

Integrin Alpha-V Beta-3-Matrix Metalloproteinase-2 (MMP-2), Cross-Talk

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Abstract

The present study aimed to detect comparative expression of integrin $\alpha_v\beta_3$ and its involvement in expression and activation of matrix metalloproteinase-2 (MMP-2) in 25 malignant human breast tumor and adjacent normal breast tissues from different clinical TNM stages (DCIS to T4) of the disease and possible involvement of known regulating parameters of MMP-2 like TIMP-2, MT1-MMP and EMPRIN. Integrin $\alpha_v\beta_3$ was highly expressed in tumors than adjacent normal breast tissues. Pro-MMP-2(72-KD) was mainly expressed in adjacent normal tissues compared to tumors. The mature forms of MMP-2 (68 KD and 64 KD) were found only in tumors. Appreciable expression of TIMP-2 and induction of MT1-MMP and EMPRIN in T2-T4 stages suggested their possible role in MMP-2 activation. Over expression Integrin $\alpha_v\beta_3$ in tumors than adjacent normal breast tissues was an indication of cancer progression with involvement of integrin signaling. We conclude that, the co-precipitation of MMP-2 with $\alpha_v\beta_3$ by anti- α_v antibody is a strong indication that integrin $\alpha_v\beta_3$ is a surface receptor for MMP-2 and $\alpha_v\beta_3$ -MMP-2 complex on the surface of tumor cells may play a very important role in determining the invasive property and malignant behavior of tumor tissues. The positive expression of endogenous inhibitor of MMP-2, TIMP-2 may have an appreciable role in activation of this protease and risk of malignancy in advanced stage of the disease. The enhanced expression of MT1-MMP and EMPRIN suggested a role for these factors in gelatinase regulation. However the exact mechanism(s) remains to be investigated. Finally, evaluation of integrin $\alpha_v\beta_3$ associated MMP-2 expression and activity may add valuable information and can possibly be therapeutic target. The clinical exploitation of integrins will provide oncologists with novel therapeutic strategies for the treatment of malignancy in breast cancer.

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Keywords

Breast Cancer, MMP-2, Alpha V Beta3, MT1-MMP, TIMP-2, EMPRIN, VEGF

1. Introduction

Integrins are transmembrane heterodimeric proteins, consisting of noncovalently associated α (120 - 180 KD) and β (90 - 110 KD) subunits [1] [2]. Integrins mediate cellular adhesion to and migration on the ECM proteins found in intracellular spaces and basement membrane [3]. They also regulate cellular entry into and withdrawal from cell cycle [4] [5]. Ligand of integrin by their ECM ligands induces a cascade of intracellular signals [6] that include tyrosine phosphorylation of focal adhesion kinase (FAK), increment of intracellular Ca^{2+} levels, inositol lipid synthesis, synthesis of cyclins [5] and expression of intermediate early genes [6]. In contrast, prevention of integrin ligand interaction suppress cellular growth and induces apoptotic cell death [7] [8]. Integrin on tumor cells are now thought to play intricate role in the progression of solid tumors. Integrin expression is altered in malignant cells as compared to their normal counter parts and altered integrin expression appears to be involved in several aspects of tumor growth, invasion and metastasis [9]. One of the most studied integrin receptors in tumor progression and metastasis is $\alpha_v\beta_3$ which is a major integrin for vitronectin and plays important role in cancer growth. In murine cells, interaction of vitronectin with its receptor provides co-mitogenic signal [10]. Malignant melanomas express these integrin receptors as they enter the vertical growth phase [11]. Upregulation of this receptor in endothelial cells facilitates their interaction with lung carcinoma cells indicating a potential role of this receptor in tumor cell extravasations [12]. Tumorigenicity in athymic nude mice strongly correlated with $\alpha_v\beta_3$ expression by tumor cells [13].

