadherin immunoexpression in the metastatic OSCC group, which was statistically significant ($P < 0.001$). There was a significantly reduced expression of membranous β-catenin in the metastatic group as compared to the non-metastatic group ($P = 0.001$). The cytoplasmic β-catenin immunoexpression showed a significant difference between the metastatic and non-metastatic OSCC groups in the age group of 31–45 years ($P = 0.02$) but did not differ significantly in the group of >45 years ($P = 0.89$) [Figure 10a]. The proportion and intensity of MMP-2 tumor cells was significantly increased in the metastatic group as compared to the non-metastatic group in the age group of >45 years, with $P = 0.002$ and 0.004, respectively. The MMP-2 stromal cell intensity was

Table 3: Relative gene expression of E-cadherin, β-catenin, matrix metalloproteinase -2, matrix metalloproteinase-9, and β-actin in oral squamous cell carcinoma tissues

Gene	Mean fold expression±SD		*P*
	Metastatic OSCC	Non-metastatic OSCC	
E-cadherin	0.50±0.11	0.74±0.18	0.04
β-catenin	0.27±0.21	0.77±0.38	0.002
MMP-2	2.89±1.97	1.84±0.69	0.07
MMP-9	5.54±1.83	2.32±0.92	0.000

OSCC: Oral squamous cell carcinoma, MMP: Matrix metalloproteinase, SD: Standard deviation

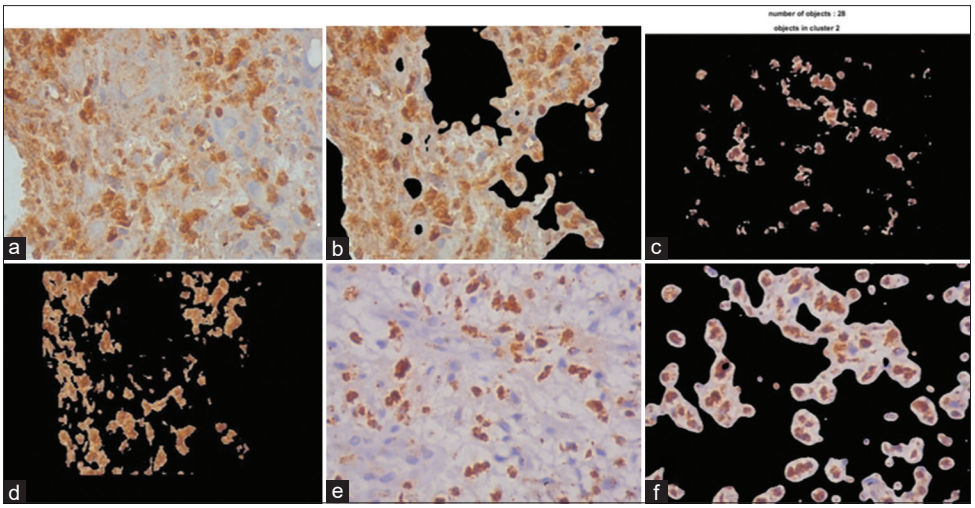

Figure 8: Photomicrographs of matrix metalloproteinase-9-immunostained oral squamous cell carcinoma showing series of images obtained by texture and color segmentation for determining proportion and intensity of tumor cells (a-d); texture segmentation of stromal cells (e-f) (immunohistochemistry; ×400)

