Effect of SALL4 on the Proliferation, Invasion and Apoptosis of Breast Cancer Cells

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Abstract

Objective: We aimed to identify the expression of Sal-like 4 (SALL4) in breast cancer tissues and to explore the role of this gene in the carcinogenesis of breast cancer cells. Methods: A total of 62 paired breast cancer and noncancerous tissue samples were obtained from patients with breast cancer. SALL4 expression patterns and their association with clinicopathological characteristics were investigated by qRT-PCR, western blotting, and immunochemistry in breast cancer tissues. After the knockdown of SALL4 by short hairpin RNAs (shRNAs), the proliferative, invasive, and apoptotic abilities of MDA-MB-435 and MDA-MB-468 cells (breast cancer cell lines) were measured by colony formation and CCK-8 assays, wound healing and transwell assays, and flow cytometry, respectively. Results: SALL4 expression was higher in breast cancer tissues than that in the paired noncancerous tissues, and increased SALL4 expression in tumor tissues was closely related to tumor size and lymphatic metastasis. Furthermore, functional experiments revealed that SALL4 knockdown inhibited the cell proliferation, induced cell cycle arrest in G0/G1 phase and apoptosis, and decreased the ability of migration and invasion in breast cancer cells. Additionally, our study first demonstrated that SALL4 played a critical role in modulating the tumorigenicity of breast cancer cells via the WNT/β-catenin signaling pathway. Conclusions: Our results suggest that the expression of SALL4 is upregulated in breast cancer, and this upregulation is involved in the regulation of cell growth, invasion, and apoptosis. Hence, SALL4 may be a promising target for diagnosis and therapy in patients with breast cancer.

Keywords

SALL4, breast cancer, proliferation, invasion, apoptosis

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Introduction

Breast cancer is one of the most common malignancies and represents a leading cause of cancer-related death in women worldwide.1-2 It has been reported that 246,660 new cases of breast cancer and 14% of deaths due to breast cancer occurred in the United States in 2016.3 Current treatment approaches such as surgery, chemotherapy, and radiotherapy, among others, have contributed to increased survival rates.4 However, tumor recurrence and metastasis frequently occur in advanced cancer with a poor prognosis. Therefore, it is important to search for molecular therapeutic targets and to identify new biomarkers for early diagnosis and treatment in breast cancer.

Sal-like 4 (SALL4) is a zinc-finger transcription factor that is a member of the SAL gene family.5 It was originally identified as a homeotic gene of the Drosophila spalt (sal), and it plays an essential role in embryonic development. In humans, mutations in SALL4 can cause Okihiro syndrome, that is characterized by limb deformities and loss of eye movement.6,7 Additionally, ectopic expression of SALL4 has been demonstrated to play a crucial role in tumorigenesis and cancer

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Table 1. Relationship Between SALL4 Expression and Clinicopathological Features of Breast Cancer Patients.

| Characteristics          | Low N | %  | High N | %  | P value |
|--------------------------|-------|----|--------|----|---------|
| Total                    | 21    | 41 |        |    |         |
| Age (years)              |       |    |        |    |         |
| ≤60                      | 16    | 76.2% | 32    | 78% | 0.868   |
| >60                      | 5     | 23.8% | 9     | 22% |         |
| Histological Type        |       |    |        |    |         |
| IDC                      | 19    | 90.5% | 38    | 92.7% | 0.762   |
| Other types              | 2     | 9.5% | 3     | 7.3% |         |
| Tumor size (cm)          |       |    |        |    |         |
| ≤2                       | 8     | 38.1% | 6     | 14.6% | 0.037a  |
| >2                       | 13    | 61.9% | 35    | 85.4% |         |
| Lymph nodes              |       |    |        |    |         |
| Positive                 | 4     | 19% | 24     | 58.5% | 0.003a  |
| Negative                 | 17    | 81% | 17     | 41.5% |         |
| TNM stage                |       |    |        |    |         |
| I                        | 0     | 0   | 4      | 9.8% | 0.182   |
| II                       | 21    | 100% | 35    | 85.3% |         |
| II-IV                    | 0     | 0   | 2      | 4.9% |         |

