Int J Clin Exp Med 2022;15(6):192-198
www.ijcem.com /ISSN:1940-5901/IJCEM0138936

Original Article

Downregulation of Sp1 suppresses cell proliferation and migration in breast cancer

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Received September 6, 2021; Accepted May 20, 2022; Epub June 15, 2022; Published June 30, 2022

Abstract: Background: Specificity protein 1 (Sp1) is a transcription factor which has been associated with metastasis in several cancers. Recent study has found that Sp1 expression was upregulated in breast cancer. However, the precise function of Sp1 in breast cancer is still unclear. Objectives: The aim of this study is to explore the expression of Sp1 and its effect on proliferation and migration in breast cancer. Methods: Expression of Sp1 in breast cancer and adjacent tissues were detected by immunohistochemistry. Quantitative real time PCR (qPCR) and western blotting assays were utilized to measure mRNA and protein expression levels of cells, respectively. Short hairpin RNA (shRNA) transfection was performed to suppress the expression of Sp1. MTT assay and Transwell assays were used to determine the proliferative and migratory ability of cells. Results: The expression of Sp1 was significantly increased in breast cancer tissues compared with adjacent normal tissues. The knockdown of Sp1 significantly inhibited the Sp1 protein and mRNA expression, and downregulation of Sp1 significantly suppressed the proliferation and migration of MCF-7 cells. Conclusions: Sp1 may be involved in metastasis of cancer cell and can be considered as potential therapeutic target for breast cancer treatment.

Keywords: Specificity protein 1, breast cancer, invasion, metastasis, transcription factor

Introduction

Breast cancer is one of the most prevalent cancers in women. Since the 1980s, the incidence of breast cancer has increased worldwide [1]. Studies showed that 12.5% of American women might be diagnosed with breast cancer during their lifetime [2]. To improve therapy and recovery, many studies have concentrated on defining the epigenetic characteristics related to tumor progression and patient prognosis [3-5]. Despite the reduced rates of mortality and metastasis in patients with breast cancer, metastases at distant sites remain the primary cause of cancer deaths [6].

Specificity protein 1 (Sp1), belonging to the Sp transcription factor family including Sp2, Sp3, and Sp4, contains C_2H_2-type zinc fingers. The Sp family can bind to cellular and viral genes with GC-rich sequences to regulate cell survival and cell growth, as well as the development and progression of tumors [7, 8]. Sp1 and Sp3 are expressed universally and have the consensus-binding sites identical to those of Sp2 and Sp4, suggesting that they can positively or negatively regulate gene expression [9, 10]. Moreover, the angiogenic potential of cancer tissue and poor patient prognosis is associated with the increased expression of Sp1 [11, 12].

Although Sp1 has been correlated with the incidence and development of cancer, it is unclear whether the expression of Sp1 can influence the biological behavior of breast cancer. Here, the invasiveness of breast cancer increased by Sp1 overexpression in clinical samples was investigated. Further, we revealed the underlying role and potential function of Sp1 in the proliferation and migration of MCF-7 cells. This study may provide insight into the recurrence and metastasis of breast cancer and suggests that Sp1 may be a potential target in the treatment of breast cancer patients.

Materials and methods

Patients and clinical samples

Fifty-four human breast cancer samples and adjacent normal tissue were obtained from

Table 1. Correlation between Sp1 protein expression and clinicopathological features in patients with breast cancer [*n* (%)]

Variable	*n*	Negative	Positive	χ^2	*P*-value
Age (years)					
<55	29	9 (31.0)	20 (69.0)	0.331	0.565
≥55	25	6 (24.0)	19 (76.0)		
Tumor size (cm)					
<3	23	7 (30.4)	16 (69.6)	0.141	0.707
≥3	31	8 (25.8)	23 (74.2)		
Histological grade					
I	11	5 (45.5)	6 (54.5)	2.310	0.315
II	24	5 (20.8)	19 (79.2)		
III	19	5 (26.3)	14 (73.7)		
TNM stage					
I+II	26	11 (42.3)	15 (57.7)	5.277	0.022
III+IV	28	4 (14.3)	24 (85.7)		
Lymph node metastasis					
No	28	12 (42.9)	16 (57.1)	6.591	0.010
Yes	26	3 (11.5)	23 (88.5)		

