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CLINICAL SIGNIFICANCE OF SALIVARY MATRIX METALLOPROTEINASE-9 IN ORAL PRECANCEROUS CONDITIONS AND ORAL CANCER

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ABSTRACT

Background: Oral cancer is a one of the major health burden worldwide and also in India. The most strenuous problem in oral cancer is metastasis, where use of newer non-invasive biomarkers for early detection of metastatic tumors would be greatly helpful. Aim: The aim was to evaluate salivary matrix metalloproteinase-9 in patients with oral precancerous conditions (OPC) and oral cancer to assess its utility in monitoring oral cancer progression. Subjects and Methods: Total 250 subjects including 100 controls, 50 patients with OPC and 100 oral cancer patients were enrolled for the study. Gelatin zymography was performed for the evaluation of matrix metalloproteinase (MMP)-9 and truncated 42 kDa MMP. Statistical Analysis: Statistical analysis like Student's independent't' test, Receiver's Operating Characteristic (ROC) curve, correlation and multivariate analysis were performed to analyze the results. Results: A truncated form of MMP i.e. 42 kDa was observed to have gelatinolytic activity. An increasing trend of salivary pro MMP-9, active MMP-9, MMP-9 activation ratio and truncated 42 kDa MMP was observed from controls to patients with OPC to oral cancer patients. ROC curve analysis revealed that all forms of MMPs could significantly discriminate controls and oral cancer patients; moreover, pro MMP-9 and active MMP-9 significantly distinguished patients with OPC and oral cancer patients. All the levels were found to be higher in patients with advanced disease and with metastasis as compared to patients with early disease and without metastasis, respectively. Conclusion: Elevated levels of salivary MMP-9 and truncated 42 kDa MMP in patients with OPC and oral cancer may be important for assessing early changes occurring during neoplastic transformation of oral cancer.

Keywords: Matrix metalloproteinase-9, Saliva, Oral precancerous conditions, Oral cancer.

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1. INTRODUCTION

Oral cancer is eleventh most common cancer in the world and account for two-third of deaths in developing countries [1]. In India 47,653 deaths due to oral cancer are registered in 2008 according to Global Cancer Statistics, 2011 [2]. The most strenuous problem in oral cancer is presence of metastasis right at the time of diagnosis. Currently lack of an easy-to-use and inexpensive sampling method and lack of an accurate and portable platform to understand the pathogenesis of the disease are the major limitations which have seriously hampered the development of appropriate clinical diagnostics. Early detection and ideal biomarkers are needed for controlling the disease progression, which may dramatically reduce the severity of its impact on the patients' life. Matrix metalloproteinases (MMPs) are the enzymes which plays important role in metastasis and invasion by proteolytic degradation of extracellular matrix (ECM), disruption of cell-cell and cell matrix adhesion, migration and angiogenesis [3-5]. Earlier reports have suggested the loss of E-cadherin, a cell adhesion molecule in oral precancerous conditions (OPC) [6]. Further MMPs are well-known for modulating the cell-cell and cell-ECM interactions affecting both cell phenotype (epithelial mesenchymal transition) and increasing cell migration[3]. Hence, understanding MMPs in oral precancerous conditions would help in monitoring early events in tumorigenesis. MMP-9, a gelatinase causes degradation of type IV collagen. MMP-9 levels are up-regulated in various malignancies including colorectal, gastric, thyroid, ovarian, bladder, lung, larynx, pancreatic, prostate cancer and oral cancer [5, 7-9]. Most of the studies correlating MMPs with cancer outcome have analyzed tissue/plasma specimen. Earlier reports have indicated elevated tissues and plasma levels of MMP-9 in oral cancer patients [7-9]. Earlier studies have indicated weak up-regulation of MMP-9 mRNA in tissues of oral lichen planus and dysplastic lesions [10].

