Silencing TGIF suppresses migration, invasion and metastasis of MDA-MB-231 human breast cancer cells

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Abstract. This study explored the potential role of TG-interacting factor (TGIF) in migration, invasion and metastasis of the human breast cancer cell line MDA-MB-231. Western blot assay, immunohistochemistry and qRT-PCR assays were applied to detect the expression of protein and mRNA. Wound healing assay, Transwell invasion assay and tail vein metastatic assay were performed to assess the migration, invasion and metastasis of stable TGIF-silenced MDA-MB-231 cell line *in vitro* and *in vivo*. The significantly higher frequency of TGIF high-expression was observed in metastatic breast cancer (62.9%) compared to that in non-metastatic breast cancer (25.8%). Silencing TGIF suppressed migration and invasion of MDA-MB-231 cells *in vitro* and tumor metastasis in nude mouse models. The expression of Snail1, matrix metalloproteinase 2 (MMP2) and β-catenin was markedly decreased in the stable TGIF-silenced MDA-MB-231 cells compared with the control cells. Our results suggest that silencing TGIF suppressed the migration, invasion and metastasis of the human breast cancer cell line MDA-MB-231 using *in vitro* and *in vivo* experiments.

Introduction

Breast cancer is the most commonly diagnosed cancer and the first leading cause of cancer-related death among women worldwide, with an estimated 1.7 million cases and 521,900 deaths in 2012. Breast cancer alone accounts for 25% of all cancer cases and 15% of all cancer-related deaths among women (1). The main reason for breast cancer-related deaths is tumor metastasis. Thus, it is of great significance that much effort should be paid to fully understand the mechanism of breast cancer metastasis and to establish an effective method to inhibit tumor metastasis.

TG-interacting factor (TGIF) is a transcriptional repressor, which is involved in the signaling pathways of retinoic acid (RA) and transforming growth factor β (TGF-β) (2,3). Increasing evidence suggests that TGIF is associated to the initiation, development and progression of several kinds of tumors, including leukemia (4,5), esophageal carcinoma and gastric carcinoma (6,7), hepatocellular carcinoma (8), lung cancer (9,10) and upper tract urothelial carcinoma (11,12). Recently, Zhang *et al* reported that TGIF drove mammary tumorigenesis and the elevated TGIF expression was correlated with high Wnt signaling and poor survival of triple-negative breast cancer (TNBC) patients (13). Kwon *et al* reported that targeted interference of SIN3A-TGIF function by SID decoy treatment inhibits Wnt signaling and the abilities of invasion in TNBC cells (14). However, it is not fully clear what the potential function of TGIF is in the metastasis of human breast cancer.

To address the potential function of TGIF in the metastasis of human breast cancer, we explored the effects of silencing TGIF on the migration, invasion and metastasis of the human breast cancer cell line of MDA-MB-231 using *in vitro* and *in vivo* experiments in the present study. We also investigated the pattern of TGIF protein expression in metastatic human breast cancer samples and non-metastatic human breast cancer samples.

Materials and methods

Cell culture. MDA-MB-231 cell line was obtained from the Cell Resource Center, Peking Union Medical College (which is the headquarters of National Infrastructure of Cell Line Resource, NSTI) and was cultured in DMEM supplemented with 2 mM of L-glutamine, 10% of fetal bovine serum (FBS), streptomycin (100 μg/ml) and penicillin (100 U/ml) at 37˚C in a humidified atmosphere containing 5% CO_2. For infection, control shRNA lentiviral particles-A (sc-108080) and

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TGIF shRNA (h) lentiviral particles (sc-36659-V) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). MDA-MB-231 cells were infected with lentiviral particles in accordance with the manufacturer's instructions and were selected with 10 μg/ml of puromycin for 28 days (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). MDA-MB-231 cells that were successfully infected with control shRNA lentiviral particles and TGIF shRNA (h) lentiviral particles were termed MDA-MB-231-control-shRNA cell and MDA-MB-231-TGIF-shRNA cell, respectively.

Biological samples. Sixty-six breast cancer tissue samples were collected at the First Affiliated Hospital of Zhengzhou University from October 2013 to December 2014. Written informed consent was obtained from individual patients. The Ethics Committee of the First Affiliated Hospital of Zhengzhou University approved this study.