Cellular invasion process involves the production of proteolytic enzymes which are capable of degrading components of the extra cellular matrix and basement membrane. One of the major groups of these enzymes is matrix metalloproteinase (MMP) family. The prognostic value of MMPs has been investigated in several malignancies. The role of MMP-2, is well established during invasive activity of many cell types [14]-[16]. In particular, the molecular interaction between MMP-2 and integrin $\alpha_v\beta_3$, via the hemopexin C (PEX) domain, was shown to be essential for efficient cell invasion and angiogenesis [17] [18]. MMP-2 overexpression has been correlated with poor survival in breast carcinoma [19] [20] especially in node-positive patients. Studies reveal that the $\alpha_v\beta_3$ integrin receptor is expressed by various cancer types. Malignant melanoma has been shown to modulate expression of proteolytic enzymes by the tumor cells. Stimulating antibodies to $\alpha_v\beta_3$ in melanoma cell line caused increased expression of MMP-2 with an enhanced ability to invade basement membrane [21]. Expression of $\alpha_v\beta_3$ on cultured melanoma cells enabled their binding to MMP-2 in a proteolytically active form, facilitating cell-mediated collagen degradation, thereby facilitating directed cellular invasion [17]. Integrin alpha V beta 3 was strongly expressed in primary invasive breast carcinomas. In contrast, this integrin heterodimer was abundant in all breast cancer cells metastatic to bone. *In situ* hybridization also revealed high levels of $\alpha_v\beta_3$ mRNA expression and suggested that integrin $\alpha_v\beta_3$ is an endothelial cell marker with significant prognostic value and potential usefulness as a target for specific anti angiogenic therapy. It also has been demonstrated that tumor-specific $\alpha_v\beta_3$ contributes to spontaneous metastasis of breast tumors to bone and suggests a critical role for this receptor in mediating chemotactic and haptotactic migration towards bone factors. Our present study aimed to detect the comparative expression and activity of integrin $\alpha_v\beta_3$ associated MMP-2 in breast tumor tissue and adjacent normal breast tissue and the possible involvement of MT1-MMP, EMPRIN and TIMP-2 in the modulation of MMP-2 activity in breast cancer.

2. Patients and Methods

2.1. Patients

The present study involving 25 breast cancer patients diagnosed among the women, who were referred to Chittaranjan National Cancer Institute, India because of clinical breast lump, suspicious mammographic finding or a breast symptom (eg. pain, nipple discharge) between 2008 and 2010. Women willing to participate in the project were interviewed and examined by a trained study nurse before any diagnostic procedures. The participation rate of patients with diagnosed breast cancer was 98%. Thus the patient series represents unselected typical

breast cancer cases of different stages from the institutional hospital catchment area. Patients were offered treatment according to the stage of the disease, either surgery followed by chemotherapy and \pm Radiotherapy or Neo-adjuvant chemotherapy followed by surgery then completion chemotherapy and radiation depending on the mode of the surgery, the patient's menopausal status, and the stage of the disease, according to the national guidelines. In brief, postoperative radiotherapy was given to all patients treated with breast-conservation surgery. Hormonal therapy was offered to receptor ER or PR positive patients with axillary node positive (pN+) or T3 and T4 tumors irrespective of the mode of surgery. All pre-menopausal patients were treated with Tablet-Tamoxifen for 5 years. Post menopausal patients were either treated for 2 - 3 years with tamoxifen followed by 2 - 3 years of aromatase inhibitor or with aromatase inhibitor for 5 years. Patients with pN+ status and some with axillary node negative (pN-) status presenting with other adverse prognostic factors such as estrogen receptor (ER)/progesterone receptor (PR) negative or poorly differentiated tumor, were given adjuvant chemotherapy (FEC/FAC cyclophosphamide, anthracyclin, taxens, methotrexate and 5-fluorouracil) for six cycles. Stage was assessed by using the TNM classification. Patients with noninvasive carcinomas, a previous history of breast cancer, metastatic disease (stage-IV), or insufficient tumor material was excluded from the present study. Thus 81 patients with sufficient primary tumors and complete clinical histories were available for the present study. The mean age of the patients was 59.2 years (median 56.8 years; range, 23.3 - 91.6 years). The mean follow up time was 55.0 months (median 57.5 months; range, 1.2 - 115.1 months). The clinicopathological data of the patients are summarized in **Table 1**.

