

Original Article

Silenced ABCE1 gene inhibited human gallbladder carcinoma GBC-SD cell proliferation, invasion and migration by electroporation method

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Abstract: To investigate the effect of silenced ABCE1 gene expression in human gallbladder carcinoma GBC-SD 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 gallbladder carcinoma GBC-SD cells by electroporation method and the ABCE1-GBC-SD, NC-siRNA-GBC-SD 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 GBC-SD cell cycle and apoptosis. CCK-8 proliferation assay, scratch healing assay, cell invasion assay were used to measure cell proliferation, migration, and invasion abilities. The expression level of ABCE1 mRNA and protein in ABCE1-GBC-SD cell group, compared with that in NC-siRNA-GBC-SD and empty vector cells group (Ctrl-GBC-SD cell group), were significantly decreased. Growth rate of the cells slowed down in ABCE1-GBC-SD group ($P<0.05$). The cell cycle was arrested in G0/G1 phase, and the number of cells in S phase were reduced; compared with empty vector group, apoptosis rate was significantly higher ($P<0.01$) in ABCE1-GBC-SD 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 gallbladder carcinoma GBC-SD cell.

Keywords: Gallbladder carcinoma, ABCE1, electroporation method, GBC-SD, cell proliferation

Introduction

Gallbladder carcinoma is one of the most common malignancy biliary system. Its early symptoms are not typical and the majority of the patients are in advanced stages before diagnosed. The prognosis is relatively poor, and the incidence of gallbladder cancer presents a rising trend year by year [1]. The comprehensive treatment program for gallbladder carcinoma are varied, but clinicians still face enormous challenges, which are due to local recurrence and distant metastasis of gallbladder cancer [2]. Genes were involved in the regulation of tumor proliferation and migration, which proposes a new effective method of controlling cancer [3]. Studies have found that ATP-binding cassette transporter E1 (ATP-binding cassetteE1, ABCE1) are closely involved in majority of tumor cell proliferation and migration [4]. At

home and abroad, literatures about ABCE1 gene inhibit Biological Characteristics of gallbladder carcinoma with breast cancer cell proliferation, invasion and migration were rare [5]. In this study, electroporation method was used to transfect the designed and targeted synthesized ABCE1 siRNA sequences (ABCE1-siRNA) into human gallbladder carcinoma GBC-SD cell. When the ABCE1 gene silenced, we investigated biological behavior of gallbladder carcinoma GBC-SD cell.

Materials and methods

Cell line and the main reagent

rAAV-hTERT and empty vector rAAV (Benyuan Zhengyang Gene Technology Co., Ltd. provided); L-DMEM medium (Hyclone US companies); fetal bovine serum (FBS) (purchased from Gibco

Company). Two-step RT-PCR kit (Beijing tournament Parkson Gene Technology Co., Ltd.), AMV reverse transcriptase kit (Beijing Tiangen Biochemical Co.), heat resistance TaqDNA polymerase (Hangzhou Bo Day), according to the NCBI gene bank hTERT the cDNA; β -actin polyclonal rabbit anti-human antibody and horseradish peroxidase labeled goat anti-rabbit IgG antibody (Thermo Corporation USA); rabbit anti-human fibroblast cytokine polyclonal antibody, goat anti-rabbit antibody (Abcam company United States). Telomerase reverse transcriptase (basic fibroblast growth factor, hTERT) (Hyclone US companies); trypsin (GibcoBRL company); Western blot protein detection kit (KPL, USA); CCK-8 kit (Beijing Us Sen). Cell Cycle Detection Kit (US Becon Dickinson Company). Flow cytometry (US company Bio-Rad).

