

DAL-1/4.1B contributes to epithelial-mesenchymal transition via regulation of transforming growth factor- β in lung cancer cell lines

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Abstract. The present study aimed to investigate the effects of the tumor suppressor gene differentially expressed in adenocarcinoma of the lung 1 (DAL-1)/4.1B on early-stage adenocarcinoma of the lung. The role of DAL-1/4.1B in the epithelial-mesenchymal transition (EMT), which is implicated in cancer metastasis, was examined using DAL-1 knockdown and overexpression, followed by polymerase chain reaction and western blot analysis of EMT markers, as well as cell counting and cell migration/invasion assays. The results showed that DAL-1/4.1B has a role in transforming growth factor (TGF)- β -induced EMT in non-small cell lung cancer cells. Silencing of DAL-1/4.1B with inhibitory RNAs altered the expression of numerous EMT markers, including E-cadherin and β -catenin, whereas overexpression of DAL-1/4.1B had the opposite effect. In addition, DAL-1/4.1B expression was induced following TGF- β treatment at the protein and mRNA level. DAL-1/4.1B deficiency impaired TGF- β -induced EMT and increased cell migration and invasion. These results suggested that DAL-1/4.1B contributed to the EMT and may be important for tumor metastasis in lung cancer. Together with the results of a previous study by our group, the present study suggested that DAL-1/4.1B acts as a tumor suppressor in the early transformation process in lung cancer, while in later stages, it functions as an oncogene affecting the biological features of human lung carcinoma cells. The results of the present study provided evidence for the feasibility of utilizing DAL-1/4.1B as a target for lung cancer gene therapy.

Introduction

Lung cancer is at present the number one cause of cancer-associated mortality of men and women. The 88%-mortality rate for non-small cell lung cancer (NSCLC) has remained unchanged

since 1985 despite advances in cytotoxic drug development, radiotherapy and patient management (1). An important step toward deciphering key intervention points for this disease is a clear understanding of its genetic pathobiology. Little information exists regarding the sequence of genetic events leading to the genesis of lung cancer, particularly for tumors such as adenocarcinomas, which occur in the peripheral airways of the lung. The human tumor suppressor gene differentially expressed in adenocarcinoma of the lung (DAL-1)/4.1B was identified using differential display polymerase chain reaction (DDPCR) as a gene whose expression was lacking in NSCLC when compared with matched normal tissue (2). This gene was determined to be a novel member of the Protein 4.1 superfamily by virtue of the presence of a 336 amino-acid N-terminal region with significant homology with the 4.1/Ezrin/Radixin/Moesin domain present in all 4.1 family proteins (3). Frequent loss of 4.1B in cervical cancer (4), laryngeal squamous cell carcinoma (5), breast cancer (6), esophageal squamous cell carcinoma (7) and lung adenocarcinoma (8) suggested that 4.1B is a potential tumor suppressor (9,10). 4.1B is a member of the 4.1-family of proteins together with 4.1R, 4.1N and 4.1G and shows significant homology with ezrin, radixin and moesin as well as merlin, which is the specific gene product of neurofibromatosis type 2. It has also been reported that loss of 4.1B expression and methylation of the 4.1B promoter are involved in the development and progression of NSCLC, providing a possible indicator of poor prognosis. Moreover, re-expression of protein 4.1B or a smaller fragment of the entire protein, termed DAL-1, resulted in growth suppression of meningioma cells (11,12).

The epithelial-mesenchymal transition (EMT) is a biological phenomenon responsible for the formation of various tissues and organs during normal metazoan development. Due to the association of the EMT with the pathogenesis of cancer, the attention of the scientific community has been directed towards the search for and identification of effective therapeutic targets to inhibit EMT-associated phenotypic changes and tumoral progression (13,14). In order to explore the role of DAL-1/4.1B in the process of EMT progression, the present study used RNA interference technology to knock-down DAL-1 expression in H460 cells and to then determine the effects on cellular proliferation and invasion. The present study attempted to elucidate the molecular mechanisms associated with the malignant progression of NSCLC, which may represent a basis for adjuvant chemotherapeutic strategies for this disease.

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Table I. Primers used in quantitative polymerase chain reaction.

