TIPE1 suppresses the invasion and migration of breast cancer cells and inhibits epithelial-to-mesenchymal transition primarily via the ERK signaling pathway

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Abstract

Tumor necrosis factor α-induced protein 8-like-1 (TIPE1) functions as an activator or a repressor in a tumor cell type-specific manner. However, the role of TIPE1 in breast cancer, especially regarding metastasis, is unknown. In this study, we aimed to investigate the TIPE1 expression in breast cancer tissues, the biological functions, and the underlying mechanisms of TIPE1 regarding the metastatic properties of breast cancer cells. The results of immunohistochemical staining and western blot analysis indicated that TIPE1 expression was associated with tumor size and lymph node metastasis, and the expression of TIPE1 was downregulated in the tissues of patients with lymph node metastasis. Transwell and wound healing assay results showed that TIPE1 inhibited the invasive and migratory capacities of breast cancer cells. Moreover, the epithelial-mesenchymal transition (EMT) was suppressed in TIPE1-overexpressing cells, as demonstrated by western blot analysis. In addition, western blot analysis also showed that TIPE1 reduced the expression levels of MMP2 and MMP9 and decreased the phosphorylation level of ERK. These results suggested that TIPE1 might suppress the invasion and migration of breast cancer cells and inhibit EMT primarily via the ERK signaling pathway. Our findings revealed the anti-tumor metastasis role of TIPE1 in breast cancer and TIPE1 might be a new candidate prognostic indicator and a potential molecular target for the treatment of breast cancer.

Key words: tumor necrosis factor α-induced protein 8-like-1 (TIPE1), breast cancer, metastasis, epithelial-to-mesenchymal transition, ERK

Introduction

Breast cancer is a malignant tumor and is known as the most common cause of cancer-related death in women [1]. Despite advances in treatment strategies involving surgery, radiotherapy, chemotherapy, endocrine therapy, and targeted therapy, many patients with breast cancer still exhibit metastasis of other organs and suffer from cancer-related death. The main reasons for therapeutic failure and mortality are cell infiltration and metastasis. Thus, exploring cancer metastasis-associated factors and genes is important for the treatment of cancer.

Epithelial-mesenchymal transition (EMT) plays a critical role in cancer metastasis by endowing tumor cells with phenotypic
variations and increased motility and invasiveness [2]. During the EMT process, epithelial cells could gain mesenchymal characteristics as well as the capacity of migration, invasion, and apoptotic resistance. EMT is characterized by the mutative expression of the epithelial marker E-cadherin, the mesenchymal marker Vimentin, and the transcription factors Snail, Twist, and Slug. Therefore, detecting EMT-related molecules is essential to further understand the cause of the malignant progression of breast cancer. In addition, matrix metalloproteinases (MMPs), especially MMP2 and MMP9, are reported to participate in EMT and function as a key pathway in tumor development and metastasis by increasing cell motility and degrading the extracellular matrix. Meanwhile, the ERK signaling pathway has been confirmed as one of the most important pathways upstream of MMP2 and MMP9, which promotes the tumor progression. Hence, identification of novel molecules regulating EMT, the expression of MMP2 and MMP9, and the ERK signaling pathway involved in the pathogenesis of breast cancer metastasis is still crucial for improving the new therapeutic approaches.

Tumor necrosis factor α-induced protein 8-like-1 (TIPE1) is a newly identified member of the TIPE family, which consists of the following four members: TNFAIP8, TIPE1, TIPE2, and TIPE3. TIPE family has been reported to be associated with cell proliferation, inflammation, and carcinogenesis. Recent studies have shown that TIPE1 has diverse functions and may act as an activator or a repressor in different cancer types. In hepatocellular carcinoma, lung cancer [3], gastric cancer [4], and osteosarcoma [5], TIPE1 functions as a negative regulator by negatively regulating Rac1 activation and reducing cell growth and metastasis. However, in cervical cancer, TIPE1 acts as an oncogene and facilitates cancer progression via binding to p53 [6]. Thus, the role of TIPE1 in cancer development is intriguing and elusive. However, whether TIPE1 is involved in the progression and metastasis of breast cancer remains unknown.

