

STC-1 expression is upregulated through an Akt/NF- κ B-dependent pathway in triple-negative breast cancer cells

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Abstract. Stanniocalcin-1 (STC-1) is a secreted glycoprotein and its expression is strongly correlated with cancer development. However, regulatory mechanism of STC-1 expression in breast cancer cells is not clear. In the present study, we investigated whether STC-1 acts as a prognostic factor in TNBC patients and the regulation of STC-1 expression in breast cancer cells. Basal levels of STC-1 were significantly higher in TNBC cells than in non-TNBC cells. Induction of STC-1 expression was also associated with poor relapse-free survival of TNBC patients. In addition, we verified the correlation between the invasiveness of TNBC cells and the STC-1 expression. We found that recombinant human STC-1 treatment increased the invasiveness of TNBC cells. In contrast, STC-1-induced cell invasiveness was completely inhibited by anti-STC-1 monoclonal antibody treatment. We found that the basal levels of STC-1 expression in TNBC cells were decreased by treatment with LY294002 or Bay11-7085, but not SB203580. In contrast, transcript levels of STC-1 and protein secretion were increased by constitutively active Akt (CA-Akt) or NF- κ B overexpression in TNBC cells. Finally, we observed that phosphorylation of NF- κ B was significantly increased by CA-Akt overexpression in TNBC cells. Taken together, elevated STC-1 expression is associated with poor clinical outcome in TNBC patients, and STC-1 is directly involved in the invasiveness of TNBC cells. STC-1 expression is upregulated through a PI-3K/Akt/NF- κ B-dependent signaling pathway in TNBC cells.

Introduction

Triple-negative breast cancers (TNBCs) account for approximately 15-20% of all diagnosed breast cancer types and are more prevalent in younger women (1-3). TNBC tumors predominantly present as invasive ductal carcinomas characterized by large tumor size, poor differentiation, high proliferation and high histologic grade (4,5). Although TNBCs respond to chemotherapy better than other breast cancer subtypes, patients with TNBCs have the worst clinical outcomes because of the higher risk of distant metastasis and significantly shorter overall survival (6,7). Treatment options for TNBC patients are currently limited to cytotoxic chemotherapy because of the absence of the well-defined molecular targets of the estrogen receptor (ER), progesterone receptor (PR) and HER2 (8). Therefore, new therapeutic targets for treating TNBC patients are urgently needed.

Stanniocalcin-1 (STC-1), a hypocalcemic glycoprotein hormone, is known to regulate plasma Ca^{2+} homeostasis (9). Mammalian STC-1 is expressed ubiquitously and is involved in numerous developmental and pathophysiological processes, including cancer, pregnancy and organogenesis (10-13). Aberrant STC-1 expression is associated with cancer development and poor prognosis in colorectal and gastric cancer (14-17). The expression of STC-1 is regulated by transcription factors NF- κ B and p53 in colon and nasopharyngeal cancer cells (18,19). Transcription factor HIF-1 α is also involved in the activation of STC-1 expression in colon, nasopharyngeal and ovarian cancer cells (20). However, the clinical significance of STC-1 expression in breast cancer has not been fully elucidated, and little is known about regulation of this protein in breast cancer cells.

In the present study, we investigated the clinical significance of STC-1 in TNBC patients and its regulation in TNBC cells. Elevated STC-1 expression was associated with poor prognosis in TNBC patients, and increased the invasiveness of TNBC cells. Furthermore, STC-1 expression was significantly higher in TNBC cells than in non-TNBC cells. Basal levels of STC-1 expression in TNBC cells were completely suppressed by blockage of PI-3K/Akt or NF- κ B signaling pathways. In contrast, CA-Akt or NF- κ B overexpression increased transcript levels of STC-1 in TNBC cells as well

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as secretion of this protein. Notably, we observed crosstalk between Akt and NF- κ B with regard to STC-1 expression. CA-Akt overexpression increased the levels of NF- κ B phosphorylation in TNBC cells. Taken together, we demonstrated that the PI-3K/Akt/NF- κ B signaling axis directly regulates STC-1 expression in TNBC cells. STC-1 may be an important therapeutic target in TNBC patients.