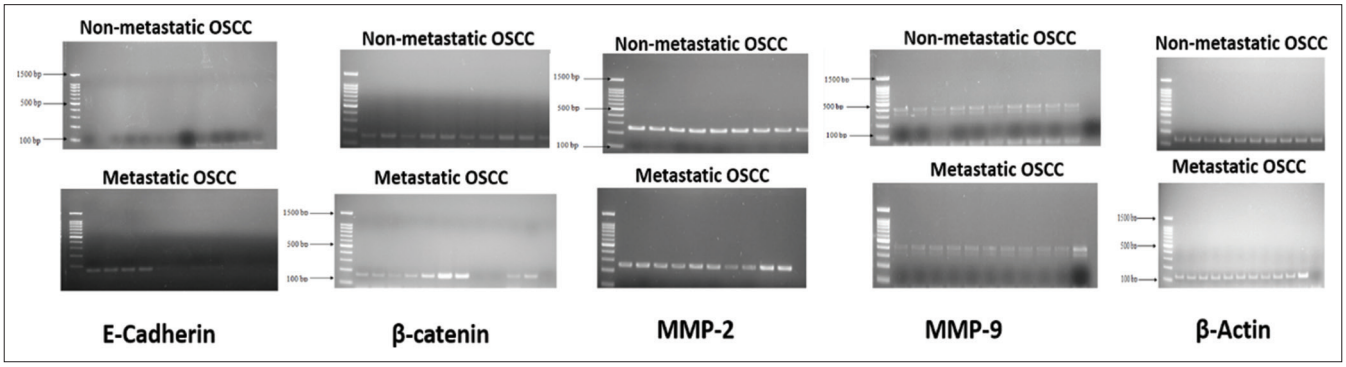

Figure 9: Amplification of E-cadherin, β-catenin, matrix metalloproteinase-2, matrix metalloproteinase-9, and β-actin (housekeeping) genes in metastatic and non-metastatic oral squamous cell carcinoma specimens

significantly high in the metastatic group in the 31–45 years' group ($P = 0.005$). Both the age groups demonstrated an increased immunoexpression of proportion of MMP-9 tumor ($P < 0.001$) and stromal ($P = 0.003$ and <0.001) cells in the metastatic OSCC [Figure 10b].

E-cadherin and membranous β-catenin immunoexpression showed a difference between the study groups in both male and female genders and was high in metastatic OSCC, with significant $P = 0.001$ [Figure 11a]. The distribution of MMP-2 tumor cells in both genders and its intensity in males was significantly found to be increased in the metastatic group ($P = 0.02$). The intensity of MMP-2 stromal cells was highly expressed in both genders in metastatic OSCC, with $P = 0.02$ and 0.04, respectively. The MMP-9 tumor and stromal cells, distribution, and intensity in both males and females were significantly increased in metastatic OSCC compared to non-metastatic [Figure 11b].

The low expression of E-cadherin in metastatic OSCC significantly shows a correlation with smokeless habit and combined smoking and smokeless tobacco habit, with $P < 0.001$ and 0.003, respectively. Patients with all three habits, smoking, smokeless, and combined tobacco users, showed a significant reduction in the membranous β-catenin expression in the metastatic group with $P = 0.008$, <0.001, and 0.02, respectively [Figure 12a]. The smokeless and combined tobacco habits of MMP-2 stromal cell distribution and combined habit for intensity in metastatic showed a significantly increased expression ($P = 0.03$, 0.02, and 0.02, respectively). The MMP-9 tumor cell intensity ($P = 0.04$, 0.04, and 0.02) and distribution ($P = 0.008$, <0.001, and 0.003), and stromal cell distribution ($P = 0.008$, 0.005, and 0.003) showed a significant increase in all three habit groups (smoking, smokeless, and combined tobacco) in metastatic as compared to non-metastatic OSCC [Figure 12b]. The MMP-9 stromal cell intensity was found to be significantly high in the smokeless group of metastatic OSCC ($P = 0.03$).

Biopsied sections from buccal mucosa and tongue showed a significantly low expression of E-cadherin in the metastatic OSCC group, with $P < 0.001$ and 0.005,

