Matrix Metalloproteinases MMP2 and MMP9 Expression in Stages II-III Breast Cancer in Iraqi Women

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ABSTRACT

Breast cancer is the most common invasive cancer in women worldwide. Metalloproteinases MMP2 and MMP9 participate in tumor invasion and metastasis by degrading extracellular matrix. In this study, we investigated the expression of MMP2 and MMP9 in breast tissues of Iraqi women with stage II and III breast cancer. The correlation between the expression levels of MMP2 and MMP9 in stage II-III breast cancer and clinicopathological features was also examined. The expression levels of MMP2 and MMP9 in the breast were determined by real-time PCR and immunohistochemistry in 64 patients with stages II-III samples and in 21 benign tumors from Iraqi women. The mRNA levels of MMP2 and MMP9 were significantly higher in breast cancer stages II-III than those in benign breast tumor tissues at $P< 0.05$. Immunohistochemistry also revealed that the protein levels of MMP2 and MMP9 were 72% and 64% in patients with stages II-III breast cancer as compared to 28% and 23% in benign breast tumor. The increased levels of MMP2 and MMP9 in stages II-III breast cancer were correlated to tumor grade ($P=0.04$ and 0.01, respectively), stage ($P=0.03$ and 0.05) and type ($P=0.004$ and 0.05) and lymph node metastasis ($P=0.009$ and 0.04), respectively.MMP2 and MMP9 expression levels were increased in stages II-III of breast cancer in Iraqi women and their levels were correlated with tumor grade, stage and type and lymph node metastasis.These metalloproteinases can be used as biomarkers for breast cancer progression and metastasis.

Key words: Breast cancer, Iraqi women, Matrix metalloproteinases, MMP2, MMP9.

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INTRODUCTION

Breast cancer is one of the most frequent cancers and it is the leading cause of death related to cancer among women worldwide (Jemal et al., 2011). Death from breast cancer results from distance metastasis rather than primary cancer (Welch et al., 2000). A key role of metastasis is achieved by degradation of extracellular matrix (ECM) allowing tumor cells to invade local tissues, enter into bloodstream and then cause development of secondary tumors in other organs (Shah et al., 2009). Under normal conditions, matrix metalloproteinases (MMPs) play important role in tissues remodeling, ovulation, wound healing and angiogenesis (Sternlicht and Werb, 2001). High MMPs expression levels participate in development of cancer (Pivetta et al.,

Table 1. The sequences of specific primers used for determination of ALDH-1, CD44, OCT3/4 and β-actin by real-time PCR.

	PCR product (bp)	Primer pair sequences (5' to 3')	Accession number
MMP2	170	F: AAGGACAGCCCTGCAAGTTT	NM_001127891.2
		R: GTTCCCACCAACAGTGGACA	
MMP9	229	F: GGTGATTGACGACGCCTTTG	NM_004994.2
		R: GGACCACAACTCGTCATCGT	
B-actin	78	F: CCGCAAATGCTTCTAGGCG	NM_001101.3
		R: TGTTTTCTGCGCAAGTTAGGT	

2011). MMPs are involved in degradation of ECM and they play important roles in breast cancer invasion and metastasis (Ala-aho and Kähäri, 2005). They are synthesized and released as zymogens which can be activated by serine proteases and/or other metalloproteinases (Shah et al., 2009). The activities of MMPs are regulated by tissues inhibitor metalloproteinases (TIMPs) and stimulated by different microenvironment signaling molecules such as growth factors and cytokines (Woessner, 1991).

Matrix metalloproteinase 2 and 9 (MMP2 and MMP9), also called gelatinases A and B, are responsible for degradation of basement membranes. MMP2 and MMP9 are implicated in breast cancer invasion and metastasis due to their ability to degrade collagen IV which is the main component scaffold protein of basal membrane (Iwasaki et al., 2002; Kato et al., 2002). When this protein is degraded, breast cancer cells are prone to infiltrate (Duffy et al., 2008). The elevated MMP2 and MMP9 expression levels in cancer provide canals during which the cancer cells spread resulting in metastasis. Therefore, the expression of high levels of MMP2 and MMP9 is associated with cancer cells invasion, metastasis and worse prognosis (Vizoso et al., 2002).

