Expression of ATP binding cassette E1 enhances viability and invasiveness of lung adenocarcinoma cells in vitro

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Abstract. ATP binding cassette E1 (ABCE1), a member of the family of ATP binding cassette transporters, has initially emerged as an RNase L inhibitor. As a highly conserved protein, it is involved in capsid assembly and translation processes of the human immunodeficiency virus as well as in tumor development and progression. Studies have shown that ABCE1 protein was overexpressed in lung carcinoma tissues and metastatic lymph nodes compared to normal lung tissues. However, little is known about the roles of ABCE1 in lung cancer. The present study investigated the biological effects of vector-mediated ABCE1 overexpression in lung cancer cells in vitro and examined the underlying molecular mechanisms. Overexpression of ABCE1 in the LTEP-a-2 lung adenocarcinoma cell line was achieved by transfection with a plasmid containing full-length ABCE1 cDNA. The ectopic expression of ABCE1 was shown to promote the viability and invasive capacity of lung cancer cells, and to in reduce p27 expression. However, overexpression of ABCE1 did not significantly affect the cell cycle distribution. In conclusion, the present study suggested that ABCE1 promotes the growth, invasion and metastasis of lung adenocarcinoma cells and may represent a potential biomarker and therapeutic target for lung cancer.

Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide, accounting for 1.38 million mortalities in 2008 (1). Non-small cell lung cancer (NSCLC) constitutes ~85% of all lung cancer cases and mainly consists of lung adenocarcinoma, squamous cell carcinoma and large cell carcinoma. The current standard treatment modalities for NSCLC, including lung adenocarcinoma, include surgery, chemotherapy, radiation therapy and targeted therapy. While surgical resection is an effective treatment option for early-stage NSCLC (2), local recurrence and distant metastases occur in 50-70% of cases, resulting in an overall five-year survival rate of only 40% (3). Tumor progression and metastasis are the most common events leading to mortality. At the molecular level, these events are regulated by upregulated oncogenes or de-activation of tumor suppressor genes as a result of genetic mutations, which may either occur spontaneously or be driven by environmental factors or oncogenes. Targeted therapies, such as use of gefitinib, bevacizumab, and crizotinib, directed against genes including EGFR, VEGF and EML4-ALK have been explored for treating advanced cancer; however, the therapeutic outcome has been shown to be restricted by inter-individual variability in drug response and development of drug resistance (4). It is therefore required to identify novel molecular mechanisms and oncogenes responsible for tumor progression and metastasis in order to provide novel approaches or strategies to effectively control lung cancer and improve the prognosis of affected patients.

The present study focused on adenosine triphosphate binding cassette (ABC) transporters, which are a large family of proteins with a variety of functions in cells. This family of proteins can be classified into seven distinct sub-families, designated as ABC A-G (5). ABC transporters are well known to contribute to drug resistance by operating as efflux pumps and their expression shows a significant inter-individual variability, therefore leading to a marked heterogeneity in patient responses to chemotherapy (6). Among these ABCs, ABCE1 protein has initially emerged as an RNase L inhibitor involved in a broad range of biological functions, including response to viral infection, cell proliferation and evasion of apoptosis (7-9). In addition, the ABCE1 protein also has an important role in the protein translation process (10,11) and multi-drug resistance. Several patient studies by other groups and ours indicated that ABCE1 expression is associated with tumor progression (12-15). The evolutionarily conserved protein ABCE1 has been identified to be essential for eukaryotic cell viability due to having central roles in protein synthesis, particularly in the process of ribosome recycling. Suppression of ABCE1 expression by small interfering (si)RNA was shown to inhibit the proliferation of HEK293 and arrest the growth.
at the gastrula stage of development of Xenopus (10). However, the current status of knowledge on the essential functions of ABCE1 identified using cell and animal models does not explain for its roles in malignant tumors. As the roles of ABCE1 in the growth and metastasis of lung cancer have not been previously elucidated, the present in vitro study assessed the effects of ABCE1 overexpression on the LTEP-a-2 human lung adenocarcinoma cell line. Following transfection of plasmid vector containing full-length ABCE1 complementary (c)DNA, the effects on cell growth, invasive capacity and cell cycle distribution were assessed.