Measurement of cell proliferation. MDA-MB-231-TGIF-shRNA cells ($4x10^4$) and MDA-MB-231-control-shRNA cells were seeded in 12-well plates. Cells were counted using a CASY Cell Counter (Scharfe System, Germany) at 24, 48, 72 and 96 h, respectively (15).

Wound healing assay. The wound healing assay was performed according to our previous report (16). Cells were wounded by scratching the surface of a 6-well plate with a 200-μl pipette tip. Floating cells were removed through washing with PBS. The cells were maintained at 37˚C for 48 h. The photographic images were taken by using a Leica DM IL LED inverted microscope at different time-points. The healing width was calculated according to our previous report (16).

Transwell invasion assay. The cell invasion assay was performed in accordance with the manufacturer's instructions (Corning Inc., Corning, NY, USA). MDA-MB-231-TGIF-shRNA cells ($5x10^4$) and MDA-MB-231-control-shRNA cells were plated into the upper chamber of a 24-well Transwell chamber with an 8-μm pore size insert (Corning Inc.) with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The lower chambers were filled with 600 μl of DMEM containing 10% FBS. After 24 h of incubation at 37˚C, the cells on the upper side of the membrane were removed, and the membranes were fixed in methanol and stained with crystal violent (Sigma-Aldrich, St. Louis, MO, USA). The cells on the lower surfaces were photographed and five randomly selected fields were counted (16).

Tail vein metastatic assay. The Ethics Committee of Henan Center for Disease Control and Prevention approved this study. All of the animal experiments were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals. Female BALB/c nude mice aged 28 days were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). MDA-MB-231-TGIF-shRNA cells and MDA-MB-231-control-shRNA cells were detached by trypsinization, washed with PBS, and then re-suspended in PBS. Cells ($6x10^5$) in 200 μl PBS were injected to each mouse (ten mice per group) by tail vein. The mice were sacrificed at 12 weeks post-injection. The lungs were examined for tumor metastasis (17,18).

Quantitative real-time polymerase chain reaction (qRT-PCR). The detailed methods of total RNA extraction, cDNA synthesis and quantitative real-time PCR were performed as previously described (9).

Western blot assay. Soluble cell lysates (30 μg protein) were loaded and separated in 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose (NC) membranes (Pall Life Sciences, Port Washington, NY, USA). The membranes were blocked with 5% bovine serum albumin (BSA), and then incubated with primary antibodies overnight at 4˚C. TGIF (sc-9084), MMP2 (sc-10736), N-cadherin (sc-7939), β-catenin (sc-7199) and β-actin (sc-8432) were purchased from Santa Cruz Biotechnology Inc. Snail1 (#3879S) and vimentin (#5741S) were purchased from Cell Signaling Technology. The membranes were washed with Tris-buffered saline-Tween-20 (TBST) and further incubated for 1 h at room temperature with corresponding horseradish peroxidase-coupled secondary antibodies (ZSGB-BIO, Beijing, China). Signals were detected by ECL kit (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry. Immunohistochemistry analysis was performed according to the manufacturer's instructions (CW2069; Beijing ComWin Biotech Co., Ltd). The primary antibody of TGIF (sc-17800) was obtained from Santa Cruz Biotechnology. The intensity of TGIF staining was scored and the scores of positive cell percentage were assigned in line with a published report (19).

Statistical analysis. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used to estimate the statistical significance. Student's t-test and Chi-square were performed. P-value <0.05 was considered to indicate statistical significance.

Results

The effects of silencing TGIF on MDA-MB-231 cell proliferation. Fig. 1 presented the silencing efficiency of the shRNA lentiviruses by determining the levels of mRNA and protein expression in the MDA-MB-231 human breast cancer cells. Our data indicated that the lower levels of protein (Fig. 1A) and mRNA (Fig. 1B) of TGIF expression were observed in MDA-MB-231-TGIF-shRNA cells compared to that in MDA-MB-231-control-shRNA cells, suggesting that a stable TGIF-silenced MDA-MB-231 cell line was successfully established.

