SREBP-1 is an independent prognostic marker and promotes invasion and migration in breast cancer

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Abstract. Re-programming of lipogenic signaling has been previously demonstrated to result in significant alterations in tumor cell pathology. Sterol regulatory element-binding protein 1 (SREBP-1) is a known transcription factor of lipogenic genes. Despite the fact that its functions in proliferation and apoptosis have been elucidated in recent studies, its role in tumor cell migration and invasion, particularly in breast cancer, remains unclear. In present study, the messenger RNA and protein expression levels of SREBP-1 in cancer tissues were observed to be overexpressed compared with those in matched para-cancerous tissues (P<0.01). SREBP-1 level was highly positively correlated with tumor differentiation (P<0.001), tumor-node-metastasis stage (P=0.044) and lymph node metastasis (P<0.001). High expression of SREBP-1 predicted poor prognosis in patients with breast cancer. Additionally, multivariate analysis revealed that SREBP-1 was an independent factor of 5-year overall and disease-specific survival in breast cancer patients (P<0.01). *In vitro* studies revealed that the suppression of SREBP-1 expression in both MDA-MB-231 and MCF7 cells significantly inhibited cell migration and invasion (P<0.01). The present data indicate that SREBP-1 plays a critical role in breast cancer migration and invasion, and may serve as a prognostic marker of this malignancy.

Introduction

Breast cancer is the most common malignant cancer and the major cause of mortality due to cancer in females worldwide (1), and the morbidity has increased gradually over recent years (2). Although the tremendous progress in diagnostic instruments and the development of standardized systematic therapy, including various combinations of surgery, radiation therapy, chemotherapy and hormone therapy (3), the prognosis for breast cancer patients is still not ideal. The high mortality rate is associated with the ability of breast cancer cells to metastasize to distant organs (4). Metastasis is a multistep process requiring cellular and environmental progresses to a secondary site (5). However, the regulatory mechanisms remain poorly understood. Therefore, the identification of critical pathways will help to discover novel therapeutic targets for breast cancer.

Increased evidence suggests that the lipogenic phenotype is a major characteristic of cancer (6,7). Current research links aberrant lipogenesis and cholesterogenesis with breast cancer development and progression (8). Sterol regulatory element-binding proteins (SREBPs) are important in regulating the gene expression of key enzymes involved in fatty acid and cholesterol biosynthesis (9,10), including SREBP-1a, SREBP-1c and SREBP-2. Previous studies have demonstrated that the aberrant expression of SREBP-1 is upregulated in several metabolic diseases, including diabetes mellitus, morbid obesity, hyperlipidemia and atherosclerosis (11). In addition to regulation by sterols, SREBP-1 has been reported to be stabilized and activated by the phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt oncogenic signaling pathway in cancer (12-14). The suppressed expression of SREBP-1 could inhibit cell growth, migration and invasion, and induce cell apoptosis in ovarian cancer (15). Pharmacological and genetic inhibition or reduction of SREBP-1 significantly induced glioblastoma cell death (16). Overexpression of SREBP-1 has been observed in atypically hyperplastic endometrium and endometrial cancer tissues, while knockdown of SREBP-1 dramatically inhibited the proliferating potential of endometrial cancer cells (17). The expression level of SREBP-1 is also elevated in certain types of tumors such as prostate and gastric cancer (18,19). However, the expression and function of SREBP-1 in human breast cancer remains to be fully elucidated.

In the present study, the expression levels SREBP-1 in breast cancer tissues were evaluated. The data indicated that the SREBP-1 level was correlated with prognosis of breast cancer, and suggested that it may serve as an independent prognostic factor in breast cancer. SREBP-1 promoted cell migration and invasion *in vitro*. Taken together, the present results demonstrated that SREBP-1 is pivotal for the tumorigenesis of breast cancer.

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Materials and methods

Ethical review. The Ethics Committee of Nanjing Medical University Affiliated Wuxi Second Hospital (Wuxi, Jiangsu, China) approved the current protocols, according to the 1975 Declaration of Helsinki. Informed consent form was signed by each patient.

Clinical tissue specimens and cell lines. The 82 female patients enrolled in the present study underwent curative surgery for breast cancer without radiation or chemotherapy prior to surgical treatment at the Nanjing Medical University Affiliated Wuxi Second Hospital, between January 2008 and December 2009. The mean age of the patients was 54.8 years (standard error of the mean, 3.2; range, 29-73 years). Tissue specimens were confirmed separately by two experienced pathologists under double-blinded conditions, and none of the patients received any therapy prior to operation. The demographic features and clinicopathological data were reviewed in the patients' medical records. The specimens were collected and immediately stored in 4% paraformaldehyde for immunohistochemistry (IHC), or in liquid nitrogen for western blotting. The prognosis concerning disease-specific survival and overall survival, which were defined as the time from surgery to first recurrence or mortality, respectively, were analyzed. A total of 60 months of clinical follow-up data were available.

