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Suppression of *ABCE1* Leads to Decreased Cell Proliferation and Increased Apoptosis in 95-D/NCI-H446 Lung Carcinoma Cells*

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Abstract ATP-binding cassette protein E (ABCE1) has been annotated as an RNase L inhibitor in eukaryotes. Previous study showed that the overexpression of ABCE1 was related with the occurrence and clinical stage of lung adenocarcinoma. As an initial investigation into the novel functions of *ABCE1*, siRNA-expressing vectors targeting sites of the *ABCE1* gene were constructed from RNAi-Ready pSIREN-DNR-DsRed-Express vector. Cultured 95-D and NCI-H446 lung carcinoma cells were transfected with the siRNA-expressing vectors using FuGENE 6 and transfection efficiency was determined by using fluorescence microscopy. The expression level of ABCE1 protein was determined by Western blot and immunofluorescence staining. Cell viability was determined by MTT, cell cycle was analysed by flow cytometry.The apoptotic rate was observed by ELISA. Fluorescence microscopy showed a satisfactory transfection efficiency which was about 42.70%. Cell viability and the growth fraction were markedly suppressed,whereas the apoptosis was significantly increased in SiRNA-95-D and SiRNA-NCI-H446 cells than controls($P < 0.05$). It can be concluded that the siRNA targeting *ABCE1* gene shows a dramatic inhibitory effect on RNA transcription and protein expression and a promoting effect on the apoptosis in 95-D/NCI-H446 cells, which offers a reliable base for the further *in vivo* experiment.

Key words ABCE 1, apoptosis, lung carcinoma cells, proliferation, RNA interference
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Tumor is an abnormal growth of local tissue resulting from uncontrolled multiplication of cells on gene level under various causes. Infiltration and metastasis are the most important biological characteristics of malignant tumors[1]. Recent progress of modern molecular biology has already announced that metastasis is a complicated multiple-step course in which many special genes are involved, including activating of metastatic gene and inactivating of metastatic suppressor and their interaction[2]. It is clearly imperative that many malignant tumors be cured if we could find real effective target genes for gene therapy. Therefore, screening for metastasis-related genes has long been one of the hotspots in modern tumor molecule biology.

ATP-binding cassette protein E(ABCE) has been annotated as an RNase L inhibitor in eukaryotes[3~6]. All eukaryotic species show the ubiquitous presence and high degree of conservation of ABCEs, however, RNase L is present only in mammals[7~11]. This indicates that ABCE1 may not only function as RNase L inhibitors, but also have other functions that have yet to be determined. Our previous study showed that ABCE1 protein and mRNA were overexpressed in 48 lung adenocarcinoma tissues samples[12]. The overexpression of ABCE1 was related with the occurrence and clinical stage of lung adenocarcinoma[12]. As an initial investigation into the novel functions of *ABCE1*, we constructed siRNA expressing vectors of the *ABCE1* gene, transfected

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them into 95-D/NCI-H446 lung carcinoma cells by FuGENE 6, selected cells by culturing the cells in the presence of G418 (Gibco-BRL). In this study, Our present data show that RNAi technology can be used to down-regulate ABCE1 expression, resulting in suppression of cell growth and induction of apoptosis in lung carcinoma cells 95-D/NCI-H446.

1 Materials and methods

1.1 Cell line

Lung carcinoma cells 95-D and NCI-H446 were obtained from Shanghai Institute of Biochemistry and Cell Biology, The Chinese Academy of Sciences.

1.2 shRNA preparation and plasmids construction

In our previous study, three hairpin *ABCE1* siRNAs were designed according to the *ABCE1* gene sequence from GenBank. Using the DNA recombination technique, the synthetic oligonucleotides containing the target sequences were inserted into the *Bgl* Ⅱ and *Hin*d Ⅲ restriction sites of the RNAi-Ready pSIREN-DNR-DsRed-Express vectors. The positive clones (Si-1, Si-2) and the negative control plasmid (Si-N) according to the *ABCE1* gene sequence were constructed completely[13].

1.3 Cell culture and plasmid transfections

Lung carcinoma cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 mg/L streptomycin at 37℃, and 5% CO_2. For transfection, the cells were seeded in 6-well plates at 1×10^6 cells/well and allowed to grow overnight to 80%～90% confluency. Lung carcinoma cells were transfected with the mixture of 1 μg plasmid DNA (Si-1 or Si-2 or Si-N) and 6 μl FUGENE6 according to the manufacturer's instructions in 93 μl serum-free medium. At 8 h after transfection 500 μl 10% FBS/well was added. At 36 h after transfection, the medium was replaced by normal medium containing 10% FBS and 1 g/L G418 (Gibco-BRL) up to 5 d(changed medium every 2 d) after transfection.