patients who had an initial surgery from January 2018 to December 2019 in the Pathology Department of the First Affiliated Hospital, Hainan Medical University, China. The inclusion criteria were female patients, aged from 18 to 80 years, who did not undergo chemotherapy or endocrine therapy and were neither pregnant nor lactating. The exclusion criteria were the lack of complete histopathological data and patients with other systematic dysfunctions. This study was approved by the Ethics Committee of Hainan Medical University (Hainan, China). The clinicopathological signatures of the cohort are shown in **Table 1**. Tumor differentiation and tumor stages were graded and classified according to the Edmondson-Steiner grading system and TNM staging system, respectively.

Immunohistochemistry

After fixation of the specimens in 10% formaldehyde for 24 h, the samples were dehydrated and embedded in paraffin. The sections were rehydrated in a graded ethanol series following cutting and dewaxing of the sections (4 μm) in xylene. Heat-induced epitope retrieval was performed using citrate buffer (pH = 6, ZSJB-Bio, Beijing, China) and a microwave oven. To block endogenous peroxidase activity, the samples were incubated with 3% hydrogen peroxide for 10 min after antigen retrieval. The samples were incubated in rabbit monoclonal Sp1 antibody (1:200, ab124804, Abcam, Cambridge, UK) overnight at 4°C, then in horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG polymer (PV-6001, ZSJB-Bio, Beijing, China) for 20 min and treated with the DAB detection kit (ZSJB-Bio, Beijing, China) to visualize positive staining. The slides were observed and captured by microscopy (CX41, Olympus, Japan).

The immunohistochemical expression was scored from 0 to 3 (score 0: negative; scores of 1 - 3: positive), and calculated as follows. The positive tumor cells were scored as 0, 1, 2, or 3 based on the percentage of positive cells in the tumor tissue (<5%, 5%-25%, 26%-50%, and >50%, respectively) and the signal intensity was scored as 0, 1, or 2, which indicated no staining, canary, and claybank staining, respectively. Lastly, the final score was obtained by multiplying the positive cell score and signal intensity score.

Cell line and cell culture

The MCF-7 human breast cancer cell line was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in Dulbecco's minimum essential medium (DMEM, Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China) at 37°C in a humidified atmosphere containing 5% CO_2.

Cell transfection

MCF-7 cells (1×10^5 cells/mL) were inoculated and grown in six-well plates. After the MCF-7 cells reached a density of approximately 80%, 5 μg/mL of shRNA targeting Sp1 (Genesil, Wuhan, China) or negative control oligonucleotides were transfected using Lipo2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Immunocytochemistry

After transfection for 72 h, 1×10^5 cells/mL of MCF-7 cells were seeded onto coverslips and

grown overnight in six-well plates. The cells were treated with 4% paraformaldehyde for 10 min, 0.5% TritonX-100 for 20 min, 3% H_2O_2 for 10 min, and sheep serum for 10 min. Then, the cells were incubated with HRP-conjugated goat anti-rabbit IgG polymer (PV-6001, ZSJB-Bio, Beijing, China) for 20 min, and rabbit Sp1 monoclonal antibody (1:200, ab124804, Abcam, Cambridge, UK) overnight at 4°C, and then treated with the DAB detection kit (ZSJB-Bio, Beijing, China) to visualize positive staining. The cells on the coverslips were observed and captured by microscopy (CX41, Olympus, Japan).

Western blot analysis

The Epiquik whole-cell extraction kit (Epigentek, NY, USA) was used to extract the total cellular protein from the MCF-7 cells. After transfection of the cells for 72 h, they were rinsed in cold phosphate-buffered saline (PBS) and 20 μg of protein extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were then blocked with 5% nonfat milk powder, and incubated overnight with rabbit Sp1 monoclonal antibody (1:1,000, ab12-4804, Abcam, Cambridge, UK). Specific reactive bands were detected using HRP-labeled anti-rabbit IgG secondary antibody (1:1,000, Beyotime, Shanghai, China) for 1 hour at room temperature. Visualization of the immunoreactive bands was performed with a DAB detection kit (ZSJB-Bio, Beijing, China). Anti-GAPDH antibody was used as a loading control (1:1,000, Goodhere, Hangzhou, China).