Materials and methods

Reagents. Cell culture media and antibiotics were purchased from Life Technologies (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT, USA). Rabbit monoclonal anti-total and phospho-Akt, NF- κ B, p38, I κ B α and STC-1 antibodies were purchased from Epitomics (Burlingame, CA, USA). β -actin antibody was purchased from AbFrontier Co., Ltd. (Seoul, Korea). Secondary horseradish peroxidase (HRP)-conjugated antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LY294002, Bay11-7085 and SB253580 were purchased from Tocris Bioscience (Ellisville, MO, USA). Recombinant human STC-1 was purchased from ProSpec HOR-259 (ProSpec, Tel Aviv, Israel). Mouse IgG and anti-STC-1 monoclonal antibodies were purchased from R&D Systems (Minneapolis, MN, USA).

Cell cultures and drug treatment. MCF7, MDA-MB-231 and Hs578T breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 IU/ml penicillin and 100 μ g/ml streptomycin. T47D breast cancer cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 IU/ml penicillin and 100 μ g/ml streptomycin. Cells were grown in a humidified atmosphere with 5% CO₂ at 37°C. In the drug treatment experiment, after serum starvation for 24 h, cells were treated with LY294002, Bay11-7085 or SB253580 for 24 h.

Analysis of public expression database. Expression data were downloaded from a public database [Kaplan-Meier plotter database (<http://kmplot.com/breast>)]. The clinical value of STC-1 in triple-negative breast cancer patients was analyzed by Kaplan-Meier analysis. The hazard ratio with 95% confidence intervals and log-rank P-values were calculated.

Western blotting. Cell lysates were prepared to detect anti-total and phospho-Akt, NF- κ B, I κ B α , STC-1 and β -actin expression. Equal amounts of proteins (50 μ g) were boiled for 5 min in Laemmli sample buffer and then electrophoresed in 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels. Separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes, and the membranes were blocked with 10% skim milk in Tris-buffered saline (TBS) containing 0.01% Tween-20 (TBS/T) for 15 min. Blots were washed three times in TBS/T and then incubated with anti-total or phospho-Akt, NF- κ B, I κ B α , STC-1 and β -actin antibodies in TBS/T buffer at 4°C overnight. Blots were washed three times in TBS/T and subsequently incubated with secondary HRP-conjugated antibodies (1:2,000 dilution) in TBS/T buffer. After an 1-h incubation at room temperature (RT), blots were washed three times in TBS/T. ECLTM prime

reagent (GE Healthcare, Buckinghamshire, UK) was used for development.

Real-time polymerase chain reaction (PCR). Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Isolated RNA samples were then used for RT-PCR. Samples of total RNA (1 μ g) were reverse-transcribed into cDNA in 20 μ l reaction volumes using a First-Strand cDNA Synthesis kit for RT-PCR, according to the manufacturer's instructions (MBI Fermentas, Hanover, MD, USA). Gene expression levels were quantified by real-time PCR using a SensiMix SYBR kit (Bioline, Ltd., London, UK) and 100 ng of cDNA per reaction. The primer sequences used for this analysis were as follows: human ACTB: forward, 5'-TCA CCA TTG GCA ATG AGC GGT T-3' and reverse, 5'-AGT TTC GTG GAT GCC ACA GGA CT-3'; human STC-1: forward, 5'-CAC ACC CAC GAG CTG ACT TC-3' and reverse, 5'-TCT CCC TGG TTA TGC ACT CTC-3'. An annealing temperature of 60°C was used for all primers. PCRs were performed in a standard 384-well plate format with an ABI 7900HT real-time PCR detection system (Applied Biosystems, Foster City, CA, USA). For data analysis, the raw threshold cycle (C_T) value was first normalized to the housekeeping gene for each sample to obtain a Δ C_T. The normalized Δ C_T was then calibrated to control cell samples to obtain $\Delta\Delta$ C_T values.

