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Original Article

Silenced ABCE1 gene inhibited human breast cancer Bcap37 cell proliferation, invasion and migration

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Abstract: To investigate the effect of silenced ABCE1 gene expression in human breast cancer Bcap37 cell on proliferation, invasion and migration by electroporation method. Targeted ABCE1 siRNA sequenced (ABCE1-siRNA) as well as the negative control sequence (NC-si RNA) was designed and synthesized. They were transfected into human breast cancer Bcap37 cells by electroporation method and the ABCE1-Bcap37, NC-siRNA-Bcap37 cells were formed. By RT-PCR, Western blotting method, expression level of ABCE1 mRNA and protein in transfected cells were detected. Flow cytometry was used to detect cell cycle and apoptosis. CCK-8 proliferation assay, scratch healing assay, cell invasion assay were used to measured cell proliferation, migration, and invasion abilities. The expression level of ABCE1 m RNA and protein in ABCE1-Bcap37 cell group, compared with that in NC-siRNA-Bcap37 and Ctrl-Bcap37 cells group, were significantly decreased. Growth rate of the cells slowed down in ABCE1-Bcap37 group. The cell cycle was arrested in G0/G1 phase, and the number of cells in S phase were reduced; compared with Ctrl-Bcap37 group, apoptosis rate was significantly higher ($P < 0.01$) in ABCE1-Bcap37 group and proliferation, migration as well as invasion were significantly decreased ($P < 0.05$). The electroporation method silenced ABCE1 gene expression may inhibit proliferation, invasion and migration ability of breast cancer cell.

Keywords: Breast cancer, ABCE1, electroporation method, Bcap37, cell proliferation

Introduction

The incidence of breast cancer is rising year by year with a trend of rejuvenation. In the early days, lymphatic or blood metastasis may occur [1] and breast cancer is a common malignancy for women. Studies have shown that tumor proliferation, invasion and migration are closely related to gene regulation, which provides a new approach for the effective treatment of tumors [2]. Studies have shown [3] that ATP combined cassette transporter E1 (ATP-binding cassetteE1, ABCE1) is a member of the ATP-binding cassette transporter gene subfamily. By inhibiting intracellular ribonuclease L (RNase L) activity it can inhibit apoptosis, promote cell proliferation and differentiation, and other biological behavior which were closely related to tumor invasion, proliferation, and migration. Therefore, in theory, if blocking ABCE1 expression have an effect on treatment of tumors [4]. At home and abroad, literatures about whether ABCE1 gene correlated with breast cancer cell proliferation, invasion and migration were rare [5]. In this study, electroporation method was used to transfected the designed and targeted synthesized ABCE1 siRNA sequences (ABCE1-siRNA) into human breast Bcap37 cells. When the ABCE1 gene silenced, we investigated its effect on the biological behavior.

Materials and methods

Cell lines and the main reagents

Bcap37 human breast cancer cells were purchased from China Center for Type Culture Collection (Wuhan University Collection). DMEM medium, Opti-MEM, Trizol, trypsin were purchased from Invitrogen Corporation USA. Fetal bovine serum (FBS) were purchased from Gibco Company USA. The plasmid pcDNA3.1 purchased from Invitrogen Corporation USA. L plasmid extraction kit, RNA extraction reagent Trizol, DNA gel recycled purification kits were purchased from Beijing Tiangen Company. DNA marker, RT-PCR related reagents were purchased from Takara Company USA. cDNA syn-

thesis kit was purchased from Japan TOYOBO Company, AMV reverse transcriptase kit was purchased from Hangzhou Bo Day, RNA interference sequence was purchased from Zhuhai English-Biological Technology Co., Ltd. PCR primer sequences was synthesized from the Shanghai GenePharma Technology Co. synthesis, cell counting kit-8 (CCK-8) by China Beyotime Biological Co. ABCE1 polyclonal rabbit anti-human antibody, mouse anti-GAPDH monoclonal antibody were purchased from Santa Cruz, USA, HRP-labeled goat anti-rabbit IgG was purchased from Beijing Zhongshan Company. ECL chemiluminescence kit was purchased from the United States Thermo company. Matrigel gel purchased from BD Biosciences, PVDF membrane was purchased from Bio-Rad Company.