Quantitative real-time PCR

The expression of Sp1 and the reference gene GAPDH was determined by quantitative real-time PCR (qPCR). Total RNA was isolated from the MCF-7 cells after transfection for 48 h using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The reverse transcription of 2 μg of RNA into cDNA was done using a reverse transcriptase kit (Beyotime, Shanghai, China). qPCR was performed using the SYBR Green kit (Tiangen, Beijing, China) in a 20-μL total reaction volume. Stratagene Mx3000P software (Agilent Technologies, Inc., Santa Clara, CA, USA) was employed to analyze the Sp1 expression levels, which are presented as fold-changes calculated based on the $2^{-\Delta\Delta Ct}$ normalization method. All the above experiments followed the instructions of the manufacturers. The primer sequences for Sp1 were forward, TGGTGGGCAGTATGTTGT and reverse, GCTATTGGCATTGGTGAA, and those for GAPDH were forward, GGAGTCAACGGATTTGGT and reverse, GTGATGGGATTTCCATTGAT.

Cell proliferation assay

MTT cell proliferation assays were performed according to the manufacturer's instructions (Beyotime, Shanghai, China) with minor modification. Briefly, 1×10^4 MCF-7 cells per well were inoculated in a 96-well plate after transfection for 24 h, 48 h, and 72 h. After 24 h, 10 μL of MTT (5 mg/mL) was added to the 96-well plate and incubated for 4 h. Then, 100 μL Formanzan dissolved liquid was added and incubated for 4 h. Plates were read by a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm absorbance.

Cell migration assay

A 24-well Transwell chamber covered with an 8-μm pore membrane (Corning, Lowell, MA, USA) was used to perform the cell migration assay. After transfection for 48 h, MCF-7 cells were trypsinized in serum-free media and added to the upper chamber along with DMEM medium supplemented with 20% FBS. After 24 h, the cells on the upper surface of the insert were removed, and the cells on the lower surface of the insert were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. The number of migrated cells was quantified in at least five random fields for each slide using a microscope (CX41, Olympus, Japan) at 100 × magnification.

Statistical analysis

Statistical analyses were carried out using SPSS 21.0 (IBM, Armonk, NY, USA). The chi-squared test was used to analyze the correlation of Sp1 expression levels with the clinicopathological variables of the patients. Differences between the two groups were analyzed by the independent-samples t-test or the general linear model (repeated measures). A *P*-value of <0.05 was considered statistically significant.