1.4 Transfection efficiency detection

The RNAi-Ready pSIRENDNR DsRed Express Vector is designed to express a small hairpin RNA (shRNA) driven by the human U6 promoter (P_{U6}; RNA Pol Ⅲ -dependent). This vector expresses a variant of *Discosoma* sp. red fluorescent protein, which has been engineered for improved solubility (excitation maximum =557 nm; emission maximum =579 nm). Using the inverted fluorescence microscopy (IX71, Olympus) and auto picture analysis system (SPOT RFKE, USA), the delivery efficiency of gene silencing construct 48 h after transfection was directly monitored.

1.5 RT-PCR analysis for *ABCE1* gene expression

Cells were harvested 48 h after transfection. Total RNA was purified using the Total RNA Isolation System (Qiagen). Reverse transcription-PCR was performed with the isolated total RNA (100 ng) using the Omniscript RT kit and HotStarTaq PCR kit (Qiagen) according to the manufacturer's instructions. The primers were: *ABCE1*(991～1 500 bp), 5′ AGCGAGTACGTTTACCTGTGAAGCCG 3′ and 5′ AGCGAGTACGTTTACCCCATCTGTGGCTTC 3′; *GAPDH*(336～1 235 bp) , 5′ CGAAGGTGAAGGTCGGAGTC 3′ and 5′ GACCACCTGGTGCTCAGTGT 3′. When RT-PCR was finished, 1 μl from the reaction mixture was withdrawn and analyzed by agarose gel electrophoresis followed by ethidium bromide staining. The 500 bp *ABCE1* bands were cut from the gel and extracted using a DNA gel extaction kit (Qiagen). DNA concentration was determined using the GeneQuant pro RNA/DNA Calculator (Biochrom Ltd). *ABCE1* gene expression was calculated by dividing the concentration of the RT-PCR product of the treated cells by the concentration of the RT-PCR product of the untreated cells (taken as 100%). Each point represents the average of triplicate tests.

1.6 Western blot analysis

Cells were harvested and lysed with RIPA buffer (0.15 mol/L NaCl, 1% NP40, 0.01 mol/L deoxycholate, 0.1% sodium dodecyl sufate [SDS], 0.05 mol/L Tris-HCl pH 8.0, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 g/L each of aprotinin, pepstatin, and leupeptin). Fifty micrograms of each soluble protein sample was separated by 12% or 15% SDS-polyacrylamide gel electrophoresis (PAGE), blocked in 2.5% skim milk/TPBS(1 phosphate buffered saline [PBS] containing 0.1% Tween 20), and probed with each primary antibody overnight at 4℃. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). The intensity of ABCE1 and GAPDH were evaluated. The relative levels of ABCE1 were as the ratio of ABCE1/GAPDH.

1.7 Cell growth and viability assay

Untreated cells or the transfected cells at 48, 72

and 96 h after transfection were harvested and reseeded at 1×10^4 cells/well in a 12-well or 1×10^3 cells/well in a 96-well plate. Cells were cultivated with RPMI 1640 medium in the CO_2 incubator at 37℃, changed medium every 2 d up to 8 d after transfectioned. The total cell number was determined every 2 d with a hematocytometer and under an inverted microscope (Olympus). Cell viability was determined by measuring the mitochondrial conversion of 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) to a colored product at 48 h, 72 h and 96 h after transfection. The cells were incubated with MTT reagent (Sigma USA) 0.5 g/L, at 37℃ for 4 h and then with isopropanol at room temperature for 1 h. The spectrophotometric absorbance of the samples was measured using an Ultra Multifunctional Microplate Reader (Tecan, Durham, NC). Results were plotted as the $\bar{x}\pm s$ of 3 separate experiments from 6 determinations per experiment for each experimental condition.

1.8 Cell cycle analysis

Cells (1×10^6) at 48 h, 72 h and 96 h after transfection were harvested and washed in cold PBS followed by fixation in 70% alcohol for 30 min on ice. After washing in cold PBS three times, cells were resuspended in 0.8 ml of PBS solution with 40 μg of propidium iodide (Sigma) and 0.1 μg of RNase A (Qiagen) for 30 min at 37℃. Samples were analyzed for DNA content by flow cytometry immediately (FACScan, BD Biosciences, San Jose, CA).

