

# Suppression of TAFI by siRNA inhibits invasion and migration of breast cancer cells

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Received November 14, 2016; Accepted May 25, 2017

DOI: 10.3892/mmr.2017.7031

**Abstract.** Human thrombin activatable fibrinolysis inhibitor (TAFI), also known as carboxypeptidase B2 (CPB2), is a procarboxypeptidase enzyme. The purpose of the present study was to observe the expression of TAFI in breast cancer (BC) and breast cancer cell (BCC) lines and to investigate the effect of TAFI suppression by small interfering (si) RNA gene silencing on invasion and migration of BCC lines. A significant increase in TAFI level was identified by immunohistochemical analysis in BC tissues compared with normal breast tissues. TAFI suppression also inhibited cell viability, invasion and migration ability as demonstrated by MTT, Transwell chamber, and wound scratch assays, respectively ( $P < 0.05$ ). The data suggested that suppression of TAFI by siRNA inhibits invasion and migration of breast cancer cells and that TAFI may be a new target for breast cancer therapy.

## Introduction

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen that functions as a molecular link between coagulation and fibrinolysis. Numerous single nucleotide polymorphisms have been identified in carboxypeptidase basic (CPB2), the gene encoding TAFI, and are located in the 5'-flanking region, in the coding sequences and in the 3'-untranslated region of the CPB2 mRNA transcript (1). It has been suggested that CBB2 serves an important role in the interactions among procoagulant, anticoagulant and fibrinolytic systems (2-4). Activated TAFI (TAFIa) attenuates fibrinolysis by removing the carboxyl-terminal lysine residues from partially degraded fibrin that mediate positive feedback in plasmin

generation (5). Pancreatic carboxypeptidase B (CPB) is a stable protease exhibiting high homology with TAFI but not to TAFIa (6).

Activation of TAFI by thrombin is increased 1,250-fold in the presence of the endothelial cell membrane protein thrombomodulin (TM) (7), which is expressed in tumors and is a prognostic factor in human cancer (8,9). Several studies have demonstrated that higher TAFI levels are associated with various types of cancer (10-13), and plasma levels of TAFI are significantly increased in breast and lung cancer, gastric carcinoma and multiple myeloma compared with healthy individuals, suggesting that TAFI may serve a role in the pathogenesis of thrombotic disorders in cancer patients (14,15). However, the role of TAFI in tumor development remains to be fully elucidated. In the present study, the TAFI gene was knocked down by small interfering (si)RNA transfection to suppress the expression of TAFI, and the effects of the TAFI signal pathway on invasion and migration were investigated in a breast cancer cell (BCC) line.

## Materials and methods

**Patients and samples.** The present study was approved by the ethics committee of The Second Hospital of Shandong University (Jinan, China) and informed consent was obtained from the participants. All eligible specimens were collected from patients with pathologically and clinically confirmed breast cancer who underwent surgical resection prior to any therapy from June 2013 to December 2016. All samples were re-evaluated by pathologists to confirm the diagnosis and to estimate the tumor cell content. Patients were aged from 25 to 65.

**Cell culture.** The human BCC lines MDA-MB-231 and MCF-7 were purchased from the American Type Cell Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated newborn calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 5 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 100 U/ml penicillin, and 100 U/ml streptomycin in 5% atmospheric CO<sub>2</sub> at 37°C.

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**Key words:** breast cancer, small interfering RNA, carboxypeptidase B2, thrombin activatable fibrinolysis inhibitor

**Immunohistochemistry.** Immunohistochemistry was performed to analyse TAFI expression in breast cancer (BC) tissues and adjacent normal tissues. In brief, human breast tissues in healthy and cancer patients were fixed in 10% formaldehyde, and then were embedded in paraffin. The sections were then deparaffinized with xylene, rehydrated and then treated with 3% hydrogen peroxide to quench the endogenous peroxidase activity. Subsequent antigen retrieval was performed by heating in citrate buffer solution (0.01 M) using a microwave oven. Sections were cut at 5  $\mu$ m and stained with a TAFI antibody at 4°C overnight (catalog no. sc-67300; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) following the manufacturer's protocol, and then sections were further incubated with 3,3'-diaminobenzidine tetrahydrochloride for 5 min at room temperature. The sections were observed and captured with a Nikon Eclipse 90i microscope (Nikon Corporation, Tokyo, Japan).