Plasmid DNA transfection. Empty vector and constitutively active-Akt (CA-Akt) plasmid DNA were a generous gift from Dr Shin Incheol (Hanyang University, Seoul, Korea). NF- κ B p65 plasmid DNA was a generous gift from Dr Lee Myung-Shik (Yonsei University, Seoul, Korea). Hs578T cells were seeded in a 6-well plate. Cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were maintained in culture media without FBS and antibiotics for 24 h while Lipofectamine transfection, and then further incubated in fresh culture media with 10% FBS for 24 h.

Enzyme-linked immunosorbent assay. ELISA assay was performed on culture media (200 μ l) collected from Hs578T breast cancer cells. Secreted protein levels of STC-1 were measured using an ELISA kit (R&D Systems) according to the manufacturer's instructions. Secreted protein levels were analyzed at the wavelength of 450 nm on a spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA).

Cell invasion assay. Cell invasive capacity was analyzed by a Boyden chamber assay, as previously described (21). Twenty-four-well Boyden chambers with Matrigel-coated filters (8 μ m pore size) were purchased from Becton-Dickinson (San Diego, CA, USA). Hs578T and MDA-MB-231 cells were resuspended in culture media (5 \times 10⁴ cells/well) and then added to the Matrigel-coated upper compartment of invasion chambers in the presence or absence of 50 ng/ml STC-1 and/or 2 μ g/ml IgG or STC-1 antibody. Fresh culture media with 5% FBS was added to the lower compartment of the invasion chamber. After a 24 or 48 h of incubation, the cells on the upper side of the filter were removed using cotton swabs. The underside of the filter was fixed in 100%