1.9 Histone DNA ELISA apoptosis assay

The Cell Death Detection ELISA Kit (Roche USA) was used for assessing apoptosis in 95-D/NCI-H446 cells at 48 h, 72 h and 96 h after transfection according to the manufacturer's protocol. Briefly, untreated cells or the transfected 95-D/NCI-H446 cells were lysed, and the cell lysates were overlaid and incubated in microtiter plate modules coated with antihistone antibody (Hyclone USA). Samples were then incubated with anti-DNA peroxidase (Sigma USA) followed by color development with ABTS™ substrate. The optical densities of the samples were determined by using the Ultra Multifunctional Microplate Reader (Tecan) at 405 nm.

1.10 Statistical analysis

Results were expressed as ($\bar{x}\pm s$) and the mean values were compared by using the ANOVA (SNK, Student-Newman-Keuls test) in the SAS 8.1 software and $P<0.05$ was considered statistically significant.

2 Results

2.1 Efficacy of siRNA express vector in transfection

The RNAi-Ready pSIREN DNR DsRed Express Donor Vector is used for targeted gene silencing when a dsDNA oligonucleotide encoding an appropriate shRNA is ligated into the vector. It can be used directly in transient transfection experiments to screen shRNA constructs for efficacy. This vector expresses a variant of *Discosoma* sp. red fluorescent protein, which allows us to directly monitor the delivery efficiency of gene silencing construct using fluorescence microscopy 48 h after transfection. 95-D and NCI-H446 cells were transfected with the siRNA plasmid (Si-1 or Si-2) respectively. Fluorescence microscopy showed that the transfection efficiency was satisfactory, which is about 42.70%(Figure 1).

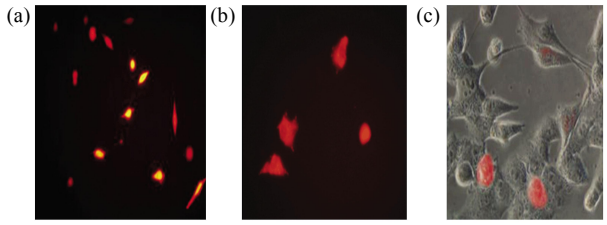

Fig. 1 Fluorescence microscopy shows satisfactory transfection efficiency in lung carcinoma cells

The RNAi-Ready pSIREN-DNR-DsRed-Express Vector expresses a variant of *Discosoma* sp. red fluorescent protein. (a) 95-D cells were transfected with positive plasmid vector (Si-1) (×200). (b) NCI-H446 cells were transfected with positive plasmid vector (Si-2) (×400). (c) 95-D cells were transfected with negative control vector (Si-N) (×400).

2.2 siRNA-expressing vector inhibiting *ABCE1* mRNA expression

We examined the siRNA-expressing vector Si-1 or Si-2 potently suppressed the synthesis of *ABCE1* mRNA in human lung carcinoma cells. The result showed that two recombinant plasmids of *ABCE1* RNAi (Si-1 and Si-2) and 1 negative control plasmid (Si-N) were constructed successfully and tested for their ability to knock down ABCE1 expression in the human lung carcinoma cells. Transfection with Si-1 and Si-2 resulted in a reproducible decrease of 60% to 70% in the expression level of both *ABCE1* mRNA (as judged by RT-PCR), whereas transfection with Si-N failed to reduce the level of *ABCE1* mRNA (Figure 2a, b).