Although MMPs are known as key mediators of cancer invasion, their involvement in oral premalignant conditions is not documented. Saliva offers an attractive non-invasive tool for assessing biomarkers in oral cancer due to its direct contact with sites of the lesions. Recently there is eye catching advancement in salivary based diagnostics [11-13]. Various gelatinolytic activities were observed in saliva and studies have suggested a 42kDa truncated form of enzyme which was due to autoactivation of gelatinolytic MMPs [14]. Gelatin zymography offers the advantage of estimating both pro and active forms of gelatinases (MMPs) [7, 9]. Hence in the present study gelatin zymography was performed which is cost effective alternative to ELISA method [7].

As per our knowledge there are no earlier reports on simultaneous evaluation in patients with OPC and oral cancer of salivary MMP-9 and truncated 42 kDa MMP. Studying MMPs in premalignant lesions might help in monitoring the early changes occurring during oral cancer progression. Hence, the present study was undertaken to evaluate salivary levels of gelatinolytic MMP-9 and truncated 42 kDa MMP in patients with OPC and oral cancer patients to understand the utility of saliva a non-invasive tool in monitoring changes occurring during neoplastic transformation of oral cancer.

2. MATERIALS AND METHODS

Study subjects: The study was approved by Institutional Review Board of The Gujarat Cancer & Research Institute, Ahmedabad, Gujarat, India. Due consent was obtained from all the subjects who participated in the study. The study enrolled 100 untreated oral cancer patients with no major disease in recent past, 50 patients with OPC as pathological controls, and 100 healthy controls. Out of 50 patients with OPC, 39 patients were with oral sub mucous fibrosis and 11 patients withhomogenous leukoplakia. Pathological tumor, node and metastasis (TNM) staging of oral cancer patients after surgical resection was determined as per American Joint Committee on Cancer (AJCC) norms [15]. The age ranges were 19-56 years of controls, 16-65 years of patients with OPC and 19-73 years of oral cancer patients. 87% (N=87) of oral cancer patients, 100% (N=50) of the patients with OPC and 50% (N=50) of controls were tobacco habituates. Most of the tobacco habituates were with tobacco chewing habit. The clinico-pathological details are as mentioned in Table 1. In 6% of the patients surgery was not performed so the pathological staging could not be evaluated.

Sample Collection: Morning fasting saliva samples were collected between 8.30 to 10.00 a.m. to avoid any possible diurnal variation in the study. For saliva collection standard protocols were followed [16]. Briefly the subjects rinsed their mouth thoroughly with water and then threw off that water. Unstimulated whole saliva was collected in 50 ml falcon tube kept on ice, and was processed immediately. The saliva was centrifuged at 2600g for 15 minutes at 4°C. The supernatant was collected in different aliquots and protease inhibitors were added as follows: 1µl Aprotinin (1mg/ml), 3µl Sodium orthovananadate (40mM), and 1µl Phenyl methyl sulfonyl fluoride (PMSF) (1mg/ml) was added in 100µl of saliva supernatant [16]. After the addition of protease inhibitors the saliva supernatants were immediately stored at - 80°C until analyzed.

Total Proteins: Total protein levels from saliva were determined using the Lowry's method [17]. Briefly, after mixing 10µl of saliva samples with 490µl of distilled water and 2.25 ml of Reagent C, the reaction mixture was incubated for 10 minutes at room temperature. Thereafter, 0.25 ml FolinCiocalteau reagent was added and after incubation for 30 minutes, the tubes were read spectrophotometrically at 750 nm. The standard curve was prepared using bovine serum albumin (Sigma, USA) as standard in concentration range of 10-60 µg.

Gelatin zymography: Gelatin Zymography was performed using SDS-PAGE (containing 0.5mg/ml gelatin) electrophoresis as described by Lorenzo, et al. [18]. For standardization, zymography was performed using a broad range of protein concentrations and finally 10µg of salivary protein was standardized. Subsequently in the experiments, 10µg of salivary protein was mixed with sample buffer dye without reducing agent and electrophoresed on 7.5% polyacrylamide gel at 4°C. Gels were washed and incubated overnight in 50mM Tris HCl pH 7.5; containing 10mL $CaCl_2$, 1uM $ZnCl_2$, 0.02% (W/V) NaN_3 and 1% (V/V) Triton X-100. Next day, the gels were stained with 0.1% (W/V) Coomassie Brilliant Blue R-250 and destained in 7% (V/V) acetic acid. The bands appeared as clear bands against a dark background. These zymograms were quantitated using gel documentation system (Alpha Innotech, USA). The

integrated density value (IDV) i.e. the sum of all the pixel values after background correction, was determined for each proteinase activity. The reproducibility of the samples was checked by running the samples in same gels as well as in different gels. Standards of pro MMP-9 (92 kDa) and active MMP-9 (83 kDa) (Calbiochem, USA) and molecular weight markers were run for molecular weight analysis.

