Tumor metastatic promoter ABCE1 interacts with the cytoskeleton protein actin and increases cell motility

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Abstract. ABCE1, a member of the ATP-binding cassette (ABC) family, is a candidate tumor metastatic promoter in lung cancer. Overexpression of ABCE1 is correlated with aggressive growth and metastasis in lung cancer cells. However, the exact mechanism remains unclear. In the present study, GST pull-down assay provided evidence of the possible interaction between ABCE1 and β-actin using GST-ABCE1 as a bait protein. Co-immunoprecipitation manifested ABCE1 formed complexes with β-actin *in vivo*. ABCE1 overexpression significantly increased the migration of lung cancer cells which may be attributed to the promotion of F-actin rearrangements. Taken together, these data suggest that overexpression of ABCE1 produces an obvious effect on the motility of lung cancer cells through cytoskeleton rearrangement.

Introduction

ABCE1, a member of the ATP-binding cassette (ABC) family (1), was initially identified as an RNaseL inhibitor (RLI) which is involved in the pathway of antiviral defense mediated by interferon (2,3). To date, several studies have revealed that ABCE1 also functions in translation initiation and termination, ribosome recycling and human immunodeficiency virus capsid assembly (4-6).

In addition, ABCE1 also plays important roles in malignant tumors. ABCE1 is highly expressed in melanoma (7), retinoblastoma (8), colorectal cancer (9), breast cancer (10,11) and esophageal cancer (12). It may possibly become a therapeutic target in colon cancer (13), hepatocellular carcinoma (14) and prostate cancer (15,16). However, the effect of ABCE1 on malignant carcinoma warrants further investigation.

In our previous study, we demonstrated that suppression of ABCE1 inhibited the proliferation and invasion of lung cancer cells *in vitro*. In addition, ABCE1 was overexpressed at both the mRNA and protein levels in lung carcinoma tissues and metastatic lymph nodes and was found to be significantly associated with advanced clinical stages (17,18). Previous studies suggest that overexpression of ABCE1 may be related to cancer metastasis [reviewed in Tian *et al* (19)]. Therefore, we aimed to ascertain whether ABCE1 is involved in lung cancer progression and metastasis. In the present study, we demonstrated that ABCE1 increases cell migration and invasion and rearranges the cytoskeleton by binding to β-actin.

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Materials and methods

Cell culture and transfection. Cell culture, LTEP-a-2 was obtained from the Chinese Academy of Science Cell Bank (CAS; Shanghai, China). Cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum at 37˚C, in a humidified atmosphere of 95% air and 5% CO_2. All culture medium and reagents were obtained from Hyclone (USA). Cell counting was performed using a hemocytometer and Beckman Coulter Cell counter, according to the manufacturer's instructions. The plasmid of pEGFP-C1-ABCE1 was constructed by YeTian.

LTEP-a-2 cells were seeded ($2x10^5$ cells/well) in 6-well plates. After 24 h of incubation, they were transfected with pEGFP-C1-ABCE1 (3 μg) or pEGFP-C1 (3 μg), in serum-free medium using Lipofectamine 2000 (Invitrogen, USA) mixed and incubated for 30 min at room temperature. The mixture was then added to the LTEP-a-2 cells. After 6 h of incubation, the mixture was replaced with full medium.

Immunoblotting. Western blotting was performed as previously described (17). Briefly, proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane for immunoblotting using a Mini-Protean Tetra system (Bio-Rad, USA). The membranes were then incubated with the respective antibodies, and developed using SuperSignal West Pico (Thermo Scientific, USA). Image analysis was carried out with ImageJ software (NIH) by calculating the mean intensity.

Expression of protein GST-ABCE1. Expression plasmid pGEX-4T-1-ABCE1 was a gift from Dr J.R. Lingappa (20).

pGEX-4T-1-ABCE1 and pGEX-4T-1 were transformed into Competent BL21 (DE3) *Escherichia coli* cells (Takara, China). The cells were induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (Tiangen, China) overnight at 20˚C on a rotating wheel. *E. coli* culture was centrifuged at 5,000 x g for 5 min, and resuspended in 1 ml of cold ProFound™ lysis buffer (Thermo Scientific) and protease inhibitors. Cells were homogenized using a sonicator (8 pulses, 10 sec each) and then centrifuged at 14,000 x g for 10 min.