The human breast cancer cell lines MCF7 and SKBR3 were cultured in complete Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), while the human breast cancer cell line MDA-MB-231 was cultured in L15 medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% FBS, in a humidified 5% CO_2 incubator at 37˚C. The cell lines were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China).

IHC staining. Paraformaldehyde-fixed, paraffin-embedded tissue sections were used for IHC with an anti-SREBP-1 (1:500; catalog no. sc-8984; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibody, according to the streptavidin-peroxidase method (15). The staining results for the SREBP-1 protein were evaluated by the staining intensity and the percentage of positive cells. The staining intensity was scored as follows: 0=none; 1=weak; 2=moderate; and 3=strong. The percentage of positive cells was scored as follows: 0, <5%; 1, 6-25%; 2, 26-50%; 3, 51-75%; and 4, >75%. A total of 10 independent, high-magnification (x400) fields were observed to calculate a mean score. A total score >1 was defined as positive staining.

Small interfering (si)RNA transfection. siRNA specific against SREBP-1 and non-silencing scrambled sequence siRNA (used as the negative control) were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection in accordance with the manufacturer's protocol. Further experiments were performed after 48 h. The specific SREBP-1 siRNA sequence was: Sense 5'-GGAAGAGUCAGU GCCACUGTT-3' and anti-sense 5'-CAGUGGCACUGACUC UUCCTT-3'.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from breast cancer tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA concentration was quantified by a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Complementary (c)DNA was synthesized using a first-strand cDNA synthesis kit (GE Healthcare Life Sciences; Chalfont, UK). cDNA (2 μl) was amplified and quantified by RT-qPCR using SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd., Dalian, China) with the following cycling conditions: 94˚C for 3 min, followed by 35 cycles of 94˚C for 30 sec and 60˚C for 30 sec. The human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was regarded as the internal control. The primers used for PCR were synthesized by Shanghai GenePharma Co., Ltd., and their sequences were as follows: 5'-CAGTCCAGCCTTTGAGGATA-3' (forward) and 5'-CAAAGGATTGCAGGTCAGAC-3' (reverse) for SREBP-1, and 5'-CAAGCTCATTTCCTGGTATGAC-3' (forward) and 5'-CAGTGAGGGTCTCTCTCTTCCT-3' (reverse) for GAPDH. All samples were normalized to GAPDH and calculated with the relative $2^{-\Delta\Delta Cq}$ method (17).

Protein extraction and western blotting. A modified radioimmunoprecipitation assay buffer with protease inhibitor was used for protein isolation (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were measured using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Total proteins (50 μg) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA), which were blocked with 5% non-fat dry milk (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in phosphate-buffered saline (PBS) with 0.1% Tween 20. Next, the membranes were incubated with primary anti-SREBP-1 (1:300; catalog no. sc-365513; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-β-actin (1:5,000; catalog no. 3700; Cell Signaling Technology, Inc.) antibodies at 4˚C overnight, followed by horseradish peroxidase-conjugated secondary antibodies (1:5,000; catalog no. bs12471; Bioworld Technology, Inc., St. Louis Park, MN, USA) for 2 h at room temperature. Bands were detected with an enhanced chemiluminescence reagent (EMD Millipore).

Wound healing assay. Cells ($5x10^5$ cells/well) were seeded into a 6-well plate and incubated to a confluent monolayer. Scratch wounds were created using a pipette tip, and washed twice with sterile PBS. Cells were then cultured in serum-free medium in a humidified 5% CO_2 incubator at 37˚C for 48 h, and visualized under an inverted microscope.

Transwell assay. Transwell chambers (8 μM pore-sized; Nalge Nunc International; Thermo Fisher Scientific, Inc.) were coated with matrigel (BD Biosciences, Franklin Lakes, NJ, USA) at 1 mg/ml on the inner layer. Cells transfected with control siRNA or SREBP-1 siRNA were seeded in the upper chamber at a density of $1x10^5$ cells/chamber in 100 μl serum-free medium. The lower chamber was filled with 600 μl DMEM containing 10% FBS. Plates were incubated for 24 h

Table I. Correlation between relative SREBP-1 expression level and clinicopathological parameters in breast cancer (n=82).

