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Silencing *ABCE1* Increases E-Cadherin Expression and Decreases Cell Invasion in 95-D/NCI-H446 Lung Carcinoma Cells*

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Abstract To investigate further the role of *ABCE1* in 95-D/NCI-H446 lung carcinoma cells, the protein of ABCE1 was knocked-down using an RNAi approach. Effect of siRNA- expressing vector on ABCE1 and E- cadherin protein expression was evaluated in 95-D/NCI-H446 lung carcinoma cells by Western blot and FACS analysis. A transwell collagen invasion assay was used to assess differences in the invasive properties of cells expressing ABCE1. Treatment of the 95-D or NCI-H446 cells with an RNAi to *ABCE1* led to near 85% knockdown of ABCE1 expression after 48 h. Interestingly, knockdown of ABCE1 expression was accompanied by a significantly higher level in E-cadherin expression. And the expression of E-cadherin in siRNA-95D/H446 was much higher than that in either of the control cell lines by FACS analysis. Moreover, cells treated with the ABCE1 RNAi displayed poor less invasion than the control cell lines too.There were close relationships between ABCE1 and E- cadherin. It has been suggested that silencing ABCE1 expression increases E-cadherin expression and decreases cell invasion in 95-D / NCI-H446 lung carcinoma cells.

Key words RNAi, *ABCE1*, E-cadherin, cell invasion
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Infiltration and metastasis are the most important biological characteristics of malignant tumors. Defective interactions between adhesion molecules have a critical role in cancer. Detachment of cells is the first step in the process of metastasis and is dependent on the presence or absence of functioning adhesion molecules. E-cadherin is a transmembrane glycoprotein involved in Ca-dependent epithelial cell-cell homophylic adhesion at the adherens junctions [1–3]. It has been demonstrated that the intracellular part of the E-cadherin molecule is also responsible for the stability and function of the extracellular domain of E-cadherin[3].

ATP-binding cassette protein E (*ABCE*) gene has been annotated as an RNase L inhibitor in eukaryotes [4–5]. Present data show that *ABCE1* may function not only as RNase L inhibitors, but also may have other functions that have yet to be determined[6–7]. Our previous study shown that ABCE1 protein and mRNA were over expressed in 48 lung adenocarcinoma tissues samples [8–10]. The over expression of ABCE1 was related with the occurrence and clinical stage of lung adenocarcinoma[8–9].

In the study, it was interesting that knockdown of ABCE1 expression was accompanied by a marked decrease in E-cadherin expression and suppresses 95-D/NCI-H446 lung carcinoma cells invasion.

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.

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1.2 shRNA preparation and plasmids construction

In our previous study, the positive clones (Si-1) and the negative control plasmid (Si-N) according to the *ABCE1* gene sequence were constructed completely[9].

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 70% ~ 80% confluence. Lung carcinoma cells were transfected with the mixture of 1 μg plasmid DNA (Si-1 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.

1.4 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/E-cadherin and GAPDH were evaluated.

1.5 Fluorescence-activated cell sorter (FACS) analysis of E-cadherin

The cells (SiRNA-95D/H446, Sicontrol-95D/H446, Control-95D/H446) were plated at a density of 3×10^6 cells in 17 cm^2 culture flasks . All cell cultures were harvested 24 h after (mock-) transfection. In short, cells were fixed with 4% paraformaldehyde and 100% methanol and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Taufkirchen, Germany). To identify E-cadherin, probes were blocked with fetal calf serum and incubated on ice for 30 min with a polyclonal rabbit-anti-E-cadherin antibody (Clone HECD-1; [IgG1]1∶400; Takara) in a concentration of 1∶25. The FITC-conjugated secondary goat-anti-rabbit-antibody (Jackson Immuno Research, Suffolk, UK) was incubated in a 1∶250 dilution for 20 min on ice. Probes were analyzed for E-cadherin content by measuring of fluorescence intensity, using a cytometer (FACScan flow cytometer, Becton Dickinson, Heidelberg, Germany) at an excitation wavelength of 525 nm. The data were analyzed with the aid of a software program (FlowJo, Tree Star, Olten, Switzerland) with dead cells gated out using pulse processing. A cell was determined as E-cadherin - positive when its fluorescence intensity (FL1-H) was greater than a certain threshold value of 5% of false positive mock transfected cells.