Target gene	Forward primer	Reverse primer
E-cadherin	5'-CAATGCCGCCATCGCTTAC-3'	5'-ATGACTCCTGTGTTCTCTGTTAATG-3'
Vimentin	5'-GAGAACTTTGCCGTTGAAGC-3'	5'-TCCAGCAGCTTCCTGTAGGT-3'
Snail1	5'-GCTCCACAAGCACCAAGAGT-3'	5'-ATTCCATGGCAGTGAGAAGG-3'
Snail2	5'-CTTTTCTTGCCCTCACTGC-3'	5'-ACAGCAGCCAGATTCCTCAT-3'
Twist1	5'-TGCATGCATTCTCAAGAGGT-3'	5'-GTTTTGCAGGCCAGTTTGAT-3'
Fibronectin	5'-ACCAACCTACGGATGACTCG-3'	5'-GCTCATCATCTGGCCATTTT-3'
β -catenin	5'-GTACGTCCATGGGTGGGACA-3'	5'-GGCTCCGGTACAACCTTCAACTA-3'
DAL-1	5'-GAGCTGCCAAGCGTTTATGGA-3'	5'-CCTGCCACTATAACGAACTTGGAA-3'
β -actin	5'-AGGTCGGAGTCAACGGATTTGGTCG-3'	5'-TGGCCAGGGGTGCTAAGCAGT-3'

Materials and methods

Cell culture and reagents. The human non-small cell lung cancer cell lines NCI H460 and A549 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI-1640 medium (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) in the absence of antibiotics at 37°C in a humidified atmosphere containing 5% CO₂. The medium of actively growing cells was replenished with medium containing 10 ng/ml TGF- β (R&D Systems, Minneapolis, MN, USA).

Plasmid pcDNA3-DAL-1 containing a full-length DAL-1 coding region (gene ID: 23136). This plasmid was verified by DNA sequencing. The control vector pcDNA3 was purchased from Invitrogen Life Technologies (Grand Island, NY, USA). Lipofectamine[®] LTX & Plus Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used for transfection. To obtain stable transfectants, cells seeded in 6-well plates were transfected with 2.5 μ g/well plasmids using 10 μ l/well Lipofectamine[®] LTX Reagent and 2.5 μ l/well Plus Reagent. After 6 h incubation in serum and antibiotic free conditions, the medium was replaced with RPMI-1640 containing 10% FBS, and the cells were cultured for 48 h prior to submitting to a 2-week selection in medium containing G418 (600 μ g/ml).

DAL-1 shRNA and transient transfection procedure. A commercially available vector, pGPU6/GFP/Neo (Shanghai Sangon Biotech Co, Shanghai, China), was used to generate short hairpin (sh)RNA specific for DAL-1. The human DAL-1 gene-coding sequence was obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>), where three sets of shRNA sequences targeting the human DAL-1 gene are listed: DAL-1-sh710 (5'-GCAGTGCAAAGTGATACTTCT-3'); DAL-1-sh1329 (5'-GCAAGTGGTCTGTTGATATAT-3') and DAL-1-sh1436 (5'-GCCGGGAGAGTTTGAACAATT-3'). A non-specific shRNA was designed as a negative control (DAL-1-shNC): (5'-CACCGTTCTCCGAACGTGTCACGTCAAGAGATTACGTGACACGTTTCGGAGAATTTTGTG-3'). All shRNAs were synthesized by Shanghai ShengGong Biotechnology Co. (Shanghai, China). These oligonucleotides were synthesized and sub-cloned into the

restriction sites of the vector at 22°C for 1h. H460 or A549 cells were plated in six-well plates at a density of 6x10⁴ cells per well and incubated overnight. Cells were then transfected with DAL-1-shRNAs (2 mg plasmid in 250 ml RPMI-1640 medium) using 5 ml Lipofectamine[™] 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Untransfected H460 cells were included as a blank control group. The success of the transfection was determined 48 h later by inverted fluorescence and phase-contrast microscopy (LEICA DMIL-PH1; Leica Microsystems, Wetzlar, Germany) using three randomly-selected fields of vision.

Reverse transcription quantitative (RT-q)PCR assays. Total RNA was isolated from H460 or A549 cells (1x10⁷ cells per well) using TRIzol reagent (Invitrogen Life Technologies). cDNA was synthesized using 2xTaq PCR MasterMix (Tiagen Biotech Co., Ltd., Beijing, China). The 25- μ l reaction mixture was composed of 1 μ l cDNA, 2 μ l of the upstream and downstream primers (MBI Fermentas, Vilnius, Lithuania), 12.5 μ l PCR-2X master mix (Tiagen Biotech Co., Ltd.) and 9.5 μ l RNase-free water (Amresco LLC, Solon, OH, USA). PCR was performed according to the manufacturer's instructions. PCR was performed using the ABI 7500 real time system (Applied Biosystems Life Technologies, Foster City, CA, USA) using SYBR Green[®] Premix Ex Taq (Takara Bio Inc., Tokyo, Japan). The cycling conditions included a holding step at 94°C for 3 min and 29 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. β -actin was used as internal control. Relative quantification was performed using the $\Delta\Delta$ CT method. Primer sequences of the target genes are listed in Table I.

