

## Evaluation of matrix metalloproteinase 9 gene expression in oral squamous cell carcinoma and its clinicopathological correlation

### ABSTRACT

**Background:** Matrix metalloproteinases (MMPs) are calcium-dependent and zinc-containing endopeptidases which enhance cancer progression by regulating angiogenesis, migration, proliferation, and invasion. Oral squamous cell carcinoma (OSCC) is one of the most common malignancies in India, and it is observed over 90% of cases. In OSCC, MMP9 which belongs to the gelatinase group promotes tumor progression by angiogenesis, disturbing tissue morphology that allows tumor growth which breaks the basement membrane and enables metastasis, and its overexpression in OSCC is proven to have prognostic value.

**Aim and Objectives:** To assess the expression of MMP9 in OSCC and to correlate the MMP9 expression with pathological staging of the OSCC.

**Materials and Methods:** Ten OSCC tissue samples and normal tissue samples were collected. Total RNA was extracted and the complementary DNA was generated. The specific primers used in the primers were synthesized. Total reaction volume was 20  $\mu$ l. The polymerase chain reaction condition included 95 C for 30 s followed by 40 cycles of two steps: 95 C for 5 s and 60 C for 30 s. The relative quantification of genes was evaluated.

**Results:** Upregulation of MMP9 gene regulation was observed in OSCC tissue samples when compared to the controls. Correlating with the pathological staging, we observed that 30% tumors were stage IVA with involvement of adjacent structures and 30% had lymph node involvement.

**Conclusion:** This study is done to assess the possibility of MMP9 gene expression as a prognostic biomarker for OSCC.

**Keywords:** Angiogenesis, gelatinase, matrix metalloproteinases, oral squamous cell carcinoma

### INTRODUCTION

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases which are secreted by macrophages, neutrophils, and fibroblasts.<sup>[1]</sup> MMPs play a crucial role in tumorigenesis by causing degradation of extracellular matrix (ECM) components.<sup>[2]</sup> This degradation of ECM in turn contributes to cancer cell migration.<sup>[2]</sup> They also participate in the release of growth-promoting signals, apoptosis, modulation of the immune response, and angiogenesis.<sup>[2]</sup> The stimuli from the transforming growth factor  $\beta$  and interleukin-8 (IL-8) are responsible for the synthesis of these enzymes.<sup>[1]</sup> Thus, MMPs maintain the bioavailability of growth factors promoting cancer proliferation.<sup>[1]</sup> MMPs cleave the FAS receptors and suppress the

natural killer cells, thereby resisting the apoptosis.<sup>[3,4]</sup> It also controls the process of angiogenesis and neovascularization

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by increasing the bioavailability of vascular endothelial growth factor (VEGF) receptor.<sup>[3,4]</sup> The role of MMPs in the cell-to-cell adhesion and cell-to-ECM adhesion is responsible for the promotion of malignancy.<sup>[3,4]</sup>

Oral cancer in India is frequently diagnosed with local or regional metastasis.<sup>[3,4]</sup> The overall survival rate for patients with oral cancer is among the lowest (<50%).<sup>[3,4]</sup> Studies have reported that only 15% of the patients are diagnosed when the disease is at a localized stage. Thus, patients with advanced disease most often reflect the spread of the disease to local, regional, and distant sites.<sup>[3,4]</sup> The treatment is either surgery/irradiation or a combination of both. The poor prognosis of the disease (recurrence and metastasis) is attributed to the extensive local invasion and spread to the lymph nodes.<sup>[3,4]</sup> Although the adjacent normal tissues of oral tumors might be clinically normal, molecular and biochemical changes can reveal vital information for prediction of tumor behavior, recurrence, and metastatic potential (concept of field cancerization).<sup>[5,6]</sup> Therefore, it is necessary to identify a better prognostic marker to categorize the patients for closer monitoring and surveillance or more intensive treatment. Although numerous efforts have been made to understand the molecular and cellular mechanisms involved in metastasis, it is documented that MMPs could predict prognosis of oral cancer.<sup>[7]</sup> MMP9 is associated with the aggressive nature of all cancers including oral squamous cell carcinoma (OSCC).<sup>[5,6]</sup> MMP9 causes type IV collagen degradation which is a major component of basement membrane (BM).<sup>[5,6]</sup> Elevated MMP9 protein expression is seen associated with recurrence, nodal, and distant metastasis.<sup>[5,6]</sup>

There are several studies that have been done previously to establish the relationship of MMPs to cancer invasion, progression, apoptosis, migration, and neovascularization in cancer.<sup>[3,4]</sup> Unlike MMP1 and MMP2 which are constitutive enzymes, MMP9 is an inducible enzyme. Hence, molecular and biochemical changes of clinically normal tissue could alter the levels of MMP9. Although various studies have been done to assess the immunohistochemical expression of MMP9, serum, and salivary levels of MMP9, gene expression of MMP9 in OSCC has not been studied. Thus, this study aimed to assess the expression of MMP9 in OSCC and to correlate the MMP9 expression with pathological staging of the OSCC. This would be the first step to ascertain if modulating MMP9 to achieve a better prognosis.

