



## ORIGINAL RESEARCH ARTICLE

### IMMUNOHISTOCHEMICAL EXPRESSION OF MMP-2 IN AN EXPERIMENTAL PROGRESSION MODEL OF ORAL CANCER

B SHRESTHA<sup>1\*</sup>, S SUBEDI<sup>1</sup>, D BAJRACHARYA<sup>2</sup>, R RADHAKRISNAN<sup>3</sup>

<sup>1</sup> Department of Oral Pathology, Chitwan Medical College, Bharatpur, Chitwan.

<sup>1</sup> Department of Oral Pathology and Microbiology, Kantipur Dental College, Kathmandu.

<sup>1</sup> Department of Oral and maxillofacial Surgery, Manipal college of dental Sciences, Manipal, Karnatak, India.

\*Correspondence to: Dr Bijayata Shrestha, Department of Oral Pathology, , Chitwan Medical College, Bharatpur, Chitwan, Nepal.  
Email: bijayata\_shr@yahoo.com

#### ABSTRACT

The progression from normal to dysplasia to carcinoma involves series of molecular events. Matrix metalloproteinase-2 (MMP-2), proteolytic enzyme causes the degradation of the basement membrane, which is a primary step in cancer invasion and progression. The aim of this study was to compare the expression of MMP-2 in normal mucosa (NOM), oral epithelial dysplasia (OED) and Oral Squamous cell Carcinoma (OSCC). MMP-2 expression was evaluated immunohistochemically in NOM (n = 10), OED in each of its histological grades (n = 26) and OSCC of the lining mucosa (n = 19). A semi quantitative retrospective analysis was carried out in 100 cells in each of 5 different fields at 40x magnification in each case and the proportion of cells expressing MMP-2 were estimated. Suitable positive and negative controls were used for all our analysis. Expression scores of basal cells as well as adjacent stromal cells of OED and tumor cells as well as adjacent stromal cells of OSCC were analyzed. Expression of MMP-2 increased from NOM to OED through different grades to OSCC. Mann Whitney U test for comparison of expression for MMP-2 was statistically significant. However, the comparison for the expression of MMP-2 in mild and moderate dysplasia (p=0.018) as well as moderate and severe dysplasia (p=0.026) was not statistically significant. Increase in the expression of MMP-2 from normal oral mucosa to dysplasia to oral cancer suggests it as an early event in oral carcinogenesis.

**Key Words:** Immunohistochemistry, Matrix metalloproteinase, Oral Cancer, Oral epithelial dysplasia.

#### INTRODUCTION

Cancer is one of the major threats to public health. Oral cancer is the most common head and neck cancer and constitutes nearly one third of all cancer cases in our part of world. Oral squamous cell carcinoma (OSCC) represents more than 90% of all oral cancers.<sup>1</sup>

Leukoplakia and erythroplakia are recognized as most common potentially malignant lesions in the oral cavity. Till date, the gold standard for the assessment of oral potentially malignant lesions is microscopic evaluation of haematoxylin and eosin stained sections for the presence of architectural and cytological changes, which are generally referred to as oral epithelial dysplasia (OED).<sup>2</sup>

The transition from dysplasia to an invasive lesion is an important step in carcinogenesis which requires a number of biological events, including the degradation of basement membrane.<sup>3,4</sup> The role of proteolytic enzyme, Matrix metalloproteinases (MMPs) have been implicated in the degradation of epithelial basement membrane. The MMPs are zinc and calcium-dependent enzymes and are secreted by both stromal and tumor cells.<sup>5,6,7</sup> Among the MMPs, the type V collagenases (MMP-2 and MMP-9) showed the highest activity against type IV collagen. Among this class, the MMP-2 has the highest collagenolytic activity and is

associated with tumor progression.<sup>4,8</sup> MMP-2 immunoexpression is shown to be increased in malignant neoplasias and immunohistochemistry overexpression is almost always related with worst prognosis.<sup>8</sup>

Therefore, the aim of the study was to compare the expression of MMP-2 in normal, OED and oral cancer. The objective was to elucidate the role of MMP-2 in the experimental progression model of oral carcinogenesis.

