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Serum and tissue expression of gelatinase and Twist in breast cancer

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Abstract. – OBJECTIVE: This study aimed to investigate the levels of matrix metalloproteinases (MMP)-2, MMP-9 and Twist in tumor tissue and serum from 46 cases of breast cancer patients and 31 cases of benign breast diseases patients by immunohistochemical staining and enzyme-linked immunosorbent assay, respectively. The association of gelatinase and Twist expression with clinicopathological factors was also analyzed in the present study.

PATIENTS AND METHODS: The studied population consisted of 46 breast cancer patients and 31 benign breast disease patients. Serum concentrations of MMP-2, MMP-9 and Twist were measured by using human enzyme-linked immunosorbent assay. The protein expression of Twist, MMP-2 and MMP-9 were determined by immunohistochemical.

RESULTS: The results show that the levels of MMP-2, MMP-9 and Twist expression were significantly increased in tissue and serum from breast cancer group, compared to the group of benign breast lesions diseases ($p < 0.05$). The pre-operative serum levels of MMP-2, MMP-9 and Twist were positively correlated with their expression in breast cancer tissues, respectively ($p < 0.05$). We, then, correlated serum and tissue levels of MMP-2, MMP-9 and Twist in breast cancer samples with patients' clinicopathologic characteristics. Compared to low expression, high serum and tissue levels of MMP-2 and Twist were associated with lymph node metastasis and higher TNM stage, high tissue MMP-9 levels were associated with lymph node metastasis and higher TNM stage, and high serum MMP-9 levels were associated with c-erbB-2 expression.

CONCLUSIONS: These data suggest that serum levels of MMP-2, MMP-9 and Twist could be as potential biomarkers for diagnosis and predicting metastasis of breast cancer.

Key Words:
Breast neoplasm, Twist, Gelatinases, Immunohistochemistry, ELISA.

Introduction

Although the therapeutic and diagnostic technologies of breast cancer have been well developed in recent years, tumor invasion and metastasis is still the most common cause of mortality among breast cancer patients[1]. Invasion and metastasis are complex, dynamic and multistep processes, the key step to cure breast cancer using combined modality therapy program is to predict and monitor the early metastasis effectively. The previous studies suggested that MMP-2 and MMP-9 play key roles in the invasion and metastasis of breast cancer[2-4], and Twist is an important tumor transfer factor[5,6]. Our early study also showed that Twist might serve as a potential regulator of gelatinases in breast cancer[7]. The serum levels of MMPs have been demonstrated to be helpful to evaluate the biological behavior of many kinds of malignant tumor[8-10]. By local secretion or inducing host cells to secrete, tumor cells produce MMPs and leach into the blood stream where their expression level can be measured[11]. Compared to tissue detection of diagnosis breast cancer, blood detection have advantages of less trauma and less cost especially to the patients whom have not been touched lump apparently. Therefore, serum MMPs might serve as useful biomarkers in early diagnosis of breast cancer. In current study, we determined serum levels of MMP-2, MMP-9 and Twist in patients with breast cancer and benign breast disease, and then detected the expression of MMP-2, MMP-9 and Twist protein in tumor tissue. Thereafter, we correlated the serum and tissue levels of MMP-2, MMP-9 and Twist, and then associated the tissue levels of MMP-2, MMP-9 and Twist in breast cancer samples with patients' clinicopathologic characteristics.

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Patients and Methods

Patients and Specimens

The studied population consisted of 46 consecutive female breast cancer patients (range, 32-83 years; mean, 52.0 years) and 31 consecutive female benign breast disease patients (range, 16-57 years; mean, 38.0 years) who underwent surgery at the First Affiliated Hospital of Anhui Medical University and the Second People's Hospital of Hefei between 2011 and 2013. Breast cancer patients who had undergone chemotherapy or radiation therapy before surgery were excluded, as were patients with rheumatic disease, acute infection, HIV or other types of cancer. The characteristics of the breast cancer patients with respect to age, tumor size, tumor lymph node metastasis, tumor grade, and estrogen and progesterone receptor and c-erbB-2 status were collected for data analyses. Our Institutional Review Board approved the protocol for the use of patient samples in our study and informed consent was obtained from all patients.