Statistical Analysis: Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) statistical software version 15.0. Student's independent't'test was performed to assess the level of significance. Pearson's correlation analysis was performed to assess the correlation between different forms of MMPs. Multivariate analysis of all the forms of MMPs i.e. Pro MMP-9, active MMP-9 and truncated 42 kDa MMP was performed to assess the correlation with various clinico-pathological parameters like stage, differentiation, metastasis, infiltration etc. Receiver's Operating Characteristic (ROC) curve is a meaningful statistical approach as it considers simultaneously both sensitivity and specificity of the parameters. It is performed to evaluate the diagnostic performance of a marker. The area under curve (AUC) AUC of 0.5 suggests valid discriminatory efficacy of the markers. In the present study, ROC curves were constructed for Pro MMP-9, active MMP-9 and truncated 42 kDa MMP to evaluate the discriminatory efficacy in distinguishing controls and patients with OPC, controls and oral cancer patients as well as patients with OPC and oral cancer patients. The values were expressed as the mean ± Standard Error of Mean (SEM). 'P' values less than 0.05 was considered to be statistically significant.

3. RESULTS

3.1. Higher Expression of Salivary pro MMP-9, Active MMP-9 and Truncated MMP 42 kDa in Patients with OPC and Oral Cancer Patients

As depicted in Fig. 1 and Fig. 2 the expression of salivary pro MMP-9 and active MMP-9 were found to be significantly higher ($P<0.001$) in oral cancer patients as compared to the controls, also the levels were found to be significantly higher ($P=0.01$ and $P=0.001$ respectively) in oral cancer patients as compared to the patients with OPC. Moreover, an increasing trend was observed from controls to patients with OPC to oral cancer patients. The truncated form of MMP i.e. of 42 kDa was observed to have gelatinolytic activity. As illustrated in Fig. 1, the expression of truncated 42kDa MMP was found to be significantly higher in oral cancer patients ($p<0.001$) as compared to the controls. In addition, the levels were found to be higher in patients with OPC as compared to controls and an increasing trend was observed from controls to patients with OPC to oral cancer patients.

Activation ratio i.e. active MMP-9/Total MMP-9 was found to be elevated in patients with OPC as compared to the controls ($P=0.07$). Also the activation ratio was found to be significantly elevated in oral cancer patients as compared to the controls ($P<0.001$). An increasing trend was observed from controls to patients with OPC to oral cancer patients.

When the levels were further compared between non-habituates (NHT) and subjects with habit of tobacco (WHT), the mean values of pro MMP-9 and active MMP-9 were found to be higher in WHT controls as compared to NHT controls, while truncated 42 kDa MMP levels were comparable. The levels were comparable between NHT oral cancer patients and WHT oral cancer patients.

3.2. Increased Expression of Salivary Pro MMP-9, Active MMP-9 and Truncated 42kDa MMP in Advanced Disease as Compared to Early Disease

As documented in Fig. 3, the levels of pro MMP-9, active MMP-9 and truncated 42 kDa were found to be significantly higher in advanced stage of disease as compared to controls (*P=0.007*, *P=0.010*, *P<0.001*, respectively). The levels of truncated 42 kDa MMP were found to be significantly elevated in early disease as compared to controls (*P=0.05*). Also, the levels of pro MMP-9, active MMP-9 and truncated 42 kDa MMP were found to be higher in advanced disease as compared to early disease (*P=0.746*, *P=0.694* and *P=0.086* respectively). The activation ratio of MMP-9 was found to be significantly higher in advanced disease as compared to the controls (*P=0.019*). The activation ratio was found to be higher in advanced disease as compared to early disease.