2.2. Materials

Gelatin Sepharose 4B beads was purchased from GE Healthcare Bio-Sciences AB, Uppsala, Sweden. All primary antibodies (anti-TIMP-2, anti-VEGF, anti-MT-1MMP, anti EMPRIN and, anti- β -tubulin) antibodies were purchased from Santa Cruz, USA. Biotinylated secondary antibodies, SuperSignal West Femto Maximum Sensitivity Substrate were purchased from Thermo Scientific, Rockford, USA. Avidin-biotinylated peroxidase complex reagent (vectastain Rabbit ABC kit) was purchased from vector laboratories, Burlingame, CA). Immobilon-P Membrane, (PVDF), was purchased from Millipore, USA.

2.3. Methods

Collection of tissue samples: Tissues from tumor and respective normal breast tissues of the same patient were collected from the operation theater during surgery. Tissues were stored at -80°C and used for the further experiments.

Immunoprecipitation: Tissues of tumor samples and respective normal breast tissues of the same patient were collected, homogenized, extracted with tissue extraction buffer (Tris-50 mM, NaCl-150 mM, NP40-1%, protease inhibitor cocktail and pH adjusted to 7.5) and the protein content of the extracts were estimated by Lowry's method. Equal amount of protein (100 μg each) of tissue lysate was pre cleared with Gelatin Sepharose 4B beads shaking for 1 hour at 4°C and then subjected to co immunoprecipitation with anti- αv monoclonal antibody (1 $\mu\text{g}/\text{ml}$) for overnight shaking at 4°C . Antigen-antibody complexes are then bound to Gelatin Sepharose 4B beads (Roche). The beads were washed with $\times 3$ with Tris-buffered saline with (0.02%) Tween-20 (TBST) and suspended in 50 μl of $1\times$ sample buffer (0.075 gm Tris, 0.2 gm SDS in 10 ml water, pH 6.8) for 30 mins at 37°C . The extract was then subjected to zymography.

Gelatin Zymography: Equal amount of protein (100 μg each) of tissue lysate was taken. The gelatinases were separated from tissue extract using Gelatin Sepharose 4B beads shaking for 2 hours at 4°C . The beads were washed $\times 3$ with Tris-buffered saline with (0.02%) Tween-20 (TBST) and suspended in 50 μl of $1\times$ sample buffer (0.075 gm Tris, 0.2 gm SDS in 10 ml water, pH 6.8) for 30 mins at 37°C . The extract was then subjected to zymography on 7.5% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) co-polymerized with 0.1% gelatin. Gel was washed in 2.5% Triton-X-100 for 30 mins to remove SDS and was then incubated overnight in reaction buffer (50 mM Tris-HCl pH 7, 4.5 mM CaCl_2 , 0.2 M NaCl). After incubation, the gel was stained with 0.5% coomassie blue in 30% methanol and 10% glacial acetic acid. The bands were visualized by destaining the gel with 30% methanol and 10% glacial acetic acid.

Immunoblot assay: The tissues were collected, extracted with cell extraction buffer (Tris-37.7 mM, NaCl-75 mM, Triton X-100-0.5%, protease inhibitor cocktail and pH adjusted to 7.5) and the protein content of the extracts were estimated by Lowry's method. Equal amount of protein (50 μg each) was taken and co-immuno-

Table 1. clinicopathological data of the patients.