#### MATERIALS AND METHODS

The material for the present study included formalin fixed paraffin embedded (FFPE) tissue sections from Normal oral mucosa (NOM) (n = 10), Oral epithelial dysplasia (OED) in each of its histological grades of mild, moderate and severe (n = 26) and Oral squamous cell carcinoma (OSCC) of the lining mucosa (n = 19) retrieved from the departmental archives, College of dental science, Manipal, from year 2007 to 2010. A retrospective analysis was carried out to study the expression of MMP-2 in all of these cases by immunohistochemistry (IHC). Tissue sections obtained from inflammatory bowel disease constituted the positive control for MMP-2 and endothelial cells were taken as internal control.

All the cases were selected on the basis of strict inclusion and exclusion criteria. Only male patients aged above forty with tobacco habits who were histologically confirmed cases of OED and OSCC and in whom the treatment had not begun at the time of biopsy were included. Other proliferative lesions like proliferative verrucous leukoplakia, verrucous carcinomas and carcinomas from sites other than the lining mucosa were excluded.

#### **Hematoxylin (H) and Eosin (E) staining for grading**

For H and E staining, the sections were first deparaffinized in xylene for 5 minutes and rehydrated in descending grades of alcohol (100%, 70% and 50% alcohol) for 1 minute each and then brought to water before staining. The sections were then stained with hematoxylin for 1 minute and washed in water. For differentiation, the sections were dipped in 1% acid alcohol for 5 seconds and then in lithium carbonate and held under running tap water for 5 minutes. The sections were then counter stained in 1% eosin and washed under running tap water. Following this step, the sections were dehydrated through two changes of absolute alcohol, cleared in xylene and mounted in DPX to observe under light microscope.

#### **Immunohistochemistry**

Immunohistochemical staining was carried out on tissue sections by polymer chain two step indirect technique. MMP-2 monoclonal antibodies were obtained from Novocastra Leica Biosystems, Newcastle Ltd. Four  $\mu\text{m}$  thick sections were obtained from FFPE tissue blocks and taken on 2 separate glass slides. The section on the first uncoated slide was stained with H and E for grading. While the second slide coated with 3-aminopropyl triethoxy silane (APES, Sigma- Aldrich Co. St. Louis, USA) was subjected to immunohistochemical (IHC) staining using mouse antiMMP-2 monoclonal antibody. For negative control, immunohistochemical staining procedure was carried out in the same manner but the primary antibody was replaced with Tris buffered saline (TBS).

The sections taken on APES coated glass slides were incubated overnight at 48°C in slide warmer to ensure complete adhesion of section on to the slide. Sections were deparaffinized through 3 changes of xylene for 5 minutes each. Sections were then hydrated through descending grades of alcohol (100%, 95%, 70% and 50%) for 5 minutes each and washed in distilled water for 3 minutes. Following which the slide around the tissue sections were dried with an absorbent wiper and encircled using a diamond marker. This marking would prevent the reagents from flowing over.

For antigen retrieval, the Tris EDTA solution (Tris- 1.2gms and EDTA 0.4gms in 1000ml of water, pH 9.0) was initially boiled at 800W in microwave oven for 5 minutes. The slides were then immersed completely in the antigen retrieval solution and the power level of 800W was maintained for 5 minutes. The temperature was lowered to 600W for 10 minutes and furthered lowered to 450W for 5 minutes. The antigen retrieval solution was allowed to cool at room temperature. The slides were washed with TBS (pH 7.6) twice for 5 minutes and wiped with tissue paper.

