# Angiogenesis and MMP-2expression in Oral Squamous Cell Carcinoma&Verrucous Carcinoma and its Correlation with Clinicopathological Parameters

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## Abstract

**Background:** An important step of tumor progression in which Matrix metalloproteinase have been implicated is angiogenesis, because these enzymes degrade the extracellular matrix and provide a permissive microenvironment for the growth of new blood vessels.
The present study conducted to evaluate the immunohistochemical expression ofMatrixmetalloproteinase -2(MMP-2) andangiogenic marker (CD34) in Oralsquamous cell carcinoma(SCC)versus verrucous carcinoma(VC) and to correlate their expressions with the clinicopathological parameters.
**Material & Methods:** MMP-2 and CD34 expression was examined immunohistochemically in twenty four paraffin tissue blocks of squamous cell carcinoma and verrucous carcinoma (twelve cases of each).
**Results:** All cases of Oral SCC exhibited positive immunostaining for MMP-2, while only one case of VC showed –ve expression. Interestingly all cases of VC showed –ve MMP-2 immunostaining of the basal cell layer. Generally lymphatic vessels were more than blood vessels in both VC&SCC cases. The mean MMP-2 immunoexpression was (59.00%) for both stage I &stage II, while the higher CD34 immuno expression was in stage I. The mean expression of MMP-2 was higher in well differentiated OSCCs, while for CD34 it was higher in poorly differentiated OSCCs followed by moderately differentiated, then well differentiated, however no statistically significant difference was found. Non- significant correlation was found concerning the expression of both markers for both lesions.
**Conclusion:** No statistical correlation was found between MMP-2 expression and angiogenesis in OSCC and OVC.
**Key word**: SCC, VC, MMP-2, CD34

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## Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the oral cavity, accounting for over 90% of the malignant neoplasms in this region. The prognosis of SCC is related to the proliferative activity of the tumour, degree of differentiation, and invasion and metastatic potential.[1] The last two processes involve multiple steps, such as degradation of the basement membrane and extracellular matrix (ECM), alterations in cell adhesiveness, tumour cell motility, and angiogenesis.[2]

Oral Verrucous carcinoma (OVC) is a low-grade variant of oral SCC. In 1948, Lauren V. Ackermann first described this neoplasm of the oral mucous membrane, which is also known as (Verrucous carcinoma of Ackermann) or Ackermann's tumor [3].

At present, therapeutic decisions in SCCs are based on clinical and pathological parameters, including age, metastasis stage and histological grade of the tumour. However, these factors frequently fail to distinguish between more or less aggressive tumours.Therefore, specificmarkers need to be identified that are related to tumour progression and thus permit the establishment of therapeutic strategies against specific antigens [4,5].

Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases that are able to degrade all ECM proteins. [6] MMPs are associated with tumour progression and a poor prognosis due to their capacity to rupture these physical barriers and to regulate angiogenesis and cell proliferation. In significant number of cases MMPs are expressed by stromal cells, a finding indicating that, in addition to endogenous production of these enzymes, tumour cells utilizes proteinases produced by stromal cells. [7]

Type IV collagen is the main component of basement membrane, and degradation of this structural protein is favored by 2 metalloproteinases, namely, the gelatinase A (MMP-2) and gelatinase-B (MMP-9). MMP-2 and MMP-9 are known to be closely associated with the malignant potential of tumor cells [8].

Angiogenesis, which is the formation of new vessels from preexisting vessels, is thought to be of crucial importance to the growth, maintenance and metastasis of solid tumors. Tumor angiogenesis are often activated during the early preneoplastic stages of tumor development and is controlled by a number of positive or negative regulators produced by cancer cells and tumor-associated leukocytes [9]**.** The fact that tumors are angiogenesis dependent and metastatic cells are only shed after the tumor establishes its microcirculation has been described in melanomas, breast carcinomas and other malignant neoplasms [10].

Microvessel density (MVD), as a prognostic indicator, has been documented in various types of human tumors.CD34 (a human progenitor cell antigen) is expressedin a wide variety of normal and neoplastic cells [11].

The objective of the present study was to evaluate the immunohistochemical expression of MMP-2 and CD34 to find out whether these molecules affect the local destructive and metastatic potentialsof OSCC versus OVC and to correlate their expressions with the clinicopathological parameters.