**siRNA transfection.** According to the principles of siRNA design, 3x19 bp sequences in TAFI cDNA, (GenBank accession number NM-001872.3) were identified as target sites using a web-based online software system (Thermo Fisher Scientific, Inc.) for computing highly effective siRNA (<https://www.thermofisher.com/order/genome-database/details/sirna/104006?CID=&ICID=uc-sirna-TAFI>). The sequences were submitted to BLAST® (National Centre for Biotechnology Information, Bethesda, MD, USA) to ensure that only the selected gene was targeted. The target sequences for the TAFI gene were: siRNA-1: 5'-AGUUAUAUGGCCUAUGAACCC-3' (sense strand) and 5'-UGGUUCAUAGGCCAUUAUACU-3' (antisense strand), siRNA-2: 5'-UGAUU GUUCGCAUAGAAAGAA-3' (sense strand) and 5'-UUCUUUCUAU GCGAACAAUCA-3' (antisense strand) and siRNA-3: 5'-UAGGUUAUAGGUUUC UGAGCC-3' (sense strand) and 5'-GGCUCAGAAACCUUA UACCUA-3' (antisense strand). Negative control siRNA: 5'-GCGUAAACGCGGGAUUUACUU-3' (sense strand) and 5'-GUAAAUUCCCGCGUACGCUU-3' (antisense strand). siRNA 3 achieved the greatest knockdown of TAFI, and therefore this was selected for further experiments. Briefly, 2  $\mu$ l siRNA was diluted with 100  $\mu$ l OPTi-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) and 10  $\mu$ l Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was diluted with 100  $\mu$ l OPTi-MEM for 30 min at room temperature. Dilutions were mixed together and incubated at room temperature for 20 min, and 200  $\mu$ l of the transfection mixture was added to each well. Experiments were repeated three times. Then, 6 h following transfection, the medium was replaced by the common complete medium again. Transiently transfected cells were harvested at 24 and 48 h following transfection for different experiments.

**Semi quantitative reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted from transfected cells after 24 h using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The purity and concentration were determined by measuring the absorbance at 260 and 280 nm (A260/A280). Reverse transcription was performed with 1  $\mu$ g of total RNA and the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). PCR primers used were as follows:

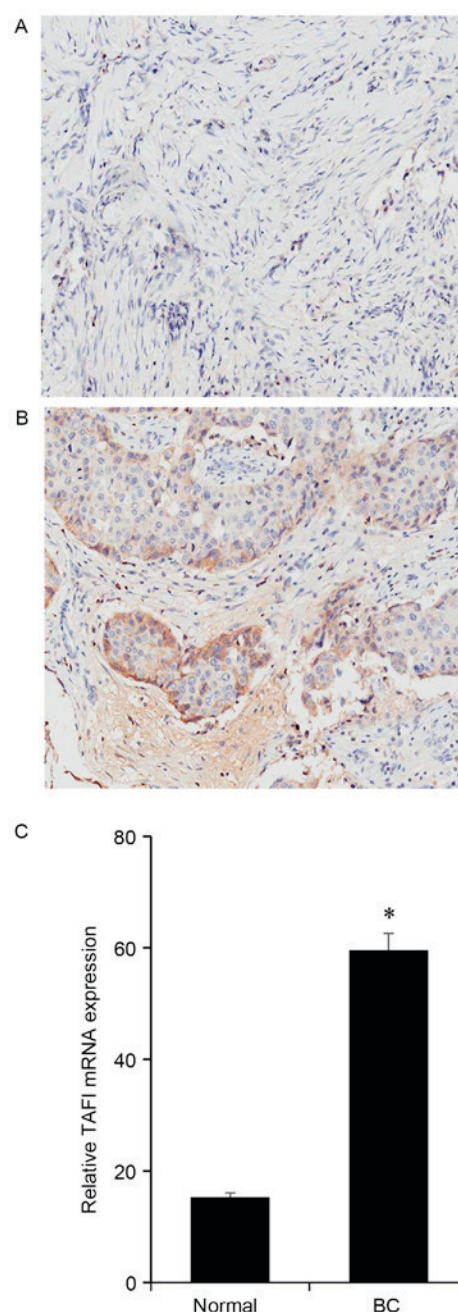


Figure 1. TAFI expression analysis in breast cancer tissues and adjacent normal tissues. Immunohistochemical staining of TAFI in (A) tumor adjacent normal tissues and (B) breast cancer tissues (original magnification, x100). (C) TAFI mRNA expression in normal tissues and breast cancer tissues, measured by semi-quantitative reverse transcription-polymerase chain reaction, relative to GAPDH expression. \*P<0.05 vs. normal. TAFI, thrombin activatable fibrinolysis inhibitor; BC, breast cancer.