*p < 0.05; IDC, invasive ductal carcinoma; TNM, tumor node metastasis.

progression. High SALL4 expression levels have been observed in numerous malignancies, including hepatocellular carcinoma, colorectal cancer, germ cell tumors, leukemias, breast cancer, and lung cancer. The SALL4 gene is a key regulator of various tumor cell behaviors. For example, the silencing of SALL4 induced cell apoptosis and cell cycle arrest in human leukemic cells, and overexpression of SALL4 in human gastric cancer cells promoted cell growth and metastasis both in vitro and in vivo. These data suggest that SALL4 plays an oncogenic role in these malignancies. However, its biological functions and clinical value in the context of breast cancer remain to be elucidated.

In this study, we first determined the expression of SALL4 in breast cancer tissues and analyzed the possible correlation of its expression with clinical outcomes. Subsequently, we investigated the effects of SALL4 knockdown on various cellular behaviors, including cell proliferation, cell cycle function, apoptosis, migration, and invasion in breast cancer cells.

Materials and Methods

Tissue Specimens

This study was approved by the Ethics Committee of China Medical University (No.2015-017) and informed consent was obtained from each patient. A total of 62 paraffin-embedded breast carcinoma samples (from January 2005 to March 2010) were collected from The First Affiliated Hospital of China Medical University. The clinical information of the patient samples, including age, histological type, tumor size, lymph nodes metastasis, and TNM stage, is summarized in Table 1. Thirty breast cancer specimens and their paired adjacent noncancerous tissues (located more than 5 cm away from cancer tissues) were frozen and stored in liquid nitrogen until further use.

Cell Lines and Transfection

The cell lines used included the human breast cancer cell lines MDA-MB-231, MDA-MB-435, MDA-MB-468, and SKBR3, which were obtained from the cell bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FBS (Gibco) and 100 units/ml penicillin-streptomycin (Gibco) at 37 °C under a 5% CO2 atmosphere. To construct shRNA vectors targeting SALL4, 2 different sequences were used in MDA-MB-468 and MDA-MB-435 cells, respectively. (i) sense: 5'-GATCCCCGTGCGCAA CATTAATGTGATCTCAAGATAGACAT CACTATTTTT-3'; antisense: 5'-AGCTAAAAGGTGGCCA ACATGAATGTATGCTC

CTTGAATCACTATTATGGTGCCACGG-3'; (ii) sense: 5'-GATCCCGCCCAT GATGATGTTCATGATTCAGATCGATGACAT CATCATGGCCTTTTTT-3'; antisense: 5'-AGCTAAAAGGAC CATGATGATGTTCATGATCTCTTGAATCATGATGACAT CATCATATGCGGG-3'. The double-stranded oligonucleotides were inserted into the pRNA-H1.1/Adeno virus vector at BamHI/HindIII restriction enzyme sites (GenScript, Nanjing, China). One control sequence (Control shRNA) was selected and cloned into vector. The cells (3 x 10^5) were plated into 6-well plates. After 24 h, a mixture of SALL4 shRNA plasmids and LipoFectamine™ 2000 (Invitrogen, Carlsbad, CA) was added to each well containing cells according to the manufacturer’s protocol. The cells were then incubated for further experiments after transfection.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from tumor tissues or cells using a simple total RNA kit (BioTeke, Beijing, China), and the RNA was then reverse transcribed into cDNA using the M-MLV Reverse Transcriptase (BioTeke, Beijing, China). Quantitative real-time PCR was performed using a SYBR-Green method (Takara, Beijing, China) in an Exicycler™ 96 real time (RT)-PCR machine (Bioneer, Daejeon, Korea). GAPDH was used as an endogenous control for normalization. The primers used for this study included: SALL4, 5'-CCGCACTGAGATTGGTGATGG-3', and 5'-CCGCACTGAGATTGGTGATGG-3'; GAPDH, 5'-GAAGGTCGGAGTCAACG-3', and 5'-CCTGGAAGATGGTGATGG-3'.