Characteristics	n (%)
No of patients	25 (100)
Age (y)	
Mean (range)	57.3 (23.1 - 77.6)
Tumor Size	
T2	7 (28)
T3	10 (40)
T4	8 (32)
Lymph Node Status	
Negative	7 (28)
Positive	18 (72)
Stage	
II	12 (48)
III	13 (52)
Histological Type	
Ductal	23 (92)
Lobular	2 (8)
Histological Grade	
II	10 (40)
III	15 (60)
ER Status	
Positive	11 (44)
Negative	14 (56)
PR Status	
Positive	9 (36)
Negative	16 (64)
Her-2 Status	
Positive	19 (76)
Negative	6 (24)
Menopausal Status	
Pre menopausal	20 (80)
Post Menopausal	5 (20)

precipitated with anti alpha-V antibody, heated with 0.1 volumes β -mercaptoethanol for 5 - 8 mins at 80°C - 90°C was subjected to electrophoresis on 10% SDS-PAGE. The proteins were electrophoretically transferred on to Immobilon-P Membrane, (PVDF). membranes. The membranes were blocked with 1% BSA and subsequently washed $\times 3$ with PBST. The membranes were reacted with anti-MMP-2(2C1) and anti- β -tubulin antibody antibody.

Equal amount of protein (50 μ g each) from tissue lysate was heated with 0.1 volumes β -mercaptoethanol for 5 - 8 mins at 80°C - 90°C and subjected to electrophoresis on 7.5% SDS-PAGE. The proteins were electrophoretically transferred on to Immobilon-P Membrane, (PVDF). membranes. The membranes were blocked with 1% BSA and subsequently washed $\times 3$ with PBST. The membranes were reacted with anti-alpha-v, anti-MT-1-MMP (L15), anti EMPRIN (8D6) and anti- β -tubulin antibody at 1/2000 dilution with 4% BSA each and kept at

37°C for 1 hour 30 mins and subsequently washed $\times 3$ with PBST. The blots were developed using respective horse redish peroxidase (HRP) coupled second antibodies at 1/2000.00 dilution and kept at 37°C for 1 hour 30 mins, the blots were then thoroughly washed $\times 6$ with PBST. Bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate [22]-[29].

Quantification of the results: Bands of zymography, western blots and RT-PCR were quantitated using Image J Launcher (version 1.4.3.67)

2.4. Statistical Analyses

The statistical analyses were carried out by using the Epi Info (TM) 3.5.3 software of the centers for disease Control and Prevention (CDC, USA) for windows 9.0 programme. The associations between MMP-2 expression and clinicopathological parameters were tested. The univariate analyses were performed using test of proportion, was carried out to find out the association of MMP-2 with molecular parameters including age, menopausal status, lymph node involvement, status of ER, PR, Her-2/neu and stage of the disease. Values of the parameters were expressed as Mean \pm s.e. and “p” values less than 0.05 were considered statistically significant.

3. Results

3.1. Integrin $\alpha_v\beta_3$ Is Highly Expressed in Breast Cancer Tissue

Comparative western blot analysis of malignant (TNM stage-T2-T4) breast tumor tissue and adjacent normal breast tissue lysate clearly shows that integrin $\alpha_v\beta_3$ surface receptors are over expressed in tumor when compared to adjacent normal tissue (Figure 1).

The tissues were collected, extracted with NP40 extraction buffer (Tris-50 mM, NaCl-150 mM, NP40-1%, protease inhibitor cocktail and pH adjusted to 7.5) and the protein content of the extracts were estimated by Lowry’s method. Equal amount of protein (100 μ g each) was taken. Pre-cleared and then subjected to co-immunoprecipitation following the method described in Methods & Materials. Bands were visualized using SuperSignal West Femto as substrate.