For endogenous peroxidase blocking, sections were incubated in 3% hydrogen peroxide/methanol for 20 minutes in a moist chamber following which the slides were dabbed with a tissue paper and agitated in TBS (pH 7.6) twice for 5 minutes. To block any non-specific antigenic sites, non specific binding sites were blocked by protein block for 10 minutes provided by Novocastra Leica Biosystems, Newcastle Ltd. In place of this, 5% casein (milk powder) in TBS (500mg of milk powder in 10ml of TBS) for 1hour was equally effective in blocking non specific antigenic sites. The slides were drained off the reagent and the sections were washed in TBS twice for 5 minutes. Incubation with primary antibody was carried out by covering the sections completely with monoclonal primary antibody diluted with Tris buffer pH 7.6 (1ml of MMP-2 reconstituted with 1ml of sterile distilled water and diluted at 1: 20) and incubated at 37°C for 3 hour in a moist chamber. Post primary block was done by a polymer penetration enhancer containing 10% (v/v) animal serum in TBS/0.09% ProClin™ 950. The sections were completely covered and incubated for half an hour in moist chamber and thereafter the sections were washed in TBS twice for 5 minutes. Incubation with secondary antibody was carried out at room temperature for 30 minutes in a moist chamber applying anti-mouse IgG-Poly-HRP (8 $\mu\text{g}$ /ml) containing 10% (v/v) animal serum in TBS. The sections were washed thoroughly with TBS twice for five minutes with gentle rocking thereafter. For visualization of this reaction, sections were incubated with chromogen containing 1.74% w/v 3, 3' - diaminobenzidine (DAB) in a stabilizer solution. DAB Chromogen required dilution up to 1/20 in NovoLink™ DAB Substrate buffer prior to use. 50 $\mu\text{l}$  of DAB Chromogen was added to 1ml of NovoLink™ DAB Substrate Buffer (Polymer) and used immediately. For visualization, sections were covered with diluted DAB chromogen for about 6 minutes. This was followed by washing the slides in running tap water and counterstained in Mayer's hematoxylin for 5 minutes after which the slides were washed in running tap water for 1 minute. Briefly, the sections were dehydrated through ascending grades of alcohol (70%, 95% and 100%) for 2 minutes each and cleared in xylene for 1 minute and mounted with a resinous media and cover slipped.

#### **Staining Interpretation**

To quantify the expression of MMP-2, we evaluated the number of MMP-2 positive cells. A positive cell demonstrated a diffuse brown signal in the cytoplasm of cells, independent of its intensity. However, tissue sections were scored based on the proportion of cells expressing MMP-2 and TIMP-2 positivity. The immunoreactivity in the tumor cells and the stromal cells was scored from 0-3 based on the criteria put forth by Roukolainen et al.<sup>9</sup> Tissue sections were considered positive even if 1% of tumor cells showed positive staining. The antibody expression was assessed in the basal, parabasal cells of dysplastic epithelium and the adjacent stroma in different grades of OED and antibody expression in tumor cells in the islands and stromal cells between the tumor islands was assessed in OSCC.

The evaluation of positive cells was assessed in five different fields at x 40 magnifications and a total of 500 cells were examined. To eliminate any inter observer bias the scoring was carried out independently by two observers. Cells were graded

as low (+) for MMP-2 when 1-25% of the basal cells/ tumor cells and/or stromal cells stained positively, moderate (++), when 26-50% of the basal cells/ tumor cells and/or stromal cells stained positively and strong (+++) when more than 50% of the basal cells/ tumor cells and/or stromal cells stained positively for MMP-2.

### Statistics

Two observers independently assessed the proportion of positive cells. Statistical analysis was carried out using SPSS (Statistical package for social service) version 16.0 for Windows. Kendall's tau-b statistics was applied to assess the measure of agreement between two observers. Frequency analysis was done to calculate the median measure of expression of MMP-2 in the normal, different grades of OED and Oral cancer. Mann Whitney U test was used to compare the median expression of MMP-2.

### RESULT

Expression of MMP-2 was observed as diffuse brown cytoplasmic staining. Expression score of basal cells as well as adjacent stromal cells of OED and tumor cells as well as adjacent stromal cells were analyzed. Kendall's tau-b measure of agreement between the two observers for MMP-2 expression showed a very high agreement (0.947,  $p < 0.001$ ). Thus the reading of only one observer was taken for statistical analysis. The expression of MMP-2 was found in the positive control (Figure.1). Expression of MMP-2 increased progressively from normal (Fig. 2) to dysplasia (Figure. 3, 4 and 5) to OSCC (Figure. 6) as shown in bar diagram (figure. 7). Median measure of expression of MMP-2 increased from NOM, OED through different grades and OSCC as shown in Figure. 8. Mann Whitney U tests for comparison of these median expressions for MMP-2 (Table 1 and 2) were statistically significant. However, the comparison for the expression of MMP-2 in mild and moderate dysplasia ( $p=0.018$ ) as well as moderate and severe dysplasia ( $p=0.026$ ) was not statically significant (Table 1 and 2).