Human gallbladder cancer GBC-SD cells were purchased from (synthetic Takara company), Opti-MEM, G418 (GibcoBRL company), L-DMEM medium (Hyclone US companies); fetal bovine serum (FBS) (purchased from Gibco Company); RNA extraction reagent Trizol, L plasmid extraction kit (GibcoBRL Co.); liposomal transfection reagent LipofectamineTM2000 (Beijing tournament Parkson gene Technology Co., Ltd.); T4 DNA, DNA marker ligase (Beijing Tiangen biochemical Co.); gel purified DNA extraction kit (Japan TOYOBO company); cDNA synthesis kit (Japan Takara company); RNA interference sequences (Hangzhou Bo Japan); CCK-8 (US Becon Dickinson company's products); PCR primer sequences (by the Shanghai flash Jing biotech companies to design and synthesis); ABCE1 polyclonal rabbit anti-human antibody (USA Thermo Corporation); mouse anti-GAPDH monoclonal antibody, HRP-labeled goat anti-rabbit IgG (USA Abcam company); HRP-conjugated goat anti-mouse IgG (Beijing Biosynthesis company); ECL chemiluminescence kit was purchased from the US Thermo company. PVDF membrane, atrigel gel (Wuhan University Collection).

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'-AATFCAAAAAGCTACAGCGAGTACGTTT AC C

CC ATCTGTGGCTTCACAGGTAAACGTACTCGCTGTAGCG-3'; no homology negative control sequence (negative control siRNA, NC-siRNA): Sense sequence 5'-GATCCGCGAGACCTCAGTATGTTACTGTGAAGCCACAGATGGGGTAAACATACTGAGGTCTCGCTTTTTTG-3', antisense sequence: 5'-AATTCAAAAAGCGAGACCTCAGTATGTTACCCATCTGTGGCTTCACAGGTAAACATACTGAGGTCTCGCG-3.

ABCE1-siRNA electric transfected GBC-SD cells

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 μ g ABCE1-siRNA plasmid and mixed, then they were placed on ice for 0.5 h. The electroporation (voltage 450 V/cm, capacitor 25 μ F, time 0.9 ms) was performed. The transfected cells were named ABCE1-GBC-SD 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% CO₂ incubator to culture. While electrical transfected with empty vector served as the control (cell named Ctrl-GBC-SD cells) and NC-siRNA electrical transfected served as the blank group. After 48 h transfection, the RNA interference effect was detected.

Expression level of ABCE1 mRNA detect by RT-PCR

10⁷ ABCE1-GBC-SD, NC-siRNA-GBC-SD, Ctrl-GBC-SD 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'-GACAAGCTCCGTTCTCAG-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 with good condition 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 (40 V 150 min transfection condition) to PVDF membrane. Through 5% nonfat dry milk, they were preserved at 37°C for 2 h. Primary antibody incubation: 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 five times with each time for 15 min; Secondary antibody incubation: 1:5000 HRP-labeled secondary antibody and GAPDH 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 were exposed in darkroom. GDSS000 gel automated imaging systems was used to photo. They were rinsed, dried and preserved in the dark. Image-Pro Plus 8.0 software was used to analysis strips gray value. The ABCE1/GAPDH ratio represented ABCE1 protein relative expression.

GBC-SD cell cycle detected by flow cytometry

Collect the GBC-SD cells in each group and adjust cell density to 1×10^6 /L. They were washed by precooled 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 was 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 GBC-SD proliferation

Exponentially growing cells were collected for three groups (experimental group, control group, the blank group). The cells were seeded in 96-well culture plate with the density of 1000/well. 10% fetal calf serum was added to 200 µl DMEM medium, and each group were six wells. At 37°C, 5% CO₂, the cells were incubated for 24 h. Then 20 µl CCK-8 were added to each hole, and they were incubated for 4 h in the incubator. The blank wells as served as the control. CCK8 assay was used to detect GBC-SD proliferation. The microplate reader detected absorbance values (D) at 490 nm. Cell growth curve was drawn with the average cell absorbance value as ordinate and time as the abscissa.