Fig. 1C demonstrates the effects of silencing TGIF on the proliferation of MDA-MB-231 cells. Our results showed that no significant difference was observed in the cell growth speed between the MDA-MB-231-TGIF-shRNA cells and the MDA-MB-231-control-shRNA cells at the indicated time-points.

The effects of silencing TGIF on the migration and invasion of MDA-MB-231 cells. Data on the effects of silencing TGIF on migration and invasion of MDA-MB-231 cells are presented in Fig. 2. Our results indicated that the MDA-MB-231-TGIF-shRNA cells migrated less quickly to close the scratched wounds than the MDA-MB-231-control-shRNA cells (Fig. 2A). Fig. 2B

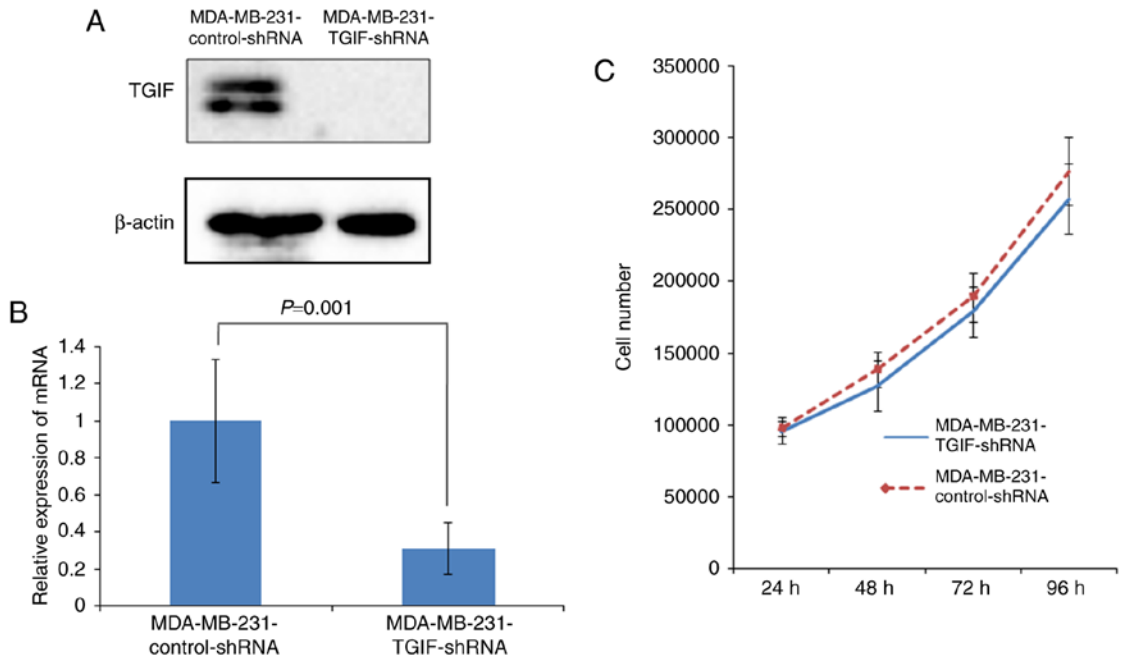

Figure 1. The silencing efficiency of shRNA targeting TGIF in MDA-MB-231 cells and the effects of silencing TGIF on MDA-MB-231 cell proliferation. (A) Western blot analysis indicated that shRNA specifically targeting TGIF markedly decreased the level of TGIF protein expression in MDA-MB-231 cells. (B) qRT-PCR analysis also indicated that shRNA specifically targeting TGIF markedly decreased the level of TGIF mRNA expression in MDA-MB-231 cells. (C) Silencing TGIF had no significant effects on the proliferation of MDA-MB-231 cells. TGIF, TG-interacting factor. qRT-PCR, quantitative real-time polymerase chain reaction (qRT-PCR)