Materials and methods

Cell line and culture. The LTEP-a-2 human lung adenocarcinoma cell line was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere containing 5% CO2. Cells were grown on sterilized glass Petri dishes and passaged with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Construction of ABCE1 cDNA plasmid and stable transfection. For ectopic ABCE1 expression, the enhanced green fluorescence protein plasmid (pEGFP-C1) carrying ABCE1 cDNA was constructed. Briefly, the full-length mRNA of the ABCE1 gene was extracted using the reverse-transcription polymerase chain reaction (RT-PCR) assay. Primers of the full length of ABCE1 were F, 5'-CTCAACGGCTTGGATACGAGCA-3' and R, 5'-CTCTGAGGTCTCCCTAATCACCAAGAAAG-3' (Takara Bio, Inc., Dalian, China). The full-length mRNA of ABCE1 and pEGFP-C1 plasmids (BioVector NTCC, Inc. Beijing, China) were digested by HindIII/BamHI (NEB Beijing, Ltd., Beijing, China) and connected by a T4 ligase (NEB Beijing, Ltd.). The pEGFP-ABCE1 recombinant plasmid was then transfected into DH5α cells (Takara Bio, Inc.) and screened overnight. The recombinant vectors were selected from single colonies and amplified, and confirmed by electrophoresis and DNA sequencing. LTEP-a-2 cells were seeded into six-well plates and incubated for attachment overnight. The pEGFP-ABCE1 plasmid or empty vector were transfected into the cells using LipoFectamin LTX & PLUS (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. After 48 h of transfection, the cells were cultured in RPMI 1640 medium containing 0.5 mg/ml G418 (Gibco) for three weeks and the ABCE1 expression was confirmed in the G418-resistant cell population using RT-PCR and western blot analyses.

RT-PCR. Total RNA of each cell sample was isolated using TRIzol reagent (Takara Bio, Inc.) and reversely transcribed into cDNA by using the PrimeScript RT-PCR kit (Takara Bio, Inc.) according to the manufacturer's instructions. Subsequently, these cDNA samples were subjected to PCR amplification. The primer sequences were as follows: ABCE1 forward, 5'-TCA AACTTCACAGGGTTGCC-3' and reverse, 5'-GATCATGTTCCACCACAATG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-GCACCGTCAAGCTGAGAAC-3' and reverse, 5'-TGGTGAAGCCAGCTGTTGA-3' (Takara Bio, Inc.). The composition of the PCR mixture (10 µl) was 0.05 µl Taq, 2 µl 5X Buffer, 1 µl dNTP, 5.5 µl ddH2O, 0.25 µl Primer F, 0.25 µl Primer R and 1 µl cDNA. The thermocycling conditions used for the PCR Thermal Cycler Dice TP600 (Takara Bio, Inc.) comprised 27 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. After electrophoresis on a 1.5% agarose gel, images of the PCR bands were captured using a Dolphin-View Image system (Watelec Corp., Sparks, NV, USA).

Protein extraction and western blot analysis. Total cellular protein was extracted from the cultured cells using lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P40, 0.5% sodium deoxycholate and phenylmethylsulfonyl fluoride (Sigma-Aldrich). The protein concentration was determined using the bicinchoninic acid method (Sigma-Aldrich). Aliquots of 100 µg protein were then separated by 5 or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis for 90 min and electrotransferred (90 mA, 90 min) onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were rinsed with Tris-buffered saline containing Tween-20 (TBS-T; Sigma-Aldrich) and blocked in 5% non-fat dry milk/TBS-T for 2 h at room temperature. Subsequently, the membranes were incubated with a rabbit polyclonal anti-ABCE1 (1:400) and p27 (1:200; Santa Cruz Biotechnology, Inc.) and GAPDH antibody (Santa Cruz Biotechnology, Inc.) was used as a loading control. Subsequently, the membranes were incubated with a rabbit polyclonal anti-ABCE1 (1:400) and p27 (1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. nos. sc-99064 and sc-528, respectively) overnight at 4°C. Following washing of the membranes three times with TBS-T, they were incubated with a goat anti-rabbit antibody at 1:5,000 dilution for 1 h. The protein bands were then visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). GAPDH antibody (Santa Cruz Biotechnology, Inc.) was used as a loading control. Subsequently, the membranes were incubated with a rabbit polyclonal anti-ABCE1 (1:400) and p27 (1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. nos. sc-99064 and sc-528, respectively) overnight at 4°C. Following washing of the membranes three times with TBS-T, they were incubated with a goat anti-rabbit antibody at 1:5,000 dilution for 1 h (Abcam, Cambridge, UK; cat. no. ab6721). The protein bands were then visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). Subsequently, the same membrane probed for GAPDH protein was eluted and probed with p27 protein (1:200; Santa Cruz Biotechnology, Inc.), and then visualized again.