3.2. Breast Cancer Tissue Has More $\alpha_v\beta_3$ Associated MMP-2 Activity

Comparative zymographic analysis of malignant (TNM stage-T2-T4) breast tumor tissue and adjacent normal breast tissue lysates, co-immunoprecipitated with anti α_v monoclonal antibody clearly shows that gelatinolytic activity of pro-MMP-2(72) has been observed mainly in adjacent normal breast tissue lysate (p = 0.00001). Where as in most of the tumor samples, subsequent proteolytic activation of this protease also has been observed

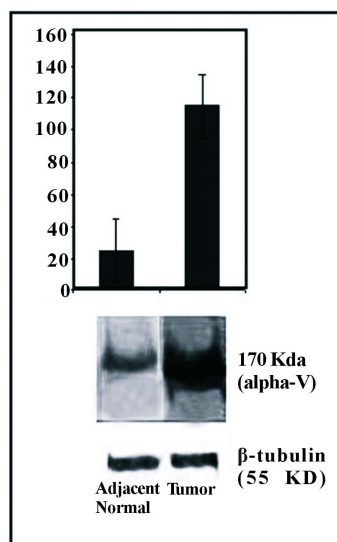


Figure 1. Expression of integrin α_v in tumor & adjacent normal breast tissue by immunoblot.

in the form of gelatinolytic band of activated MMP-2 (68 KD and 64 KD). Though there was no significant difference of MMP-2 activity in TNM stage-II and III (T2-T4) (**Figure 2**).

Equal amount of protein (100 µg each) from Tissues of tumor samples (lane-T) and respective adjacent normal breast tissues (lane-N) of the same patient were taken. Pre-cleared with Gelatin Sepharose 4B beads shaking for 1 hour at 4°C and then subjected to co immunoprecipitation with anti- α_v monoclonal antibody (1 µg/ml) following methods described in the text. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using image J Launcher (version 1.4.3.67).

3.3. Breast Cancer Is Associated with Enhanced Expression of Integrin $\alpha_v\beta_3$ Associated MMP-2

Comparative western blot analysis of malignant (TNM stage-T2-T4) breast tumor tissue and adjacent normal breast tissue lysates, co-immunoprecipitated with anti- α_v monoclonal antibody clearly shows that total protein expression of pro-MMP-2 (72KD) has been appreciably increased in tumor tissue lysates as compared to adjacent normal breast tissue (**Figure 3**).

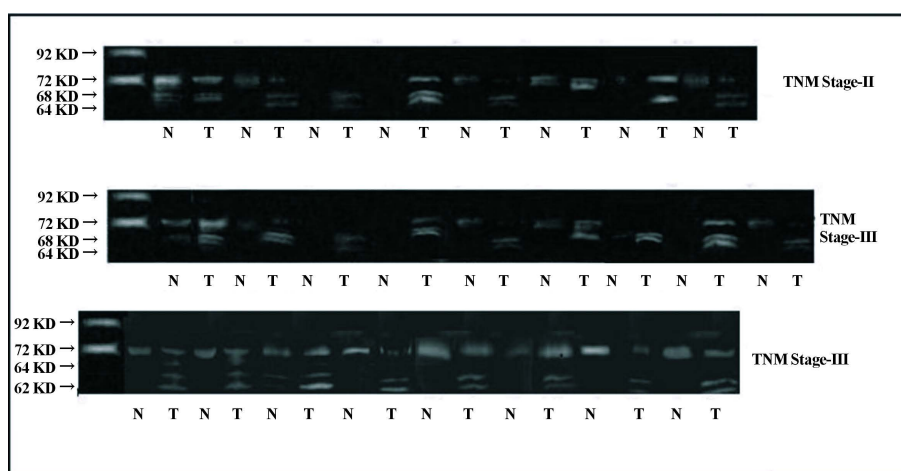


Figure 2. Zymographic analysis for gelatinolytic activity of $\alpha_v\beta_3$ associated MMP-2 in breast tumor and adjacent normal tissue.

