

# NTN4 is associated with breast cancer metastasis via regulation of EMT-related biomarkers

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**Abstract.** Metastasis is the leading cause of death for breast cancer patients. Nerve guidance factor 4 (Netrin-4, NTN4) is reduced in variety of malignancies and involved in tumor metastasis. However, the functions of NTN4 and related molecular mechanisms in breast cancer are poorly understood. Oncomine data revealed that NTN4 was decreased in breast cancer compared with normal breast tissues. For fresh frozen breast cancer samples, significantly depressed expression of NTN4 mRNA was observed in lesion tissues compared with that in adjacent tissues. Afterwards, NTN4 protein level was evaluated in 52 paired breast cancer tissues, and the results were consistent with that in fresh frozen samples. NTN4 expression was upregulated using NTN4-pcDNA3.1 plasmid in MDA-MB-231 cells and silenced using NTN4 small interfering RNA (siRNA) in Hs578T cells. Then the effects of NTN4 overexpression or knockdown on breast cancer cell migration and invasion were investigated. The results manifested that NTN4 overexpression attenuated cell migration and invasion, and induced N-cadherin and vimentin downregulation, while NTN4 siRNA-transfected cells had a significant increase in migration and invasion, as well as upregulation in N-cadherin and vimentin expression. These results demonstrate that NTN4 is reduced in breast cancer tissues and NTN4 is associated with breast cancer cell migration and invasion via regulation of epithelial mesenchymal transition (EMT)-related biomarkers.

## Introduction

Breast cancer is the most frequently diagnosed malignant tumor for female patients, which seriously threatens the health

of women. It accounts for 25% of all cancer cases and 15% of all cancer deaths among women, with an estimated 1.7 million cases and 521.9 thousand deaths in 2012 (1). Although comprehensive treatments based on surgical operation have certain effects on breast cancer, the majority of patients still inevitably die of tumor recurrence and metastasis. The estimated number of breast cancer deaths in 2010 in China was approximately 55,500 (2). According to the statistics by the Ministry of Health in 2011, approximately 37,000 people die of breast cancer recurrence and metastasis in mainland China each year (data from 145 population-based cancer registries).

Nerve guidance factors (netrins) are the earliest discovered soluble nerve guidance factor family, including three secreted proteins (netrin-1, 3 and 4) and two anchored membrane proteins (netrin-G1 and G2). Their secondary structures are similar, all consist of an amino-terminal signal sequence, a laminin-type globular domain, three laminin-type epidermal growth factor repeats, and a carboxyl-terminal that is enriched in amino acids (3).

NTN4, the new member of netrin family, is a kind of secreted protein. Several studies have indicated that NTN4 is widely detected in non-nervous systems and plays a vital role in tissue morphogenesis, angiogenesis, apoptosis, tumorigenesis, cell migration and invasion (4-9). Besides, emerging evidence have testified that NTN4 expression is decreased in variety of malignancies, including breast, pancreatic and colon cancers (10-13). *In vitro* studies exhibited that NTN4 was involved in the development of multiple types of cancers by inhibiting proliferation in a concentration-dependent manner (11,13-15). Consequently, NTN4 is considered as a tumor suppressor. Nevertheless, NTN4 promoted tumor cell proliferation at relatively low concentrations (13,16,17). *In vivo* studies demonstrated that NTN4 overexpression induced inhibitory effect in metastasis and recurrence of colorectal cancer (12), while metastasis was elevated in NTN4-overexpressing breast cancer models (18). Moreover, NTN4 is upregulated in the effusions compared with corresponding solid tumors (19), suggesting that NTN4 may be involved in tumor metastasis. However, the functions and molecular mechanisms of NTN4 in breast cancer have not been investigated thoroughly. In the present study, we firstly investigated the effects of NTN4 on metastasis of breast cancer and analyzed the underlying molecular mechanisms.

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**Key words:** nerve guidance factor 4, breast cancer, migration, invasion, epithelial mesenchymal transition

## Materials and methods

**Patient samples and tissue microarrays.** Breast cancer fresh frozen tissues were obtained from the Pathology Department of Shaoxing People's Hospital (Shaoxing, China). Briefly, samples were from 14 breast cancer patients at initial diagnosis for breast cancer, and were rapidly frozen using liquid nitrogen. The use of all samples for this study was approved by the Ethics Committee of the hospital and informed consents were obtained from all patients. Tissue microarrays were purchased from Alenabio Biotechnology, Co., Ltd. (Xi'an, China).

**Immunohistochemical staining.** Immunohistochemical (IHC) staining was performed using Polink-2 Plus<sup>®</sup> Polymer horseradish peroxidase (HRP) detection system (ZSGB-BIO, Beijing, China). The tissue slides were heated at 65°C for depa-  
raffination, endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide for 10 min after antigen retrieval in a citrate buffer (pH 6.0) heated to 121°C for 90 sec. Then, slides were washed in phosphate-buffered saline (PBS) and incubated with anti-NTN4 antibody (goat anti-human, diluted 1:1800, AF1254; R&D Systems, Minneapolis, MN, USA) at 37°C for 1.5 h. Then, the tissue sections were incubated with polymer adjuvant and anti-goat IgG polymer labeled with HRP both for 15 min. The staining was visualized using 3,3'-diaminobenzidine (DAB) substrate-chromogen (ZLI-9017; ZSGB-BIO) according to the manufacturer's instructions and was counterstained with hematoxylin. All sections were evaluated by two pathologists who were unaware of the study contents. Three randomly selected views were observed per case and 100 cells were observed per view at x400 magnification. NTN4 expression was divided into four groups based on staining range: we consider <10% as -, 10-24% as +, 25-49% as ++, and ≥50% as +++.