GST pull-down assays. The pull-down assay was performed according to the protocol of the ProFound™ Pull-Down GST Protein:Protein Interaction kit (21516; Pierce). Bacterial lysate was clarified by centrifugation in a 12124 rotor (Sigma) at 14,000 x g for 10 min, and 800 µl of the resulting supernatant was incubated with 50 µl of settled immobilized glutathione resin for 2 h at 4˚C. LTEP-a-2 cell lysates were harvested in ProFound™ lysis buffer and incubated with washed GST-ABCE1 glutathione-Sepharose columns for 2 h at 4˚C on a rotating wheel. After incubation with LTEP-a-2 cell lysate, glutathione-Sepharose was washed five times with 400 µl 1:1 wash solution of TBS:ProFound™ lysis buffer. Fifty-microliter elution was carried out using buffer containing 100 mM glutathione and boiled in SDS sample buffer, and loaded onto an SDS-PAGE gel. Gels were stained using Coomassie Brilliant Blue R350 (GE Healthcare, USA) and protein bands were excised and collected in 96-cell plates.

In-gel digestion. Gel slices were incubated in destaining buffer (25 mM NH_4HCO_3, 50% CH_3CN) at 37˚C for 20 min. Destaining was repeated with fresh buffer until the gel turned colorless. Gel slices were dehydrated in 100 µl acetonitrile until the gel turned white. Then the gels were reduced in a buffer containing 10 mM DTT soluble in 25 mM ammonium bicarbonate at 37˚C for 1 h. Protein alkylation was performed by incubation of the gel slices in 25 mM ammonium bicarbonate for 45 min in darkness at room temperature. Afterwards, the gel slices were washed using 100 µl of 50% CH_3CN and dehydrated by acetonitrile. Three microliters of 10 ng/l trypsin (Promega, USA) was added to each gel slice and incubated at 4˚C for 30 min. Ammonium bicarbonate (10 µl 25 mM) was added to the gels at 37˚C overnight.

Mass spectrometry analysis. Mass analysis was performed by using a MALDI TOF/TOF analyzer (Bruker Daltonic, Germany). Data were searched in AutoFlex3 against SwissProt databases. Mascot software was used to analyze Mass data. Mascot search parameters were set as follow: taxonomy, *Homo sapiens;* fixed modification, carbamidomethyl (C); variable modification, oxidation (M); MS/MS fragment tolerance, 0.7 Da; precursor tolerance, 100 ppm; peptide charge, +1, monoisotopic. Proteins were accepted when scored greater than 56 ($P<0.05$)

Co-immunoprecipitation assays. LTEP-a-2 cell lysate was harvested in a immunoprecipitation lysis buffer (Thermo) with Halt™ Protease Inhibitor Cocktail (Thermo Scientific). After sonication, the lysates were clarified by centrifugation at 14,000 x g for 10 min. Co-immunoprecipitation (co-IP) was conducted following the manufacturer's protocol (co-IP Kit, 26149; Thermo Scientific Pierce). Briefly, the ABCE1 antibody was first immobilized for 2 h using AminoLink Plus coupling resin. After washing, the resin was incubated with the LTEP-a-2 lysate overnight at 4˚C. After incubation, the resin was again washed and the protein was eluted using elution buffer. A negative control resin that was provided with the IP kit to assess nonspecific binding received the same treatment as the co-IP samples, including the ABCE1 antibody. In this control, the coupling resin was not amine-reactive preventing covalent immobilization of the primary antibody onto the resin. Another control was used coupling resin without the ABCE1 antibody. Antibodies used were: rabbit monoclonal anti-ABCE1 (Abcam, USA) and rabbit polyclonal anti-β-actin (Santa Cruz Biotechnology, USA).