Design and synthesis of targeted ABCE1 siRNA

According to the literature [6], ABCE1 gene targeted ABCE1-siRNA sequence was designed. Sense sequence: 5'-ATCCGCTACAGCGAGTACGTTTACCTGTGAAGCCACAGATGGGGTAAACGTACTCGCTTAGCTTTTTTG-3', antisense sequence: 5'-AATFCAAAAAAGCTACAGCGAGTACGTTT AC C CC ATCTGTGGCTTCACAGGTAAACGTACTCGCTG-TAGCG-3'; no homology negative control sequence (negative control siRNA, NC-siRNA): Sense sequence 5'-GATCCGCGAGACCTCAGTATGTTACCTGTGAAGCCACAGATGGGGTAACATACTGAGGTCTCGCTTTTTTG-3', antisense sequence: 5'-AATTCAAAAAAGCGAGACCTCAGTATGTTACCCAT CTGTGGCTTCACAGGTAACATACTGAGGTCTCGCG-3'. All the sequences were synthesized by Ying Ping Zhuhai Biotechnology Co., Ltd.

ABCE1-siRNA electric transfected Bcap37 cells

Breast cancer cell lines Bcap37 were cultured in DMEM medium containing 10% fetal bovine serum at 37°C 5% CO_2 incubation chamber. Take the cell when they were in logarithmic growth phase to make them into a single cell suspension. They were washed with PBS, trypsin digested and centrifuged. Electroporation solution was added and they were resuspended. The cells were centrifuged at 800 g for 5 min. The cells were washed for 3 times and resuspended. Then they were centrifuged in electroporation solution and transferred to cuvette. Adding 10 μg ABCE1-siRNA plasmid and mixed, then they were placed on ice for 0.5 h. The electroporation (voltage 450 V/cm, capacitor 25 μF, time 0.9 ms) was performed. The transfected cells were named ABCE1-Bcap37 cells and they were set at room temperature for 0.5 h. The cells were transferred to petri dish pulsing DMEM medium (containing 10% fetal calf serum, 1% double antibody). They were placed in 37°C, 5% CO_2 incubator to culture. While electrical transfected with empty vector served as the control (cell named Ctrl-Bcap37 cells). After 48 h transfection, the RNA interference effect was detected.

Expression level of ABCE1 mRNA detect by RT-PCR

10^7 ABCE1-Bcap37, NC-siRNA-Bcap37, Ctrl-Bcap37 cells were collected separately. They were washed by PBS three times. Total RNA was extracted by TRIzol reagent. cDNA was obtained by reverse transcription, and PCR amplification was performed. ABCE1 upstream primer was: 5'-TTGGTTGTGGGAAGTCGT-3', and downstream primer was: 5'-GCTTATGTAGTTAATGGGAGGT-3'. The amplification product was 415 bp. GAPDH primer sequence: upstream primer was: 5'-GAGTCAACGGATTGGTCGT-3', and downstream primer was: 5'-GACAAGCTTCCCGTTCTCAG-3'. The amplification product was 185 bp. RT-PCR reaction conditions were as follows: 95°C pre-denaturation for 5 min, 95°C denaturation for 30 s, 60°C annealing for 30 s, 72°C extension for 60 s, with a total of 35 cycles. Through 1.2% agarose gel electrophoresis, PCR products were observed by gel imager. UVI gel imaging systems was used to photo. Image-Pro Plus 7.0 software was used to analysis strips gray value. The ABCE1/GAPDH ratio represented ABCE1 mRNA relative expression.

Expression of ABCE1 protein detected by western blotting

1×10^7 cells were collected from each group. They were washed by PBS for three times. The total protein was extracted and protein concentration was determined. 60 μg total protein, after 10% SDS-polyacrylamide gel electrophoresis, were electro-transferred to PVDF membrane. Through 5% nonfat dry milk, they were preserved at 37°C for 2 h. 1:1000 diluted polyclonal ABCE1 polyclone was the primary antibody and combined with the membrane to incubate for 1 h. TBST was used to wash membrane four times with each time for 15 min; 1:5 000 HRP-labeled secondary antibody and GAPDH