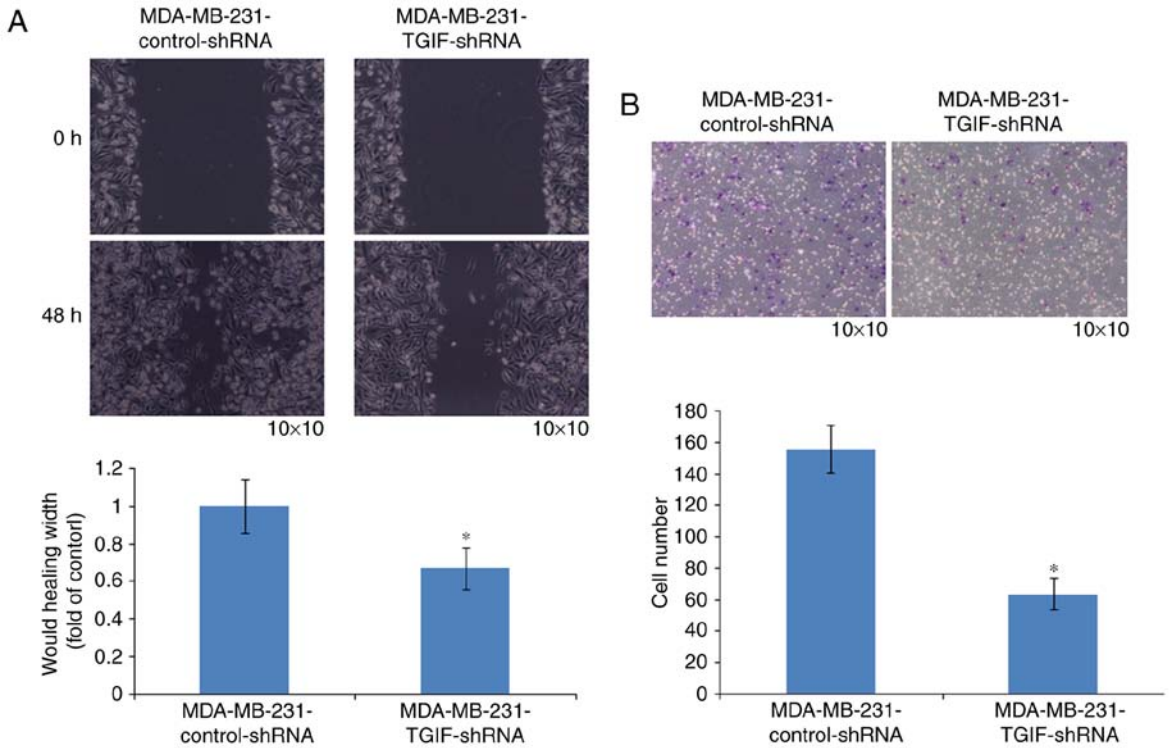

Figure 2. The effects of silencing TGIF on the migration and invasion of MDA-MB-231 cells. The cells migrated less quickly to close the scratched wounds in MDA-MB-231-TGIF-shRNA cells as compared to MDA-MB-231-control-shRNA cells (A). The significantly decreased number of cells invading through the Matrigel was observed in MDA-MB-231-TGIF-shRNA cells as compared to MDA-MB-231-control-shRNA cells (B). *P<0.05. TGIF, TG-interacting factor.

showed that the decreased number of cells invading through the Matrigel was observed in the MDA-MB-231-TGIF-shRNA cells compared with the MDA-MB-231-control-shRNA cells. Taken together, our findings suggested that silencing TGIF was able to inhibit migration and invasion of human breast cancer cell line of MDA-MB-231 in *in vitro* experiments.