## MATERIALS AND METHODS

### Ethical approval and informed consent

The study was approved by the Institutional Ethics Committee of Saveetha Dental College and Hospitals (IHEC/

SDC/OPATH-1802/21/31). All patients were included after obtaining their voluntary informed consent.

### Patient description

After informed consent, tumor tissue was collected at the time of surgical excision from 10 treatment-naive OSCC patients from patients undergoing resection surgery, at the Department of Oncology after concordant histopathological diagnosis of the incisional biopsy. Adjacent clinically apparent normal tissues at least 1 cm away from the core of the lesion malignant tissues were taken from free margins. The staging was done according to the American Joint Committee on Cancer norms. Clinical details of the patients are presented in Table 1. These patients were treated by combinational therapy involving surgery, radiotherapy, and chemotherapy.

### Tissue samples

Tissue samples were immediately deep-frozen at  $-20^{\circ}\text{C}$ .

### Total RNA isolation, complementary DNA conversion, and real-time polymer chain reaction

Using a Total RNA Isolation Reagent Invitrogen (TRIR) kit, total RNA was isolated from healthy and OSCC patients samples. In brief, to 100 mg fresh tissue, 1 ml of TRIR was added and homogenized. The contents were transferred to a microcentrifuge tube instantly and 0.2 ml of chloroform was added, vortexed for 1 min, and then kept at  $4^{\circ}\text{C}$  for 5 min. Later, the contents were centrifuged at 12,000 g for 15 min at  $4^{\circ}\text{C}$ . The aqueous phase (upper layer) was carefully transferred to a fresh microfuge tube, and equal volume of isopropanol was added vortexed for 15 s and placed on ice for 10 min. After centrifugation of the content at 12,000 g for 10 min at  $4^{\circ}\text{C}$ , the supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol by vortex. The isolated RNA was estimated spectrometrically. The RNA concentration is expressed in micrograms ( $\mu\text{g}$ ).

### mRNA expression

Using the reverse transcriptase kit from Eurogentec (Seraing, Belgium), complementary DNA (cDNA) was synthesized from 2  $\mu\text{g}$  of total RNA as stated in the manufacturer's protocol. To perform real-time polymerase chain reaction (RT-PCR), the reaction mixture containing  $\times 2$  reaction buffer (Takara SYBR Green MasterMix), forward and reverse primers of the target gene, and housekeeping gene, water, and  $\beta$ -actin [the primer sequences are listed in Table 1] in total volume of 45  $\mu\text{l}$  except the cDNA was made, mixed intensively, and spun down. In individual PCR vials, about 5  $\mu\text{l}$  of control DNA for positive control, 5  $\mu\text{l}$  of water for negative control, and 5  $\mu\text{l}$  of template cDNA for samples were taken and reaction mixture (45  $\mu\text{l}$ ) was added. Forty cycles ( $95^{\circ}\text{C}$  for 5 min,  $95^{\circ}\text{C}$  for 5 s,  $60^{\circ}\text{C}$  for 20 s, and  $72^{\circ}\text{C}$  for 40 s) was set

**Table 1: Clinical details of patients with oral squamous cell carcinoma**

Age	Gender	Site	Grade of OSCC	Tumor size (cm)	Nodal involvement	pTNM staging
42	Male	Tongue	MDSCC	2-4	Absent	pT2N0M0
40	Male	Gingivobuccal sulcus	MDSCC	>4	Absent	pT4aN0M0
37	Male	Gingivobuccal sulcus	WDSCC	2-4	Present	pT2N1M0
46	Male	Buccal mucosa	MDSCC	2-4	Absent	pT2N0M0
44	Female	Tongue	MDSCC	2-4	Present	pT2N1M0
56	Male	Buccal mucosa	WDSCC	2-4	Present	pT2N1M0
58	Male	Buccal mucosa	MDSCC	>4	Absent	pT4aN0M0
43	Male	Buccal mucosa	MDSCC	2-4	Present	pT2N1M0
45	Male	Gingivobuccal sulcus	MDSCC	>4	Absent	pT4aN0M0
50	Male	Gingivobuccal sulcus	MDSCC	2-4	Absent	pT2N0M0

OSCC: Oral squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, pTNM: Pathological tumor-node-metastasis

up for the reaction, and the obtained results were plotted by the PCR machine (Stratagene MX3000P, Agilent Technologies, 530I, Stevens Creek Blvd, Santa Clara, CA, 95051, USA) on a graph. Relative quantification was calculated from melt curve and amplification curve analysis.

