

A small interfering ABCE₁-targeting RNA inhibits the proliferation and invasiveness of small cell lung cancer

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Received November 13, 2009; Accepted December 22, 2009

DOI: 10.3892/ijmm_00000392

Abstract. Small cell lung cancer (SCLC) is a highly aggressive lung neoplasm. To study the pathogenesis of SCLC, we investigated roles of ABCE1, a member of the ATP-binding cassette (ABC) superfamily, in the development of small cell lung cancer. RNA interference was used to knock down ABCE1 expression in human small cell lung cancer cell lines (NCI-H446). Then we examined the effects of ABCE1 knock-down in cancer cells, including proliferation, invasiveness, apoptosis, and gene expression. We found that ABCE1 could be efficiently knocked down by siRNA, and the ABCE1 silence inhibited the proliferation, invasiveness of small cell lung cancer cell lines NCI-H446. In conclusion, our results suggest that ABCE₁ play an important role in the pathogenesis of human small cell lung cancer cell. ABCE₁ may be used as a potential target of gene therapy for small cell lung cancer in future.

Introduction

ABCE1 is a member of the ATP-binding cassette (ABC) multigene family and is composed of two nucleotide binding domains and an N-terminal Fe-S binding domain. The ABCE1 gene encodes a protein originally identified for its inhibition of ribonuclease L, a nuclease induced by interferon in mammalian cells (1). The function of ABCE1 in translation initiation in vertebrate implies that this highly conserved gene might play important roles in gene regulation (2). Previous studies have revealed that ABCE1 gene expression was amplified in 19 drug-resistant cell types (3). As deregulation of gene translation is related to the pathologies of cancer, whether ABCE1 is involved in the development of SCLC is of interest.

Small cell lung cancer (SCLC) is a highly aggressive lung neoplasm, which accounts for 20% of annual lung cancer

cases. SCLC is generally unsuitable for surgical resection. Although radiotherapy and chemotherapy have produced modest benefits for some patients, it could lead to recurrent and multidrug resistance (4). Recently gene therapy, as one prevalent strategy, has been considered and employed in practice. Several genes have been explored for treating cancer, including tumor necrosis factor, p53, tumor suppressor gene, Herpes Simplex Virus Type-1 (HSV-1) and bacterial cytosine deaminase (CD) gene. However, clinical trials have shown that the therapeutic outcome was severely restricted by the poor efficiency of current gene transfer vector systems. In this sense, more novel tumor related gene candidates need to be found.

ABCE1 gene, as a member of the ATP-binding cassette (ABC) family, encodes a protein originally identified for its inhibition of ribonuclease L, a nuclease induced by interferon in mammalian cells. Ribonuclease L plays a key role in apoptosis pathway, cell proliferation and is also a candidate for tumor-suppressor protein (5,6). Therefore, it is valuable to study whether ABCE1 is responsible for cell metastasis and the cell proliferation in small cell lung cancer.

RNA interference (RNAi) is a process of sequence-specific post-transcriptional gene silencing initiated by double-stranded RNA (dsRNA) homologous with the target gene. Previous studies elucidated the molecular mechanisms underlying RNAi. dsRNA introduced into cells is processed by Dicer, a cellular RNA polymerase III (RNaseIII) that cleaves to generate duplexes of 21-23 nucleotides with two base-3' overhangs (small interfering RNA, siRNA), and can extend up to several hundred nucleotides. Thus, RNAi mediates sequence-specific mRNA degradation and exerts an interfering effect (7,8). RNAi represents evolutionary technology that has been adopted in functional genomic analysis, cellular signaling pathway studies, and the development of highly specific gene-silencing therapies to treat tumors and virus infections (9,10). siRNA expression mediated by a vector system named pSUPER enables an efficient and specific down-regulation of the gene expression in mammalian cells and the maintenance of stable loss-of-function phenotypes of the target gene (11).

Here, using RNAi technology, we investigated the roles of ABCE1 in the pathogenesis of SCLC by specially knocking down the expression of ABCE1 in NCI-H446 cells. Phenotypic changes resulting from the reduction in ABCE1 expression, including the protein levels of ABCE1, and cell

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Key words: ABCE1, small cell lung cancer, siRNA, NCI-H446

proliferation, invasiveness and apoptosis of NCI-H446 cells were investigated.

