EVI-1 acts as an oncogene and positively regulates calreticulin in breast cancer

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Abstract. Ecotropic viral integration site-1 (EVI-1) is an important transcription factor involved in oncogenesis. Aberrant EVI-1 expression has been reported to be a characteristic of multiple types of malignancies; however, very little is known about how EVI-1 regulates breast cancer. Current knowledge of how target genes mediate the biological function of EVI-1 remains limited. In the present study, overexpression of EVI-1 promoted cell proliferation, migration, and invasion, and inhibited apoptosis in breast cancer. By contrast, silencing of EVI-1 inhibited cell proliferation, migration and invasion, and enhanced apoptosis in breast cancer. In addition, the results also revealed that the aberrant expression of EVI-1 regulates genes associated with the apoptotic pathway in breast cancer. Furthermore, EVI-1 was also likely to target the promoter region of calreticulin (CRT) in vitro. It was concluded that EVI-1 may be a potential effective therapeutic target in breast cancer.

Introduction

Breast cancer is a highly prevalent malignancy in women that is associated with high rates of morbidity and mortality worldwide, and these rates have continued to increase over the past few years (1). Although breast cancer is traditionally treated using various methods including surgery, chemotherapy and endocrine therapy, recurrence and metasatosis still remain a serious issue in advanced stage patients (2).

Calreticulin (CRT) is a multifunctional calcium-binding protein that is predominantly expressed in the endoplasmic reticulum (3). This protein contains three functional domains: N-, P- and C-domains (4). It is involved in a variety of cellular processes, including protein folding, calcium homeostasis and cell adhesion (5). Extensive research has revealed that the expression of CRT is markedly increased in various types of cancers (6). A previous study demonstrated that the expression of CRT was positively associated with lymph node metastasis and clinical stages in breast cancer (7). An earlier study reported epithelial-mesenchymal transition (EMT)-like changes in the cellular phenotype in CRT-overexpressing Madin-Darby canine kidney cells (8). Furthermore, the mRNA expression of the EMT marker E-cadherin was reduced when CRT was overexpressed. Overexpressing CRT regulated EMT marker characteristics in gastric cancer cells (9). These results suggested that there may be a direct association between CRT and EMT.

Ecotropic viral integration site-1 (EVI-1) has a critical role in oncogenesis as a transcription factor (10). Overexpression of EVI-1 has frequently been observed in hematological malignancies (11-13) and in several types of solid tumor (14-16). However, very little is known about how EVI-1 regulates the oncogenesis of breast cancer.

Previous studies have demonstrated that EVI-1 is a master regulatory element in EMT (17, 18). Nayak et al. (19) reported that the expression of EVI-1 was strongly correlated with E-cadherin and N-cadherin in stage IV of colon cancer. Whether EVI-1 regulates the process of EMT in breast cancer via CRT remains unknown. In the present study, the overexpression of EVI-1 promoted cell proliferation, migration and invasion, and inhibited apoptosis in breast cancer cells. In addition, EVI-1 positively regulated the expression of CRT in breast cancer. Furthermore, a novel mechanistic pathway was investigated for how EVI-1 induced CRT activation in breast cancer. The results revealed that EVI-1 may be a potential effective therapeutic target in breast cancer.

Materials and methods

Cell lines and culture. The human breast cancer cell line MDA-MB-231 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells
were cultured in L-15 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) in a humidified atmosphere at 37°C without CO₂.