### Statistical analysis

One-way ANOVA using Tukey's multiple comparison test was used to compare the mRNA expression levels between different groups in both cases and controls. Comparisons were made between categorical groups by Chi-square test. A two-tailed  $P < 0.05$  was considered statistically significant.

## RESULTS

The clinical details of patients with OSCC are summarized in Table 1.

In our study, we observed that nearly 90% were male and 10% were female. We noted that nearly 60% of the patients were between 40–50 years of age and 90% were male patients. 40% of OSCCs were involving gingivobuccal sulcus and buccal mucosa each. Nearly 80% of the cases were graded histopathologically as moderately differentiated squamous cell carcinoma. 70% reported with tumor size of 2–4 cm, 40% had nodal involvement, and 30% of the tumor involved the adjacent structures such as skin and bone.

To measure the mRNA expression of MMP9 gene, 100 mg tissue from control and OSCC patients was homogenized and total RNA was isolated, and cDNA was converted from total RNA using reverse transcriptase enzymes. mRNA expression analysis of MMP9 was done using real-time PCR analysis using gene-specific primers.

MMP9 mRNA showed higher expression in OSCC tissue samples. Comparison of the expression of MMP9 mRNA among OSCC tissue samples and adjacent normal tissue

revealed statistically significant differences, with a  $P = 0.005$  [Figures 1 and 2].

## DISCUSSION

India has one-third of oral cancer cases in the world. Globacom in 2018 stated that 119,992 new cases were reported in India of which 72,616 deaths were reported.<sup>[8]</sup> The most significant characteristics of OSCC are invasion into adjacent structures and distant metastasis.<sup>[9]</sup> This is caused by degradation of the ECM by the cancer cells.<sup>[9]</sup> MMPs are zinc-dependent endopeptidases that efficiently degrade the components of the ECM and BMs which are secreted by these tumor cells.<sup>[10]</sup> MMP9 is expressed in OSCC cells and inflammatory cells around carcinoma islands.<sup>[10]</sup> The increased expression of MMP9 may be associated with a shortened disease-free survival and a high metastatic frequency.<sup>[10]</sup> Thus, this study was done to assess the MMP9 gene expression in OSCC tissue samples.

In our study, we observed that nearly 60% of the patients were between 40 and 50 years of age and 90% were male patients. 40% of OSCCs were involving gingivobuccal sulcus and buccal mucosa. Nearly 80% of the cases were graded histopathologically as moderately differentiated squamous cell carcinoma. We observed elevated expression of MMP9 gene expression in OSCC tissue when compared to the adjacent normal tissue. Various studies have reported that MMP9 is associated with the aggressive nature of OSCC.<sup>[11,12]</sup> Ikebe *et al.* stated that the gelatinolytic activity and increased expression of both MMP2 and MMP9 in OSCC tumors were related to the invasiveness, but not to the metastatic potential of OSCC tumors.<sup>[13]</sup> Few studies have reported that high levels of MMP9 expression in OSCC patients were correlated with regional lymph nodes or distant metastases.<sup>[11,12]</sup> This contributed to the poor prognosis in OSCC tissue samples.<sup>[11,12]</sup> Kato *et al.* demonstrated that MMP9 expression was high in OSCC and was associated with