Materials and methods

Cell lines and culture conditions. The human small cell lung cancer cell line (NCI-H446) was obtained from Shanghai Biological Sciences Institute in China. Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 10 U/l penicillin G, and 100 mg/l streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Vector construction. Based on the ABCE1 cDNA sequence in Gene Bank, we used BLAST to designed 3 pairs of oligonucleotide synthesized by Dalian Biotechnologies (Dalian, China).

The sequences used were: (Si-1) F: 5'-GATCCGCTACAGCGAGTACGTTTACCTGTGAAGCCACAGATGGGGTAAACGTACTCGCTGTAGCTTTTTTG-3'; (Si-1) R: 5'-AATTCAAAAAGCTACAGCGAGTACGTTTACCCCATCTGTGGCTTCACAGGTAAACGTACTCGCTGTAGCG-3'; (Si-2) F: 5'-GATCCGAGTACGATGATCCTCCTGACTGTGAAGCCACAGATGGGTCAGGAGGATCATCGTACTCTTTTTTG-3'; (Si-2) R: 5'-AATTCAAAAAGAGTACGATGATCCTCCTGACCCATCTGTGGCTTCACAGTCAAGGAGTATCGTACTCG-3'; (Si-N) F: 5'-GATCCGCGAGACCTCAGTATGTTACCTGTGAAGCCACAGATGGGGTAACATACTGAGGTCTCGCTTTTTTG-3' (control group). Oligonucleotide was annealed and ligated with RNAi-ready pSIREN-DNR-DsRed-express using T4 DNA ligase. We constructed three recombinant plasmids: ABCE1-siRNA-1, ABCE1-siRNA-2, ABCE1-siRNA-N, and they were verified by sequencing and identified by restriction endonuclease digestion (Fig. 1).

Transfection of siRNA. NCI-H446 cells were seeded (2x10⁵ cells/well) in 6-well plates. After 24 h of incubation, they were transfected with ABCE1-siRNA-1, ABCE1-siRNA-2, ABCE1-siRNA-N, in serum-free medium using FuGENE HD (Roche, Germany). Vector (4 µg) and 10 µl of FuGENE HD were mixed and incubated for 15 min at room temperature. The mixture was then added to wash NCI-H446 cells, after 6 h of incubation, the mixture was replaced with full medium.

Western blot analysis of ABCE1. Cells were washed thrice with ice-cold PBS, and then lysed in lysis buffer for 30 min in ice-bath. Protein samples were electrophoresed on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane. Non-specific reactivity was blocked in 5% non-fat dry milk in TBST for 1 h at room temperature. The membrane was then immunoblotted with rabbit anti-human ABCE1 antibody (1:250; Jingmei Biotech Corp, Beijing, China) overnight at 4°C, followed by reaction with goat anti-rabbit antibody (1:500; Jingmei Biotech Corp). Each sample was also probed with β-actin antibody (Sigma-Aldrich Corp) as a loading control.

Cell proliferation assay. Cells were treated with siRNA as described above for 48 h. Then cells (2x10³/200 µl) were plated in 96-well micro plates. After 24, 48 and 72 h of culture, 20 µl of MTT solution (5 mg/ml in PBS) was added,

and the cells were incubated at 37°C for 4 h, then the supernatant was removed, and 150 µl of DMSO was added into each well. The dark-blue crystals of MTT were dissolved by shaking the plates at room temperature for 10 min and absorbance was then measured on a Bio-Rad Micro plate Reader (Bio-Rad, Hercules, CA) using a test wavelength of 490 nm and a reference wavelength of 570 nm.

FACS analysis. Cells were treated with siRNA as described above. After 48 h, cells were isolated and stained with annexin V-EGFP and propidium iodide (PI) (both from BD Biosciences Clontech, Palo Alto, CA). Cells were analyzed using FACS for fluorescence of annexin V positive and PI negative (apoptosis) cells. The fraction of apoptotic cells in the siRNA treated population was determined using the super-enhanced DMax method of WinList software (Verity Software House, Topsham, ME).