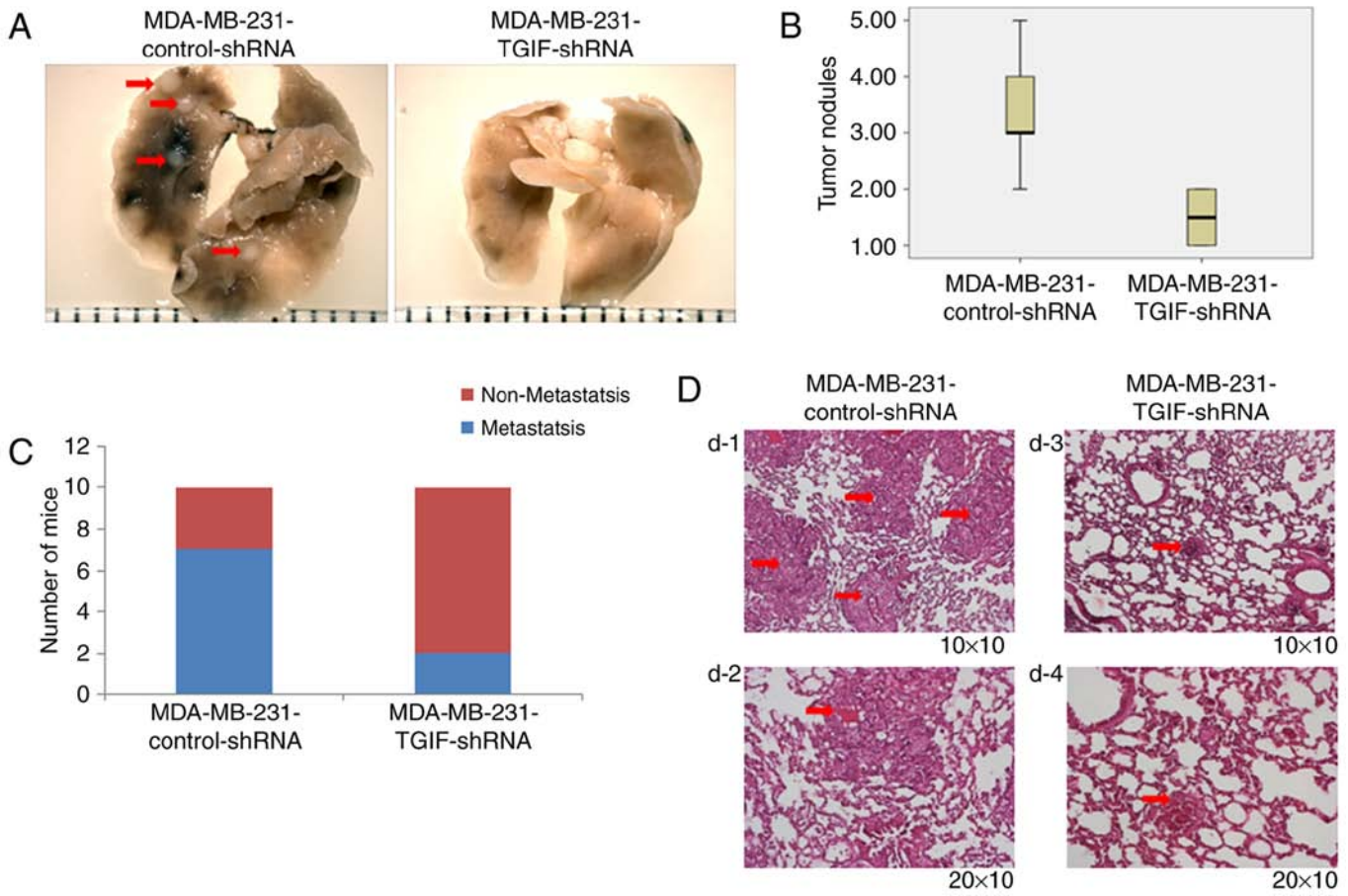

Figure 3. The effects of silencing TGIF on the ability of the metastasis of MDA-MB-231 cells *in vivo*. (A) The representative images of tumor nodules of lung surface are indicated. The number of metastatic lung nodules was significantly reduced in MDA-MB-231-TGIF-shRNA group as compared to the control group (B). The incidence rate of metastasis in mouse lung was lower in MDA-MB-231-TGIF-shRNA group than that in the control group (C). (D) The representative images of histological examinations are shown. The red arrows indicated the tumor nodules. TGIF, TG-interacting factor.

The effects of silencing TGIF on MDA-MB-231 cell metastasis in vivo. Fig. 3 shows the effects of silencing TGIF on MDA-MB-231 cell metastasis *in vivo*. The representative images of tumor nodules are demonstrated in Fig. 3A. Our data indicated that the significantly reduced number of metastatic lung nodules was observed in the group of MDA-MB-231-TGIF-shRNA cells compared with the control group (Fig. 3B). The mice injected with MDA-MB-231-TGIF-shRNA cells had markedly decreased incidence of lung metastasis compared with those injected with MDA-MB-231-control-shRNA cells (P=0.029; Fig. 3C). The representative images of histological examinations are shown in Fig. 3D.