Antibodies and western blot analysis. The following antibodies were used in the present study: Mouse monoclonal anti-4.1B (1:200; cat. no. 514386; Santa Cruz Biotechnology, Dallas, TX, USA.); mouse monoclonal anti-vimentin (1:1,000; cat. no. 3390; Cell Signaling Technology, Inc., Danvers, MA, USA); mouse monoclonal anti- β -catenin (1:1,000; cat. no. 2698; Cell Signaling Technology, Inc.); mouse monoclonal anti-Snail (1:1,000; cat. no. 3895; Cell Signaling Technology, Inc.); mouse monoclonal anti-fibronectin (1:1,000; cat. no. F7387; Sigma-Aldrich, St Louis, MO, USA); mouse monoclonal anti- β -actin (1:10,000; cat. no. A5441; Sigma-Aldrich).

Cells (1×10^7 cells per well) were lysed in pre-cooled lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS, pH 7.4) and the protein content of the lysates was assessed using a Bicinchoninic Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Briefly, cells were lysed using lysis buffer [20 mM Tris/HCl, pH 7.5, 137 mM NaCl, 1% (v/v) Triton X-100, protease inhibitor cocktail (Complete™, EDTA-free; Roche Applied Science Indianapolis, IN, USA) and a phosphatase inhibitor cocktail (PhosSTOP; Roche Applied Science)] and sonicated on ice. Bradford protein assay was used to determine the protein concentration of lysates (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Lysates were heated at 70°C with lithium dodecyl sulfate sample buffer (Invitrogen Life Technologies) and dithiothreitol (Sigma-Aldrich) for 10 min. Equal amounts of cell lysate (20 mg) were separated by 10% SDS-PAGE and were electroblotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Blotted membranes were blocked with 5% skimmed milk in Tris-buffered saline containing Tween (TBST; pH 7.6) at room temperature for 1 h. Blots were then incubated overnight at 4°C with the primary antibodies followed by incubation for 1 h at room temperature with a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse monoclonal antibody (Santa Cruz Biotechnology). After incubation, membranes were washed three times each for 15 min using 0.05% TBST. Protein bands were visualized using a diaminobenzidine coloration kit and the Fusion FX7 electrochemiluminescence analysis system (Wuhan Boster Biological Technology, Wuhan, China) according to the manufacturer's instructions. Relative band intensities were detected using ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA). The density of each band was normalized against that of β -actin.

Cell counting kit-8 (CCK8) assay for assessment of cell proliferation. Exponentially growing H460 cells were seeded into 96-well microtiter plates at a concentration of 5×10^4 cells per well and allowed to attach overnight prior to DAL-1 shRNA transfection. Following 24, 48, 72 and 96 h of culture, cells were incubated with 20 μ l CCK8 (5 mg/ml; Sigma-Aldrich) at 37°C for 4 h after which the medium was decanted and the reaction stopped by addition of 150 μ l dimethylsulfoxide (Sigma-Aldrich). The spectrometric absorbance of each sample was measured at 490 nm using an automatic microplate reader (iMark™ microplate absorbance reader; Bio-Rad Laboratories, Inc.).

Wound-healing assays. Cells were seeded into six-well plates and cultured until >90% confluent. Three straight wounds were scratched in each well using a sterile 200- μ l pipette tip. Cells were rinsed gently with phosphate-buffered saline (PBS) and 2 ml media (without FBS or containing 10% FBS) was added. Images were captured at 40x magnification immediately after scratching and again after 24 h and 48 h. All assays were performed in triplicate and all experiments repeated three times.

Matrigel™ invasion assay. Transwell chambers with 8-mm pore size polycarbonate membranes (Corning-Costar, Corning, NY, USA) were used to perform the cell invasion assay. Following 24 h of transfection, 2×10^4 cells were

suspended in 200 μ l serum-free medium and inoculated into each extracellular matrix-coated upper compartment of the 24-well plates that were pre-coated with 50 μ l 1 μ g/ μ l Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The lower compartment of each chamber was filled with 10,000 μ l RPMI-1640 with 30% FBS and incubated for 48 h at 37°C with 5% CO₂. The cells on the upper surface were then removed using cotton tips and the cells that had migrated to the lower side of the membrane were fixed with methanol for 30 min, stained with 0.1% crystal violet (Tianjin Yixin Hengxin Chemical Co., Ltd., Tianjin, China) for 30 min and washed with PBS three times. The number of invaded tumor cells was calculated in five random fields at a magnification of x200, using an inverted microscope (DP70; Olympus, Tokyo, Japan) and expressed as the average number of cells/field of view.