Therefore, in this study, we aimed to investigate the expression patterns of TIPE1 in breast cancer tissues (lymph node metastasis or non-lymph node metastasis) and explore its effects on cell invasion, migration, and EMT and at same time to explore the potential signaling molecules related to TIPE1.

Materials and Methods

Breast cancer tissue samples
This study was approved by the local ethics committee of the Zibo Central Hospital, Shandong Province. All patients signed an informed consent form prior to participation in the study. Breast cancer specimens from 102 patients who underwent surgical resection at the Zibo Central Hospital between 2017 and 2018 were collected. The samples were dehydrated through a graded alcohol series and embedded in paraffin. Then, tissue sections (4-μm thick) were prepared. The samples were stained with hematoxylin and eosin (Zhongshan Company, Beijing, China) and classified histologically against the criteria of the seventh edition of American Joint Committee on Cancer/International Union against Cancer Tumor Node Metastasis (TNM) classification system. A total of 30 moderately differentiated tumor tissues were selected for further study. The clinicopathological characteristics of the patients are summarized in Table 1.

Immunohistochemistry staining
Immunohistochemistry staining was performed according to the instructions of the Power Vision Histostain Streptavidin-Peroxidase kit (Zhongshan Company). The sections were deparaffinized in xylene and hydrated through a graded alcohol series. The activity of endogenous tissue peroxidase was blocked with 3% H2O2 (Zhongshan Company), followed by heat-induced epitope retrieval. After treatment with 1% bovine serum albumin (Zhongshan Company) for 20 min to block non-specific binding, the sections were incubated with anti-TIPE1 antibody (1:100 dilution; Santa Cruz Biotech, Santa Cruz, USA) at 4°C overnight. Biotinylated donkey anti-goat immunoglobulin G (IgG) (Zhongshan Company) was applied as secondary antibody to incubate the sections at a density of 105 cells per well. Then, the sections were exposed to streptavidin-peroxidase conjugate (Zhongshan Company) at 37°C for 25 min. Diaminobenzidine (DAB; Zhongshan Company) solution was added to visualize localization. Finally, the sections were lightly counterstained with hematoxylin and dehydrated through a graded alcohol series. Immunohistochemistry staining of TIPE1 was observed under a microscope (Olympus, Tokyo, Japan). The integrated optical density (IOD) of TIPE1 staining was quantitatively analyzed using Image-Pro Plus 6.0 software. TIPE1 expression was determined by randomly selecting five tumor cell areas of each specimen.

Cell lines and infection
The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the Chinese Academy of Sciences Committee Type Culture Collection Cell Bank (Shanghai, China). Cells were cultured at 37°C with 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, USA) containing 10% fetal bovine serum (FBS; Gibco). The TIPE1 and TIPE1 shRNA lentiviral vectors were constructed by Genechen Company (Shanghai, China). Cells were transfected with the TIPE1 or TIPE1 shRNA (TIPE1 shRNA: 5′-GCATCTG TAGCAGCTGTTT-3′; NC: 5′-TTCTCCGAA CGTGCAGT-3′) lentiviral vector according to the manufacturer’s protocols. Cells were seeded the day before transfection at 1 × 105 cells/well in 24-well plates. Briefly, lentivirus-based TIPE1 overexpression and silencing systems were employed to infect MCF-7 and MDA-MB-231 cells. Puromycin (2 μg/ml; Beyotime, Shanghai, China) was added to select stable TIPE1-overexpressing and TIPE1-silenced cells.