Silencing TGIF downregulates the expression of Snail1, MMP2 and β-catenin. Since the proteins such as MMP2, Snail1, N-cadherin, vimentin and β-catenin are involved in the progression and metastasis of human breast cancer (20-24), we tentatively sought to observe whether silencing TGIF could affect the protein expression of Snail1, MMP2, N-cadherin, vimentin and β-catenin. Western blot analysis indicated that the obviously decreased levels of Snail1, MMP2 and β-catenin protein expression were observed in MDA-MB-231-TGIF-shRNA cells compared with MDA-MB-231-control-shRNA cells (Fig. 4).

Table I. The main clinical characteristics of human breast cancer.

	No.
Age (years)	
≤45	22
>45	44
Histology type	
Ductal carcinoma	11
Invasive carcinoma	55
TNM stage	
I	11
II	40
III	15
Metastasis	
Yes	35
No	31

TGIF expression in human breast cancer tissues. The main clinical characteristics of human breast cancer patients are listed in Table I. Fig. 5 demonstrates the difference of TGIF expression between metastatic human breast cancer samples

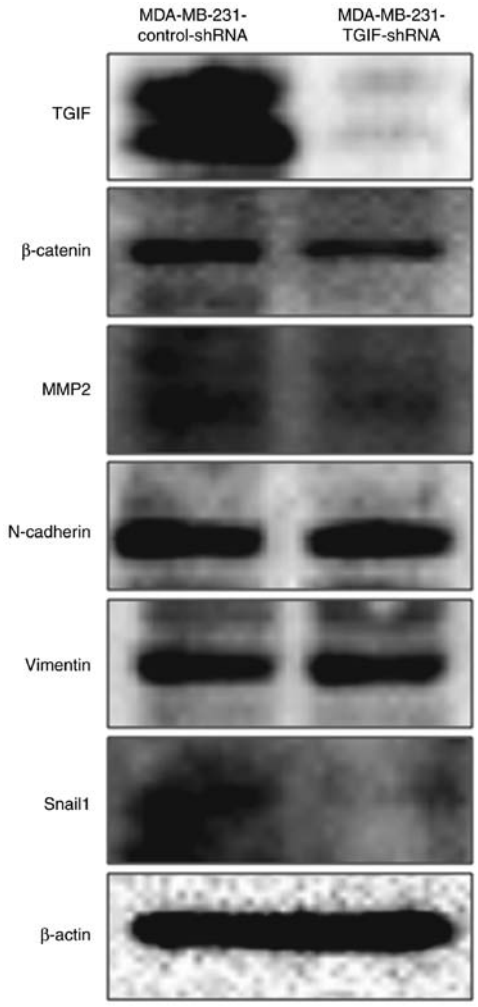

Figure 4. The effects of silencing TGIF on the expression of the studied proteins. Western blot analysis showed that silencing TGIF decreased the protein expression of Snail1, MMP2 and β-catenin. TGIF, TG-interacting factor.

and non-metastatic human breast cancer samples. The representative images of TGIF low-expression (a-1, 10x10; a-2, 10x20) and TGIF high-expression (a-3, 10x10; a-4, 10x20) are shown in Fig. 5A. Our results indicated that the significantly higher percentage of TGIF high-expression was observed in metastatic human breast cancer (62.9%, 22 of 35) than that in non-metastatic human breast cancer (25.8%, 8 of 31) (P=0.003, Fig. 5B).

Discussion

To our knowledge, previous studies have shown that overexpression of TGIF is correlated to metastasis and worse progression in non-small cell lung cancer (NSCLC) (10) and upper urinary tract urothelial carcinoma (11,12). Xiang *et al* reported that overexpression of TGIF increased the ability of migration of NSCLC cells, while suppressing TGIF expression inhibited the ability of migration of NSCLC cells. Moreover, knocking down TGIF impaired metastasis of NSCLC cells (10). Yeh *et al* reported that overexpression of TGIF could significantly increase the capabilities of migration and invasion of RT4 cells and TSGH8301 cells, on the contrary, knocking down TGIF inhibited the ability of invasion of T24 cells (11).