3.3. Higher Expression of Pro MMP-9, Active MMP-9 and Truncated MMP 42 kDa in Patients with Lymph-Node (LN) Metastasis as Compared to the Patients without LN Metastasis

As documented in Fig. 4, the levels of pro MMP-9, active MMP-9 and truncated 42 kDa MMP were found to be significantly higher in patients with LN metastasis (*P=0.007*, *P=0.026* and P=0.004 respectively) and without LN metastasis (*P=0.022*, *P=0.042* and *P=0.085* respectively) as compared to controls. Also, the levels were found to be higher in patients with LN metastasis as compared to patients without LN metastasis. The activation ratio of MMP-9 was found to be higher in patients with metastasis as compared to patients without metastasis. Multivariate analysis depicted significant positive correlation of truncated 42 kDa MMP with infiltration (F=4.165, *P=0.049*).

3.4. Significant Positive Correlation between All Forms of MMPs

Pearson's correlation analysis was performed to evaluate the correlation between different forms of MMPs. The results revealed that salivary pro MMP-9, active MMP-9 and truncated 42 kDa MMP were significantly (*P<0.0001*) positively correlated as shown in Table 2.

3.5. ROC Curve Analysis Revealed Good Discriminatory Efficacy of proMMP-9, Active MMP-9 and Truncated 42 kDa MMP in Discriminating Controls, Patients with OPC and Oral Cancer Patients

ROC curve takes into account both sensitivity and specificity, therefore is a meaningful statistical approach to evaluate discriminatory efficacy of the marker. The area under curve (AUC) above 0.5 suggests a statistically valid discriminatory efficiency of the marker. The ROC curves were constructed for pro MMP-9, active MMP-9 and truncated 42 kDa MMP. The analysis showed that salivary pro MMP-9, active MMP-9 and truncated 42 kDa MMP could significantly discriminate between controls and oral cancer patients (*P=0.006*, *P=0.016*, *P=0.001*), respectively (Table 3). Moreover, pro MMP-9 and active MMP-9 significantly distinguished patients with OPC and oral cancer patients (*P=0.004* and *P=0.003* respectively). The AUC of truncated 42 kDa MMP was 0.622 (*P=0.069*) in distinguishing patients with OPC and oral cancer. The highest AUC (0.702) was of truncated 42 kDa MMP (*P=0.001*), which significantly distinguished controls and oral cancer patients.

4. DISCUSSION

Saliva can serve as an excellent fluid to study salivary MMPs, as it is in direct contact with oral cancer lesions. MMPs have been known to be involved in disruption of cell-cell and cell-ECM interactions. Earlier reports have indicated truncation of E-cadherin, a cell adhesion molecule in patients with OPC [6]. As loss of E-cadherin is an early event in oral cancer progression, understanding MMPs in precancerous conditions might help in predicting metastatic behavior of the disease. Gelatin zymography is a cost effective technique which is able to detect both pro and active forms of gelatinases. There is a dearth of study on salivary MMP-9 in patients with OPC and oral cancer, hence the present study was undertaken to understand utility of salivary MMP-9 in monitoring changes occurring during neoplastic transformation of oral cancer. An increasing trend of salivary pro and active MMP-9 was observed from controls to patients with OPC to oral cancer patients. Earlier studies have indicated elevated levels of tissues MMP-9 in benign, premalignant and malignant laryngeal lesions [19]. Earlier salivary MMP-9 levels have been examined in patients with periodontitis [20] and have indicated bands corresponding to higher molecular mass complexes as well as low molecular sized components representing truncated MMP species. Earlier reports have suggested that 42 kDa MMP is the truncated form of MMP which is expressed due to autoactivation of various other gelationolytic MMPs [14]. Moreover, salivary protease activity was very high in saliva, in the present investigation. Hence, a truncated species of 42 kDa MMP was observed which might be due to proteolytic activities of other MMPs. High molecular weight gelatinases and truncated lower molecular weight species of gelatinases were also observed by Ingman, et al. [21] in periodontitis patients, which represented *invivo* proteolytically activated truncated enzymes. In the present study, an increasing trend of truncated 42 kDa MMP was observed from controls to patients with OPC to oral cancer patients. The levels of pro MMP-9, active MMP-9 and truncated MMP 42