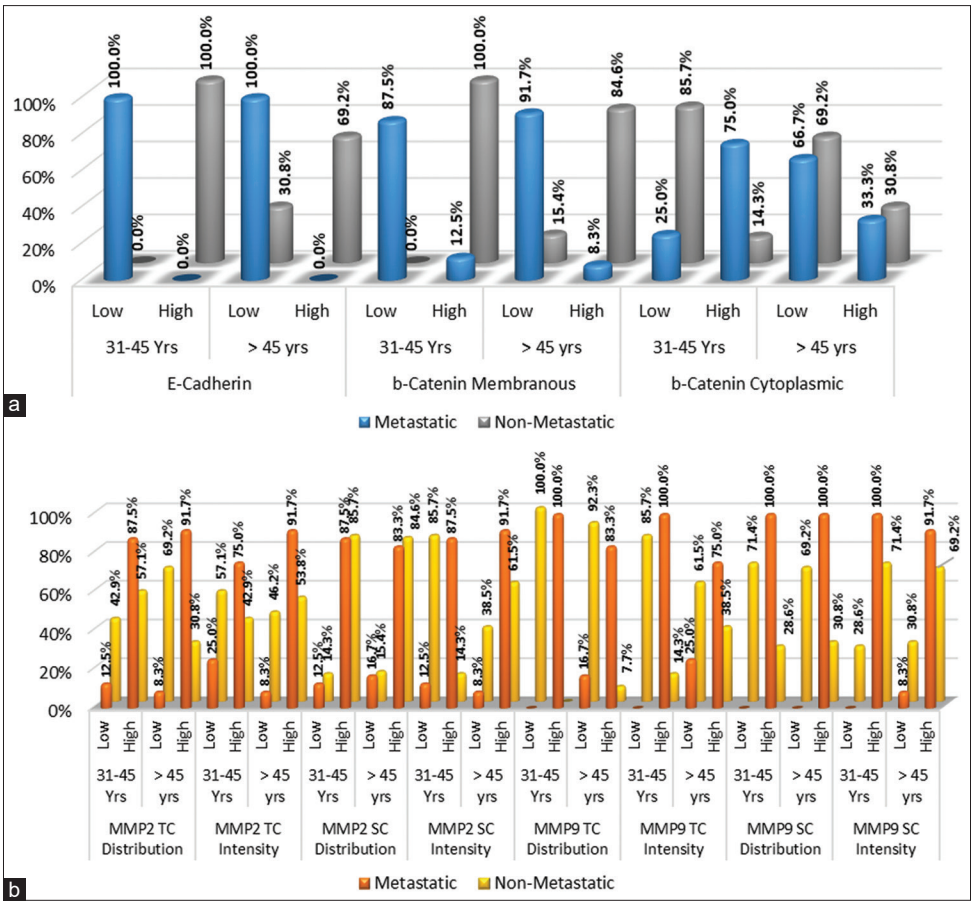

Figure 10: Bar graphs showing the age-wise comparison of protein immunoexpression of E-cadherin and membranous and cytoplasmic β-catenin in (a) and tumor and stromal cell distribution and intensity of matrix metalloproteinase-2 and matrix metalloproteinase-9 biomarkers between metastatic and non-metastatic oral squamous cell carcinomas in (b)

respectively. All the three sites of biopsy, buccal mucosa, tongue, and alveolar mucosa showed a significantly reduced expression of membranous β-catenin, with $P = 0.001$, 0.005, and 0.04, respectively, in the metastatic group compared to non-metastatic OSCC. However, cytoplasmic β-catenin did not show a significant difference in its expression between the study groups when comparison was made based on the site of the lesion [Figure 13a]. An increased proportion of positive MMP-2 tumor cells were observed in metastatic OSCC tissue sections obtained from buccal mucosa and alveolus ($P = 0.01$ and 0.04). MMP-2 stromal cell intensity of the metastatic OSCC group from tongue was significantly high compared to non-metastatic, with $P = 0.005$. MMP-9 tumor and stromal cell distribution was significantly high in metastatic OSCC from biopsied sections of buccal mucosa and tongue, with P values of each of <0.001 and 0.005, respectively. The tissue sections from buccal mucosa and alveolus showed a significant increase in MMP-9 tumor cell intensity, with $P = 0.04$ and 0.008, respectively [Figure 13b].

A significantly low E-cadherin protein expression was observed in high number of clinical Stage III and IV patients of the metastatic OSCC group, with $P = 0.03$ and <0.001, respectively. Low expression of membranous β-catenin was noted in 16 cases of Stage IV metastatic OSCC, with $P = 0.001$ [Figure 14a]. MMP-2 tumor cell distribution and intensity was significantly high in metastatic OSCC as compared to the non-metastatic group ($P = 0.02$ and 0.009). MMP-2 stromal cell intensity ($P = 0.03$ and 0.02) and MMP-9 tumor cell distribution of Stages III and IV ($P = 0.03$ and <0.001) was found to be significantly high in metastatic OSCC compared to non-metastatic [Figure 14b].