Venous blood samples were obtained from all patients before surgery. The sera were separated from the blood and frozen at –80°C until use. Surgical tissue specimens from breast cancer or benign breast disease patients were obtained, fixed in 10% formaldehyde, and embedded in paraffin. Consecutive 4 μm sections were cut from the paraffin block and then sticked to the 10% poly lysine pre-treated slides. All the hematoxylin and eosin (H&E)-stained sections from each formalin-fixed, paraffin-embedded block were assessed to identify target areas. All tissue diagnoses were confirmed by permanent histology.

The pathological tumor stage was defined according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer. Tumor differentiation was defined according to the World Health Organization classification of tumors (WHO)[12]. Tumor size was classified as ≤ 2 cm, 2-5 cm or > 5 cm; both tumor size and lymph node metastasis status were evaluated separately. Estrogen and Progesterone receptor and c-erbB-2 data were obtained from patients' pathology records.

Enzyme-Linked Immunosorbent Assay

Serum concentrations of MMP-2, MMP-9 and Twist were determined by using human MMP-2, MMP-9 and Twist enzyme-linked immunosorbent assay (ELISA) kits from Biosource International (Camarillo, CA, USA) according to the manufacturer's instructions. In brief, 100 μL of serum (diluted at 1:50) was used, in duplicate, to measure MMP-2, MMP-9 and Twist levels. The calibrations on each microtiter plate included recombinant standards. Optical densities were determined by using a microtiter plate reader (ELX800; Bio-Tek Instruments, Winooski, VT, USA) at 492 nm. Concentrations are reported as nanogram per milliliter. Serum MMP-2, MMP-9 and Twist levels were determined with no knowledge of clinical data.

Immunohistochemical Analysis

We performed immunohistochemical (IHC) analyses of Twist, MMP-2, MMP-9 protein expression in paraffin-embedded tissue sections (4 μm thick) by using the Two-Step histostaining kit (Changdao Biotech Co., Ltd, Shanghai, China) with polyclonal antibody Twist (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), monoclonal antibodies against human MMP-2 (1:200; Maixin, Fuzhou, China), MMP-9 (1:200; Maixin, Fuzhou, China). The sections were deparaffinized in xylene and rehydrated in a graded series of ethanol solutions. For antigen retrieval, slides were heated in a microwave oven in 0.01 M sodium citrate buffer (pH 6.0) for 20 min. Then, the slides were allowed to cool down in the same buffer and then immersed in 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. After being washed in phosphate-buffered saline (PBS) (2 minutes, 3 times), slides were incubated with primary antibody at 4°C overnight; Rinse in PBS as above; Incubate for 20 min with universal horseradish peroxdase-conjugated detection reagent (Changdao, Shanghai, China); Rinse in PBS as above; Incubated with 3,3'-Diaminobenzidine tetrahydrochloride (Changdao, Shanghai, China) and then all IHC slides were counterstained with hematoxylin stain solution. Using known positive samples as positive controls. For negative controls, the primary antibody was replaced with 0.01 mol/L PBS.

Scoring of Stained Sections

The stained sections were reviewed and scored for expression of Twist, MMP-2 and MMP-9 protein independently by two expert pathologists under double-blind conditions. The sum of the extent and intensity score was used as the final staining score for Twist, MMP-2 and MMP-9. The extent of staining, defined as the percentage of positive staining areas of tumor cells in relation to the whole tissue area, was scored on a

scale of 0 to 3 as follows: 0, no staining; 1, < 1/3; 2, 1/3-2/3; 3, > 2/3. The staining intensity was scored as 0 (no staining), 1 (weakly stained), 2 (moderately stained), and 3 (strongly stained). For the evaluation of Twist expression, the final staining score < 6 were considered to be weak expression, the final staining score ≥ 6 were considered to be high expression[13]. For the evaluation of MMP-2 and MMP-9, the final staining score ≥ 3 were considered to be positive[14].