kDa were found to be significantly higher in oral cancer patients as compared to the controls. Earlier reports have indicated elevated levels of pro and active MMP-9 in tissues and plasma of oral cancer patients as compared to the controls [7-9]. Moreover, in the present study a band of 125 kDa was observed which possessed gelatinolytic activity. It has been earlier reported that MMP-9 is associated with 25 kDa protein (microglobulin), giving a band at 125 kDa [22]. Studies have indicated elevated levels of MMP-9 after areca quid chewing in healthy volunteers and an elevation in oral cancer patients who are betel quid users [23, 24].

However, in the present study no significant difference was observed between tobacco habituates as compared to non-habituates for pro and active MMP-9. The results indicated that pro MMP-9, active MMP-9 and truncated MMP 42 kDa levels were higher in patients with LN metastasis as compared to patients without LN metastasis. Earlier reports have indicated elevated levels of tissue and plasma MMP-9 in patients with metastasis as compared to the patients without metastasis [7-9, 25]. All forms of MMPs i.e. Pro MMP-9, active MMP-9 and truncated 42 kDa were found to be higher in patients with advanced disease as compared to early disease. Previous investigations have documented positive correlation of plasma MMP-9 with stage of disease [5].

In conclusion, the results indicated increasing trend of pro MMP-9, active MMP-9, MMP-9 activation ratio and truncated 42 kDa MMP from controls to patients with OPC to oral cancer. This clearly represents significant involvement of MMPs (gelatinases) in sequential changes occurring during oral cancer progression. The results exhibited potential utility of saliva, a non invasive tool in monitoring changes occurring during oral cancer progression and also to predict metastatic behavior. The present study revealed salivary MMPs as an ideal tumor marker with necessary characteristics including its non-invasiveness, cost-effectiveness and reproducibility, which can be ideally used in clinics; outpatient doors (OPDs)/screening programmes with minimum basic facilities and primary infrastructure.

5. ACKNOWLEDGEMENT

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Table-1. Clinical details of oral cancer patients

Clinical characteristics	Oral cancer patients (N=100)
Disease site	
Buccal mucosa	45
Oral tongue	21
Alveolus	08
Others	18
Multiple sites	08
Histopathology	
Squamous cell carcinoma	97
Others	03
Lymph node metastasis	
No	56
Yes	34
Undefined	10
Stage of disease	
I	16
II	16
Early disease (I + II)	32
III	08
IV	54
Advanced disease (III + IV)	62
Undefined	06
Tumor differentiation	
Well	33
Moderate	57
Poor	05
Undefined	05

Table-2. Correlation analysis between all forms of salivary MMPs

	Salivary pro MMP-9	Salivary active MMP-9	Truncated 42 kDa MMP
Salivary pro MMP-9	---	r=0.903 *P<0.0001*	r=0.683 *P<0.0001*
Salivary active MMP-9	r=0.903 *P<0.0001*	----	r=0.723 *P<0.0001*
Truncated 42 kDa MMP	r=0.683 *P<0.0001*	r=0.723 *P<0.0001*	----

Table-3. ROC curve analysis of pro MMP-9, active MMP-9 and truncated 42 kDa MMP

Groups compared		Pro MMP-9	Active MMP-9	Truncated 42kDa MMP
Controls vs. patients with OPC	AUC Significance	0.478 *P=0.735*	0.442 *P=0.379*	0.576 *P =0.248*
Controls vs. oral cancer patients	AUC Significance	0.664 *P=0.006*	0.644 *P=0.016*	0.702 *P=0.001*
Patients with OPC vs. oral cancer patients	AUC Significance	0.694 *P=0.004*	0.700 *P=0.003*	0.622 *P=0.069*