Immunofluorescence microscopy. The cells were grown on glass coverslips for 36 h before treatment. The cells were washed in PBS and fixed in 4% paraformaldehyde for 20 min. The cells were rinsed for 5 min in 0.5% Triton X-100 three times, and then incubated with the primary antibodies at 4˚C overnight in a wet box. The coverslips were rinsed three times in PBS. The following steps were operated in darkness. The cells were incubated with the secondary antibodies for 2 h at 37˚C. The coverslips were rinsed three times in PBS and mounted on glass slides. Antibodies used were: rabbit monoclonal anti-ABCE1 (Abcam), mouse polyclonal anti-β-actin (Santa Cruz Biotechnology), and conjugated secondary antibodies (EarthOx, USA), and Hoechst 33342 (Sigma, USA). ABCE1 was detected using anti-rabbit IgG, Rhodamine Fluor (red fluorescence), and actin was detected using anti-mouse IgG Dylight 649 (yellow fluorescence). Nuclei were observed by Hoechst 33342 staining. Images were obtained utilizing the Olympus FV1000S-SIM/IX81 confocal system.

F-actin staining. Cells were grown on glasses and fixed with 4% paraformaldehyde solution. The cells were stained with 100 µM Rhodamine Phalloidin (cytoskeleton) for 30 min before the addition of Hoechst 33342. Images were also obtained on the Olympus FV1000S-SIM/IX81 confocal system. Microspikes were counted in 3 different fields; 5 cells were chosen from each field. The quantification was repeated by three individuals.

Transwell cell migration assay. Cell migration assay was performed using a 24-well Transwell chamber (8.0-µm pore size; Corning). The cells ($4x10^4$) were seeded in the upper chamber which was inserted into a 24-well plate and cultured for another 48 h. Then, the cells were allowed to migrate forward to DMEM containing 15% FBS in the bottom chamber. The non-migratory cells on the upper membrane surface were removed with a cotton tip, and the migratory cells that had attached to the lower membrane surface were fixed with 4% paraformaldehyde and stained with crystal violet. The number of migrated cells was counted in five randomly selected high power fields under a microscope. Data presented are representative of three individual wells.

Statistical analysis. All the statistical analyses were performed with SPSS13.0 using one-way ANOVA test. $P<0.05$ is indicative of a significant difference.

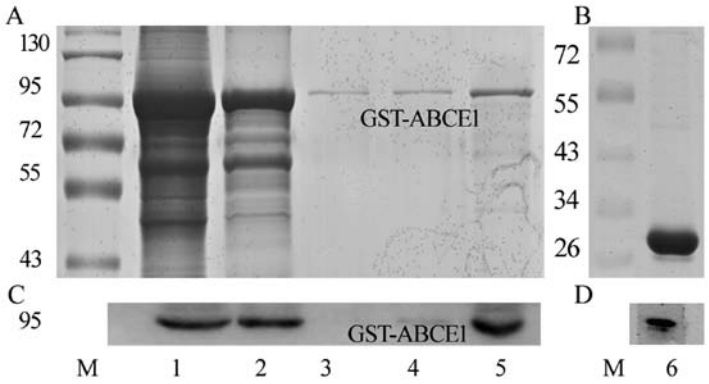
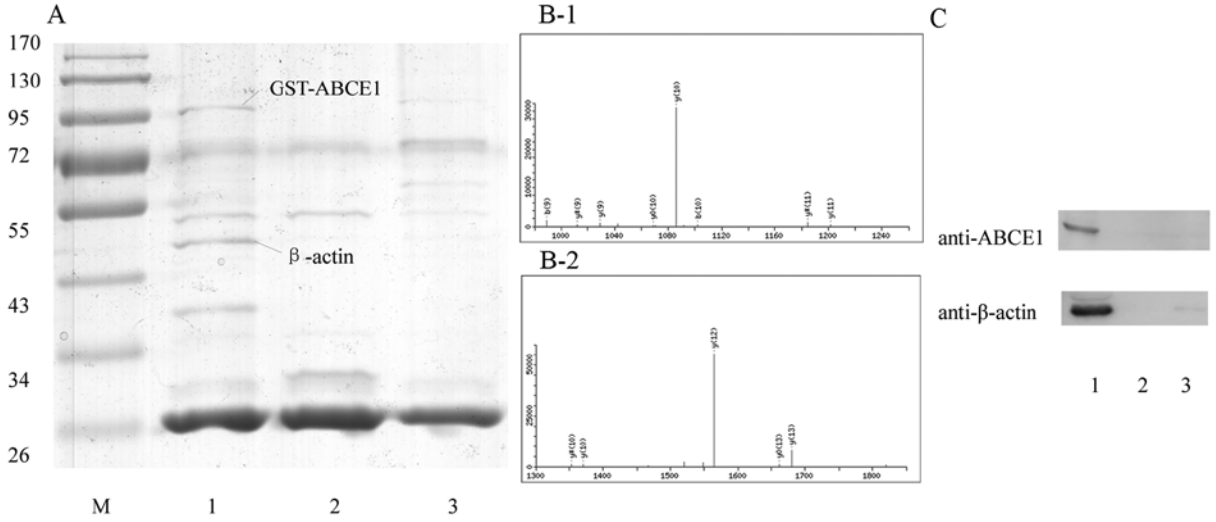