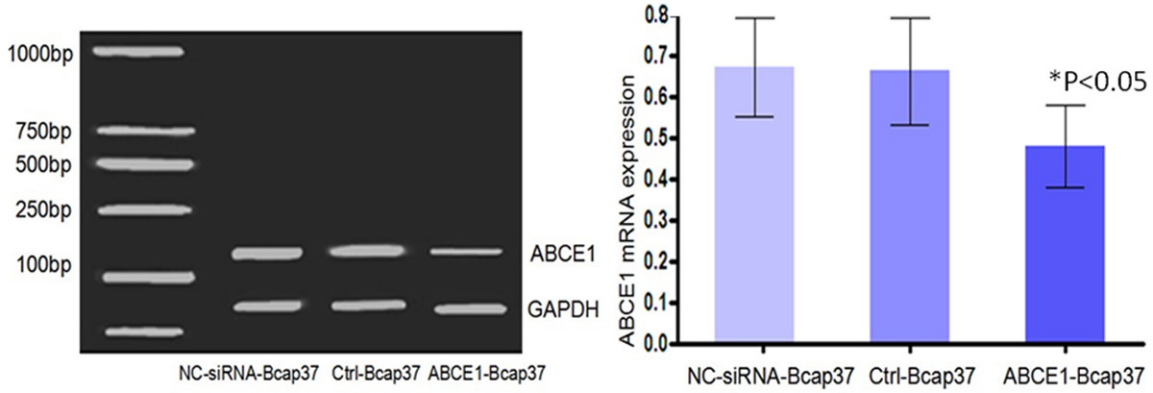

Figure 1. ABCE1 mRNA expression was reduced in ABCE1-Bcap37 cells.

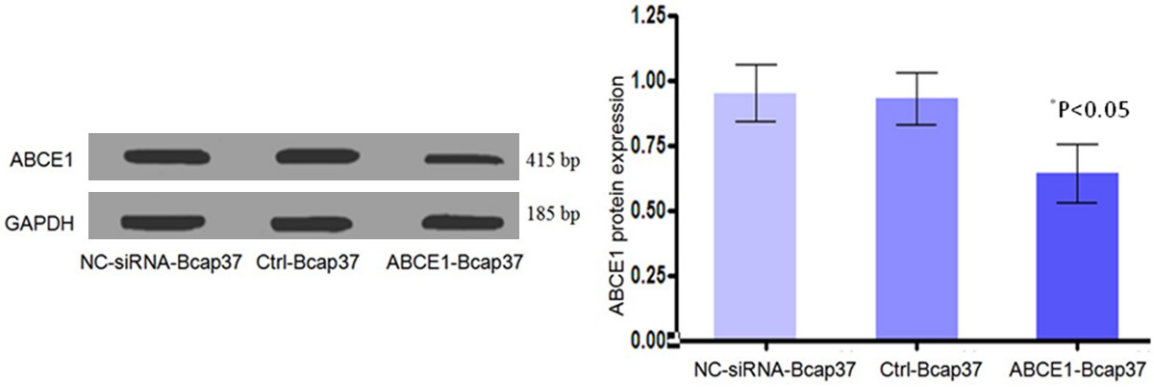

Figure 2. ABCE1 protein expression was reduced in ABCE1-Bcap37 cells.

Table 1. Cell cycle distribution and apoptosis rate % in each group

Group	G0/G1	S	G2/M	apoptosis rate
NC-siRNA-Bcap37	56.3 ± 2.2	35.4 ± 2.6	8.5 ± 1.2	0.58 ± 0.26
Ctrl-Bcap37	54.6 ± 2.3	36.1 ± 2.2	9.2 ± 1.0	0.60 ± 0.25
ABCE1-Bcap37	77.7 ± 2.1*	16.1 ± 1.3*	8.3 ± 1.2	17.80 ± 3.56

VS. Blank group and Control group, *P < 0.05.

were added, and they were incubated at 37°C for 2 h. Then they were washed by PBS. ECL detection kit was used to perform chemiluminescence. X-rays was used for tabletting, developing and fixing. UVI gel imaging systems was used to photo. Image-Pro Plus 7.0 software was used to analysis strips gray value. The ABCE1/GAPDH ratio represented ABCE1 protein relative expression.

Bcap37 cell cycle detected by flow cytometry

Collect the cells in each group and adjust cell density to 1 × 10^6/L. They were washed by pre-cooled PBS for two times. The cells were precipitated with 70% 4°C ice ethanol and mixed. The cells were washed, and the cell density was adjusted to 1 × 10^6/ml by PBS. They were incubated for 30 min with Tris-HCl buffer (Ph 7.4) which containing 50 μg/ml RNA enzyme. After the DNA were stained by 1 μg.ml^{-1} propidium iodide, they were stored in the dark at room temperature for 30 min. Flow cytometry was used to detect cell cycle and apoptosis. The experiment was repeated three times.