CPB2, forward 5'-AACTGCTTCACTGGCTACTA-3' and reverse 5'-TATGCTTACAAAAATCCACA-3' GAPDH forward 5'-CCACCCATGGCAAATTCCATGGCA-3' and reverse 5'-TCTAGACGGCAGGTCCAGGTCCACC-3'. PCR was carried out in a 20  $\mu$ l reaction volume containing 50 ng of genomic DNA, 10  $\mu$ mol of each primer, 200  $\mu$ mmol/l of each dNTP, 4  $\mu$ l 5 x PCR buffer and 2 u taq DNA polymerase. Amplification was carried out with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 sec, extension at 72°C for 50 sec with a final extension at

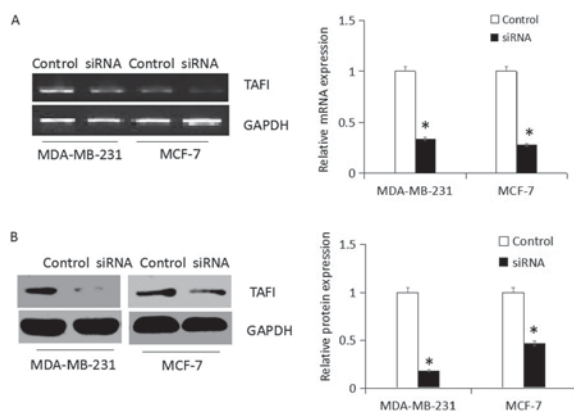


Figure 2. TAFI expression in MDA-MB-231 and MCF-7 cells transfected with the specific siRNA targeting TAFI for 48 h. (A) Semi-quantitative reverse transcription-polymerase chain reaction analysis of CPB2 mRNA expression, relative to GAPDH. (B) Western blot analysis of TAFI protein expression levels, relative to GAPDH. \* $P<0.05$  vs. control. TAFI, thrombin activatable fibrinolysis inhibitor; siRNA, small interfering RNA; CPB2, carboxypeptidase B2.

72°C for 10 min. A densitometric scanning instrument (model GS-800; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to quantify the bands and the relative amount of CPB2 and TAFI gene mRNA expression was estimated relative to the GAPDH mRNA detected in the same sample.

**Western blot analysis.** Cytoplasmic proteins of different groups were extracted using cytoplasmic extraction reagents (Beyotime Institute of Biotechnology, Haimen, China). Protein concentration in the supernatants was determined by bicinchoninic acid assay. A total of 20  $\mu$ l protein was separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked in 5% skimmed milk in TBST buffer at room temperature for 1 h with gentle agitation and then incubated overnight at 4°C with mouse anti-human TAFI immunoglobulin (Ig)G antibody (catalog no. sc-67867; 1:200; Santa Cruz Biotechnology, Inc.) or rabbit anti-human GAPDH IgG antibody (catalog no. sc-25778; 1:1,000; Santa Cruz Biotechnology, Inc.). Following washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-human IgG antibody (catalog no. ab109489; 1:1,000; Abcam). The bound antibodies were visualized using an enhanced chemiluminescence reagent (EMD Millipore, Billerica, MA, USA) and quantified by densitometry using ChemiDoc XRS+ image analyzer (Bio-Rad Laboratories, Inc.). The expression levels of TAFI and GAPDH protein were quantified by densitometry analysis (model GS-800, Bio-Rad Laboratories, Inc.). The signal strength of each TAFI signal was normalized against the corresponding GAPDH control.