Clinical parameters	Cases (n)	SREBP-1 expression		P-value
		Positive (n=58)	Negative (n=24)	
Age (years)				0.071
<50	50	39	11	
≥50	32	19	13	
Tumor size (cm)				0.776
<2	39	27	12	
≥2	43	31	12	
Tumor location				0.953
Left	44	31	13	
Right	38	27	11	
Differentiation				<0.001[a]
Moderate/high	34	11	23	
Poor	48	47	1	
T stage				0.806
I/II	67	47	20	
III/IV	15	11	4	
Lymph node metastasis				<0.001[a]
Negative	29	6	23	
Positive	53	52	1	
TNM stage				0.044[a]
I/II	55	35	20	
III/IV	27	23	4	
ER status				0.585
Negative	44	30	14	
Positive	38	28	10	
HER2 status				0.161
Negative	38	24	14	
Positive	44	34	10	
PR status				0.866
Negative	49	35	14	
Positive	33	23	10	

[a]P<0.05. SREBP-1, sterol regulatory element-binding protein 1; TNM, tumor-node-metastasis; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

at 37˚C, and cells in the top surface of the matrigel membrane were swabbed carefully. The adherent cells on the undersurface of the insert were stained with 0.3% crystal violet and counted under a light microscope. A total of four fields were randomly selected to calculate the mean cell number.

Statistical analysis. All data are presented as the mean ± standard deviation. SPSS version 13 (SPSS, Inc., Chicago, IL, USA) was used for Pearson χ^2 test and multivariate Cox regression analysis. Two-tailed Student's *t* test or Kaplan-Meier method was used to calculate the survival, and log-rank test or analysis of variance was used to analyze the difference in multivariate Cox regression using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Increased SREBP-1 protein expression in breast cancer and its correlation with clinicopathological features. To evaluate the potential role of SREBP-1 in human breast cancer, RT-qPCR, IHC staining and western blotting were performed to investigate SREBP-1 messenger (m)RNA and protein expression in tissue samples of 82 patients with primary breast cancer, together with matched para-cancerous tissue samples. Notably, the SREBP-1 mRNA and protein level was robustly increased in the majority of the cancer samples compared

Table II. Multivariate Cox regression analysis on 5-year overall and disease-specific survival of 82 breast cancer patients.

	Overall survival			Disease-specific survival		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
SREBP-1	2.976	1.109-7.987	0.030	2.327	1.093-4.955	0.029
Age	0.993	0.741-1.352	0.836	0.854	0.651-1.223	0.885
Tumor size	2.731	1.945-3.786	0.003	2.912	1.883-4.147	0.001
Lymph node metastasis	3.183	1.991-4.505	0.001	3.962	2.975-4.756	<0.001
Histology grade	1.842	1.185-2.846	0.023	1.954	1.512-2.513	0.032

HR, hazard ratio; CI, confidence interval; SREBP-1, sterol regulatory element-binding protein 1.