1.6 Cell invasion assays

Cells(5×10^4/chamber) were used for each invasion assay. The upper parts of the Transwell were coated with 70 μl of a bovine collagen matrix. Cells were plated onto the collagen coated transwell in the presence of serum-free DMEM. In the lower chamber, 500 μl of 10% serum DMEM was added. The inserts were incubated for 4 days at 37℃.Wiping collagen matrix and the upper cells with a cotton swab, cells that had invaded the lower 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 $\bar{x} \pm s$ deviation of 3 separate experiments from 6 determinations per experiment for each experimental condition.

2 Results

2.1 Effect of siRNA-expressing vector on ABCE1 and E-cadherin protein expression

We evaluated the effect of siRNA-expressing vectors(Si-1) on target protein ABCE1 and E- cadherin by Western blotting analysis. Treatment of the 95-D or NCI-H446 cells with an RNAi to ABCE1 led to near 85% knockdown of ABCE1 expression after 48 h (Figure 1a, left). In contrast, a control RNAi construct had no effect on ABCE1 expression (Figure 1a, right). Interestingly, knockdown of ABCE1 expression was accompanied by a significantly increase in E-cadherin expression (Figure 1a, b). Again, like ABCE1, the control RNAi had no effect on E-cadherin expression.

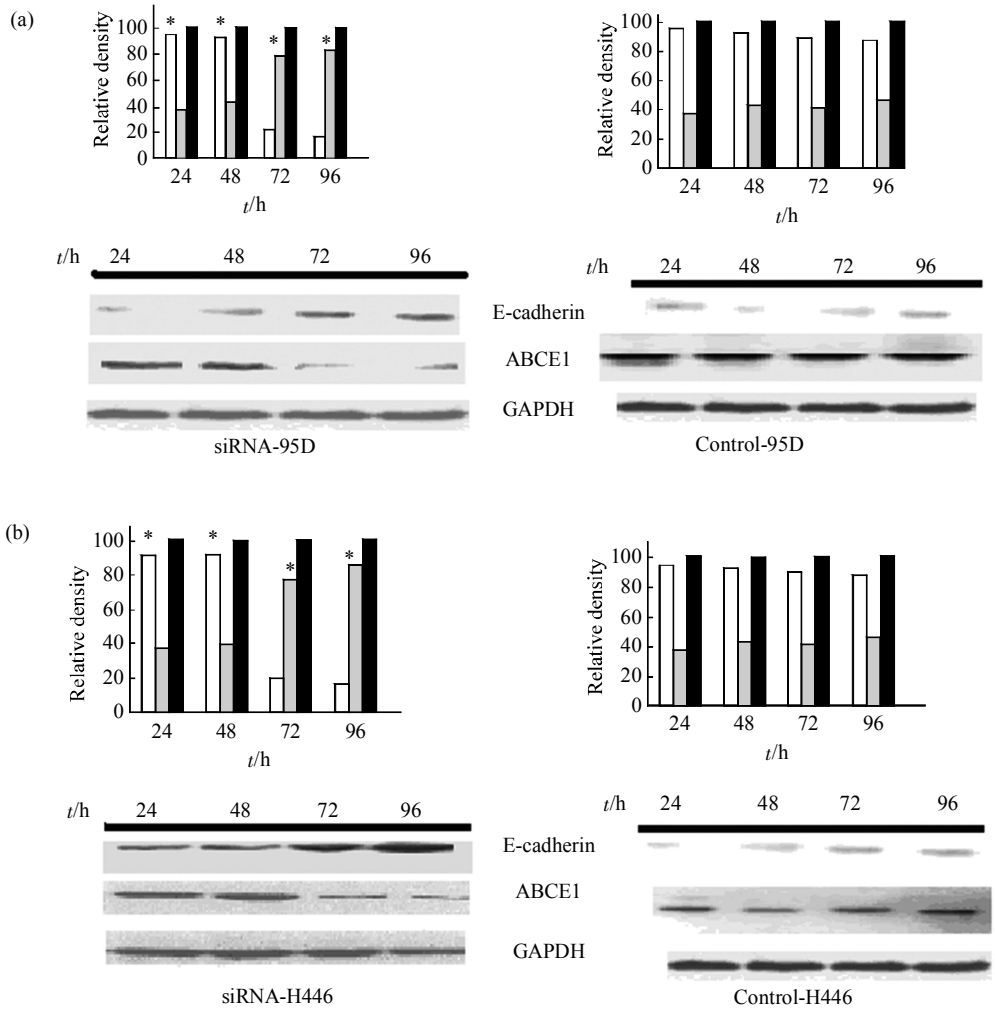

Fig. 1 Western blotting analysis to show effect of siRNA-expressing vectors in a time course dependent manner

(a) 95-D cells. (b) NCI-H446 cells. Control lane: 95-D/ NCI-H446 cells were transfected with negative control Si-N vector. The average signal intensity of ABCE1/E-cadherin protein was standardized to GAPDH. The data represent ($\bar{x} \pm s$) of 4 independent experiments. $P < 0.05$ (asterisk) was considered to have statistical significance. □: ABCE1; ▨: E-cadherin; ■: GAPDH.

