Original Article

Association between Expression of Tissue Inhibitors of Metalloproteinases-1, Matrix Metalloproteinase-2, and Matrix Metalloproteinase-9 Genes and Axillary Lymph Nodes Metastasis in Patients with Breast Cancer

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Abstract

Background: Certain enzymatic biomarkers such as matrix metalloproteinase (MMPs) are instrumental in the breast cancer. Hence, they are viewed as predictive biomarkers in the primary prognosis of this type of cancer. Furthermore, they enjoy a predictive value in the evaluation of the disease, recurrence of tumor, invasion of tumor cells to other areas as well as therapeutic outcomes. The present study aimed to determine the association between the expression of the three tissue inhibitors of metalloproteinases-1 (TIMP1), MMP2, and MMP9 genes and axillary lymph nodes involvement in patients with breast cancer. **Methods:** Patients in this study were categorized into two groups, first with axillary lymph nodes involvement (as the case group) and second group without the involvement of axillary lymph nodes (as the control group) referred to Cancer Institute at Imam Khomeini Hospital in Tehran in 2016. The gene expression was assessed using the reverse transcription polymerase-chain reaction technique. **Results:** There was no significant difference in the mRNA level of MMP2 and MMP9 genes between the cancer tissues with and without axillary lymph node metastasis in comparison with normal samples. However, the mRNA level of TIMP1 gene was considerably higher in the cancer tissue with axillary lymph node metastasis as compared to the samples without metastasis. In other words, the presence of axillary lymph node metastasis induced a 77.8-fold increase in mRNA expression when compared to condition without metastasis. **Conclusions:** The expression of TIMP1 gene is strongly associated with axillary lymph node metastasis in breast cancer patients.

Keywords: *Breast cancer, lymph nodes, matrix metalloproteinase-2, matrix metalloproteinase-9, tissue inhibitors of metalloproteinases-1*

Introduction

The appearance of metastatic disease in the regional lymph node chain provides vital information to define staging, treatment, and prognosis of breast cancer. The gold standard in evaluating the involvement of lymph nodes is still axillary lymphadenectomy associated with histological assessment.[1] However, because of its associated relevant morbidity, the use of valid biomarkers is now discovering by researchers whole of the word.[2] As advances have been made in the imaging the diagnosis of breast and subsequently, the incidence of cases of early stage disease increases, the presence of axillary lymph nodes metastasis declines, and meantime, there is need for a less invasive option.[3,4]

Identifying new prognostic markers, a better understanding of the behavior of tumors and technological developments in the field of imaging, are potentially able to change the axillary staging in the future through selecting patients eligble for less aggressive intervention.[5]

However, neither of clinical examination and mammography presents appropriate accuracy in the process of identifying metastasized axillary lymph nodes and several studies recommend other modalities and imaging methods.[6] In total, no method of imaging is of sufficient negative predictive value to avoid a surgical approach to the axilla when no lymph node involvement has been identified.[7,8]

As clinical and epidemiological studies have already show, some enzymatic biomarkers such as matrix metalloproteinase (MMPs) are instrumental in breast cancer.[9,10] They

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may be viewed as predictive biomarkers for the primary diagnosis of breast cancer. They also enjoy a predictive value in evaluating the disease, recurrence of tumor, invasion to other areas as well as therapeutic outcomes.[11] It has been recently suggested that the presence of mutations in the genes encoding some of these enzymes such as MMP-1,-2,-3,-9 may be involved in progression of breast cancer,[12-14] however, some others could not demonstrate such role for these gene polymorphisms. In addition, the critical role of MMPs in predicting lymph nodes involvements and metastasis has remained unknown. The present study aimed to determine the association between expression of the three tissue inhibitors of metalloproteinases-1 (TIMP1), MMP2, and MMP9 genes and axillary lymph nodes involvement in patients with breast cancer.

Methods

Study population

This cross-sectional study was conducted on the two groups of cancer patients. The first group with axillary lymph nodes involvement (as the case group) and the second group without involvement of axillary lymph nodes (as the control group) referred to Cancer Institute at Imam Khomeini Hospital in Tehran in 2016. All cancer patients were finally diagnosed by physical examination, histological assessment, and also mammography. Those with the history of receiving radiotherapy or chemotherapy were excluded from the study. All study protocols were approved by the Tehran University of Medical Sciences and the written informed consent were received from all subjects after explaining the study details and before participating in the study.

