RESEARCH ARTICLE

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Upregulation of Matrix Metalloproteinases in the Metastatic Cascade of Breast Cancer to the Brain

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Abstract

Background: In patients with triple-negative breast cancer (TNBC), brain metastasis is a fatal consequence. Matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9 as the major members of the MMP family, are involved in many different facets of breast cancer metastasis. **Aims:** In this study, we sought the MMPs expression in the metastatic cascade of TNBC. **Methods and results:** Primary breast cancer cells known as 4T1T were extracted from the tumor mass following the creation of an animal model of TNBC. The brain metastasis lesions of malignant mice were used to extract highly brain metastatic tumor cells known as 4T1B. Gelatinase zymography and real-time polymerase chain reaction (RT-PCR) were used to examine the expression of MMPs at the proteomic and transcriptomic levels in 4T1T and 4T1B. Our results indicated; brain metastatic tumor cells greatly increased their expression of MMPs. In 4T1B, MMP-2 and MMP-9 gene expression were upregulated by 4 and 3.4 folds respectively. Zymographic analysis found MMP activity only in 4T1B. **Conclusion:** These results offer significant information about the massive alteration of MMPs expression in the brain metastasis of TNBC. By analyzing the molecular characteristics of brain metastatic tumor cells, we can understand the molecular and genetic features of brain metastasis and develop tailored therapeutic strategies to combat TNBC brain metastasis.

Keywords: Triple Negative Breast Cancer- brain metastasis- matrix metalloproteinase- zymography

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Introduction

Breast cancer is the most prevalent cancer among women worldwide (Siegel et al., 2016). The most aggressive and invasive kind of breast cancer, triple-negative breast cancer (TNBC), has a terrible prognosis (Yao et al., 2017). Up to 70% of individuals with TNBC experience recurrence and metastases (Isakoff, 2010).

Brain metastasis affects 15–30% of breast cancer patients (Rusciano and Burger, 1992). Studies using patient samples (Palmieri et al., 2009) and brain metastasis animal model systems (Bos et al., 2009; Fidler et al., 2010) are advancing our knowledge of the pathobiology of brain metastasis. A subline of tumor cells with increased brain metastatic capacity was identified using experimental models developed to explore the process of brain metastasis. The function of numerous genes influencing the emergence of brain metastases has been examined using these chosen sublines (Yoneda et al., 2001; Kim et al., 2004).

Matrix metalloproteinases (MMPs) are a large family of zinc-dependent proteinases that have been linked to a variety of pathologic events, including cancer, and play a critical role in the breakdown of the extracellular matrix. These matrix-degrading enzymes may be secreted by tumor cells or may be induced in host cells by tumor cells (Stetler-Stevenson et al., 1993). MMPs are believed to be crucial in the invasion, metastasis, and tumor angiogenesis in breast cancer (Hynes, 2003). Most studies indicate that a worse prognosis for breast cancer patients is correlated with higher MMP-2 and MMP-9 protein expression (La Rocca et al., 2004). Human cancers' capacity for invasion has been linked to MMP-2 overexpression and activation. Malignant breast carcinomas were more frequently found to have active MMP-2 and MMP-9 than benign breast carcinomas (Hynes, 2003). MMPs have been thoroughly investigated in relation to the prognosis of breast cancer. The majority of studies done so far have used breast cancer cell lines or human tissue taken from patients who have been diagnosed with the disease. In this regard, more in vivo research defining MMPs expression in metastasis is required. MMPs expression and activity in breast cancer brain metastasis have been described in some research (Mendes et al., 2005; Liu et al., 2012; Harati et al., 2020); however, none of these studies focused on the modification of MMPs in the metastatic cascade of breast cancer. Here,

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we focused on the mouse model of TNBC's breast cancer brain metastasis to assess the expression and activity of MMP-2 and MMP-9 involved in the metastatic cascade of breast cancer to the brain.

