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Elevated expression of both MDR1 and MMP-2 genes in metastasized lymph node of invasive ductal breast cancer

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Abstract. – BACKGROUND: There is emerging evidence that matrix metalloproteinase (MMP)-2 plays a crucial role in cancer invasion/metastasis. However, little evidence is available about the connections of multidrug resistance protein 1 (MDR1) and cancer invasion/metastasis so far.

AIM: To investigate the expression of MDR1 and MMP2 in primary breast tumors and their corresponding metastasized lymph nodes.

MATERIALS AND METHODS: Only lymph nodes which were pathologically identified as metastases were included in this study to compare with the corresponding primary tumor. We determined the expression of MDR1 and MMP2 in primary breast tumor and its metastasized lymph node specimens of 21 patients. The quantitative real-time polymerase chain reaction (Q-RT-PCR) technique was used to assess the MDR1 and MMP2 RNA expression levels in primary breast tumor and lymph nodal specimens. Target gene copies were normalized using beta-actin (beta-actin) gene copies. Tumor characteristics and number of metastatic lymph nodes were gathered from the pathology reports.

RESULTS: The Q-RT-PCR data showed that MDR1 expression in metastasized lymph node was higher than that of their corresponding primary tumors ($p < 0.05$), MMP2 expression in metastasized lymph nodes was also even higher compared with their matched primary tumors ($p < 0.01$). But SPSS bivariate correlation analysis revealed that MDR1 expression in lymph node was not correlated with MMP-2 expression in lymph node, number of metastasized lymph nodes and tumor size ($p > 0.05$). MDR1 expression in primary tumors was highly correlated with in corresponding lymph node metastases ($p < 0.01$ r = 0.795).

CONCLUSIONS: All those indicated that MMP-2 should play an important role in the lymph node metastasis. However, further clinical studies with larger sample size need to be performed to verify these findings.

Key Words:
Breast tumor, MMP-2, RNA expression, Lymph node metastasis.

Introduction

Tumor metastasis and drug resistance are the main causes of treatment failure and mortality in cancer patients[1]. Matrix metalloproteinase-2 (MMP-2) and multidrug resistance protein 1 (MDR1: ABCB1) gene as representations of invasion/metastasis and multidrug resistance (MDR) were studied extensively in these decades. MMP-2 (gelatinase A; 72-kDa gelatinase; type IV collagenase) is an important enzyme of the MMP family, which is able to degrade collagen IV, a basic component of constitutive basement membranes[2]. The role of MMP-2 as essential for metastasizing tumor cells has been considered. Because of its ability to degrade the basement membrane, it has been postulated as a potential marker of tumor progression and prognosis[3]. MDR1 (ABCB1) gene was one of the most important genes in drug resistance. The produced P-glycoprotein is a transmembrane ATP-dependent transporter, which could be associated with poor outcomes in cancer patients. The result is presumably due to imparting resistance to cancer treatment[4-6].

Although these two properties of malignant tumors have been widely studied, most investigations have proceeded along separated pathways. Recently, there are some indirect evidences which show that these two phenotypes may be interrelated. For example, Meyers and Biedler[7]. found that in highly resistant multi-drug resistant (MDR) cell lines, P-glycoprotein over expression

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led to decreased tumorigenicity. Weinstein et al[8] also found that P-glycoprotein may have other activities that led to a poor prognosis. One possibility is that P-glycoprotein (+) cells may acquire a propensity towards invasion and metastasis, as suggested in colon cancer cells[8,9]. A number of other studies have also shown the connections between MDR and invasion/metastasis[10].

As mentioned above, most studies about the relationship between drug resistance and metastasis/invasion were performed *in vitro*. In an *in vivo* study carried out by Bradley et al[11], the results showed that distinct elevation of MDR1 mRNA occurred during stepwise liver carcinogenesis, suggesting that the progressed malignant phenotype in liver carcinogenesis was associated with increased P-glycoprotein expression. However, this study was performed in rats. Some other *in vivo* studies were only about MDR or about cancer invasion/metastasis[5,11].

In this study, we employed the quantitative real-time polymerase chain reaction (Q-RT-PCR) technique to assess the MDR1 gene and MMP-2 gene RNA expression levels in invasive ductal breast cancer patients and their corresponding metastatic lymph nodes[12]. We further studied the relationship among MDR1, MMP-2 expression levels and tumor size, ER, PR and numbers of metastatic lymph node. The purpose of the present study is to investigate whether or not a linkage exists between MDR and metastatic potential *in vivo*.