## Materials and Methods

Twenty four formalin- fixed, paraffin-embedded (FFPE) tissue blocks (twelve squamous cell carcinoma and twelve verrucous carcinoma) obtained from the archieves of oral pathology department /college of dentistry/ Baghdad university, were included in this study. Data concerning patients' age, sex, location, clinical presentation, clinical staging and histopathological grading were obtained from the associated surgical reports. Tumor histology was reviewed blindly by two pathologists, and the representative paraffin blocks were selected.

**Immunohistochemistry (IHC)**

Two 4µm thickness sections were cut and mounted on positively charged slides (biocare USA) to be stained Immunohistochemically with anti CD34 and anti MMP-2 monoclonal antibodies (Mab) (US Biological-C2386-10).

Sections were sequentially deparaffinized and rehydrated. Then they were immersed in (0.3%) H2O2 to block the endogenous peroxidase activity, washed by phosphate – buffered saline (PBS) and incubated in (10%) normal goat serum to block the non specific antibody binding. The sections were

incubated with monoclonal mouse antihuman MMP-2 (diluted at 1:50) and anti CD34 monoclonal antibody (diluted at 1:40) over night, the bounded antibodies were detected by streptavidin-biotin complex and then counterstained with Harris hematoxylin.

In each IHC run positive and negative controls were included. Tissue blocks of colon cancer and tissue blocks of pyogenic granuloma served as positive control for MMP-2 and CD34 Mabs respectively(according to the manufacture).

**Evaluation of Immunohistochemistry Results: Assessment of MMP-2 immunostaining**

Tumor cells with clear brown cytoplasmic staining pattern were considered positive for MMP-2 immunostaining (according to the manufacturer's data sheets). MMp-2 scoring was performed by examining at least 1000 cells per section and 200 cells per field in five representative areas and immunoreactivity was scored as follows: -(0) point for negative staining of the considered cells, (1+) <10%, (2+) 10-50%, (3+) 51-100% (Naka et al, 2004).

**Quantification of microvessel density (MVD)**

Microvessel density (MVD) was determined by immunostaining of endothelial cells with anti-CD34 monoclonal antibody.

The number of microvessels in four fields under a light microscope (Olympus BX41TF, Tokyo, Japan) at a magnification of 400× in the area of the most intense vascularization (hot spot) was counted, and the average count in each case was recorded. Any endothelial-lined vessel appearing reddish brown and clearly separated from adjacent stromal and tumoral cells was considered to be a single countable microvessel. [13]

All variables were compared using Chi-square test. While Pearson correlation coefficient was applied to plot a correlation .P values of less than 0.05 were considered statistically significant.

## Results

The study sample consisted of twenty two FFPE blocks, of which ten cases were diagnosed as VC and twelve cases as OSCC. The age range for VC was from (50 -83) while for SCC it was from (32-86) with almost an equal mean age for both lesions (62.10 and 62.63) respectively.

From the total (10) cases of VC the most common sites were the buccal mucosa and the mandible (3) cases for each, while for OSCC it was the buccal mucosa (7 cases) followed by the tongue (5 cases).

**Assessment of MMP-2 immunostaining and MVD in OSCC& VC**

All cases of OSCC exhibited positive immunostaining for MMP-2 (Fig.1,2,3,4), and from the total of VC cases only one case showed –ve MMP-2 expressionInterestingly all cases of VC showed –ve MMP-2 immunostaining of the basal cell layer (Fig 5,6).

Generally lymphatic vessels were more than blood vessels in both VC&SCC cases Microvessel density (MVD) was determined by reddish brown immunostaining of endothelial cells lining the blood vessels in both OSCC&VC(Fig 7,8).

The results of this study showed a higher mean of MMP-2 (51.16%), &CD34 (37.66%) immunoexpression in OSCC than VC (35.25% and 29.25%) respectively, however, non significant results revealed by t-test.( table 1)

Regarding VC, females showed higher MMP-2 &CD34 mean of expression (43.00%, 35.33% respectively) than males (36.75%, 28.12% respectively), and the lip showed the highest mean for both MMP-2&CD34 (71.50%, 45.00%) but it was statistically non-significant .

Oral squamous cell carcinoma mean MMP-2 & CD34 immunoexopression was

higher in females (76.00%, 40.00% respectively)than males (42.88%, 36.88% respectively). The highest mean of MMP-2 expression was in the lip (68.00%), while for CD34 it was in the tongue (52.75 %).).Yet, statistically non- significant results were found.