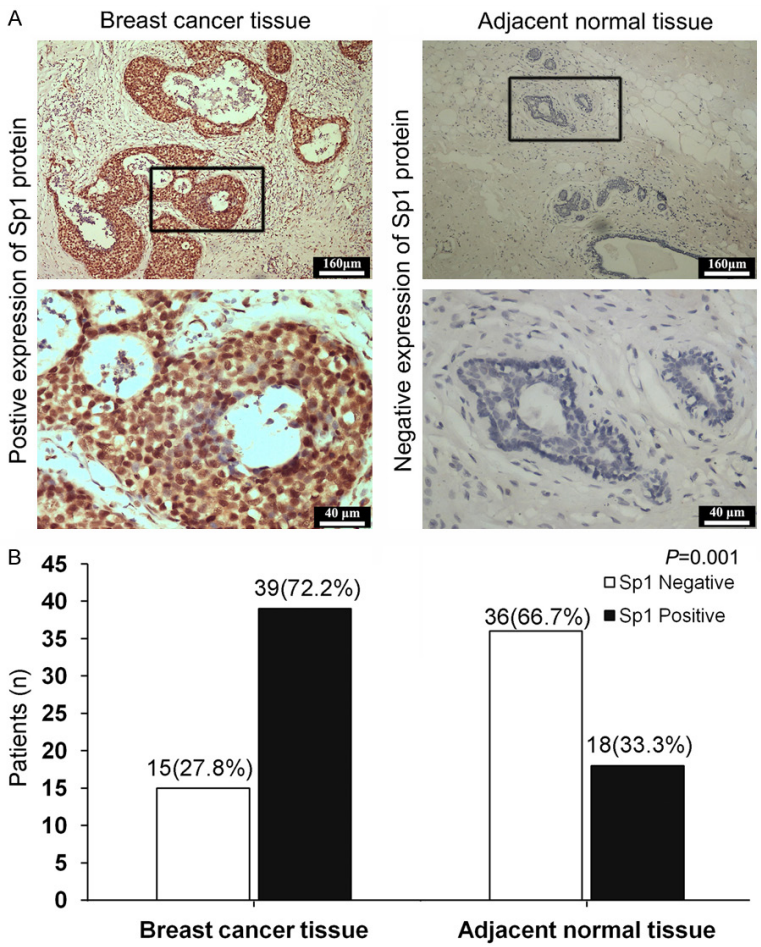

Figure 1. Sp1 protein expression of breast cancer and adjacent normal tissues. A. Representative photograph of Sp1 protein expression in breast cancer and adjacent normal tissues were detected by immunohistochemistry (100 ×, 400 ×). B. Sp1 protein of positive expression was significantly higher in the breast cancer than that in the adjacent normal tissues.

Results

High Sp1 expression in breast cancer

Initially the expression level of Sp1 in breast cancer and adjacent normal tissues was detected, and positive Sp1 proteins were seen as brown particles mostly distributed in nuclei of the cancer cells (**Figure 1A**). As shown in **Figure 1B**, the Sp1 expression level increased in the breast cancer tissues compared to the adjacent normal tissues (P = 0.001). The expression level of Sp1 was related to the clinical stage (P = 0.022) and lymph node metastasis (P = 0.010). Moreover, there was no correlation with age (P = 0.565), tumor size (P = 0.707), or histological grade (P = 0.315) (**Table 1**).

Low Sp1 expression in MCF-7 cells after transfection

The Sp1 expression level in the Sp1 knockdown group was decreased compared to the negative control group (**Figure 2A**). Sp1 protein and mRNA expression were also significantly decreased in the Sp1 knockdown group compared to the negative control group (**Figure 2B**, **2C**).

Sp1 knockdown suppresses the proliferation of MCF-7 cells

Compared to the negative control group, the MTT assays showed that Sp1 knockdown highly decreased the proliferation of MCF-7 cells after transfection for 48 h and 72 h, and no significant differences were observed among the groups after transfection for 24 h (**Figure 3**).

Sp1 knockdown represses the migration of MCF-7 cells

Compared to negative control group, the Transwell assays showed that Sp1 knockdown decreased the number of cells that migrated into the lower side of the Transwell membranes (**Figure 4**).

Discussion

Cancer progression can be induced by uncontrolled cell proliferation or aggressive tumor cell metastasis, which leads to tumor growth and promotes tumor transfer to distant sites [13, 14]. Tumors are often caused by multiple mutations of oncogenes, as well as their products of signal transduction pathways, which regulate the onset and development of tumors [15-17]. Sp1 is a type of extensive nuclear transcriptional regulatory element, which exists in nearly all cells. It regulates the expression of genes containing proximal GC/GT-rich promo-