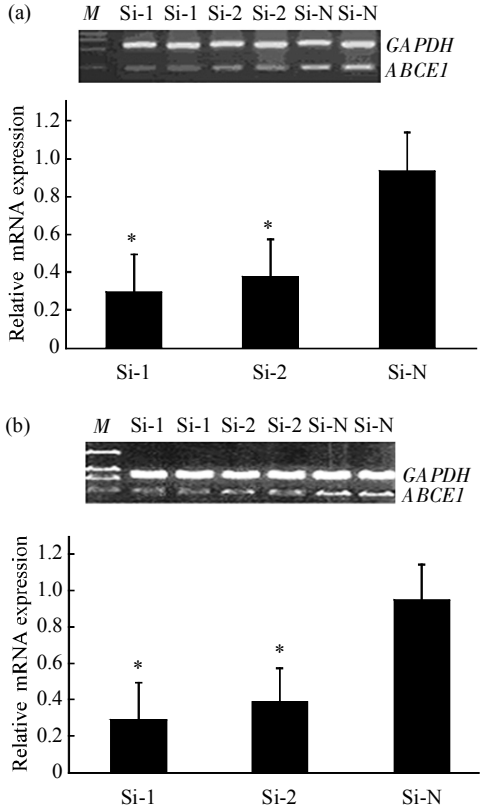

Fig. 2 *ABCE1* mRNA expression after transfection treatment siRNA-expressing vectors

(a) 95-D cells. (b) NCI-H446 cells. (a) and (b) were illustrated inhibition of *ABCE1* mRNA at 48h after transfection treatment siRNA-expressing vectors (Si-1, Si-2) in human lung carcinoma cancer cells (95-D and NCI-H446). Control lane: Control cells were treated with negative control plasmid (Si-N). The mRNA levels in 95-D and NCI-H446 cells treated with siRNA-expressing vector were assessed by using RT-PCR analysis. The level of *ABCE1* mRNA was standardized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as a loading control.The data represent the mean ± standard error of 3 independent experiments. $P < 0.05$ (asterisk) was considered to have statistical significance. *M*: Molecular mass marker.

2.3 Effect of siRNA-expressing vector on ABCE1 protein expression

We evaluated the effect of siRNA-expressing vectors (Si-1, Si-2) on target protein ABCE1 by Western blotting analysis. 95-D or NCI-H446 cells were transfected with varying concentrations of siRNA ABCE1 (10 nmol/L, 50 nmol/L, and 100 nmol/L). The result showed that siRNA-expressing vectors (Si-1, Si-2) markedly down-regulated the expression of ABCE1 in human lung carcinoma cells as compared with untreated cells. However, treatment with negative control plasmid (Si-N) vector did not change the expression of ABCE1 in human lung carcinoma cells as compared with untreated cells (Figure 3a, b).

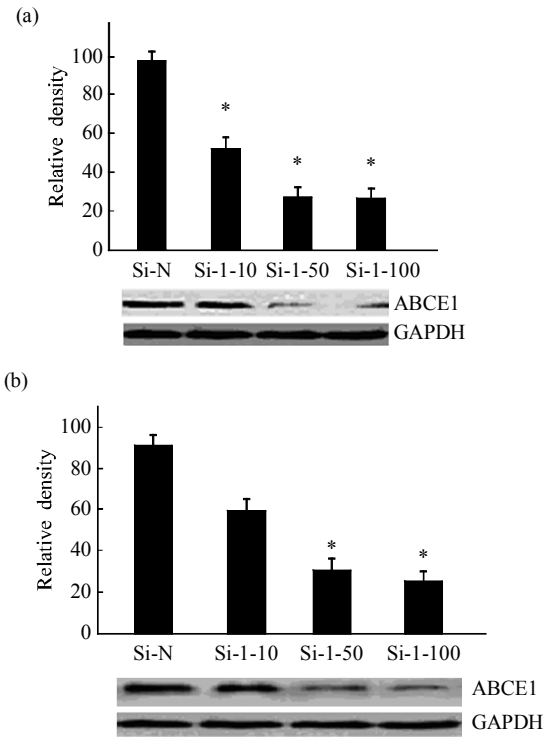

Fig. 3 Western blotting analysis to show effect of siRNA-expressing vectors in a concentration-dependent manner

(a) 95-D cells. (b) NCI-H446 cells. Control lane: 95-D/NCI-H446 cells were transfected with negative control Si-N vector; Si-1-10: Human lung carcinoma cancer cells were transfected with 10 nmol/L siRNA (Si-1); Si-1-50: Human lung carcinoma cancer cells were transfected with 50 nmol/L siRNA (Si-1); Si-1-100: Human lung carcinoma cancer cells were transfected with 100 nmol/L siRNA (Si-1). The average signal intensity of ABCE1 protein was standardized to GAPDH. The data represent the mean ± standard error of 4 independent experiments. $P < 0.05$ (asterisk) was considered to have statistical significance.