Protein Extraction and Western Blotting

Proteins were extracted from breast cancer tissues or cells using RIPA lysis buffer (Beyotime, Shanghai, China). The
concentration of the extracted protein was measured using a bicinchoninic acid (BCA) kit (Thermo Fisher Scientific). A total of 40 μg proteins from each sample were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with the appropriate primary antibody, and this was followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Specific primary antibodies included anti-SALL4 (1:1000, Proteintech, Wuhan, China), anti-cleaved caspase-3, anti-cleaved-PARP (1:1000, Abcam, Cambridge, UK), anti-Bcl-2, Bax, anti-Wnt3a (1:1000, Sangon, Shanghai, China). Immune complexes were visualized using an enhanced chemiluminescence system (Beyotime, Shanghai, China).

**Immunohistochemistry**

Expression of SALL4 in paraffin-embedded breast cancer tissues was measured by immunohistochemistry as described previously.\(^{16}\) The staining intensity was scored from grade 0 to 3 (0, negative; 1, pale yellow staining; 2, moderate yellow staining; 3, brown staining). The percentage of the stained area was scored as 2 (51%–100%), 1 (11%–50%), 0 (0–10%). The final score (range 0–5) for SALL4 expression was calculated according to the sum of the intensity scores and area scores. Cancer tissues possessing final scores of 0–2 and 3–5 were classified as exhibiting low and high expression, respectively. All specimens were independently evaluated by a pathologist.

**Proliferation Assay**

For the colony formation assay, cells were seeded into 35-mm dishes at a density of 300 cells per dish, and they were then incubated with suitable culture medium for 14 days. The cells were then fixed and stained with Giemsa solution. Colonies consisting of at least 50 cells were counted under a microscope. For the CCK-8 assay, cells were seeded into 96-well plates at a density of 3 × 10^3 cells per well. The CCK-8 reagent (10 μl) was added to each well at the indicated time point (24, 48, 72, or 96 h), and this was followed by incubation at 37 °C for 1 h. The absorbance at 490 nm was determined using an automatic microplate reader (BioTek, Vermont, USA).

**Flow Cytometric Analysis**

For cell cycle analysis, the collected cells were fixed in 70% cold ethanol at 4 °C for 2 h, following which, they were incubated in staining buffer containing RNase A and propidium iodide (PI) in the dark at 37 °C for 30 min. Cell cycle analysis was performed using a FACS Calibur Flow cytometer (BD, Franklin Lakes, NJ, USA). Cell apoptosis was detected using an Annexin V-FITC/PI apoptosis detection kit (KeyGen Biotech, Nanjing, China) according to the manufacturers’ instructions, and apoptosis was analyzed by flow cytometry.

**Wound Healing Assay**

Cells were grown to 90% confluence in the plates. Then the wounds were scratched using a sterile pipette tip. The remaining cells were washed twice in serum-free culture media, and wound closing was then observed. Images at 12 and 24 h were captured using a phase contrast microscope and the percent of wound closure was calculated.

**Transwell Assay**

The invasive ability of tumor cells was determined using a transwell system (Corning Incorporated, Corning, NY, USA). Cells were seeded into the upper chamber at a density of 1 × 10^4 per chamber. The lower chamber was filled with 800 μL of medium containing 30% FBS. After 24 h incubation, the cells remaining in the upper chamber were removed using cotton-tipped swabs, and the cells that migrated to the bottom of the filter were fixed and stained with crystal violet dyes. The number of invaded cells was photographed and counted under an inverted phase contrast microscope.