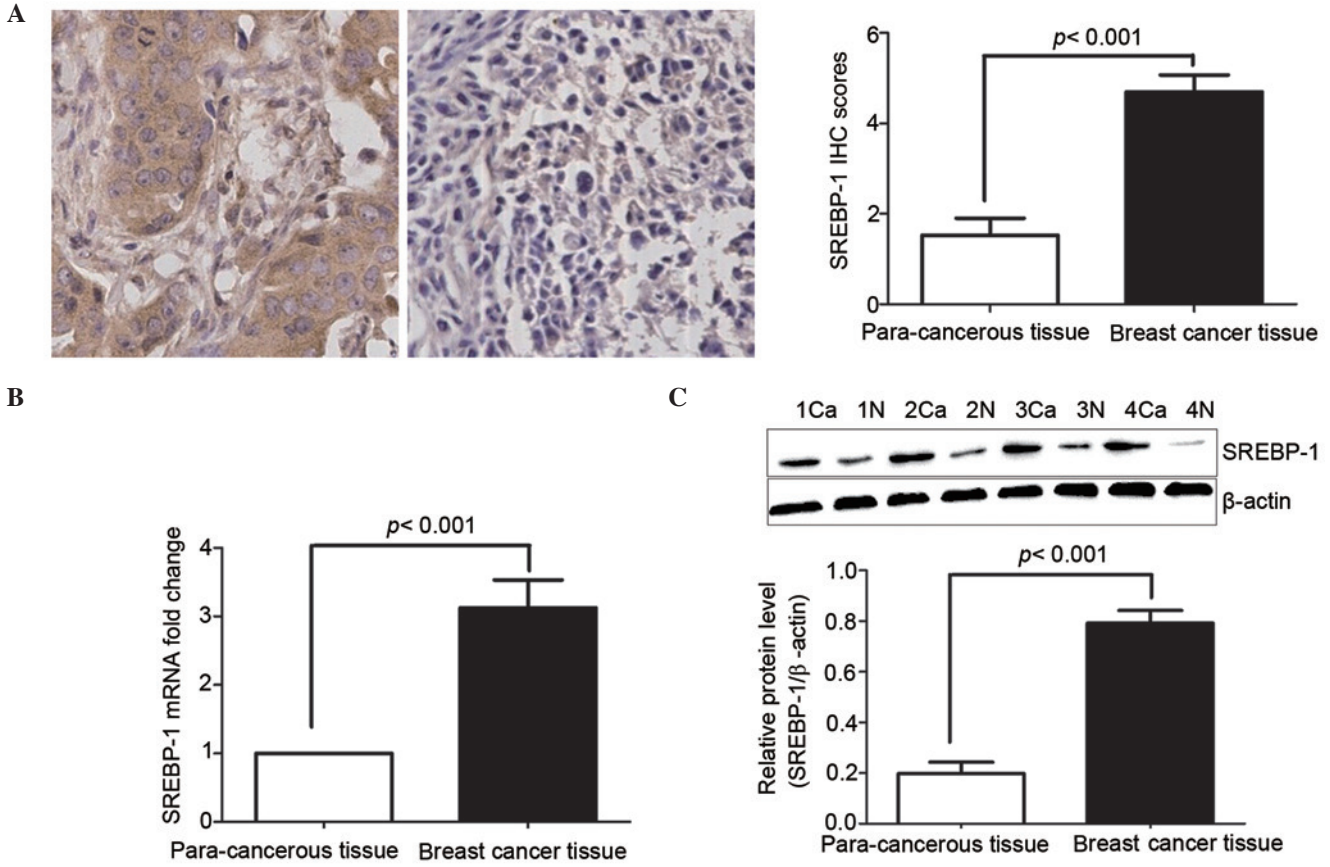

Figure 1. SREBP-1 expression in breast cancer cases. (A) Representative immunostaining of SREBP-1 in tissues and immunohistochemistry scores: Left, positive expression of SREBP-1; right, negative expression of SREBP-1 (original magnification, x400). Expression of SREBP-1 (B) messenger RNA and (C) protein in breast cancer and para-cancerous tissues (n=82). Values are depicted as the mean ± standard deviation. SREBP-1, sterol regulatory element-binding protein 1; IHC, immunohistochemistry; N, normal; Ca, cancer; mRNA, messenger RNA.

with para-cancerous tissues (P<0.01; Fig. 1A and B). SREBP-1 was positively stained in both the nucleus and the cytoplasm. Compared with malignant cells, SREBP-1 immunostaining in benign cells was negative or relatively weak (Fig. 1A). According to the criteria of semi-quantitative assessment employed, SREBP-1 was highly expressed in 58 (70.7%) of 82 breast cancers and in 24 (29.3%) of 82 para-cancerous tissues. Statistical analysis of SREBP-1 staining scores confirmed increased staining in malignant cells compared with benign cells (P<0.01; Fig. 1A). Western blotting further confirmed the IHC results (P<0.01; Fig. 1C).

Next, the association between the expression of SREBP-1 protein and clinicopathological features was analyzed (Table I). Pearson χ^2 test suggested that high SREBP-1 expression was strongly correlated with tumor differentiation, tumor-node-metastasis (TNM) stage and lymph node metastasis (P<0.01). However, there was no significant association between SREBP-1 expression and other clinicopathological variables. Taken together, these data reveal that the expression of SREBP-1 in breast cancer is elevated, and increased SREBP-1 expression is correlated with poor clinicopathological features in breast cancer.