**Cell culture and reagents.** Human SK-BR-3, T-47D, BT-474, MCF-7, MDA-MB-231 and Hs578T cell lines were obtained from the Cell Bank of Chinese Academy of Medical Science (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, SH30243.01; HyClone Laboratories, Logan, UT, USA) with 10% fetal bovine serum (FBS, 10099-141; Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (15140122; Life Technologies) at 37°C in a moist environment containing 5% CO<sub>2</sub>. Cells were maintained in sterilized culture dishes and passaged every 3 days with 0.25% trypsin (SH30042.01; HyClone Laboratories).

**Quantification of NTN4 in cell supernatant by ELISA.** NTN4 concentration of cell supernatant was measured using an enzyme linked immunosorbent assay (ELISA) kit (CSB-E11900h; CusaBio, Wuhan, China). Diluted cell supernatant and standard samples (100 µl) were added to 96-well plates and incubated at 37°C for 2 h, then biotin-conjugated antibody against NTN4 was added and incubated at 37°C for 1 h. After incubated with avidin-conjugated HRP for 1 h, 3,3',5,5'-tetramethylbenzidine (TMB) substrate reaction was performed and was stopped by adding stop buffer. Absorbance was measured at a wavelength of 450 nm with plate reader (Anthos Fluido 2010).

**Construction and transfection of NTN4 expressive plasmid.** The human NTN4 cDNA amplified by PCR (the primers: F, 5'-ATACTCGAGACCATGGGGAGCTGCGCGCGGCTG-3' and R, 5'-CACGGATCCCTACTTGCCTCTCTTTTAAATATCC-3') was cloned into the pcDNA3.1 vector and the sequence of recombinant plasmid was confirmed by Platinum Biological Technology, Co., Ltd. (Shanghai, China). The cells were seeded in 6-well plates and then transfected with the recombinant plasmid and negative control plasmid with Lipofectamine 2000 (11668-019; Invitrogen) according to the manufacturer's instructions.

**Transfection of siRNA.** Cells were seeded in 6-well plates and transfected with siRNA using Lipofectamine 2000 (11668-019; Invitrogen) according to the manufacturer's instructions. The sequences of siRNAs are as follows: NTN4 siRNA-1: 5'-GGCGCUAUUUGUACUUCUAdTdT-3'; NTN4 siRNA-2: 5'-GUCCAUGGGAAGUGUAUGUdTdT-3'; NTN4 siRNA-3: 5'-CAGGCAAACUAAUUGUGAAdTdT-3'. The siRNAs were chemically synthesized by Biotend Bio-Technique, Co., Ltd. (Shanghai, China).

**Quantification of NTN4 and EMT-related biomarkers by RT-qPCR.** Total RNA was extracted from breast cancer tissues and cells using TRIzol (113702; Life Technologies). RT-PCR was performed with the First-Strand cDNA Synthesis kit (RT0212-03; Biomiga, San Diego, CA, USA) and the cDNA was amplified by SYBR-Green PCR Master Mix (RT0411-02; Biomiga) according to the manufacturer's instructions in the LightCycler 480 PCR apparatus (Hoffman-La Roche Ltd., Basel, Switzerland). PCR reactions were performed under the following conditions: 95°C for 10 min, 35 cycles of 95°C for 15 sec, and 60°C for 1 min. Levels of gene expression relative to β-actin were evaluated by the 2<sup>-ΔΔCT</sup> method. PCR primers were used as follows: NTN4 F, 5'-GTACTTTGCGACTAACTGCTCC-3' and R, 5'-TCCAGTGCATGGAAAAGGACT-3'; N-cadherin F, 5'-CTGCTTCAGGCGTCTGTAGA-3' and R, 5'-AGGCTCACTGCTCTCATATTGT-3'; vimentin F, 5'-ATCTGGATTCACTCCCTCTG-3' and R, 5'-AAGGTCATCGTGATGCTGAG-3'; β-actin F, 5'-ACCCACACTGTGCCCATCTAC-3' and R, 5'-TCGGTGAGGATCTCATGAGGTA-3'.

**Wound healing assay.** Transfected cells were seeded into 12-well plates, when the monolayer confluence was 90-95%, cells were wounded using a sterile 200 µl pipette tip and washed using sterile PBS 2 times. Then, fresh medium with 0.5% FBS was added to the 12-well plates and images were taken at 0-48 h using microscope (Leica DMI3000B). The gap sizes were analyzed with the ImageJ program and measured as the percentage of the original time-point (0 h).

**Transwell migration and Matrigel invasion assay.** The invasion was evaluated in Transwell chambers coated with 50 µl Matrigel (356234; BD Biosciences, San Jose, USA; Leica DMI3000B) in serum-free DMEM at a dilution of 1:5, the upper and lower chambers were separated by polycarbonate membranes with 8-µm pore size (3422; CoStar Group, Inc., Cambridge, MA, USA). For the migration assay, uncoated Transwell chambers were used. The lower chambers contained 600 µl of DMEM with 10% FBS as a chemo-attractant.