Figure 1. Purification and identification of recombinant GST-ABCE1 protein. (A) Coomassie Blue staining of GST-ABCE1. M, standard protein marker. Lane 1, recombinant GST-ABCE1 cell lysate; lane 2-4, wash solution 1-3; lane 5, eluant of purified recombinant GST-ABCE1. (B) Coomassie Blue staining of GST, lane 6. (C) Western blot analysis corresponding to lane 1-5 in A. (D) Western blot analysis of GST.



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	Description	Peptide	Score	Range	MW（kDa）	pI
1	β-actin	SYELPDGQVITIGNER	114	239-254	42318	5.39
2	ABCE1_human	YDDPPDWQEILTYFR	92	134-148	68240	8.63

Figure 2. ABCE1 interacts with β-actin. (A) ABCE1 interacts with β-actin in the GST pull-down assay. GST-ABCE1 was used to probe LTEP-a-2 cell lysate. The β-actin band only appeared in lane 1. M, standard protein marker; lane 1, purified GST-ABCE1 as bait protein, β-actin was detected; lane 2, purified GST as a bait protein; lane 3, control agarose beads. (B-1) MS/MS spectrum identified the peptide unique to ABCE1. (B-2) The MS/MS spectrum identified the peptide unique to β-actin. (C) ABCE1 co-precipitates with β-actin. Lane 1, resin coupling the anti-ABCE1 antibody, lane 2, control resin coupling anti-ABCE1 antibody, lane 3, resin without any antibody. (D) Characteristics of the identified proteins by using mass spectrometry.

Results

Expression and identification of recombinant GST-ABCE1. SDS-PAGE analysis with Coomassie staining showed that recombinant GST-ABCE1 was expressed with a molecular mass of ~94 kDa (Fig. 1A, lane 1) which was consistent with the ABCE1 predicted size as the increased 26 kDa is related to GST tag. The purified GST-tagged fusion protein appeared as nearly one single band, indicating the high purity of the preparation (Fig, 1A, lane 5). The protein was confirmed by western blot analysis (Fig. 1C) using the anti-ABCE1 antibody. The purified GST protein is shown in Fig. 1B, lane 6. It was also confirmed by western blot analysis (Fig. 1D) using the anti-GST antibody.

ABCE1 interacts with β-actin. GST pull-down assays were used to screen the target protein interacting with ABCE1. To eliminate unspecific binding of non-target proteins, the LTEP-a-2 cell lysate was also incubated with GST and agarose beads as the control (Pierce protocol). Several protein bands that interacted with the GST-ABCE1 protein were found after Coomassie Blue staining. But the most distinct specific peptide that was confirmed by mass spectrometry was β-actin (Fig. 2A). The peptide and the score of β-actin