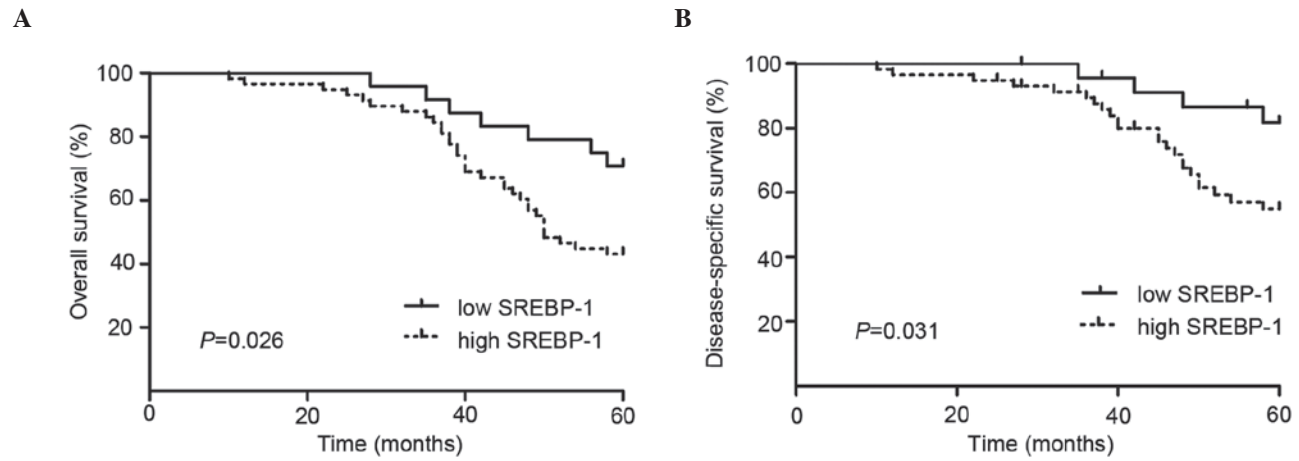

Figure 2. Kaplan-Meier 5-year (A) overall and (B) disease-specific survival curves of breast cancer patients according to the status of SREBP-1 protein expression. In the high expression group (n=58), the IHC score of SREBP-1 was ≥1, while in the SREBP-1 low expression group (n=24), the IHC score of SREBP-1 was 0. SREBP-1, sterol regulatory element-binding protein 1; IHC, immunohistochemistry.

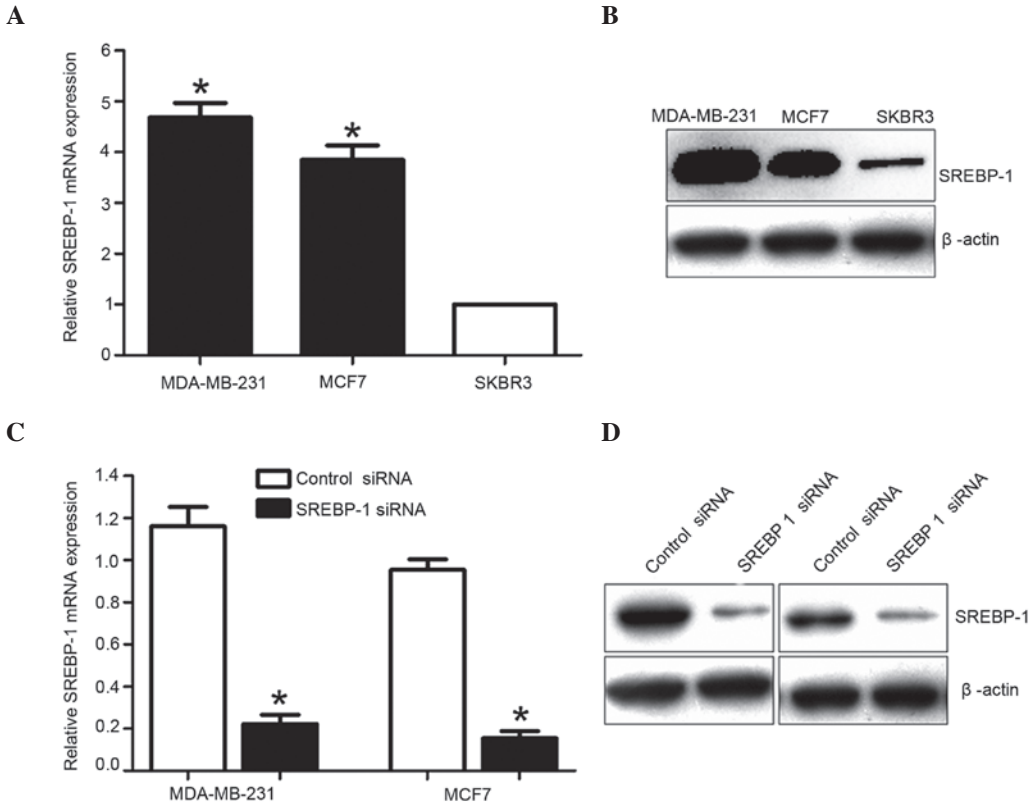

Figure 3. Downregulation of SREBP-1 expression in MDA-MB-231 and MCF7 cells by specific siRNA transfection. (A) RT-qPCR and (B) western blot analysis of SREBP-1 expression in different breast cancer cell lines. (C) Relative expression levels of SREBP-1 mRNA were detected by RT-qPCR. (D) Expression of SREBP-1 protein was detected by western blotting. The results demonstrated that SREBP-1 siRNA could remarkably inhibit the expression of SREBP-1 mRNA and protein in MGA-MB-231 and MCF7 cells. Data are expressed as the mean ± standard deviation (*P<0.001). SREBP-1, sterol regulatory element-binding protein 1; mRNA, messenger RNA; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

High SREBP-1 expression correlates with a poor 5-year survival for breast cancer patients. To determine the role of SREBP-1 in predicting the prognosis of patients, Kaplan-Meier survival curves were constructed using 5-year overall and disease-specific survival to analyze cases with high and low SREBP-1 expression (n=82; follow-up time, 60 months). The results indicate that high SREBP-1 staining predicts a poor overall and disease-specific patient survival (P<0.01, log-rank test; Fig. 2A and B).