2.4 Effect of siRNA-expressing vector on cell proliferation

We have analyzed siRNA-expressing vector Si-1 on the cell proliferation of 95-D or NCI-H446 lung carcinoma cells. It showed that siRNA-expressing vector Si-1 targeting *ABCE1* significantly inhibited the cell growth in lung carcinoma cells as compared with the untreated cells ($P < 0.05$) (Figure 4a, b). Cells treated with control siRNA expressing vector Si-N showed only slight cell growth inhibition and had no difference as compared with the untreated cells. Cell viability was determined by MTT at 48, 72 and 96 h after transfection. Cell lines of siRNA-expressing

vector Si-1 targeting *ABCE1* after transfection showed significantly inhibited proliferation compared with the controls cells ($P < 0.05$)(Figure 4c, d).

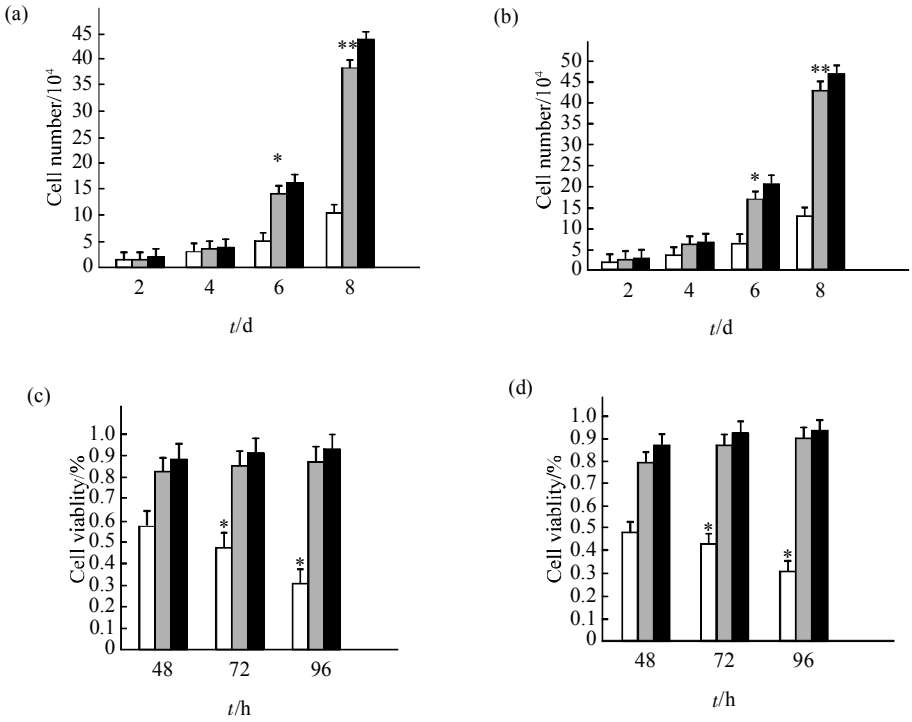

Fig. 4 Effect of siRNA-expressing vector on cell proliferation

(a, b) Treated and untreated 95-D(a)/NCI-H446(b) cells 48 h after transfection. (c, d) Cell viability of 95-D(c)/ NCI-446(d) cells. Cells were cultured in a 96-well plate. Cell viability was determined by MTT at 48, 72 and 96 h after transfection. Data are the $\bar{x} \pm s$ of 3 independent experiments (*$P < 0.05$ and **$P < 0.01$). □: Si-1; ▒: Si-N; ■: N.

2.5 Effect of treatment on cell cycle distribution

Cell cycle analyses were carried out to characterize the treatment effect on the growth fraction (S + G2-M) distribution by flow cytometry. The percentage of cycling cells were relatively constant (40% ～44%) in untreated cells over 96 h, whereas transfected cells exhibited a significantly inhibited growth fraction. As shown in a representative study (Figure 5), S + G2-M cycling cells constituted (22.41± 0.41)% of Si-95 cells, (20.12± 2.13)% of Si-446 cells at 72 h after transfection, and (18.54±2.52)% of Si-95,(17.50 ± 4.30)% of Si-446 at 96 h. Cell lines of siRNA-expressing vector Si-1 target *ABCE1* after transfection (Si-95 cells and Si-446 cells) showed significantly inhibited growth fraction compared with the controls cells ($P < 0.05$).