Statistical analysis. Each experiment was performed in triplicate. Statistical analyses were performed using SPSS statistical software, version 19.0 (SPSS, Inc. Chicago, IL, USA) for Windows. Values are expressed as the mean \pm standard deviation. Analysis of variance (ANOVA) experiments (one-way, factorial and repeated-measures ANOVA), followed by the Student-Newman-Keuls test were performed, with $P < 0.05$ considered to indicate a statistically significant difference between values.

Results

Downregulation of DAL-1/4.1B expression effectively suppresses DAL-1/4.1B protein expression in lung cancer cells. To examine the possible roles of DAL-1/4.1B in lung cancer cells, DAL-1/4.1B was knocked down using small interfering (si)RNA. Following transfection with DAL-1/4.1B siRNA, the levels of DAL-1/4.1B mRNA were reduced compared with those of the blank control group. At 48 h after transfection, DAL-1/4.1B mRNA expression was effectively knocked down by sh1329, while sh710 and sh1436 had no obvious effect (Fig. 1A). In addition, western blot analysis demonstrated that DAL-1/4.1B shRNA exerted a silencing effect on DAL-1/4.1B expression *in vitro* (Fig. 1B). These results confirmed that sh1329 effectively interfered with DAL-1/4.1B expression.

DAL-1/4.1B shRNA promotes cell proliferation. Compared with untransfected H460 cells (blank control group), the cell proliferation significantly increased in cells transfected with DAL-1-sh1329 (Fig. 1C) ($P < 0.05$ at 48 h). There were no significant differences in cell proliferation between the control groups ($P > 0.05$; blank controls and shNC-transfected cells).

Downregulation of DAL-1/4.1B decreases the migration of lung cancer cells *in vitro*. The role of DAL-1/4.1B in cell migration and invasion was evaluated by a wound-healing assay and the Matrigel-based Transwell invasion assay. Cell migration is a critical step in metastasis, and the results demonstrated that DAL-1/4.1B has a critical role in the metastatic behavior of cancer cells. The effects of DAL-1/4.1B shRNA on the characteristics of lung cancer cells were examined. Transfection of DAL-1/4.1B shRNA into H460 cells resulted in increased migration capacity compared with that of the blank control, as was evident from the migration assay (Fig. 1D and E). Culture