Materials and Methods

Cell culture

The Pasteur Institute of Iran's cell bank provided the 4T1 cell line (C604). The cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 2% Penicillin-Streptomycin (both from Gibco, USA) at 37°C in a humidified 5% CO_2 environment.

Development of the syngeneic animal model of breast cancer

20-to-25-grams female BALB/c mice were purchased from Royan Institute (Iran). At a 12-hour photoperiod, the animals were kept in cages with free access to food and water. The Shahroud University of Medical Sciences Ethics Committee accepted this work, and all animal experiments were performed in accordance with the applicable regulations (registration number: IR.SHMU.REC.1400.112). 105 4T1 cells suspended in 100 microliters (µl) phosphate buffer saline (PBS) were subcutaneously injected into the mice's flanks (or right hind limbs) using an insulin syringe (32G needle). The appearance and behavior traits of the mice were observed every day.

Extraction of primary and brain metastatic tumor cells

For pathological confirmation, collected tumoral tissue, was fixed, sectioned, and stained with haematoxylin and eosin (H&E), and examined under a light microscope. The extraction of primary and metastatic tumor cells was done in accordance with earlier research by our lab (Kamalabadi-Farahani et al., 2022). After 35 days of tumor induction in mice, the primary tumor and brain were surgically removed, and surface blood was washed away using PBS. Slices were cut into smaller pieces using scissors and added to a 50 ml conical tube. The main tumor and the brain were digested using enzymatic methods in 10 mg/ml collagenase type IV at 37°C for 75 minutes on a platform rocker. The enzymes were all bought from Sigma (St Louis, MO, USA). The digested organs were rinsed with PBS and passed through 70-um cell strainers. The following stage involved resuspending cleaned cells in media containing 10% FBS, 100 units per milliliter of penicillin, and 100 ug/ml of streptomycin (all from Gibco, USA). In the end, the cells were grown in 5% CO2 at 37°C.

Real-Time Polymerase Chain Reaction (RT- PCR) quantification of MMP-2 and MMP-9

In 24-well plates with complete media, primary and brain metastatic tumor cells (1×10^4) were plated in each well. Using QIAzol Lysis Reagent, Total RNA was recovered from these cells after 48 hours (QIAGEN). Utilizing electrophoresis and spectrophotometry (NanoDrop-ThermoFisher), the quality, quantity, and size of the isolated RNA were examined. A reverse transcription technique was used to create the first strand of cDNA (Easy cDNA Synthesis Kit for RNA or mRNA to cDNA - pars tous). All samples were subjected to real-time PCR using 1 ul of cDNA. According to the manufacturer's instructions, all gene transcripts were quantified using the StepOnePlus RT-PCR System and SYBR Green Real-Time PCR Master Mix (Amplicon A/S, Denmark). The following steps were used in the amplification process: one cycle at 95°C for 15 minutes, 40 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The level of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) expression was used to normalize the precise mRNA expression. The following formula was used to compute the relative changes in gene expression, and fold up-regulation/down-regulation was used to represent the results.

Fold change = $2^{-\Delta\Delta Ct}$, where ΔΔCt = [Ct of MMPS (in 4T1B cells) - Ct of GAPDH (in 4T1B cells)] - [Ct of MMPS (in 4T1T cells) - Ct of GAPDH (in 4T1T cells)].

The software AlleleID version 6 was used to create the primers (Premier Biosoft Inc.). The following are the used primers:

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

On 9% polyacrylamide gels that had been cast while containing gelatin, zymography was conducted. Briefly, samples (100 µl) were redissolved in a loading buffer before being separated on a 9% SDS-PAGE gel with 0.5 mg/ml gelatin. Following electrophoresis, the gels were washed to remove SDS and renaturing buffer (50 mM Tris, 5 mM $CaCl_2$, and 1% Triton X-100) was added for 30 min of room temperature incubation. The gels were incubated for 48 hours at 37 degrees Celsius in a developing buffer that contained Coomassie Brilliant Blue G-250, 30% methanol, 5 mM $CaCl_2$, 0.15 M NaCl, and 1% Triton X-100 to detect the secretion of gelatinase.