**Cell transfection.** MDA-MB-231 breast cancer cells were cultured in a 6-well plate for 24 h at 37°C and then transfected with plasmids pcDNA3.1-EVI-1 and pSilencer-2.1-EVI-1 [EVI-1-short hairpin RNA (shRNA)]; both synthesized by Jrdun Biotechnology Co., Ltd., Shanghai, China). The following primers were used to amplify the EVI-1 sequence for cloning into the pcDNA vector: pcDNA3.1-EVI-1 forward, 5'-CGG GAATTCTGATCTTCTAGCAAGTTTACA-3'; pcDNA3.1-EVI-1 reverse, 5'-CGCGGATCCATACGTTGGGATA GCACTGG-3'. The short hairpin RNA sequence (shRNA) in pSilencer-2.1-EVI-1 (EVI-1-shRNA) was forward, 5'-CCT AGCGACTGCTTACCA-3' and reverse, 5'-TGGTACGGAC TGATCGTACGGG-3'. For EVI-1 expression, 2.5 µg pcDNA3.1 control vector or pcDNA3.1-EVI-1 or pSilencer-2.1 control vector/pSIlencer-2.1-EVI-1 were transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) into 6-well plates (3x10⁵ cells/well). For CRT expression, a small interfering RNA (siRNA) targeting CRT was used (synthesized by Jrdun Biotechnology Co., Ltd.), which had the following sequence: siRNA-CRT, 5'-GGA GCA GUU UCU GGA AGA TTC ATG ATC TTA GAC GAA TTT TAC A-3'; pcDNA3.1-EVI-1 forward, 5'-CCG CGGATCCATACGTTGGGATA GCACTGG-3'. The short hairpin RNA sequence (shRNA) in pSilencer-2.1-EVI-1 (EVI-1-shRNA) was forward, 5'-CCT AGCGACTGCTTACCA-3' and reverse, 5'-TGGTACGGAC TGATCGTACGGG-3'. For EVI-1 expression, 2.5 µg pcDNA3.1 control vector or pcDNA3.1-EVI-1 or pSilencer-2.1 control vector/pSIlencer-2.1-EVI-1 were transfected into cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For all plasmids, 2 µg was used for transfection and cultured in a 6-well plate for 24 h at 37°C.

**Dual luciferase reporter system.** The MDA-MB-231 cells were transfected psiCHECK2-CRT-WT and pcDNA3.1 empty vector, psiCHECK2-CRT-WT (the fragments of the promoter region of CRT) pcDAN3.1-EVI-1, psiCHECK2-CRT-MUT and pcDAN3.1 empty vector, or pcDAN3.1-EVI-1 and psiCHECK2-CRT-MUT using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For all plasmids, 2 µg was used for transfection and were synthesized by Jrdun Biotechnology Co., Ltd. After 48 h, the luciferase activity was measured using a Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Firefly luciferase activities were normalized by *Renilla* luciferase activities to control for transfection efficiency.

**Electrophoresis mobility shift assay (EMSA).** EMSA was performed using the LightShift EMSA kit (Pierce; Thermo Fisher Scientific, Inc.). Nuclear protein extracts from breast cancer cells were prepared using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc.). Protein concentration was determined using bicinchoninic acid assay. Oligonucleotides were synthesized for the CRT EVI-1 binding site and the mutation, and labeled with biotin by Beijing Genomics Institute (Shenzhen, China). The oligonucleotide sequences were as follows: EVI-1 binding site from CRT promoter region, 5'-GCTGGTTCTCTCAATGT CAAGATAAGAGCTGG-3'; and EVI-1 binding site-mutant from CRT promoter region 5'-GCTGGTTCTCATCGATCT GATAAGAGCTGG-3'. The binding reaction mixtures (2 µl 5X Gel shift buffer, 2 µl nuclear extracts, 1 µl labeled DNA probe, nuclease-free water to 10 µl) were incubated according to the manufacturer's instruction. For competition experiments, the corresponding unlabeled probe (cold probe) was used at 100-fold excess concentrations when compared with the labeled probe in the binding reaction. Reaction products were separated by 5% non-denaturing polyacrylamide gels in 0.5X Tris/Borate/EDTA buffer, and then the bands were transferred to a nylon membrane. The nylon membrane was visualized using the LightShift™ Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

**Cell viability assay.** Cell viability was assessed by a Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Haimen, China) assay according to the manufacturer's instructions. Transfected cells were seeded into a 96-well plate at 5,000 cells/well. After transfection for 24, 48 and 72 h, 10 µl CCK-8 assay solution was added to each well and incubated for 1 h. Absorbance was then measured at 450 nm using a microplate reader.

**Flow cytometry analysis of cell apoptosis.** Cells were collected after transfection for 48 h, then an Annexin V-FITC Apoptosis Detection Kit (Beyotime Institute of Biotechnology) was used to assess apoptosis according to the manufacturer's instructions. The rate of apoptosis was analyzed by flow cytometry with BD FACSDiva software version 8.0 (BD Biosciences, San Jose, CA, USA).