CCK8 assay to detect Bcap37 proliferation

Bcap37 cells were seeded in 96-well culture plate with the density of 4000/well. 10% fetal calf serum was added to 200 μl DMEM medium, and each group were six wells. The blank wells as served as the control. 20 μl CCK-8 were added to each hole, and they were incu-

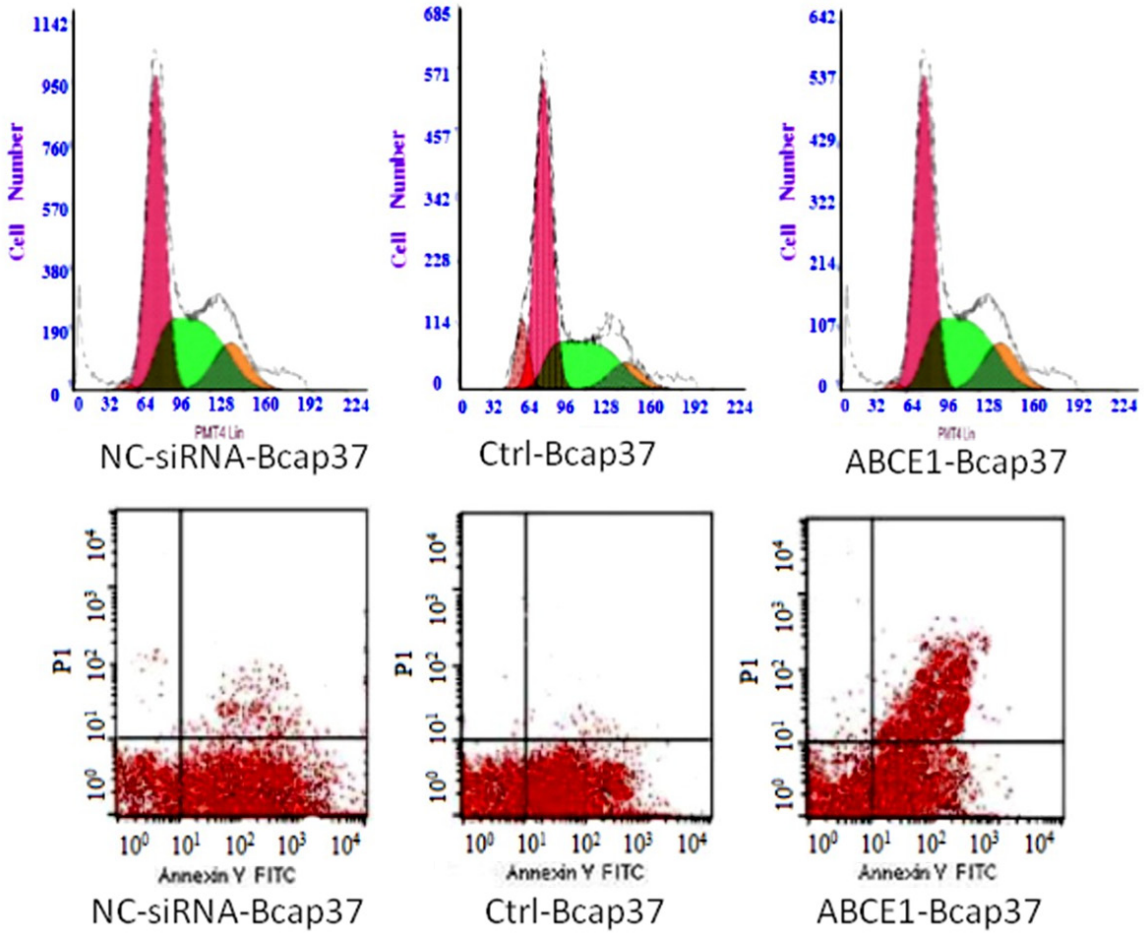

Figure 3. Flow cytometry results for cell cycles and apoptosis.

Table 2. Absorbance at 490 nm ($\bar{x}$ ± s, n = 6)

Group	48 h	72 h	96 h	120 h
NC-siRNA-Bcap37	0.6 ± 0.04	1.8 ± 0.15	2.5 ± 0.16	2.9 ± 0.13
Ctrl-Bcap37	0.6 ± 0.06	1.7 ± 0.11	2.4 ± 0.12	2.8 ± 0.14
ABCE1-Bcap37	0.6 ± 0.04*	1.4 ± 0.12	1.8 ± 0.11	2.2 ± 0.10

VS. Blank group and Control group, *P < 0.05.

bated for 4 h in the incubator. The microplate reader detected absorbance values (D) at 490 nm and cell growth curve was drawn.