Tanaka *et al*. assessed the expression of E-cadherin, α-catenin, and β-catenin with metastasis and correlated the clinicopathologic findings in metastatic and non-metastatic OSCC. They found no significant association between age, gender, site of tumor, or histological differentiation and the existence of lymph node metastasis. However, lymph node metastasis was found more frequently in the cases with T3 or T4 tumor than in those with T1 or T2 tumor.[15] Studies have also shown that reduced expression of E-cadherin, β-catenin, MMP-2 and MMP-9 correlates with poor prognosis.[23,24] Moreover, there are meager studiesin

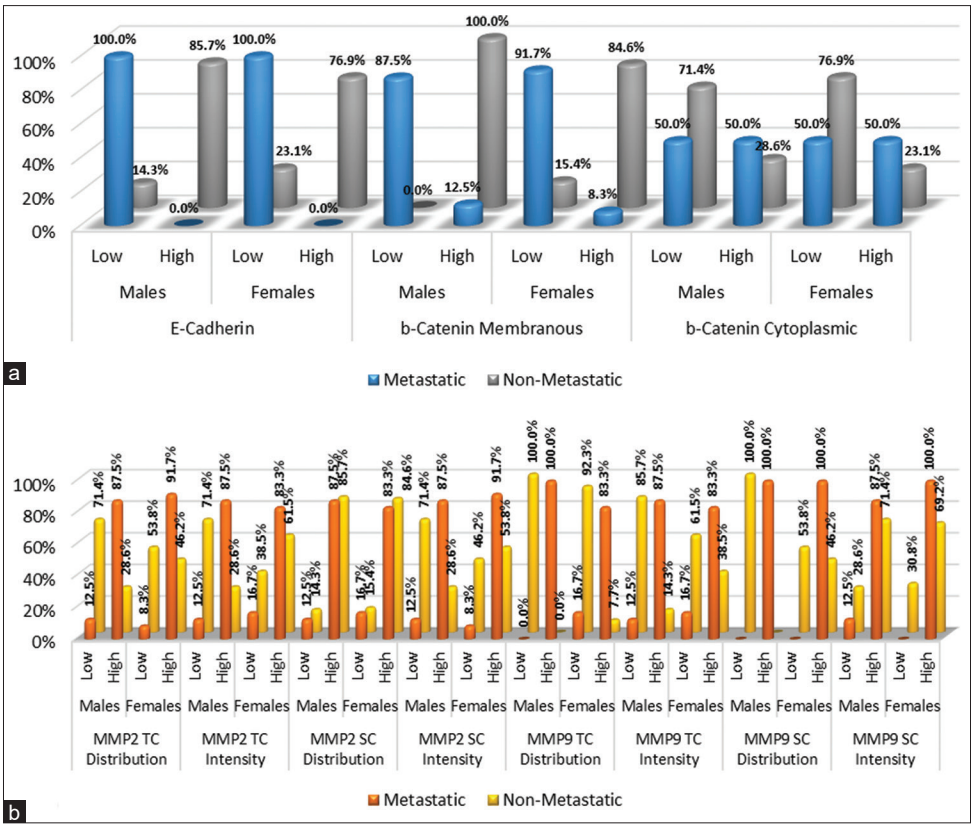

Figure 11: Bar graphs showing the gender-wise comparison of protein expression of E-cadherin and membranous and cytoplasmic β-catenin in (a) and tumor and stromal cell distribution and intensity of matrix metalloproteinase-2 and matrix metalloproteinase-9 biomarkers between metastatic and non-metastatic oral squamous cell carcinomas in (b)

the literature that have observed the association between the expression of these EMT markers with the clinical parameters of age, gender, habits, site of the lesion, and staging.