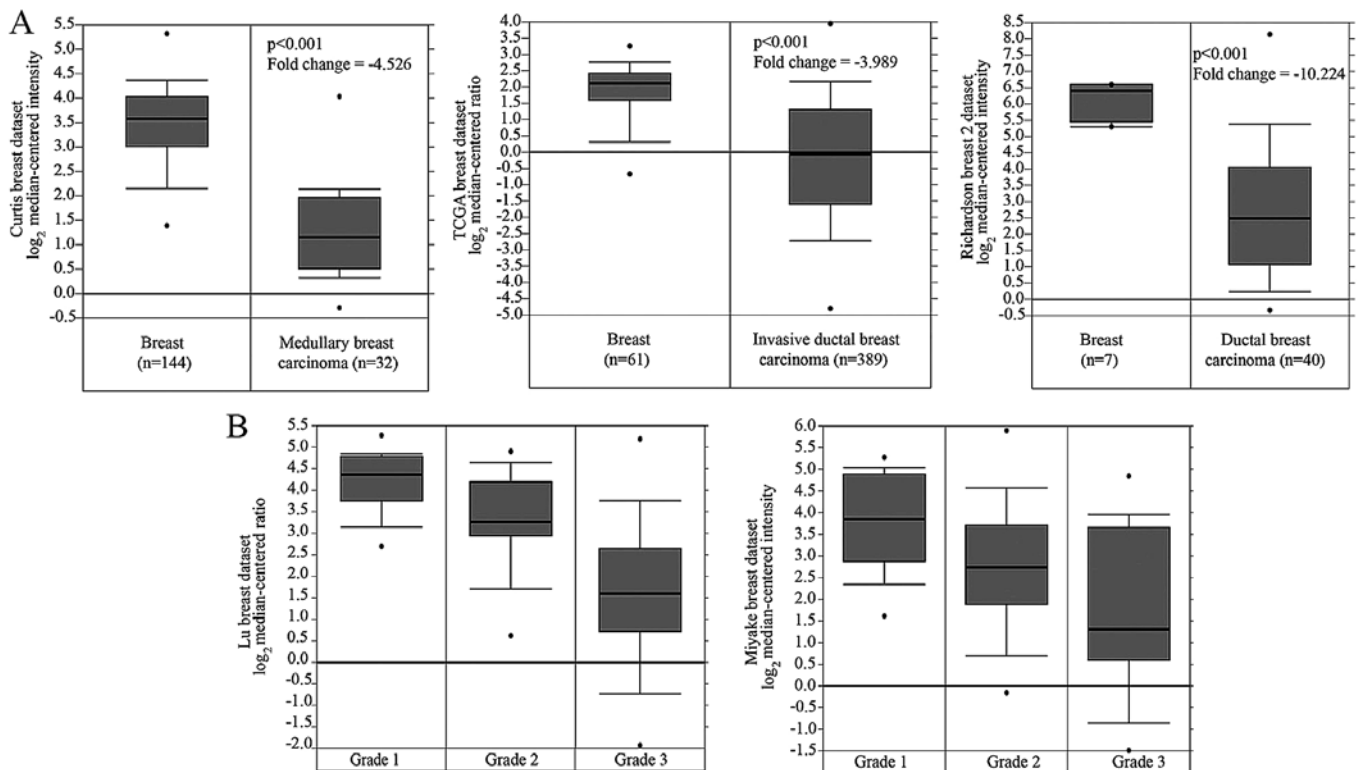


Figure 1. Expression of NTN4 in breast cancer analyzed by Oncomine data. (A) Expression of NTN4 in cancer tissues was markedly downregulated compared with normal breast tissues. (B) Expression of NTN4 in different grades of breast cancer.

Transfected cells ( $6 \times 10^4$ ) were seeded in the upper chambers with serum-free DMEM containing 0.1% albumin from bovine serum (BSA). After 24 h (migration assay) or 48 h (invasion assay) of cultivation, cells on the upper surface of the membranes were removed with a cotton swab and cells migrated or invaded to the bottom surface were fixed with 95% alcohol, stained with 1% crystal violet, and counted in five random fields at  $\times 400$  magnification.

**Western blotting.** Cells transfected with recombination plasmids or siRNAs for 48 h were washed by 4°C PBS, lysed with cell lysis buffer (P0013B; Beyotime Institute of Biotechnology, Jiangsu, China) with inhibitors of proteases (Po100; Solarbio, Beijing, China), and protein concentrations were quantified with an Enhanced BCA protein assay kit (Beyotime Institute of Biotechnology). Protein electrophoresis was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (P1200; Solarbio) and immunoblotting was performed on polyvinylidene fluoride (PVDF) membranes (IPVH00010; Millipore, Billerica, MA, USA). After non-specific antigen blocking with 5% non-fat milk, the PVDF membranes were incubated at 4°C overnight with the following antibodies:  $\beta$ -actin (mouse anti-human, diluted 1:5000, BS6007M; Bioword Technology, Bloomington, MN, USA), N-cadherin (rabbit anti-human, diluted 1:2000, 22018-1-AP; Proteintech Group Inc., Chicago, IL, USA) and vimentin (rabbit anti-human, diluted 1:1000, 10366-1-AP; Proteintech Group Inc.). After 3 washes with Tris-buffered saline with tween (TBST), the PVDF membranes were incubated with secondary antibodies

conjugated with HRP [diluted 1:5000, BS13278 (BS12478); Bioword Technology] and were visualized using ECL-plus (32106; Thermo Fisher Scientific, Grand Island, NY, USA). Then the blots were imaged with Tanon 5200 chemiluminescence imaging system.