In addition, SREBP-1 was an independent prognostic marker for both 5-year overall survival [hazard ratio, 2.976; 95% confidence interval (CI), 1.109-7.987; P=0.030; Table II) and disease-specific survival (hazard ratio, 2.327; 95% CI, 1.093-4.955; P=0.029; Table II) according to multivariate Cox regression analysis. These results definitely confirmed that high SREBP-1 expression is associated with poor prognosis, suggesting that SREBP-1 may function as a prognostic marker for breast cancer.

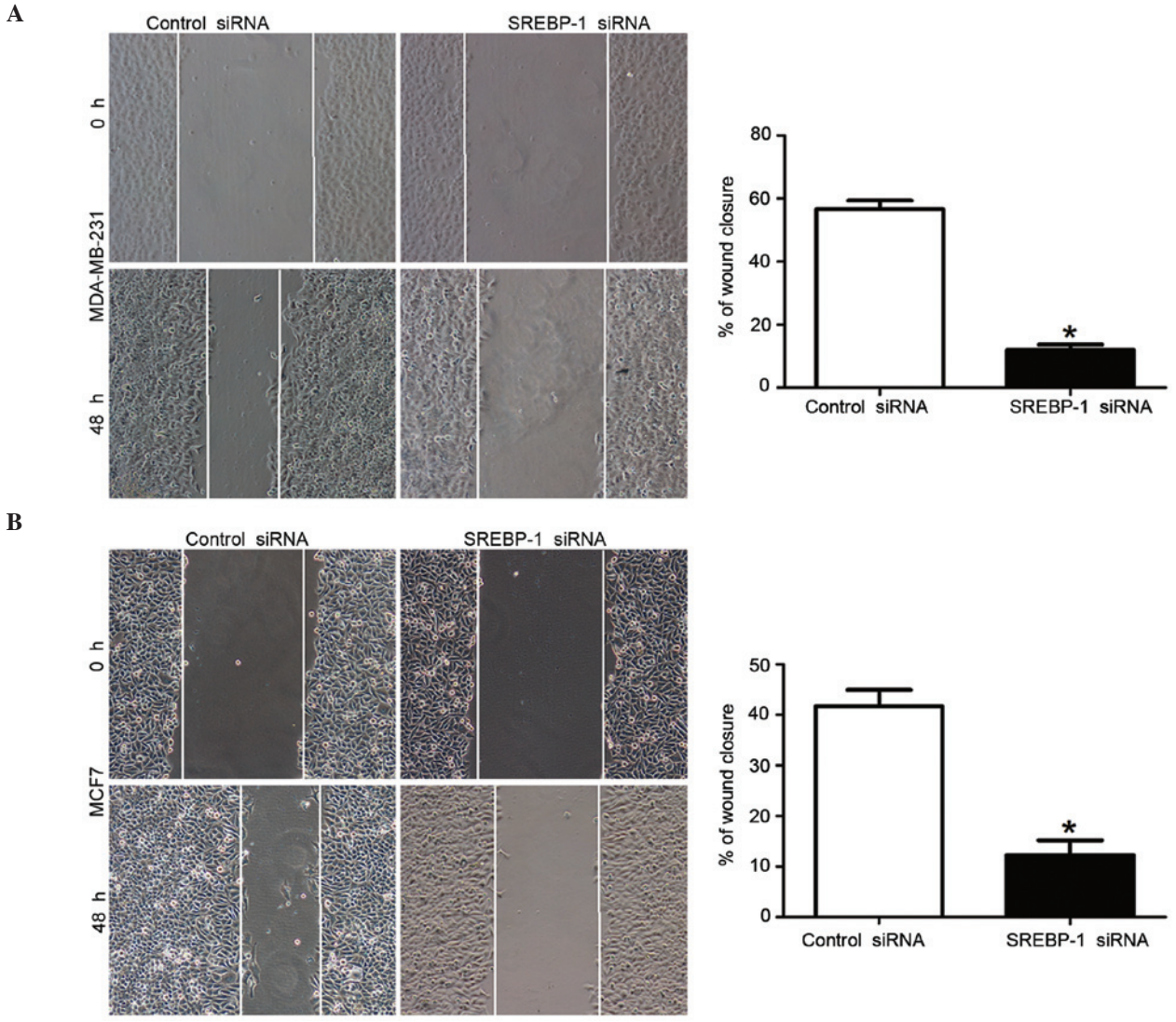

Figure 4. SREBP-1 regulates migration of MGA-MB-231 and MCF7 cells. Wound healing assays demonstrated that SREBP-1 knockdown reduced the migration of (A) MGA-MB-231 and (B) MCF7 cells (original magnification, x100). Data correspond to representative results of replicate experiments (n=3). Values are depicted as the mean ± standard deviation (*P<0.01). SREBP-1, sterol regulatory element-binding protein 1; siRNA, small interfering RNA.