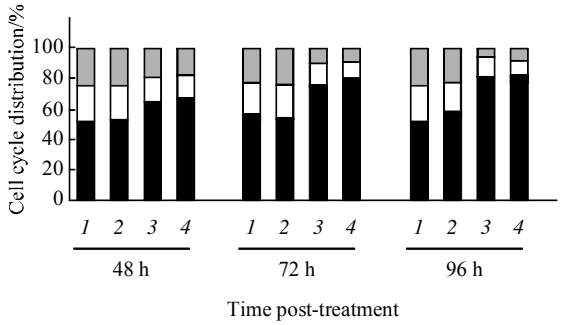

Fig. 5 Effect of treatment on cell cycle distribution

Data are $\bar{x} \pm s$ of 3 independent experiments. *1*: N-95; *2*: N-446; *3*: Si-95; *4*: Si-446. ■: G0/G1; □: S; ▒:G2/M.

2.6 *ABCE1* shRNAs induced cell apoptosis

The apoptosis rate in lung carcinoma cells (95-D/NCI-H446) was observed by ELISA at 48 h, 72 h and 96 h after transfection. As shown in Figure 6a, the apoptosis in 95-D cells was(0.512±0.013, 0.961±0.012, 1.344±0.011) for siRNA express (Si-1), (0.201±0.011, 0.242±0.012, 0.270±0.011) for negative control (Si-N) and (0.052 ±0.013, 0.094 ±0.011, 0.103 ±0.011) for control 95-D cells (N), respectively. The apoptosis in NCI-H446 cells was (0.502 ±0.015, 0.963 ±0.011, 1.544±0.011) for siRNA express (Si-1), (0.211±0.011, 0.244±0.013, 0.307±0.011) for negative control (Si-N) and (0.082 ±0.013, 0.097 ±0.012, 0.113 ±0.012) for control NCI-H446 cells (N), respectively (Figure 6b).

From the above results, apoptosis was much higher in cells with *ABCE1* silencing than the controls ($P<0.05$).

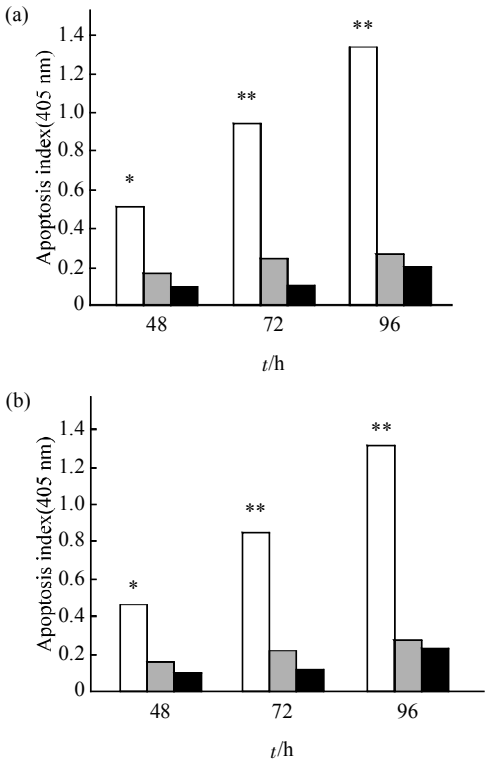

Fig. 6 ABCE1 shRNAs induced cell apoptosis

(a) 95-D cell. (b) NCI-H446 cell. Apoptosis in 95-D/NCI-H446 cells was measured with the histone/DNA fragment analysis by using an enzyme-linked immunoadsorbent assay at 48 h, 72 h and 96 h after transfection, respectively. Si-1 and Si-N were treated with transfection cell lines, N was the untreated 95-D/NCI-H446 cell line. Data are $\bar{x} \pm s$ of 3 independent experiments, (*$P < 0.05$ and **$P < 0.01$). □ : Si-1; ▩ : Si-N; ■ : N.

3 Discussion

The ATP-binding cassette enzyme *ABCE1* (also known as RNase-L inhibitor, Pixie and HP68), one of the evolutionary most sequence conserved enzymes in evolution and present in all archaea and eukaryote[3, 4]. All eukaryotic species show the ubiquitous presence and high degree of conservation of ABCEs, however, RNase L is present only in mammals[7~11]. This indicates that *ABCE1* may function not only as RNase L inhibitors, but also may have other functions that have yet to be determined. Our previous study showed that ABCE1 protein and mRNA were over expressed in 48 lung adenocarcinoma tissue samples. And, the over expression of ABCE1 was related with the occurrence and clinical stage of lung adenocarcinoma.

