RESEARCH ARTICLE

Effects of Tissue Factor, PAR-2 and MMP-9 Expression on Human Breast Cancer Cell Line MCF-7 Invasion

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Abstract

Objective: This study aimed to explore the expression of tissue factor (TF), protease activated receptor-2 (PAR-2), and matrix metalloproteinase-9 (MMP-9) in the MCF-7 breast cancer cell line and influence on invasiveness. Methods: Stable MCF-7 cells transfected with TF cDNA and with TF ShRNA were established. TF, PAR-2, and MMP-9 protein expression was analyzed using indirect immunofluorescence and invasiveness was evaluated using a cell invasion test. Effects of an exogenous PAR-2 agonist were also examined. Results: TF protein expression significantly differed between the TF cDNA and TF ShRNA groups. MMP-9 protein expression was significantly correlated with TF protein expression, but PAR-2 protein expression was unaffected. The PAR-2 agonist significantly enhanced MMP-9 expression and slightly increased TF and PAR-2 expression in the TF ShRNA group, but did not significantly affect protein expression in MCF-7 cells transfected with TF cDNA. TF and MMP-9 expression was positively correlated with the invasiveness of tumor cells. Conclusion: TF, PAR-2, and MMP-9 affect invasiveness of MCF-7 cells. TF may increase MMP-9 expression by activating PAR-2.

Keywords: Breast cancer - tissue factor - protease activated receptor-2 - matrix metalloproteinase-9

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Introduction

Tissue factor (TF) is an important clotting promoter that is involved in intracellular signal transduction and tumorigenesis. TF is also closely related to tumor growth, invasion, and metastasis (Förster et al., 2006; Kasthuri et al., 2009; Ruf et al., 2010). The combination of TF with factor VIIa, as well the combination of TF with factor VII and protease activated receptor-2 (PAR-2) (Hjortoe et al., 2004; Morris et al., 2006) induces tumor cells to form new blood vessels and promote metastasis. In addition, TF changes the expression of cancer extracellular matrix proteins through a variety of signaling pathways. Matrix metalloproteinases (MMPs) are enzymes important for degrading the extracellular matrix (ECM) that can promote cancer cell invasion and metastasis by degrading ECM proteins, thereby allowing tumor cells to penetrate the basement membrane (Tryggvason et al., 1987). MMP-9 (gelatinases B) decomposes the type IV collagen in the basement membrane, and is attracting increasing attention as a specific enzyme for degrading the basement membrane (Wieczorek et al., 2012). Although the invasion and metastasis of breast cancer is a major cause of death, its etiology remains unclear. Previous clinical studies (Zhao et al., 2008) found that TF expression is significantly correlated with MMP-9 expression in breast cancer, and that MMP-9 serves as an independent prognostic factor. TF has been found to regulate MMP expression through PARs in human colon cancer cells (Hu et al., 2013; Wu et al., 2013a; Wu et al., 2013b); however, no corresponding research has been conducted on breast cancer cells. Therefore, this study investigates TF, PAR-2, and MMP-9 expression in the MCF-7 breast cancer cell line and its influence on tumor cell invasiveness.

Materials and Methods

Cell culture

Human MCF-7 breast cancer cells (Union of Basic Medical Cell Center, Beijing, China) were seeded in 50 ml culture flasks with Dulbecco's modified Eagle's medium (DMEM) complete medium (containing 10% fetal bovine serum (FBS) and 10% green streptomycin) and incubated at 5% CO_2 and 37 °C until the cells covered the bottom. The cells were digested with 0.25% trypsin, harvested, and placed in a 12-well cell culture plate. After adding 1 ml of complete medium, the culture medium was replaced with serum-free medium, and the cells were incubated for 24 h.