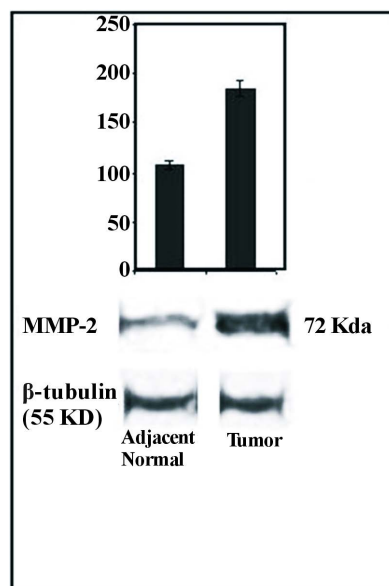


Figure 3. Expression of integrin $\alpha_v\beta_3$ associated MMP-2 in tumor and adjacent normal tissue.

The tissues were collected, extracted with NP40 extraction buffer (Tris-50 mM, NaCl-150 mM, NP40-1%, protease inhibitor cocktail and pH adjusted to 7.5) and the protein content of the extracts were estimated by Lowry's method. Equal amount of protein (100 µg each) was taken, pre cleared with Gelatin Sepharose 4B beads shaking for 1 hour at 4°C and then subjected to co-immunoprecipitation with anti- α_V monoclonal antibody (1 µg/ml) for overnight shaking at 4°C following methods described in the text. Bands were visualized using Super-Signal West Femto as substrate. The accompanying graph represents the comparative densitometric/quantitative analysis of the band intensities using image J Launcher (version 1.4.3.67).

4. Discussion

In this present communication we have tried to elucidate the role of $\alpha_V\beta_3$ integrin receptor in binding and activation of MMP-2 from 25 screened invasive breast cancer patients. The most pronounced difference was seen in zymographic analysis where we found gelatinolytic activity of activated forms of MMP-2 (*i.e.*, the 68-kDa and 64 kDa) were more frequent in tumor than the precursor form and were more intense than that corresponding to pro-gelatinase A (72 kDa). This fully active form of MMP-2 was not observed in adjacent normal breast tissue samples. Our findings are in agreement with other studies suggesting that the activation of MMP-2 (gelatinase A) is a more common event in aggressive breast cancer and integrin $\alpha_V\beta_3$ is also responsible for this proteolytic activation of MMP-2 [30]-[33]. We observed progelatinase A in very few of tumor samples. There is strong evidence that shows integrin ligand interaction initiates a cascade of signaling reactions which induces the release of different proteases that dissolve the basement membrane and help in cell invasion. The enhanced expression of MMP-2 upon $\alpha_V\beta_3$ ligation was demonstrated by Bufetti *et al.* [34]. As the cancer progressed from DCIS into T4stage, there was a tendency towards an increment of the gelatinolytic activity of MMP-2 which becomes significantly higher with subsequent activation of this protease in comparison to non-malignant breast tissue indicating the involvement of surface receptor integrin $\alpha_V\beta_3$ in different stages of tumor progression and metastasis. MMP activity is tightly regulated at several levels, from transcription, to proenzyme activation and finally by inhibition with TIMPs. Most MMPs are secreted in a proenzyme form that is later activated by cleavage of the amino-terminal 80 amino acids. This processing to an active form is accomplished by one of the five membrane-type (MT) MMPs residing on the cell surface, by activated protein C, or by the plasminogen activator-plasminogen cascade [35]-[37]. A final step in regulating active MMPs is inhibition by small inhibitory proteins called TIMPs. MMP-2 is secreted in association with TIMP-2, which mediates cell surface binding of the latent complex [38]. Several proteases, MMP2, MT1-MMP, TIMP2, and integrin $\alpha_V\beta_3$ were shown to colocalize in caveolae in human endothelial cells [39]. In this present study positive expression of TIMP-2 has been observed in tumors and also in normal tissues by means of ELISA and RT-PCR in both stage-II and stage-III cases. In line with our results Garbett E A *et al.* (2000) [40] showed increased expression of TIMP-2 in invasive breast cancer. In a previous study Ree A.H *et al.* 1997 correlated increased amount of TIMP-1 and TIMP-2 with distant metastasis [41]. So TIMP-2 may also play a crucial role in activation of MMP-2 in advanced stages of human breast cancer (Table 2).