In this study, the expression levels of MMP2 and MMP9 in Iraqi women with stages II-III breast cancer and benign breast tumors were investigated using real-time PCR and immunohistochemistry. In addition, correlations between the expression levels of MMP2 and MMP9 and patient's clinicopathological features were investigated.

MATERIALS AND METHODS

Patients and tumor characteristics

This study involved 64 breast cancers as well as 21 benign tumors surgically harvested from women admitted to Alkarama Teaching Hospital in Baghdad, Iraq between June 2013 and April 2014. This study was approved by the Institutional Review Board at Beirut Arab University (BAU), Lebanon. All patients with breast cancer recruited in this study were diagnosed as stages II-III, where 60.9% of patients were stage II and 39.1% were stage III breast cancer. The mean age of patients was 64 years

(range was 36 to 77 years). The fine needle aspiration (FNA) technique, mammogram and histophathological examination were used for the diagnosis of all cases. All the patients enrolled in this study have not received chemo or radiation therapy.

Fresh breast cancer and benign breast tissue samples were collected and divided into two parts: one part was fixed in 10% formalin and embedded in paraffin to be used in immuno histochemisty staining and pathological examination for the determination of tumor grade, type and stage, lymph node metastasis and ER, PR and HER2/Neu hormones receptor status. Tumor stages were evaluated according to modified bloom-Richardson grading system. The other parts of fresh samples were stored in liquid nitrogen for subsequent use for RNA extraction.

The details for histophathological examination were described previously (Mahmood et al., 2015). In brief, 75% of the cases were pure invasive ductal carcinomas, 15.6% were invasive lobular carcinomas and 9.4% were other cancers (invasive medullary carcinoma and mixed infiltrative ductal carcinoma and lobular carcinoma).

RNA extraction and cDNA preparation

To determine the mRNA levels of MMP2 and MMP9, total RNA was extracted from frozen tissues using an RNA miniprep kit (Agilent biotechnology Inc., USA) according to manufacture instructions. Pure extracted RNA was used for the synthesis of cDNA using a Revert Aid First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc., USA) according to instructions.

qRT-PCR

The mRNA levels of MMP2, MMP9 and β-actin in breast tissues from breast cancer and benign tumors were determined by real-time PCR. The mRNA accession numbers for the mRNA sequences were obtained from NCBI database. Primer-Blast tool at NCBI was used to design specific primers for the genes examined in this study. The sequences of these primers were used are shown in Table 1.

The PCR amplification reaction was carried out by AccuPower GreenStar qPCR Premix (Bioneer, Korea) using Stratagene Mx3005PqPCR system (Agilent Technologies, CA, USA). The thermal profile reaction used was 5 minutes at 95° C for 1 cycle, followed by 40 cycles at 94° C for 15 seconds, 55° C for 30 seconds and 72° C for 30 seconds. The calculation of relative amount of gene expression was performed by using the equation $2^{-\Delta\Delta CT}$.The Mann-Whitney U test was used to compare the median expression levels.

Immunohistochemistry staining

Protein expression of MMP2 and MMP9 was determined by immunohistochemically. Four mm of paraffin embedded sections were cut and mounted on positively charged glass slides and deparaffinized in xylene and rehydrated by series of ethanol solution. Primary antibodies: anti-MMP2 antibody (M2420-52Q US-bio, USA), or anti-MMP9 antibody (M2420-01R US-bio USA) were added for overnight at 4°C. The slides were incubated with horseradish peroxidase conjugate detection reagent (DAKO biotechnology) and with complex avidin-biotin for 30 minutes at room temperature for each. The sections were visualized using diaminobenzidine (DAB) and counter-stained with hematoxiline, dehydrated with ethanol and with zylene. Finally the slides were mounted with water-free mounting medium (DPX) and analyzed by light microscope at (400x). Placental tissue was used as a positive control. The negative control was treated with all the above steps except the incubation with primary antibody.

Evaluation of immunohistochemical staining was carried out blind to the patient's data and pathological features. The percentage and intensity of the staining were considered in this study. Normal cells that present in the whole tissue were scored as negative 0 percentage (no positive staining), score 1: (1-10%), score2: (11-50%) and score 3 (51-100 %). The positive intensity was considered as 0 (none), weak positive, moderate' and strong positive intensity. Both 0 and score 1 were considered as low expression and while score 2 and score 3 were considered as high expression. Expression of each gene over 10 % was considered positive.

