Upregulation of MMPs in Metastatic Cascade of Breast Cancer To Brain

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Abstract

Background

Brain metastasis is a lethal complication in triple negative breast cancer (TNBC) patients. Many factors including tumor cell molecular characteristics and biological environment are the main determinant in the brain metastasis process. Matrix metalloproteinases (MMPs) play a key role in extracellular matrix degradation, implicated in numerous aspects of metastasis processes of breast cancer.

Methods

After development of syngenic animal model of TNBC, primary breast cancer cells named 4T1T were isolated from tumor mass. Highly metastatic tumor cells named 4T1B were isolated and expanded from brain metastasis lesions of cancerous mice. Quantitative real-time polymerase chain reaction and gelatinase zymography were performed to analyze the expression of MMPs in transcriptomic and proteomic level in 4T1T and 4T1B.

Results

Our data revealed that, expression of MMPs was significantly upregulated in brain metastatic tumor cells. In transcriptomic level, MMP-2 and MMP-9 genes expression were up-regulated 4 and 3.4 folds in 4T1B, respectively. Zymographic analysis could be detect MMPs activity only in 4T1B.

Conclusion

These findings provided important insights regarding the gross alteration of MMPs expression in brain metastatic cascade of TNBC for the first time. Analysis of molecular properties of brain metastatic tumor cells can be used for understanding of molecular and genetic aspects of brain metastasis and also designing a targeted therapeutic strategies in combat with brain metastasis of TNBC.

Background

Breast cancer is the most common cancer in women worldwide[1]. Triple-negative breast cancer (TNBC) is the most aggressive and invasive type of breast cancer with poor prognosis [2]. The recurrence and metastasis of TNBC due to chemoresistance takes place in up to 70% of the patients [3]. The metastatic process of TNBC has been the subject of intense scrutiny. The brain is one of the most common organs affected in the spread of TNBC that ultimately results in fatal development of the disease.

Brain metastasis is an increasingly common complication in breast cancer patients. Approximately 15–30% of breast cancer patients develop brain metastasis [4]. The exact role of the brain environment to the

development of the metastatic process has yet to be clarified. A suitable specific environment is important to the development of tumor cells [5]. Many theories have been developed to study and understand metastatic behavior. Factors such as neoplastic cell molecular and genetic characteristics and biological environment are thought to be determinant in the metastatic process[6]. Investigations using patient samples[7] and animal model systems of brain metastasis[8, 9] are leading to improved understanding of the pathobiology of brain metastasis. Experimental models created to study the process of brain metastasis were used to isolated variants of tumor cells with enhanced brain metastatic ability. These selected variants have been used to identify and investigate the function of various genes contributing to the development of brain metastasis[10, 11].

Matrix metalloproteinases (MMPs) are a broad family of zinc-dependent proteinases that play a key role in extracellular matrix degradation, implicated in numerous pathogenic processes including cancer. Tumor cells are thought to secrete these matrix-degrading enzymes and/ or induce host cells to elaborate them[12]. In breast cancer, MMPs are thought to play an important role in invasion, metastasis and tumor angiogenesis[13]. Most reports suggest that increased expression of MMP-2 and MMP -9 proteins correlates with worse prognosis of breast cancer patients[14]. MMP2 over-expression and activation have been associated with the invasive potential of human tumors. Active MMP2 and MMP9 were detected more frequently in malignant than benign breast carcinomas [13]. MMPs have been extensively studied in the context of breast cancer prognosis. Most studies to date have been performed in human tissue collected from patients diagnosed with breast cancer or in breast cancer cell lines. In this regard, additional in vivo studies that characterize MMP expression in metastasis are needed. Few studies are available on the expression of MMPs within breast cancer metastasis [15–18]. According to our knowledge, only three studies[19–21] have characterized the expression and activity of these molecules in brain metastasis of breast cancer but alteration of MMPs in metastatic cascade of breast cancer was not focus of any of these research. It is important to determine if MMPs have different effects/roles in the development of metastasis in different organs because this may help to understand why breast cancer cells metastasize to preferential organs. Here, we focused on the metastatic process of breast cancer to the brain in a mouse model of TNBC. This model consistently produces brain metastasis to evaluate the expression and activity of MMP-2 and MMP-9 involved in metastatic cascade of breast cancer in the brain.