Transfection of MCF-7 cells using pcDNA3.1/ZeoTF (+)

Single cell suspensions of MCF-7 cells were prepared by adding 7.5×10^5 cells into a 25 ml cell culture flask with DMEM complete culture medium until 70% to 80% confluence. A total of 4 μg of successful cloning plasmid pcDNA3.1/ZeoTF (+) (Colorectal Cancer Laboratory,

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Table 1. Experiment Grouping and Disposal

Groups	Cell line	Disposal
1	The MCF-7 Transfection of pcDNA3.1/ZeoTF (+)	PBS
2	The MCF-7 Transfection of pcDNA3.1/ZeoTF (+)	PAR-2 agonist
3	The MCF-7 Transfection of pGCsi/TF (-) shRNA	PBS
4	The MCF-7 Transfection of pGCsi/TF (-) shRNA	PAR-2 agonist

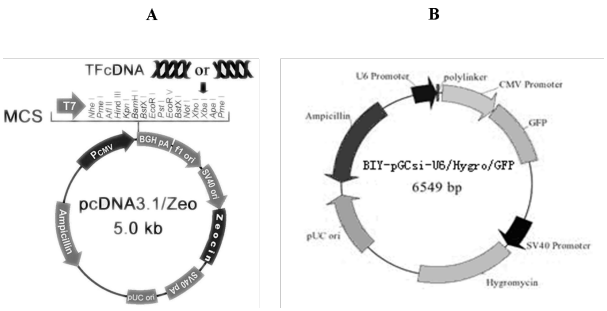

Figure 1. Plasmid Construction of Sense and Antisense TF. A: pcDNA3.1/ZeoTF (+); B: pGCsi/TF (-) shRNA

Peking University First Hospital, Beijing, China) (Figure 1A) was diluted and mixed with Lipofectamine 2000 (Invitrogen, New York, USA). The cells were incubated at room temperature for 20 min to form DNA/liposome complexes. The complexes were then added to the medium. The cells were incubated at 37 °C and 5% CO_2 for 24 h. The filter medium was replaced with DMEM containing FBS and Zeocin (50 μg/ml) (Invitrogen, New York, USA). The cells were cultured at 37 °C and 5% CO_2, and the screening medium was replaced every 2 d to 3 d until the cell clones appeared. Screening was performed for 1 wk to 2 wk to maintain stable growth and allow the subculturing of the cells. The cells were observed under an inverted fluorescence microscope (Leica DM IL LED, Wetzlar, Germany). Under fluorescence microscopy, successfully transfected cells would emit green fluorescence. The transfection efficiency was also calculated.

Transfection of MCF-7 cells using TF-ShRNA

Single cell suspensions of MCF-7 cells were prepared by adding 7.5 × 10^5 cells into a 25 ml cell culture flask with DMEM complete culture medium until 70% to 80% amalgamation was achieved. A total of 4 μg of successful cloning plasmid pGCsi/TF (-) shRNA (Beijing Yixin Industrial Co., Ltd., Beijing, China) (Figure 1B) was diluted, mixed with Lipofectamine 2000 (Invitrogen, New York, USA) dilution, and incubated them at room temperature for 20 min to form DNA/liposome complexes. The complexes were added to the medium. The cells were incubated at 37 °C and 5% CO_2 for 24 h. The filtered medium was replaced with DMEM containing FBS and hygromycin B (Bio-Rad Company, Hercules, USA). The concentration of the culture medium was 200 μg/ml. The cells were cultured at 37 °C and 5% CO_2, and the screening medium was replaced every 2 d to 3 d until the cell clones appeared. The cells were screened for 1 wk to 2 wk to maintain stable growth during subculturing. The cells were observed under an inverted fluorescence microscope, with successfully transfected cells emitting green fluorescence. Transfection efficiency was also calculated.

TF protein expression

Western blot analysis was used to detect the MCF-7 human breast cancer cells. The cells were transfected with the plasmid pcDNA3.1/ZeoTF (+), and the cells transfected with TF ShRNA. The cells transfected with the same plasmid were cultured in one plate. TF protein expression was then detected. Gel Doc 1000 imaging system (Bio-Rad, Hercules, USA) and analysis software were used to record relative absorbance. Each experiment was repeated three times.