SREBP-1 knockdown is established in breast cancer cell lines. In order to identify the function of SREBP-1, the SREBP-1 mRNA and protein levels were analyzed in three different breast cancer cell lines. Consistent with the clinical results, the expression levels of SREBP-1 were significantly higher in MCF7 and MDA-MB-231 cells than in SKBR3 cells (Fig. 3A and B). Based on such difference in SREBP-1 expression, MCF7 and MDA-MB-231 cells were selected, and SREBP-1 was knocked down by transfecting specific SREBP-1 siRNA into these cells. The RT-qPCR and western blotting results demonstrated that SREBP-1 was markedly downregulated by SREBP-1 siRNA in these cell lines (Fig. 3C and D).

Effect of SREBP-1 knockdown on the invasion and migration of breast cancer cells. To elucidate effect of SREBP-1 knockdown on breast cancer cells migration and invasion, mechanical scrape wound healing and transwell assays were performed. The wound healing assay revealed that the loss of SREBP-1 expression could significantly reduce the migration rate in both MDA-MB-231 and MCF7 breast cancer cells (P<0.01; Fig. 4A and B, respectively). Consistently, the transwell assay also confirmed that the cell migration rate was significantly decreased by SREBP-1 knockdown in these cells (P<0.01; Fig. 5A). Furthermore, compared with those in the control groups, the number of invaded cells in the SREBP-1 siRNA group was significantly reduced (P<0.01; Fig. 5B). These data, therefore, indicate that SREBP-1 has an impact on the migration and invasion of breast cancer cells.

Discussion

Numerous types of cancer exhibit increased *de novo* lipogenesis irrespective of the extracellular lipid availability (20). Exacerbated lipogenesis has been demonstrated to be one of main characteristics of cancer (19). The maintenance of intracellular lipid homeostasis depends on the balance between lipid biosynthesis and degradation (21). The upregulated lipogenesis in tumor cells is reflected by a marked increase in lipogenic enzymes, which is partly