Scratch assay to detect GBC-SD 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 4×10^3 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 "-". 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% CO₂. 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 3×10^5 /L GBC-SD cells in each group were added (total number of cells was 3×10^5 /L); After incubated in an incubator for 24 hours, the tumor cells which did not pass through membrane were carefully scraped off with a cotton swab; the chamber was fixed in 95% ethanol for 5 minutes, gently rinsed with PBS for three times, stained with hematoxylin for 10 min, rinsed with PBS cell, and dried naturally. The polycarbonate membrane of the upper chamber was carefully removed with a scalpel blade along the edge, secured on the

ABCE1 gene inhibits human gallbladder carcinoma

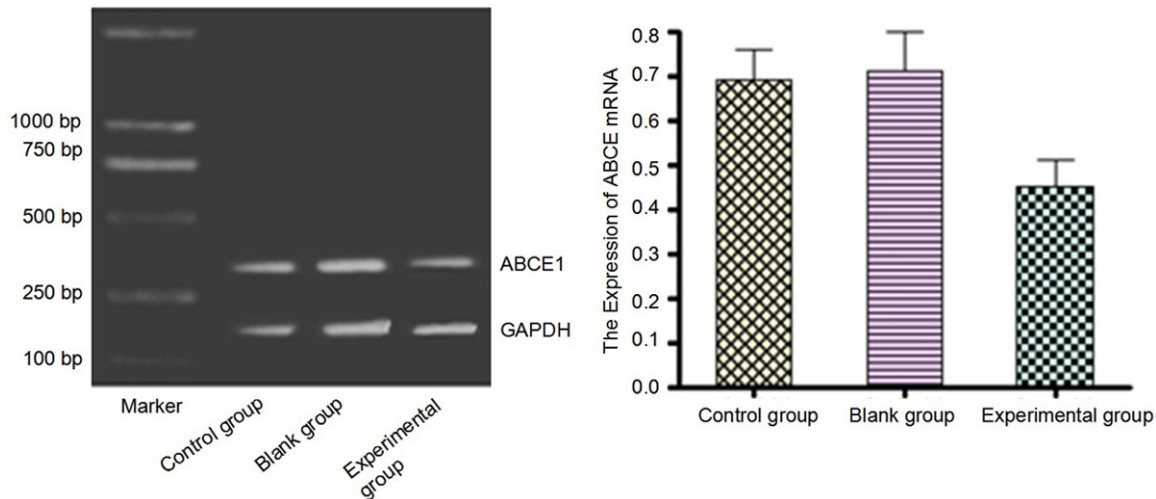


Figure 1. Detection of the mRNA expression of ABCE1 in the GBC-SD cells by reverse transcription quantitative polymerase chain reaction. Values are expressed as the mean \pm standard deviation. ABCE1, ATP-binding cassette protein E1. Compared with the control group and blank group, the ABCE1 mRNA level was significantly decreased in the experimental group when ABCE1 gene silencing.