Scratch assay to detect Bcap37 cells migration

Take the three kinds of cells when they were in logarithmic growth phase, and they were seeded in 6-well plates with density 5 × 10^3 cells/well. When the confluent of cells reached 90% in each group. 10 μl pipette tip or sterile toothpick was used to scratch in the monolayer with the style "-". 0.01 mol/L PBS was used to wash for three times to remove the floating cells. 2 ml cell culture medium was used to resuspended. They were cultured at 37°C, 5% CO_2. After 48 h, cell migration was observed under an inverted microscope and photographed.

Transwell chamber invasion assay

In Polycarbonate microporous membrane, Matrigel was capped (50 μg/hole); in the well-polymerized lower chamber, 10% fetal bovine serum was added as the conditioned medium; in the upper chamber, 100 μl suspended solutions of above Bcap37 cells in each group were added (total number of cells was 3 × 10^5/L); after incubated in an incubator for 24 hours, the chamber was fixed in 4% paraformaldehyde for 10 minutes, stained with hematoxylin for

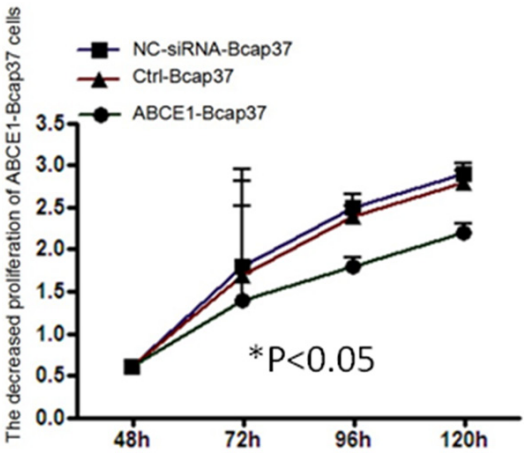

Figure 4. CCK-8 to detect the decreased proliferation of ABCE1-Bcap37 cells.

20 min. Under an optical microscope, invasion cells (cells on the lower surface of the film) in five random horizons in each film were counted respectively, and the average was calculated. In each group, three chambers were arranged in parallel; the experiment was repeated three times. Cell invasion rate (%) = the number of cells across membrane/the total number of cells inoculated in upper chamber × 100%.

Statistical analysis

SPSS16.0 statistical software was used; measurement data were expressed as $\bar{x} \pm s$; differences among many groups were compared using One-way ANOVA and q test; $P < 0.05$ or $P < 0.01$ indicated a statistically significant difference.

Results

ABCE1-siRNA transfection suppressing ABCE1 mRNA expression in Bcap37 cells

RT-PCR test results (**Figure 1**) showed that compared with NC-siRNA-Bcap37 group and Ctrl-Bcap37 group, the expression of ABCE1 mRNA in ABCE1-Bcap37 group was significantly reduced; the differences were statistically significant (0.45 ± 0.05) vs (1.29 ± 0.09), (1.32 ± 0.12, $P < 0.05$). No significant difference had been found in the expression of ABCE1 mRNA between NC-siRNA-Bcap37 group and Ctrl-Bcap37 group ($P > 0.05$), indicating that ABCE1-siRNA transfection can effectively inhibit Bcap37 ABCE1 mRNA expression.

siRNA inhibiting ABCE1 protein expression

ABCE1 protein assay results showed (**Figure 2**) that, compared with the NC-siRNA-Bcap37 group and Ctrl-Bcap37 group, the expression of ABCE1 protein in ABCE1-Bcap37 group was significantly decreased, and the differences were statistically significant (0.63 ± 0.12) vs (0.95 ± 0.14), (0.97 ± 0.16, $P < 0.05$). No significant difference had been found in ABCE1 protein expression between Ctrl-Bcap37 and NC-siRNA-Bcap37 group ($P > 0.05$), indicating that ABCE1-siRNA transfection can effectively inhibit the expression of ABCE1 protein in Bcap37 cells.

Flow cytometry to detect the cell cycle of ABCE1-Bcap37

Effect of ABCE1-siRNA on cell cycle: flow cytometry analysis results showed that, there were statistically significant differences in the cells in G0/G1 phase and S phase between ABCE1-Bcap37 group and the NC-siRNA-Bcap37 group and Ctrl-Bcap37 group ($P < 0.05$); while there was no statistically significant difference between NC-siRNA-Bcap37 group and Ctrl-Bcap37 group ($P > 0.05$), showing that ABCE1-siRNA arrested the cell cycle in G0/G1 phase (**Table 1**; **Figure 3**).

