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Original Article

Expression of Tim-3 in breast cancer tissue promotes tumor progression

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Abstract: T-cell immunoglobulin mucin-3 (Tim-3) plays a pivotal role in immune regulation and tolerance induction as a negative regulatory molecule on innate versus adaptive immune cells, especially in antitumor immunity. However, the mechanism of Tim-3 expression on tumor cells and the mechanism that inhibits anti-tumor immunity are obscure. In this present study, we aimed to investigate the functions of Tim-3 in breast cancer and to explore its correlation to tumor prognosis. In a total of 42 clinical samples of invasive breast cancer, Tim-3 was semiquantitatively scored based on both distribution and intensity of immunohistochemistry staining and was found to correlate with clinicopathological parameters. Western blotting was used to detect the expression of Tim-3 in breast cancer cells. Furthermore, the effect of Tim-3 in breast cancer cells was evaluated after overexpression by ADV-Tim-3 and downregulation by Tim-3-siRNA. High immunoreactivity of Tim-3 was found to be significantly correlated with clinical stage, metastasis, KI67, and a lower 5-year survival rate. We supported this finding by confirming the presence of Tim-3 protein in the breast cell lines. Downregulation of Tim-3 significantly inhibited the proliferation, migration, and invasion of breast cancer cells while it promoted apoptosis. Overexpression of Tim-3 promoted the proliferation, migration, and invasion of breast cancer cells and inhibited apoptosis. Taken together, as a valuable marker of breast cancer prognosis, Tim-3 in breast cancer cells play an important role in the progression of breast cancer and may be an effective novel target in tumor prevention and treatment.

Keywords: Breast cancer, Tim-3, Ki67, overexpression, downregulation, SiRNA

Introduction

Breast cancer is currently the most common female malignancy worldwide [1]. Although the wide applications of early detection and treatment improvement have led to a reduction in mortality from breast cancer, it is still the second leading cause of cancer-related deaths in women [2]. Its rapid recurrence and poor survival rates are caused by sustained proliferation, activated invasion and metastasis, and resistance to cell death. Therefore, identification of novel molecular mechanisms that suppress the proliferation and metastasis of cancer cells may provide novel targets for clinical treatment.

T-cell immunoglobulin mucin-3 (Tim-3), a member of the Ig superfamily, was initially identified as a specific cell surface marker of Th1 $CD4^+$ T-cells [3]. However, it has been confirmed that TIM-3 is also expressed on other cell types such as cytotoxic $CD8^+$ T-cells, regulatory T-cells, monocytes, dendritic cells, and mast cells [4-7]. Despite its differential expression on innate versus adaptive immune cells, Tim-3 is regarded as a negative regulatory molecule that plays a crucial role in antitumor immunity [8-13].

Recent studies have confirmed that TIM-3 expression is not limited to immune cells but has also been detected in a series of normal and malignant tissues. TIM-3 is expressed as a surface molecule on leukemia stem cells in most types of acute myeloid leukemia and TIM-3 positive leukemia cells are able to reconstitute human AML in immunodeficient mice [14, 15]. Cao et al. confirmed the presence of TIM-3 mRNA and protein in cervical cell lines and in clinical cervical tissues. Patients with high TIM-3 expression had significantly increased metastatic potential, advanced cancer grades, and shorter overall survival than those

Table 1. Tim-3-siRNA sequence

Sequence	Sense 5'-3'	Antisense 5'-3
Tim-3-siRNA-750	GAGCCUCCCUGAUAUAAAUTT	AUUUAUAUCAGGGAGGCUCTT
Tim-3-siRNA-452	GUGCUCAGGACUGAUGAAATT	UUUCAUCAGUCCUGAGCACTT
Tim-3-siRNA-311	GGUCCUCAGAAGUGGAAUATT	UAUUCCACUUCUGAGGACCTT
NC	UUCCUCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

with lower expression and blocking Tim-3 can inhibit cell migration and invasion ability [16]. It has also been shown that TIM-3 is expressed on tumor cells in non-small cell lung cancers and that expression levels of TIM-3 may correlate with patient survival as a potential independent prognostic factor [10].