Cell viability assay. Stably pEGFP-ABCE1-transfected LTEP-a-2 cells were seeded into 96-well plates (8x10³ cells/well in 100 µl) were cultured for 2, 24, 48, 72 or 96 h. The growth medium containing G418 (0.5 mg/ml) was replaced every three days. At the end of each experiment, 50 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was added to each well and the cells were incubated for an additional 4 h. The supernatant was then removed and 150 µl dimethyl sulfoxide (Nanjing KeyGen Biotech Co., Ltd.) was added to each well to dissolve the formazan crystals. The optical density was measured using a spectrometer
(Tecan Sunrise, Tecan, Männedorf, Switzerland) at 570 nm. The experiment was performed in triplicate with two wells for each experimental condition.

**Flow cytometric cell cycle analysis.** Stably pEGFP-ABCE1-transfected LTEP-a-2 cells were detached with 0.5% trypsin, washed with cold phosphate-buffered saline (PBS) and fixed with 70% cold ethanol (Novatech Enterprise Co., Ltd., Jiangsu, China) at 4°C overnight. Subsequently, the cells were rehydrated with PBS containing RNase A (Nanjing KeyGen Biotech Co., Ltd.) at 37°C for 30 min. Following staining with propidium iodide (Nanjing KeyGen Biotech Co., Ltd.) for 45 min in the dark, cells were subjected to cell cycle analysis using a flow cytometer (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). The percentage of cells in each phase of the cell cycle was determined using the ModFit LT 3.0 software (Verity Software House, Inc., Topsham ME, USA).

**Transwell invasion assay.** Stably pEGFP-ABCE1-transfected LTEP-a-2 cells were detached with 0.5% trypsin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), washed with cold PBS and counted. For the tumor cell invasion assay, 4x10⁴ cells were suspended in serum-free medium and seeded into the upper chambers of Transwell inserts with Matrigel (BD Biosciences)-coated 8-µm porous polycarbonate membranes (Corning, Inc., Corning, NY, USA). The lower chambers were then filled with medium containing 10% fetal bovine serum. After 48 h of incubation at 37°C, the cells on the upper surface of the chambers were completely removed using cotton swabs and the cells on the bottom surface that had migrated through the pores were fixed with 4% paraformaldehyde (Novatech Enterprise Co., Ltd.) for 30 min and stained with crystal violet (Sigma-Aldrich). The experiments were performed in triplicate wells and repeated at least once. To quantify tumor cell invasion, 10 high-power microscopic fields (magnification, x400) were counted and averaged for comparison to the control cells using the IX71 microscope with a DP70 Color Camera (Olympus Corporation, Tokyo, Japan).

**Statistical analysis.** Values are expressed as the mean ± standard deviation. All statistical calculations were performed using SPSS software, version 17.0 for windows (SPSS, Inc., Chicago, IL, USA). Differences for each experimental group were compared using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Ectopic expression of ABCE1 in lung adenocarcinoma cells.** First, the pEGFP-ABCE1 vector was constructed and stably transfected into LTEP-a-2 cells. The induced expression of ABCE1 mRNA and protein was confirmed by RT-PCR and western blot analysis, respectively. As shown in Fig. 1A, transfection with pEGFP-ABCE1 recombinant plasmid markedly increased the expression of ABCE1 mRNA in LTEP-a-2 cells. Furthermore, western blot analysis showed that the expression of ABCE1 protein was also increased compared to that in the parental and empty vector-transfected cells (Fig. 1B). The presence of a ~95 KDa recombinant protein corresponded to the fusion of the ~27 KDa EGFP and the ~68 KDa ABCE1 protein.

**Overexpression of ABCE1 results in reduced p27 expression in lung adenocarcinoma cells.** To explore the effects of ABCE1 in LTEP-a-2 cells, the expression of p27 was assessed using western blot analysis. As shown in Fig. 1C, p27 protein levels were markedly reduced in cells overexpressing ABCE1 compared to that in the parental cells and empty vector-transfected cells.