**Cell viability assay.** Viable cells were quantified by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The tetrazolium salt MTT (Sigma-Aldrich; Merck KGaA) was used to compare the cellular metabolic capacities. Each well of a 96-well plate was seeded with  $1 \times 10^4$  cells and preincubated in 5% atmospheric CO<sub>2</sub> at 37°C. Then, 48 h following transfection, the MTT

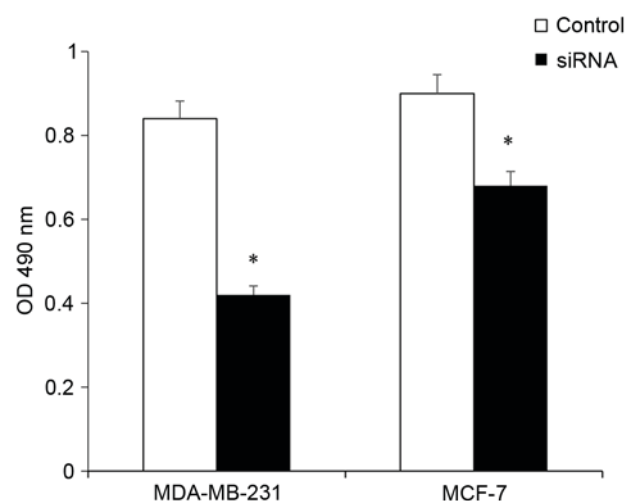


Figure 3. Breast cancer cell viability detected using MTT assay. TAFI siRNA-treated MDA-MB-231 and MCF-7 cells exhibited a significant decrease in cell viability. \* $P<0.05$  vs. control. TAFI, thrombin activatable fibrinolysis inhibitor; siRNA, small interfering RNA.

assay was performed by adding 20  $\mu$ l of MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA) for 4 h. The supernatant was removed and 150  $\mu$ l DMSO added to each well. Optical density (OD) of the solution was measured at 490 nm. Triplicate experiments with triplicate samples were performed.

**Cell migration assay.** Wound healing assays were performed to quantify the rate of cell migration. A density of  $5 \times 10^5$  cells/well were plated onto 24 well plates and were grown for 24 h to >90% confluence. The medium was removed and a wound line, that is a cell-free area, was created on the cell monolayers by manually scraping the cells with a plastic pipette tip. Debris was removed from the culture by washing with PBS twice and then the cells were cultured in RPMI-1640 (Sigma-Aldrich; Merck KGaA) containing 1% FBS. Wound sizes were verified using ImageJ software version 1.43 (National Institutes of Health, Bethesda, MD, USA). Cell migration = 0 h wound width (1 mm) - 6 h wound width. Images (original magnification, x40) were obtained using a Nikon Eclipse 90i microscope (Nikon Corporation; Tokyo, Japan). These experiments were repeated three times.

**Cell invasion assay.** The cell invasion ability was evaluated using a modified Boyden chamber (BD Biosciences, San Jose, CA, USA) with a polycarbonate filter with 8  $\mu$ m pores placed between the upper and lower chambers coated with 70  $\mu$ l Matrigel (1 mg/ml) as previously described (16). Briefly,  $1 \times 10^5$  cells in 0.1 ml of serum-free DMEM medium were placed in the upper chamber and the lower chamber was filled with DMEM medium containing 15% newborn calf serum. Following incubation for 24 h at 37°C in a 5% CO<sub>2</sub> incubator, the cells on the top surface of the insert were removed by wiping with a cotton swab. Cells that migrated to the bottom surface of the insert were fixed in 100% methanol for 2 min, stained in 0.1% Crystal Violet for 20 min, rinsed in PBS and then subjected to microscopic inspection (original magnification, x100). The magnitude of cells migration was evaluated by counting the migrated cells in 10 random high-power microscope fields.