Statistical Analysis

All statistical analyses were performed by using SPSS software system for Windows (version 13.0; SPSS, Chicago, IL, USA). ELISA data are presented as the mean±standard deviation and were compared using the Student's *t* test. Statistical significance was set as $p < 0.05$. The chi-square test was used to examine the difference in the positive expression rate between the groups. The correlation between the positive expression rate and the different clinicopathological parameters was examined using the non-parameter Spearman's rank correlation analysis.

Results

Serum Expression Levels of MMP-2, MMP-9 and Twist

The minimum, maximum and mean serum levels of MMP-2, MMP-9 and Twist proteins in patients with breast cancer and benign breast disease were shown in Table I.

Significantly higher levels of MMP-2, MMP-9 and Twist were observed in breast cancer patients than in benign breast disease patients ($p < 0.05$) (Figure 1).

We set the median value as the cut-off values for MMP-2, MMP-9 and Twist levels, which is identical to that used in previous publication[15]. The cut-off values for MMP-2, MMP-9 and Twist were defined as 21.2 ng/mL, 47.5 ng/mL and 39.8 ng/mL, respectively. We categorized the serum levels as high or low by compared with the cut-off values. Twenty-eight breast cancer patients (61%) had high serum levels of MMP-2, thirty-nine (85%) of MMP-9 and forty-two (91%) of Twist.

Tissue Expression of MMP-2, MMP-9 and Twist

MMP-2, MMP-9 and Twist proteins were mainly expressed in the cytoplasm of tumor cells (Figure 2). Our results showed that 33 (72%) of the 46 breast cancer specimens stained positive for MMP-2, 35 (76%) stained positive for MMP-9, and 32 (70%) stained strongly or very strongly for Twist.

Table I. Serum levels of MMP-2, MMP-9 and Twist proteins in patients with breast cancer and benign breast disease.

Patient group	Serum level (ng/mL)	
	Range	Mean ± SD
MMP-2		
Breast cancer	18.0-32.8	24.05 ± 4.79
Benign disease	11.7-28.2	19.82 ± 4.13
MMP-9		
Breast cancer	45.9-60.8	52.62 ± 4.77
Benign disease	10.8-23.5	18.90 ± 3.78
Twist		
Breast cancer	30.4-67.5	47.62 ± 9.51
Benign disease	16.4-37.8	26.21 ± 6.00

Clinical Significance of MMP-2, MMP-9 and Twist Expression in Breast Cancer Patients

We compared tissue and serum levels of MMP-2, MMP-9 and Twist protein expression with the patients' clinicopathologic parameters, the results are shown in Table II. A positive association was found between tissue levels of MMP-2 and patients' age, lymph node metastasis and TNM stage ($p = 0.006$, $p = 0.000$ and $p = 0.001$, respectively); We also found a significant correlation between tissue levels of MMP-9 and lymph node metastasis ($p = 0.004$) or TNM stage ($p = 0.013$); Twist protein level in tumor tissues was associated with lymph node metastasis and TNM stage ($p = 0.001$ and $p = 0.004$, respectively).

As shown in Table II, high serum MMP-2 levels were significantly associated with patient age, lymph node metastasis and TNM stage ($p = 0.000$, $p = 0.007$ and $p = 0.034$, respectively); High serum MMP-9 levels were associated with c-erbB-2 expression ($p = 0.033$), but no significant associations between serum levels of MMP-9 and lymph node metastasis and TNM stage ($p = 0.107$ and $p = 0.220$, respectively); Meanwhile, significant association was found between serum levels of Twist and lymph node metastasis and TNM stage ($p = 0.024$ and $p = 0.037$, respectively).