**Cell migration and invasion assays.** The migration and invasion assays were performed using Transwell inserts. For the migration assay, at 48 h after transfection the indicated cells were starved for 24 h, 1x10⁵ cells resuspended in serum-free medium and then added to the upper chamber of Transwell plates (Corning Incorporated, Corning, NY, USA). The lower chamber was filled with L15 medium and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). After incubation for 24 h, the cells attached to the lower surface of the membrane were fixed in 95% ethanol at 37°C for 30 min, and stained with 0.5% crystal violet for 30 min at room temperature; cells were then counted in five randomly selected fields under a light microscope (Olympus Corporation, Tokyo, Japan). For the cell invasion assay, the indicated cells were plated in Transwell polycarbonate membrane inserts precoated with a layer of diluted Matrigel (BD Biosciences). The remaining experimental procedures were consistent with those described for cell migration experiments.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from MDA-MB-231 breast cancer cells using RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China) according to manufacturer's instructions. cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China) according to manufacturer's instructions. The expression of target genes was...
normalized to GAPDH. The reaction thermocycling conditions for qPCR were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, and 60°C for 31 sec. The sequences of the primers used for RT-qPCR are presented in Table I. All of the qPCR data were processed using the 2^ΔΔCq method (20).

Western blot analysis. Total protein was isolated from MDA-MB-231 breast cancer cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and protein concentration was measured using a bicinchoninic acid assay. Equal amounts of protein (20 µg/ lane) separated by SDS-PAGE on 8% gels and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk for 2 h at room temperature and then incubated with rabbit polyclonal anti-EVI-1 (1:1,000; cat. no. ab28457), rabbit polyclonal anti-CRT (1:1,000; cat. no. ab2907), rabbit polyclonal anti-E-cadherin (1:1,000; cat. no. ab15148), rabbit polyclonal anti-SNAI1 (1:1,000; cat. no. ab53154), rabbit polyclonal anti-Slug (1:1,000; cat. no. ab53154), rabbit polyclonal anti-EVI-1 (1:1,000; cat. no. ab18203), rabbit polyclonal anti-apoptosis regulator BAX (BAX; 1:1,000; cat. no. ab55154), rabbit polyclonal anti-capase-3 (1:1,000; cat. no. ab13847), rabbit polyclonal anti-zinc finger protein SNAI1 (Snail 1; 1:1,000; cat. no. ab10490), rabbit polyclonal anti-zinc finger protein SNAI2 (Slug; 1:1,000; cat. no. ab27568) and rabbit polyclonal anti-apoptosis regulator Bcl-2 (BCL-2; 1:1,000; cat. no. ab59348) primary antibodies at 4°C overnight. GAPDH (1:3,000; cat. no. ab9485) was used as the internal control to ensure equal protein loading. All antibodies were purchased from Abcam (Cambridge, UK). The signal was developed with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.) following incubation with the corresponding goat anti-rabbit horseradish peroxidase-conjugated (IgG H&L) secondary antibody at room temperature for 1 h (1:2,000; cat. no. ab205718; Abcam).

Statistical analysis. All experiments were repeated three times independently, and the data are presented as the mean ± standard deviation. One-way analysis of variance followed by Bonferroni’s multiple comparisons procedure was used to compare multiple groups and paired t-test was used to compare two groups. Analysis was performed using SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

EVI-1 may target the CRT promoter region in vitro. The expression of EVI-1 was markedly upregulated following transfection with pcDNA3.1-EVI-1, whereas EVI-1 was down-regulated after transfection with pSilencer-2.1-EVI-1 (Fig. 1A). Subsequently, the expression of CRT was analyzed at the mRNA and protein levels. CRT mRNA expression was increased following transfection with pcDNA3.1-EVI-1; however, pSilencer-2.1-EVI-1 reduced CRT mRNA expression (Fig. 1B). In addition, a similar effect on CRT protein expression was observed (Fig. 1C). To further demonstrate that CRT was a target of EVI-1, dual luciferase reporter and EMSA assays were performed. Luciferase activity was increased in cells co-transfected with pcDNA3.1-EVI-1 and the psiCHECK2-CRT-WT reporter vector. However, pcDNA3.1-EVI-1 had no effect on the luciferase activity of cells co-transfected with the psiCHECK2-CRT-MUT reporter vector (Fig. 1D). Based on the EMSA gel shifts, a protein bound to the CRT promoter region, and this protein may be EVI-1. No specific gel shift was observed in lane 1; however, a marked shift was observed in lane 2. In addition, no shift was observed with the 100-fold excess of the unlabeled EVI-1 probe (cold probe) in lane 3, and there was no obvious interaction between the nuclear extract and the mutated EVI-1 probe in lane 4 (Fig. 1E). In summary, these results indicated that EVI-1 positively regulated CRT expression, and EVI-1 is likely to regulate its expression by binding to the promoter region of CRT in vitro experiment.