Material And Methods

Cell culture

4T1 cell line was obtained from the cell bank of Pasteur Institute of Iran (C604). The cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS (fetal bovine serum) and 2% Penicillin-Streptomycin (all from Gibco, USA) in humidified atmosphere of 5% CO₂ at 37°C.

Induction of syngeneic animal model of breast cancer

Female BALB/c mice weighing 20 to 25 gram obtained from Royan institute (Iran). The animals were housed in cages at 12-h photoperiod while they had free access to food and water. All animal experiments were in compliance with the relevant laws, and this study was approved by the Ethics Committee of Shahrood University of Medical Sciences (registration number: IR.SHMU.REC.1400.112). 4T1 cells were subcutaneously injected to the flank (or the right hind limb) of the mice (10^5 cells suspended in 100 μ L PBS) using an insulin syringe with 32G needle. The mice were monitored daily for the appearance and behavior characteristics.

Brain metastatic and primary breast tumor cell extraction

Primary and metastatic tumor cell extraction, was performed according to our and other group previous works[22-25]. Briefly primary tumor and brain of cancerous mice were excised after 35 days of tumor induction in mice, and surface blood was removed by rinsing it in PBS. After mincing with scissors, fragments were placed to 50 ml conical tube. For enzymatic digestion, primary tumor and the brain were digested in 10 mg / ml collagenase type IV at 37°C for 75 min on a platform rocker. All enzymes were purchased from Sigma (St Louis, MO, USA). The digested organ filtered through 70-um cell strainers, and washed with PBS. In the next step, washed cells were resuspended in medium containing 10% FBS, 100 U/ml Penicillin, and 100 ug/ml Streptomycin (all from Gibco, USA). Ultimately, the cells were cultured at 37°C in 5% CO₂.

Quantification of MMP-2 and MMP-9 by RT-qPCR

Primary and brain metastatic tumor cells (1×10^4) were seeded in each well of 24-well plates in complete medium. After 48 hours Total RNA was extracted from these cells using QIAzol Lysis Reagent (QIAGEN). The quality, yield, and size of extracted RNA were analyzed using spectrophotometry (NanoDrop-ThermoFisher) and electrophoresis. The first strand cDNA synthesis was performed using reverse transcription system (Easy cDNA Synthesis Kit for RNA or mRNA to cDNA - pars tous). Real-time PCR procedure was executed based on the 1 μ L cDNA in all samples. Quantization of all gene transcripts was done by SYBR Green Real time PCR Master Mix (Amplicon A/S, Denmark) using StepOnePlus™ Real-Time PCR System, according to the manufacturer's instruction. The amplification procedure was as follows: 1 cycle of 95°C for 15 min, 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The exact mRNA expression was normalized to the expression level of GAPDH. Relative changes of gene expression were calculated by the following formula, and the data was represented as fold up-regulation/down-regulation.

Fold change = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ = [Ct of MMPS (in treated cells) - Ct of GAPDH (in treated cells)] - [Ct of MMPS (in control cells) - Ct of GAPDH (in control cells)].

Primers were designed using AlleleID version 6 software (Premier Biosoft Inc.).

The used primers are as follows:

For MMP-2, Forward 5'-TTTATTTGGCGGACAGTGAC-3', Reverse 5'- AGTTAAAGGCAGCATCTACTTG -3';

For MMP-9, Forward 5'-TCCAGTATCTGTATGGTCGTG-3', Reverse 5'- CATAGTGGGAGGTGCTGTC -3';

For GADPH, Forward 5'-CCTGGAGAAACCTGCCAAGTA-3', Reverse 5'-GGCATCGAAGGTGGAAGAGT -3'.