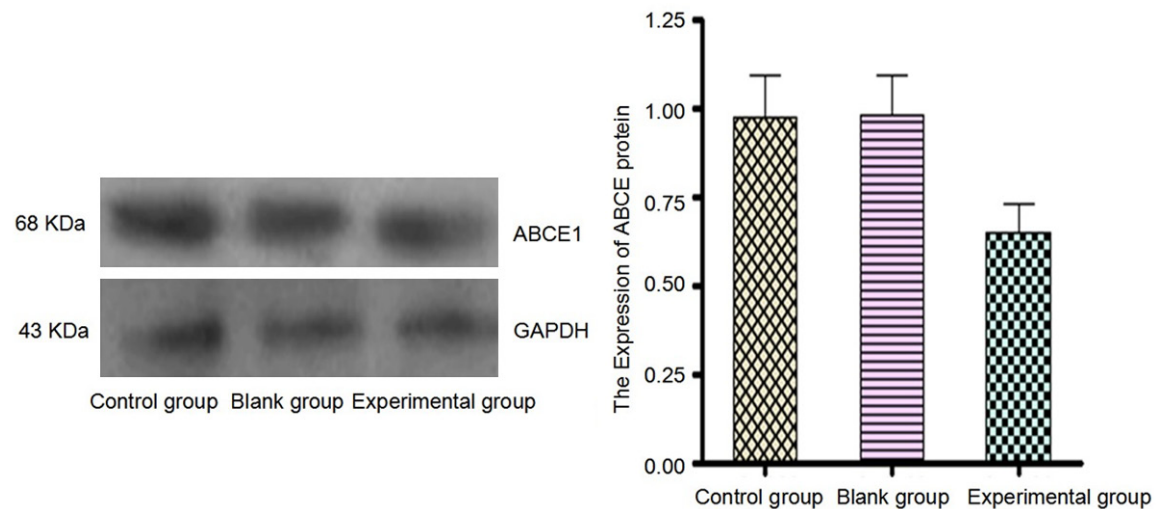


Figure 2. Detection of the protein expression of ABCE1 in the GBC-SD cells by western blot analysis. Values are expressed as the mean \pm standard deviation. ABCE1, ATP-binding cassette protein E1. Compared with the control group and blank group, the ABCE1 protein level was significantly decreased in the experimental group when ABCE1 gene silencing.

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

Group	G0/G1	S	G2/M	Apoptosis rate
Control	56.2 \pm 2.11	35.4 \pm 2.5	8.4 \pm 1.6	0.57 \pm 0.25
Blank	54.4 \pm 2.0	36.7 \pm 2.6	9.5 \pm 1.2	0.68 \pm 0.25
Experimental	77.5 \pm 2.2*	16.5 \pm 1.4*	8.5 \pm 1.0	16.80 \pm 3.58

*P<0.05 vs. control and blank group.

high time optical microscope, invasion XB1702 cells in five horizons (upper, lower, left, right, middle) 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.

Statistical analysis

SPSS13.0 statistical software was used; measurement data were expressed as $\bar{x} \pm s$; the con-

slides with a resin glue (with the inner surface side up), and mounted; after drying, under a