Univariate logistic regression found an odds ratio (OR) of 1.16 (95% CI: 0.27 ± 4.92) for metastatic patients above 45 years relative to those between 31 and 45 years, with *P* = 0.84. OR for metastasis in males was 0.86 (95% CI: 0.20 ± 3.68) relative to females, with *P* = 0.74. OR for metastasis in patients with smokeless and combined tobacco habits relative to smoking was 1.33 (95% CI: 0.24 ± 7.28) and 1.67 (95% CI: 0.23 ± 12.22), respectively. There was an OR of 0.27 (95% CI: 0.04 ± 1.65) and 0.67 (95% CI: 0.08 ± 5.88) for metastasis from buccal mucosa and tongue relative to alveolar mucosa site (*P* = 0.25), respectively. OR for metastasis of Stages II, III, and IV were 1.95 (95% CI: 0.84 ± 4.32), 3.67 (95% CI: 1.24 ± 6.71), and 6.28 (95% CI: 3.75 ± 10.92), respectively, relative to Stage I, with significant *P* < 0.001 [Table 4].

Univariate logistic regression analysis revealed OR of 3.18 and 3.30 for low E-cadherin and membranous β-catenin protein expression to undergo metastasis relative to high expression (95% CI: 0.94–6.37), with significant P = 0.001 and 0.02, respectively. Regression analysis also revealed

Table 4: Univariate regression model for the assessment of metastatic risk of oral squamous cell carcinoma using the clinical parameters

Characteristic	OR	95% CI		*P*
		Lower	Upper	
Age (years)				
31-45	Reference			
>45	1.16	0.27	4.92	0.84
Gender				
Females	Reference			
Males	0.86	0.20	3.68	0.74
Habits				
Smoking	Reference			
Smokeless	1.33	0.24	7.28	0.88
Combination	1.67	0.23	12.22	
Site				
Alveolus	Reference			
Buccal mucosa	0.27	0.04	1.65	0.25
Tongue	0.67	0.08	5.88	
Staging				
Stage I	Reference			
Stage II	1.95	0.84	4.32	<0.001*
Stage III	3.67	1.24	6.71	
Stage IV	6.28	3.75	10.92	

CI: Confidence interval, OR: Odds ratio. *Statistically significant

OR for metastasis of high MMP-2 and MMP-9 intensity of positive tumor cells and MMP-9 proportion of tumor