**Statistical Analysis**

Data are presented as mean ± standard deviation (S.D) of 3 independent experiments and all data were processed using GraphPad Prism 5.0 software (San Diego, CA, USA). A paired t-test was used to identify the differential expression of SALL4 in breast cancer tissues and paired noncancerous tissues. The Chi-square test was used to verify the relationship between SALL4 expression and the clinicopathological features of breast cancer patients. One-way ANOVA analysis was performed for all other statistical analyses. A value of P < 0.05 was considered statistically significant.

**Results**

**SALL4 Was Highly Expressed in Breast Cancer Tissues**

We first examined SALL4 expression in 30 pairs of breast cancer tissues and their adjacent noncancerous tissues. qRT-PCR results revealed that SALL4 mRNA levels were significantly upregulated in 30 breast cancer tissues compared to levels in the paired adjacent noncancerous tissues (P < 0.05, Figure 1A), and western blot results also confirmed this elevation of SALL4 expression (Figure 1B, Figure S1). We next performed immunohistochemical analyses of SALL4 expression in 62 breast cancer samples. As shown in Figure 1C, 41 of the 62 cancer samples (66%) possessed high-level SALL4 staining expression. According to the SALL4 expression levels, the 62 samples were divided into 2 groups (highly expressed group [n = 41] vs. low expressed group [n = 21]). We further investigated the association between SALL4 expression and clinicopathological characteristics. As shown in Table 1, there is a significant difference between the 2 groups with respect to the degree of tumor size (P = 0.037) and lymphatic metastasis (P = 0.003). However, with respect
to age, histological type, and TNM stage, no significant differences were observed. These results suggest that the expression of SALL4 significantly correlated with the degree of tumor size and lymphatic metastasis in breast cancer.

**SALL4 Expression Is Markedly Inhibited by siRNA in Breast Cancer Cell Lines**

To verify the biological functions of SALL4 in breast cancer cells, we first examined the mRNA levels of SALL4 in 4 breast cancer cell lines, including MDA-MB-231, MDA-MB-435, MDA-MB-468, and SKBR3. The qRT-PCR results revealed that SALL4 expression was higher in MDA-MB-435 and MDA-MB-468 cells relative to that in MDA-MB-231 and SKBR3 cells (Figure 2A). Thus, MDA-MB-435 and MDA-MB-468 cells were transfected with SALL4 shRNA or control shRNA. Figure 2B-E shows that both the mRNA and the protein levels of SALL4 were markedly downregulated in SALL4 shRNA-transfected cells compared to levels in the parental or control shRNA-transfected cells (P < 0.01), thus indicating an efficient inhibition of SALL4 expression by RNA interference in breast cancer cells.

**SALL4 Knockdown Inhibited Proliferation and Induced Cell Cycle Arrest in Breast Cancer Cells**

MDA-MB-435 and MDA-MB-468 cells were transfected with SALL4 shRNA, and we subsequently investigated the effect of SALL4 on cell proliferation. The results of a colony formation assay showed that fewer colonies were presented in the SALL4-silenced cells compared to colony numbers in the control shRNA-transfected cells (Figure 3A, B). Additionally, the CCK-8 assay results demonstrated that the cell proliferation rate was remarkably decreased after SALL4 knockdown (Figure 3C, D; P < 0.01). Furthermore, the influence of SALL4 on cell cycle distribution was detected using flow cytometry. Knockdown of SALL4 resulted in cell cycle arrest in the G0/G1 phase, as evidenced by increased cell numbers in the G0/G1 phase and decreased numbers in the S and G2/M phases (Figure 3E, F; P < 0.01 vs. control). There was no significant difference observed between parental and control shRNA-transfected cells. These results indicated that the downregulation of SALL4 expression can inhibit proliferation and induce cell cycle arrest.
Silencing of SALL4 Induced Apoptosis in Breast Cancer Cells