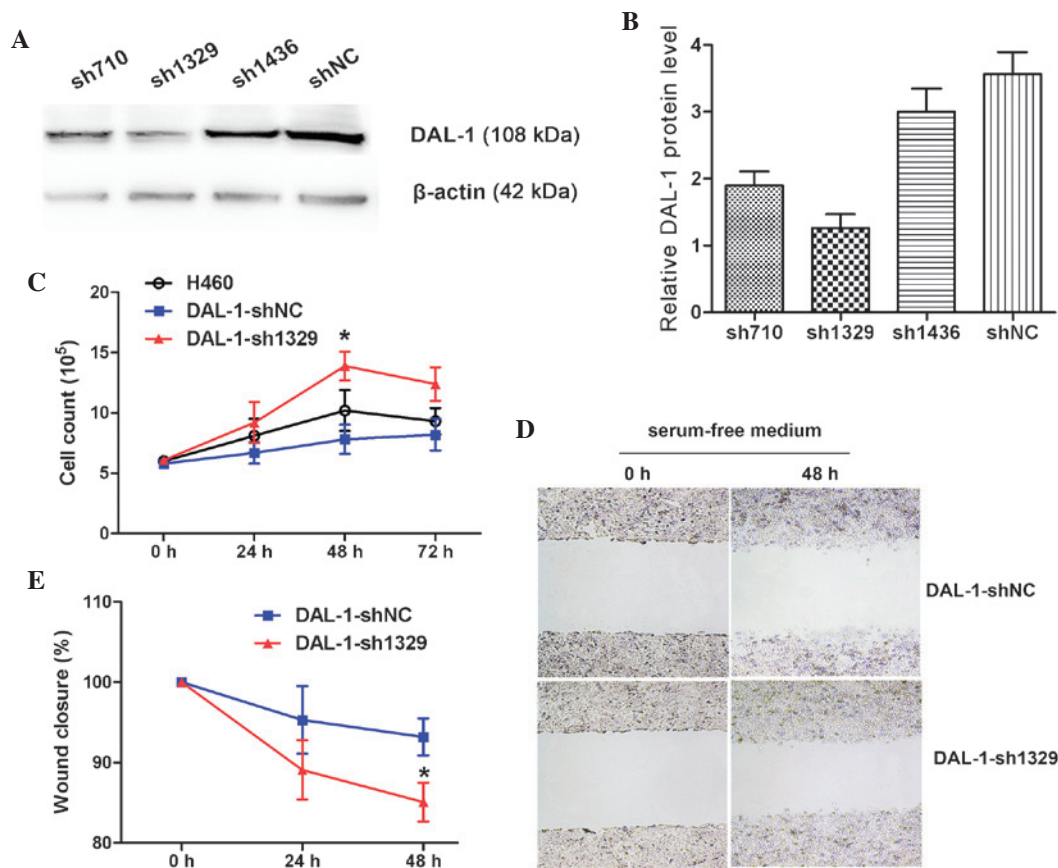


Figure 1. Detection of the efficiency of DAL-1/4.1B shRNA transfection and RNA interference. (A) Representative western blot of DAL-1 protein expression in H460 cells 48 h after transfection with shRNA sequences specific for DAL-1/4.1B. DAL-1/4.1B was effectively knocked down by sh1329, while sh710 and sh1436 had no obvious effect. (B) Quantitative analysis of the relative DAL-1/4.1B protein levels as determined by western blot analysis. (C) The proliferation of H460 cells was assessed using a cell counting kit-8 assay. The cell proliferation was determined by cell counting 0, 24, 48 and 72 h following transfection. (D and E) DAL-1 knockdown attenuates cell migration. Control and DAL-1/4.1B knockdown H460 cells were subjected to a wound-healing assay in the absence of serum. Images of the cell layers were captured immediately after generation of the scratch wound and 48 h thereafter (magnification, $\times 40$). Values are expressed as the mean \pm standard deviation ($n=3$). * $P<0.05$ vs. DAL-1-shNC group. shRNA, small hairpin RNA; NC, negative (scrambled) control; DAL-1, differentially expressed in adenocarcinoma of the lung 1.

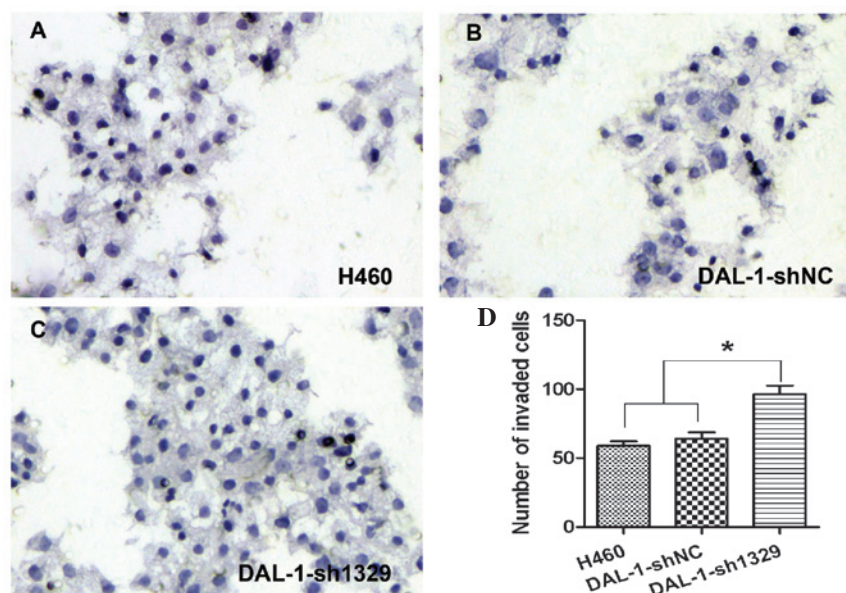


Figure 2. DAL-1/4.1B shRNA increases the invasive capacity of H460 cells. (A) H460 cells, (B) DAL-1-shNC and (C) DAL-1-sh1329 cells were seeded into the Matrigel-coated upper chambers of Transwell plates. The cells were counted under a microscope in five random fields (magnification, $\times 200$). (D) Statistical analysis showed that DAL-1 knockdown increased the invasive capacity of H460 cells compared with that of the control. * $P<0.05$, vs. H460, DAL-1-shNC and DAL-1-sh1329 cells. shRNA, small hairpin RNA; NC, negative (scrambled) control; DAL-1, differentially expressed in adenocarcinoma of the lung 1.