**Table 1: Comparison of expression of MMP-2 in NOM, OED, OSCC**

Variable	Cases		Median Q2(Q1-Q3)	P Value
MMP-2	Oral Epithelial Dysplasia	Normal mucosa Oral Sqamous Cell Carcinoma	15(8-20)	<0.001
MMP-2	Oral Squamous Cell Carcinoma	Normal Oral Epithelial Dysplasia	33.95 (19.55-53.75)	<0.001

**Table 2: Comparison of expression of MMP-2 in mild, moderate and severe OED and in OSCC**

Variable	Cases		Median Q2(Q1-Q3)	P Value
MMP-2	Mild dysplasia	Moderate Dysplasia	6(0-15)	=0.018 (NS)
		Severe Dysplasia		<0.008
		Oral Squamous cell Carcinoma		<0.008
MMP-2	Moderate Dysplasia	Severe Dysplasia	15(10-19)	=0.026 (NS)
		Oral Squamous cell Carcinoma		<0.008
MMP-2	Severe Dysplasia	Oral Squamous cell Carcinoma	33.95(19.55-53.75)	<0.008

### DISCUSSIONS

Oral carcinogenesis is a multistep phenomenon whose progression is segregated into the early and late stages. Earliest morphological changes are potentially malignant oral lesions of Leukoplakia and Erythroplakia with dysplasia. <sup>2</sup> A series of molecular events bring about this progression from normal to dysplasia to carcinoma in situ to invasion and eventual metastasis. <sup>10</sup> Tumor invasion and metastasis is associated with controlled proteolysis, and involves interactions between the tumor cells and the extracellular matrix (ECM). <sup>11</sup> During this process, the cells acquire the invasive phenotype because of the ability to secrete or induce lytic enzymes, such as MMP-2 which destroys the ECM barriers facilitating the transition from OED to OSCC. MMP-2 mediates the generation of promigratory signals by disrupting the cell to cell contact. MMP-2 also participates in the activation of latent GF, VEGF and TGF, thus promoting angiogenesis and tumor growth. <sup>12,13</sup> MMP expression is tightly regulated at transcriptional level by several cytokines, growth factors and EMMPRIN and at the post transcriptional level by cell surface activation of proMMP-2 which forms a ternary complex with MT1MMP & TIMP-2. <sup>12, 13, 14, 15, 16</sup>

MMPs are considered to be secreted as proenzyme form by tumor as well as stromal cells and the activation of the proenzymes is one of the critical steps that lead to ECM breakdown. <sup>17</sup> It is apparent that stromal cells have the ability to synthesize proforms of MMPs and may provide them to the neoplastic epithelial cells. Thus it seems possible that the neoplastic cells receiving proenzymes from surrounding stromal cells activate them by their own MT1-MMP. This suggests that MMPs produced by tumor cells and stromal cells are involved in tumor progression and metastasis. <sup>18</sup> Our study thus confirms previous findings and provides further evidence that both tumor

cells and the intervening stromal cells are the major sources of MMPs.

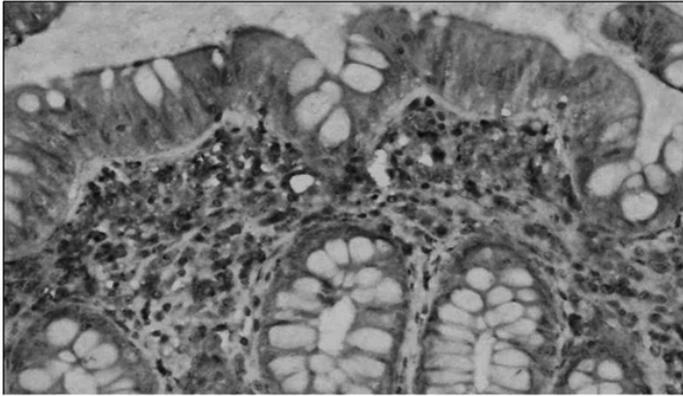


Figure 1: Inflammatory bowel disease showing positive expression of MMP-2 (20x).