**Statistical analysis.** All data were expressed as mean  $\pm$  standard deviation and SPSS 20.0 was used for statistical analyses. The independent-samples two-tailed Student's t-test and Mann-Whitney U test were used to analyze the significance between the groups. For all the results, three levels of significance ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ ) were used.

## Results

**NTN4 expression is decreased in breast cancer.** Through application of RNA sequencing (RNA-Seq) technology and bioinformatic analyses for 5 paired breast cancer and adjacent tissues, we achieved 871 differential genes. Then we found that NTN4 expression is markedly varied in different stages by combining differential genes with clinicopathologic data (data not shown). In addition, Oncomine data analyses showed that NTN4 expression in breast cancer was reduced compared with normal breast tissues (Fig. 1A), and NTN4 expression in grade 2 and grade 3 was significantly lower than grade 1 (Fig. 1B).

Then, we detected NTN4 mRNA expression in 14 paired fresh frozen breast cancer tissues, and the results manifested that NTN4 expression was significantly decreased in majority of breast cancer lesions compared with adjacent tissues (Fig. 2A). To further verify the results, NTN4 protein expression in 52

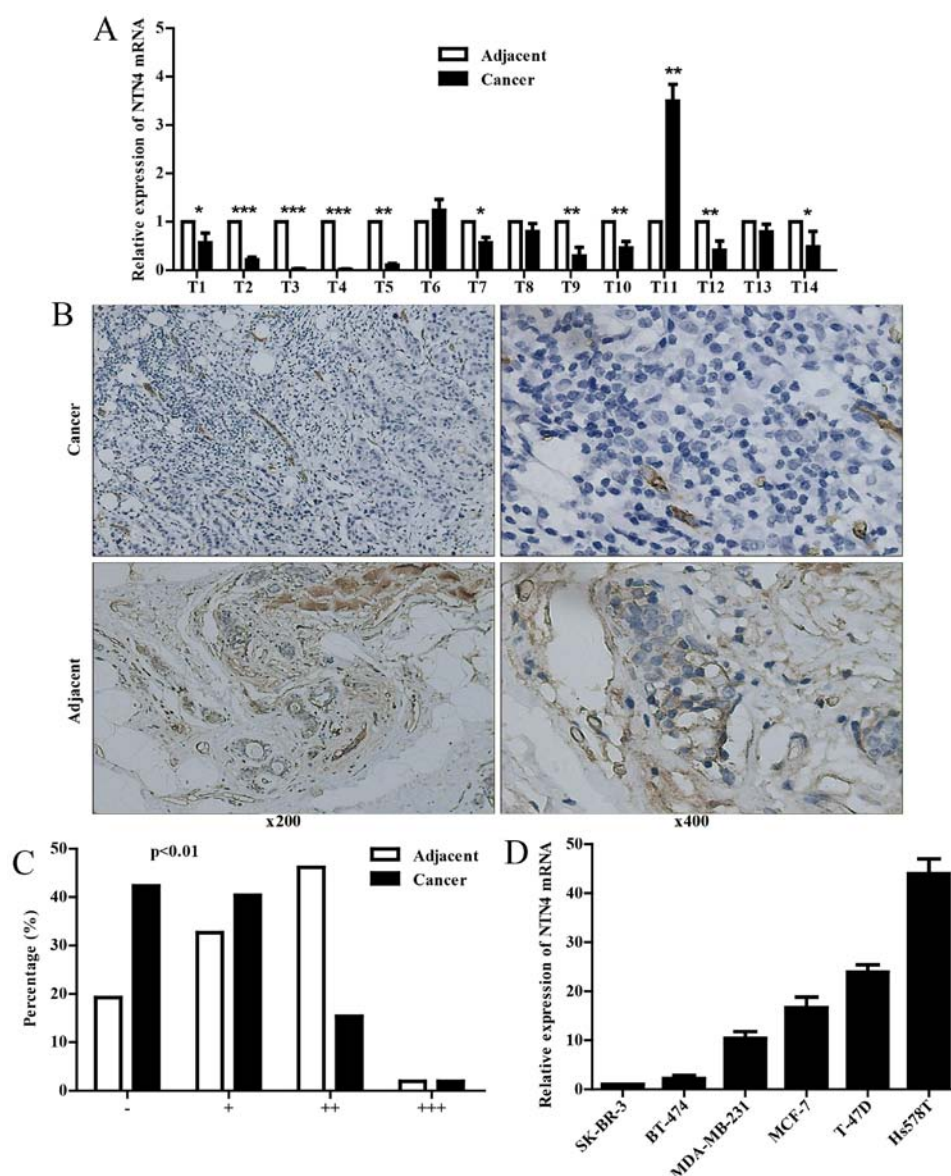


Figure 2. NTN4 is decreased in breast cancer compared with adjacent tissues. (A) NTN4 mRNA expression was significantly lower in breast cancer lesions than in adjacent tissues. (B) IHC staining of NTN4 in breast cancer tissues. Weak staining is observed in the breast cancer lesions (upper panel) compared with the strong staining in adjacent tissues (lower panel). (C) Mann-Whitney U test of IHC results in breast cancer and adjacent tissues (<10% as -, 10-24% as +, 25-49% as ++, ≥50% as +++, n=52; P<0.01). (D) NTN4 mRNA expression in 6 breast cancer cell lines. Mean ± SD, n≥3. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

Table I. The correlation between NTN4 expression and breast cancer clinicopathological features.

Clinicopathological features	N	P-value
Age (years)		
<50	36	0.249
≥50	16	
TNM stage		
I+II	39	0.736
III	13	
Node metastasis		
Positive	24	0.138
Negative	28	

NTN4, nerve guidance factor; TNM, tumor node metastasis; N, number.

paired paraffin sections (Table I) was measured by IHC, and the difference between lesions and adjacent tissues (Fig. 2B and C and Table II) was consistent with that in fresh frozen tissues. Thus, we speculated that NTN4 may be closely associated with breast cancer metastasis.

Next, we tested the NTN4 mRNA expression in six breast cancer cell lines. As the results showed, NTN4 expression in Hs578T cells was significantly higher than other cell lines (Fig. 2D). Based on the characteristic and the NTN4 expression of breast cancer cell lines (20), we selected the highly metastatic MDA-MB-231 cells with relatively low NTN4 expression and moderately metastatic Hs578T cells with high NTN4 expression to conduct *in vitro* experiments.

*NTN4 overexpression inhibits cell migration and invasion in MDA-MB-231 cells.* To explore the role of NTN4 in breast cancer metastasis, the migration and invasion abilities of