Study protocol

The tumor tissue-containing samples we collected for this study are archived formalin-fixed, paraffin-embedded (FFPE) (FFPE tissue was used to diagnosis of histology and lymph node involvement, and fresh frozen tissue was used for RNA extraction). It was confirmed that tumor grades were using the Modified Scarff-Bloom-Richardson-Elston-Ellis grading system. We also obtained normal breast tissues to serve as internal controls. The pathological information including tumor grading, tumor type, and its staging, as well as metastasis to axillary lymph nodes were collected by pathological assessment. The expression of MMP9, MMP2, and TIMP1 by normal mammary and breast cancer cells was studied following cell cultures. Extraction of total RNA from frozen tissues was done through the use of an RNA miniprep kit (Agilent biotechnology Inc., USA), following manufacture instructions. Moreover, pure extracted RNA was used in the synthesis of cDNA by using a Revert Aid First Strand cDNA synthesis kit. Quanitification of the amount of isolated RNA was done through measurement of its absorbance at 260 nm. RNA preparations were totally free of DNA contamination as seen after reverse transcription polymerase-chain reaction (RT-PCR) analysis and the absorbance ratios (A260/A280). The expression of mRNAs encoded for MMP2, MMP9, and TIMP1 was examined by RT-PCR. RT of RNA was performed using the Revert Aid First Strand cDNA synthesis kit. To design specific primers for the genes studies in this survey, Primer-Blast tool at NCBI was used. The sequence of the primers and subsequent conditions were used for MMP2: Upstream 5'-AAGGACAGCCCTGCAAGTTT-3' downstream 5'-GTTCCCACCAACAGTGGACA-3'; for MMP9: Upstream 5'-GGTGATTGACGACGCCTTTG-3' downstream 5'-GGACCACAACTCGTCATCGT-3'; and for TIMP1: Upstream 5'-TTCCGACCTCGTCATCAGGG-3' downstream 5'-ATTCAGGCTATCTGGGACCGC-3'.

Accu Power Green Starq PCR Premix (Bioneer, Korea) was used to carry out the PCR amplification reaction. That was done by applying Stratagene MX 3005PqPCR system (Agilent Technologies, CA, USA). Thermal profile reaction was first used at 95°C for 5 min for 1 cycle. Then, it was used by 40 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s.

Glyceraldehyde phosphate dehydrogenase (GAPDH) was amplified as an internal control. After the performance of arrays, the values were obtained for the threshold cycle (Ct) for the genes. They were then normalized using the housekeeping gene (GAPDH) on the same array. The change (ΔCt) between the genes in the states with and without metastasis and controls was calculated. The fold-change was specified through use of the formula, fold-change = $2^{[-\Delta\Delta Ct]}$. The obtained values were reported as fold-change; the only genes to be considered where those that had shown two-fold or greater change. The study end-point was to first compare gene expressions in the two types of cancer tissues with and without axillary lymph node metastasis with normal tissue and then to assess the relationship between the gene expressions with tumor patterns including vascular invasion, number of lymph nodes, positive axillary lymph node, and extension of tumor cells to adjacent tissues.

Statistical analysis

For statistical analysis, the results were reported as mean ± standard deviation for quantitative variables before being summarized by absolute frequencies and percentages for categorical variables. The Kolmogorov–Smirnoff test was used to analyze the normality of data. Using Chi-square test or Fisher's exact test m categorical variables were compared. That was when >20% of cells with expected count of <5 were observed. Furthermore, quantitative variables were compared through *t*-test or Mann–Whitney U-test. The association between the variables was examined using the Pearson's or Spearman's

correlation test. For the statistical analysis, the statistical software SPSS version 22.0 for Windows (SPSS Inc., Chicago, IL) was used. $P = 0.05$ or less were considered statistically significant.

Results

The mean value of MMP2, MMP9, and TIMP1 gene expression in breast cancer patients (with and without axillary lymph node involvement) is shown in Table 1. There was no significant difference in mRNA level of MMP2 and MMP9 in patients with and without axillary lymph node involvement. By the way, there is a significant decrease in MMP2 gene expression levels in tumor sample in comparison to normal adjacent tissue. The mRNA level of TIMP1 gene was considerably higher in the cancer tissue of patients with axillary lymph node involvement in comparison with patients without metastasis. In another word, the presence of axillary lymph node involvement induced a 77.8 fold increase in mRNA expression level when compared to condition without metastasis.

Association between the mRNA expression level and histopathologic characteristics are described in Table 2. There is no significant relationship between VI and TIMP1 ($P = 0.341$), MMP9 ($P = 0.398$), and MMP2 ($P = 0.414$) expression levels in our study population, expression level of TIMP1 and MMP9 had no relationship with tumor extension ($P = 0.535$ and 0.185, respectively.) but MMP 2 expression level had significant negative relationship with tumor extension ($P = 0.002$). Axillary lymph node involvement also negatively related to the expression level of TIMP1 ($P = 0.30$) but not related to the expression level of MMP9 ($P = 0.112$) and MMP2 ($P = 0.441$). There was no correlation between number of positive lymph node and expression level of TIMP1, MMP9, and MMP2.