RNAi is characterized by high efficiency, high specificity and low toxicity. siRNA has become a powerful tool for studies on gene function, carcinoma and viral disease therapy[14]. In this study, we designed and constructed the siRNA plasmid expression vector. Its transcript can form a short hairpin RNA (shRNA) with inverted repeat sequence separated by a short loop sequence, then the shRNA is processed into functional siRNA to degradate target mRNA and silences its expression. The siRNA plasmid expression vector constructed in this study contains the *ABCE1* gene that makes it convenient to observe the result of transfection. The RNAi-Ready pSIREN-DNR-DsRed-Express Vector expresses a variant of *Discosoma* sp. red fluorescent protein. The DsRed-Express fluorescent marker allows us to directly monitor the delivery efficiency of our gene silencing construct using either fluorescence microscopy or flow cytometry at 8 h after transfection. For our study, we designed and successfully constructed 2 *ABCE1* gene expression vectors of double-stranded RNAi and verified that both of them (Si-1 and Si-2) were effective in that they knocked down *ABCE1* gene expression by 60% to 70% in lung carcinoma cells. In contrast, the negative sequence (Si-N) was not effective. From these results, we conclude that the RNAi constructs cause selective degradation of *ABCE1* mRNA and thereby decrease ABCE1 protein expression levels in lung carcinoma cells and inhibit the cells growth in a certain degree.

Current data suggest that *ABCE1* has key cellular roles in translation or ribosome biogenesis, the assembly of HIV1 capsids and inhibition of RNase L[15~18]. By promoting efficient capsid formation in cells, *ABCE1* may act as a molecular chaperone in concert with RNA to ensure Gag multimerization under circumstances where assembly is not favored[19]. Some data provide new and direct evidence for a more general role of RNase L in the regulation of cellular mRNAs, and RNase L and RLI(ABCE1) could regulate the antiproliferative effect of IFN [20]. Mitochondria play a central role in apoptosis by releasing cyto-cytochrome c [20]. On the other hand, activation of RNase L causes caspasedependent apoptosis accompanied by cytochrome c release[20]. These results show that the regulation of mitochondrial mRNAs by RNase L and its inhibitor RLI(ABCE1) is at the center of the pathway leading from IFN to apoptosis. In the study, it shows that the siRNA targeting *ABCE1* gene

shows a dramatic inhibitory effect on RNA transcription and protein expression and a promoting effect on the apoptosis in 95-D/NCI-H446 cells. It is possible that cancer cells are more sensitive to inhibition of protein translation through *ABCE1* than are normal cells.

In summary, we show that RNAi knock-down of ABCE1 leads to suppression of cell growth and induction apoptosis, and down-regulation aggressiveness and invasiveness in the 95-D/ NCI-H446 lung carcinoma cells. All these investigations suggest that *ABCE1* may serve as a potential target metastasis-related gene.

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RNA 干扰 *ABCE1* 基因后可抑制肺癌 95-D/NCI-H446 细胞的增殖和诱导细胞凋亡 *

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摘要 ABCE1 作为 RNase L 抑制剂首先是在脊椎动物中被发现的. 前期研究结果显示 ABCE1 与肺腺癌的发生率及临床分期显著相关. 为了进一步研究 ABCE1 的新功能，构建了 *ABCE1* 基因的 siRNA 表达质粒(RNAi-Ready pSIREN-DNR-DsRed-Express vector)，培养肺癌细胞(95-D 和 NCI-H446)，用 FuGENE 6 作为转染试剂转染后，使用荧光显微镜观察转染效果，RT-PCR 分析 *ABCE1* 基因表达，Western blot 分析 ABCE1 蛋白的表达，MTT 法检测细胞的活性，流式细胞仪分析细胞周期，ELISA 法检测细胞凋亡. 结果显示：质粒的转染效果较满意，阳性率约为 42.70%；在实验组，细胞活性和生长指数明显受到抑制，细胞凋亡明显增加，与对照组比较差异显著($P < 0.05$). 上述结果显示，RNA 干扰 *ABCE1* 基因可显著抑制肺癌细胞(95-D/NCI-H446) RNA 的转录、蛋白质的表达，并增加细胞凋亡，为进一步研究 *ABCE1* 基因提供必要的基础.

关键词 ABCE1, 凋亡，肺癌细胞，增殖，RNA 干扰

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