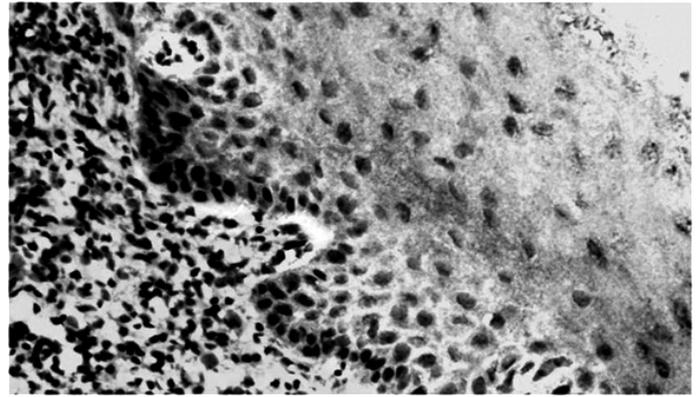


Figure 4: Moderate OED showing moderate (++) expression of MMP-2 in basal and adjacent stromal cells (20x).

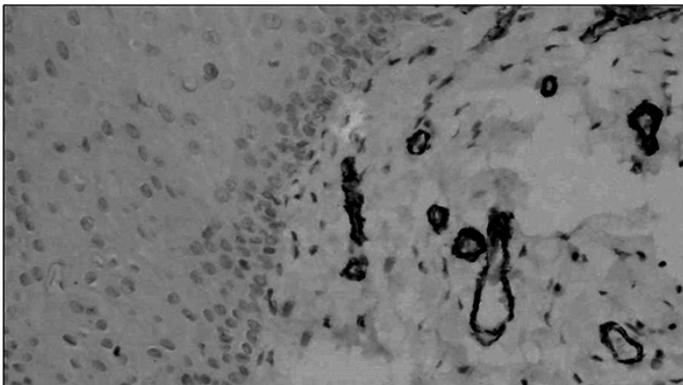


Figure 2: Normal oral mucosa showing positive expression of MMP-2 in endothelial cells (20x).

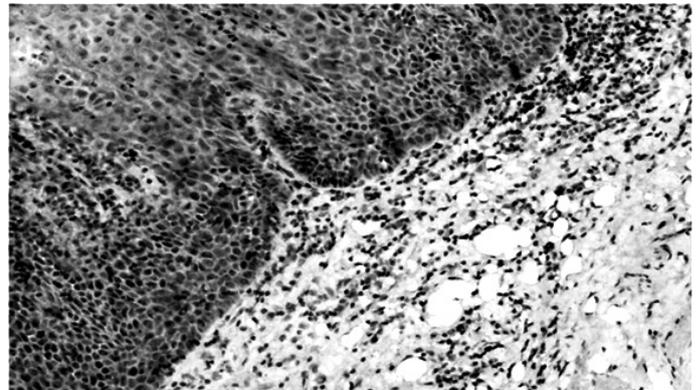


Figure 5: Severe OED showing moderate (++) expression of MMP-2 in basal and adjacent stromal cells (20x).

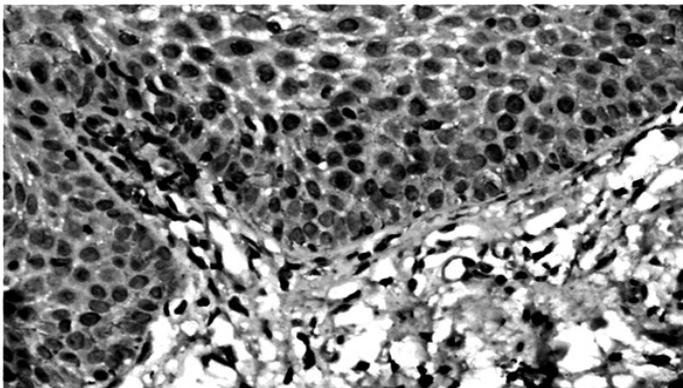


Figure 3: Mild OED showing low (+) expression of MMP-2 in basal and adjacent stromal cells (20x).