Zymography

Zymography was performed on 9% polyacrylamide gels that had been cast in the presence of gelatin. Briefly, samples (100 µl) were resuspended in loading buffer and separated on a 9% SDS-PAGE gel containing 0.5 mg/ml gelatin without prior denaturation. After electrophoresis, the gels were washed to remove SDS and incubated for 30 min at room temperature in a renaturing buffer (50 mM Tris, 5 mM CaCl₂, and 1% Triton X-100). The gels were incubated for 48 h at 37°C in a developing buffer (50 mM Tris-HCl [pH 7.8], 5 mM CaCl₂, 0.15 M NaCl, and 1% Triton X-100) and then stained with Coomassie Brilliant Blue G-250, destained in 30% methanol, and flooded with 10% acetic acid to detect gelatinase secretion.

Statistical analysis

Results are expressed as the mean ± standard deviation. Data were analyzed with GraphPad Prism statistical software 6.0 (GraphPad Software, La Jolla, CA, USA) using Paired Samples t Test. P <0.05 was considered statistically significant.

Results

Primary and metastatic tumor cells extraction

Metastatic animal model of breast cancer was generated after 35 days following tumor induction in Balb/c mice (Figure 1A). When injected into BALB/c mice, 4T1 spontaneously produces highly metastatic tumors that can metastasize to the brain while the primary tumor is growing in situ. The primary tumor does not have to be removed to induce metastatic growth. H and E staining and pathological confirmation were performed on tumor tissues and brain metastatic lesions (Figure 1C, B). We properly extracted primary and brain metastatic tumor cells from subcutaneous primary tumor and brain of cancerous mice, respectively (Figure 1C, B). The metastatic tumor cells in the brain, after primary isolation, form colonies in the culture medium. Due to the high rate of growth and proliferation, the tumor cells in these colonies are purified after 3 passages. These tumor cells are called brain metastatic tumor cells or 4T1B while tumor cells that are obtained in the same way, from the original tissue of the tumor, are primary tumor cells called 4T1T(Figure 1C, B).

Significant Upregulation of MMPs in brain metastatic tumor cells in mRNA level

The expression of matrix metalloproteinase (MMP-2 and MMP-9), was analyzed in 4T1T and 4T1P. The quality, yield, and size of extracted RNA, synthesized cDNA, and PCR products were confirmed using nanodrope and gel electrophoresis. As shown in fig 2 the expression of MMP-9 was up-regulated 3.4

times in 4T1P compared with 4T1T. About MMP-2, the expression of these MMP was involved in higher alteration in metastatic cascade of breast cancer and up-regulated 4 times in 4T1P compared with 4T1T (Figure 2).

Zymographic analysis could be detect MMPs protein expression only in 4T1B

Gelatin zymography was used to evaluate gelatinase activity, especially MMP-9 and MMP-2. MMPs activity were observed based on the white band formed on the SDS-page gel, which indicated the degradation of gelatin by MMPs. The result shows that detection of gelatinase activity of MMPs was feasible only in 4T1B (Figure 3). As showed in fig 3 results indicated that secretion of MMP proteins in conditioning media (CM) of 4T1T was not as enough as to detect in zymography but in 4T1B, secreted MMPs in CM could be create a white band on SDS-page.

Discussion

In the present study, we revealed significant increase in MMP-2 and MMP-9 expression in brain metastatic tumor cells in mRNA and protein levels. Our works report the gross alteration of MMPs expression in brain metastatic cascade of TNBC for the first time and suggesting that these molecules may be relevant in the metastatic process of breast cancer to the brain. To our knowledge, this is the first report of characterization of these molecules in brain metastasis cascade of breast cancer.

Many reports correlating MMP activity with metastatic and invasive behavior of tumor cells [12]. Previous studies that describe MMP expression correlated with breast cancer metastasis reveal that MMPs may be important for the metastatic process[16]. About breast cancer brain metastasis, incidence of metastasis to the brain was increased in animals injected intracardiac with clones of breast cancer cells transfected with MMP2[18]. Results of research by Mendes et al in 2005 showed that MMP-2, -3 and -9 proteins expressions are significantly higher in neoplastic brain tissue compared to normal brain tissue[21].