Statistic evaluation

The mean and standard deviation are used to express the results. The Paired Samples T Test was used to analyze the data using GraphPad Prism statistical software, version 6.0 (GraphPad Software, La Jolla, CA, USA). Statistics were judged significant at P 0.05.

Results

Primary and metastatic tumor cells extraction

After 35 days following the injection of 4T1 in BALB/c mice, a metastatic animal model of breast cancer was created (Figure 1A). 4T1 develops highly metastatic tumors that can spontaneously spread to the brain. To promote metastatic growth, the initial tumor

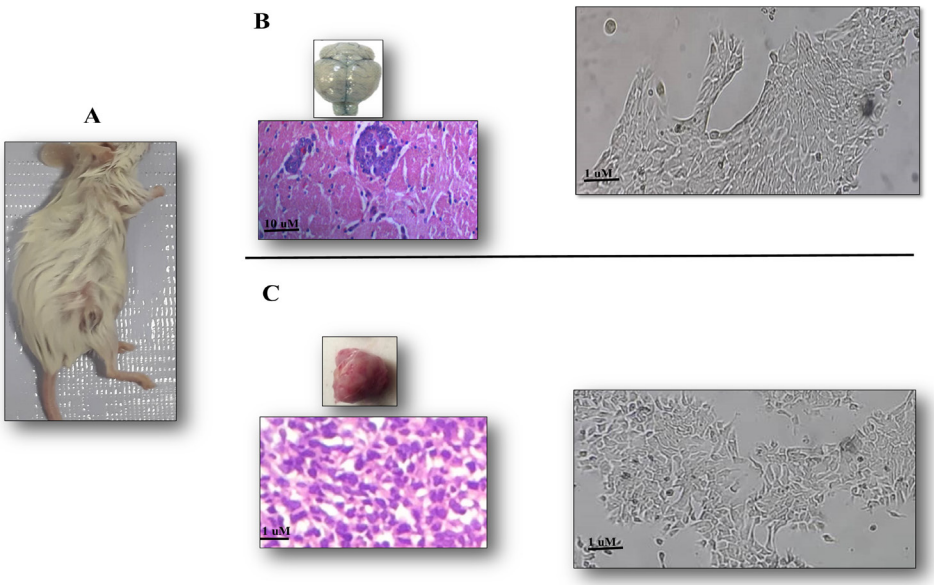

Figure 1. Primary and Highly Metastatic Tumor Cells Isolation. A. Metastatic animal model of triple-negative breast cancer was generated after 35 days of tumor induction in BALB/c mice. B. Brain metastatic tumor isolation, H&E staining and metastatic tumor cell extraction were performed on the brain of cancerous mice. C. Primary tumor isolation, H&E staining and primary tumor cell extraction were performed on primary tumor tissues.

need not be removed. Brain metastatic lesions and tumor samples were examined using H and E staining and pathological confirmation (Figure 1C, B). From the subcutaneous primary tumor and the brain of malignant mice, respectively, we correctly retrieved primary and brain metastatic tumor cells (Figure 1C, B). After initial isolation, the brain's metastatic tumor cells organize into colonies in the culture medium. After three passages, the tumor cells in these colonies are isolated due to rapid growth and proliferation. These tumor cells are known as brain metastatic tumor cells or 4T1B, whereas primary tumor cells, which are obtained in the same manner from the initial tumor tissue, are known as 4T1T (Figure 1C, B).

MMPs are overexpressed at the mRNA level in brain metastatic tumor cells

In 4T1T and 4T1B, the expression of matrix metalloproteinase (MMP-2 and MMP-9) was examined. Using nanodrop and gel electrophoresis, the efficacy, yield, and size of isolated RNA, generated cDNA, and PCR products were verified. MMP-9 expression was increased 3.4 times in 4T1B compared to 4T1T, as shown in figure 2. Regarding MMP-2, this MMP's expression was four times higher in 4T1B than in 4T1T, and it was associated with a higher alteration in the metastatic cascade of breast cancer.