However, the role of Tim-3 on cancer cells has not been conclusively documented. Our previous work has indicated that that +4259T/G SNP of the TIM-3 gene may be a risk factor for progression and prognosis of invasive breast cancer in Han population of northern china. In our present study, we aimed to investigate Tim-3 expression in breast cancer cells and to explore its relationship with tumor progression.

Materials and methods

Patients

We randomly selected 42 invasive ductal carcinoma patients in Shandong Provincial Hospital Affiliated to Shandong University between January 2011 and October 2012. All of the patients were histopathologically diagnosed by two pathologists using the *World Health Organization's Classification of Tumors: Pathology and Genetics of Tumors of Breast and Female Genital Organs, Fourth Edition.* Patients who had a history of other types of cancer or that had received chemotherapy or radiotherapy prior to surgery were excluded from the study. Breast cancer tissues and their corresponding adjacent non-tumor tissues (≥2 cm from tumor margin) were collected. In addition, clinicopathologic parameters were obtained from medical records and all of the patients were regularly followed up by telephone interviews. The last follow up assessment was conducted in November 2016. Each patient's overall survival (OS) was calculated as the period from the date of surgery until the date of death. Approval for the study was granted by the Ethics Committee of the Shandong Provincial Hospital Affiliated to Shandong University with written informed consent from all participants.

Immunohistochemical staining of Tim-3

Immunohistochemical detection was performed on paraffin-embedded breast tissue sections, as previously described [17]. Anti-Tim-3 goat polyclonal antibody (Proteintech, Chicago, USA) and biotinylated secondary antibody (Proteintech, Chicago, USA) were used in the present study. Tim-3 expression were observed under a microscope and evaluated by two pathologists who were blinded to the clinical and laboratory patient information and carried out evaluation of the specimens independently.Tim-3immunoreactivitywassemiquantitatively scored based on both distribution and intensity of staining. The distribution of stained cells was categorized as follows: ≤5%=0, >5% but ≤30%=1, >30% but ≤70%=2, and >70%=3. Intensity of staining was designated as follows: no staining =0, weak staining =1, moderate staining =2, and strong staining =3. The staining scores were calculated by multiplying the intensity and percentage of positive cells. The sample with a score of 0-1 points was considered as negative expression of Tim-3, otherwise it was designated as positive.

Cell lines and culture

The human breast cancer cell lines MDA-MB-231, MCF-7, and MCF-7/ADM were obtained from American Type Culture Collection (ATCC, USA). The cell lines were maintained in DMEM media or RPMI 1640 medium (Life Technologies, Beijing, China) with 10% fetal bovine serum and cultured at 37°C in 5% CO_2.

Transfection

For overexpression, the recombinant adenovirus vectors respectively named ADV-Tim-3 containing a fragment of Tim-3 cDNA and ADV-GFP containing a GFP gene used as a control were constructed by Sangon Biotech (Shanghai, China). For downregulation of Tim-3, Tim3-siRNAs (Tim3-siRNA-750, Tim3-siRNA-451, Tim3-siRNA-311) and nonspecific siRNA (as a negative control, NC) used in this study were provided by Sangon Biotech (Shanghai, China) (summarized in **Table 1**). We seeded the breast cell lines at a density of 3×10^5 per well in a 6-well culture plate. Transfections were per-

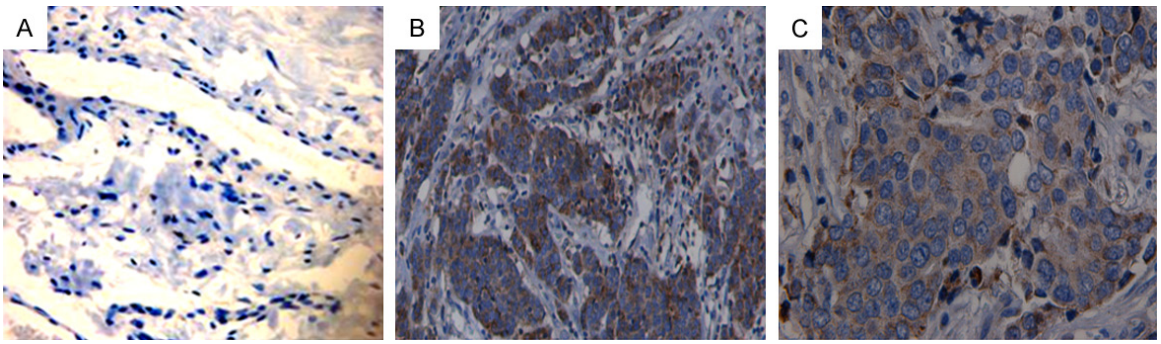

Figure 1. Representative immunohistochemical staining for Tim-3 in mammary tissues. A. Adjacent tissues: ×100. B. Breast cancer tissues: ×100. C. Breast cancer tissues: ×400.