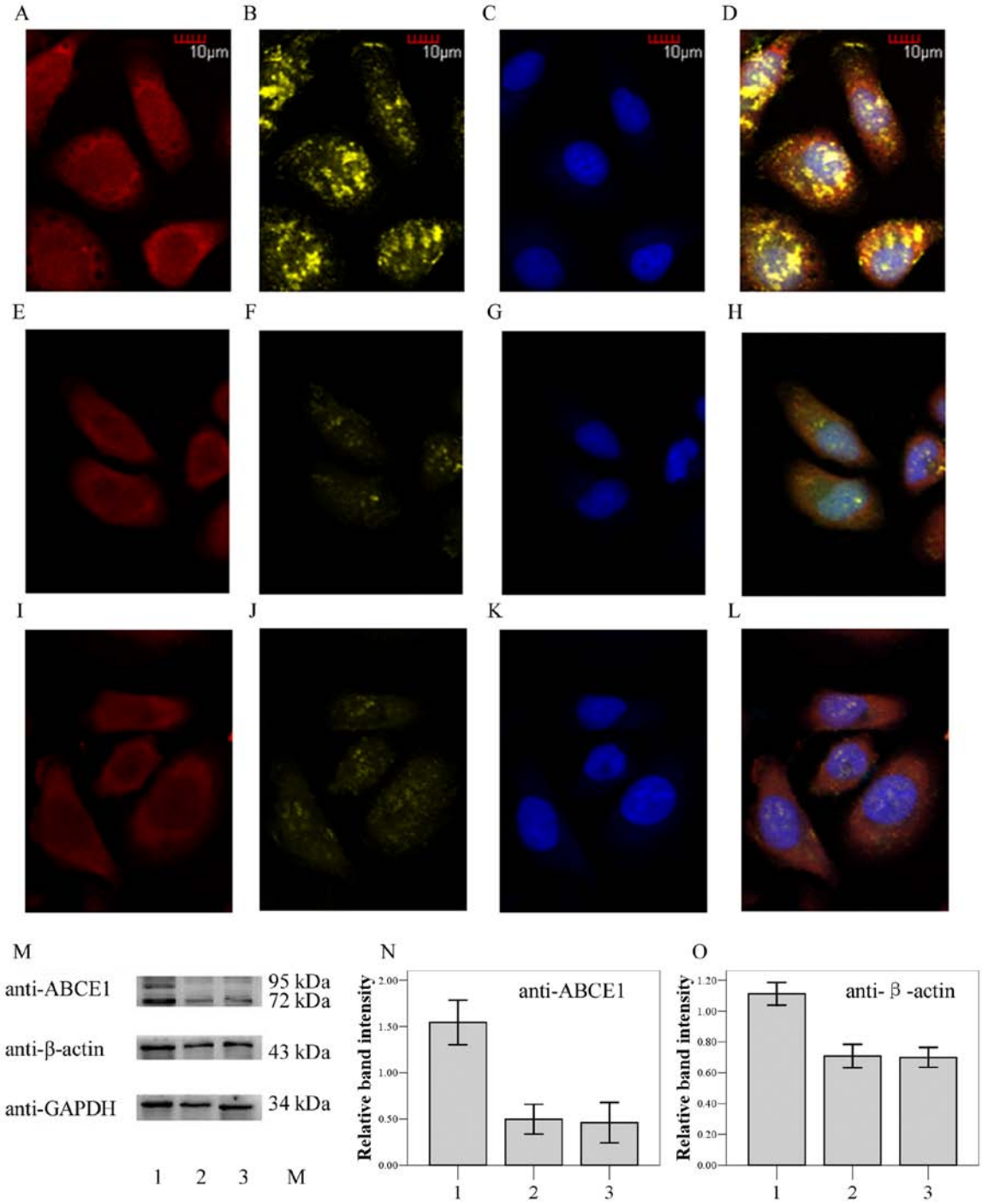

Figure 3. Expression and localization of ABCE1 and β-actin in the LTEP-a-2 cells. Column 1 (A, E and I), ABCE1 staining red. Column 2 (B, F and J), β-actin staining yellow. Column 3 (C, G and K), cell nuclei staining blue. Column 4 (D, H and L), merged image. Row 1 (A-D), LTEP-a-2 cells transfected with pEGFP-ABCE1 (group 1). Row 2 (E-H), LTEP-a-2 cells transfected with pEGFP (group 2). Row 3 (I-L), non-transfected LTEP-a-2 cells (group 3). (M-O), Western blot analysis (M) also showed that both ABCE1 (N, the relative band intensity is the ratio of GST-ABCE1 plus ABCE1 to GAPDH) and β-actin (O, the relative band intensity is the ratio of β-actin to GAPDH) were more strongly expressed in the transfected group 1 than group 2 and group 3.