Invasion assays. The invasiveness of small cell lung cancer cells was assayed using modified transwell chambers. Polycarbonate filter (pore size, 8 µm) separating the upper and lower compartments was coated with 50 µg of reconstituted basement membrane (Matrigel, Peking University Health Science Center, China). At 48 h after transfection, serum-free RPMI-1640 containing 2.0x10⁵ cells in 300 µl were introduced into the upper compartment; the lower compartment contained RPMI-1640 supplemented with 15% FCS. After 48 h of incubation at 37°C, cells on the upper surface of the filter that had not invaded through the Matrigel were removed completely with cotton swabs. Cells that had invaded remained on the filter. Cells on the polycarbonate filter were fixed with Hoechst 33258.

Statistical analysis. The SPSS 13.0 software was applied to complete data processing. Data analysis was done by one-way ANOVA. Results were considered statistically significant at p<0.05

Results

Identification of ABCE1-siRNA-1, ABCE1-siRNA-2, ABCE1-siRNA-N. When annealed, oligonucleotide dsRNAs were ligated with pSUPER, ABCE1-siRNA-1, ABCE1-siRNA-2, ABCE1-siRNA-N were obtained. The results of restriction endonuclease-digestion of recombinant plasmids are shown in Fig. 1A. The sequence of the inserts was identical to that of the synthesized siRNA oligos (Fig. 1B).

ABCE1-RNAi induces morphology changes. After transfection with ABCE1-siRNA-1 or ABCE1-siRNA-2, red fluorescence was observed in the cytoplasm. We observed that some of the cells treated with ABCE1-siRNA were less confluent or became smaller and orbicular compared to control (Fig. 2). Consistently there were fewer cells in both ABCE1-siRNA-1 and ABCE1-siRNA-2 as compared to ABCE1-siRNA-N and control cells cultured 72 h after transfection. The ABCE1-siRNA treatment-decreased cell number of NCI-H446 implied that ABCE1 could participate in cell cycle progression and cell survival.