EVI-1 promotes cell proliferation and inhibits cell apoptosis in human breast cancer. The overexpression of EVI-1 markedly increased cell proliferation, while downregulation of EVI-1 inhibited cell proliferation (Fig. 2A). Overexpression of EVI-1 also decreased the apoptosis of breast cancer cells, while EVI-1 silencing increased the rate of apoptosis in breast cancer cells (Fig. 2B). Subsequently, expression of apoptotic

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
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<tr>
<td>EVI-1</td>
<td>TTATAGAAGCGGATACAAGGAGGAG</td>
<td>CGCCGTCTTGATTATCTTGATGAG</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>AAGGAGCGATTTCTTGAGCGG</td>
<td>GCGGACAGAGCATAAAGGCG</td>
</tr>
<tr>
<td>Snail 1</td>
<td>TGCTGTCCCGGCCGATATT</td>
<td>GTAGCTTCGCTTGATGAG</td>
</tr>
<tr>
<td>Slug</td>
<td>TTCCTCGCGTGGATGGTAAGACA</td>
<td>TCTGCTCTGGAGACCAGGTGC</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>TCAGGCGGCAAGCCATCTCCT</td>
<td>TTTCCCCAGCGCTTCCTCACC</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>CGGCCGCGTGGCCAACGAGT</td>
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<td>GGTCGCTTGTGCCCCTTTTTC</td>
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<tr>
<td>Caspase-3</td>
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<tr>
<td>GAPDH</td>
<td>CTCTTCCTGGCCCTCCTGT</td>
<td>GCTGTACCTTCCACGGT</td>
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EVI-1, ectropic viral integration site-1; Snail 1, zinc finger protein SNAI1; Slug, zinc finger protein SNAI2; BAX, apoptosis regulator BAX; BCL-2, apoptosis regulator Bcl-2.
pathway-associated genes, including caspase-3, Bax and Bcl-2, was investigated. As indicated in Fig. 2C and D, a marked decrease in caspase-3 and Bax expression, and a marked increase in Bcl-2 expression was observed when EVI-1 was overexpressed. By contrast, knockdown of EVI-1 increased the expression of caspase-3 and Bax, and reduced the expression of Bcl-2.
Figure 2. Effect of EVI-1 on the proliferative and apoptosis abilities of breast cancer cells. (A) A Cell Counting Kit-8 assay was conducted to assess cell proliferation in breast cancer cells. (B) Cell apoptosis was detected by flow cytometry. The expression of apoptotic pathway-associated genes and proteins was measured by (C) reverse transcription-quantitative polymerase chain reaction and (D) western blot analysis (*P<0.05 vs. empty vector). The data are presented as the mean ± standard deviation. *P<0.05. EVI-1, ecotropic viral integration site-1; PI, propidium iodide; FITC, fluorescein isothiocyanate; BAX, BAX apoptosis regulator; BCL-2, Bcl-2 apoptosis regulator.
EVI-1 promotes cell migration and invasion in human breast cancer. The present study further investigated the effect of EVI-1 on cell migration and invasion. The results revealed that overexpression of EVI-1 effectively promoted cell migration and invasion, and silencing EVI-1 expression markedly suppressed cell migration and invasion in human breast cancer cells compared with the respective empty vector controls (Fig. 3).

EVI-1 regulates EMT-related gene expression in breast cancer. Previous studies have indicated that the abnormal expression of CRT regulates the expression of EMT-associated genes (8,9), and the present study revealed that EVI-1 positively regulated CRT expression. As indicated in Fig. 4A and B, knockdown of CRT using siRNA-CRT decreased the expression of Snail 1, Slug and N-cadherin, and increased the expression of E-cadherin.

To determine whether EVI-1 can regulate EMT-associated gene expression, RT-qPCR and western blot analyses were performed to investigate the expression of EMT-associated genes. As demonstrated in Fig. 4C and D, a marked increase in Snail 1, Slug, and N-cadherin expression, and a marked decrease in E-cadherin expression were observed when EVI-1 was upregulated. By contrast, silencing EVI-1 decreased the expression of Snail 1, Slug and N-cadherin, and increased the expression of E-cadherin. These results suggested that EVI-1 may influence the process of EMT in breast cancer cells.