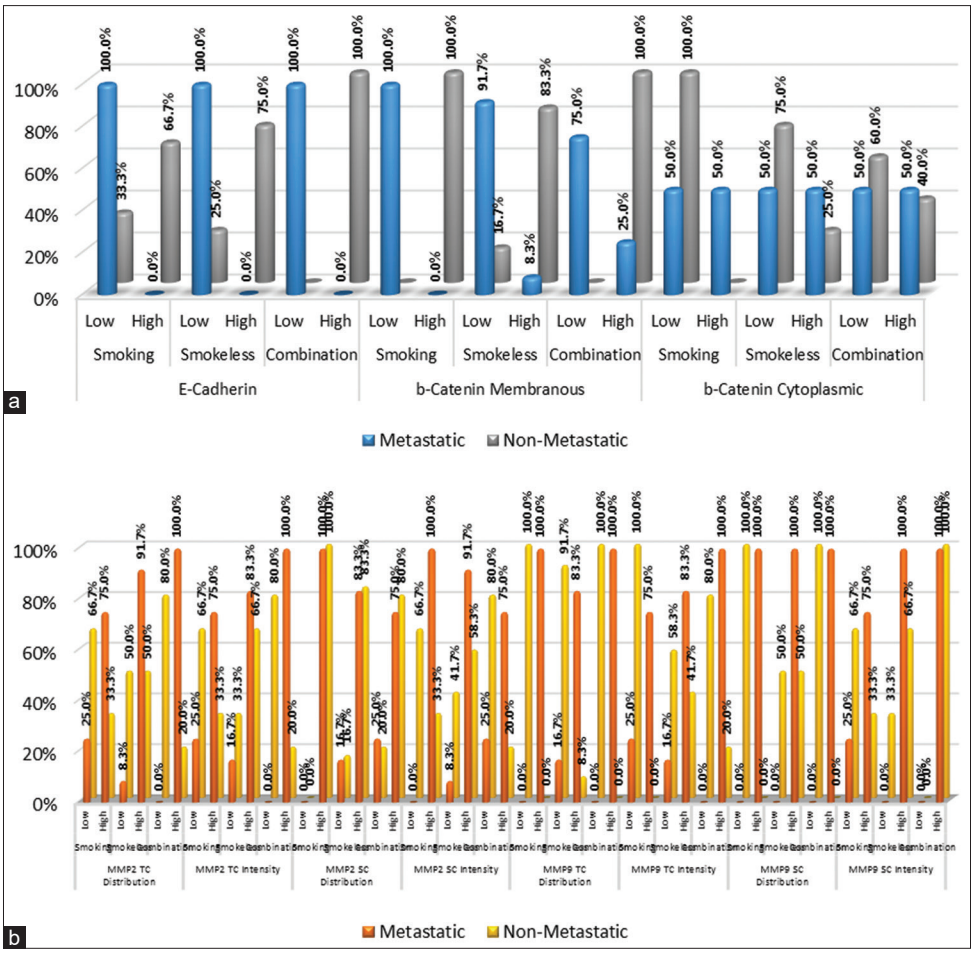

Figure 12: Bar graphs showing comparison of habits with the protein expression of E-cadherin and membranous and cytoplasmic β-catenin in (a) and tumor and stromal cell distribution and intensity of matrix metalloproteinase-2 and matrix metalloproteinase-9 biomarkers in (b) between metastatic and non-metastatic oral squamous cell carcinomas

Table 5: Univariate regression model for the assessment of metastatic risk of oral squamous cell carcinoma based on the protein expression of epithelial-mesenchymal transition biomarkers

Characteristic	OR	95% CI		*P*
		Lower	Upper	
E-cadherin protein expression				
Low	3.18	0.94	6.37	0.001*
High	Reference			
Membranous β-catenin protein expression				
Low	3.30	1.80	4.98	0.02*
High	Reference			
Cytoplasmic β-catenin protein expression				
Low	Reference			
High	1.71	0.11	4.87	0.71
MMP-2 protein expression of number of tumor cells				
Low	Reference			
High	1.08	0.71	2.87	0.01*
MMP-2 protein expression of intensity of tumor cells				
Low	Reference			
High	1.00	0.11	8.91	0.79
MMP-2 protein expression of number of stromal cells				
Low	Reference			
High	0.16	0.01	0.85	0.38

Contd...

Table 5: Contd...

Characteristic	OR	95% CI		*P*
		Lower	Upper	
MMP-2 protein expression of intensity of stromal cells				
Low	Reference			
High	7.04	4.50	11.54	0.04*
MMP-9 protein expression of number of tumor cells				
Low	Reference			
High	2.80	1.01	4.88	0.02*
MMP-9 protein expression of intensity of tumor cells				
Low	Reference			
High	4.89	1.30	5.54	0.02*
MMP-9 protein expression of number of stromal cells				
Low	Reference			
High	4.71	1.57	7.26	0.01*
MMP-9 protein expression of intensity of stromal cells				
Low	Reference			
High	0.21	0.03	14.56	0.47

MMP: Matrix metalloproteinase, CI: Confidence interval, OR: Odds ratio. *Statistically significant