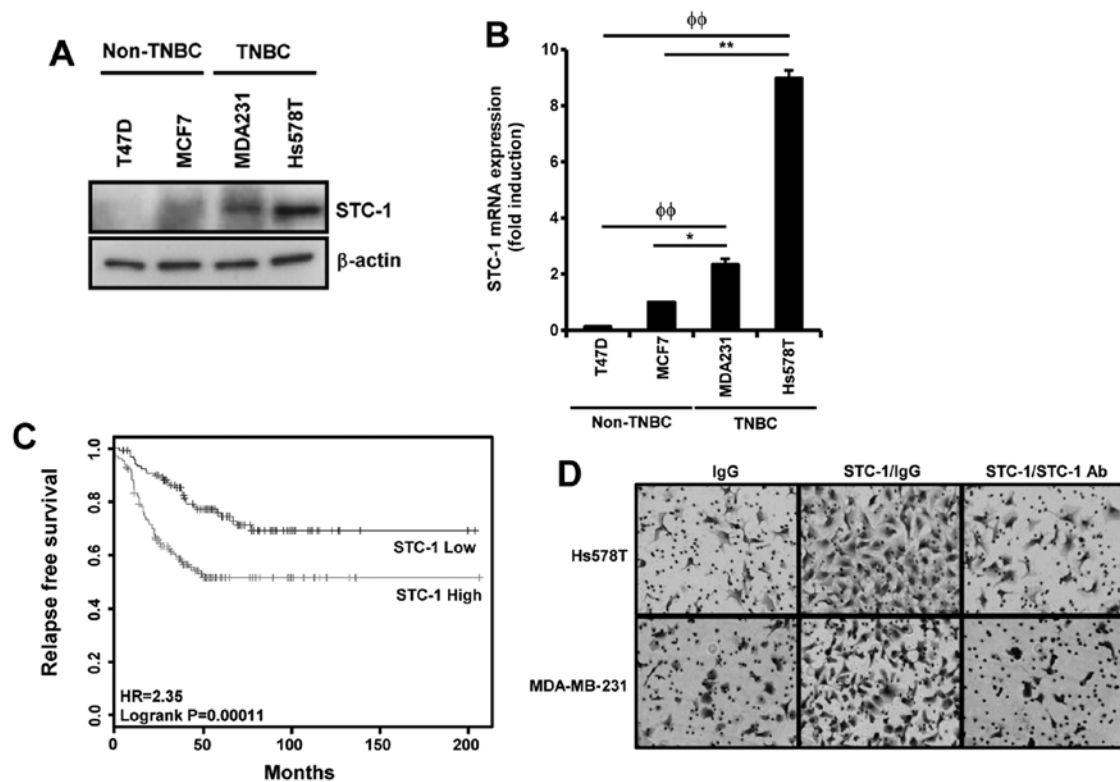


Figure 1. STC-1 expression in breast cancer cells and its clinical significance in TNBC patients. Under the serum-free conditions, cells were harvested and levels of STC-1 protein (A) and mRNA (B) expression were analyzed by western blotting and real-time PCR, respectively. (C) STC-1 expression data were obtained from a public database [Kaplan-Meier plotter database (<http://kmplot.com/breast/>)]. The clinical value of STC-1 in triple-negative breast cancer patients (relapse-free survival) was analyzed by a Kaplan-Meier survival plot. (D) After serum starvation for 24 h, TNBC cells (Hs578T and MDA-MB-231 cell lines) were pretreated with 2 μ g/ml STC-1 and IgG antibodies for 5 h and then treated with 50 ng/ml STC-1 for 24 or 48 h. Cell invasion rate was analyzed by a Boyden chamber assay, as described in Materials and methods. Results are representative of three independent experiments. Values shown are means \pm SEM. $\phi\phi$ P<0.01 vs. T47D cells; *P<0.05, **P<0.01 vs. MCF7 cells.

methanol, washed in 1X PBS and stained using hematoxylin and eosin (H&E). Cells that had invaded through the matrigel were located on the underside of the filter. These cells were analyzed using a ScanScopeXT apparatus (Aperio Technologies, Vista, CA, USA).

Statistical analysis. Statistical significance was determined using Student's t-test. Results are presented as means \pm SEM. All quoted P-values were two-tailed and differences were considered statistically significant when the P-value was <0.05. Statistical analyses were performed using Microsoft Excel.

Results

STC-1 expression in breast cancer cells and its clinical significance in TNBC patients. To evaluate the clinical significance of STC-1 expression, we examined the levels of STC-1 expression in breast cancer cells. As shown in Fig. 1A, STC-1 protein expression was higher in TNBC cells than in non-TNBC cells. STC-1 protein expression was particularly high in Hs578T TNBC cells (Fig. 1A). Under the same conditions, STC-1 mRNA expression was also increased in TNBC cells (Fig. 1B). STC-1 transcript levels were increased to 2.34 ± 0.21 -fold (MDA-MB-231 cells) and 8.97 ± 0.29 -fold (Hs578T cells) of the control cells (MCF7 cells) (Fig. 1B).

In addition, we investigated the co-relation between STC-1 expression and clinical outcomes in breast cancer patients

and the prognostic value of STC-1 in breast cancer patients using a Kaplan-Meier plotter database (<http://kmplot.com/breast/>). We found that induction of STC-1 was involved with a poor prognosis in TNBC patients (Fig. 1C). Patients with high STC-1 levels showed poorer relapse-free survival than patients with low STC-1 levels ($P=0.00011$) (Fig. 1C). However, STC-1 expression level did not affect the relapse-free survival of luminal-type or HER2-type breast cancer patients (data not shown).

We also investigated the role of STC-1 in the invasiveness of TNBC cells. As shown in Fig. 1D, invasive rates of Hs578T and MDA-MB-231 TNBC cells were significantly increased by STC-1 treatment. In contrast, STC-1-induced cell invasiveness was completely blocked by STC-1 antibody treatment in Hs578T and MDA-MB-231 TNBC cells. Based on these results, we concluded that elevated STC-1 expression is associated with poor prognosis in TNBC patients and that it enhances the invasiveness of TNBC cells.

Basal levels of STC-1 expression are decreased by LY294002 or Bay11-7085 treatment in TNBC cells. Next, we compared the phosphorylation levels of signaling molecules such as Akt, NF- κ B and p38 between MCF7 non-TNBC and Hs578T TNBC cells. As shown in Fig. 2A, the phosphorylation levels of Akt and NF- κ B were significantly higher in Hs578T TNBC cells than MCF7 non-TNBC cells. Furthermore, we investigated regulation of STC-1 expression using MDA-MB-231 and