Table 2. Expression of Tim-3 in mammary tissues

Group	Cases	Tim-3	
		Positive cases (%)	Score[a]
Breast cancer	42	18 (42.9)	0.902±0.916*
Adjacent tissues	42	4 (18.2)	0.304±0.401

[a]Expression score (mean ± SD). *P=0.005.

formed using Lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer's instructions.

Quantitative RT-PCR (qRT-PCR) analysis

Total RNA was collected from the three breast cancer cell lines using TRIzol reagents (Invitrogen, CA, USA), according to the instructions. cDNA was then synthesized by PrimeScript II 1st Strand cDNA (Takara, Tokyo, Japan). PCR products were detected by agarose gel electrophoresis. After PCR, 10 μL aliquots of the reaction mixtures were resolved on 1% agarose gel containing ethidium bromide to identify the DNA amplicons generated. The primer for TIM-3 was 5'-TGTGATTGTGGAGTAGACAGTTG-3' and 5'-GGTGTAGAAGCAGGGCAGATAG-3'.

Western blot

Total protein was extracted using RIPA lyses buffer and protein concentration was measured by the BCA assay kit (Thermo Fisher, CA, USA). Equal amounts of protein were separated by SDS-PAGE, electrotransferred to PVDF membranes, and blocked in 5% non-fat dry milk. Immunoreactivity was detected by enhanced chemiluminescence. β-actin was used as the loading control.

Cell proliferation assay

Cell proliferation was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Transfected breast cancer cells were plated in 96-well plates at 5-8×10^3 cells/well and incubated at 37°C. Proliferation rates were determined at 0, 24, 48, and 72 hours with 10 μl CCK after transfection.

Invasion and migration assays

Cell migration was assayed using Wound Healing Assay. The cells were seeded in a 6-well culture plate. A linear wound was made by scratching the monolayer with a sterile 10-ul pipette tip. The wounded monolayers were washed 2 times with regular medium and incubated in fresh serum-free medium. Photographs were taken at 0 hours and 24 hours after wounding by phase-contrast microscopy. Cell invasion was assayed using Transwell chambers (Corning, Beijing, China) with Matrigel. For the determination of cells invasion, Transwell chambers were placed into 24-well plates and coated with 40 μl Matrigel, then incubated at 37°C for 2 hours. Cells were trypsinized and seeded into the upper chambers in serum-free medium at a density of 2×10^4 per well at 24 hours after transfection and 800 μl of 30% fetal bovine serum containing medium was placed in the lower chamber as a chemo-attractant. After 24 hours, the migrated cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet solution. Cells on the upper surface of the filter were removed with cotton buds. Invaded cells on the underside of the filter were photographed and counted by phase-contrast microscopy for a minimum of 10 random visual fields.

Table 3. Expression of Tim-3 in breast cancer tissues correlates with clinicopathologic features

Clinicopathological Variables		Cases	Tim-3 score[a]	*t*	*P*
Age	<55	20	1.06±0.870		
	≥55	22	0.76±0.950	1.0637	0.2939
Clinical stage	I	12	0.49±0.58		
	II, III, IV	30	1.17±0.96	2.283	0.0278*
Histological grade	I	16	0.84±0.706		
	II, III	26	0.930±1.02	0.3096	0.7585
Metastasis	No	16	0.46±0.620		
	Yes	26	1.12±0.980	2.4059	0.0207*
Tumor diameter	≤2 cm	18	1.11±1.11		
	>2 cm	24	0.75±0.75	1.2510	0.2182
KI67	Negative	18	0.62±0.69		
	Positive	24	1.12±0.98	2.0355	0.0444*
PR	Negative	19	1.02±1.08		
	Positive	23	0.81±0.81	0.7198	0.4759
ER	Negative	17	0.85±1.02		
	Positive	25	0.93±0.89	0.2695	0.7889
P53	Negative	16	1.29±1.07		
	Positive	26	0.55±0.59	0.3782	0.7074

[a]Expression score (mean ± SE). *P<0.05.