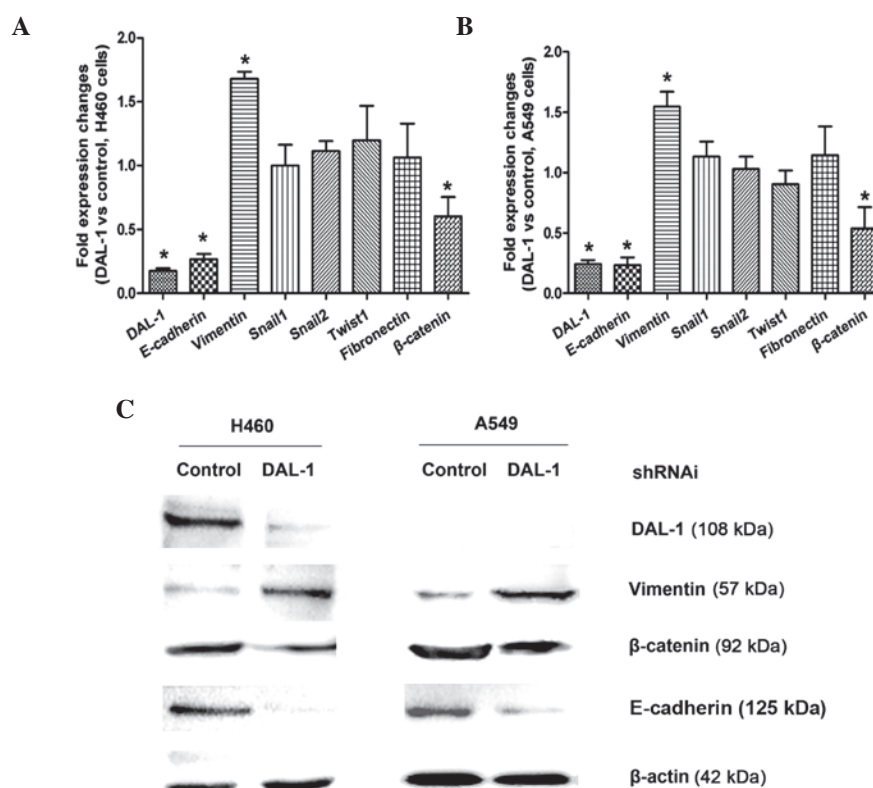


Figure 3. DAL-1/4.1B deficiency alters the expression of EMT markers. RNA extracts of control and DAL-1/4.1B-knockdown (A) A549 and (B) H460 cells were subjected to polymerase chain reaction analysis to determine the expression of target genes as indicated. The mRNA expression levels of E-cadherin and β-catenin decreased in the two cell lines, whereas the mRNA expression levels of vimentin increased. * $P < 0.05$ vs. control. (C) The protein expression of EMT markers was assessed in control and DAL-1/4.1B-knockdown A549 and H460 cells by western blotting. Representative blots show that the protein expression levels vimentin increased, while E-cadherin and β-catenin expression levels decreased. EMT, epithelial-mesenchymal transition; shRNAi, small hairpin RNA interference; DAL-1, differentially expressed in adenocarcinoma of the lung 1.

media without FBS was used to exclude the contribution of cell proliferation in the determination of migration of H460 cells. The average number of migrated cells in the experimental group (DAL-1-sh1329) was significantly lower than that in the blank control group (DAL-1-shNC) ($P < 0.05$).

The numbers of cells which transgressed the Matrigel™ and adhered to the lower side of the membrane in the blank control (Fig. 2A), DAL-1-shNC (Fig. 2B) and DAL-1-sh1329 (Fig. 2C) groups were 59.5 ± 6.52 , 64.0 ± 8.16 , 96.67 ± 10.33 , respectively. An increase in the number of invaded cells in the DAL-1-sh1329 group was observed compared with that in the control groups (Fig. 2D; $P < 0.05$), while there was no significant difference in the invasive potential between the two control groups.

DAL-1/4.1B deficiency alters the expression of EMT markers. To address the role of DAL-1/4.1B in H460 and A549 non-small cell lung cancer cell lines, control and DAL-1/4.1B-knockdown cells were generated by transfection with scrambled shRNA as a control and shRNA targeting DAL-1/4.1B. RT-qPCR and western blot analysis were used to examine the expression of EMT markers in control and DAL-1-knockdown H460 and A549 cells. As shown in Fig. 3A and B, E-cadherin and β-catenin mRNA were decreased, while vimentin was increased in DAL-1/4.1B-knockdown cells ($P < 0.05$). However, changes in the expression of fibronectin, Snail and Twist1 following DAL-1/4.1B knockdown were not significant ($P > 0.05$). Western blot analysis confirmed that DAL-1/4.1B knockdown resulted

in a decrease in E-cadherin and β-catenin expression and an increase in vimentin expression at the protein level (Fig. 3C).

DAL-1/4.1B overexpression alters the expression of EMT markers. To assess the effect of DAL-1/4.1B overexpression on EMT marker expression, A549 and H460 cells stably expressing DAL-1 were generated along with control cells transfected with empty vector. As expected, DAL-1 overexpression resulted in increased E-cadherin and β-catenin expression levels, whereas vimentin levels were reduced at the mRNA as well as at the protein level (Fig. 4). Collectively, these results suggested that DAL-1 is involved in the EMT process in lung cancer cell lines.