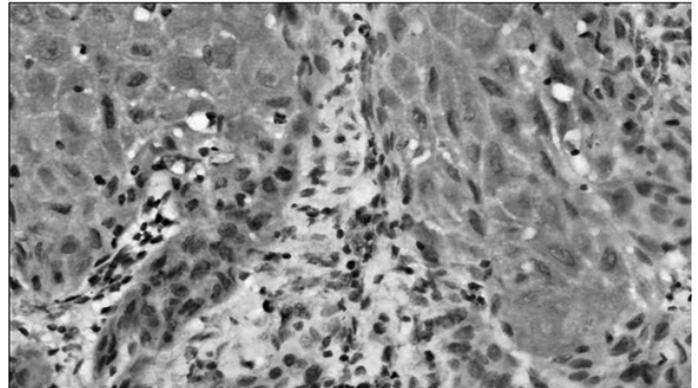


Figure 6: OSCC showing strong (+++) expression of MMP-2 tumor cells and adjacent stromal cells (20x).

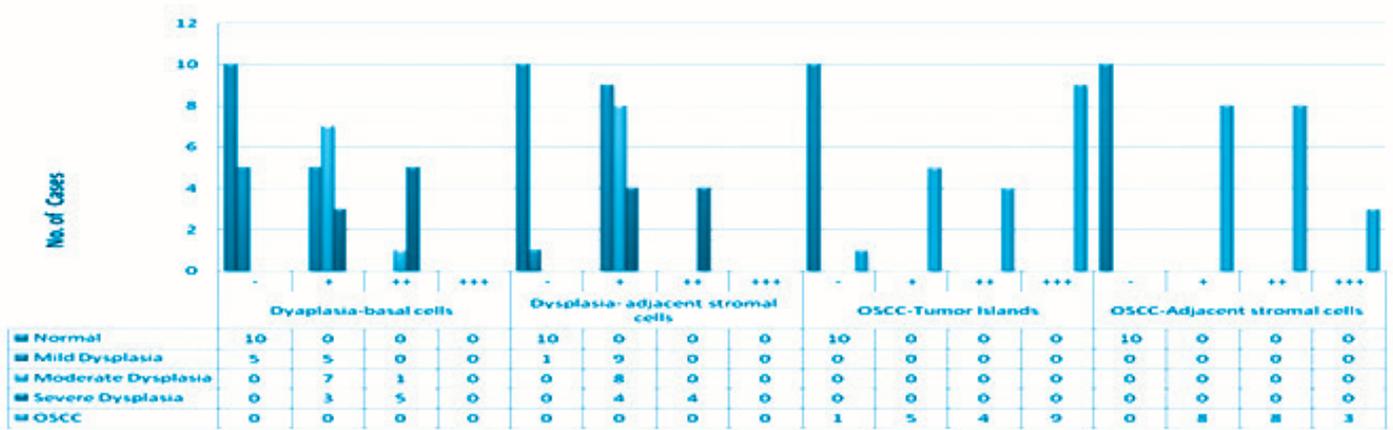


Figure 7: Bar diagram showing expression score of MMP-2 in normal, mild, moderate and severe dysplasia and OSCC.