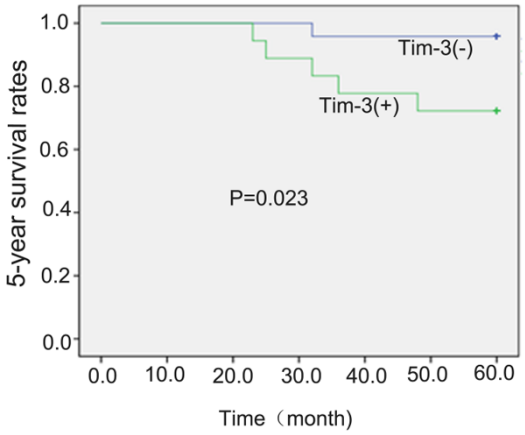

Figure 2. Comparison of five-year cumulative-survival curve of breast cancer patients between Tim-3 positive expression and Tim-3 negative expression. P<0.05.

Apoptosis assay

Apoptotic cells after transfection were detected using the PI/Annexin V staining kit on a FACS (BD, CA, USA). Briefly, 1.5×10^6 transfected cells/well were seeded into 6-well plates and incubated 24 hours, harvested and washed with PBS, then stained by PI (2 mg/ml) and Annexin V (100 μg/ml) and incubated in the dark for 15 minutes. Cells were collected and subjected to FACS analysis of subG1 population.

Statistical analysis

The results were analyzed using software SPSS version 18.0 (SPSS, Chicago, USA). Each experiment was done three times and the data were expressed as mean ± SD. A two-tailed Student's t-test and χ^2-test was used when indicated to determine statistical significance. Survival curves were analyzed using Kaplan-Meier curves and survival differences were examined using the log-rank test. P<0.05 was considered statistically significant.

Results

Tim-3 is highly expressed in breast cancer tissues

In order to clarify the relationship between Tim-3 and breast cancer progression, we first analyzed sections of tumor tissue and adjacent tissues from 42 patients that underwent primary surgery for breast cancer. As shown in **Figure 1**, positive Tim-3 staining was identified in 42.9% (18 out of 42) of breast cancer but only 18.2% (4 out of 42) of adjacent tissues. When expression of Tim-3 protein was further compared by semiquantitative immunoreactivity scoring, breast cancer displayed a much higher Tim-3 score than adjacent tissues (0.902±0.916 vs 0.304±0.401, P=0.0050) (**Table 2**).

Tim-3 expression was associated with progression and prognosis of breast cancer

We first determined the relationship of Tim-3 with the clinicopathologic characteristics. As shown in **Table 3**, high immunoreactivity of Tim-3 was found to be significantly correlated with clinical stage (P=0.0278), metastasis (P=0.0207), and Ki67 (P=0.0444) while showing no significant correlation with age (P= 0.294), histological type (P=0.7585), tumor diameters (P=0.2182), and PR, ER, P53 (P= 4579, 0.7889, 0.7074, respectively). To evaluate whether expression of Tim-3 is prognostic

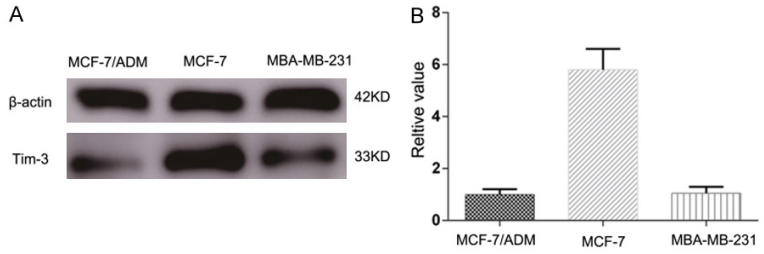

Figure 3. Expression of Tim-3 was detected by Western blotting in three different breast cancer cells.