Flow cytometry to detect ABCE1-Bcap37 apoptosis

Flow cytometry analysis showed that, in ABCE1-Bcap37 group, apoptosis rate was significantly higher than that in the NC-siRNA-Bcap37 group and Ctrl-Bcap37 group; the difference was statistically significant ($P < 0.01$); There was no significant difference in apoptosis rate between NC-siRNA-Bcap37 group and Ctrl-Bcap37 group ($P > 0.05$) (**Table 2**).

CCK-8 assay to detect ABCE1-Bcap37 proliferation

The growth curve drawn by CCK-8 test results showed that, the curve of ABCE1-Bcap37 group was significantly lower than that of NC-siRNA-Bcap37 group and Ctrl-Bcap37 (**Figure 4**); the

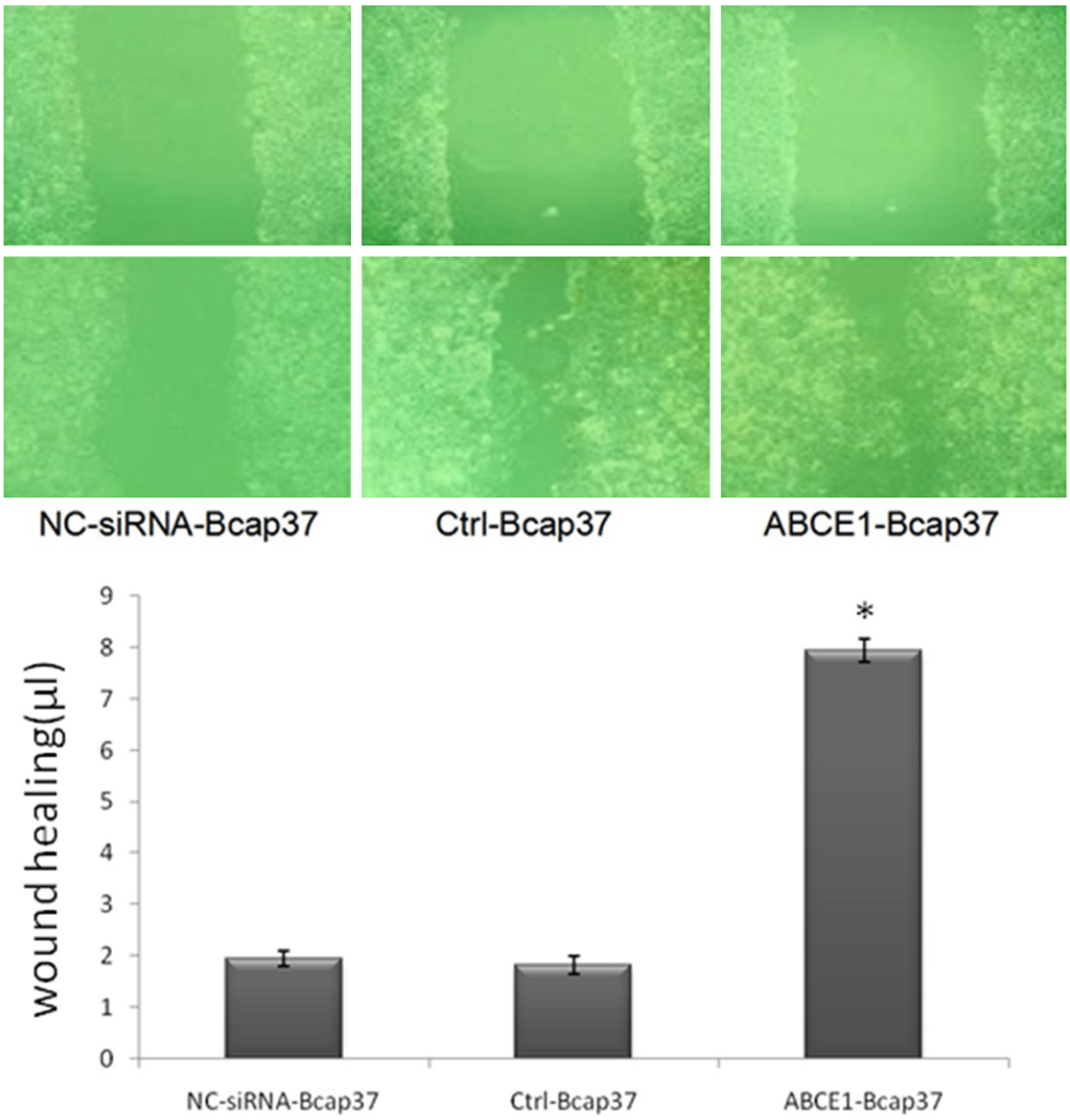

Figure 5. Cell scratch wound experiment showed slow healing of ABCE1-Bcap37 cells. 48 hours later, scratches of ABCE1-Bcap37 cells were slowly healed, while scratches in NC-siRNA-Bcap37 group and Ctrl-Bcap37 group had been basically covered, indicating that migration of the experimental group was significantly decreased (× 100), *P < 0.05.

difference was statistically significant (P < 0.05).