No significant association was found between serum and tissue levels of MMP-2 and tumor size, histological grading, c-erbB-2 expression,

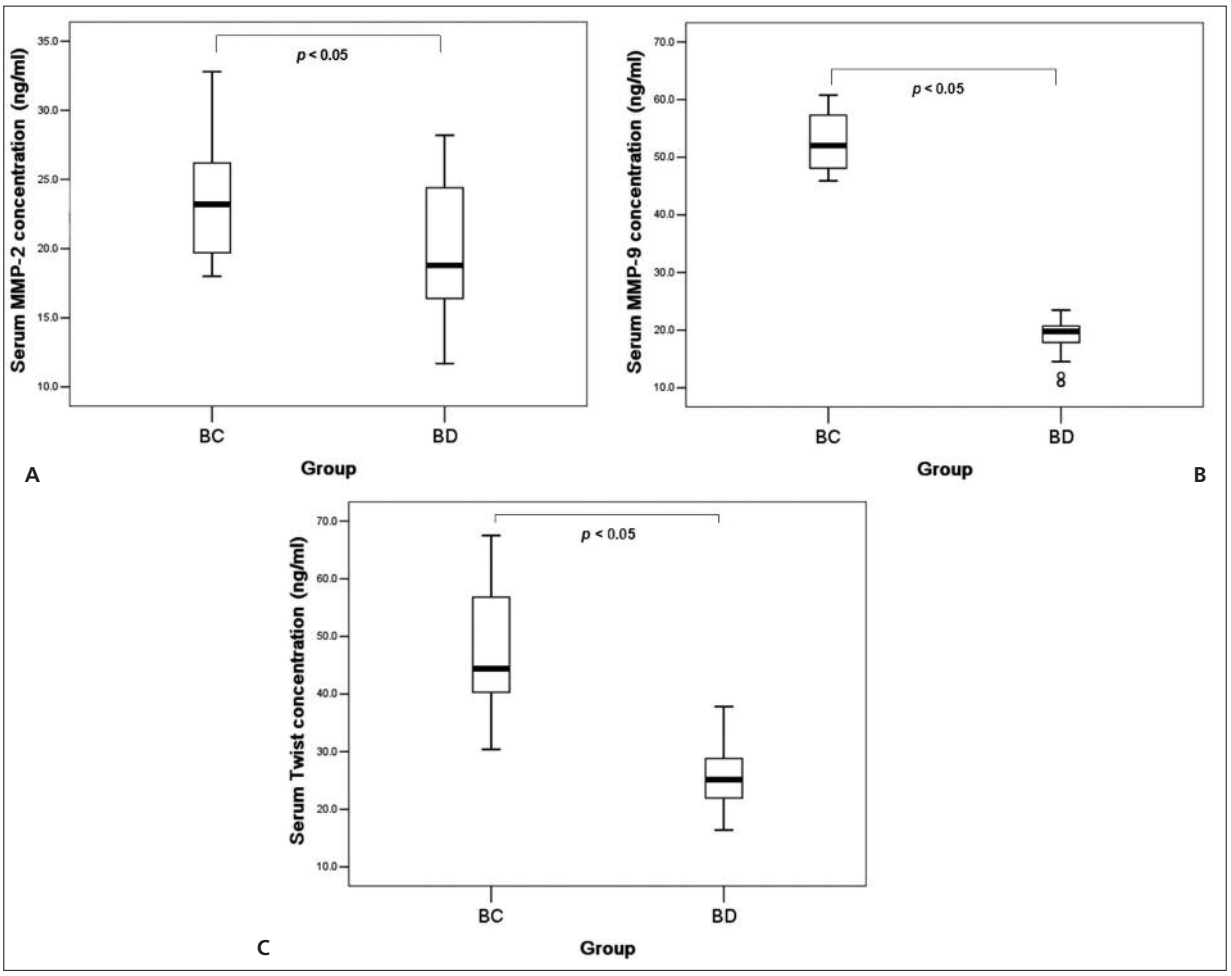

Figure 1. Serum concentrations of MMP-2 ***(A)***, MMP-9 ***(B)*** and Twist ***(C)*** proteins in breast cancer and benign breast disease. We used 100 μL serum to determine MMP-2, MMP-9 and Twist levels by using ELISA. The data are summarized as the mean±standard deviation from a duplicate analysis. Both MMP-2, MMP-9 and Twist concentrations were significantly higher in breast cancer patients (BC group) than in benign breast disease (BD group).

estrogen receptor and progesterone receptor status. No significant association was observed between serum and tissue levels of MMP-9 or Twist and patients' age, tumor size, histological grading, estrogen receptor and progesterone receptor status. No significant associations between tissue levels of MMP-9 and c-erbB-2 expression, and no significant associations between serum and tissue levels of Twist and c-erbB-2 expression.