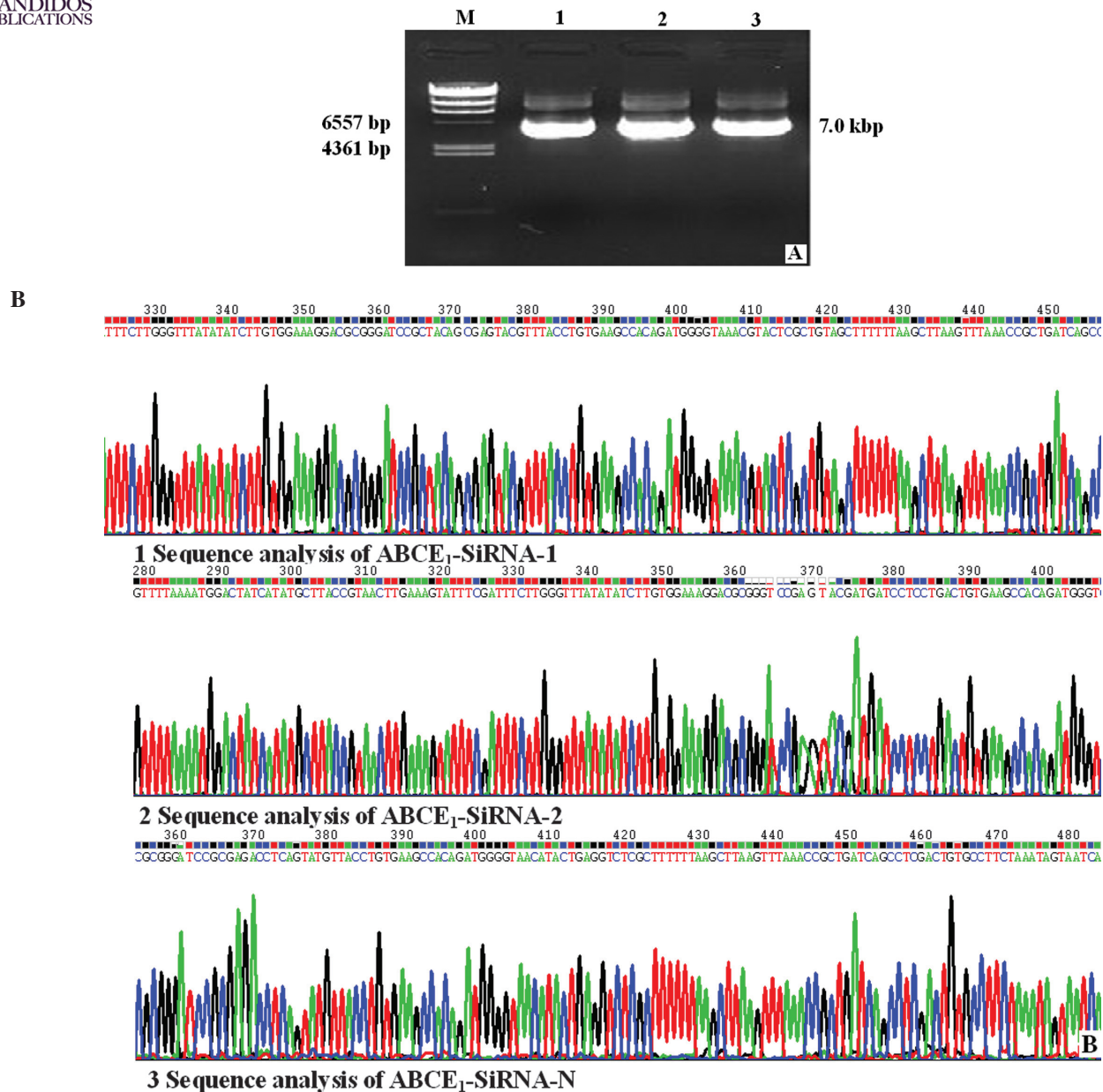


Figure 1. (A) Identification of recombinant plasmid ABCE₁-siRNA. M, λ -Hind III DNA marker; 1, recombinant plasmid ABCE₁-siRNA-1; 2, recombinant plasmid ABCE₁-siRNA-2; 3, recombinant plasmid ABCE₁-siRNA-N. (B) Sequencing analysis of recombinant plasmids and of the synthesized siRNA oligonucleotides.

Western blot analysis of ABCE1. Proteins of NCI-H446 cells were extracted. Cell lysate was analyzed for ABCE1 protein expression levels by immunoblot analysis with ABCE1 antibody. As shown in Fig. 3 transfected with ABCE₁-siRNA-1 and ABCE₁-siRNA-2 resulted in a significant inhibition of ABCE1 protein, respectively. ABCE1 expression was significantly down-regulated at protein level after transfection with ABCE₁-siRNA-1, ABCE₁-siRNA-2 compared with the untransfected or transfected with ABCE₁-siRNA-N cells. ABCE1-siRNA significantly knocked down the expression of ABCE1 in human small cell lung cancer cell line (NCI-H446).

Proliferation assays. Then we determined the proliferation of NCI-H446 cells with MTT, as shown in Fig. 4, compared

with NCI-H446 cells transfected with ABCE₁-siRNA-N. The proliferation of NCI-H446 cells transfected with ABCE₁-siRNA-1 and ABCE₁-siRNA-2 were significantly inhibited to between 75.54 and 71.60% ($P < 0.05$), 72.29 and 73.24% ($P < 0.05$), and 63.94 and 62.59% ($P < 0.01$) after 24, 48 and 72 h, respectively. There was no significant deviation between non-treated NCI-H446 and NCI-H446 cells transfected with ABCE₁-siRNA-N ($P > 0.05$).

Invasion assays. We used a transwell invasion assay to determine the possible function of ABCE1-siRNA in the invasiveness of small cell lung cancer cells. NCI-H446 cell, NCI-H446 cell transfected with ABCE₁-siRNA-1, ABCE₁-siRNA-2, ABCE₁-siRNA-N, were placed for 48 h on Matrigel-coated filters, and the filters were stained with Hoechst 33258 and