Figures

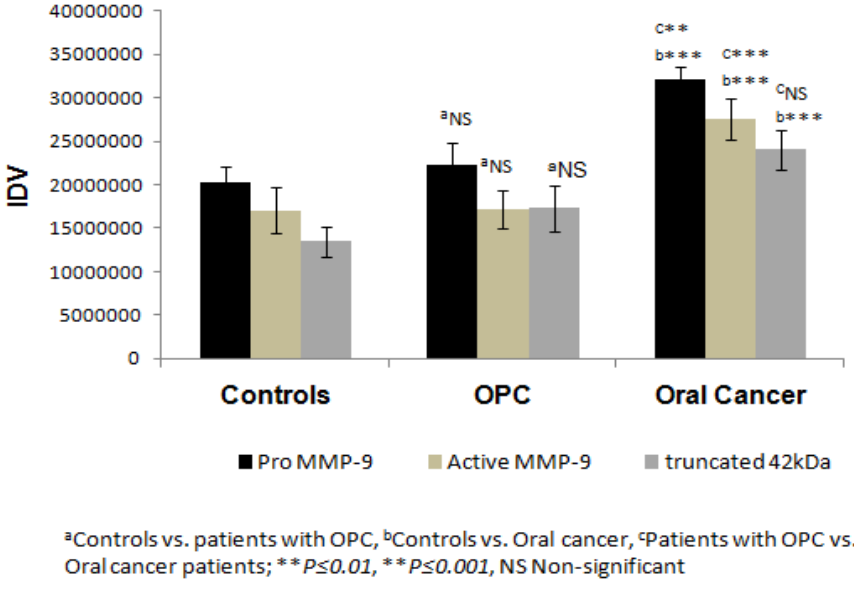

Fig-1. Expression of pro MMP-9, active MMP-9 and truncated 42 kDa MMP in controls, patients with OPC and oral cancer patients. Values are expressed as mean ± SEM. IDV: Integrated density value; OPC: oral precancerous conditions

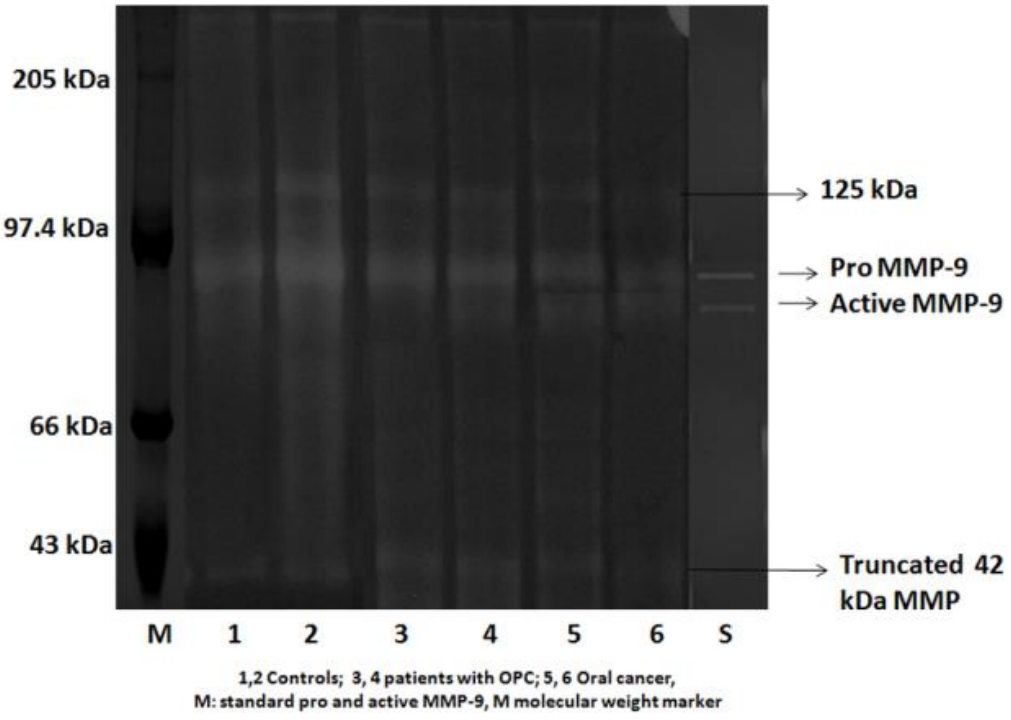

Fig-2. Representative pattern of gelatin zymogram from saliva. OPC: Oral precancerous conditions