PAR-2 agonist treatment

The PAR-2 agonist SLIGKV (Industrial Technology Co., Ltd., Beijing, China) (10 μmol/L) was added to the cells transfected with pcDNA3.1/ZeoTF (+) and those transfected with TF-ShRNA. The cells were co-cultured with the agonist for 24 h. An equal volume of PBS was added to the control group, as shown in Table 1.

TF, PAR-2, and MMP-9 protein variation

Treated cells were rinsed with serum-free medium to produce a single cell suspension with a cell density of 5 × 10^5 cells/ml. Then, 400 μl (2 × 10^5) of the treated cells was allowed to react with the dose-saturated primary antibodies (mouse anti-human TF monoclonal antibodies at 1:100 dilution, mouse anti-human PAR-2 monoclonal antibodies at 1:200 dilution, and mouse anti-human MMP-9 monoclonal antibodies at 1:100 dilution from the Santa Cruz Corporation, Dallas, USA) in an ice bath for 40 minutes, centrifuged, and washed twice. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Beijing Zhongshan Golden Bridge Technology Co., Ltd., Beijing, China) were added. The cells were placed on ice. After 40 min, the cells were centrifuged, washed, and fixed. A flow cytometer (BD Company, Franklin Lakes, USA) was used to detect the fluorescence of 10 000 counts per tube of cells, with three replicates.

Cell invasion assay

The treated cells were rinsed with serum-free medium to produce a single cell suspension with a cell density of 5 × 10^5 cells/ml. A total of 100 μl (5 × 10^4) of the treated cells was seeded in the upper chamber of Transwell culture plates (Corning Inc., New York City, USA). FBS (500 μl) was added into the lower chamber of Transwell culture plates. The cells were incubated for 24 h at 37 °C and 5% CO_2. The base polycarbonate film in the upper chamber was obtained, and the number of cells attached to its surface was recorded. Six high power fields (400×) were randomly selected and their cell counts were averaged, with three replicates.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. The cell counts were recorded as mean ± standard deviation (SD). The differences between groups were analyzed using t-tests. The correlations among factors were determined using regression analysis. Differences with $P < 0.05$ were considered statistically significant.

Table 2. The Invasive Experiment Result in 4 Groups

Group	Result (/400×)			$\overline{\chi}$±SD	t	*P*
1	15	17	21	17.67±3.06	-24.3631	0.000*
2	72	68	69	69.67±2.08	1.7712	0.151
3	99	109	116	108.00±8.54		
4	95	101	100	98.67±3.21		

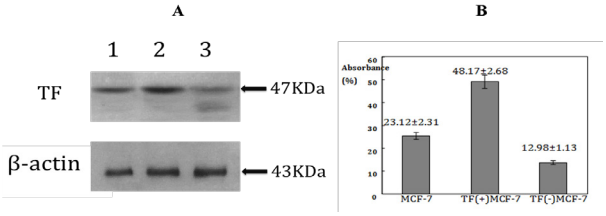

Figure 2. The TF Expression among TF (+) Group, TF (-) Group and Control Group. A: The TF expression (Western blot); B: The TF expression (comparative absorbance).

Results

TF protein

Lane 1 in Figure 2A represents MCF-7 human breast cancer cells, lane 2 represents the MCF-7 cells transfected with TF cDNA, and lane 3 represents the MCF-7 cells transfected with TF shRNA. The results show the TF expression in each group. Compared with the TF expression in MCF-7 cells, that in MCF-7 cells transfected with TF cDNA increased, whereas that in MCF-7 cells transfected with TF-shRNA decreased. The TF relative absorbance intensities of the three groups are shown in Figure 2B. The differences were analyzed using a t-test. The T value was 20.967, and the *P* value was 0.040.