The mean MMP-2 immmunoexpression was (59.00%) for both stage I &stage II, while the higher CD34 immmunoexpression was in stage I (73.00%) (table 4). The mean expression of MMP-2 was higher in well differentiated OSCCs (61.00%), while for CD34 it was higher in poorly differentiated OSCCs (44.33%) followed by moderately differentiated (39.50) then well differentiated (27.33) still no statistically significant results were found, according to t-test.(table 3).

Correlating the expression of both markers for both lesions, statistically non-significant results were found, as shown in tables (4&5).

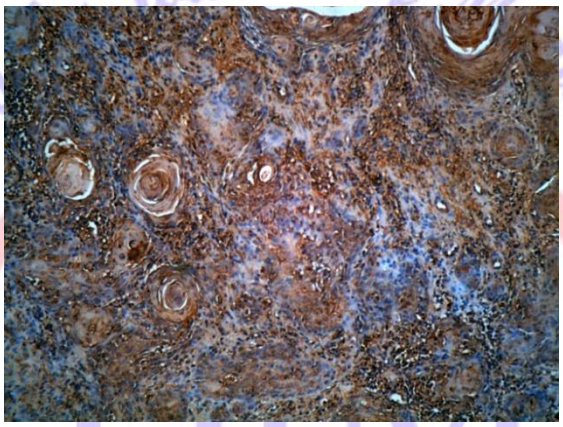

**Figure (1):** Positive cytoplasmic MMP-2 immunostaining in WDSCC (X10).

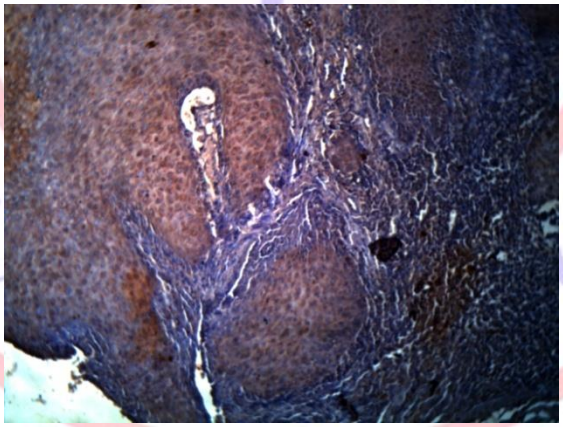

**Figure (2):** Positive cytoplasmic MMP-2 immunostaining in MD SCC (X10)**.**

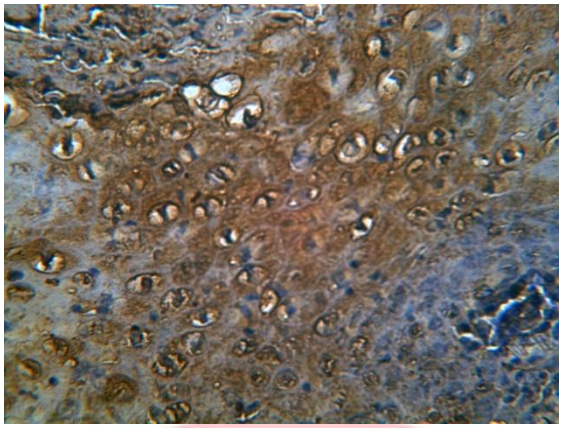

**Figure (3):** Positive cytoplasmic MMP-2 immunostaining in MD SCC (X40).

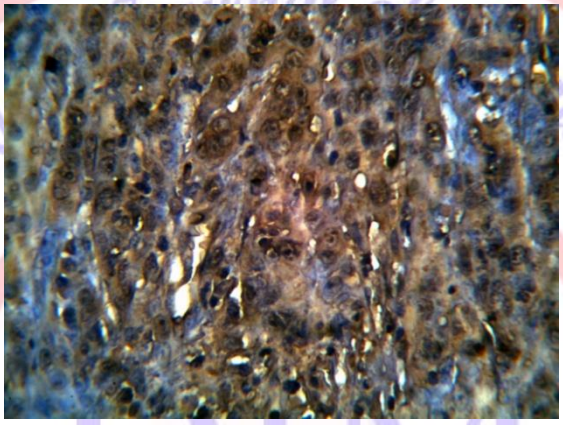

**Figure (4):** Positive cytoplasmic MMP-2 immunostaining in PD SCC (X40).

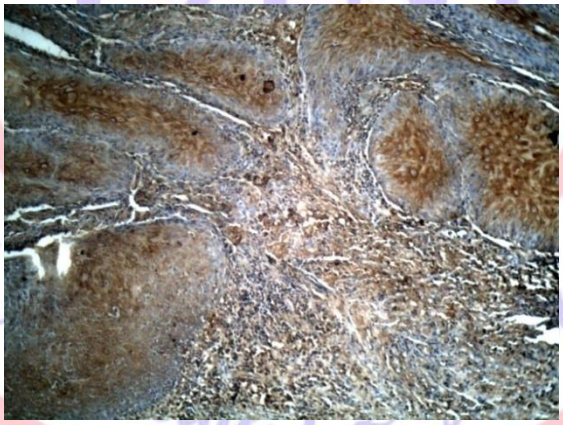

**Figure (5):** Positive cytoplasmic MMP-2 immunostaining in VC(X10).

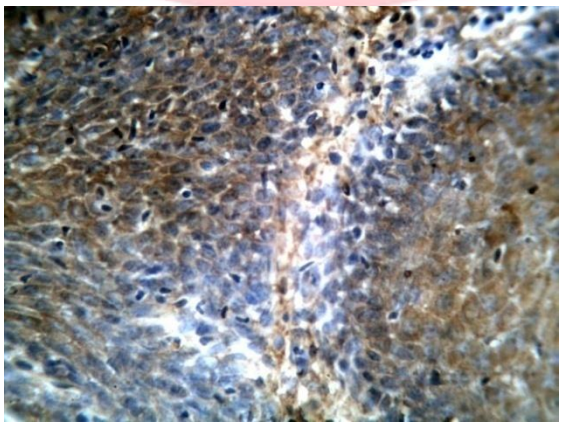

**Figure (6):** Positive cytoplasmic MMP-2 immunostaining in VC(X40). The basal cell layer is negative

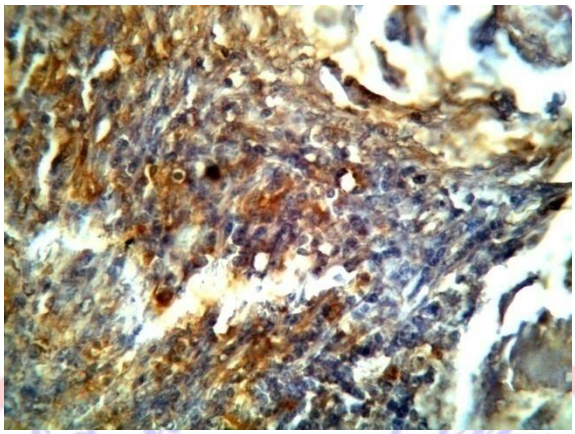