Materials and Methods

Patients Selection and Specimens

A total of 21 patients with operable breast tumors and metastatic lymph nodes were included in this study for the present investigation. All the patients underwent Modified Radical Mastectomy for Breast Cancer between 2003 and 2004 without induction chemotherapy. Patients were diagnosed by core-tissue biopsy (CTB) using Bard-Magnum Gun (MG1522, Bard Magnum Biopsy Instrument, CR Bard, Inc., Covington, Georgia, United States) with 14-gauge 13-cm-long biopsy needles (Bard Magnum Core Tissue Biopsy Needle, CR Bard, Inc.). The excised primary tumor tissues and axillary lymph nodes were kept in liquid nitrogen. The axillary lymph nodes were pathologically identified as the metastases. Estrogen receptor (ER), progesterone receptor (PR), and proto-oncogene erbB2 (cerbB2) expressions on the primary tumors were routinely examined by immunohistochemistry method. Tumor characteristics and number of metastatic lymph nodes were gathered from the pathology reports.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from 15-20 mg liquid-nitrogen-frozen breast cancer tissue and metastatic lymph node tissue using Total RNA Extraction Miniprep System (Cat. No.: GR1001, VIOGENE Inc., Taiwan, China). The procedure was according to the manufacturer's protocols. Tumor and lymph node sample RNA was diluted in diethyl pyrocarbonate (DEPC)-treated RNase-free ultrapure water (DEPC; Sigma-Aldrich, the Netherlands) and stored at -80 °C.

Reverse transcription was carried out with the SuperScript First-Strand Synthesis System (Shinegene Inc., Shanghai, China) for RT-PCR. The following procedure was based on manufacture's protocol. The RNA/primer mixture including total RNA 5 μg, random primers (50 ng/μl) 3 μl, and 10 mM deossi-nucleotide-tri-phosphate (dNTP) 1 μl in each tube was prepared. The samples were incubated at 65°C for 5 min and then transferred on ice for at least 1 min. Then, the reaction master mixture was prepared. For each reaction: 10 × reverse transcriptase (RT) buffer 2 μl, 25 mM $MgCl_2$ 4 μl, 0.1 M dithiothreitol (DTT) 2 μl, and RNAaseOUT 1 μl were used. The reaction mixture was added to the RNA/primer mixture and allowed to mix briefly. The mixture was then placed at room temperature for 2 min before adding 1 μl (50 units) of SuperScript II RT to each tube. The mixture was incubated at 25°C for 10 min, and 42°C for 50 min. After heat inactivated at 70°C for 15 min, the mixture was cooled on ice. Subsequently, 1 μl RNase H was added and the mixture was incubated at 37°C for 20 min. The 1st strand cDNA was stored at -20°C until it was to be used for real-time PCR.

Quantitative Real-Time PCR

The mRNA level of MDR1 and MMP-2 were measured by Q-RT-PCR method using a Hot Start Fluorescent PCR Core Reagent Kit for SYBR Green I. Q-RT-PCR (FTC2000 Detect System, Funglyn Biotech, Toronto, ON, Canada) was carried out with 5 μl cDNA in a 30 μl PCR reaction system. In addition, gene *β-actin* was used as control to normalize the mRNA level of E-cadherin gene. Primer pairs were designed by Oligo 6.0 primer analysis software (Medprobe, Oslo, Norway) as shown in Table I. Real-time PCR was performed in 50 μl of reaction mixture system, including 25 μl 1 × Hotstart Fluo-PCR

Table I. Primer sequences for MDR1, MMP-2 and β-actin genes, respectively.

Gene	Primers	Tm (°C)
MDR1	Forward: CCACAGAGGGGATGGTCAG	58
	Reverse: TAGGCATTGGCTTCCTTGAC	58
MMP-2	Forward: CTCCTGGCTCATGCCTTCG	61
	Reverse: TACTCCCCATCGGCGTTCC	61
β-actin	Forward: TGACGTGGACATCCGCAAAG	
	Reverse: TGGAAGGTGGACAGCGAGG	

mix, about 300 nM forward and reverse primers, and containing about 2 μl of tumor sample cDNA as a template. Reaction conditions were as follows: 50°C for 2 min for uracil-N-glycosylase (UNG) activation and 94°C for 4 min for TaqDNA polymerase activation, followed by 35 cycles of 94°C for 30 s for denaturation, 55°C for 30 s for annealing and 72°C for 1 min for extension. For every transcript measured, serial dilutions (1: 10, 1: 100, 1: 1000, 1: 10 000) of standard-concentration sample were used to generate a standard curve. Data were analyzed with FTC2000 software (Funglyn Biotech, Toronto, ON, Canada) according to the above standard curve.