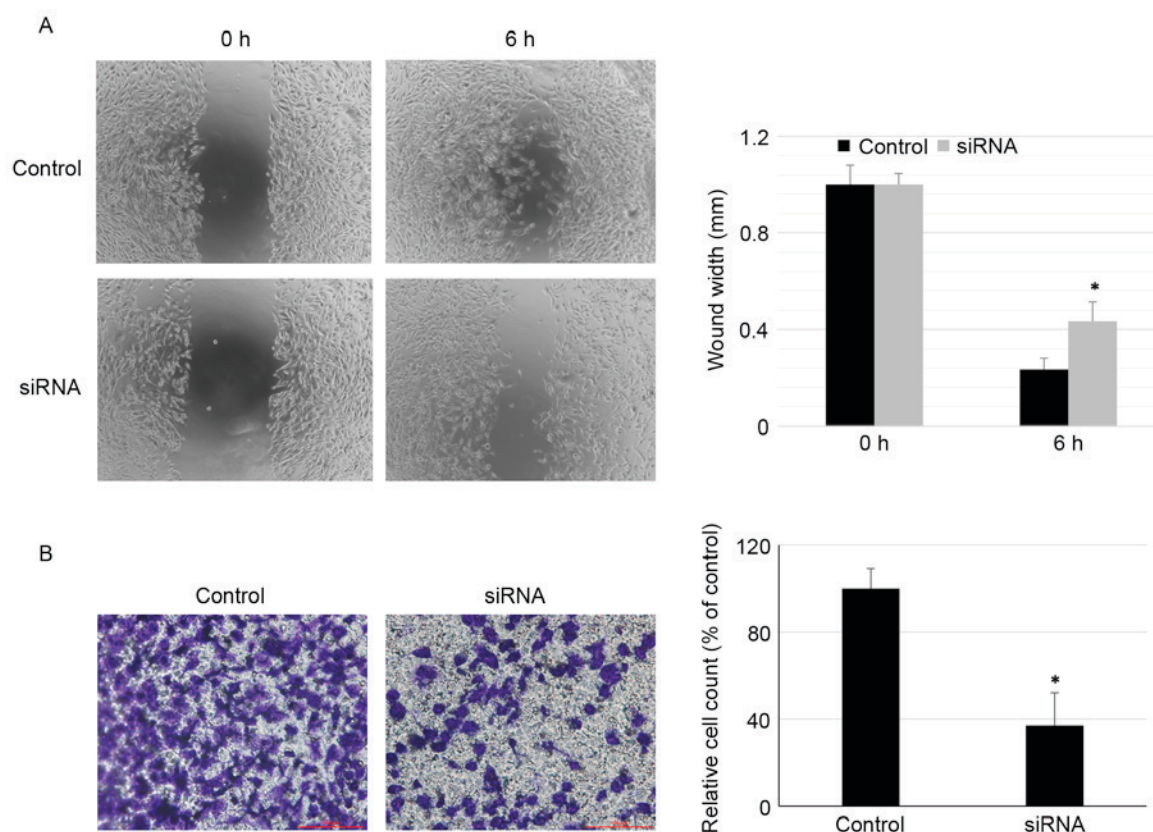


Figure 4. Effects of TAFI suppression on cell migration and invasion. (A) Transfection with TAFI siRNA significantly inhibited cell migration at 6 h, as detected by wound healing assay (original magnification, x40). (B) Transfection with TAFI siRNA significantly inhibited invasion at 24 h, as revealed by Transwell assay (original magnification, x100). \* $P < 0.05$  vs. control. TAFI, thrombin activatable fibrinolysis inhibitor; siRNA, small interfering RNA; TAFI, thrombin activatable fibrinolysis inhibitor.

Migration activity was expressed as a percentage of the control group.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation. Comparisons between treatments were analyzed by one-way analysis of variation followed by S-N-K post hoc test using SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**TAFI is expressed in BC tissues.** BC samples, together with their adjacent normal tissues, from 10 cases breast cancer patients were analyzed. Immunohistochemistry demonstrated that in tumor-adjacent normal tissues the expression of TAFI was relevantly low (Fig. 1A), while in BC tissues TAFIs were visibly elevated (Fig. 1B). The expression of TAFI mRNA was assessed using RT-PCR. TAFI was expressed at higher levels in BC tissues than in matched normal tissues (Fig. 1C).

**siRNA-mediated CPB2 knockdown.** Specific siRNA targeting CPB2 was transfected in MDA-MB-231 and MCF-7 cells for 48 h, then TAFI protein expression levels were detected. In the present study, RT-PCR detection demonstrated that siRNA blocked the expression of CPB2 gene expression, and western blot analysis confirmed that CPB2 gene silencing successfully suppressed expression of the corresponding TAFI protein

compared with non-transfected group (control;  $P < 0.05$ ; Fig. 2A and B).

**TAFI depletion reduces BCC viability.** The effect of TAFI depletion on cell viability was evaluated using the MTT assay. Following treatment with siRNA for 48 h, the cell viability exhibited a significant decrease in MDA-MB-231 and MCF-7 cells ( $P < 0.05$ ; Fig. 3). This indicated that TAFI has an effect on the regulation of BCC viability.