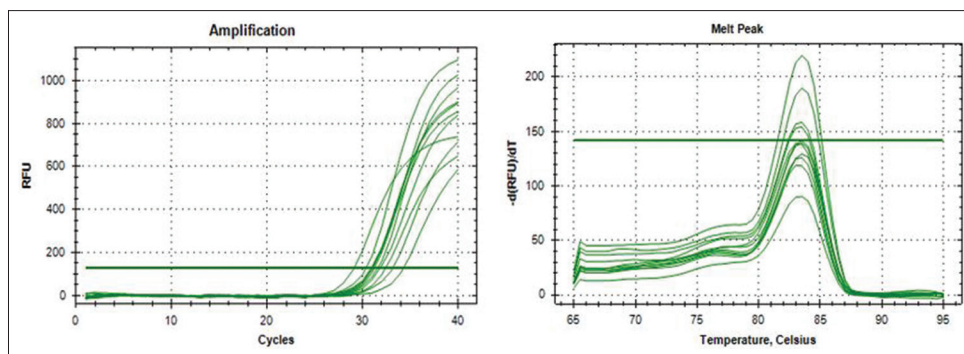


Figure 1: Real-time polymerase chain reaction analysis showing the mRNA expression of matrix metalloproteinase 9 in oral squamous cell carcinoma tissue samples. The left graph indicates the amplification curve and the right graph indicates the melt peak, X-axis denotes the number of cycles while Y-axis denotes the intensity of fluorescence. The melt curve analysis was performed in order to check the single product of matrix metalloproteinase 9

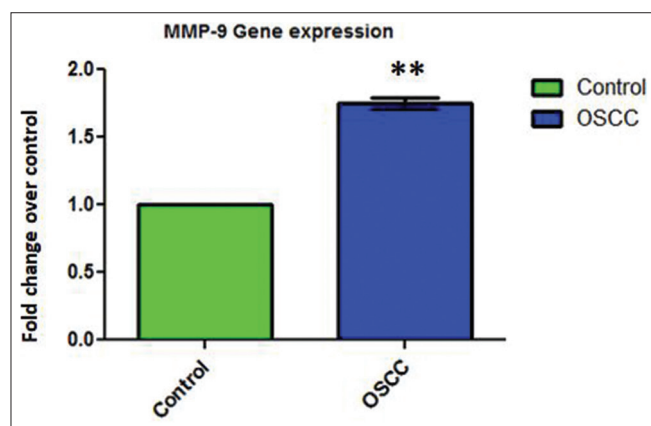


Figure 2: As we could see in Figure 2, X-axis indicates groups and Y-axis denotes expression of MMP9, mRNA is represented in fold change over control. The result shows that matrix metalloproteinase 9 expression was significantly increased (\*\* $P = 0.05$ ) in oral squamous cell carcinoma when compared to control

advanced stages of disease.<sup>[14]</sup> However, many researchers have presented contradictory results. Guttman *et al.* reported no significant correlation between MMP9 expression and the size of the primary tumor or the neck metastasis in tongue SCC patients.<sup>[15]</sup> de Vicente *et al.* concluded that MMP9 expression was not associated with clinical variables, such as tumor stage or recurrence rate.<sup>[16]</sup>

MMP9 causes type IV collagen degradation which is a main component of BMs, thereby attributing to the aggressive nature of the OSCC.<sup>[10-12]</sup> It is believed that MMP9 is upregulated by SNAIL (transcription factor) which triggers epithelialmesenchymal transition which facilitates migration of the carcinoma cells by altering the morphology and reducing the cell adhesion molecules.<sup>[10]</sup> MMP9 inhibits angiogenesis by releasing antiangiogenic factors from their precursors and enhances angiogenesis by releasing and activating VEGF from extracellular proteoglycans.<sup>[10-12]</sup> Thus, it is believed to play a dual role in the regulation of angiogenesis. The proteolytic degradation of ECM

components such as collagen III, IV, and V collagens, as well as gelatin by MMP9, facilitates OSCC cell invasion which facilitates the release of growth factors, such as VEGF, that enhance angiogenesis and tumor progression.<sup>[10-12]</sup> It is also believed that anti-angiogenic endostatin, angiostatin, and tumstatin are released.<sup>[10-12]</sup> MMP9 mediates proinflammatory chemokines (CXCL-1, -4, -8, -9, -11, and -12), proforms of cytokines (proTNF- $\alpha$ , proIL-1 $\beta$ ), and cell adhesion proteins such as intercellular adhesion molecule-1 that triggers an inflammatory reaction and modulates transcription factors, leading to an epithelial-to-mesenchymal transition and enhanced carcinogenesis.<sup>[10]</sup>

#### Limitations

The limitations of this study include the small sample size. Only gene expression of MMP9 was assessed; however, correlation with the serum MMP9 levels was not assessed. The MMP9 gene expression along with survival of the patients was not done.

#### CONCLUSION

Significant elevation of the MMP9 gene was observed in OSCC. These elevated MMP9 levels were seen associated with the advanced stage of OSCC. The study needs to be expanded on a larger scale.

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Nil.

#### Conflicts of interest

There are no conflicts of interest.

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