In this study, we investigated the effects of silencing TGIF on the migration and invasion of the human breast cancer cell line MDA-MB-231 *in vitro*. Our findings indicated that silencing TGIF inhibited the migration and invasion of MDA-MB-231 cells in wound healing assay and Transwell invasion assay, which suggested that TGIF might be involved in the metastasis of human breast cancer. Our results are consistent with data of a previous report, Kwon *et al* reported that a marked 80% reduction of cell invasion was observed in MDA-MB-231-luc-D3H2LN cells transfected with TGIF-targeting siRNA, compared with control cells (14). Our

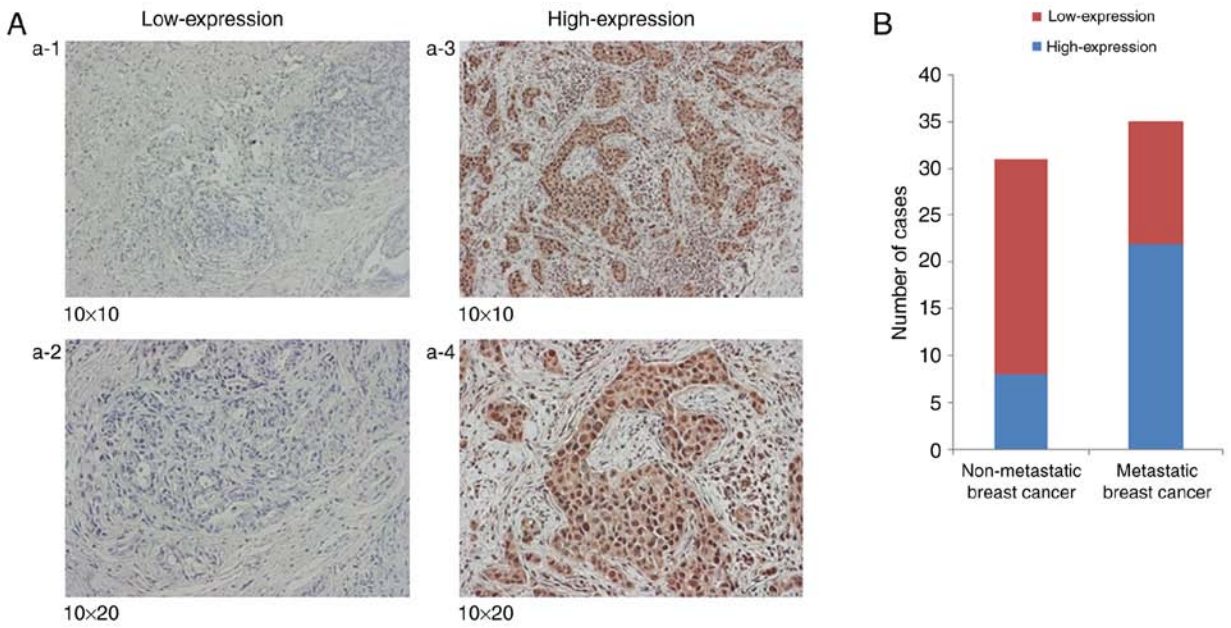

Figure 5. The expression of TGIF in human breast cancer tissues detected by immunohistochemistry. The representative images showed low-expression (a-1, 10x10; a-2, 10x20) and high-expression (a-3, 10x10; a-4, 10x20) of TGIF in human breast cancer tissues (A). The higher percentage of TGIF high-expression was observed in metastatic human breast cancer than that in non-metastatic human breast cancer (B). TGIF, TG-interacting factor.

findings also indicated that silencing TGIF inhibited metastasis of MDA-MB-231 cells to lung *in vivo*, which verified the potential role of TGIF in the metastasis of breast cancer in the animal model. Furthermore, we observed the rate of TGIF high-expression in metastatic human breast cancer samples was significantly higher than that in non-metastatic human breast cancer samples, which supported the notion that TGIF was involved in breast cancer metastasis in the population level.