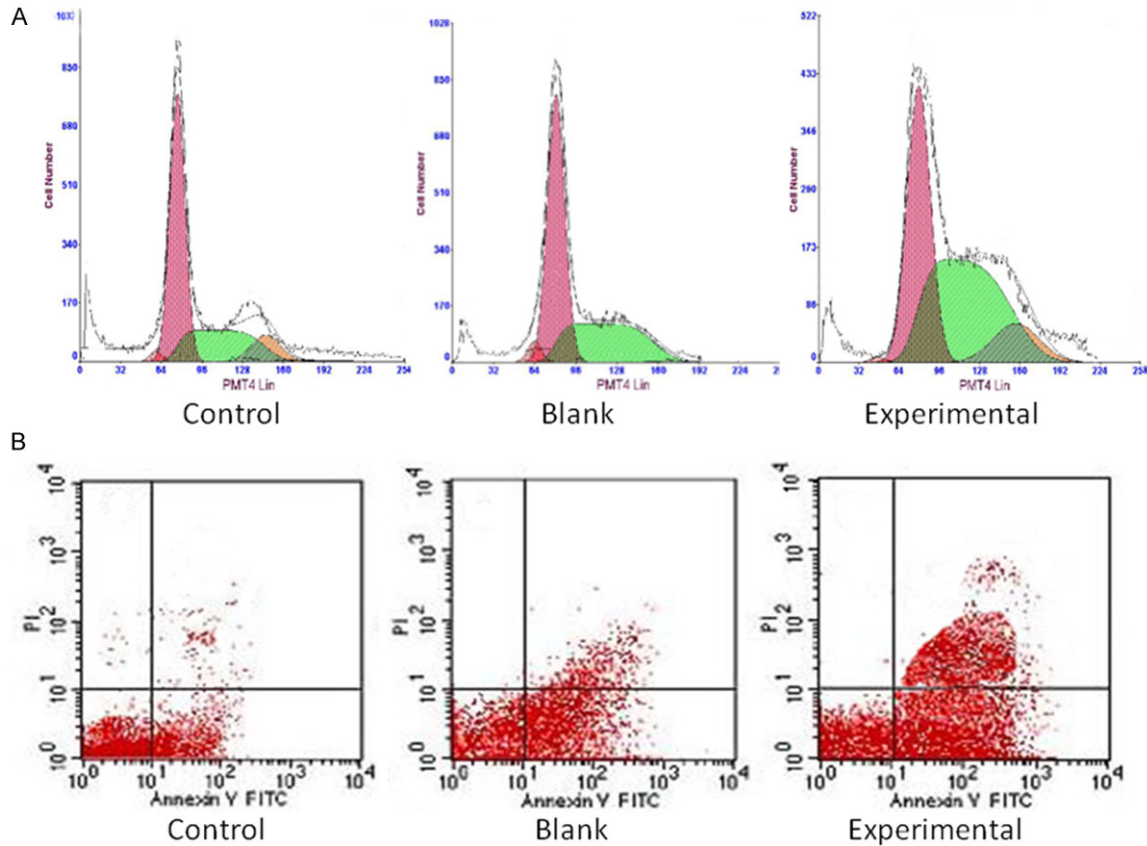


Figure 3. Flow cytometry results for cell cycle (A) and apoptosis (B).

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

Group	48 h	72 h	96 h	120 h
Control	0.6±0.04	1.8±0.15	2.5±0.16	2.9±0.13
Blank	0.6±0.06	1.7±0.11	2.4±0.12	2.8±0.14
Experimental	0.6±0.04*	1.4±0.12	1.8±0.11	2.2±0.10

*P<0.05 vs. control and blank group.

(0.45±0.06) vs (0.69±0.07), (0.71±0.09), P<0.05). No significant difference had been found in ABCE1 mRNA expression between Ctrl-GBC-SD group (control group) and NC-siRNA-GBC-SD group (blank group) (P>0.05), indicating that ABCE1-siRNA transfection can inhibit ABCE1 mRNA expression in GBC-SD cells.

tinuous variables between two groups were compared using t-test; differences among many groups were compared using One-way ANOVA and q test; P<0.05 indicated a statistically significant difference.

Results

ABCE1-siRNA transfection inhibiting ABCE1 mRNA expression in GBC-SD cells

RT-PCR test results (**Figure 1**) showed that compared with NC-siRNA-GBC-SD group (blank group) and Ctrl-GBC-SD (control group), ABCE1 mRNA expression in ABCE1-GBC-SD group (experimental group) was significantly reduced; the differences were statistically significant

siRNA inhibiting ABCE1 protein expression

Protein detection (**Figure 2**) showed that compared with NC-siRNA-GBC-SD group (blank group) and Ctrl-GBC-SD (control group), ABCE1 protein expression in ABCE1-GBC-SD group (experimental group) was significantly reduced; the differences were statistically significant ((0.65±0.10) vs (0.97±0.12), (0.98±0.11), P<0.05). No significant difference had been found in ABCE1 protein expression between Ctrl-GBC-SD group (control group) and NC-siRNA-GBC-SD group (blank group) (P>0.05), indicating that ABCE1-siRNA transfection can effectively inhibit ABCE1 protein expression in GBC-SD cells.

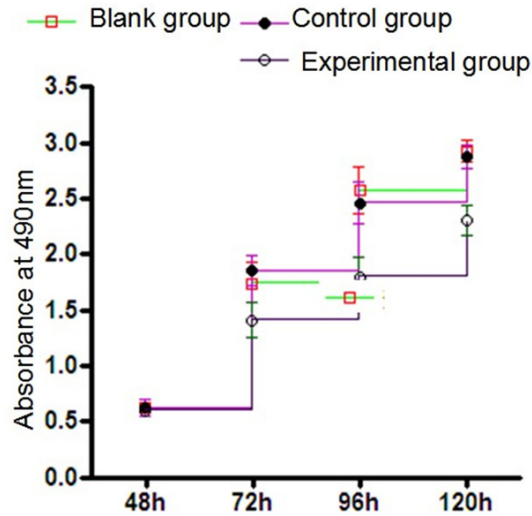


Figure 4. Growth curve of the three treatment groups revealing changes in absorbance with increasing duration. Compared with control group and blank group, GBC-SD cell proliferation of experiment group was significantly inhibited ($P < 0.05$).