**Figure (7):** PositiveCD34 immunostaining in SCC(X40).

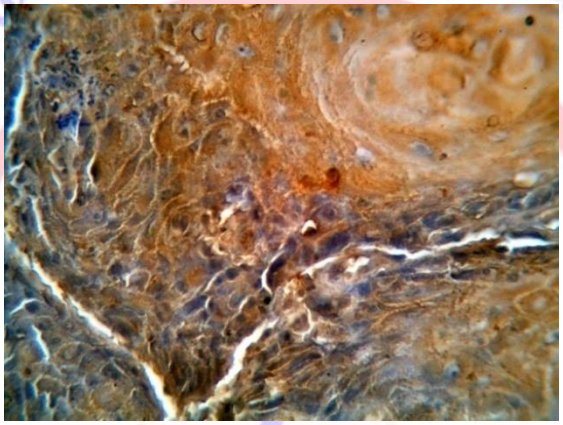

**Figure (8):** Positive CD34 immunostaining in VC(X40).

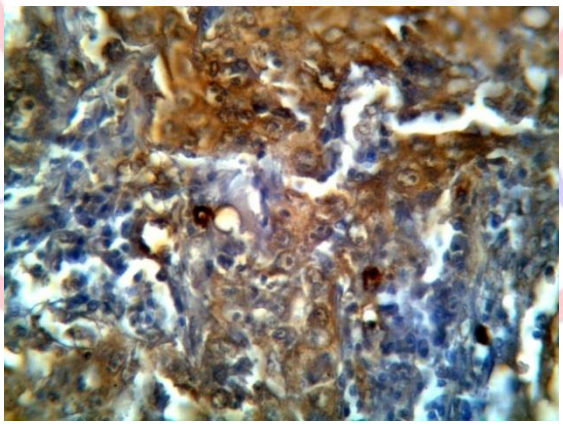

**Fig.9: Positive CD34 immunostaining in VC(X40)**

**Table (1):** MMP2 and CD34 expression in OSCC and VC.

| | Type | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| MMP2 | VC | 12 | 35.2500 | 24.77948 | 7.15322 |
| | SCC | 12 | 51.1667 | 20.39088 | 5.88634 |
| CD34 | VC | 12 | 29.2500 | 17.31066 | 4.99716 |
| | SCC | 12 | 37.6667 | 21.17174 | 6.11175 |
| **t-test for Equality of Means** | | | | | |
| | t | | df | Sig. (2-tailed) | |
| MMP2 | -1.718- | | 22 | .100 | |
| CD34 | -1.066- | | 22 | .298 | |

**Table (2):** MMP2 and CD34 expression in relation to the tumor stage in OSCC**.**

| Stage | | MMP2 | CD34 |
|---|---|---|---|
| I | Mean | 59.0000 | 73.0000 |
| | N | 1 | 1 |
| | Std. Deviation | . | . |
| II | Mean | 59.0000 | 3.0000 |
| | N | 1 | 1 |
| | Std. Deviation | . | . |
| III | Mean | 49.8571 | 43.2857 |
| | N | 7 | 7 |
| | Std. Deviation | 23.08989 | 12.60574 |
| IV | Mean | 53.5000 | 13.0000 |
| | N | 2 | 2 |
| | Std. Deviation | 33.23402 | 1.41421 |
| Total | Mean | 52.1818 | 36.8182 |
| | N | 11 | 11 |
| | Std. Deviation | 21.06570 | 21.99008 |

**Table (3):** MMP2 and CD34 expression in relation to the tumor grade in OSCC.

| Grade | | MMP2 | CD34 |