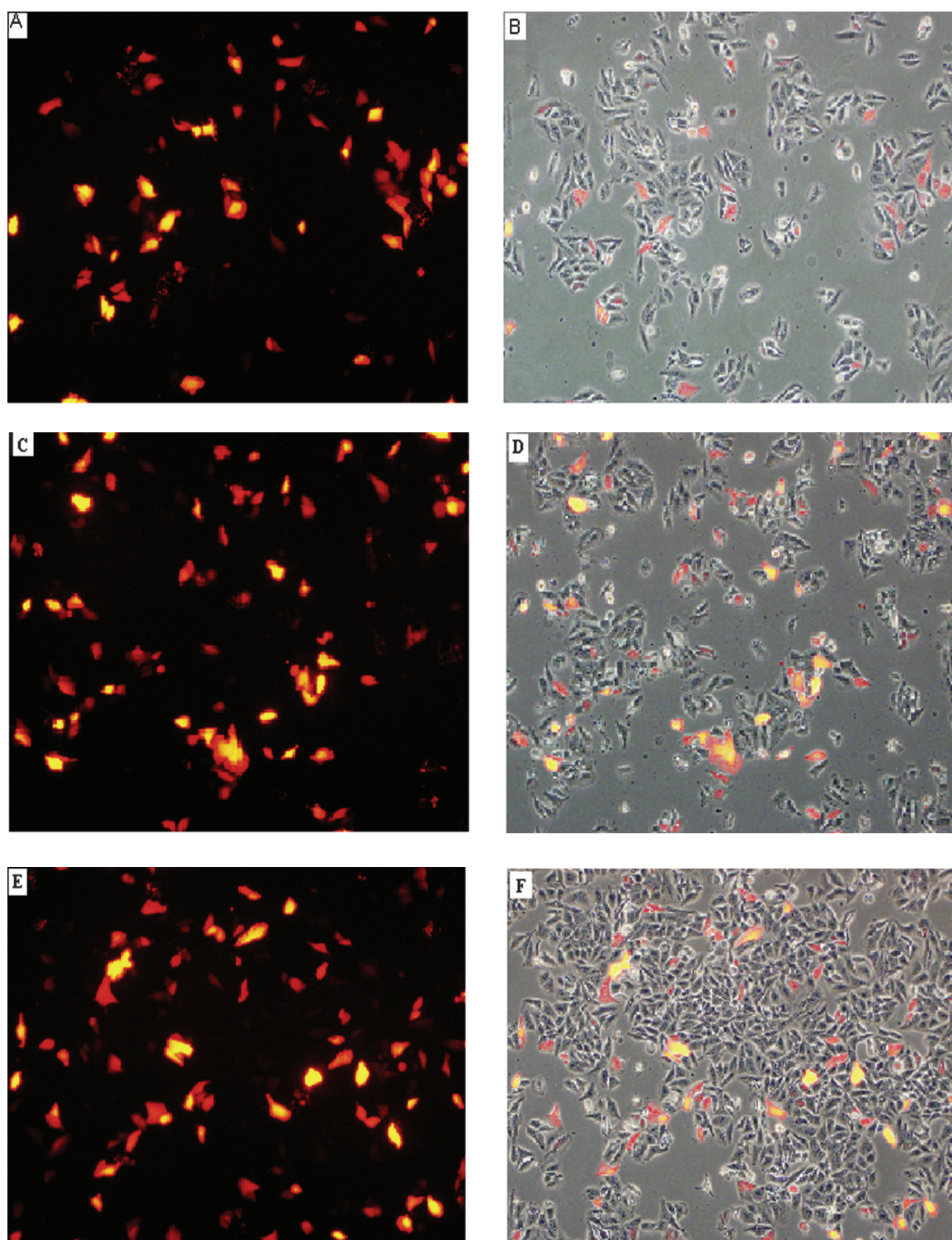


Figure 2. NCI-H446 cells transfected with ABCE₁-siRNA. (A and B) NCI-H446 cells transfected with ABCE₁-siRNA-1 (x100). (C and D) NCI-H446 cells transfected with ABCE₁-siRNA-2 (x100). (E and F) NCI-H446 cells transfected with ABCE₁-siRNA-N (x100).

inspected under fluorescence microscope. The cell number in the filter of NCI-H446 cell transfected with ABCE₁-siRNA-1 and ABCE₁-siRNA-2 was significantly decreased compared with the siRNA-N group and control group ($P < 0.05$). There was no significant difference between the siRNA-N group and control group ($P > 0.05$) (Fig. 5). These data demonstrated that the knockdown of ABCE1 by transient transfection of

ABCE1-siRNA was able to inhibit the invasiveness of small cell lung cancer cells *in vitro*.