Statistical analysis

The comparison between breast carcinoma stage II-III and benign tumors was performed by using prism pad graph version 6 (Graphic pad Software Inc., San Diego CA, USA). Comparison of expression values was performed by Mann-Whitney U test. A chi-square (χ^2) statistic was used to investigate whether expression values differed between breast cancer tissues stage II-III and benign tumors. A P<0.05 value was considered as statistically significant.

RESULTS

MMP2 and MMP9 gene expression in breast cancer and benign tumors

Figure 1 shows the expression levels of MMP2 and MMP9 in breast tissues from stage II-III breast carcinomas and benign tumors as determined by real-time PCR. The mRNA level of MMP2 was increased in stages III and II of breast carcinomas (median= 42.97, P=0.0006 and 21.81, P= 0.04, respectively) as compared to that of benign tumors (median= 5.94) (Figure 1A). The mRNA level of MMP9 was increased in stages III and II of breast cancer tissues (median= 9.31, P=0.0006 and 4.78, P= 0.01, respectively) as compared to that in benign tumors (median= 2.83) (Figure 1B).

Protein expression levels of MMP2 and MMP9 as determined by immunohistochemistry

MMP2 expression was localized in the cytoplasm and nuclei of stage III breast cancer cells (Figure 2). The high expression of MMP2 was observed in 71.8% of cancerous tissues compared to 28.6% of benign tumors (P= 0.001) (Table 2). Immunohistochemistry analysis of breast cancer tissues showed high expression of MMP9 in the cytoplasm of stage II-III breast cancer tissues (Figure 3). MMP9 high expression was found in 74.1% of cases in cancer tissues and 23.8% of benign tumors (P value= 0.001) (Table 2).

Relation between MMP2 and MMP9 expression and clinical pathological data

Expression levels of MMP2 and MMP9 were significantly increased with tumor stage (P=0.03 and 0.05, respectively), tumor grade (P=0.04 and 0.01, respectively), and with tumor type (P=0.004 and 0.05) (Table 3). MMP2 and MMP9 expression levels were higher in patients with lymph node metastasis when compared to the negative metastatic group (P=0.009 and 0.04). There was no significant variation between the expression levels of MMP2 and MMP9 with age or hormone receptor status (Table 3).

DISCUSSION

Distance metastasis is the main cause of mortality among women with breast cancer worldwide (Pantel and Brakenhoff, 2004). Degradation of extracellular matrix by
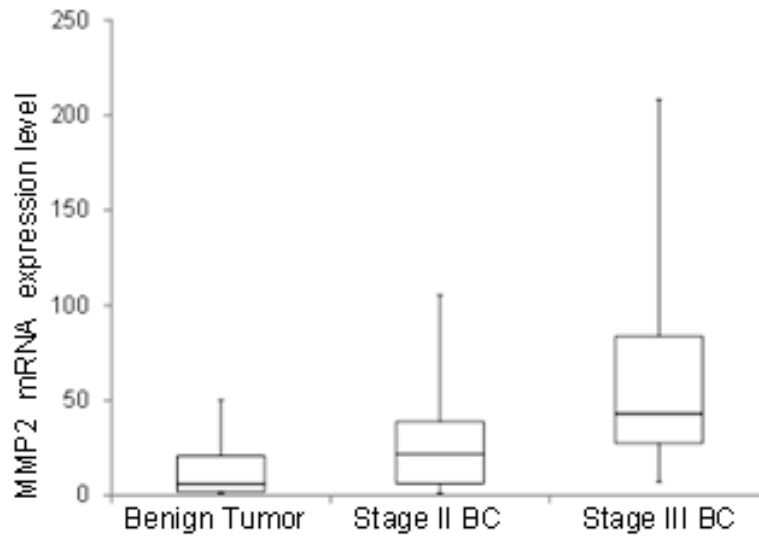
Figure 1A. Boxplot analysis of mRNA levels of MMP2 in benign breast tumors (n=17) and stage II (n=32) and stage III (n=18) breast cancer in Iraqi women as determined by real-time PCR. The expression level of each mRNA was normalized to the corresponding expression of β-actin. The horizontal line within the box represents the median value, and the lines extending from the box indicate the maximum and minimum expression levels.
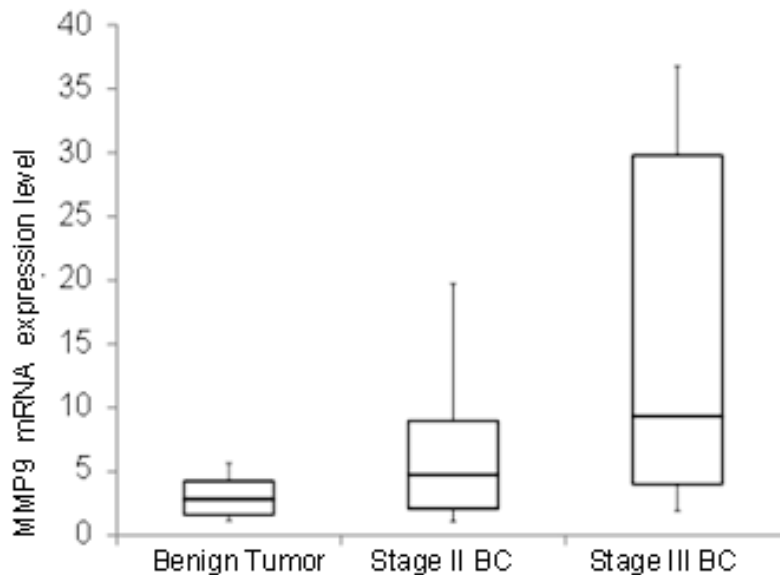