Correlation of Serum and Tissue Levels of MMP-2, MMP-9 and Twist in Breast Cancer

Serum and tissue levels of MMP-2, MMP-9 and Twist protein expression were correlated and the results revealed a significant correlation between serum and tissue levels of MMP-2 protein (r_s = 0.471, $p < 0.05$), and between serum and tissue levels of MMP-9 protein (r_s = 0.432, $p < 0.05$) and between serum and tissue levels of Twist protein (r_s = 0.365, $p < 0.05$).

Discussion

In our previous studies, the expression and relationship of matrix metalloproteinases (MMP)-2, MMP-9 and Twist protein has been identified in breast cancer tissues. However, the serum levels of gelatinase and Twist in breast cancer patients and its relationship with tumor expression are still unknown. Several studies have demonstrated that gelatinases induced proteolytic degradation of extracellular matrix (ECM) components and basement membranes to facilitate the invasion of diverse tumors[16-18]. MMPs play a central

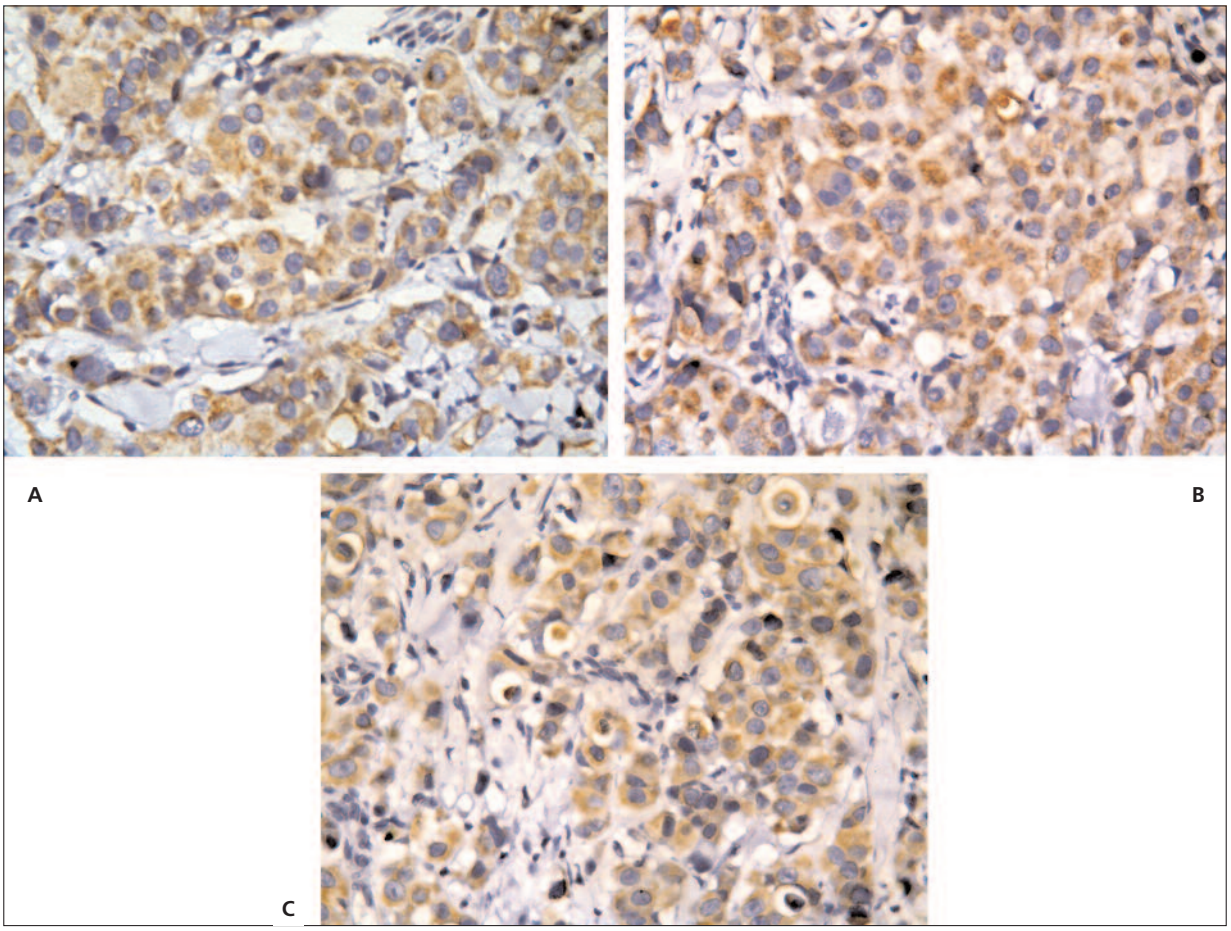

Figure 2. Immunohistochemical analysis of MMP-2, MMP-9 and Twist protein expression in breast cancer. ***A,*** MMP-2 protein expression in the cytoplasm of breast cancer cells. ***B,*** MMP-9 protein expression in the cytoplasm of breast cancer cells. ***C,*** Twist protein expression in the cytoplasm of breast cancer cells. Original magnification, ×400.

role in tumor invasion and metastasis by mediating extracellular matrix proteolysis[19,20]. MMP-2 and MMP-9 are two members of MMP family that has been implicated in tumor cell invasion and metastasis because of their unique ability to degrade Type IV collagen (a major component of the basement membrane) and