|---|---|---|---|
| W | Mean | 61.0000 | 27.3333 |
| | N | 3 | 3 |
| | Std. Deviation | 16.52271 | 13.27906 |
| M | Mean | 43.1667 | 39.5000 |
| | N | 6 | 6 |
| | Std. Deviation | 14.93207 | 26.00577 |
| P | Mean | 57.3333 | 44.3333 |
| | N | 3 | 3 |
| | Std. Deviation | 32.59346 | 19.00877 |
| Total | Mean | 51.1667 | 37.6667 |
| | N | 12 | 12 |
| | Std. Deviation | 20.39088 | 21.17174 |

**Table (4):** Correlation between MMP2 and CD34 in VC.

| | | MMP2 | CD34 |
|---|---|---|---|
| MMP2 | Pearson Correlation | 1 | .367 |
| | Sig. (2-tailed) | | .241 |
| | N | 12 | 12 |
| CD34 | Pearson Correlation | .367 | 1 |
| | Sig. (2-tailed) | .241 | |
| | N | 12 | 12 |

**Table (5):** Correlation between MMP2, CD34 in OSCC**.**

| | | MMP2 | CD34 |
|---|---|---|---|
| MMP2 | Pearson Correlation | 1 | .142 |
| | Sig. (2-tailed) | | .661 |
| | N | 12 | 12 |
| CD34 | Pearson Correlation | .142 | 1 |
| | Sig. (2-tailed) | .661 | |
| | N | 12 | 12 |

## Discussion

Tumor angiogenesis is a complex event mediated by angiogenic factors released from cancer cells and or by host immune cells.

No statistical relationship was found regarding MVD in respect to the age, sex and tumor location in OSCC,which is in accordance with the results of Ascani et al,Chowdhur et al, Gao et al,Mayumiet al([14,15,16,17], as well as in VC, in accordance with the results of Gaoetal[16].