In the present study, we observed that silencing TGIF reduced the MMP2 protein expression in the human breast cancer cell line MDA-MB-231. Previous reports suggested that TGIF could regulate the MMP2 expression in urothelial carcinoma cell lines (11,12). Together, these studies supported that TGIF could regulate the MMP2 expression in different types of cell lines. MMP2 is one of the matrix-degrading enzymes, which plays a key role in the invasion and metastasis of breast cancer (25,26). Published reports showed that the elevated MMP2 expression was related to poor prognostic clinical pathological factors in breast cancer (25,27-29). Pei *et al* reported that plantamajoside (PMS) inhibited the metastasis of breast cancer by decreasing the activity of MMP2 (30). Ni *et al* reported that downregulation of miR-106b induced breast cancer cell invasion and motility in association with overexpression of MMP2 (31). Accompanied with previous studies, our data suggested that TGIF silencing might inhibit metastasis of breast cancer via regulating MMP2. However, the underlying molecular mechanism should be explored in detail in future studies.

Snail1 is a zinc-finger transcription factor, which plays a significant role in the progression and metastasis of breast cancer (32,33). Moody *et al* reported that the higher levels of Snail1 expression predicted decreased relapse-free survival of breast cancer patients and Snail1 was sufficient to promote mammary tumor recurrence *in vivo* (34). Geradts *et al* reported that nuclear Snail1 expression in early stage breast lesions might predict future development of invasive breast cancer (35). Results from Tran *et al* study showed that Snail1 expression in primary human breast cancer correlated with higher rates of metastasis (36). Zhang *et al* found the collagen receptor discoidin domain receptor 2 stabilized Snail1 protein to promote the metastasis of human breast cancer (37). Tran *et al* reported that transient overexpression of Snail1 in primary breast tumors increased breast cancer metastasis in MMTV-NeuNT mice (38). In this study, we observed that silencing TGIF repressed Snail1 protein expression in the human breast cancer cell line of MDA-MB-231, which suggested that the reduced abilities of the migration and invasion of human breast cancer cell line of MDA-MB-231 induced by silencing TGIF might be through partially downregulating Snail1 protein expression. The inference should be verified in further studies through constructing TGIF-overexpressed and Snail-silenced cell models. Xiang *et al* observed that silencing TGIF reduced Snail1 expression in NSCLC cells (10). However, the molecular mechanism of TGIF regulating the expression of Snail1 was not addressed in this study, which should be a focus of future study.

β-catenin plays a significant role as a key mediator in the Wnt/β-catenin signaling pathway, which is involved in the progression and metastasis of human breast cancer (22,39,40) and modulates the sensitivity of breast cancer to ionizing radiation (41). Li *et al* reported that aberrant β-catenin expression was related to a poor clinical outcome in invasive human breast cancer (42). Lin *et al* reported that the elevated activity of β-catenin was significantly associated with poor prognosis of the breast cancer patients and was an independent prognostic factor (43). The higher levels of β-catenin expression had a significantly decreased overall survival of breast cancer patients (44). SiRNA-medicated decrease in the levels of β-catenin protein expression resulted in a significant inhibition of migration and invasion in the MDA-MB-231 cells (39). Stably knocking down β-catenin significantly suppressed the ability of migration of breast cancer cells compared to its corresponding control cells (45). In this study, silencing TGIF repressed the expression of β-catenin protein in the human breast cancer cell line MDA-MB-231, which suggested that TGIF silencing might suppress cell migration and invasion of breast cancer partially by repressing Wnt/β-catenin signaling pathway. Two previous reports indicated that TGIF regulated the β-catenin expression in NSCLC cells and breast cancer cells (10,13). The molecular mechanism of TGIF regulating the expression of β-catenin was reported showing TGIF was associated with and diverted Axin1 and Axin2 from the β-catenin destruction complex, therefore allowing β-catenin accrual (13). Together, these studies verified the regulation of β-catenin expression by TGIF.

In conclusion, our results suggest that silencing TGIF inhibited migration, invasion and metastasis of the human breast cancer cell line of MDA-MB-231 *in vitro* and *in vivo*. Therefore, this study extends our knowledge of the progression of breast cancer and suggests that TGIF might be a potential therapeutic target for human breast cancer.

Acknowledgements

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