other essential extracellular matrix components[21]. The Twist protein is a highly conserved transcription factor that belongs to the family of basic helix-loop-helix proteins and it plays an important role in the embryogenesis. Twist was regarded as an oncogene protein, regulating the apoptosis of tumor cells[22]. Epithelial-mesenchymal transition (EMT) is a characteristic of the most aggressive metastatic cancer cells, and is critical for induction of invasiveness and metastasis of human cancers[23,24]. It has been shown that Twist is a key regulator of epithelial-mesenchymal transition and responsible for the invasion and metastasis of tumor. Increasing evidence suggests that Twist emerges as one of the major EMT inducers by regulating E-cadherin expression to promote cancer progression[25-28].

In our current and previous study[7], we demonstrated that increased MMP-2, MMP-9 and Twist protein expression in breast cancer tissues were associated with increased lymph node involvement and higher TNM stage. Wu et al[2] measured serum and tissue levels of MMP-9 by enzyme-linked immunosorbent assay and, immunohistochemistry and in *situ* hybridization in 60 breast cancer patients. They found that high serum and tissue levels of MMP-9 were associated with lymph node metastasis, higher tumor stage and lower relapse-free and overall survival rates. Yang et al[29] detected Twist expression in several human breast tumor cell lines. They observed that invasive and metastatic cell lines expressed Twist while nonmetastatic breast tumor cell lines

Table II. Association of MMP-2, MMP-9 and Twist protein expression with the breast cancer patients' clinicopathologic parameters.