MVD appeared to increase with the increase in histological grading, i.e,the higher MVD detected in tumors showing lower degree of differentiation (poorly differentiated SCC).This agrees with several studies [14, 18, 19], but disagrees with the study of Astekar etal and shintani etal whom showed decreased MVD score from WDSCC to MDSCC to PDSCC [20, 21]. However, increased mean MVD scores was observed histopathologically from normal oral mucosa to dysplasia to OSCC by many studies[22,23,24,25,26,27] Conflicting results may be due to the subjective variation in the classification of OSCC, the use of different pan-endothelial markers and the different methodologies used in the assessment of the parameters, besides the interobserver variations.

The current results revealed higher MVD in OSCC than VC in accordance with ELRouby, who found MVD significantly lower in OVC compared with OSCC (19). This may imply that high MVD values predict more aggressive tumor behavior in OSCC. However contrasting result showed by Gaoetal[16].

Low neovessel activity was seen within the tumor cell areas, in contrast the adjacent peritumoral stromal area showed intense new vessel formation ,hence the mean MVD for SCC in the peritumoral area was higher than the intra tumoral area(21.1,16.5)respectively, identical results shown by Hollemann etal in HNSCC [10].

These data show a high activity of new vessel formation in peritumoral stroma, with endothelial precursor cells being incorporated into these structures. [10]

The process of angiogenesis in the peritumoral tissue follows several sequential

steps, beginning with degradation of the basement membrane of the initial vessel and extracellular matrix.

No statistically significant results were found regarding the expression of CD34 with the stage of OSCC similar to the findings of other studies [16, 28], but disagrees with the result of Miyahara etal [17]. However, it must be emphasized that neovascularization permits, but does not guarantee, progressive tumor spread [29].

Moreover, an adequate vascular response is essential for initial development as well as continued growth of solid tumors and experimental evidences suggest that tumor growth can be stunted by a variety of agents that have the ability to inhibit angiogenesis.

Many studies have shown that gelatinases have significant clinical usefulness in tumor progression. In view of this, the study of MMP-2 in OSCCs could be a valuable approach for management of these patients. The current results revealed higher MMP-2 expression in OSCC than VC.This may imply that higherMMP-2 values dictate the metastatic behavior of OSCC. Besides, the -ve MMP-2 basal cell layer immmuno staining revealed in all cases of VC also confirms its non - metastatic behavior. No previous studies concerned this marker in both lesions together to compare with. However, Patel etal&Tokumaru et al have found that active MMP-2 was significantly elevated in malignant head and neck SCC tissues as compared with normal tissues[30,31]and a Chinese study found that the expression ofMMP-2 mRNA in VC was significantly higher than that in SCC[32].

Regarding the results of this study, no statistically significant correlation was found between MMP-2 expression and any of the clinicopathological parameters including age, tumor stage and tumor differentiation. Similar results were also been noted in OSCC[33],Tongue andLarangeal SCC[34,35] respectively.

Considering the cases of SCC evaluated in this study, although high expression of MMP-2 was observed in low grade malignancy but statistically non significant correlation was found in regard to the degree of tumor differentiation. This finding is in agreement with previous studies related tothe tongue, lip and laryngeal carcinomas, [36, 34, 37].

Although an important step of tumor progression in which MMPs have been implicated is angiogenesis and these enzymes degrade the extracellular matrix and provide a permissive microenvironment for the growth of new blood vessels. However in the present study no correlation was found between MMP-2 expression and MVD, in accordance with the study of Franchietal(38).This could be due to the fact that most of the cases included in the present study were node negative and metastasis negative except one case which was N1.
Moreover,Although angiogenesis plays a crucial role in hematogenous and lymphatic metastases for which studies have suggested that lesions that entered a higher angiogenic state have an increased probability of metastasis. However, certain tumors with marked angiogenesis surprisingly had no evidence of metastasis and were associated with a good prognosis. Thus, an understanding the complex biology of MMPs requires understanding that MMPs are responsible for promoting cancer so that targeted MMP therapy can be developed [39].

Furthermore, MMPs have both tumor promoting and inhibitory effects. Recent evidence suggests that MMPs can have a protective effect on tumor development. For example, MMPs have been found to negatively regulate neovascularization. MMP upregulation can increase conversion of

plasminogen to angiostatin, which can decrease vascularization of transplanted xenographs.

Thus, unclear relationship exists among angiogenesis, metastasis and prognosis [40]. Finally, the expression of MMP-2 and CD34revealed in the present study suggests that these markers play a role in the process of tumorigenesis.

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