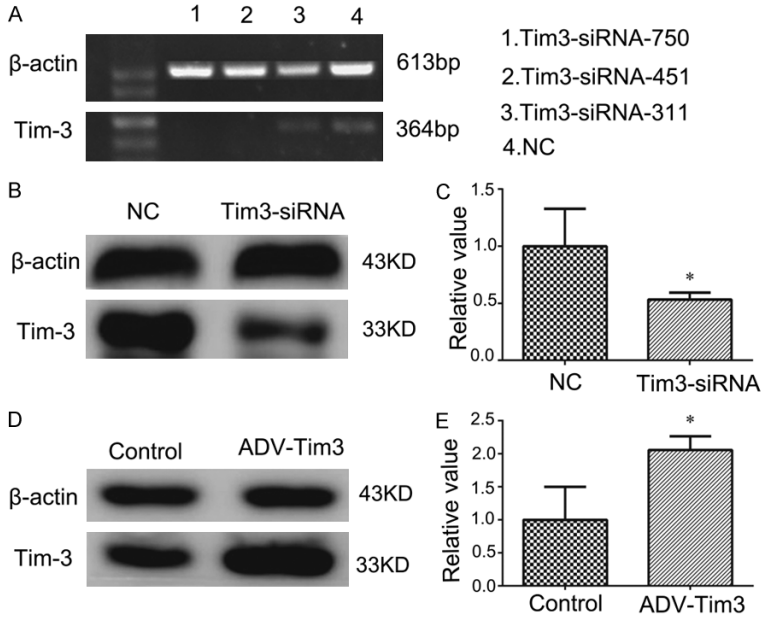

Figure 4. Effect of TIM-3 suppression and overexpression in breast cancer cells was detected by RT-PCR and Western blotting. A. Inhibitory effects of three kinds of small interference was detected by RT-PCR; B, C. Inhibitory effects transfected by the mixture with Tim3-siRNA-750 and Tim3-siRNA-451 were detected by Western blotting; D, E. Overexpression effects transfected by ADV-Tim3 were measured by Western blotting. *P<0.05.

in breast cancer, we evaluated Tim-3 expression with respect to the overall survival (OS) at the end of the 5 year follow up period. As shown in **Figure 2**, 5 patients died in Tim-3 positive group while only 1 in Tim-3 negative group. The 5-year survival rate was 72.2% vs 95.8% respectively (P=0.023).

Tim-3 is expressed in different breast cancer cell lines and effects of TIM-3 suppression and overexpression in breast cancer cells

Western blot was used to detect Tim-3 protein levels in three human breast cancer cell lines MCF-7/ADM, MCF-7, and MDA-MB-231. As shown in **Figure 3**, Tim-3 was confirmed to be present in these three cell lines. In MCF-7, the expression of Tim-3 was higher and the expression in MDA-MB-231 and MCF-7/ADM was relatively lower. In order to explore additional functions of Tim-3 in breast cancer, we transfected MDA-MB-231 cells with ADV-Tim3 and MCF-7 cells with Tim3-siRNAs. By assessing transfection efficiency of three kinds of Tim3-siRNAs, we found that the effects of Tim3-siRNA-750 and Tim3-siRNA-451 were better (**Figure 4A**), so we chose the mixture of Tim3-siRNA-750 and Tim3-siRNA-451 by 1:1 for subsequent experiments. As shown in **Figure 4B**, **4C**, MDA-MB-231 cells transfected with ADV-Tim3 showed significantly increased Tim-3 protein expression compared with control cells and MCF-7 cells transfected with mixed small interference showed significantly decreased Tim-3 protein expression compared with control cells.

Tim-3 promotes proliferation, migration, and invasion of breast cancer cells and inhibits apoptosis

We explored the potential impact of Tim-3 on proliferation, invasion, and migration in breast cancer cell lines. As shown in **Figure 5A**, CCK-8 proliferation assay indicated that cell proliferation was inhibited in the Tim3-siRNA transfected MCF-7 cells compared with negative control (P<0.05). Conversely, overexpression of Tim-3 in the ADV-Tim3 transfected MDA-MB-231 cells significantly promoted the proliferation (P<0.05) (**Figure 5B**). Since cancer cell migration and invasion are directly related to metastasis, a wound healing assay and a cell invasion assay were performed to determine whether repression of Tim-3 expression inhibits cell migration and invasion. We found that the migration distance of Tim3-siRNA group was significantly lower than that in NC group