**Overexpression of ABCE1 enhances the proliferation of lung adenocarcinoma cells.** Next, the present study determined the effects of the induced ABCE1 expression on the proliferation of LTEP-a-2 cells in vitro. Cells transfected with pEGFP-ABCE1, empty vector or parental cells were cultivated for 2, 24, 48, 72 or 96 h and quantified using an MTT assay. The growth curves revealed that the proliferation of LTEP-a-2 cells transfected with pEGFP-ABCE1 was significantly enhanced to 119.63, 126.55, 121.58 and 128.13% of that of the control cells after 24, 48, 72 and 96 h, respectively (P<0.05) (Fig. 2). However, the proliferative rates of the parental cells (Control) and empty vector-transfected cells (Mock) were similar (P>0.05). These results clearly indicated that ABCE1 promoted the growth of lung adenocarcinoma cells.

**ABCE1 does not affect the cell cycle of lung adenocarcinoma cells.** To further determine the mechanisms by which ABCE1 enhances the proliferation of lung adenocarcinoma cells, the cell cycle distribution of LTEP-a-2 cells stably transfected with pEGFP-ABCE1 was assessed by flow cytometry. As shown in Fig. 3, overexpression of ABCE1 did not affect the cell cycle distribution of LTEP-a-2 cells compared with that of empty vector-transfected and parental cells.

**ABCE1 expression promotes lung adenocarcinoma cell invasion.** The effects of the ABCE1 overexpression on the invasive capacity of LTEP-a-2 cells were assessed using a Transwell assay. After 48 h of incubation, the number of cells transgressed through the Matrigel-coated membrane was significantly higher in the ABCE1 overexpression group (93±5; P<0.05) compared with that in the empty vector-transfected group (38±4) and parental cells (37±5) (Fig. 4). This result demonstrated that ABCE1 enhances the invasive capacity of lung adenocarcinoma cells.

**Discussion**

The present study determined the effects of ABCE1 overexpression on the proliferation and invasiveness of lung adenocarcinoma in vitro. The results showed that ectopic expression of ABCE1 promoted tumor cell growth and invasion, and reduced the expression of p27 protein in LTEP-a-2 cells. However, flow cytometric analysis did not show any effect of ABCE1 overexpression on the cell cycle distribution of LTEP-a-2 cells. Overall, these results demonstrated that ABCE1 exerts tumor-promoting effects in lung adenocarcinoma cells.

Tumor progression and metastasis are important factors that contribute to cancer mortality and poor survival of...
the clonogenicity and anchorage tumor lymph node metastasis (13). A recent study by our group demonstrated that expression of ABCE1 protein was associated with advanced clinical stages of lung cancer and oral cancer (18). In addition, a previous study by our group showed that expression of ABCE1 protein was highly expressed in numerous tumor types, including colon and mitochondria (16). It has been reported that ABCE1 is expressed in normal tissues and its protein is abundant in the cytoplasm of endogenous ABCE1 in LTEP-a-2 cells was found to result from the G1 towards the S phase, and further are required to confirm this fact.

Figure 1. Induction of ABCE1 expression and regulation of p27 expression after transfection of pEGFP-ABCE1. ABCE1 overexpression in LTEP-a-2 lung adenocarcinoma cells stably transfected with ABCE1 expression vector was confirmed using (A) reverse-transcription polymerase chain reaction and (B) western blot analysis. The ABCE1 antibodies were specific for the 95 kDa and 68 kDa proteins of ABCE1. (C) Overexpression of ABCE1 led to a reduction of p27 protein expression, as indicated by western blot analysis. Overexpression, cells transfected with pEGFP-ABCE1; Mock, cells transfected with empty vector; EGFP, enhanced green fluorescence protein; ABCE1, adenosine triphosphate binding cassette E1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Figure 2. ABCE1 overexpression enhances the proliferation of LTEP-a-2 cells. Growth curves determined by MTT assays are shown. Values are expressed as the mean ± standard deviation. Experiments were performed in triplicate with two wells per experimental condition each. *P<0.05 vs. mock and control groups. Overexpression, cells transfected with ABCE1 overexpression plasmid; Mock, cells transfected with empty vector; Control, parental cells; ABCE1, adenosine triphosphate binding cassette E1.