The statistical analyses were carried out by using the Epi Info (TM) 3.5.3 software of the centers for disease Control and Prevention (CDC, USA) for windows 9.0 programme. The associations between MMP-2 expression and the regulating parameters of MMP-2 were tested. The univariate analyses were performed using chi-square analysis, and the independent prognostic value of variables was further examined with their corresponding Probability values.

Recently, the expression of MT1-MMP in various human cancer tissues has been associated with pro-MMP-2 activation. Deryugina *et al.* (2001), have demonstrated that MT1-MMP:TIMP2:MMP2: $\alpha_V\beta_3$ complex was shown to promote maturation of MMP2 in carcinoma cells [42]. In our present study increased expression of MT1-MMP has been observed in tumor as compared to adjacent normal tissue, confirming its possible role in activation of MMP-2 in breast cancer. Extracellular matrix metalloproteinase inducer (EMMPRIN) (also known as CD 147) is a 58 kDa glycoprotein, originally purified from the plasma membrane of cancer cells and was designated tumor collagenase stimulating factor (TCSF) because of its ability to stimulate collagenase-1 (MMP-1) synthesis by tumor stromal fibroblast cells [43]. In our present study, increased expression of EMMPRIN has been observed in tumor tissue as compared to adjacent normal tissue, indicating its role in MMP-2 activation. VEGF has been identified as a predominant regulator of tumor angiogenesis. Expression of the VEGF ligand has been observed across a range of tumor types and has been widely correlated with tumor development and/or poor prognosis [44]-[48]. In ductal carcinoma *in situ*, increased pathologically aggressive lesions was associated with increased

Table 2. Gelatinase expression and statistically significant clinicopathological variables of breast cancer patients in univariate analysis.

Variables	High expression in tumor	High expression in normal tissue	P
MMP-2	25	0	0.00001
TIMP-2	17	8	0.0413
MT1-MMP	12	8	0.0374
EMPRIN	17	0	0.0001
VEGF	25	0	0.00001

VEGF protein levels [49]. VEGF protein content is also increased in invasive breast cancer and this overexpression has prognostic significance in patients with either node-positive or node-negative disease for both relapse-free and overall survival. In patients with human epidermal growth factor receptor (HER)-2 overexpressing tumours, a higher VEGF content was demonstrated, confirming that VEGF is a downstream target of HER-2 activation. It is observed, that over expression of VEGF significantly correlated with MMP-2 expression and activity in various cancer types including breast cancer. In our present study, enhanced expression of VEGF protein has been observed in tumors, compared to adjacent normal tissues indicating its correlation with enhanced MMP-2 expression. In line with our result, Li Hao *et al.* (2007) has shown that VEGF and MMP-2 co-expression is associated with primary tumor progression, histological grade and lymph node status in patients of breast cancer. In a previous study from our lab, we investigated whether $\alpha_v\beta_3$ and MMP-2 are associated on the membranes of a human cervical cell, SiHa and the possible involvement of MT1-MMP and TIMP-2 in the modulation of MMP-2 activity. SiHa cells expressed all the molecules which are reported to form a complex to activate pro-MMP-2. Active MMP-2 associated with $\alpha_v\beta_3$ may regulate matrix degradation and thereby modulate directed motility of SiHa cells. In this present study we confirm that breast cancer tissue shows more $\alpha_v\beta_3$ associated MMP-2 compare to matched control tissue. The components of the MMP-2 activation complex (MT1-MMP, EMMPRIN, TIMP-2) and VEGF are all in increased amount in cancer tissues than the matched controls. The findings may help to understand the role of $\alpha_v\beta_3$ intrigin associated MMP-2 in breast cancer progression. The findings may have potential use in clinical management of breast cancer.

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