ABCE1-RNAi induces apoptosis in NCI-H446 cells. The significant decrease of cell number as above suggested that NCI-H446 cells treated with ABCE1-siRNA may have undergone apoptosis. To test this, we used annexin V and

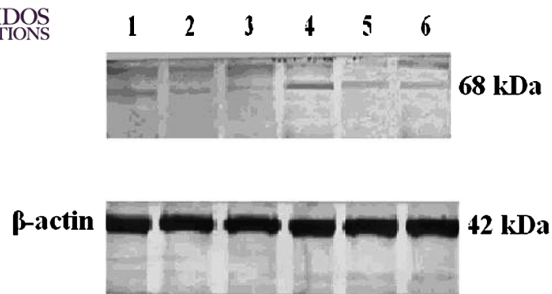


Figure 3. Western blotting of ABCE1. 1, control group; 2 and 3, NCI-H446 cells transfected with ABCE1-siRNA-1; 4, NCI-H446 cells transfected with ABCE1-siRNA-N; 5 and 6, NCI-H446 cells transfected with ABCE1-siRNA-2.

propidium iodide (P.I) to stain cells followed by fluorescence activated cell sorting analysis. Further, we confirmed that the cells had undergone apoptosis (Fig. 6), and this result indicated that ABCE1 could regulate an anti-apoptotic program in NCI-H446 cells and the inactivation of ABCE1 could lead to induction of apoptosis.

Discussion

Small cell lung cancer, which always has lymph metastasis in early stage, is a lung cancer unsuitable for surgical resection. Although radiotherapy and chemotherapy have produced modest benefits in some patients, after traditional therapies it could recur leading to multidrug resistance. It is important to investigate its mechanism and develop therapies for this cancer.

ABCE1 gene (HP68) locates in chromosome 4q31 and the full-length cDNA encodes 599 amino acids. ABCE1, which contains a unique N-terminal region with eight conserved cysteines, is an ATP-binding cassette transporter gene and locates in cytoplasm and mitochondria. ABCE1 is universally expressed in human tissues and organs (12), but at different expression forms and levels. For example, it is highly expressed in the brain, kidney and prostate while in the lung, liver, spleen, heart, pancreas, only slightly. In bone and spinal cord there is no expression at all. Studies have revealed ABCE1 gene expression was amplified in 19 drug-resistant cell types.

ABCE1 was formerly named RNase L inhibitor, which specifically combines with RNase L and inhibits its activity. In gene function studies, the specific knock-down of target genes without affecting other genes is critically important, such as RNAi. RNAi mediated by siRNA is a specific gene-silencing technology. Using this technology we constructed the ABCE1-siRNA expression vector and transfected ABCE1 into the small cell lung cancer cell line NCI-H446. We found that both ABCE1-siRNA-1 and ABCE1-siRNA-2 could inhibit ABCE1 expression in small cell lung cancer. Previous studies have confirmed (13) that RNase L could specifically bind with RNA and degrade it, while preventing the translation of the protein and leading to cell apoptosis. When RNase L amount increased, cell apoptosis rate significantly increased. On the contrary when ABCE1 gene was transfected into cells, RNase L's activity significantly

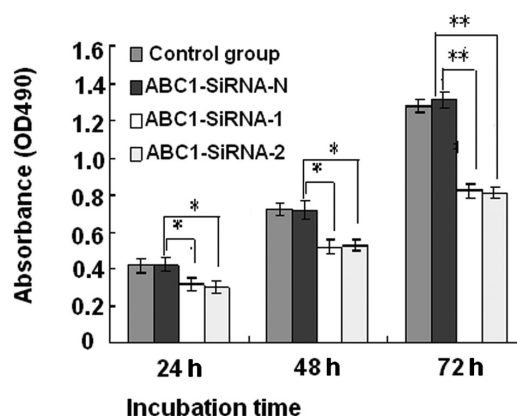


Figure 4. Decrease in the proliferation potential of cells transfected with ABCE1-siRNA. Small lung cancer cells seeded in 96-well micro plates were cultured for 24, 48 and 72 h and their number was determined by absorbance as described in Materials and methods. *P<0.05; **P<0.01; compared with ABCE1-siRNA-N (ANOVA). Data are representative of one of 3 independent assays.

decreased and apoptosis pathway was inhibited. The cell proliferation and differentiation capabilities were enhanced (5). Variation in the RNase L has been recently reported to be associated with an increased risk of prostate cancer (14,15); the lack of RNase L also may trigger prostate cancer. Another study (16) showed that ABCE1 participated in the original immune response of colon cancer. Chen *et al* (2) showed that when ABCE1-siRNA was transfected into HEK239 cells, cell proliferation was significantly inhibited, which suggest that ABCE1 expression was related to cell proliferation. Our results shown that after transfected with ABCE1-siRNA, cell proliferation significantly decreased, which was consistent with the conclusions of Chen *et al* (2).