and ABCE1 are shown in Fig. 2B and D. As a result, GST pull-down interaction screening provided the evidence of the original possible interaction between ABCE1 and β-actin.

ABCE1 co-immunoprecipitates with β-actin. Co-IP using ABCE1 antibody was used to determine the interaction between ABCE1 and β-actin; control resin and resin without antibodies were used as negative controls. ABCE1 was immunoprecipitated from the LTEP-a-2 cell lysates, and β-actin was detected in the precipitates with ABCE1 by Western blot analysis (Fig. 2C). This finding indicated that the interaction between ABCE1 and β-actin may happen at endogenous protein levels.

ABCE1 overexpression leads to aggregation of β-actin. After transfection with pEGFP-C1-ABCE1, ABCE1 (red) was overexpressed in both the cytoplasm and membrane-proximal areas (Fig. 3A compared with Fig. 3E and I). As a result, the

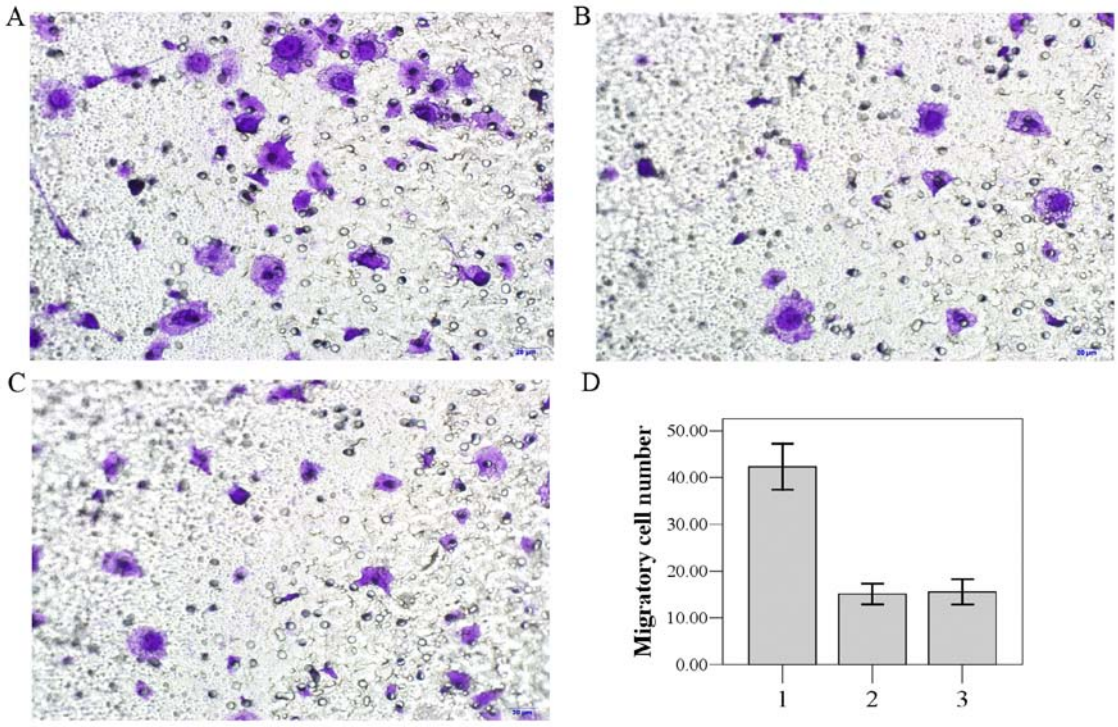

Figure 4. ABCE1 promotes cell migration. (A) LTEP-a-2 cells transfected with pEGFP-C1-ABCE1 (group 1). (B) LTEP-a-2 cells transfected with pEGFP-C1 (group 2). (C) Non-transfected LTEP-a-2 cells (group 3). The number of LTEP-a-2 cells transfected with pEGFP-C1-ABCE1 which migrated in the filter (group 1, 42.11±3.14) was higher than the number in group 2 (16.22±1.72) and group 3 (18.67±2.12) (P<0.05). There was no significant difference between group 2 and group 3 (P>0.05).