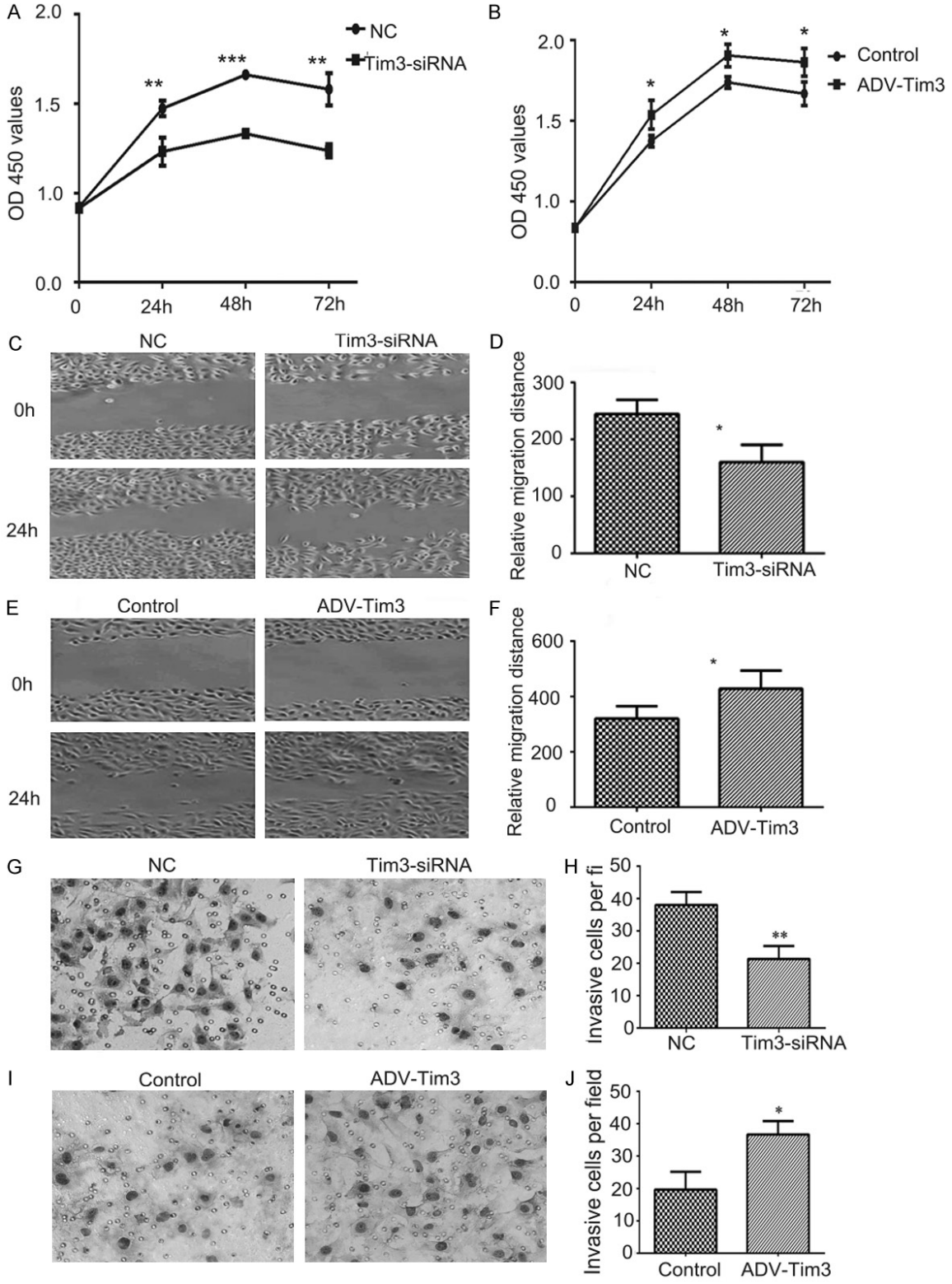

Figure 5. Detection of the proliferation, migration, and invasion ability of breast cancer cells. A. Growth curves of MCF-7 transfected by Tim3-siRNA and NC; B. Growth curves of MDA-MB-231 transfected by ADV-Tim3 and Control; C-F. Detection of the migration ability by wound healing test. G-J. Detection of the invasion ability by Transwell assay. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