Cell scratch wound experiment showed slow healing of ABCE1-Bcap37 cells

48 hours later, scratches of ABCE1-Bcap37 cells were slowly healed, while scratches in NC-siRNA-Bcap37 group and Ctrl-Bcap37 group had been basically covered, shown in **Figure 5**.

Vitro invasiveness of ABCE1-Bcap37 cells was reduced

As shown in **Figure 6**: the number of cells across the membrane in NC-siRNA-Bcap37 group and Ctrl-Bcap37 group were higher (53.34 ± 3.25) and (54.27 ± 4.21); penetrating cells in ABCE1-Bcap37 group were significantly reduced (31.80 ± 3.78); the difference was statistically significant (P < 0.01). Results showed

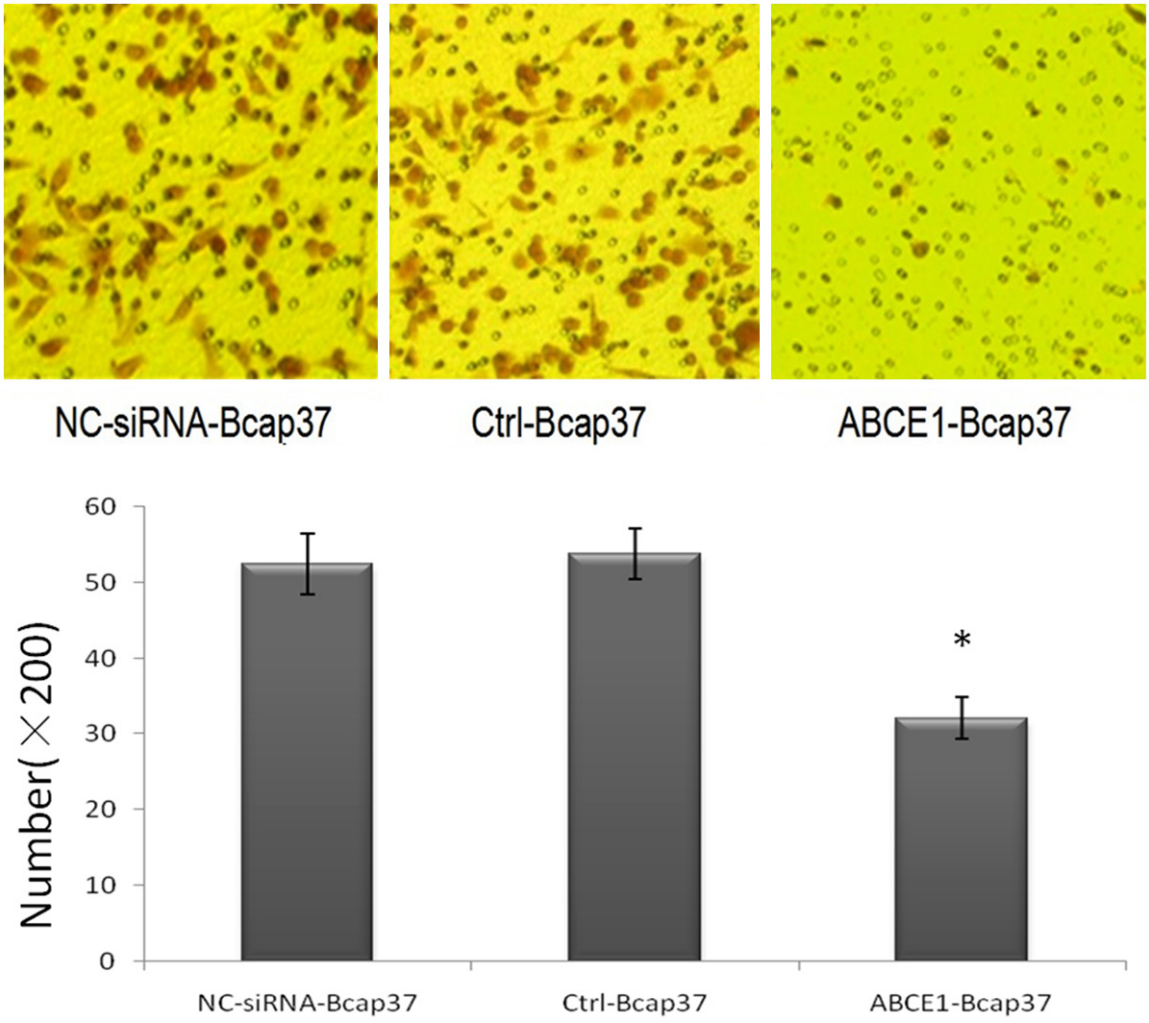

Figure 6. Vitro invasiveness of ABCE1-Bcap37 cells was reduced (× 200), *P < 0.05.

that specifically interfering with ABCE1 gene expression may effectively reduce the invasiveness of Bcap37 cells.

Discussion

Breast cancer is a systemic disease; its incidence rate is increasing year by year, and the age of onset is gradually getting younger and younger; lymphatic or hematogenous metastasis may occur early; it is a serious threat to women's health [7]. Clinicians at home and abroad prefer surgical treatment for breast cancer patients, supplemented by radiotherapy, endocrine, biological molecules targeting therapy; but local recurrence and distant metastasis of breast cancer are still the huge clinical problems currently. With the development of functional gene science and the completion of Human Genome Project, the gene therapy in oncology filed gets medical attention [8]. Breast cancer proliferation, invasion and migration are related with the abnormality in the structure and function of many genes; identifying the roles of key genes in breast cancer incidence is significant for clinical diagnosis, treatment and prevention of breast cancer [9].

ABCE1 is a member of the ATP-binding cassette multigene family; the gene is located on autosome 4q31; full-length cDNA sequence encodes 599 amino acids and encodes a protein with a relative molecular weight of 68 kDa; it is expressed in every human tissues and organs through the lifetime [10]. 2-5 oligonucleotide content (2-5A/RNase L) increase in the normal cells results in activated ribonuclease L (RNase L); activated RNase L could specifically