The tumor invasion and metastasis are the chief causes of treatment failure. Invasion and destruction of tumor cells, migration into the vascular system is the key step in the process of tumor invasion and metastasis. To some extent, invasion and migration of tumor cells represent its malignant potential (17,18). In the transwell chamber experiments our results showed that after transfection with ABCE1-siRNA, cell invasion declined and the malignancy degree was reduced.

RNase L induced apoptosis and prevented uncontrolled cell proliferation and the inhibition of apoptosis is the key characteristic in malignant tumors. It has been confirmed (19,20) that transfected RNase L cells induced cell apoptosis. ABCE1, is an inhibitor of RNase L, thus ABCE1-siRNA may also increase the RNase L of cells. Our results showed that when NCI-H446 cells were transfected with ABCE1-siRNA-1, ABCE1-siRNA-2, cell apoptosis rate significantly increased, which was consistent with that expected. We inferred that this would be one of the mechanisms of biological behavior change after transfection of ABCE1-siRNA into small cell lung cancer cells.

Treatment of human small cell lung cancer cell lines with ABCE1-siRNA resulted in morphologic and biochemical changes. Although ABCE1-siRNA inhibited the amount of ABCE1 in NCI-H446, it had no significant effect on their viability. This suggested that ABCE1 may be an ideal target for cancer therapy.