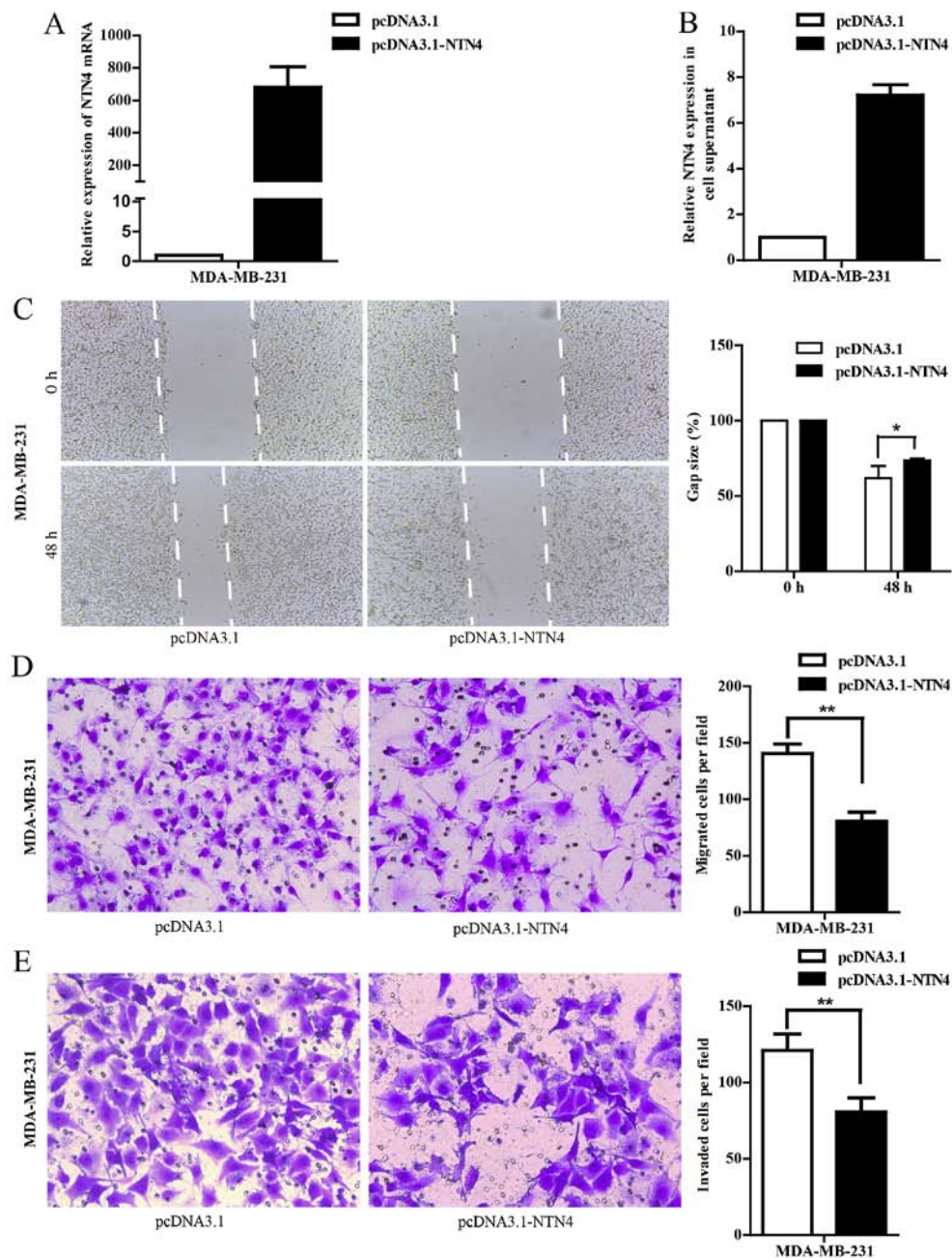


Figure 3. Overexpression of NTN4 inhibits the migration and invasion of MDA-MB-231 cells. (A and B) The overexpression of NTN4 in MDA-MB-231 cells by transient transfection of pcDNA3.1-NTN4 and pcDNA3.1 was confirmed with RT-qPCR (A) and ELISA (B) after transfection for 48 h. (C) NTN4 overexpression reduced the wound healing rate of MDA-MB-231 cells. (D and E) Overexpression of NTN4 inhibited the migration and invasion of MDA-MB-231 cells. Cells transfected with pcDNA3.1 or pcDNA3.1-NTN4 were subjected to the migration and invasion assays, and the migrated and invaded cells were fixed at 24 and 48 h, respectively. Magnification,  $\times 400$ . Mean  $\pm$  SD,  $n \geq 3$ . \* $P < 0.05$ , \*\* $P < 0.01$ .

Table II. NTN4 expression in cancer and adjacent tissues.

Results	Adjacent (n)	Cancer (n)	Total
-	10	22	32
+	17	21	38
++	24	8	32
+++	1	1	2
Total	52	52	104

NTN4, nerve guidance factor; n, number.

MDA-MB-231 cells were investigated. NTN4 overexpression was achieved by transiently transfecting with pcDNA3.1-NTN4. NTN4 overexpression at the mRNA (Fig. 3A) and protein level (Fig. 3B) were confirmed by RT-qPCR and ELISA, respectively. The wound healing assay demonstrated that the migration rate of cells with pcDNA3.1-NTN4 transfection was significantly reduced at 48 h compared with control groups (Fig. 3C). The results from the Transwell migration and the Matrigel invasion assays showed that the cells in the pcDNA3.1-NTN4 groups had a significant decrease in migration and invasion activity compared with control cells (Fig. 3D and E). These results indicate that