Transwell assay
Transwell analysis was performed as previously described [7]. Cells (TIPE1-overexpressing and control cells at a density of 1 × 105 cells per well; TIPE1-silenced and negative control cells at a density of 1 × 105 cells per well) in serum-free medium were added to the

<table>
<thead>
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<th>Characteristics</th>
<th>n</th>
<th>TIPE1 expression (IOD)</th>
<th>P</th>
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<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>12</td>
<td>0.15 ± 0.009</td>
<td>0.924</td>
</tr>
<tr>
<td>≥60</td>
<td>18</td>
<td>0.15 ± 0.005</td>
<td></td>
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<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt;1 cm</td>
<td>8</td>
<td>0.17 ± 0.007</td>
<td>0.005</td>
</tr>
<tr>
<td>≥1 cm</td>
<td>22</td>
<td>0.14 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Absent</td>
<td>15</td>
<td>0.17 ± 0.005</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Present</td>
<td>15</td>
<td>0.14 ± 0.006</td>
<td></td>
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Table 1. Association between TIPE1 expression and clinicopathological factors in patients with breast cancer
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Table 2. Sequence of primers used in RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Prime sequence (5′→3′)</th>
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<tr>
<td>E-cadherin</td>
<td>F: CTTAGAGGTAGGTCAGCGTGTGA</td>
</tr>
<tr>
<td></td>
<td>R: CGAAGAAACAGGAAGAGGAGGACGC</td>
</tr>
<tr>
<td>Vimentin</td>
<td>F: ATTGCGAGGAGGAGATTCTCA</td>
</tr>
<tr>
<td></td>
<td>R: TTTCCACTTTTGGCTCAAGGTCG</td>
</tr>
<tr>
<td>Snail</td>
<td>F: CCTTGTGGTCTGAGCGACCTGA</td>
</tr>
<tr>
<td></td>
<td>R: CACTGGTATCTTCTCACATCCGG</td>
</tr>
<tr>
<td>Slug</td>
<td>F: ACCCTGTGTTGCTCAAGGACA</td>
</tr>
<tr>
<td></td>
<td>R: CTTACATCAGAATGGGGTCGTG</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F: AAATCGTGTCCTGACATCAAAGAA</td>
</tr>
<tr>
<td></td>
<td>R: GGCCATCTCCTGCTCGAA</td>
</tr>
</tbody>
</table>

upper chambers, and the bottom chambers were covered with the medium containing 10% FBS. For ERK signaling pathway analysis, the ERK inhibitor SCH772984 (Selleck Chemicals, Houston, USA) dissolved in dimethyl sulfoxide was added to the medium. After 24 h of incubation, the remaining cells on the bottom of the basement membrane were fixed with methanol for 10 min and stained with 10% crystal violet for 20 min. Cells remaining on the membrane were counted under the microscope.

Wound healing assay
TIPE1-overexpressing and control cells at a high density were seeded in six-well plates and cultured routinely until a cell monolayer was formed. Then, a scratch was created on the cell monolayer with a sterile micropipette tip. The cells were cultured in serum-free medium for 24 h. Images were finally taken under the microscope to measure cell migration using the width of the wound in cell monolayer.

Western blot analysis
Cellular protein was isolated from tissues and cells. Protein concentrations were measured using a Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, USA). Then, the protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene fluoride membranes (Millipore, Billerica, USA). The membranes were incubated with various primary antibodies (anti-TIPE1, anti-E-cadherin, anti-Vimentin, anti-Snail, anti-Slug, anti-β-actin, anti-MMP2, anti-MMP9, anti-p-MEK, anti-MEK, anti-p-ERK, and anti-ERK) (1:1000; Cell Signaling Technology, Beverly, USA) at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:5000; Cell Signaling Technology) for 1 h at room temperature. Protein bands were then visualized using an Enhanced chemiluminescence (ECL) kit (Millipore).