Expression of TF, PAR-2, and MMP-9 proteins

Each group was treated with a PAR-2 agonist. Indirect immunofluorescence was used to detect TF, PAR-2, and MMP-9 protein expression. The results are shown in Figure 3A. The PAR-2 agonist (group 1 and group 2) significantly increased the MMP-9 expression rate (*P* = 0.002). However, TF and PAR-2 expression was not significantly different. Adding the PAR-2 agonist to the control group was without significant affect.

Cell invasiveness

Invasiveness was tested by treating the cells in each group with the PAR-2 agonist. The results are shown in Table 2. Group 1 exhibited the weakest invasiveness (17.67 ± 3.06/400× field). The PAR-2 agonist significantly increased the invasiveness of group 2 compared with group 1. The Transwell assay revealed that group 2 had 69.67 ± 2.08 cells/400× field, which is significantly greater than that in group 1 ($P < 0.05$). The invasiveness of group 3 (108.00 ± 8.54 / 400 × field) did not significantly differ from that of group 4 (98.67 ± 3.21 / 400 × field).

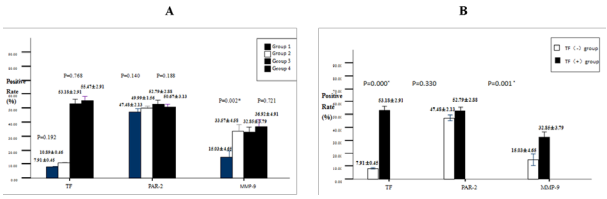

Figure 3. The Positive Expression of TF, PAR-2 and MMP-9 Comparison among Groups. A: The positive expression of TF, PAR - 2 and MMP - 9 in 4 Groups; B: The positive expression of TF PAR - 2 and MMP - 9 between TF (+) group and TF (-) group

TF, PAR-2, and MMP-9 expression

The TF, PAR-2, and MMP -9 protein expression in groups 1 and 3 (both control groups) was compared. The results are shown in Figure 3B. The TF (+) group expressed higher TF and MMP-9 protein level than the TF(-) (P = 0.000 and 0.001, respectively). PAR-2 expression significantly differed between the two groups.

Relationship of TF, PAR-2, and MMP-9 protein expression with cell invasion

The relationship of the protein expression of TF, PAR-2, and MMP-9 with cell invasion in the all four groups is shown in Table 3. The PAR-2agonist significantly increased MCF-7 and MMP-9 expression in the TF (-) group, and the protein expression consistent changed with invasion assay cell counts. Meanwhile, the positive rate of TF and PAR-2 slightly increased. The PAR-2 agonist slightly increased TF and MMP-9, but slightly decreased the cell count. The regression analysis showed that the invasion assay cell counts were correlated with TF and MMP-9 expression. The difference had statistical significance (P = 0.039 and 0.004, respectively).

Discussion

TF is an important factor in various malignant tumors (van den Berg et al., 2012). TF has become one of the targets of targeted therapy for breast cancer cells (Cole et al., 2013), and is considered an important aspect of breast cancer research (Santos et al., 2013). Studies have shown that TF and MMP - 9 expression is significantly correlated with breast cancer cells in humans and dogs (Zhao et al., 2008; Hu et al., 2013). In human colon cancer cells (Hu et al., 2013; Wu et al., 2013a; Wu et al., 2013b), TF promotes the invasion and metastasis of tumor cells by upregulating MMP-9 (Hu et al., 2013). Thus, MMP-9 may be the effector molecule of TF. A study on TF signaling pathways revealed that a TF–FVII complexes directly or indirectly pass through the downstream product and activate PAR-2 (Hjortoe et al.,

Table 3. The Relationship Between Protein Expression and Invasive Experiment Result

	Group 1	Group 2	Group 3	Group 4	Regression coefficient	t	*P*
TF	7.91±0.45	10.89±0.46	53.18±2.91	55.47±2.91	0.439	2.473	0.039*
PAR-2	47.48±2.13	49.99±1.56	52.79±2.88	50.67±3.13	0.074	0.463	0.656
MMP-9	15.03±4.65	33.57±4.58	32.85±3.79	36.91±4.91	0.551	3.916	0.004*
The invasive result	17.67±3.06	69.67±2.08	108.0±8.54	98.67±3.21			

*$P < 0.05$

2004; Wilson et al., 2004; Morris et al., 2006; Disse et al., 2011).