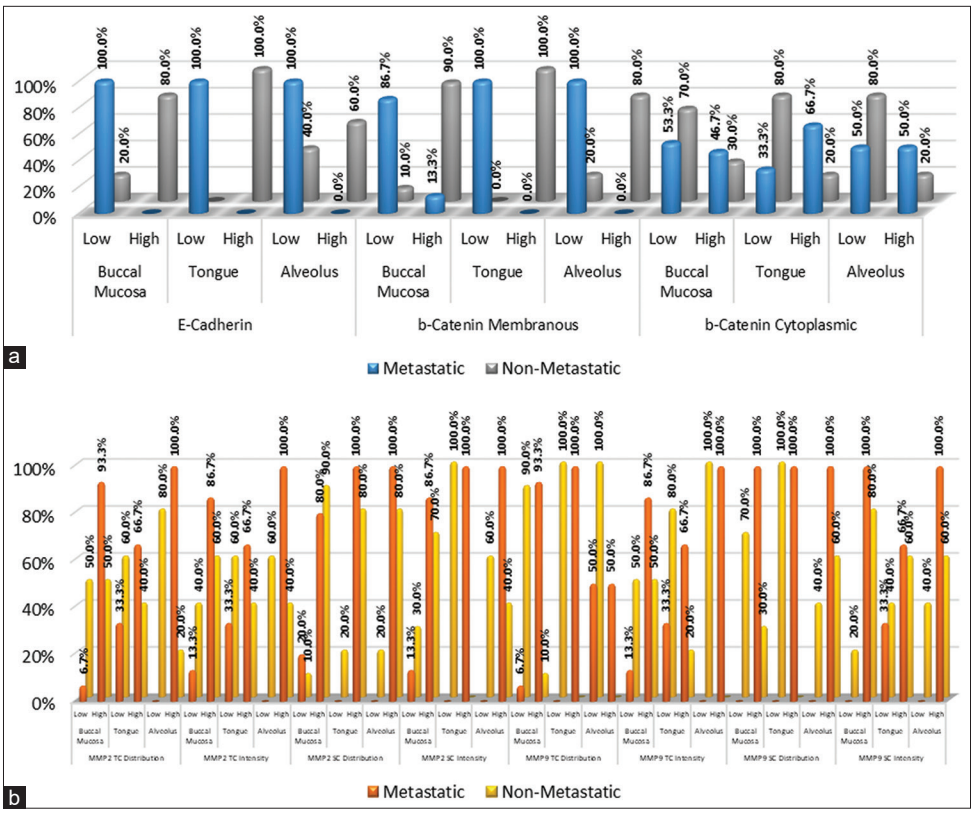

Figure 13: Bar graphs showing comparison of site of lesion with the protein expression of E-cadherin and membranous and cytoplasmic β-catenin in (a) and tumor and stromal cell distribution and intensity of matrix metalloproteinase-2 and matrix metalloproteinase-9 biomarkers in (b) between metastatic and non-metastatic oral squamous cell carcinomas

and stromal cells to be 7.04 (95% CI: 4.50–11.54) (*P* = 0.04), 4.89 (95% CI: 1.30–5.54) (*P* = 0.02), 2.80 (95% CI: 1.01–4.88) (*P* = 0.02), and 4.71 (95% CI: 1.57–7.26) (*P* = 0.01), respectively, relative to low expression [Table 5].

Metastasis of OSCC for MMP-9 gene expression showed a significant OR of 8.60 (95% CI: 1.30 ± 57.12) relative to non-metastatic cases (*P* = 0.03). Although the other EMT biomarkers showed a significant gene expression difference between the study groups, the univariate regression analysis did not reveal a significant difference [Table 6].

Studies have correlated the gene expressions of MMP-2 and MMP-9 with clinical features and have shown a