A previous microarray analysis by our group revealed that a number of genes were differentially expressed after ABCE1 knockdown (13). Among these, p27 was selected for further study of the mechanisms induced by ABCE1 in lung adenocarcinoma in the present study. Overexpression of endogenous ABCE1 in LTEP-a-2 cells was found to result in a reduced expression of p27 protein, which confirmed the result of our previous study. P27 protein is a well-characterized tumor suppressor and is frequently inactivated in a large variety of cancer types with increased aggressiveness and poor clinical outcome (24,25). In NSCLC, low levels of p27 protein are associated with cancer development and poor prognosis (26). Numerous studies have shown that p27 protein has a dual function in suppressing cell proliferation and affecting motility and invasiveness (27-29). In accordance with these findings, the present study confirmed that overexpression of ABCE1 promoted lung cancer cell proliferation and invasion, directly or indirectly by downregulation of p27 protein. The cell cycle was not demonstrated to be directly affected by the p27 protein; however, this protein serves a central role in regulating the Cdk activity in cell progression from the G1 towards the S phase, and further are required to confirm this fact.

Furthermore, previous studies have reported that ABCE1 not only appeared to negatively regulate the 2-5A/RNase L-associated apoptotic pathway, but also promoted translational processes in eukaryotes (7-11). However, unexpectedly, the results of the present study indicated that ABCE1 overexpression did not affect the cell cycle distribution of LTEP-a-2 cells, which is not in line with the findings of enhanced proliferation and downregulation of p27 protein.
of p27 following ectopic expression of ABCE1. Therefore, it remains to be fully elucidated how ABCE1 contributes to tumor progression.

The ABCE1 protein is an ABC transporter whose physiological function has not been well characterized; ABC transporters are widely and constitutively expressed in various cell types other than cancer cells. Functionally, ABC transporters bind to and hydrolyze ATP to transport various molecules across the plasma membrane as well as to intracellular membranes of the endoplasmic reticulum, peroxisome and mitochondria. ABC transporters are thought to participate in the absorption and secretion of endogenous and exogenous substances (5,7-11). Altered expression of these genes contributes to various types of disease, including cancer (13-16). The observations of the

Figure 3. Effects of ABCE1 overexpression on the cell cycle distribution of LTEP-a-2 cells. Following transfection for 72 h, cells were stained with propidium iodide and subjected to flow cytometric analysis. Values are expressed as the mean ± standard deviation of three independent experiments. Overexpression, cells transfected with ABCE1 overexpression plasmid; Mock, cells transfected with empty vector; Control, parental cells; ABCE1, adenosine triphosphate binding cassette E1.

Figure 4. ABCE1 overexpression enhances the invasiveness of LTEP-a-2 cells. A Transwell invasion assay showed that the number of invasive cells in the overexpression group was significantly increased compared to that in the mock and control groups. Representative images of crystal violet-stained cells on the lower surface of the membrane are shown (magnification, x400). Values are expressed as the mean ± standard deviation of ten microscopic fields for each group. *P<0.05 vs Mock/Control group. Overexpression, cells transfected with ABCE1 overexpression plasmid; Mock, cells transfected with empty vector; Control, parental cells; ABCE1, adenosine triphosphate binding cassette E1.
present study supported the hypothesis that increased ABCE1 expression is critical in lung cancer progression. Apart from this role, several lines of evidence suggested that ABCE1 is a primary cellular target responsible for the attenuation of damage through reactive oxygen species in diverse aerobic organisms (30,31). This reactive oxygen species-mediated damage is linked to various diseases, including cancer. It is likely that normal levels of ABCE1 are required to maintain healthy cells, while aberrant expression may impact cell fate to possibly drive tumorigenesis and cancer progression. It is therefore essential to determine whether the increased expression of ABCE1 has a significant effect on lung cancer progression, or whether a lower expression leads to increased ROS damage, and possibly carcinogenesis.

The present study reported on the effects of ABCE1 overexpression on lung adenocarcinoma cells in vitro, demonstrating its enhancing effect on cell proliferation and invasiveness with simultaneous downregulation of p27 protein expression. However, further study is required to more precisely define the exact underlying mechanisms of the observed effects of ABCE1 in NSCLCs.

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References