degrades RNA, inhibit 68 kDa protein, make the cells cannot properly synthesize protein, induce apoptosis, prevent the spread of the virus and prevent uncontrolled cell proliferation. Inhibition of apoptosis is the key of proliferation, invasion and migration of malignant tumors. Research shows that [11], ABCE1 gene is the RNase L inhibitor, which can inhibit intracellular RNase L activity, block interferon-mediated 2-5A/RNase L antiviral pathway, interfere with the biological metabolism of normal cells, and inhibit apoptosis [12]; it also can promote cell proliferation, differentiation and protein synthesis; therefore it may closely related with the proliferation, invasion and migration and other biological behaviors of malignant tumors. Studies have shown that when silenced ABCE1 gene was transfected into tumor cells, the proliferation and apoptosis changed significantly. Studies have also shown [13] that overexpression of ABCE1 gene existed in lung cancer, colorectal cancer and prostate cancer cells. But its effect on the biological behaviors of breast cancer has been reported rarely worldwide.

Electroporation is to make tiny holes in the cell membrane instantly under the influence of high-voltage electric field pulses, so that exogenous DNA can enter the cell, which is an ideal transfection method to achieve high efficiency in laboratory [14]. In this study, the ABCE1-siRNA sequence and negative control (NC-siRNA) sequence were designed, synthesized and transfected into human breast cancer cell Bcap37 by electroporation to obtain ABCE1-Bcap37 and NC-siRNA-Bcap37 cells; RT-PCR and Western blotting detection found that after transfection, ABCE1 mRNA and protein expressions were blocked, which was caused by the targeted-silencing of ABCE1 gene; flow cytometry was used to detect cell cycle and apoptosis; CCK-8 proliferation assay , scratch healing assay, and cell invasion assay were used to detect the proliferation, migration, and invasion of human breast cancer Bcap37 cells, respectively. The biological behavior of Bcap37 cells was observed; the results showed that in ABCE1-Bcap37 cells, ABCE1 mRNA and protein expression significantly decreased; growth of ABCE1-Bcap37 cells was significantly slowed; cell cycle was arrested in G0/G1 phase; cells in S phase were reduced; the proliferation, invasion and migration of ABCE1-Bcap37 cells significantly decreased; apoptosis rate increased significantly.

The results of this study showed that in human breast cancer, ABCE1 gene silencing can inhibit tumor cell proliferation, invasion, migration and other biological behaviors, so as to lay an experimental foundation for gene therapy of breast cancer.

Disclosure of conflict of interest

None.

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