Flow cytometry to detect the cell cycle of ABCE1-GBC-SD

Flow cytometry to detect the effect of ABCE1-siRNA on cell cycle showed that, there were statistically significant differences in cell distribution in G0/G1 phase and S phase between ABCE1-GBC-SD group (experimental group) and Ctrl-GBC-SD group (control group), ABCE1-GBC-SD group (experimental group) and NC-siRNA-GBC-SD (blank group), (all $P < 0.05$); No significant difference had been found between Ctrl-GBC-SD group (control group) and NC-siRNA-GBC-SD group (blank group) ($P > 0.05$), indicating that ABCE1-siRNA arrested cell cycle in G0/G1 phase (**Table 1**, **Figure 3**).

Flow cytometry to detect ABCE1-GBC-SD apoptosis

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

CCK-8 assay to detect ABCE1-GBC-SD proliferation

The growth curve drawn by CCK-8 test results showed that, the curve of ABCE1-GBC-SD group was significantly lower than that of NC-siRNA-GBC-SD group (blank group) and Ctrl-GBC-SD (control group) (**Figure 4**); the difference was statistically significant ($P < 0.05$).

Cell scratch wound experiment showed slow healing of ABCE1-GBC-SD cells

48 hours later, scratches of ABCE1-GBC-SD group were slowly healed, while scratches in NC-siRNA-GBC-SD group (blank group) and Ctrl-GBC-SD (control group) had been basically covered, shown in **Figure 5**.

Vitro invasiveness of ABCE1-GBC-SD cells was reduced

As shown in **Figure 6**: the number of cells across the membrane in Ctrl-GBC-SD group and NC-siRNA-GBC-SD group were higher (53.34 ± 3.25) and (54.27 ± 4.21); penetrating cells in ABCE1-GBC-SD group were significantly reduced (31.80 ± 3.78); the difference was statistically significant ($P < 0.01$). Results showed that specifically interfering with ABCE1 gene expression may effectively reduce the invasiveness of GBC-SD cells.

Discussion

Currently comprehensive treatment of gallbladder cancer varied, but the surgery rate of gallbladder is low; it is insensitive to radiotherapy and chemotherapy; invasion and metastasis in surrounding vital tissues and organs occur early; the prognosis is poor; local recurrence and distant metastasis are the huge challenges in clinical [7, 8]. Therefore, exploring effective methods to prevent and treat gallbladder cancer has extremely important clinical significance and social value.

Now with the successful completion of the Human Genome Project, the developing scientific research of gene function shows that [9], gene regulation is involved in the proliferation, invasion and migration of a variety of tumor cells (such as liver cancer, lung cancer, prostate cancer, etc.), so effectively inhibiting tumor