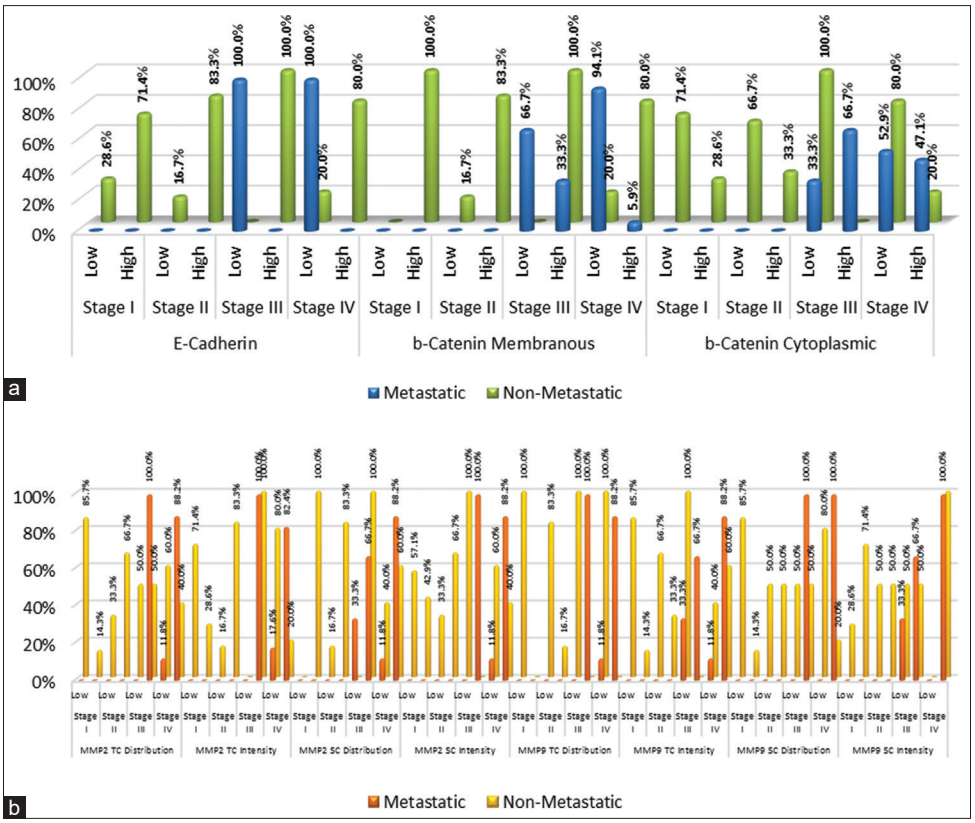

Figure 14: Bar graphs showing comparison of clinical staging with the protein expression of E-cadherin and membranous and cytoplasmic β-catenin in (a) and tumor and stromal cell distribution and intensity of matrix metalloproteinase-2 and matrix metalloproteinase-9 biomarkers in (b) between metastatic and non-metastatic oral squamous cell carcinomas

Table 6: Univariate regression model for the assessment of metastatic risk of oral squamous cell carcinoma based on the gene expression of epithelial-mesenchymal transition biomarkers

PCR	Mean±SD		*P*	OR	95% CI	
	Metastatic OSCC	Non-metastatic OSCC			Lower	Upper
E-cadherin	0.50±0.12	0.74±0.19	0.15	0.10	0.01	0.94
β-catenin	0.27±0.22	0.77±0.39	0.08	1.36	0.21	1.85
MMP-2	2.89±1.97	1.84±0.69	0.21	2.06	0.67	6.30
MMP-9	5.54±1.83	2.32±0.93	0.03*	8.60	1.30	57.12

OSCC: Oral squamous cell carcinoma, PCR: Polymerase chain reaction, MMP: Matrix metalloproteinase, SD: Standard deviation, CI: Confidence interval, OR: Odds ratio. *Statistically significant

high expression of genes with the highest incidence of metastasis and advanced pathological stages of cancer. However, they did not find a significant difference between expression of MMPs with age, sex, site of primary tumor, and histological grade.[22] These results correlate well with the current study findings.

The limitations of the study include smaller sample size, unequal distribution of samples from different oral sites, gender, and age groups. Future research needs attention on large samples involving tissues with equal distribution from different oral sites, gender, and age and correlates its relationship with metastasis.

Conclusions

This research focuses on the combined assessment of quantitative protein and gene expressions for E-cadherin, β-catenin, MMP-2, and MMP-9 biomarkers to differentiate the metastatic and non-metastatic groups of OSCC. Immunoexpression of EMT biomarkers – E-cadherin, membranous β-catenin, MMP-2, and MMP-9 – using FFPE samples may help in the prediction of OSCC cases with high metastatic risk. The gene expression of MMP-9 may be employed as an early predictor for metastasis in OSCC. This study is the first of its kind to employ texture and color segmentation in MATLAB as an objective tool to assess the protein expression of EMT biomarkers. This research is instrumental in studying the protein and gene expressions of EMT markers with clinical correlation. The low immunoexpression of E-cadherin and β-catenin and the high expression of MMP-2 and MMP-9 correlate with Stages III and IV showing high metastatic risk. Furthermore, the upregulated MMP-2

and MMP-9 mRNA expressions in advanced clinical stages of OSCC have high metastatic potential.

Acknowledgments

The authors sincerely thank Dr. Sheela SV, PhD, Professor in Information Science and Technology, B.M.S. College of Engineering, for providing expertise and training in MATLAB. We would like to thank Dr. Santhosh Kumar and Dr. Suvi Kanchan for the statistical analysis. We also thank Mr. Chetan Kumar and Mr. Raju, technicians, for performing staining procedures. We are extremely grateful to Dr. Anand Sreenivasan, Dr. Yogisha. S and the team of Skanda Life Sciences Pvt. Ltd for carrying out PCR work for the study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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