MMPs protein expression could only be found by zymographic analysis in 4T1B

Gelatin zymography was utilized to assess the activity of gelatinases, particularly MMP-9 and MMP-2. The white band that appeared on the SDS-page gel, which indicated the breakdown of gelatin by MMPs, served as evidence of MMPs activity. The outcome demonstrates that only 4T1B was capable of detecting the gelatinase

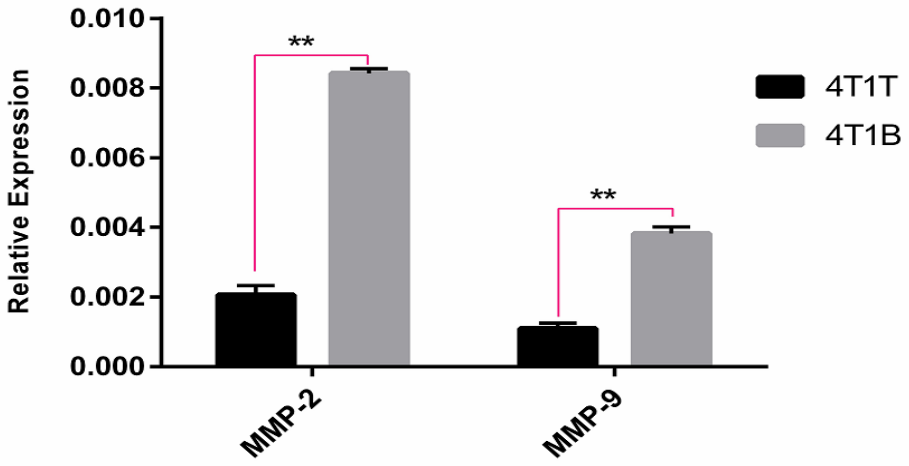

Figure 2. Enhanced Expression of MMPs in Brain Metastatic Tumor Cells Using Real-Time PCR. Both MMP-2 and MMP-9 were significantly upregulated in Brain Metastatic Tumor Cells. All results are expressed as mean ± SD from at least three independent experiments analyzed by the Two-tailed T-test (**P < 0.001).

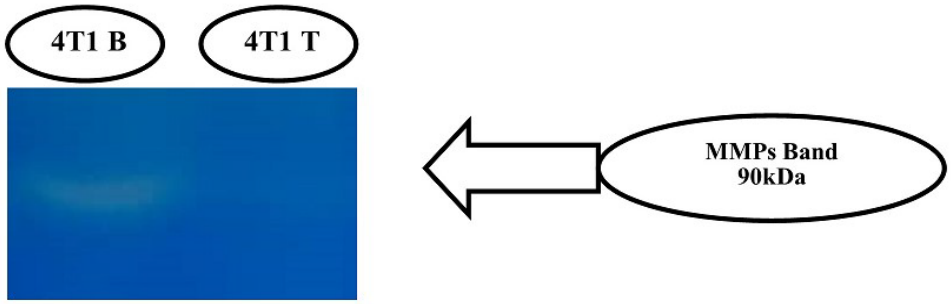

Figure 3. Gelatinolytic Activity of MMPs in Brain Metastatic Tumor Cells. Detection of gelatinase activity of MMP2 was feasible only in 4T1B.

activity of MMPs (Figure 3). According to the results, the secretion of MMPS proteins in conditioning media (CM) of 4T1T was insufficient to be detected in zymography, but in 4T1B, it was sufficient to produce a white band on SDS page.

Discussion

In the current investigation, we found a considerable rise in the mRNA and protein levels of MMP-2 and MMP-9 expression in brain metastatic tumor cells. For the first time, our research documents a severe shift in these MMPs' expression in the brain metastatic cascade of TNBC, indicating that these molecules may be important in the spread of breast cancer to the brain. According to our knowledge, this is the first account of the characterization of these enzymes in the breast cancer brain metastatic cascade.