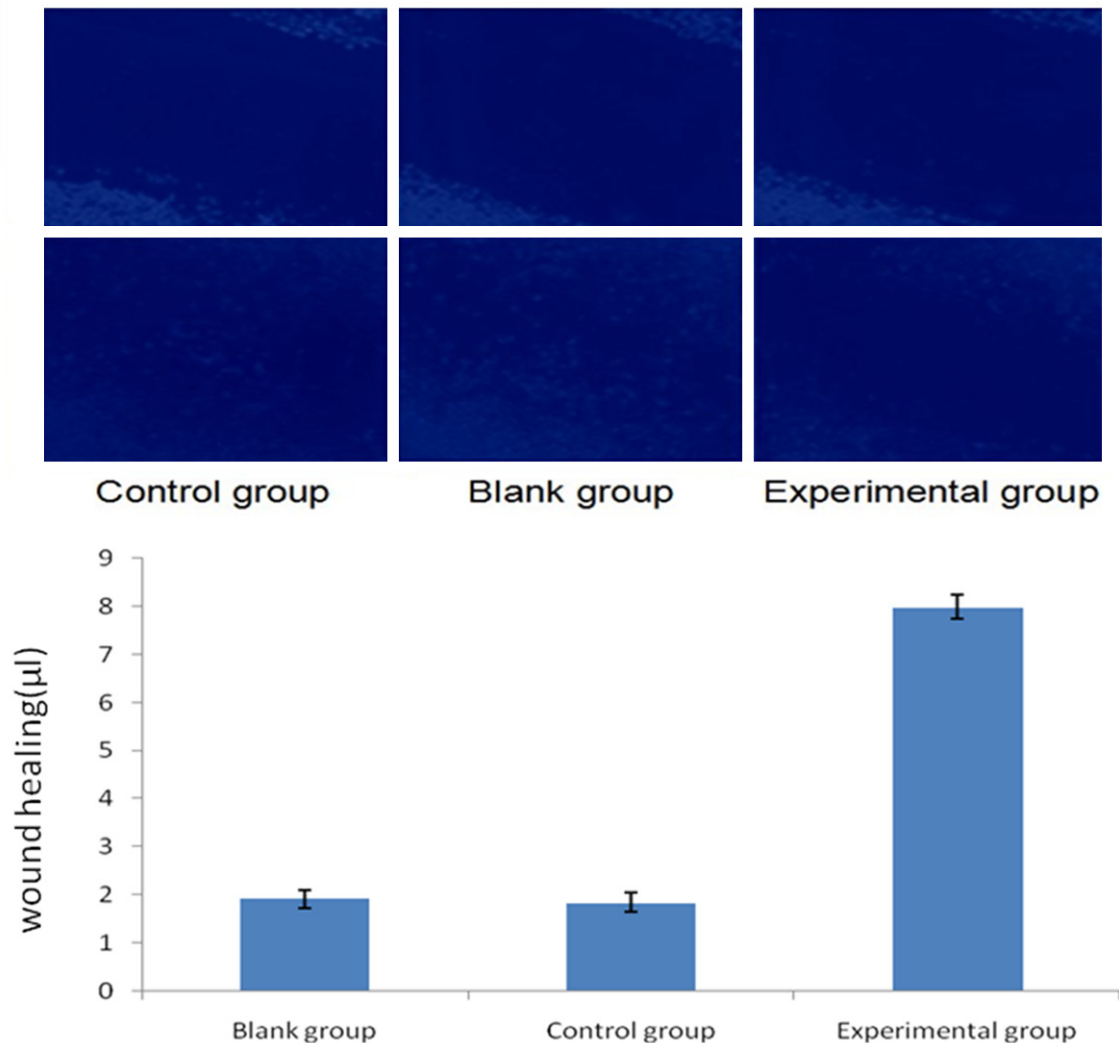


Figure 5. Wound-healing assay results following scratch injury. After 48 h, the cells in the experimental group exhibited relatively slow healing, however, the cells in the control group and the blank group had almost entirely covered the scratches, indicating that cell migration in the experimental group was decreased significantly.

development at gene level has become a new focus of research; medical researchers attach great importance to this area [10]. Current study shows that the key of inhibiting tumor cell apoptosis is to inhibit tumor cell proliferation, invasion and migration [11]. ATP binding cassette transporter E1 gene locating on autosomal 4q31 encodes a protein with a molecular weight of about 68kDa, which is one of the members of the ATP-binding cassette transporter gene subfamily; it has sustained expression in human tissues and organs; the full-length cDNA sequences were encoded by 599 amino acids, involved in tumor cell proliferation, differentiation, invasion, migration, and protein synthesis; it plays its role mainly by

inhibiting ribonuclease L (RNase-L) activity to inhibiting apoptosis [12]. Research shows that [13], in normal cells, RNase L was activated by the increased 2-5A/RNase L; the activated RNase L can specifically inhibit 68 kDa protein synthesis, degrade RNA, prevent uncontrolled cell proliferation and induce apoptosis; Theoretically blocking ABCE1 expression in tumor cells can effectively inhibit the 2-5A/RNase L activity and the development of tumor cells, which may be a new and effective method for the gene therapy of malignant tumors [12-14]. Studies have also shown [11-14] that overexpression of ABCE1 gene existed in lung cancer, colorectal cancer and prostate cancer. ABCE1 gene silencing could affect tumor cell proliferation