2.2 Results of E-cadherin expression by FACS analysis

E-cadherin was marked with a FITC-conjugated antibody as described above. E-cadherin expression of siRNA cells ranged from 67% of E-cadherin -positive siRNA- 95D cells to 73% in siRNA-H446 cells 24 h after (mock-) transfection. E-cadherin expression of siControl cells ranged from 7% of E-cadherin -positive siControl-95D cells to 5% in siControl-H446 cells 24 h after (mock-) transfection (Figure 2). There was significant difference in expression of E-cadherin-positive cells between siRNA-95D/H446 and control groups. However, there was no significant difference in expression of E-cadherin-positive cells between siControl-95D/H446 and Control-95D/H446 24 h after (mock-) transfection.

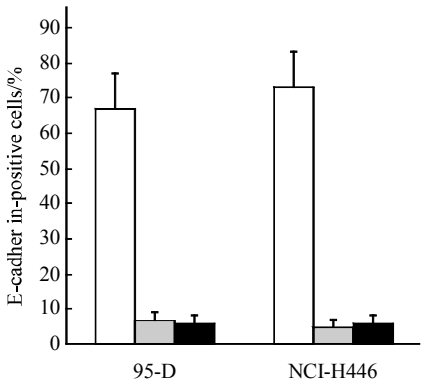

Fig. 2 The percentage of E-cadherin-positive cells was determined by FACS analysis

FACS analysis of E-cadherin expression in lung carcinoma cells siRNA-95D/H446, siControl——95D/H446 and control——95D/H446 24 h after (mock-) transfection. □: siRNA; ▨: siControl; ■: Control.

2.3 Cell invasion

To determine whether ABCE1 up-regulation influenced cell invasion, we used a transwell collagen invasion assay to assess differences in the invasive properties of cells expressing ABCE1 (control and Sicontrol) and those treated with ABCE1 RNAi (siRNA-95D/H446) . Cells treated with the ABCE1 RNAi displayed poor less invasion than either of the control cell lines(Figure 3). It has been suggested that silencing ABCE1 expression decreases tumor cell invasion.

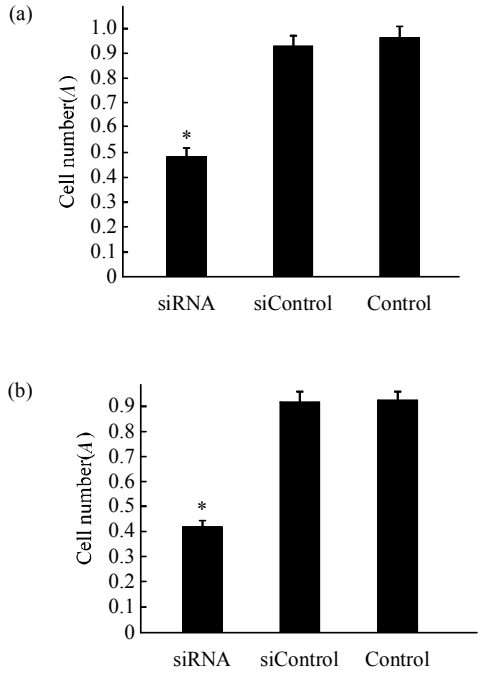

Fig. 3 Knockdown of ABCE1 decreases cell invasion

(a) 95D. (b) NCI-H446. Cells that invaded the collagen were counted by MTT. This figure shows a significant difference (*$P < 0.05$) in invasion when comparing 95-D/NCI-H446 cells transfected and the control cells. Results show the ($\bar{x} \pm s$) of three independent experiments.