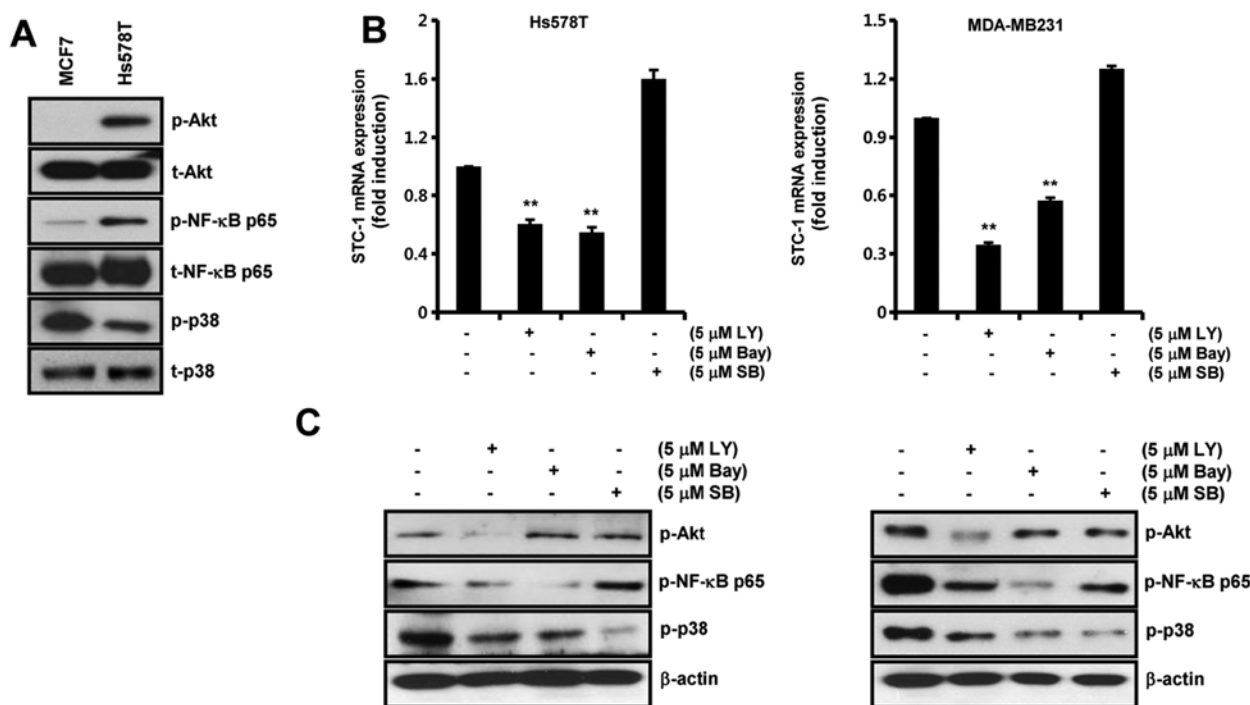


Figure 2. Basal levels of STC-1 expression are decreased by LY294002 or Bay11-7085 treatment in TNBC cells. (A) After starvation for 24 h, cells were harvested and expression of p-Akt, t-Akt, p-NF-κB, t-NF-κB and β-actin was assessed by western blotting. (B) After serum starvation for 24 h, Hs578T (left panel) and MDA-MB-231 (right panel) TNBC cells were treated with 5 μM LY, Bay, or SB for 24 h. STC-1 transcript levels were determined by real-time PCR. (C) Under the serum-free conditions, alterations of Akt, NF-κB and p38 expression in response to specific inhibitors were analyzed by western blotting. Results are representative of three independent experiments. Values shown are means ± SEM. **P<0.01 vs. control. LY; LY294002, Bay; Bay11-7085, SB; SB253580.