Figure 1B. Boxplot analysis of mRNA levels of MMP9 in benign breast tumors (n=17) and stage II (n=32) and stage III (n=18) breast cancer in Iraqi women as determined by real-time PCR. The expression level of each mRNA was normalized to the corresponding expression of β-actin. The horizontal line within the box represents the median value, and the lines extending from the box indicate the maximum and minimum expression levels.

metalloproteinases allows tumor cells to escape from their primary tumor, invade local tissues, enter into the blood stream and reach the secondary sites causing formation of new tumors on other organs. Metalloproteinases play key roles in metastasis process due to their ability to degrade matrix proteins (Rowe and Weiss, 2008).

Cancer cells invasion and metastasis are controlled by genetic variation in tumor cells and by stromal cells that secret MMPs (Jezierkska and Motyl, 2009). Interaction
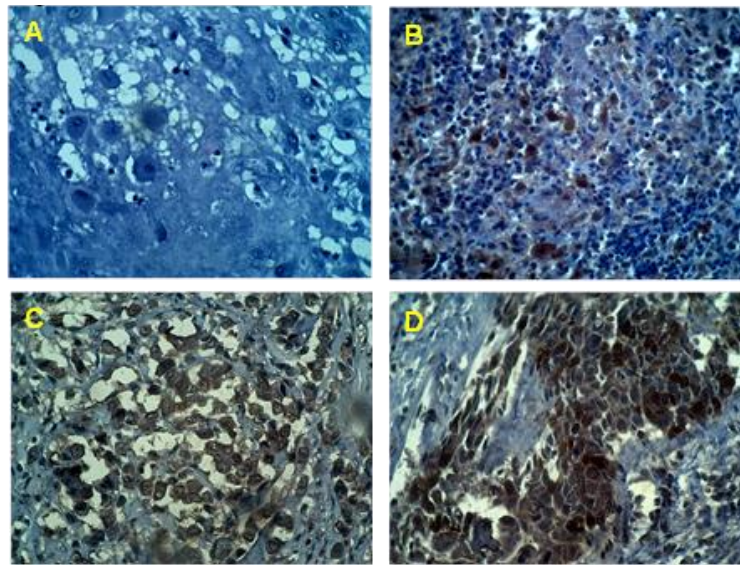
Figure 2. Immunohistochemistry staining of MMP2 in benign tumors, stage II and stage III breast cancer tissues (400X). **(A)** Negative control showing no expression of MMP2. **(B)** Benign tumor with slight MMP2 protein expression. **(C)** Stage II breast carcinoma section showing infiltrative tumor sheeths with strongly positive MMP2 protein expression. **(D)** Stage III breast carcinoma with high MMP2 protein expression. Tumor masses replace normal epithelia and adipose tissue with positive expression of MMP2 in the nuclei of cancer cells.

Table 2. MMP2 and MMP9 expression levels in breast carcinoma tissues (n=64) and benign tumors (n=21).