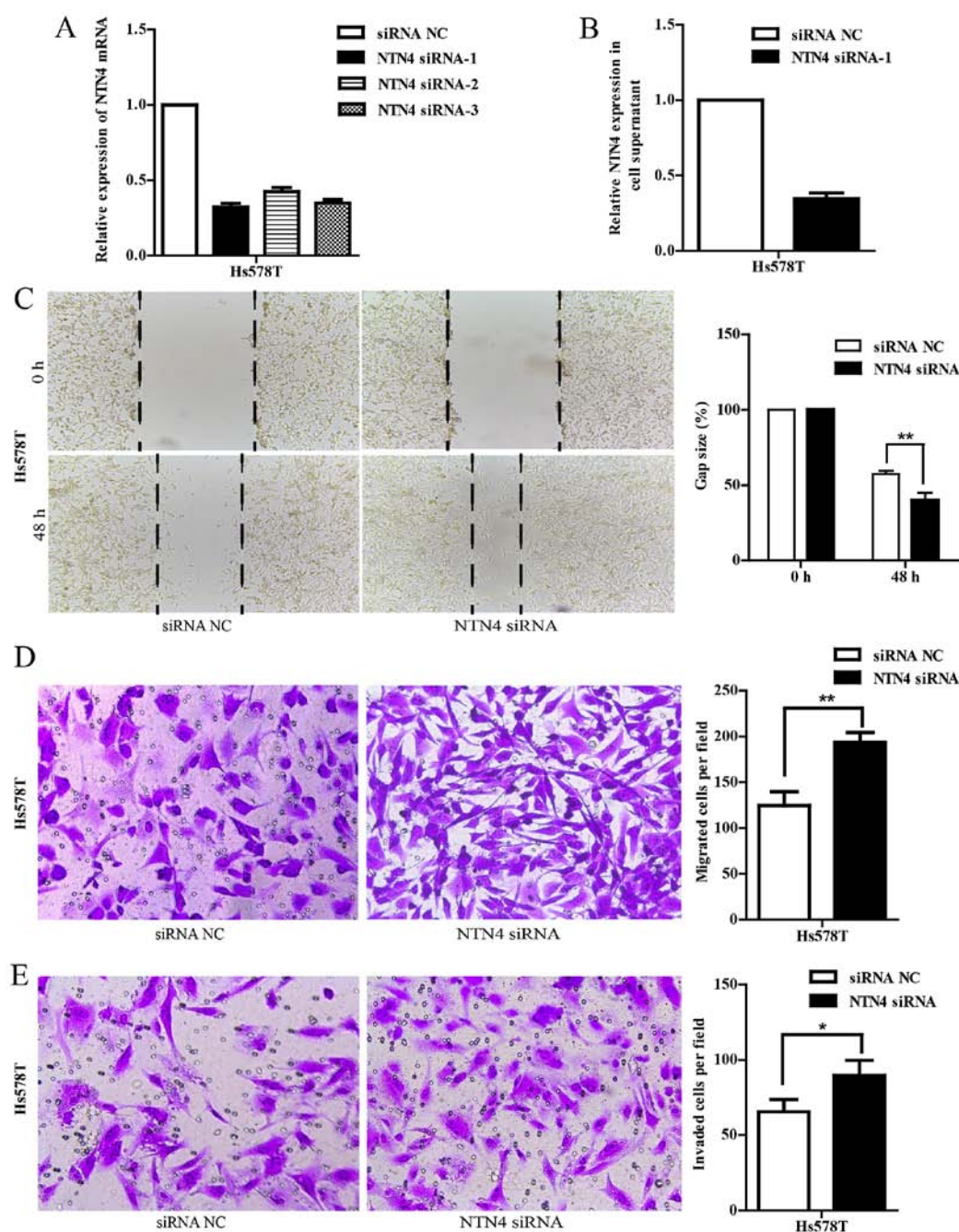


Figure 4. Silencing of NTN4 promotes migration and invasion of Hs578T cells. (A) qRT-PCR and (B) ELISA revealed that siRNA knockdown of NTN4 reduced mRNA and protein levels of NTN4 in Hs578T cells after transfection for 48 h. (C) NTN4 knockdown accelerated the wound healing rate in Hs578T cells. (D and E) NTN4 silencing enhanced cell migration and invasion activity. Cells transfected with siRNA NC or NTN4 siRNA were subjected to the migration and invasion assays, and the migrated and invaded cells were fixed at 24 and 48 h, respectively. Magnification, x400. Mean  $\pm$  SD,  $n \geq 3$ . \* $P < 0.05$ , \*\* $P < 0.01$ .

NTN4 overexpression inhibits cell migration and invasion of breast cancer cells.

*NTN4 silencing promotes cell migration and invasion in Hs578T cells.* To further validate the function of NTN4, we knocked down NTN4 expression in Hs578T cells using NTN4-specific siRNA targeting 5'-GGCGCTATT TGTACTTCTA-3' (NTN4 siRNA-1). As shown in Fig. 4A and B, NTN4 expression was efficiently silenced in Hs578T cells at both mRNA and protein levels compared with the controls. We also evaluated whether NTN4 knockdown could influence cell migration and invasion. Wound healing assay showed that siRNA-mediated