Statistical Analysis

A two-tail *t* test was performed to compare the difference of expression of MDR1 and MMP-2 gene between primary breast cancer and their corresponding metastatic lymph nodes. The correlation between MDR1 and MMP-2 expression and pathological parameters were analyzed by bivariate correlation analysis using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered significant.

Results

Clinical and Pathological Features

All the patients were females, and all the pathological types were the invasive ductal breast cancers. The tumor characteristics are presented in Table II. The media age at diagnosis of primary invasive breast cancer was 50.8 years, ranging from 35 to 79.

Gene Expression in Primary Breast Cancers and their Corresponding Metastatic Lymph Nodes

The intactness of total RNA was confirmed by two sharp bands which were 28 s rRNA and 18 s rRNA separated on denaturing agarose gels.

The Q-RT-PCR was used to assess the mRNA level of MDR1 gene and MMP-2 gene in primary breast cancers and their corresponding metastatic lymph nodes. The expressions of our target genes are shown in Figures 1 and 2. The result of statistical analysis is given in Table III. The results indicated that both of MMP-2 and MDR1 gene expression in metastatic lymph nodes were much stronger than that in primary tumors. (4.231 ± 3.573 vs 2.584 ± 2.757, $p < 0.05$; 2.111 ± 1.610 vs 0.910 ± 0.510, $p < 0.05$. respectively).

Correlation of Target Gene Expression Between Metastatic Lymph Node and their Corresponding Primary Tumor

The bivariate correlation analysis was employed to analyze the correlations between target gene expressions in metastatic lymph nodes and that in primary breast cancer. As shown in Table III, there was a statistically significant positive correlation between MDR1 expression in metastatic lymph node and in primary breast cancer ($r = 0.439$ $p = 0.047$).

Table II. Clinical and pathological characteristics of patients.

Clinical and pathological characteristics	
Number of patients	21
Pathological stage at diagnosis [n (%)]	
Stage II (T2N1M0)	12 (57)
Stage III (T2N2M0)	9 (43)
Tumor size (range) (cm)	3.4 (2-5)
Histological type	
Ductal	21
Number of invaded lymph node (range)	8.8 (1-18)
Estrogen receptor status [n (%)]	
Negative	11 (52)
Positive	10 (48)
Progesterone receptor status [n (%)]	
Negative	8 (38)
Positive	13 (62)
CerbB2 receptor status [n (%)]	
Negative	9 (43)
Positive	12 (57)

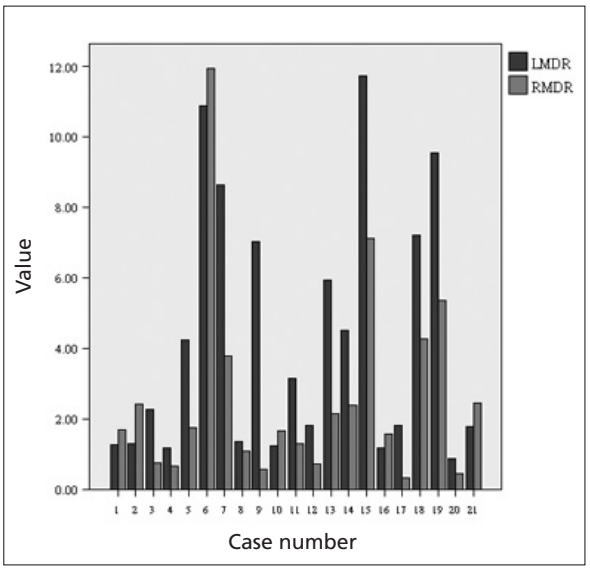

Figure 1. MDR1 gene expression levels in paired samples. Blue bars represent metastasis samples, and green bars represent primary tumors. Vertical axes show normalized densities for the indicated genes.