3 Discussion

Current data suggest that ABCE1 has key cellular roles in translation or ribosome biogenesis or the assembly of HIV1 capsids and inhibition of RNase-L and interacts with several translation initiation factors (eIF3/Hcr1, eIF5,eIF2B) and ribosomal subunits [11–13]. 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 [8]. Meanwhile, we constructed siRNA expressing vectors of the *ABCE1* gene, transfected them into 95-D/NCI-H446 lung carcinoma cells by FuGENE 6. The results show that RNAi technology can be used to down-regulate ABCE1 expression, suppress cell growth and induce apoptosis . To study further the role of ABCE1 in 95-D/NCI-H446 lung carcinoma cells, we knocked-down the protein of ABCE1 using an RNAi approach. Interestingly, knockdown of ABCE1 expression was accompanied by a significantly increase in E-cadherin expression . However, like ABCE1, the control RNAi had no effect on E-cadherin expression.

Tumor cell homotypic adhesion is important in cellular aggregation and metastatic potential. E-cadherin intracellular trafficking between plasma membrane and cytoplasm is critical for the maintenance of epithelial cell-cell junctions and functional integrity of the paracellular barrier in the epithelium [14–15]. Epithelial mesenchymal transition (EMT), a process associated with loss of epithelial polarity and cell-cell adhesion, has been found to be operative in both embryonic development and carcinogenesis. Loss of E-cadherin, a major hallmark of the EMT process, is frequently observed at sites of EMT during cancer development and progression[16–17]. E-cadherin promotes cell-cell interactions in the cell membrane, whereas reduced E-cadherin expression has been postulated to play a role in cell migration and metastasis, as well as anchorage independence [18–19]. The present data have shown that tumor cells with limited E-cadherin expression are more likely to detach from a tumor mass than that with high E-cadherin- expressing tumor cells , thus suggesting a mechanism whereby limited cell-cell adhesion could lead to tumor metastasis [13–18]. Additionally, reduced expression of E-cadherin in several carcinomas is significantly correlated with increased lymphogenous metastasis, tumor dedifferentiation and poor survival[1, 16]. Although other factors involving the motility of cancer cells without affecting E-cadherin should also be considered, low E-cadherin expression seems to have a crucial role in cell-cell detachment and increased invasiveness[14–16].

Our previous study shown that RNAi technology can be used to down-regulate ABCE1 expression, suppress cell growth and induce apoptosis[9–10]. To determine further whether ABCE1 down-regulation influenced cell invasion, we used a transwell collagen

invasion assay to assess differences in the invasive properties of cells expressing ABCE1 (control and siControl) and those treated with ABCE1 RNAi (siRNA95-D/NCI-H446).Our study shown that cells treated with the ABCE1 RNAi displayed poor less invasion than either of the control cell lines. It has been suggested that ABCE1 expression increases lung carcinoma cell invasion. It is likely that the effects of ABCE1 expression on invasion are linked to the regulation of E-cadherin, which is known to be a suppressor of tumor cell invasion. It is hoped that a more in-depth understanding of these processes will shed light on the early events involved in the oncogenic transformation of lung carcinoma keratinocytes.

It is noteworthy that the destruction of specific RNA using siRNA is a powerful tool in the analysis of protein function. To our knowledge, this is the first *in vitro* study to show that silencing ABCE1 increase E-cadherin expression and decreases cell invasion in 95-D/NCI-H446 lung carcinoma cells. These results demonstrate the potential for using siRNA to knock down the oncogenic protein ABCE1 to treat patients with lung carcinoma.

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RNA 干扰 *ABCE1* 基因后可增加肺癌 95-D/NCI-H446 细胞的 E-钙黏附蛋白表达并减低细胞侵袭力 *

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摘要　研究 *ABCE1* 对肺癌(95-D 和 NCI-H446)细胞的作用．使用 RNA 干扰技术，抑制 *ABCE1* 基因的表达，通过 Western blot 分析及 FACS 检测，观察 *ABCE1* 基因对 E- 钙黏附蛋白在 95-D/NCI-H446 细胞表达的影响；运用 transwell 侵袭实验，观察 M95-D/ NCI-H446 细胞侵袭力的变化．RNA 干扰 *ABCE1* 基因后，实验组与对照组相比，在 48 h 后可显著抑制肺癌(95-D 和 NCI-H446)细胞 ABCE1 蛋白的表达，同时，伴随 E- 钙黏附蛋白的高表达，以及细胞侵袭力的降低．*ABCE1* 基因与 E- 钙黏附蛋白相关，抑制 *ABCE1* 基因可增加肺癌 95-D/NCI-H446 细胞的 E- 钙黏附蛋白的表达，减低细胞的侵袭力．

关键词　RNA 干扰，*ABCE1* 基因，E- 钙黏附蛋白，细胞侵袭力

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