**Effects of RNA interference TAFI on cell migration and invasion.** Next, the role of TAFI in migration and invasion of BCCs was determined using wound scratch and Transwell chamber assays, respectively. As demonstrated in Fig. 4A and B, transfection with TAFI siRNA significantly inhibited migration and invasion in MDA-MB-231 cells compared with non-transfected group (control;  $P < 0.05$ ). The present study revealed similar effects of TAFI inhibition on cell migration and invasion in the less metastatic MCF-7 cells compared with the more metastatic MDA-MB-231 cells (data not shown). Together these results demonstrated that knockdown of TAFI markedly inhibited BCC migration (at 6 h) and invasion (at 24 h).

## Discussion

BC is one of the most frequent malignancies worldwide. There is an upward trend in incidence; the prognosis of patients

with advanced BC is closely associated with metastasis with few effective treatments (17). BC initiation and progression is a complicated process which is associated with the loss of the normal regulatory pathways between cell proliferation, differentiation and apoptosis (18,19). In recent years, the incidence of BC in China has been increasing, according to the national cancer centre statistics (20), BC is the predominant female malignant tumor (21). Tumor metastasis is the basic characteristic of malignant tumors, and also the main difficulty in curing them. Previous studies have demonstrated that an abnormal increase in circulating coagulation protein conducive to thrombophilia is associated with different types of cancer (12,13). Coagulation-associated abnormality in cancer patients is an increased plasma fibrinogen level. Thrombotic and hemorrhagic complications are among the common causes of mortality in cancer patients. Impairment of coagulation may be an important early symptom of tumor progression (22). Studies have identified that cancer patients with tumor growth, angiogenesis and end-organ damage, may also experience coagulation activation (15,23).

TAFI represents the important molecular link between the coagulation and fibrinolytic pathways (24). TAFI activity is generated in the process of coagulation through binding to thrombin, thrombin-TM complex and or plasmin, which in turn cleave TAFI protein at Arg114 (residue Arg92 following removal of the signal peptide) into N-terminal activation peptide and catalytic domains, leading to exposure of the active site cleft of activated TAFIa (25-27). The plasma concentration of TAFI is controlled by genetic and non-genetic factors. It is suggested that a variety of diseases are positively associated with plasma TAFI levels (28). Reports showed that (12,13) databases indicate a high expression of TAFI levels in breast, ovarian and hepatic cancer cell lines. Plasma levels of TAFI are also increased in a number of types of cancers, including BC (10,29). In addition, higher levels of TAFI have been associated with more advanced stages of cancer (30). The fibrinolytic system, more appropriately referred to as the plasminogen activator system, controls not only the intravascular fibrin deposition but is also involved in a wide variety of physiologic and pathologic processes. These components are involved in tumor growth, invasion and metastasis in cancer cells (31). The pathogenesis of the hypercoagulable state of the patients is extremely complex. However, the enhanced coagulation of tumor cells serves an important function in it. Malignant tumors are involved in the hemostasis system by several pathways. Studies have demonstrated that the cytokines which tumor cells excrete contribute to the adhesiveness of the epithelium, thus aiding metastasis (32,33). Anticoagulant drugs like acetaminophen (34) may reduce the migration of tumor cells by inhibiting coagulation. Cancer cell invasion and metastasis require the degradation of the extracellular matrix during local invasion, angiogenesis, intravasation and extravasation. Invasive tumor cells express a plasminogen activation system composed of a urokinase- and/or tissue-type plasminogen activator, plasminogen and protease receptors on their membrane surface. There is a negative correlation between the TM expression level and cell malignancy; patients with low TM expression in BC tissues have a significantly lower rate of disease-free survival compared with patients with high TM expression (35). However, soluble type TM reduced invasion

and metastasis of cancer cells, indicating that TM is not only an anticoagulant protein but also a regulator of tumor invasion and metastasis, (36,37). However, the mechanisms are not completely understood and the importance of TAFI in homeostasis and fibrinolysis remains to be elucidated.

The present study inhibited TAFI expression using siRNA in BCC lines and the results demonstrated that decreasing TAFI expression may reduce cell growth, migration and invasion in two types of BCC cells. Although the mechanism of TAFI synthesis in the BCC cells remains to be elucidated, the present study may offer a novel therapeutic approach for the treatment of BC metastasis.

## Acknowledgements

We are grateful to Central Research Laboratory, the Second Hospital of Shandong University for technical assistance and the generous support. This study was supported by a grant from the Youth Foundation of the Second Hospital of Shandong University (grant no. Y2014010044) and the Natural Science Foundation of Shandong Province (grant no. ZR2014HM023)..

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