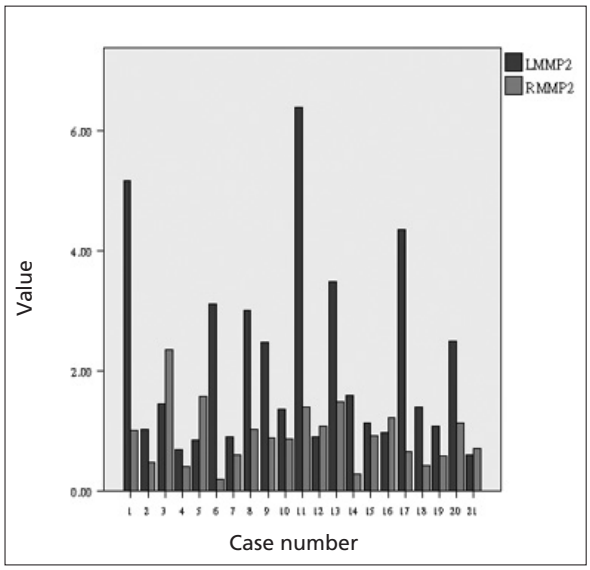

Figure 2. MMP-2 gene expression levels in paired samples. Blue bars represent metastasis samples, and green bars represent primary tumors. Vertical axes show normalized densities for the indicated genes.

There was no significant correlation between MMP-2 expression in metastatic lymph node and in primary breast cancer (r = 0.107 p = 0.645). Interestingly, we found that there was a statistically significant positive correlation between MMP-2 and MDR1 expression in primary tumors.

Correlations with Different Clinicopathologic Parameters

The relationships between the target gene expression and clinicopathologic parameters are summarized in Table IV.

Discussion

Invasion and metastasis are the most insidious and life-threatening aspects of breast cancer. The process of metastasis is a cascade of linked sequential steps involving multiple host-tumor interactions. One important step in tumor invasion is the penetration of the basement membrane[13]. The basement membrane is a strong barrier to the movement of tumor cells. The invasion of the basement membrane proceeds through a series of discrete steps[14].

In the current study, we detected the expression of MMP-2 gene and MDR1 gene in metastatic lymph nodes and their corresponding primary breast cancers. Furthermore, we analyzed the correlations between these two genes and clinicopathological parameters (tumor size, histological type, nuclear and histological grade, stage, lymph nodes status, ER, PR and CerbB2).

One of the primary observations of the current study was that MMP-2 gene expression in metastatic lymph nodes was much stronger than in primary breast cancer. MMP-2 (gelatinase A; 72-kDa gelatinase; type IV collagenase) is an important enzyme of the MMP family which is able to degrade collagen IV, a basic component of constitutive basement membranes. Like other members of the MMP family, MMP-2 is secreted in a latent form which requires cleavage of the

Table III. MDR1 and MMP2 expression in primary breast cancer and corresponding metastatic lymph node.

	Gene copy (X ± SD)	*t*-test (t)	*p*	Correlation (r)	*p*
Tumor MDR1	2.584 ± 2.757	3.481	0.003	0.795	0.001
Lymph MDR1	4.231 ± 3.573				
Tumor MMP-2	0.910 ± 0.510	3.418	0.002	0.159	0.490
Lymph MMP-2	2.111 ± 1.610				

Table IV. The relationships between the target gene expression and clinicopathologic parameters.

	T MDR1 (r)	p	L MDR1 (r)	p	T MMP-2 (r)	p	L MMP-2 (r)	p
Tumor Size	-0.091	0.69	-0.13	0.58	-0.287	0.21	-0.265	0.25
Num of lymph node	-0.086	0.71	-0.05	0.83	-0.012	0.958	-0.392	0.08
ER	0.001	0.99	-0.159	0.49	0.118	0.609	0.278	0.22
PR	0.076	0.74	-0.185	0.42	-0.047	0.839	-0.150	0.52
CerbB2	0.23	0.92	-0.163	0.48	-0.034	0.884	-0.101	0.66

N-terminal 80 amino acids to become active. The activation and enzymatic activity of MMP-2 is regulated by the tissue inhibitor of metalloproteinases-2 (TIMP-2). The role of MMP-2 as essential for metastasizing tumor cells has been considered. These results in gene level suggest that MMP-2 gene may play a role in development of lymph node metastases. Monig et al[15] have detected the MMP-2 expression in 114 gastric carcinoma patients to investigate the correlation between MMP-2 expression and lymph node metastasis. They found that the expression of MMP-2 was strongly correlated with tumor progression and lymph node metastasis. Nakopoulou et al[16] pointed out the strong relationship between MMP-2 expression and patients' overall survival. Hao et al[17] have studied 8 pairs of breast and lymph node specimens. They found that MMP-2 mRNA level was decreased in many lymph node metastasis specimens; the expression level of corresponding protein was elevated in most metastases. The Authors explained that extracellular matrix protein expression and nuclear gene expression were associated via a negative feedback regulatory mechanism. This finding was consistent with our research.