Activation of PAR-2 in fibroblasts (Wang et al., 2007) and lung epithelial cells (Vliagoftis et al., 2000) by specific agonists increases MMP-9 expression. Therefore, this experiment aims to develop breast cancer cell lines that have different TF expression to study the PAR-2 and MMP-9 protein expression and the change in cell invasiveness in different breast cancer cells with different TF expression.

Western blot analysis verified that the TF expression in the MCF-7 of the TF (-) group (transfected with TF ShRNA) and the TF (+) group (transfected with TF cDNA) was significantly different ($P = 0.04$). PAR-2 and MMP-9 protein expression in the cells of the two groups was compared by performing indirect immunofluorescence experiments. MMP-9 expression in the MCF-7 cell of the TF (-) group was significantly weaker than that in the MCF-7 cell of the TF (+) group ($P = 0.001$), whereas the PAR-2 expression did not significantly differ between the two groups ($P = 0.330$). Analysis indicated that TF protein expression is positively correlated with the MMP-9 expression, which is similar to the performance of Lovo large intestine cancer cells (Kasthuri et al., 2009). By contrast, PAR-2 expression was unaffected by TF, which suggest thatPAR-2 expression may be independent of TF and MMP-9 in MCF-7 cells.

The specific PAR-2 agonist SLIGKV was used to study the downstream product of PAR-2–specific activation in MCF-7 breast cancer cells. The results indicated that breast cancer cells with no endogenous agonists (TF) have significantly increases MMP-9 expression and slightly increases TF and PAR-2 expression. However, the TF protein expression in breast cancer cells was unaffected. The PAR-2 agonist SLIGKV simulates the function of TF as an endogenous activator of PAR-2 receptors, which increases MMP-9 expression, which suggests that MMP-9 is the downstream product of TF-activated PAR-2. In the cell invasion experiment, the invasiveness of the MCF-7 cells in the TF (-) group was significantly lower than that in the MCF-7 cells in the TF (+) group. Removing the PAR-2 agonist significantly enhanced the invasiveness of the MCF-7 cells in the TF (-) group, but did not change the invasiveness of those in the TF (+) group. The invasiveness of the MCF-7 cells was positively correlated with TF and MMP-9 expression, which suggests that TF and MMP -9 affect the invasiveness of MCF-7 breast cancer cells. Moreover, PAR-2 activation promoted the invasiveness of MCF-7 breast cancer cells, with MMP-9 acting as the effector. Recent studies on arthritic chondrocytes (Christelle et al., 2007) found that the PAR-2 agonist SLIGKV simulates TF mainly by activating MMP transcription and expression through the MAPK pathway. A study by Wang (Wang et al., 2007) on fibroblasts suggested that PAR-2-induced secretion of MMP-9 precursors occurs via the JAK/STAT3 pathway. These findings provide insight into the signaling pathway of MMP-9 secretion triggered by the PAR-2 activation in MCF-7 breast cancer cells, which will be our next research direction.

References

Christelle B, Nathalie A, Martel-Pelletier J, et al (2007). Activation of proteinase-activated receptor 2 in human osteoarthritic cartilage upregulates catabolic and proinflammatory pathways capable of inducing cartilage degradation: a basic science study. *Arthritis Res Ther*, **9**, R121.

Cole M, Bromberg M (2013). Tissue factor as a novel target for treatment of breast cancer. *Oncologist*, **18**, 14-8.

Disse J, Petersen HH, Larsen KS, et al (2011). The endothelial protein C receptor supports tissue factor ternary coagulation initiation complex signaling through protease-activated receptors. *J Biol Chem*, **286**, 5756-67.