Discussion

EVI-1, an oncogene located on chromosome 3q26, specifically binds to promoter DNA sequences and has a role in transcriptional regulation (10). Multiple studies have revealed that EVI-1 is upregulated in leukemia, ependymoma, ovarian cancer, breast cancer and colon cancer, and the downregulation of EVI-1 expression inhibited the proliferation of tumor cells (11-13). In addition, increased EVI-1 expression has also been revealed to be a risk factor for poor prognosis in patients with leukemia (21-23). However, whether EVI-1 exerts specific regulatory effects on breast cancer has not been reported thoroughly. In the present study, overexpression of EVI-1 increased the proliferative ability of breast cancer.

c-Jun N-terminal protein kinase (JNK) has a critical role in the process of cell apoptosis and the mechanism of JNK-mediated apoptosis is associated with the regulated expression of apoptosis-associated proteins, including Bax and Bcl-2 (24-26). However, EVI-1 inhibits the activity of JNK and the subsequent cell apoptosis by interfering with the interactions between JNK and its physiological substrates (27). It was hypothesized that EVI-1 may regulate breast cancer apoptosis and the present study demonstrated that cell apoptosis was inhibited by the overexpression of EVI-1 and enhanced by the downregulation of EVI-1. Bcl-2 has a major role in the signal transduction pathways of cell apoptosis (28). Bcl-2 and Bax are the most representative genes for inhibiting and promoting apoptosis, and Bax is the main regulator of Bcl-2 (29). Bcl-2 can bind to Bax to form a heterodimer, and the increased expression of Bax can antagonize the effect of Bcl-2 and promote cell apoptosis (30-32). Caspase-3 is the key mediator of cell apoptosis and has a role in a variety of apoptotic signaling pathways (33-35). Previous studies have reported that overexpression of Bcl-2 can effectively inhibit the activation of caspase-3, thus inhibiting the occurrence of apoptosis (36). In the present study, RT-qPCR and western blotting revealed that EVI-1 overexpression/silencing altered the expression of the aforementioned proteins associated with apoptosis, and subsequently the process of apoptosis, in breast cancer. When EVI-1 was highly expressed, the expression of Bax and caspase-3 was reduced, and Bcl-2 was increased.

A number of previous studies investigating CRT demonstrated that the abnormal expression of CRT leads to changes in certain biological processes, including cell invasion and proliferation, and may be associated with the cancer occurrence, development and prognosis (3,6,37-39). However, research on the association between EVI-1 and CRT is rarely reported.
EMSA is an invaluable tool to study interaction of proteins with DNA. The experiment can simulate the specific binding of protein and DNA in vitro. EMSA has some limitations in the process of reconstructing the binding between proteins and DNA in vivo. However, EMSA is still a good method to predict the binding between EVI-1 and CRT in vitro in this study. By using EMSA and dual luciferase assays, the present study revealed that EVI-1 may bind to the promoter region of CRT, and positively regulate its expression. Thus, it is reasonable to suggest that EVI-1 may affect some of the biological functions of breast cancer by regulating the expression of CRT.

EMT is a process by which epithelial cells gain the phenotype of mesenchymal cells, and the close connection between cells with the extracellular matrix and neighboring...
cells weakens or is completely abolished; consequently, cell migration and invasion is enhanced (40). In the process of EMT, the expression of several proteins, including E-cadherin, N-cadherin, Snail 1 and Slug, is altered (41-44). Previous studies have revealed that the overexpression of CRT can inhibit the expression of E-cadherin and enhance cell migration abilities (8,45). Liu et al (9) reported that the overexpression of CRT promoted cell invasion and metastasis in gastric cancer by regulating the expression of Snail 1 and E-cadherin. In the present study, the results demonstrated that increased expression of EVI-1 significantly reduced the expression of E-cadherin and enhanced the expression of N-cadherin, Snail 1 and Slug. When the expression of EVI-1 was suppressed, the expression of E-cadherin was increased, and N-cadherin, Snail 1 and Slug were reduced. It was speculated that EVI-1 may regulate EMT-associated genes via the CRT pathway in breast cancer.

In conclusion, the results of the present study revealed that EVI-1 overexpression promotes cell proliferation, migration and invasion, and inhibits apoptosis in breast cancer cells. EVI-1 is likely to bind to CRT and positively regulate the expression of CRT in vitro environment; it was concluded that EVI-1 may affect EMT-associated genes by regulating the expression of CRT in breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LW designed the study, performed the experiments and drafted the manuscript. TW, DH and XL analyzed and interpreted the experimental data. YJ conceived the study and participated in the manuscript. TW, DH and XL analyzed and interpreted the experimental data. YJ conceived the study and participated in its design and coordination.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References