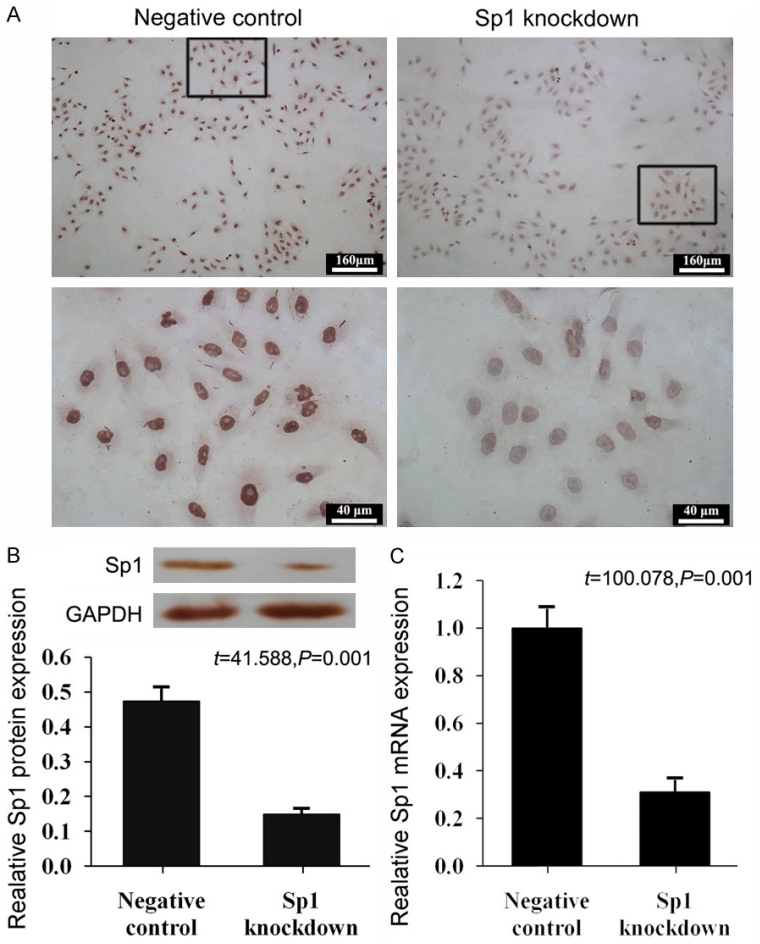

Figure 2. Sp1 expression of MCF-7 cells after transfection. A. Representative photograph of Sp1 protein expression in MCF-7 cells were detected by Immunocytochemistry (100 ×, 400 ×). B. Sp1 protein expression of MCF-7 cells by Western blot. C. Sp1 mRNA expression of MCF-7 cells by qPCR.

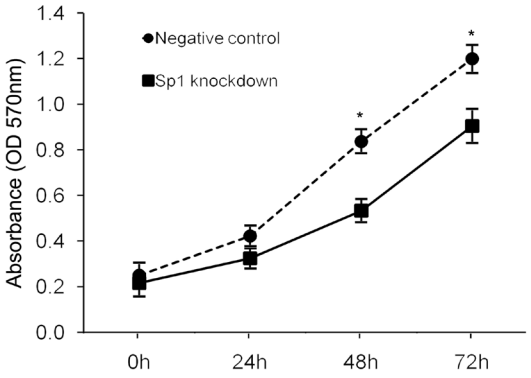

Figure 3. Sp1 knockdown suppresses the proliferation of MCF-7 cells. Cell proliferation of MCF-7 was determined by the MTT assay. Data are representative of three independent experiments, and presented as the mean ± SD; significant differences between groups were shown as *P<0.05.

ter elements involved in essential cell cycling, differentiation and apoptosis process, thus controlling the number of cellular processes [18]. The current evidence indicates that Sp1 acts as a signal transducer in the occurrence, development, and prognosis of multiple tumors. Sp1 as an upstream gene, regulated the expression of E-cadherin [19] and MALAT1 [20] in lung tumors and SHIP2 [21] in gastric cancer, while Sp1 as a downstream target gene was mediated by miR-520d-5p [22] in colorectal cancer, miR-205 [23] in esophageal squamous cell carcinoma, and miR-634 [24] in gastric cancer. Previous reports presented that the Sp1 expression levels were remarkably improved in hepatocellular carcinoma (HCC) tissues compared to adjacent normal tissues [25]. Further, Sp1 expression increased in HCC cell lines compared to normal hepatic cell lines [26]. These findings suggest that Sp1 may functionally participate in HCC occurrence and development.