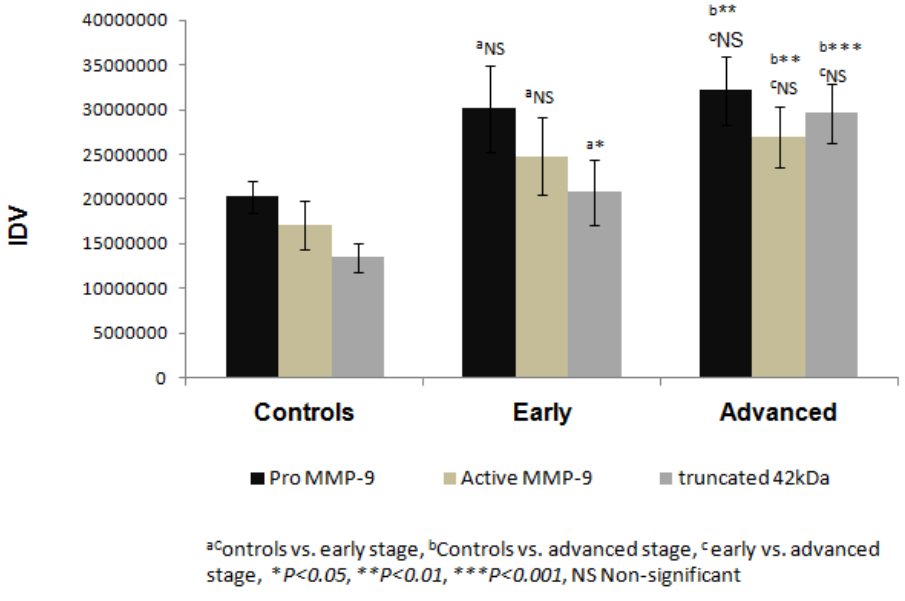

Fig-3. Expression of pro MMP-9, active MMP-9 and truncated 42 kDa MMP in early and advanced stage of disease.Values are expressed as mean ± SEM. IDV: Integrated density value

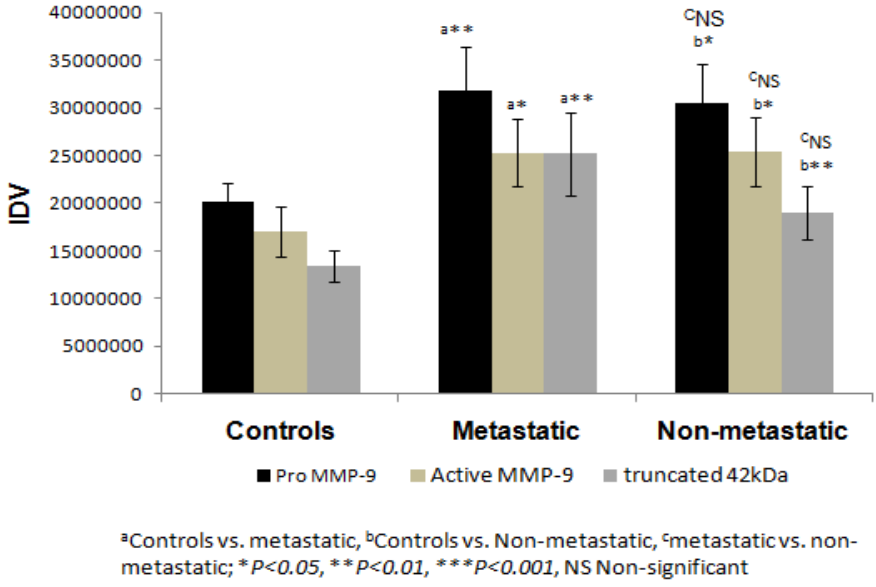

Fig-4. Expression of pro MMP-9, active MMP-9 and truncated 42 kDa MMP in patients with LN metastasis and without LN metastasis. Values are expressed as mean ± SEM. IDV: Integrated density value; LN: Lymph node