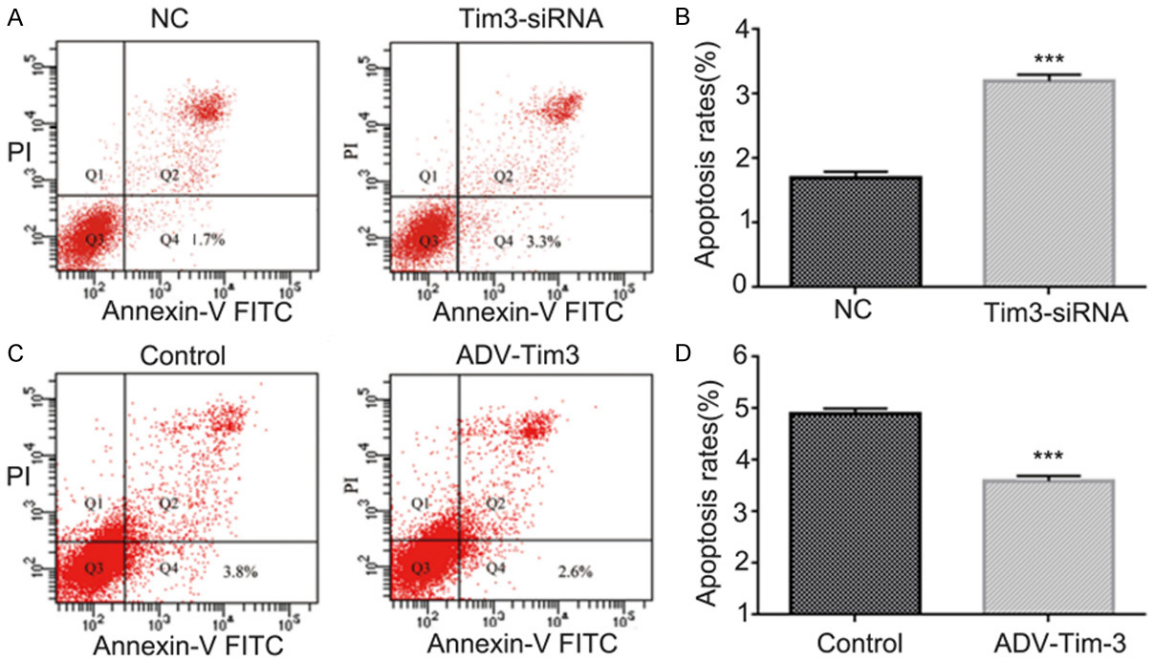

Figure 6. Detection of the apoptosis rates by Annexin V/FITC. A, B. NC group vs Tim3-siRNA group; C, D. Control group vs ADV- Tim3 group. ***P<0.001.

(P<0.05) and the migration distance of cells in ADV-Tim3 group was significantly greater than that of the control group (P<0.05) (**Figure 5C**, **5D**). As shown in **Figure 5E**, **5F**, the number of cells in the Tim3-siRNA group across the membrane was significantly lower than NC group whereas the number of cells in ADV-Tim3 group through the membrane was higher than that control group (P<0.05). These results indicate that blocking Tim-3 can inhibit the proliferation, migration, and invasion of breast cancer cells and that upregulation of Tim-3 can enhance the cell proliferation, migration, and invasion abilities.

Annexin V/FITC staining results showed that although the apoptosis rate is not high, the early apoptosis rate in Tim-3-siRNA group was significantly higher than that of NC group (3.27±0.16 vs 1.7±0.05, P<0.05) (**Figure 6A**, **6B**) and the apoptosis rate in ADV-Tim-3 was significantly lower than that of control group (2.60±0.10 vs 3.90±0.15, P<0.05) (**Figure 6C**, **6D**). These results suggest that Tim-3 can inhibit apoptosis of breast cancer cells.