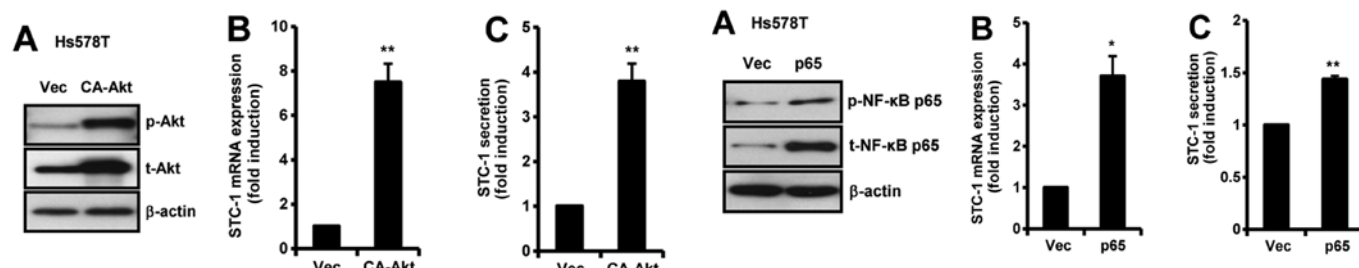


Figure 3. STC-1 expression is increased by CA-Akt overexpression. Hs578T TNBC cells were transfected with either empty or CA-Akt-containing vector in media without FBS for 24 h, and then further incubated in fresh media with 10% FBS for 24 h. (A) p-Akt, t-Akt and β-actin expression were analyzed by western blotting. (B) STC-1 mRNA expression and (C) STC-1 protein secretion were analyzed by real-time PCR and ELISA, respectively. These results are representative of three independent experiments. Values shown are the means ± SEM. **P<0.01 vs. Vec. Vec; empty vector.

Figure 4. STC-1 expression is increased by NF-κB overexpression. Hs578T TNBC cells were transfected with either empty or NF-κB-containing vector in media without FBS for 24 h, and then further incubated in fresh media with 10% FBS for 24 h. (A) p-NF-κB, t-NF-κB and β-actin expression was analyzed by western blotting. (B) STC-1 mRNA transcript levels and (C) STC-1 protein secretion were analyzed by real-time PCR and ELISA, respectively. These results are representative of three independent experiments. Values shown are the means ± SEM. *P<0.05, **P<0.01 vs. Vec. Vec; empty vector, p65; NF-κB p65.

Hs578T TNBC cells. Each cell type was treated with various specific inhibitors for 24 h. After 24 h, we harvested cell lysates to detect the levels of STC-1 mRNA expression. Basal STC-1 mRNA expression in TNBC cells was decreased by LY (a specific PI-3K inhibitor) or Bay (a specific NF-κB inhibitor), but not by SB (a specific p38 inhibitor) (Fig. 2B). LY and Bay decreased STC-1 mRNA expression to 0.60 ± 0.03 -fold and 0.55 ± 0.01 -fold of the control level, respectively, in Hs578T TNBC cells (Fig. 2B, left panel). In addition, STC-1 levels were decreased to 0.35 ± 0.01 -fold (LY) and 0.57 ± 0.01 -fold (Bay) of the control level in MDA-MB-231 TNBC cells (Fig. 2B, right panel). Under the same conditions, we examined the effect of specific inhibitors on the phosphorylation levels of signaling

molecules. As expected, phosphorylation levels of Akt, NF-κB and p38 were significantly decreased by LY, Bay and SB, respectively, in Hs578T and MDA-MB-231 TNBC cells (Fig. 2C). These results demonstrated that STC-1 expression is regulated by PI-3K/Akt and/or NF-κB signaling pathways in TNBC cells.

STC-1 expression is increased by CA-Akt overexpression. Next, we investigated whether Akt directly regulates STC-1 expression in Hs578T TNBC cells. Constitutively active-Akt (CA-Akt) was transfected into Hs578T TNBC cells for 48 h. We confirmed the levels of phospho and total-Akt expression