DAL-1/4.1B expression is induced by transforming growth factor (TGF)-β. It is known that TGF-β signaling is increased in a variety of cancer types, including lung cancer (15). To determine whether TGF-β and DAL-1/4.1B are associated in lung cancer, the non-small cell lung cancer cell line H460, which is known to undergo EMT upon TGF-β treatment, was utilized as a model. DAL-1/4.1B-knockdown H460 cells and scrambled control-transfected cells were incubated with TGF-β. As shown in Fig. 5A, DAL-1/4.1B protein levels were increased upon TGF-β treatment in H460 cells. Although the basal levels of DAL-1/4.1B were markedly low in DAL-1/4.1B-knockdown cells, its expression was induced following incubation with TGF-β. RT-qPCR showed a similar effect on the mRNA levels of DAL-1/4.1B (Fig. 5B).

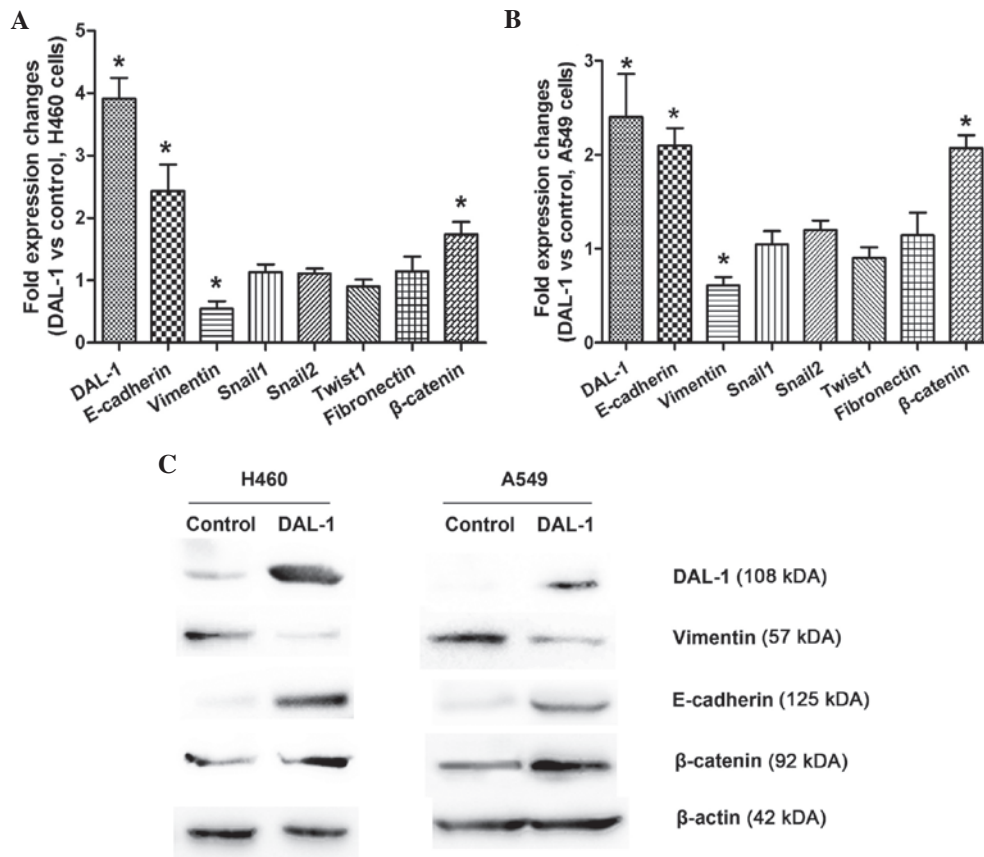


Figure 4. DAL-1/4.1B proficiency alters the expression of EMT markers. RNA extracts of (A) A549 and (B) H460 cells stably expressing control (pcDNA3) or Flag-DAL-1/4.1B were analyzed for EMT markers levels by polymerase chain reaction analysis. The mRNA expression levels of E-cadherin and β -catenin increased in the two cell lines, whereas the mRNA expression levels of vimentin decreased. * $P < 0.05$ vs. control. (C) The protein expression of target genes was determined by western blotting. Representative blots show that the protein expression levels vimentin decreased, while E-cadherin and β -catenin expression levels increased. DAL-1, differentially expressed in adenocarcinoma of the lung 1; EMT, epithelial-mesenchymal transition.