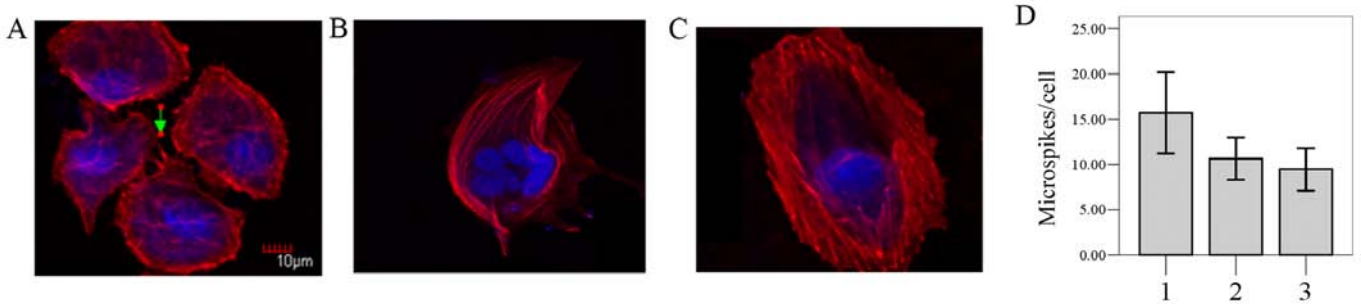

Figure 5. Overexpression of ABCE1 influences actin cytoskeletal organization. (A) LTEP-a-2 cells transfected with pEGFP-C1-ABCE1 (group 1). (B) LTEP-a-2 cells transfected with pEGFP-C1 (group 2). (C) Non-transfected LTEP-a-2 cells (group 3). In group 2 and group 3 cells, F-actin was concentrated in the cell periphery and in fine stress fibers that traverse in the cytoplasm. In contrast, cells in group 1 showed more filopodia-like protrusions (green arrow) and few regular cytoplasmic stress fibers. (D) Microspike quantification is shown as the mean microspike number per cell, P<0.05.

β-actin (yellow) obviously aggregated into plaque (Fig. 3B compared with Fig. 3F and J). Western blot analysis (Fig. 3M) also showed that both ABCE1 (Fig. 3N) and β-actin (Fig. 3O) were more strongly expressed in transfected group 1 than levels in group 2 and group 3. Moreover, they presented in roughly equal amounts in group 2 and group 3.

ABCE1 promotes cell migration. Transwell invasion assay was used to investigate the role of ABCE1 in the invasion of lung cancer cells. LTEP-a-2 cells transfected with the pEGFP-C1-ABCE1 and pEGFP-C1 plasmids and non-transfected cells were plated, respectively, on Matrigel-coated filters. After incubation for 48 h, the filters were stained with crystal violet and inspected under a microscope. The number of LTEP-a-2 cells transfected with pEGFP-C1-ABCE1 found in the filter (group 1, 42.11±3.14) was higher than the number in group 2 (16.22±1.72) and group 3 (18.67±2.12) (P<0.05) (Fig. 4). There was no significant difference between group 2 and group 3 (P>0.05).