	High expression n (%)	Low expression n (%)	P value
MMP2			
Cancer Tissues	46 (71.8 %)	18 (28.2%)	
Benign Tumors	6 (28.6%)	15 (71.4%)	0.001
MMP9			
Cancer Tissues	41 (74.1%)	23 (25.9%)	
Benign Tumors	5 (23.8%)	16 (76.2%)	0.001

between these factors may promote cancer cells invasion and metastasis. The basement membranes that surround tumor nests play important roles in preventing cancer cells from invasion and spread (Hai-Xia et al., 2012). Inwell differentiated tumors (grade 1), the tumor cells have low invasion properties due to their relatively low expression of matrix metalloproteinases. However, in poor differentiated breast cancer (grade III), there is a high invasion and malignant activity resulting from the overexpression of MMPs (Hai-Xia et al., 2012). In breast cancer, the most important matrix metalloproteinases are MMP2 and MMP9 that catalyze the breakdown of gelatin IV which is the main component of extracellular matrix. Therefore, MMP2 and MMP9 are implicated in breast cancer cells invasion and metastasis (Eiseler et al., 2009).

In this study, we investigated the expression levels of MMP2 and MMP9 in breast cancer tissues as well as in benign tumor tissues in Iraqi women. We also evaluated the correlation between the expression levels of MMP2 and MMP9 and clinical pathological parameters of Iraqi women with stage II-III breast cancer.

Our results showed significant increased MMP2 and MMP9 expression levels in the breast tissues at the mRNA and protein levels in 64 cases of stage II-III breast cancer as compared to that in benign tumors in Iraqi women.

Other studies also showed that MMP2 and MMP9 expression levels were significantly increased in breast cancer as compared to that in benign tumors (Shah et al., 2009; Jinga et al., 2006).

In this study, the increased mRNA levels of MMP2 and MMP9 were found to be stage-dependent. Higher expression levels of MMP2 and MMP9 were found in stage III breast cancer tissues as compared to that in stage II. The increased expression levels of these
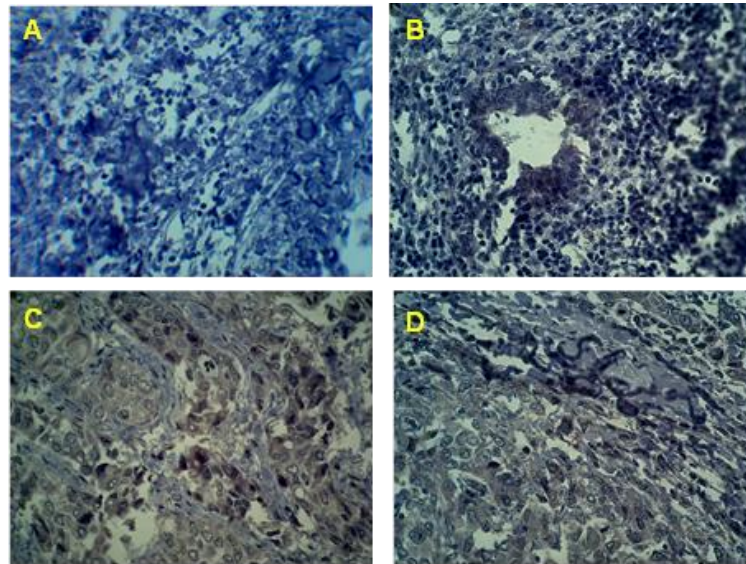
Figure 3. Immunohistochemistry staining of MMP9 in benign tumor tissues, stage II and stage III breast cancer tissues (400X). **(A)** Negative control. **(B)** Benign tumor section with low positive expression. **(C)** Stage II breast cancer section showing infiltrated tumor cell within stromal and epithelial cells. **(D)** Stage III breast cancer section showed highly expressed of MMP9 in cytoplasm of cancer cells.

Table 3. Correlation between the MMP2 and MMP9 expression levels and patients clinic pathological data features.