Clinical and pathological features	n	MMP-2		MMP-9		Twist	
		Tissue level (positive), n (%)	Serum level (> 21.2 ng/mL), n (%)	Tissue level (positive), n (%)	Serum level (> 47.5 ng/mL), n (%)	Tissue level (++/+++), n (%)	Serum level (> 39.8 ng/mL), n (%)
Age (years)							
≤ 35	3	**2 (66.7)**[a]	**3 (100)**[d]	2 (66.7)	2 (66.7)	2 (66.7)	3 (100.0)
35-55	24	**22 (91.7)**	**20 (83.3)**	20 (83.3)	22 (91.7)	19 (79.2)	22 (91.7)
> 55	19	**9 (47.4)**	**5 (26.3)**	13 (68.4)	15 (78.9)	11 (57.9)	17 (89.5)
Tumor size (cm)							
≤ 2	6	2 (33.3)	2 (33.3)	4 (66.7)	6 (100.0)	4 (66.7)	6 (100.0)
2-5	38	29 (76.3)	24 (63.2)	29 (76.3)	31 (81.6)	27 (71.1)	34 (89.5)
> 5	2	2 (100.0)	2 (100.0)	2 (100.0)	2 (100.0)	1 (50.0)	2 (100.0)
Lymph node metastasis							
–	19	**8 (42.1)**[b]	**7 (36.8)**[e]	**10 (52.6)**[g]	14 (73.7)	**8 (42.1)**[j]	**15 (78.9)**[l]
+	27	**25 (92.6)**	**21 (77.8)**	**25 (92.6)**	25 (92.6)	**24 (88.9)**	**27 (100.0)**
Histological grading							
I-II	37	29 (78.4)	23 (62.2)	27 (73.0)	31 (83.8)	26 (70.3)	33 (89.2)
III	9	4 (44.4)	5 (55.6)	8 (88.9)	8 (88.9)	6 (66.7)	9 (100.0)
TNM stage							
I -II	21	**10 (47.6)**[c]	**9 (42.9)**[f]	**12 (57.1)**[h]	16 (76.2)	**10 (47.6)**[k]	**17 (81.0)**[m]
III -IV	25	**23 (92.0)**	**19 (76.0)**	**23 (92.0)**	23 (92.0)	**22 (88.0)**	**25 (100.0)**
Estrogen receptor							
Negative	16	10 (62.5)	10 (62.5)	10 (62.5)	14 (87.5)	10 (62.5)	15 (93.8)
Positive	30	23 (76.7)	18 (60.0)	25 (83.3)	25 (83.3)	22 (73.3)	27 (90.0)
Progesterone receptor							
Negative	20	12 (60.0)	10 (50.0)	14 (70.0)	18 (90.0)	14 (70.0)	19 (95.0)
Positive	26	21 (80.8)	18 (69.2)	21 (80.8)	21 (80.8)	18 (69.2)	23 (88.5)
c-erbB-2							
Low	26	19 (73.1)	16 (61.5)	19 (73.1)	**25 (96.2)**[i]	16 (61.5)	24 (92.3)
High	20	14 (70.0)	12 (60.0)	16 (80.0)	**14 (70.0)**	16 (80.0)	18 (90.0)

[a]$p = 0.006$; [b]$p = 0.000$; [c]$p = 0.001$; [d]$p = 0.000$; [e]$p = 0.007$; [f]$p = 0.034$; [g]$p = 0.004$; [h]$p = 0.013$; [i]$p = 0.033$; [j]$p = 0.001$; [k]$p = 0.004$; [l]$p = 0.024$; [m]$p = 0.037$.

did not. In addition, they found that suppression of Twist expression inhibits tumor metastasis and reduces the presence of tumor cells in the blood circulation in a mice model. Consistent with their results, our present study demonstrated that MMP-2, MMP-9 and Twist protein expression were correlated with tumor lymph node involvement and TNM stage, suggesting that MMP-2, MMP-9 and Twist might be evaluated as biomarkers for predicting progression and prognosis of breast cancer.

Our data also suggest that serum levels of MMP-2, MMP-9 and Twist protein were positively associated with their tissue expression. Furthermore, significantly higher serum levels of MMP-2, MMP-9 and Twist were found in breast cancer patients than in benign breast disease patients, high serum MMP-2 levels and serum Twist levels were significantly associated with lymph node metastasis and TNM stage. Consistent with our results, Rashad et al[30] found that serum MMP-9 was increased significantly in metastatic breast cancer patients as compared to non-metastatic patients. Ruokolainen et al[31,32] reported that serum and tissue MMP-2 and MMP-9 levels were associated with clinical behaviors in head and neck squamous cell carcinoma.

Conclusions

Our study suggests that MMP-2, MMP-9 and Twist may play essential roles in breast cancer invasion and metastasis. Serum and tissue levels of them may serve as potential novel prognostic factors for breast cancer patients. Further studies are required to confirm Twist and gelatinases as biomarkers for detection of breast cancer progression.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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