Quantitative real-time polymerase chain reaction (PCR)
Cells were lysed with TRIzol reagent (Invitrogen, Carlsbad, USA). Total RNA was extracted and reverse transcribed into cDNA by using Prime Script™ RT Master Mix (Takara, Otsu, Japan). Real-time PCR was carried out on the 7900HT Fast Real-Time PCR System (Life Technologies Corporation, Carlsbad, USA) by using

Figure 1. TIPE1 was significantly reduced in breast cancer tissues with lymph node metastasis (A) Positive TIPE1 staining was found in breast cancer tissues. Moreover, TIPE1 staining in breast cancer tissues without lymph node metastasis was stronger than that in tissues with lymph node metastasis. (B) Western blot analysis showed that the expression of TIPE1 was downregulated in the tissues of patients (n = 3; *, #1, #2, and #3) with lymph node metastasis compared with that in the tissues of patients (n = 3; #1, #2, and #3) without lymph node metastasis. Data are presented as the mean ± SEM. *P < 0.05.
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Figure 2. TIPE1 inhibited the invasion and migration of breast cancer cells

(A) TIPE1 significantly inhibited MCF-7 and MDA-MB-231 cell invasion and migration. The number of invading and migrating cells in the TIPE1-overexpressing (LV-TIPE1) cells was significantly decreased compared with that in the control (LV-CON) cells. A larger number of cells invaded to the lower surface of the membrane in the TIPE1-silenced (LV-shTIPE1) cells compared to the control (LV-shNC) cells. 

(B) Statistical analysis of the migration distance showed that cells transfected with TIPE1 (LV-TIPE1) migrated at a lower rate than the control (LV-CON) cells. Data are presented as the mean ± SEM. *P < 0.05.

SYBR Green Master Mix (Tiangen, Beijing, China). The reaction condition was as follows: an initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 5 s, 55°C for 30 s, and 72°C for 20 s. The specific primers for E-cadherin, Vimentin, Snail, Slug, and β-actin genes are listed in Table 2. The ΔΔCt method was used for quantifying gene expression relative to β-actin.

Statistical analysis

Data were collected from experiments in triplicate. Data are presented as the mean ± SEM. Significant differences between groups were evaluated using one-way ANOVA or Student’s t-test with P < 0.05.

Results

TIPE1 expression was significantly decreased in breast cancer tissues with lymph node metastasis

To investigate the expression level of TIPE1 in different breast cancer tissues and the correlation between TIPE1 expression level and clinicopathological parameters in patients with breast cancer, immunohistochemistry staining and western blot analysis were performed. Immunohistochemistry staining results showed that TIPE1 protein was positively expressed in breast cancer tissues and primarily localized in the cytosol of tumor cells. TIPE1 expression level was positively associated with both tumor size (P = 0.005) and lymph node metastasis (P < 0.001). However, no significant associations were found between the TIPE1 expression level and patient age (P = 0.924; Table 1). In addition, the expression level of TIPE1 in cancer tissues without lymph node metastasis was higher than that in cancer tissues with lymph node metastasis (P < 0.001; Fig. 1A). Furthermore, western blot analysis confirmed the downregulation of TIPE1 in breast cancer tissues with lymph node metastasis (Fig. 1B). These data indicated that TIPE1 might be a tumor suppressor gene in breast cancer development and metastasis.

TIPE1 inhibited the invasive and migratory capacities of breast cancer cells

The effects of TIPE1 on the invasion and migration of MCF-7 and MDA-MB-231 cells were examined using a Transwell chamber with or without Matrigel coating, respectively. Our results indicated that TIPE1 overexpression significantly inhibited MCF-7 and MDA-MB-231 cell invasion and migration. The number of tumor cells passing through the membrane in TIPE1-overexpressing cells was less than that in the control cells. Meanwhile, TIPE1 silencing obviously increased the invasive and migratory capacities of tumor cells. Compared with the negative control cells, more cells were found on the lower surface of the membrane in TIPE1-silenced cells (Fig. 2A). In addition, the wound healing assay also showed that...
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TIPE1 suppressed the EMT phenotype in breast cancer cells