silencing of NTN4 significantly promoted migration at 48 h, as shown by an accelerated wound closure (Fig. 4C). Furthermore, the Transwell migration results also determined that NTN4 siRNA-transfected cells had a significant increase of migrated cells compared with control scramble RNA transfected cells (Fig. 4D). Similar results of invasion assay were obtained (Fig. 4E). These findings demonstrate that NTN4 silencing promotes the migration and invasion of breast cancer cells.

*NTN4 upregulation or downregulation influences expression of EMT-related biomarkers.* Gene-set enrichment analysis demonstrated that the downregulated genes, NTN4 included,

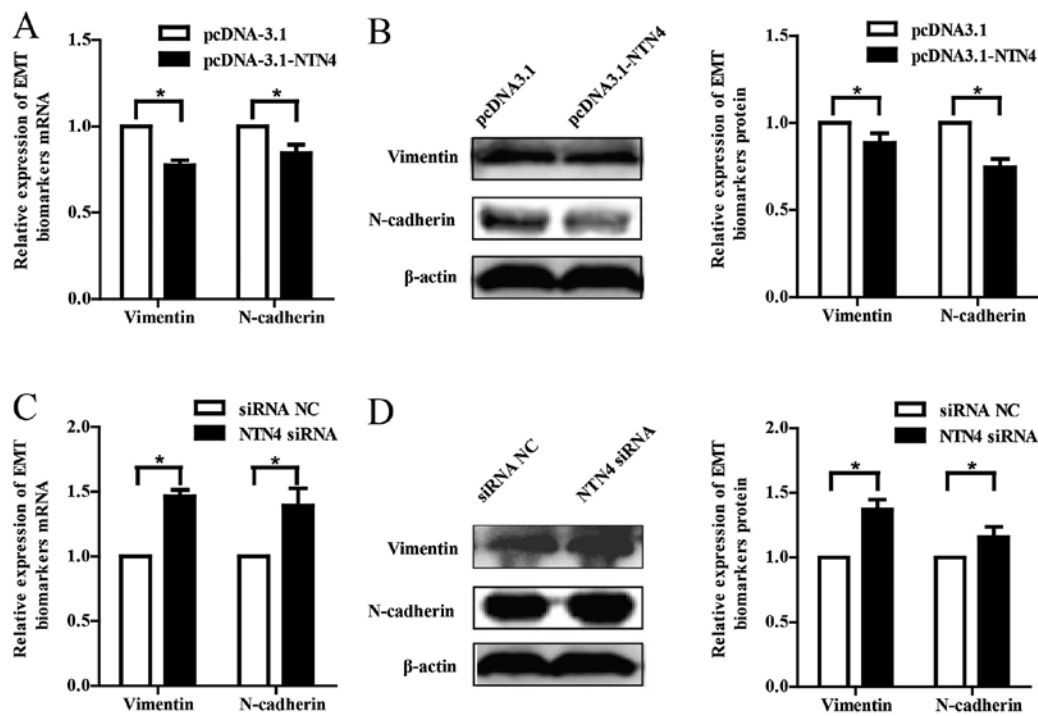


Figure 5. Effects of NTN4 on EMT-related biomarkers (vimentin and N-cadherin). (A and B) NTN4 overexpression downregulated the mRNA and protein expression of vimentin and N-cadherin in MDA-MB-231 cells. (C and D) NTN4 knockdown upregulated the mRNA and protein expression of vimentin and N-cadherin in Hs578T cells. Mean  $\pm$  SD,  $n \geq 3$ . \* $P < 0.05$ .