The mechanisms involved in lymph node metastasis remain unclear. It has been hypothesized that dramatic genetic changes occur in a small population of cells in the primary tumor and that these changes led to the emergence of lymph node metastases[18,19]. This hypothesis would predict a greater degree of difference in gene expression profiles between primary tumors and metastases. Our findings strongly supported this hypothesis. Yonemura et al[20], in order to demonstrate the role of membrane type 1-metalloprotease (MT1-MMP) on the lymph node metastasis using *in vivo* experimental model of lymph node metastasis by orthotopic implantation of MT1-MMP, transfected gastric cancer cell lines in the stomach of nude rats. They clearly demonstrated that MT1-MMP gene transfection would promote the lymph node metastasis on TMK-1 cells with little ability for lymph node metastasis. In the TMK-MT gastric tumor in nude rats, lymphatic invasion was frequently found in the submucosa around primary tumor, and regional lymph nodes of stomach were involved in 5 (63%) of 8 nude rats. This finding suggested that MT1-MMP plays a role in the lymph node metastasis.

In the clinical specimens of gastric cancer, Bando et al[21] reported the close implication between MT1-MMP expression and the activation of MMP-2. By the immunohistochemistry, MT1-MMP was colorized in the gastric cancer cells in almost all MMP-2 positive cells. Furthermore, the percentage of MT1-MMP staining-positive tumors was significantly higher in lymphatic invasion-positive cases. Bando et al[22] also reported that tissue status of MT1-MMP on the primary tumors of gastric cancer was an independent predictor for lymph node metastasis.

The second noteworthy finding of the current study is that MDR1 expression in lymph node metastasis was also stronger than in corresponding primary tumors. All the patients did not undergo induction chemotherapy before operation. As it is well known, both *in vitro* and *in vivo* studies have shown that some metastases can be more drug resistant and invasive/metastasized than their primary tumors. High expression of both MDR1 and MMP-2 gene in metastatic lymph nodes may explain this phenomenon[1]. Interestingly, there was a correlation between MMP-2 expression and MDR1 expression in primary tumors. This suggested that there is a connection between MDR and invasion/metastasis.

Three recent reports involving in vitro studies make the connection between these two phenotypes more apparent. Yang et al[10] reported that the extracellular matrix metalloproteinase inducer (EMMPRIN), a cell membrane glycoprotein involved in invasion and metastases[23], was over expressed in MDR cells and not in drug-sensitive parental cell lines. Misra et al[24] reported that MDR in cancer cells could be reg-

ulated by a ubiquitous extracellular matrix component, hyaluronan, a major ligand for the metastases-related CD44 receptor[25]. Miletti-Gonzalez et al[26] found that there was a close interaction between P-glycoprotein (MDR) and CD44 (motility, invasion, and metastases)[25,26]. CD44 expression in sensitive cells promoted the expression of P-glycoprotein and the MDR phenotype. Our group compared the biological alterations of the drug-resistant MCF-7/Adr cells with their parental control and found that drug-resistant cells have acquired enhance invasive ability in addition to its acquired MDR phenotype. Although there are many studies on the topic, very few studies produced evidence from *in vivo* studies. This *in vivo* study provides evidence to the connection between MDR and invasion/metastasis.

The consequence of this analysis about the correlation between gene expressions and clinicopathologic parameters is interesting. There is a statistically significant positive correlation between MMP-2 expression and MDR expression in primary tumors and between tumor size and the number of metastatic lymph nodes. However, there is no significant correlation between gene expressions and other clinicopathological parameters. These findings supported the notion that there was a connection between MDR and metastasis/invasion. However, the sample size used in this study is too small to get a definite conclusion. Further clinical studies with larger sample size need to be performed to verify these findings.

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