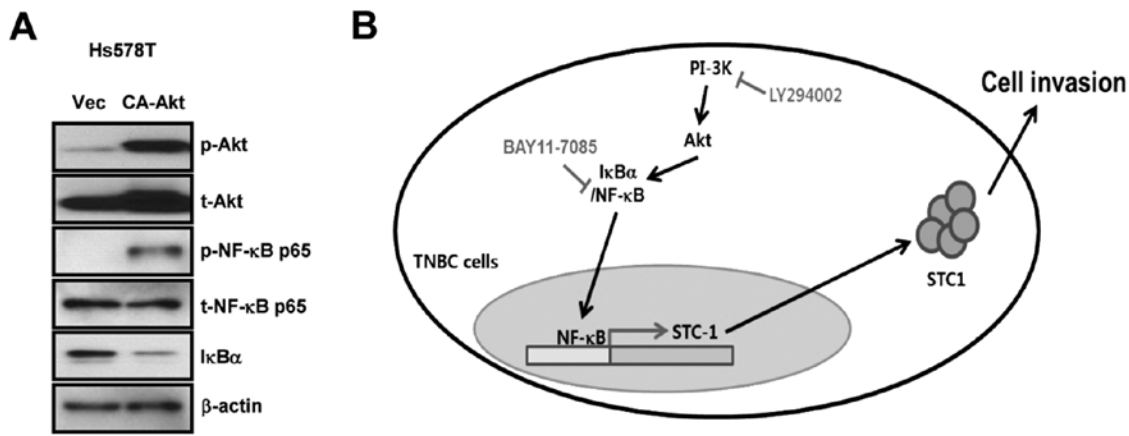


Figure 5. CA-Akt enhances NF- κ B activity and the degradation of I κ B α in Hs578T TNBC cells. Hs578T TNBC cells were transfected with either empty or CA-Akt-containing vectors in media without FBS for 24 h, and then further incubated in fresh media with 10% FBS for 24 h. (A) p-Akt, t-Akt, p-NF- κ B, t-NF- κ B, I κ B α and β -actin levels were analyzed by western blotting. Results are representative of three independent experiments. (B) Schematic model of STC-1 regulation in TNBC cells.

in Hs578T cells (Fig. 3A). Under the same conditions, STC-1 mRNA expression was significantly increased by CA-Akt overexpression in Hs578T cells (Fig. 3B). Transcript levels of STC-1 increased to 7.49 ± 0.83 -fold of the control level in CA-Akt overexpressing cells (Fig. 3B). In addition, we observed that the amount of secreted STC-1 was increased by CA-Akt overexpression (Fig. 3C). The amount of CA-Akt-induced STC-1 secreted protein was 3.79 ± 0.40 -fold that of the control (Fig. 3C). Together, these results indicate that the PI-3K/Akt signaling pathway plays an important role in STC-1 expression in TNBC cells.

STC-1 expression is increased by NF- κ B overexpression. To further investigate the role of NF- κ B in STC-1 expression, we overexpressed NF- κ B in Hs578T cells for 48 h. As shown in Fig. 4A, we confirmed the levels of phospho and total-NF- κ B expression in Hs578T cells. STC-1 mRNA expression was significantly increased by NF- κ B overexpression in Hs578T cells (Fig. 4B). STC-1 mRNA expression was 3.71 ± 0.48 -fold of the control level in NF- κ B overexpressing cells (Fig. 4B). In addition, levels of secreted STC-1 were increased by NF- κ B overexpression (Fig. 4C). Level of NF- κ B-induced STC-1 secreted protein was 1.44 ± 0.03 -fold of the control level (Fig. 4C). These results demonstrated that STC-1 expression is upregulated through an NF- κ B-dependent pathway in TNBC cells.

CA-Akt enhances NF- κ B activity and the degradation of I κ B α in Hs578T TNBC cells. As shown in Figs. 3 and 4, basal STC-1 expression was upregulated by CA-Akt or NF- κ B overexpression in Hs578T TNBC cells. We therefore investigated if cross-talk between the Akt and NF- κ B pathways affected STC-1 expression in Hs578T TNBC cells. First, we transfected Hs578T TNBC cells with CA-Akt for 48 h and then harvested whole cell lysates to measure Akt, NF- κ B and I κ B α expression. CA-Akt overexpression in Hs578T cells significantly increased phosphorylation of NF- κ B (Fig. 5A). Degradation of I κ B α was also increased in CA-Akt overexpressing cells (Fig. 5A). However, the phosphorylation level of Akt was not altered by NF- κ B overexpression (data not shown). Akt activity

therefore appears to trigger the phosphorylation of NF- κ B and the degradation of I κ B α , resulting in upregulation of STC-1 expression in TNBC cells.