The apoptosis rates of MDA-MB-435 and MDA-MB-468 cells were analyzed by flow cytometry. As shown in Figure 4A, B, the proportion of apoptotic cells in SALL4-silenced cells (19.74 ± 2.69% in MDA-MB-435 cells and 17.574 ± 3.03% in MDA-MB-468 cells, respectively) was significantly higher than that of the control groups (3.53 ± 0.74% in MDA-MB-435 cells and 4.46 ± 0.66% in MDA-MB-468 cells, respectively), indicating an enhanced cell apoptosis in SALL4-silenced cells. Additionally, the expression levels of apoptotic-related proteins, including bcl-2, bax, cleaved-caspase-3, and cleaved-PARP, were further examined. Western blot results demonstrated that the silencing of SALL4 markedly upregulated the levels of bax, cleaved-caspase-3, and cleaved-PARP, and downregulated bcl-2 levels (Figure 4C, D, P < 0.01). These results indicated that the silencing of SALL4 expression can induce apoptosis in breast cancer cells.

SALL4 Knockdown Inhibited Migration and Invasion of Breast Cancer Cells

We further investigated the functional role of SALL4 in cell migration and invasion. Results from wound-healing assays revealed that knockdown of SALL4 in both MDA-MB-435 and MDA-MB-468 cells resulted in significantly reduced cellular mobility at 12 h and 24 h compared to that of controls (Figure 5A-D, P < 0.01). The results of a transwell invasion assay also revealed that knockdown of SALL4 reduced cell invasion in breast cancer cells compared to that observed in controls (Figure 5E, P < 0.01). Together, these results suggest that the silencing of SALL4 inhibits cell migration and invasion in breast cancer cells.

Down-Regulation of SALL4 Inhibited the Wnt/β-Catenin Signaling Pathway in Breast Cancer Cells

The Wnt/β-catenin signaling pathway is known to play a critical role in tumorigenesis and cell proliferation. Previous...
studies revealed that SALL4 may enhance the activity of the Wnt/β-catenin signaling pathway in cervical cancer cells. Wnt3a is a crucial molecule in the Wnt/β-catenin signaling pathway. Therefore, we measured the expression of Wnt3a using western blotting in MDA-MB-468 cells and MDA-MB-435 cells. As shown in Figure 6A-B, down-regulation of SALL4 in those cells remarkably decreased the expression of Wnt3a in the cytoplasm compared to that of control cells, indicating that SALL4 modulates the tumorigenicity of breast cancer cells via the Wnt/β-catenin signaling pathway.

Discussion

SALL4 has been reported to be highly expressed in malignancies and to be involved in cancer progression. In this study, we found that SALL4 expression was upregulated in breast cancer tissues relative to that in the paired adjacent noncancerous tissues. High SALL4 expression was associated with increased breast cancer malignancy. Using transfection of SALL4 knock-down constructs, we demonstrated that SALL4 silencing inhibited the proliferation of breast cancer cells, caused cell cycle arrest at the G0/G1 phase, and induced cell apoptosis. Furthermore, knockdown of SALL4 suppressed the migratory and invasive properties of breast cancer cells.

Additionally, we found that high expression of SALL4 was significantly correlated with tumor size and lymph node metastasis. This result suggests that SALL4 expression may be associated with the progression of breast cancer. However, no statistical correlation was found between SALL4 expression and other parameters such as age, histological type, and TNM stage. One reason for this may be that most of the collected cases were of breast infiltrating duct carcinoma (IDC), that
presented in the TNM II stage. The sample size must be expanded for further verification.

It has been demonstrated that the genesis and development of tumors are both associated with the imbalance between cell proliferation and apoptosis in tumor cells. The tumor grows when apoptosis is arrested or the proliferation exceeds apoptosis. The cell cycle is the fundamental process of cell proliferation, and it is regulated by a series of cell cycle-related genes and proteins, such as cyclins and cyclin-dependent kinases. Apoptosis is regulated by multiple apoptosis-related proteins, such as Bcl-2 and Bax. Previously, SALL4 has been shown to be essential for the regulation of cell