Discussion

In recent years, Tim-3 has been given more and more attention in tumor immunity as an important immune regulatory molecule. After binding of Tim-3 with ligand Gal-9, it has been reported to promote the induction of T-cells and induce apoptosis of TIM-3-expressing cells *in vitro* and *in vivo* [18]. A higher level of TIM-3 might lead to an elevated level of CD80 expression on cells resulting in CD80 preferentially interacting with the inhibitory molecule CTLA-4 (cytotoxic T lymphocyte-associated antigen-4) and causing local immunosuppression [19]. Huang et al. found that TIM-3 was also expressed on B cell lymphoma on endothelial cells by inhibiting $CD4^+$T-cell activation and polarization of Th1 cells to promote the development of lymphoma, growth, and metastasis [20]. A recent study has revealed that TIM-3 is highly upregulated on both $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes from human lung cancer tissues which reveals a new role for TIM-3 as an important immune regulator in tumor microenvironment via its predominant expression in regulatory T-cells [21]. Tim-3 facilitates osteosarcoma proliferation and metastasis through the NF-κB pathway and epithelial-mesenchymal transition [22]. Blockade of the Tim-3 signaling pathway could increase not only the virus-specific $CD8^+$ T-cell response in patients with chronic HBV infection [23] but also the functionality of tumor infiltrating Tim-3^+ T-cells in HBV-related HCC [24]. It has been demonstrated that T-cell exhaustion could promote tumorigenesis and

tumor progression in various cancers [25]. These results suggest that TIM-3 might affect tumor progression through multiple pathways.

However, the relationship between Tim-3 and breast cancer has been poorly reported. In our preliminary work, the study indicated that the rs1036199 (T>G) SNP mapped to exon 3 (at position +4259) of the TIM-3 gene was the most closely associated with lymph node- or distant-metastasis of invasive breast cancer and may be a risk factor for progression and prognosis of breast cancer in Han population of northern china. In our present study, we found that Tim-3 was preferentially expressed in breast cancer tissues instead of adjacent tissues and correlated with clinical stage, metastasis, KI67 labeling index, and shorter survival.

It's very interesting that we have also observed a correlation between Tim-3 expression and KI67 labeling index in breast cancer tissues. The proliferation rate of breast carcinoma cells has been recognized as a marker for both prognosis [26, 27] and tumor response to preoperative chemotherapy [28, 29]. The proliferation biomarker Ki67 is expressed in all phases of the cell cycle other than the G0 phase 30] and is considered to be a prognostic factor for breast cancer [31, 32]. The association between a high Ki67 labelling index, poor differentiation of tumors, and large tumor size in breast carcinoma have been demonstrated in many studies [31, 33]. Studies have shown correlation between Ki67 and overall survival and disease-free survival, with an increased risk of recurrence in patients with a high Ki67 [34, 35]. Thus, expression of Tim-3 in breast cancer may be related to tumor proliferation and progression. This discovery prompted us to further investigate expression of Tim-3 in human breast cancer cells and its effects on proliferation, migration, invasion, and apoptosis.

Although the expression level is different, our research confirms the presence of Tim-3 protein in three breast cancer cell lines (MCF-7/ADM, MCF-7, and MDA-MB-231). In order to explore the link between Tim-3 and tumor progression, we used breast cancer cells transfected by Tim3-siRNA and ADV-Tim3 to do CCK assay, a wound healing assay, and Transwell invasion assay. These results strongly suggest that downregulating expression of Tim-3 inhibits the proliferation, migration, and invasion of breast cancer cells significantly and that upregulation of Tim-3 can enhance the cell proliferation, migration, and invasion. The results are consistent with other studies in NSCLC [10], cervical cancer [13], and prostate cancer [36]. By apoptosis assay, we also found that downregulating expression of Tim-3 can enhance the early apoptosis rate of breast cancer cells and upregulation of Tim-3 can decrease the early apoptosis rate, suggesting that Tim-3 could inhibit apoptosis of breast cancer cells. These results suggest that Tim-3 may have an important role in recurrence, metastasis, and prognosis of breast cancer. In addition, we found that inhibition of Tim-3 protein expression can prevent tumor progression. Thus, Tim-3 may represent a valid therapeutic target.

Taken together, we have associated for the first time, expression of Tim-3 in tumor cells with worse clinical pathological parameters in breast cancer. Tim-3 can be expressed on breast cancer cells and promote proliferation, migration, and invasion of breast cancer cells and inhibit apoptosis. In addition, we found that inhibition of Tim-3 protein expression can prevent tumor metastasis. Therefore, this provides new insights into the mechanisms underlying breast cancer and Tim-3 may be an effective novel target in breast cancer prevention and treatment.

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Disclosure of conflict of interest

None.

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