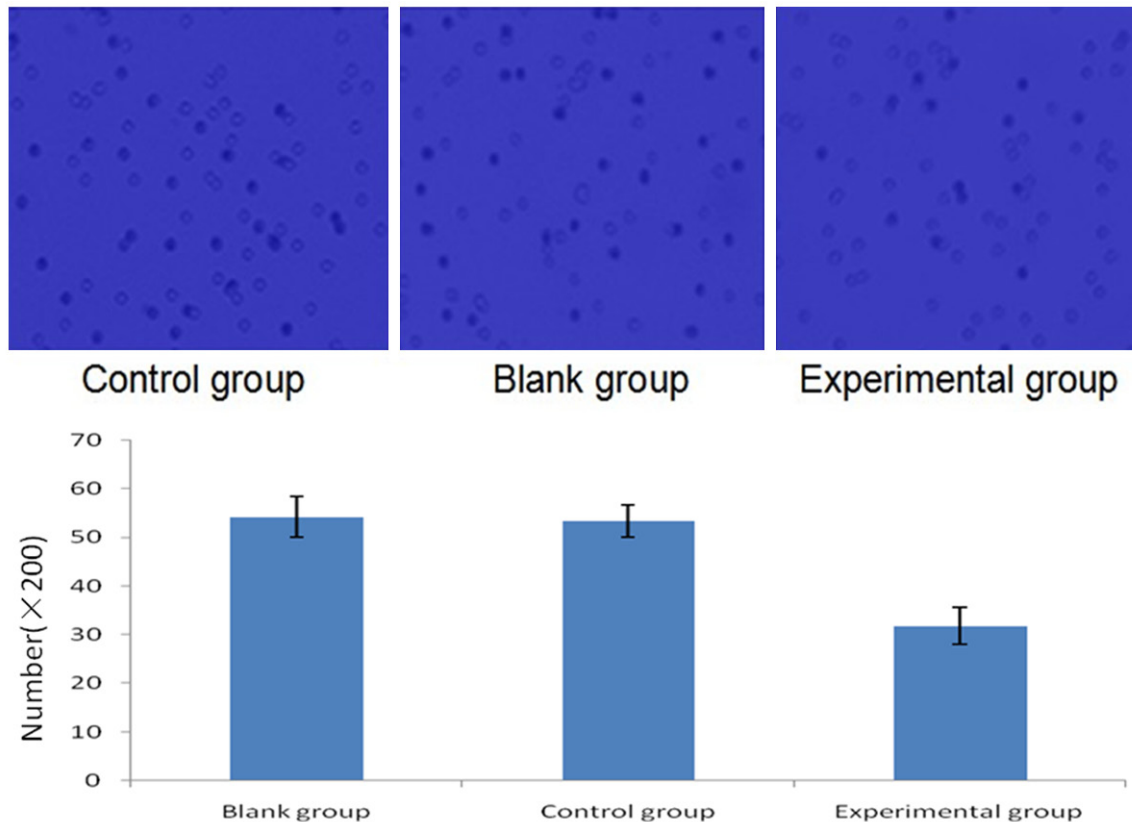


Figure 6. Transwell chamber invasion assay. After 48 h, the number of cells which transferred through the membrane in the experimental group was significantly lower compared with the number in the control group and the blank group (31.80 ± 3.78 ; $P < 0.05$).

and apoptosis. But its effect on the biological behaviors of gallbladder cancer has been reported rarely worldwide.

In recent years, the efficient and ideal transfection method used in relevant experimental studies is to make tiny holes in the cell membrane instantly under the influence of high voltage electric field and electrical pulses to transfect exogenous DNA [15, 16]. In this study, the ABCE1-siRNA sequence and negative control (NC-siRNA) sequence were designed, synthesized and transfected into human gallbladder cancer cell GBC-SD by electroporation to obtain ABCE1-GBC-SD and NC-siRNA-GBC-SD cells; RT-PCR and Western blotting detection showed that after transfection, ABCE1 mRNA and protein expressions were blocked; the effect of targeted-silencing of ABCE1 gene was tested and confirmed; 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 gallbladder cancer GBC-SD cells, respectively. The biological behavior of GBC-SD cells was observed; the results showed that in ABCE1-GBC-SD cells, ABCE1 mRNA and protein expression significantly decreased; growth of ABCE1-GBC-SD 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-GBC-SD cells significantly decreased; apoptosis rate increased significantly.

The results of this study showed that in human gallbladder 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 gallbladder cancer.

Disclosure of conflict of interest

None.

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