Förster Y, Meye A, Albrecht S, Schwenzer B (2006). Tissue factor and tumor: Clinical and laboratory aspects. *Clinica Chimica Acta*, **364**, 12-21.

Hjortoe GM, Petersen LC, Albrektsen T, et al (2004). Tissue factor factor VIIa-specific up-regulation of IL-8 expression in MDA-MB-231 cell s is mediated by PAR-2 and result s in increased cell migration. *Blood*, **103**, 3029-37.

Hu L, Xia L, Zhou H, et al (2013). TF/FVIIa/PAR2 promotes cell proliferation and migration via PKCα and ERK-dependent c-Jun/AP-1 pathway in colon cancer cell line SW620. *Tumour Biol*, **34**, 2573-81.

Kasthuri RS, Taubman MB, Mackman N (2009). Role of tissue factor in cancer. *J Clin Oncol*, **27**, 4834-8.

Morris DR, Ding Y, Ricks TK, Gullapalli A, Wolfe BL, Trejo J (2006). Protease-activated receptor-2 is essential for factor VIIa and Xa-induced signaling, migration, and invasion of breast cancer cells. *Cancer Res*, **66**, 307-14.

Morris DR, Ding Y, Ricks TK, et al (2006). Protease-activated receptor-2 is essential for factor VIIa and Xa-induced signaling, migration, and invasion of breast cancer cells. *Cancer Res*, **66**, 307-14.

Ruf W, Yokota N, Schaffner F (2010). Tissue factor in cancer progression and angiogenesis. *Thromb Res*, **125**, S36-8.

Santos AA, Lopes CC, Ribeiro JR, et al (2013). Identification of prognostic factors in canine mammary malignant tumours: a multivariable survival study. *BMC Vet Res*, **9**, 1.

Tryggvason K, Höyhtyä M, Salo T (1987). Proteolytic degradation of extracellular matrix in tumor invasion. *Biochem Biophys Acta*, **907**, 191-217.

van den Berg YW, Osanto S, Reitsma PH, Versteeg HH (2012). The relationship between tissue factor and cancer progression: insights from bench and bedside. *Blood*, **119**, 924-32.

Vliagoftis H, Schwingshackl A, Milne CD, et al (2000). Proteinase-activated receptor -2-mediated matrix metalloproteinase-9 release from airway epithelial cells. *J Allergy Clin Immunol*, **106**, 537-45.

Wang L, Luo J, He S (2007). Induction of MMP-9 release from human dermal fibroblasts by thrombin: involvement of JAK/STAT3 signaling pathway in MMP-9 release. *BMC Cell Biol*, **8**, 14.

Wieczorek E, Reszka E, Gromadzinska J, Wasowicz W (2012). Genetic polymorphism of matrix metalloproteinases in breast cancer. *Neoplasma*, **59**, 237-47.

Wilson SR, Gallagher S, Warpeha K, Hawthorne SJ (2004). Amplification of MMP-2 and MMP-9 production by prostate cancer cell lines via activation of protease-activated receptors. *Prostate*, **60**, 168-74.

Wu B, Zhou H, Hu L, Mu Y, Wu Y (2013). Involvement of PKCα activation in TF/VIIa/PAR2-induced proliferation, migration, and survival of colon cancer cell SW620. *Tumour Biol*, **34**, 837-46. (a)

Wu Y, Zhang X, Zhou H, et al (2013). Factor VIIa regulates the expression of caspase-3, MMP-9, and CD44 in SW620 colon cancer cells involving PAR2/MAPKs/NF-ϰB signaling pathways. *Cancer Invest*, **31**, 7-16. (b)

Zhao JX, Lin ZM, Yao HW, Wan YL (2008). Analysis of the relationship between expressions of TF and MMP-9 and prognosis of breast cancer patients. *Chin J Clin Oncol*, **5**, 141-5.