participated in the focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK) and transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling pathways (data not shown). In addition, recent studies have reported that these signaling pathways are involved in EMT and tumor metastasis (21-24).

To obtain insight into the molecular mechanisms of NTN4 on breast cancer cells metastasis, the expression of EMT-related biomarkers (vimentin and N-cadherin) in mRNA and protein levels were examined. We found that vimentin and N-cadherin were downregulated at both mRNA and protein levels in MDA-MB-231 cells with transfection of NTN4 expressive plasmid (Fig. 5A and B). Conversely, NTN4 silencing increased the expression of vimentin and N-cadherin in Hs578T cells (Fig. 5C and D). These results manifest that NTN4 overexpression or silencing is associated with breast cancer migration and invasion via regulation of EMT-related biomarkers.

## Discussion

Metastasis is the major cause of deaths for breast cancer patients. It is necessary to discover effective factors to hinder the metastasis of breast cancer. NTN4, a nerve guidance factor, exerts an influence on physiological functions such as neurocyte growth and migration through binding to its various receptors (25,26). Recent studies have revealed that NTN4 is involved in tumor proliferation, as well as metastasis (12,18).

In the present study, we found that NTN4 was decreased in breast cancer lesion tissues compared with matched adjacent tissues, which was in accordance with the results observed by others (10). In addition, previous studies have

demonstrated that NTN4 inhibited tumor growth and angiogenesis (11,12,27), and promoted tumor cell proliferation at relatively low concentrations (13,16,17). Similarly, we found that NTN4 overexpression reduced the migration and invasion activity in MDA-MB-231 cells, in contrast, NTN4 silencing enhanced migration and invasion in Hs578T cells. Therefore, we conclude that NTN4 is associated with breast cancer metastasis. However, a study in gastric cancer demonstrated that NTN4 overexpression accelerated cell proliferation and invasion, and NTN4 knockdown had the opposite effects. Investigations have manifested that high concentration NTN4 inhibited proliferation and migration through combining with Unc5B, while low concentration NTN4 promoted proliferation and migration through binding to integrin  $\beta 4$ , that is why NTN4 exerted different functions on tumor proliferation and metastasis (17). Hence, NTN4 may have diverse functions at different concentrations in different tumors.

Besides, studies have reported that NTN4 knockout suppressed proliferation and motility through reducing the phosphorylation of signal transducer and activator of transcription 3 (Stat3), extracellular regulated protein kinases (ERK), Akt and p38, and inhibited invasion through decreasing matrix metalloproteinase 2 (MMP2) expression and increasing tissue inhibitor of metalloproteinase 1 (TIMP1) expression in gastric cancer cells, while overexpression or adding exogenous NTN4 can reverse the effects (8). In addition, proliferation and migration was hindered with decreased expression of p-Akt-1, p-Jnk-2 and p-c-Jun when pancreatic cancer cells were treated with NTN4 (13). However, the molecular mechanisms involved in NTN4-mediated metastasis in breast cancer are less understood.

NTN4 participates in the FAK, MAPK and TGF- $\beta$  signaling pathways. Moreover, these signaling pathways are involved in EMT and tumor metastasis (21-24). FAK activation led to numerous cell processes including cell adhesion, migration, invasion and proliferation (28), and activation of TGF- $\beta$  promoted the occurrence of EMT (29). EMT is a major mechanism to explain metastatic events in breast cancer. During EMT, epithelial cells display reduced expression of epithelial markers (E-cadherin) and enhance mesenchymal traits (upregulation of vimentin and N-cadherin). Expression of N-cadherin and vimentin was significantly higher in metastases than in the related primary breast tumors (30-32). In order to further understand the molecular mechanisms that NTN4 influences the migration and invasion in breast cancer cells, we investigated the effects of NTN4 on vimentin and N-cadherin expression. In the study, NTN4 overexpression reduced the expression of vimentin and N-cadherin, while NTN4 knockdown facilitated vimentin and N-cadherin expression. Studies have demonstrated that expression of N-cadherin would confer on breast cancer cells the capacity to invade (33,34), and elevated vimentin expression was correlated with increased migration and invasion of breast cancer cells (35). Given that upregulation of N-cadherin and vimentin are mesenchymal traits during EMT, we consider that NTN4 is associated with breast cancer migration and invasion via regulation of EMT-related biomarkers.

In conclusion, this study demonstrates that NTN4 is decreased in breast cancer tissues and that NTN4 is associated with breast cancer migration and invasion via regulation of EMT-related biomarkers.

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