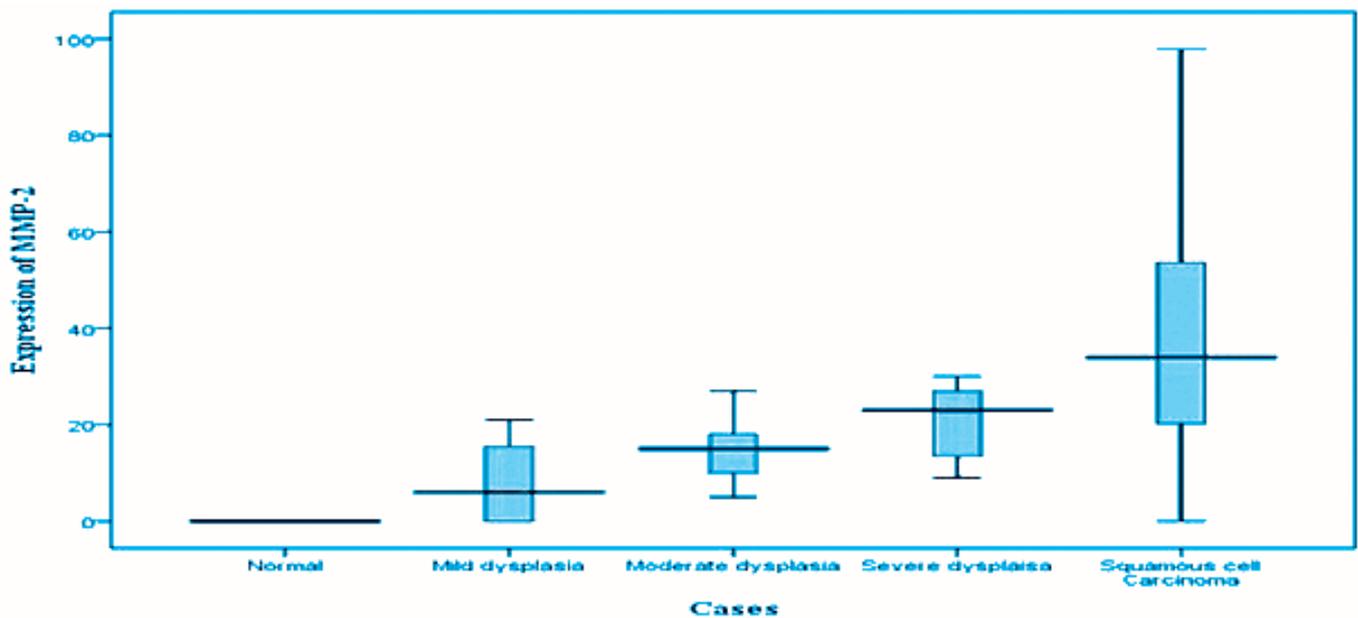


Figure 8: Box plot showing median expression of MMP-2.

In this study the expression of MMP-2 was absent in normal oral mucosa, which is in agreement with Bindhu et al.<sup>3</sup> and Sutinen et al.<sup>4</sup> Endothelial cells showed positive expression which is in accordance to studies that indicated the endothelial cells secrete relatively high concentrations of immunoreactive, but functionally inactive metalloproteinases. This difficulty in demonstrating metalloproteinase activity by the endothelial cells was attributed to production of large amounts of TIMP-1 and -2 by the endothelial cells which form a complex with and inhibits MMP activity.<sup>5</sup>

The MMP-2 expression was noted in dysplastic cells/ cancer cells as well as the stroma adjacent to it. Tumor cells interact with fibroblasts via extracellular matrix metalloproteinase inducer (EMMPRIN) and leads to fibroblast-induced secretion of MMP-2. In addition to the stimulation of stromal cells, EMMPRIN may also facilitate tumor cell dissemination by stimulating MMP production by both tumor cells and adjacent stroma.<sup>13</sup>

The expression of MMP-2 was significantly higher in OSCC compared to OED and NOM which is in agreement with Qu hong et al.<sup>6</sup> and Sutinen et al.<sup>4</sup> Although the difference in the median score of NOM, different grades of OED and OSCC was demonstrable, the expression of MMP-2 was not statistically significant between the histological grades of mild and moderate dysplasia or moderate and severe dysplasia suggesting the expression of MMP-2 does not correlate with histological grades of dysplasia. Our result thus indicates that increase in the expression of MMP-2 occurs in early stage of oral carcinogenesis.

**CONCLUSION**

Increase in the expression of MMP-2 from normal oral mucosa to dysplasia to oral cancer suggests it as an early event in oral carcinogenesis. It may act as a potential marker to identify the subset of oral epithelial dysplasia which progress to oral cancer.

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