To elucidate the role of Sp1 in the development of breast cancer, we measured Sp1 expression in breast cancer and normal tissues. We discovered that Sp1 expression in breast cancer was significantly increased compared to that in normal tissues and that the increased expression of Sp1 was related to the invasiveness of breast cancer in the clinical samples. Our results also showed that Sp1 knockdown in MCF-7 cells strongly hindered cell proliferation and cell migration *in vitro*, indicating that Sp1 expression was closely related to the biological behavior of MCF-7 cells. However, the possible molecular mechanism remains unclear. Sp1 can be used as an upstream regulatory gene or as a downstream target gene in the development of breast cancer. MiR-212-3p could inhibit vascular endothelial growth factor-A (VEGFA)

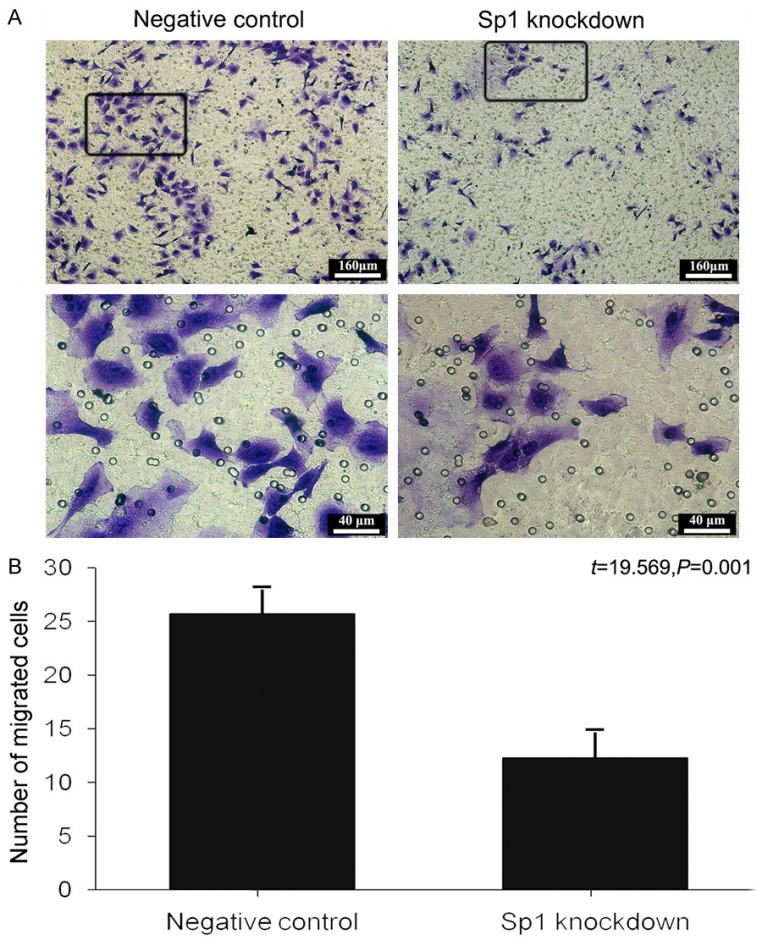

Figure 4. Sp1 knockdown represses the migration of MCF-7 cells. Cell migration of MCF-7 was determined by the transwell assay. The number of migrated cells through a transwell chamber was quantified under a light microscope at 100 × magnification (crystal violet staining).

expression through Sp1, thereby inhibiting angiogenesis and the progression of breast cancer [27]. Oncoprotein hepatitis B X-interacting protein (HBXIP) controlled the transcriptional regulation of ZEB1 by co-activating Sp1, thereby accelerating breast cancer growth [28]. Long non-coding RNA (lncRNA) terminal differentiation-induced non-coding RNA (TINCR) was aberrantly upregulated by SP1, which in turn, stimulated cell proliferation, anchorage-independent growth, and suppressed cell apoptosis in breast cancer [29]. Therefore, in the next step of our work, we will explore the molecular mechanisms of Sp1 in breast cancer.

In summary, this study provides novel insight into the mechanism of the growth regulation of MCF-7 cells and offers new targets and strategies for the treatment of breast cancer. Further studies are required to more fully elucidate the underlying molecular mechanisms of Sp1 in breast cancer development.

Disclosure of conflict of interest

None.

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