Overexpression of ABCE1 influences actin cytoskeletal organization and the morphology of LTEP-a-2 cells. We showed that ABCE1 can bind β-actin. To investigate the cellular function of ABCE1 binding to β-actin, we analyzed the effect of the overexpression of ABCE1 on cell morphology. Phalloidin staining of F-actin showed that there were irregular edges and numerous irregular stress fibers in the LTEP-a-2 cells transfected with pEGFP-C1-ABCE1 (Fig. 5A). In contrast, cells in

group 2 (Fig. 5B) and group 3 (Fig. 5C) appeared to have a rounded morphology with few regular stress fibers. Quantitative analysis confirmed that the number of filopodia-like microspikes in group 1 (15.69±4.47) was more than that in group 2 (10.64±2.33) and group 3 (9.47±2.35) (Fig. 5D, $P<0.05$). These results indicated that overexpression of ABCE1 induced morphological and actin-related cytoskeletal changes in the LTEP-a-2 cells.

Discussion

In our previous study, Ren *et al* (17) confirmed that downregulation of ABCE1 significantly inhibited the growth of lung cancer cells, and accompanied by downregulation of ABCE1, it was determined that expression of several lung cancer-related genes was also changed. Huang *et al* (18) demonstrated that downregulation of ABCE1 may inhibit the proliferation and invasiveness of lung cancer cells. Therefore, we speculated that the ABCE1 gene is involved in lung cancer metastasis.

In this present study, β-actin was firstly screened as an interacting protein of ABCE1 by GST-pull-down assay. Although there are several other candidates such as IFNA1-66, AKAP9, CD63, and elongin (data not shown), we aimed to ascertain how the ABCE1 protein promotes the movement of cancer cells by identifying β-actin *in vitro* and *in vivo*. The complex was next testified by co-immunoprecipitation assays. We were interested in researching how the ABCE1 protein *in vitro* relates to the movement of cancer cells in living tumor tissue.

In the immunofluorescence analysis, we found that β-actin expression was significantly increased and aggregated into plaque when ABCE1 was overexpressed. More exactly, more G-actin was detected when ABCE was overexpressed.

Transwell invasion assay next demonstrated that upregulation of ABCE1 promoted the invasiveness of lung cancer cells *in vitro*. After labeling F-actin, overexpression of ABCE1 induced actin-related cytoskeletal changes in the LTEP-a-2 cells.

Obviously, our results showed that overexpression of ABCE1 in the LTEP-a-2 cells led to upregulation of G-actin expression and aggregation, increased cell migration and increased invasiveness of lung cancer cells.

The concept of a multi-stage process of cancer metastasis, involves invasion into surrounding tissue, intravasation, transit in the blood or lymph, extravasation, and growth at a new site (21). There is little doubt that invasion into surrounding tissue is the prerequisite of cancer metastasis. Dove *et al* (22) reported that cell motility is driven by cycles of actin polymerization, cell adhesion and acto-myosin contraction. The actin polymerization acts as the initial forces for translocation (23,24). In addition, the protrusive structures from the cell membrane such as lamellipodia and filopodia act as morphologic markers for cell motility. In fact, the actin polymerization requires energy. Notable, ABCE1 belongs to a protein family which can transfer ATP.

There are several classical pathways regulating actin polymerization: FH protein and Ena/WASP directly promote actin binding and extension in the positive terminal (25-27), ARP2/3 can promote the extension of existing actin filaments (28); p38 MAPK can also promote actin polymerization and actin formation (29). Cofilin can cut off existing filaments, increasing barbed (plus end) side for polymerization (30). Yet, we believe that ABCE1 can influence the cytoskeleton by its own mechanism in lung cancer cells.

Karcher *et al* found that ABCE1 has an N-terminal iron-sulfur (FeS) domain in contrast to all other ABC enzymes (31). Thus, further investigation should focus on the function of the FeS domain in the interaction of ABCE1 and β-actin.

In summary, ABCE1 may be a new interaction protein of β-actin, and it can increase the motility of lung cancer cells through cytoskeleton rearrangement. ABCE1 localizes to the cytosol, and is enriched at the cell periphery. Upregulation of ABCE1 stimulates β-actin polymerization and promotes migration. In summary, our findings offer the first insight on the biological role of ABCE1 in lung cancer biology. Increased expression of ABCE1, which is clinically correlated to aggressive tumor growth and invasion, may increase the G-actin pool to increase the formation of protrusions in lung cancer cells and increased their invasive ability.

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