**Figure 4.** Silencing of SALL4 induced apoptosis in breast cancer cells. A and B, Flow cytometry analysis of cell apoptosis in different groups of MDA-MB-435 and MDA-MB-468 cells. Cells were double stained with FITC-conjugated anti-Annexin V antibody and PI, and then analyzed by flow cytometry. The cells in the lower and upper right quadrants were considered as early and late apoptosis. C and D, Western blot analysis of apoptotic-related bcl-2, bax, cleaved-caspase-3, and cleaved-PARP in both MDA-MB-435 and MDA-MB-468 cells. GAPDH was served as the internal control for grayscale comparison (**P < 0.01).
proliferation and apoptosis in tumor cells. The silencing of SALL4 resulted in cell cycle arrest and induced apoptosis in chronic myeloid leukemia cells. Forced SALL4 expression in liver cancer cells increased cell proliferation and reduced the G1 phase cell population. To further evaluate the functional roles of SALL4 in breast cancer, we performed knockdown experiments and functional assays in breast cancer cells. Consistent with previous data, our results showed that the silencing of SALL4 in breast cancer cells inhibited cell proliferation and induced cell cycle arrest in the G0/G1 phase. These results indicate that SALL4 may regulate the cell cycle in breast cancer cells, thereby modulating proliferation and tumor growth. Additionally, we found that silencing of SALL4 increased the number of apoptotic cells, reduced the expression of anti-apoptotic Bcl-2, and increased the expression of Bax, cleaved caspase-3, and cleaved-PARP. These results indicate that SALL4 may play an inhibitory role in the apoptosis of breast cancer cells, to thereby modulate tumor growth.

The migration and invasion of tumor cells are associated with cancer metastasis. Previous studies have shown that the SALL4 level is highly correlated with lymph node metastasis of gastric cancer and predicts a malignant phenotype of breast invasive ductal carcinoma. The silencing of SALL4 inhibited the migration and invasion properties of endometrial cancer cells in vitro. Additionally, SALL4 was involved in the regulation of epithelial-mesenchymal transition (EMT) in breast cancer cells.

Figure 5. Silencing of SALL4 inhibited breast cancer cell migration and invasion in breast cancer cells. (A and B) Cell migratory abilities in both MDA-MB-435 and MDA-MB-468 cells were determined by wound-healing assays and (C, D) quantification of the wound closure capacities (scale bar = 200 μm). (E) Cell invasive abilities in both MDA-MB-435 and MDA-MB-468 cells were determined by transwell assays. Representative images (left) and quantification (right) were shown (scale bar = 100 μm) (* P < 0.05; ** P < 0.01).
In agreement with these findings, our results indicated that knockdown of SALL4 impeded the migratory and invasive abilities of breast cancer cells. Furthermore, the expression of SALL4 was closely correlated with lymphatic metastasis in breast cancer. These results suggest that SALL4 promotes the motility of breast cancer cells to thereby promote metastasis.

It had been reported that SALL4 promotes intrahepatic cholangiocarcinoma cell proliferation by activating Wnt/β-catenin signaling. Previous studies demonstrated that SALL4 can regulates Wnt3a expression, which promote tumor progress and metastasis. Our studies have demonstrated for the first time that down-regulation of SALL4 decreased the expression of Wnt3a at protein levels in breast cancer cells, indicating that SALL4 promotion of Wnt/β-catenin signaling is a mechanism for SALL4 regulation of breast cancer. Though we found that SALL4 function in breast cancer was associated with the Wnt/β-catenin signaling pathway, more studies are required to further elucidate the specific mechanism between SALL4 and Wnt3a regulation in breast cancer.

In summary, our study provides clinical and experimental evidence to support the idea that SALL4 may be associated with tumor progression in breast cancer. The biological functions of SALL4 in breast cancer cells are involved in the regulation of cell proliferation, apoptosis, cell cycle progression, and cell invasion. Therefore, SALL4 may represent a promising target for breast cancer diagnosis and therapy.

Authors’ Note
The datasets used during the current study are available from the corresponding author on a reasonable request. This study was approved by the Ethics Committee of China Medical University (No. 2015-017).

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material
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