Figure 3. TIPE1 suppressed the EMT phenotype in breast cancer cells. (A) Representative western blots and summarized data revealed the upregulation of E-cadherin and downregulation of Vimentin, Snail, and Slug expression in MCF-7 and MDA-MB-231 cells overexpressing TIPE1 (LV-TIPE1). (B) TIPE1 silencing (LV-shTIPE1) led to downregulation of E-cadherin expression and upregulation of Vimentin, Snail, and Slug expression compared with that in the control cells (LV-shNC). (C) RT-PCR results showed the increased mRNA level of E-cadherin and the decreased mRNA levels of Vimentin, Snail, and Slug in breast cancer cells transfected with TIPE1 (LV-TIPE1). (D) TIPE1 silencing (LV-shTIPE1) decreased the mRNA level of E-cadherin and increased the mRNA levels of Vimentin, Snail, and Slug. Data are presented as the mean ± SEM. * P < 0.05.

TIPE1 suppressed EMT in breast cancer cells

To examine whether TIPE1 was associated with the EMT progression of breast cancer cells, we evaluated the protein expression patterns and mRNA levels of the epithelial marker E-cadherin, the mesenchymal marker Vimentin, and the transcription factors Snail and Slug by western blot and RT-PCR, respectively. Western blot analysis indicated that the expression of E-cadherin protein was upregulated in TIPE1-overexpressing cells, whereas the expressions of Vimentin, Snail, and Slug proteins were downregulated compared to those in the control cells (Fig. 3A). In addition, the opposite expression patterns of EMT-associated proteins were observed in TIPE1-silenced cells (Fig. 3B). Moreover, similar changes of mRNA levels of these genes were detected via RT-PCR analysis (Fig. 3C,D). These data demonstrated that TIPE1 suppressed the EMT of breast cancer cells.

TIPE1 reduced the expression of MMP2 and MMP9 in breast cancer cells

To explore the possible mechanism of TIPE1 in breast cancer cells, the expression patterns of some MMP family members (MMP2, MMP9) were detected by western blot analysis. As shown in Fig. 4, TIPE1
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Figure 4. TIPE1 reduced the expressions of MMP2 and MMP9 in breast cancer cells

(A) Representative western blots and summarized data revealed the down-regulation of MMP2 and MMP9 in breast cancer cells overexpressing TIPE1 (LV-TIPE1). (B) TIPE1 silencing (LV-shTIPE1) led to the upregulation of MMP2 and MMP9 expression compared with that in the control cells (LV-shNC). Data are presented as the mean ± SEM. *P < 0.05.

overexpression significantly downregulated the expression levels of MMP2 and MMP9 in breast cancer cells (Fig. 4A), and TIPE1 silencing obviously upregulated the expression levels of MMP2 and MMP9 (Fig. 4B). These data indicated that TIPE1 might inhibit the invasion and migration of breast cancer cells and prevent the EMT by downregulating the expressions of MMP2 and MMP9.

TIPE1 negatively regulated the ERK signaling pathway in breast cancer cells

The ERK signaling pathway is known as a key upstream of MMPs. Thus, we investigated the phosphorylation levels of MEK and ERK by western blot analysis. Our results showed no significant fluctuation in the expression of p-MEK. However, the phosphorylation level of ERK in the TIPE1 overexpression cells was significantly decreased compared with that in the control cells. Moreover, TIPE1 silencing obviously upregulated the expression levels of p-ERK (Fig. 5A,B). Based on this result, we hypothesized that ERK might be involved in the regulation of TIPE1 on the metastasis of breast cancer cells. To further confirm this hypothesis, we added an ERK inhibitor, SCH772984 (final concentration of 1 mM). The Transwell assay showed that the ERK inhibitor SCH772984 decreased the number of invading cells regardless of TIPE1 silencing (Fig. 5C). These data indicated that the regulation of TIPE1 on the metastasis of breast cancer cells might be related to the ERK signaling pathway.