Discussion

Aberrant STC-1 expression is correlated with poor clinical outcomes in colorectal, lung and gastric cancer, and has been reported to be a promising target in various human malignancies (14,15,22). STC-1 expression level is directly related to tumor growth, angiogenesis, and metastasis in gastric, breast and ovarian cancers (15,23,24). In the present study, TNBC patients with high STC-1 levels had a poorer prognosis than those with low STC-1 levels with regard to relapse-free survival. However, the level of STC-1 expression did not affect the relapse-free survival of patients with other breast cancer types, such as luminal- and HER2-type breast cancer (data not shown). We also observed that STC-1 augmented the invasiveness of TNBC cells, while STC-1-induced cell invasiveness was completely suppressed by treatment of cells with an STC-1 monoclonal antibody. These findings indicate that STC-1 expression is associated with survival in TNBC patients and is directly involved in the invasiveness of TNBC cells.

STC-1 expression has been reported to be higher in hepatocellular carcinoma and colorectal cancer tissues than cancer-free tissues (16). STC-1 expression can be upregulated by various extracellular stimuli such as vascular endothelial growth factor (VEGF), hypoxia, and glial cell line-derived neurotrophic factor (GDNF), based on studies in several tumor tissues and cell lines (16,20,25). Moreover, STC-1 expression can be regulated by a variety of transcription factors, including NF- κ B, p53 and HIF-1 α , in colon and nasopharyngeal cancer cells (18,19,26). To date, the regulatory mechanism of STC-1 expression in breast cancer has not been elucidated. Here, we observed that STC-1 mRNA and protein expression were significantly increased in TNBC cells compared with non-TNBC cells. In addition, the activities of Akt and NF- κ B were also higher in Hs578T TNBC cells than control cells. Thus, we investigated the roles of Akt and NF- κ B in STC-1 expres-

sion. LY (a specific PI-3K inhibitor) or Bay (a specific NF- κ B inhibitor) decreased expression of STC-1 mRNA and protein in TNBC cells. In contrast, CA-Akt or NF- κ B overexpression increased STC-1 expression in TNBC cells. Based on these data, we suggest that STC-1 expression is upregulated through PI-3K/Akt and/or NF- κ B-dependent pathways in TNBC cells.

In a previous study, Ozes *et al* (27) reported that Akt triggers TNF-induced NF- κ B activation through phosphorylation of IKK- α . IL-1-induced PI-3K activation leads to phosphorylation and activation of the NF- κ B p65/RelA subunit (28). Therefore, the PI-3K/Akt signaling pathway is regulated by multiple mechanisms, and there is often crosstalk with other signaling pathways. As shown in Fig. 5B, we also found crosstalk between Akt and NF- κ B in TNBC cells. When CA-Akt was overexpressed in Hs578T TNBC cells, phosphorylation of NF- κ B and degradation of I κ B α significantly increased. In addition, Akt and NF- κ B activities were suppressed by LY294002 treatment of Hs578T TNBC cells. However, Bay11-7085 only suppressed the activity of NF- κ B, but not Akt. These results demonstrated that Akt is located upstream of NF- κ B and that it directly upregulates NF- κ B activity through degradation of I κ B α in TNBC cells.

The aim of the present study was to investigate the regulatory mechanism of STC-1 expression in TNBC cells. Elevated STC-1 expression was associated with a poor prognosis in TNBC patients, and also increased the invasiveness of TNBC cells. STC-1 expression was higher in TNBC cells than non-TNBC cells. In addition, basal levels of STC-1 expression in TNBC cells were completely suppressed by LY294002 or Bay11-7085 treatment. In contrast, STC-1 expression was increased by CA-Akt or NF- κ B overexpression in Hs578T TNBC cells. Interestingly, we found that CA-Akt overexpression also triggered the phosphorylation of NF- κ B and the degradation of I κ B α in Hs578T TNBC cells. We conclusively demonstrated that the PI-3K/Akt/NF- κ B signaling pathway plays a pivotal role in STC-1 expression in TNBC cells. Our results also suggest that STC-1 may be a promising therapeutic target in TNBC patients.

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