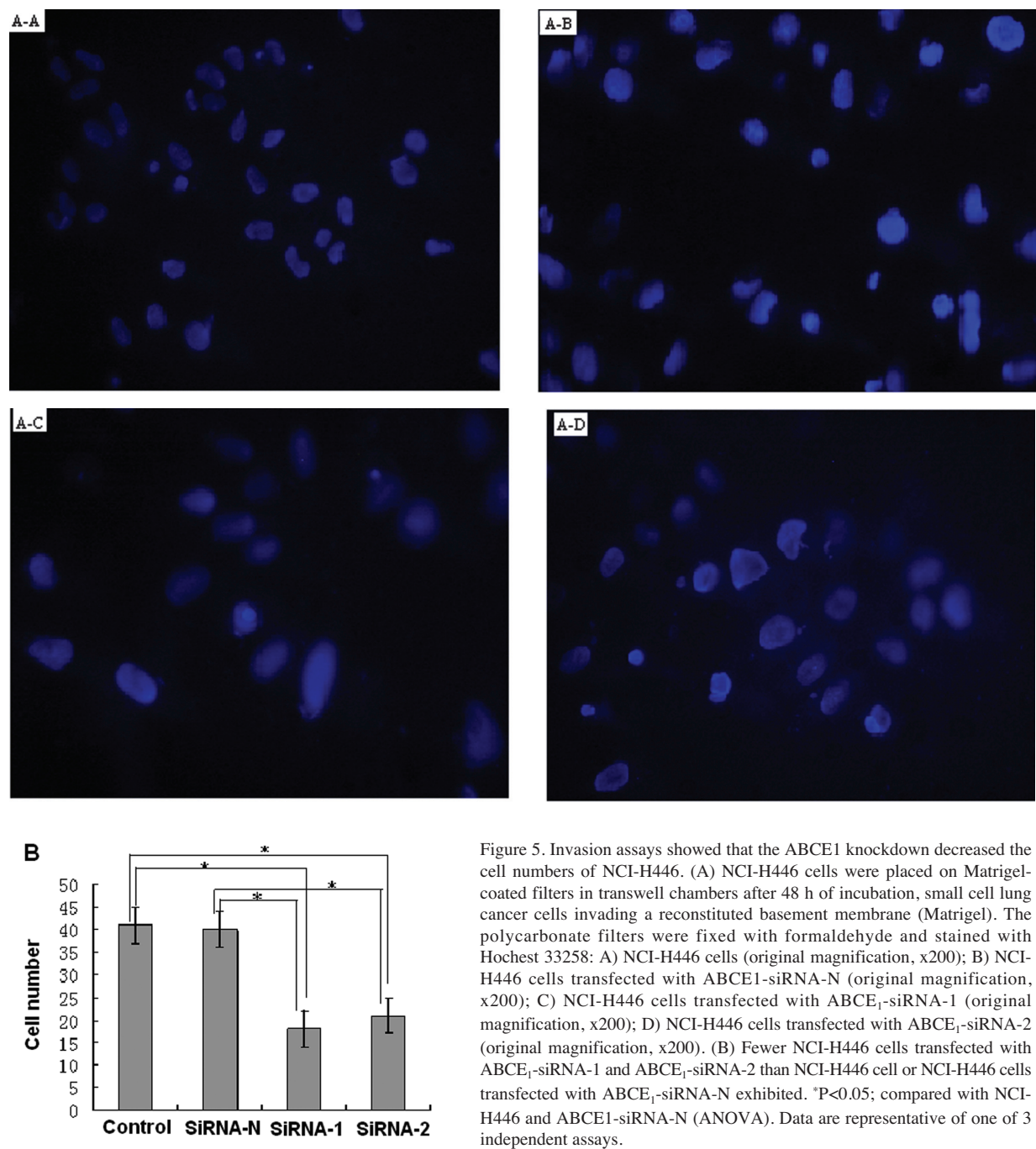


Figure 5. Invasion assays showed that the ABCE1 knockdown decreased the cell numbers of NCI-H446. (A) NCI-H446 cells were placed on Matrigel-coated filters in transwell chambers after 48 h of incubation, small cell lung cancer cells invading a reconstituted basement membrane (Matrigel). The polycarbonate filters were fixed with formaldehyde and stained with Hoechst 33258: A) NCI-H446 cells (original magnification, x200); B) NCI-H446 cells transfected with ABCE1-siRNA-N (original magnification, x200); C) NCI-H446 cells transfected with ABCE1-siRNA-1 (original magnification, x200); D) NCI-H446 cells transfected with ABCE1-siRNA-2 (original magnification, x200). (B) Fewer NCI-H446 cells transfected with ABCE1-siRNA-1 and ABCE1-siRNA-2 than NCI-H446 cell or NCI-H446 cells transfected with ABCE1-siRNA-N exhibited. * $P<0.05$; compared with NCI-H446 and ABCE1-siRNA-N (ANOVA). Data are representative of one of 3 independent assays.

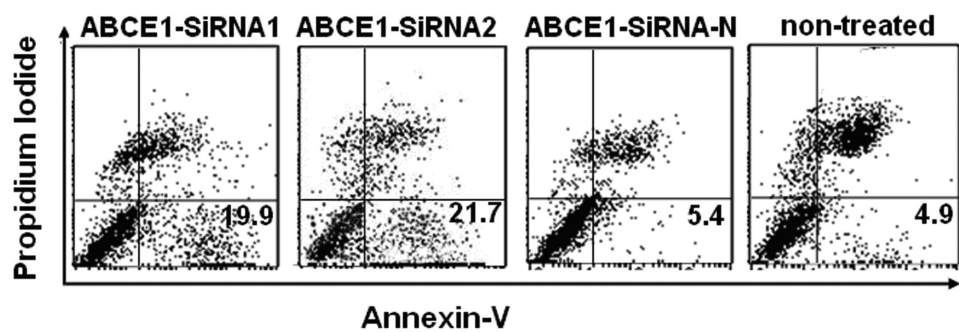


Figure 6. ABCE1 knockdown decreased the apoptosis of NCI-H446 cells. NCI-H446 cells transfected with ABCE1-siRNA-1 , ABCE1-siRNA-2, ABCE1-siRNA-N respectively or left untreated. Then cells were collected and stained with annexin-V and PI. Data are representative of one of 3 independent assays.



Summary, our research showed that ABCE1-targeting could significantly down-regulate the ABCE1 level in small cell lung cancer cells and also it could inhibit cell proliferation, invasiveness, and induce cell apoptosis. Our findings could provide novel insights for the development of gene therapy technology to treat patients with small cell lung cancer.

Acknowledgements

This study was supported by National Natural Science Foundation of China (30973520) and the Educational Department of Liao Ning Province (20060966, 2008768).

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