Discussion

Here, we presented the first study on the function of TIPE1 in breast cancer metastasis. We found that the expression of TIPE1 was downregulated in the tissues of patients with lymph node metastasis. Furthermore, TIPE1 might function as a metastasis suppressor to inhibit the invasive and migratory capacities and the EMT of tumor cells. TIPE1 reduced the expression of MMP2 and MMP9 of breast cancer cells. In addition, the ERK signaling pathway might be involved in the regulation of TIPE1 on breast cancer metastasis.

TIPE1 exhibits diverse expression patterns in different cancer tissues [3–6]. These different expression patterns led to our hypothesis that TIPE1 may have dual functions as an oncogene and also as a tumor suppressor gene. In this study, the downregulated expression of TIPE1 in breast cancer tissues with lymph node metastasis indicated that TIPE1 might be a candidate tumor suppressor gene and a good prognosis marker for patients with breast cancer.

In addition, we established stable TIPE1-overexpressing and TIPE1-silenced transfectants to evaluate the function of TIPE1 on the biological behaviors of breast cancer cells. Biological behaviors, including cell proliferation, cell invasion, migration, and apoptosis, are important factors that affect the development, progression, and prognosis of tumors [8]. The invasion and migration of tumor cells are closely related to tumor progression [9]. Our results showed that TIPE1 overexpression significantly inhibited the invasion and migration of breast cancer cells, and TIPE1 silencing increased the invasive and migratory capacities of tumor cells. These data suggested that TIPE1 might act as a repressor in breast cancer metastasis.

EMT is widely recognized as a vital biological process for tumor cell metastasis. The migration and invasion capabilities of tumor cells might be enhanced by phenotypic variations in the process of EMT. In this study, the upregulated expression of an epithelial marker (E-cadherin) and the downregulated expression of a mesenchymal marker (Vimentin) and transcription factors (Snail, Slug) in TIPE1-overexpressing cells demonstrated that TIPE1 suppressed the
EMT of breast cancer cells. This conclusion was further verified in TIPE1-silenced cells. MMPs, especially MMP2 and MMP9, could increase cell motility and degrade the extracellular matrix [10–15] to accelerate cancer metastasis and are implicated in EMT. Our results showed that the expression levels of MMP2 and MMP9 were markedly downregulated in TIPE1-overexpressing and upregulated in TIPE1-silenced cells. Taken together, our data indicated that TIPE1 might inhibit the invasion and migration of breast cancer cells by preventing the EMT phenotype and the expressions of MMP2 and MMP9.

The ERK signaling pathway is known to be a key upstream of MMP2 and MMP9 [11,16]. Our results indicated that TIPE1 obviously decreased the phosphorylation level of ERK in breast cancer cells. Thus, we speculated that TIPE1 downregulated the expression of MMP2 and MMP9 primarily via the ERK signaling pathway in breast cancer cells. For further validation, an ERK inhibitor SCH772984 was used. Consistently, the ERK inhibitor obviously inhibited tumor cell invasion regardless of TIPE1 silencing. These findings suggested that TIPE1 might suppress the invasion and migration of breast cancer cells and inhibit EMT primarily via the ERK signaling pathway.

In conclusion, our study showed that TIPE1 was downregulated in breast cancer tissues of patients with lymph node metastasis. TIPE1 inhibited the invasion and migration of breast cancer cells and prevented the EMT phenotype and the expressions of MMP2 and MMP9, which might be primarily mediated by the ERK signaling pathway. This finding demonstrated that TIPE1 might be a tumor suppressor gene in breast cancer progression. Therefore, TIPE1 might be a new candidate prognostic marker and a potential molecular target for the treatment of breast cancer.
Funding
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References