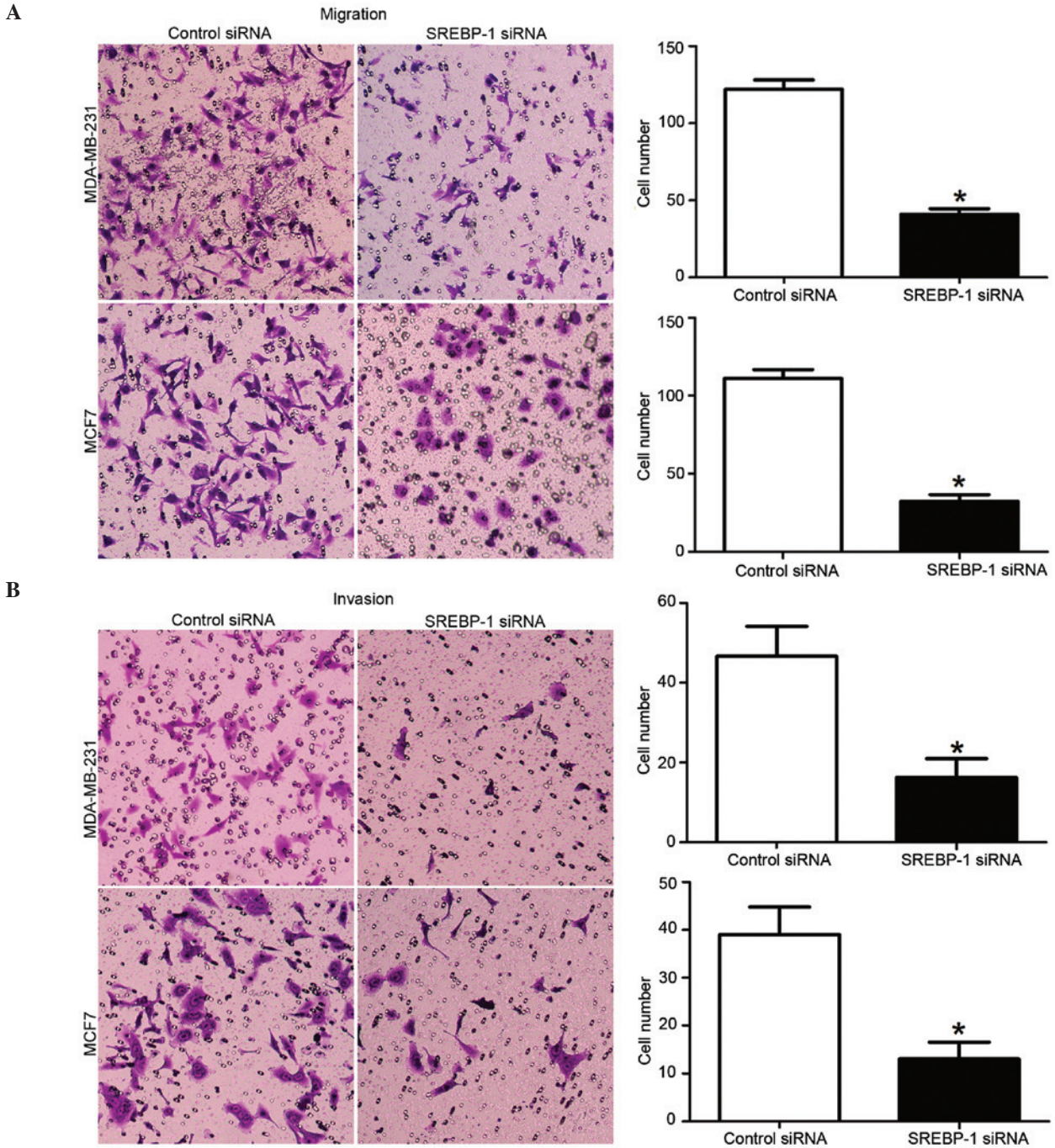

Figure 5. Migration and invasion of cells were measured by Transwell assay. Migrating and invasive cells were stained with crystal violet and imaged by microscopy (original magnification, x100). SREBP-1 small interfering RNA significantly reduced the migration and invasion of (A) MGA-MB-231 and (B) MCF7 cells. Data are expressed as the mean ± standard deviation (*P<0.01). SREBP-1, sterol regulatory element-binding protein 1; siRNA, small interfering RNA.

due to the transcriptional activation mediated by SREBP-1 in cancer cells (6,21). Consistent with the fundamental role of SREBP-1 in regulating multiple types of cancer, elevated SREBP-1 expression had a great impact on cancer progression and metastasis (22,23). However, the impact of SREBP-1 on the progression of breast cancer remains largely unknown, and the underlying mechanisms require to be elucidated.

The present study demonstrated that both the mRNA and protein levels of SREBP-1 were significantly overexpressed in breast cancer tissues compared with para-cancer tissues. In addition, upregulated SREBP-1 expression was highly correlated with tumor differentiation, TNM stage and lymph node metastasis. Kaplan-Meier analysis revealed that breast cancer patients with SREBP-1 positive expression had a poorer survival following surgery than those without SREBP-1 expression. These findings are consistent with previous data reporting that the level of SREBP-1 is associated with the tumorigenesis and prognosis of cancer (24). Additionally, the Cox proportional hazards model employed in the present study revealed that SREBP-1 was an independent factor for predicting the 5-year survival of patients. Therefore, these

results demonstrate that SREBP-1 is a critical molecule for prognosis determination in breast cancer.

To investigate the pathological function of SREBP-1 in breast cancer, several biophysiological experiments were further conducted in SREBP-1-silenced cell lines, which were established by being transfected with specific siRNA targeting SREBP-1. The results revealed that SREBP-1 knockdown in breast cancer cells restrained cell migration and invasion, which are the cytological fundament of tumor metastasis. Previous studies have reported that the downregulation of SREBP-1 induces a decrease in the expression of several enzymes in the fatty acids signaling pathways, including acetyl-CoA carboxylase, fatty-acid synthase and stearoyl-CoA desaturase 1, which are involved in lipid metabolism, lipogenesis, proliferation, apoptosis and survival in breast cancer (25,26). Through approaches of either siRNA or small molecule inhibitors, depletion of the expression and activity of these genes suppressed tumor cell proliferation and growth (27,28). Therefore, SREBP-1 may be important in tumorigenesis via these molecular signaling pathways.

In conclusion, the present study found that SREBP-1 is upregulated in breast cancer tissues and cells, and that its elevated expression is associated with poor prognostic features. The *in vitro* studies indicated that SREBP-1-knockdown inhibits breast cancer cell migration and invasion. Therefore, SREBP-1 has the potential to be a valuable diagnostic and prognostic biomarker for breast cancer.

Acknowledgements

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