The determination of what cell component of the tumor mass expresses MMPs is important in order to understand the role of these molecules in tumor development. Some studies have localized MMP2 to neoplastic epithelial cells. Others, however, associate them with different components of the tumor stroma[26] and/or angiogenic blood vessels[27]. MMP9 has been associated with neoplastic cell plasma membrane[28], non-neoplastic ducts and acini, stromal fibroblasts; endothelial cells, and tumor-infiltrating inflammatory cells including neutrophils, macrophages, and lymphocytes. Expression of MMP3 was observed in both tumor and stroma cells[29]. In concurrence with previous reports we observed high level of MMP2 and MMP9 expression in metastatic tumor cells.

MMP inhibitors are being investigated as an important tool for cancer treatment [30, 31]. In a study for determination of MMPs role in breast cancer brain metastasis development, application of a selective MMPs inhibitor induce slight but significant decrease in in vitro ENU 1564 invasion behavior when cells were in presence of MMPs inhibitor. Additionally development of brain metastasis in animals treated with MMPs inhibitor was decreased dramatically. According to in vivo results, this group strongly suggests

that MMPs are important in the brain metastatic process of breast cancer. But for interpretation of disparity observed in vivo vs in vitro results this group emphasized low levels of in vitro MMP expression [21]. In opposition with these results, our work in, in vitro level detect a high level of MMPs expression in metastatic breast tumor cells.

Liu et al in a xenograft model revealed the role of MMP-1 in breast cancer growth and metastasis to the brain. In this study two variants of the MDA-MB-231 human breast cancer cell line selected for enhanced ability to form brain metastases in nude mice (231-BR and 231-BR3 cells) were found to express high levels of matrix metalloproteinase-1 (MMP-1) [19]. In a recent study, after isolation of brain metastatic tumor cells from brain of cancerous mice, results indicated that silencing mir-202-3p increases MMP-1 and promotes a brain invasive phenotype in these cells [20].

In conclusion our study, for the first time, use a mouse model for distant breast cancer metastasis to the brain to successfully study expression and activity of MMP-2 and MMP -9. The use of animal models to study in vivo tumor progression and metastatic behavior is important to understand the mechanism of metastasis development. It is also an important tool for pharmacological evaluation of cancer therapy. Several synthetic MMP inhibitors are under investigation for clinical trials in patients with cancer. They are thought to inhibit both primary tumor invasion and metastasis[30, 31]. Our results indicate that MMPs may be involved in breast cancer metastasis to the brain. By targeting these molecule we can overcome these resistant and preventing recurrence of the disease in patients with metastatic breast cancer. This understanding may be utilized in the development of the current therapeutic approach to metastatic cancer.

Declarations

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Compliance with Ethical Standards:

Conflict Of Interest

The author declares that they have no competing interests.

Ethical statement

This study was approved by the Ethics Committee of Shahroud University of Medical Sciences (registration number: IR.SHMU.REC.1400.112).

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Author contributions:

Conceptualization, M-KF; methodology, M-KF, AA, FS-B, RK and MM-E; formal analysis M-KF, RK and MM-E; writing-original draft preparation, MKF; writing-review and editing, AA; supervision, M-KF.

Data availability:

All data generated or analysed during this study are included in this published article.

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Figures

Figure 1

Primary and Brain Metastatic Tumor Cells Isolation. A. Metastatic animal model of triple negative breast cancer was generated after 35 days of tumor induction in Balb/c mice. B. Brain metastatic tumor

isolation, H&E staining and metastatic tumor cell extraction was performed on brain of cancerous mice.
C. Primary tumor isolation, H&E staining and primary tumor cell extraction was performed on primary tumor tissues.

Figure 2

Enhanced Expression of MMPs in Brain Metastatic Tumor Cells Using Real-Time PCR. Both MMP-2 and MMP-9 was significantly upregulated in Brain Metastatic Tumor Cells. All results are expressed as mean $\pm$ SD from at least three independent experiments analyzed by Two-tailed T test. **P < 0.001.

Figure 3

Gelatinolytic Activity of MMPs in Brain Metastatic Tumor Cells. Detection of gelatinase activity of MMPs was feasible only in 4T1B.