Statistic analysis

The difference between expression level two or more were analyzed by nonparametric tests using Mann–Whitney U-test and Kruskal–Wallis test. Correlation between the number of positive axillary lymph nodes and the expression levels of TIMP1, MMP9, and MMP2 have been down using bivariate correlation and Pearson test. $P < 0.05$ has been considered as statistically significant.

Discussion

The assessment of gene expression using RT-PCR technique is fundamentally performed by determining the level of mRNA related to the pointed gene and thus calculating the fold change between the targeted and control samples. In this study, the changes in gene expression levels of TIMP1, MMP9, and MMP2 in breast cancer patients with and without axillary lymph node involvement were evaluated and relationship between expression fold change and histopathologic characteristics of tumors were investigated. The result of this study indicated that the expression of TIMP1 is significantly associated with lymph node involvement. In another word, upregulation of TIMP1 is related to the risk of axillary lymph node involvement. In the second step, the association between genes expression and tumor characteristics such as vascular invasion, extent to adjacent tissues, and number of positive lymph nodes were evaluated. Reviewing the literature indicates paradoxical findings in association between expressions of three genes with lymph node metastasis or tumor prognosis. In a study by Wu *et al*.,[15] the level of TIMP1 coded mRNA in the patients with metastatic disease was significantly higher than that reported in those without lymph node metastasis. In addition, expression of the gene was correlated to tumor grade, tumor recurrence as well as lower patients' long-term survival. In another study by Lu *et al*.,[16] the MMP9/TIMP1 ratio was considerably in malignant than in benign breast masses. Contrarily, in another study by Thorsen *et al*.,[17] the MMP9/TIMP1 ratio was not associated with disease prognosis. Zheng *et al*.[18] also revealed that the expression of both MMP2 and MMP9 could increase the risk for lymphangiogenesis and metastasis to regional lymph nodes. In a study by Sullu *et al*.,[19] although MMP9 was not associated with lymphatic metastasis, it could predict poor disease prognosis. Stankovic *et al*.[20] indicated that the increase in the level of both MMP2 and MMP9 was associated with tumor size and lymph nodes involvement. In final, Radenkovic *et al*.[21] showed that the breast cancer patients with axillary lymph node metastasis had higher level of active forms of MMP2 and MMP9 than those without metastasis. Comparing our study with previous studies strongly shows paradoxical results on association between TIMP1, MMP9, and MMP2 genes expression and lymph node metastasis emphasizing the ethnical and geographical differences in the genes

Table 1: Tissue inhibitors of matrix metalloproteinase 1, matrix metalloproteinase 9 and matrix metalloproteinase 2 mean expression fold change in breast cancer patients with and without axillary lymph nodes involvement

Gene	Mean±SD		Fold-change	*P*
	Sample with axillary lymph nodes involvement	Sample without axillary lymph nodes involvement		
TIMP1	61,744.39±348,962.40	793.52±1372.21	77.8	0.003
MMP9	133,615.82±1,026,051.72	191,607.52±1,471,311.09	0.70	0.08
MMP2	2070.45±11,929.17	2118.35±11,974.40	0.98	0.61

$P<0.05$ have considerate as significant. TIMP1=Tissue inhibitors of matrix metalloproteinase 1, MMP=Matrix metalloproteinase, SD=Standard deviation

Table 2: The association between tissue inhibitors of matrix metalloproteinase 1, matrix metalloproteinase 9 and matrix metalloproteinase 2 mean expression fold change and tumor histopathologic characteristics in breast cancer patients

	Mean±SD		
	TIMP1	MMP9	MMP2
Vascular invasion			
Present	3142.70±10,857.99	71.85±150.60	122.69±231.56
Absent	417.01±1178.63	33.98±88.10	183.58±227.59
P	0.341	0.398	0.419
Tumor extension			
Present	442.85±531.32	26.25±22.60	23.75±26.93
Absent	956.15±1611.42	41.47±21.20	158.07±237.68
P	0.535	0.185	0.002
Positive axillary lymph node			
Present	188.10±321.82	11.43±21.85	111.14±201.46
Absent	1136.79±1662.96	73.58±146.67	163.10±226.37
P	0.030	0.112	0.441
Number of lymph nodes (*R*, *P*)	−0.086, 0.565	−0.063, 0.803	−0.106, 0.510

P<0.05 have considerate as significant. TIMP1=Tissue inhibitors of matrix metalloproteinase 1, MMP=Matrix metalloproteinase, SD=Standard deviation

expressions. In other word, the triggering or inhibiting role of these genes is potentially affected by geographical and ethnical variations.

Conclusions

The expression of TIMP1 gene is strongly associated with axillary lymph node metastasis in breast cancer patients.

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Conflicts of interest

There are no conflicts of interest.

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