Pathological Data	Number of patients	MMP2 Expression		P value	MMP9 Expression		P value
		High	Low		High	Low	
Age of patients							
≤ 50 years	17	12(70%)	5(30%)	0.21	10(58%)	7(42%)	0.31
≥ 50 years	47	25(53%)	22(47%)		21(44%)	26(56%)	
Tumor Stages							
Stage II	39	22(56%)	17(44%)	0.03*	20(51%)	19(49%)	0.05*
Stage III	25	21(84%)	4(16%)		19(76%)	6(24%)	
Tumor grades							
Grade I	8	3(38%)	5(62%)	0.04*	2(25%)	6(75%)	0.01*
Grade II	36	23(64%)	13(36%)		19(53%)	17(47%)	
Grade III	20	16(80%)	4(20%)		17(85%)	3(15%)	
Tumor types							
IDC	48	36(75%)	12(25%)		32(67%)	16(33%)	0.05*
ILC	10	4(40%)	6(60%)	0.004**	3(30%)	7(70%)	
Others	6	1(16%)	5(84%)		2(33%)	4(67%)	
Lymph node metastasis							
Positive	27	21(77%)	6(23%)	0.009**	19(70%)	8(30%)	0.04*
Negative	37	14(37%)	23(63%)		15(41%)	22(59%)	
ER status							
Positive	49	14(28%)	35(72%)	0.08	17(36%)	32(64%)	0.43
Negative	15	6(46%)	9(54%)		8(47%)	7(53%)	
PR status							
Positive	47	20(42%)	27(58%)	0.28	27(57%)	20(43%)	0.33
Negative	17	9(52%)	8(48%)		12(71%)	5(29%)	
Her2/neu status							
Positive	24	14(58%)	10(42%)	0.9	13(54%)	11(46%)	0.97
Negative	40	28(60%)	12(40%)		22(55%)	18(45%)	

IDC: invasive ductal carcinoma, **ILC**: invasive lobular carcinoma, **ER**: estrogen receptor, **PR**: progesterone receptor, **HER2/Neu**: epidermal growth factor receptor.*P≤0.05, **P≤0.01.

markers may accelerate tumor growth by induced local invasion and metastasis by degradation of extracellular expression in breast cancer (Peng et al., 2007; Yu and Stamenkovic, 2000).

We have previously demonstrated that CD44 was highly expressed in stage II-III breast cancer tissues as compared to that in the benign breast tumors (Mahmood et al., 2015). In this current study, we found that MMP9 expression level was also increased in stage II-III breast cancer patients. CD44 may act as a docking site location for MMP9 to form CD44-MMP9 complex in breast cancer and this complex may play important roles of MMP9 in progression and metastasis of breast cancer (Fáblo et al., 2009). A previous study demonstrated elevated MMP9 expression levels in stage III breast cancer with no significant variation in MMP2 expression levels (Köhrmann et al., 2009). In contrast to

the latter study, our results showed highly expression levels of MMP2 in stage II and stage III breast cancer tissues from Iraqi women.Invasive ductal carcinoma is considered as a poor prognosis with low overall survival rate compared to well prognosis in mucinous and tubular carcinoma with high overall survival (Del Caser et al., 2010). In this study, there was a significant correlation between MMP2 and MMP9 expression levels and tumor type of invasive ductal carcinoma.

These results confirm the role of MMP2 and MMP9 in invasion and aggressiveness of breast cancer (Gonzalez et al., 2010). Our results showed that MMP2 and MMP9 expression levels were significantly higher in Iraqi women with positive lymph node metastasis as compared to negative lymph node metastasis patients indicating that MMP2 and MMP9 participate in tumor cells spread and metastasis of breast cancer in Iraqi women. It has been shown that the activities of latent and active forms of MMP2 and MMP9 in patients with positive lymph node metastasis were higher than that in patients with negative lymph node metastasis (Sandra et al., 2013). MMP2 is localized in the nuclei of breast cancer cells while it is expressed only in the cytoplasm of normal breast epithelial cells (Noemi et al., 2013). In this study, MMP2 was highly expressed in the cytoplasm and nuclei of stage III breast cancer cells.

This inter localization of MMP2 may play a role in the aggressiveness and metastasis of breast cancer and requires further investigation.

In conclusion, these results indicate that the expression levels of MMP2 and MMP9 were increased in stages II-III breast cancer in Iraqi women. MMP2 and MMP9 can be used as biomarkers for diagnosis of breast cancer stage, grade, type and metastasis. Localization of MMP2 in the nuclei of breast cancer cells may play important roles in breast cancer progression and metastasis.

The use of specific inhibitors targeting these metalloproteinases for the treatment of breast cancer metastasis requires further investigation.

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