Much research has been focused on the roles of MMP-2 and MMP-9 expression in breast cancer prognosis (Li et al., 2017; Jiang and Li, 2021). There are numerous studies linking MMPs activity to the ability of tumor cells to spread and invade (Stetler-Stevenson et al., 1993). MMPs may be important in the metastatic process, according to previous research (Ohshiba et al., 2003).

Regarding breast cancer brain metastasis, there is research that describes the expression and activity of MMPs in breast cancer brain metastasis. However, none of these studies focused on the modification of MMPs in the metastatic cascade of breast cancer. In research by Tester et al. animals given intracardiac injections of breast cancer cell clones that had been MMP-2 transfected showed an increased incidence of brain metastasis (Tester et al., 2004). In a rat model, MMP-2, -3, and -9 protein expressions were much higher in neoplastic brain tissue compared to normal brain tissue, according to research by Mendes et al. from 2005 (Mendes et al., 2005). In accordance with these results, our work clearly defined MMP-2 and MMP-9 overexpression in brain metastatic tumor cells.

According to Liu et al. in a xenograft model, MMP-1 is involved in the growth and spread of breast cancer to the brain. In this work, it was shown that two variations of the MDA-MB-231 human breast cancer cell line (231-BR and 231-BR3 cells) expressed high amounts of matrix metalloproteinase-1 (MMP-1) due to their heightened capacity to develop brain metastases in nude mice (Liu et al., 2012). In a recent study, it was discovered that silencing mir-202-3p increased MMP-1 and promoted a brain-invasive phenotype in brain metastatic tumor cells isolated from the brain of malignant mice (Harati et al., 2020).

Understanding the function of MMPs in tumor formation requires knowledge of which cells within the tumor mass express MMPs. MMP-2 has been linked to neoplastic epithelial cells in several studies. Others, however, link them to various tumor stroma elements (Caudroy et al., 1999) and/or angiogenic blood vessels (Bartsch et al., 2003). MMP-9 has been linked to tumor-infiltrating inflammatory cells, such as neutrophils, macrophages, and lymphocytes, as well as non-neoplastic ducts and acini, stromal fibroblasts, and endothelial cells. MMP-3 expression was seen in stromal and tumor cells, respectively (Brummer et al., 1999; Balduyck et al., 2000). We saw high levels of MMP-2 and MMP-9 expression in metastatic tumor cells, which is consistent with other results.

MMPs inhibitors are being researched as a key cancer therapeutic technique (Lee et al., 2003; Nozaki et al., 2003). Application of a selective MMPs inhibitor resulted in a minor but significant reduction in vitro ENU1564 invasion behavior in a study to determine the involvement of MMPs in the development of breast cancer brain metastasis. Additionally, MMPs inhibitor treatment significantly reduced the development of brain metastases in rats. This group's in vivo findings strongly imply that MMPs are crucial to the process by which breast cancer spreads to the brain. However, this group focused on modest levels of in vitro MMPs expression to analyze the discrepancy between in vivo and in vitro results (Mendes et al., 2005). In contrast to these findings, our in vitro research found that metastatic breast tumor cells had a high level of MMPs expression.

In conclusion, our study is the first to successfully examine the expression and activity of MMP-2 and MMP-9 in a mouse model of breast cancer metastasis to the brain. To comprehend the mechanisms of metastasis, it is crucial to research in vivo tumor growth and metastatic behavior using animal models. Our findings suggest that MMPs could play a pivotal role in the brain metastasis of breast cancer. These findings may be helpful in designing targeted therapies to improve the current treatment

strategy for metastatic breast cancer.

Author Contribution Statement

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

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Ethical statement

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

Data availability

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

Conflict Of Interest

The author declares that they have no competing interests.

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