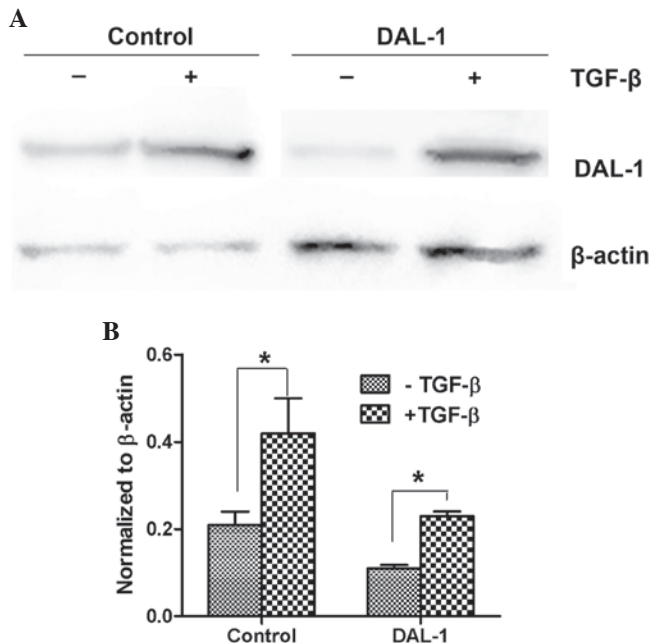


Figure 5. DAL-1/4.1B expression is induced by TGF- β . H460 stably expressing scrambled shRNA and DAL-1/4.1B shRNA were treated with TGF- β (2 ng/ml) for 7 days. Whole-cell lysates and RNA extracts were subjected to (A) western blotting and (B) polymerase chain reaction analysis. * $P < 0.05$ vs TGF- β -untreated group. DAL-1, differentially expressed in adenocarcinoma of the lung 1; TGF, transforming growth factor; shRNA, small hairpin RNA.

Discussion

Lung cancer is one of the most common malignant tumor types, and their morbidity and mortality have increased in recent years. Lung cancer has the highest mortality rate in males, and the second highest mortality rate in females amongst all tumor types. According to a recent study (16), the global number of newly diagnosed lung cancer cases was as high as 120 million people per year, and 100 million people succumbed to this disease. Lung cancer has become one of the most significant threats to human health. Although the early diagnosis and treatment of non-small cell lung cancer have significantly improved in recent years, the five-year survival rate remains at 10-20% only (17).

The tumor suppressor gene DAL-1/4.1B was shown to be located on chromosomal fragment 18p11.3 and was inactivated in certain types of tumor (18,19). The protein it encodes, 4.1B, belongs to the protein 4.1 superfamily of scaffold proteins. DAL-1/4.1B is normally expressed at high levels in the brain, while its expression is low in the kidneys, intestine and testes. As other 4.1 family member proteins, DAL-1/4.1B has been identified to be localized in regions of the plasma membrane at points of cell-to-cell contact by immunocytochemistry (20).

A previous study suggested that aberrant 4.1B expression is involved in progression of breast cancer, particularly in invasion into the stroma and metastasis (3). The 4.1B protein

is downregulated in several carcinoma types, including prostate cancer (21). Cavanna *et al* (22) identified a sub-set of genes with significantly altered expression levels between non-metastasizing and metastasizing cells in tissue culture and in primary tumors. Cells with reduced 4.1B expression displayed an altered F-actin morphology, with significantly fewer stress fibres. DAL-1/4.1B-knockdown cells migrated at twice the speed of the untreated cells. Examination of the expression of the 4.1B protein in human intestinal mucosa showed that DAL-1 was also expressed in matured epithelial cells in human colons, with a definite expression gradient along the crypt axis (23).

The EMT is characterized by a loss of cell-cell adhesion and polarity, downregulation of epithelial markers, as well as acquisition of mesenchymal markers and phenotype (24). E-cadherin, another tumor suppressor of the transmembrane adhesion molecule, also interacts with the cytoskeleton and is involved in the invasion or metastasis of gastric cancer as well as several other cancer types (25,26). Accumulating evidence from studies on the EMT have indicated the involvement of numerous signaling pathways, including TGF- β , Notch, Wnt, epidermal growth factor and fibroblast growth factor (27,28). Among these, TGF- β efficiently induces EMT in a variety of model cell lines and *in vivo* (29,30).

In conclusion, the present study showed that shRNA targeting DAL-1/4.1B significantly downregulated DAL-1/4.1B mRNA and protein expression in lung cancer cells, and inhibited cell proliferation as well as migratory and invasive potential. The results also indicated that downregulation of DAL-1/4.1B decreased the expression of E-cadherin and β -catenin in H460 and A549 cells. The findings of the present study provided novel insight into the underlying molecular mechanisms of NSCLC associated with the EMT, indicating that the tumor suppressor gene DAL-1/4.1B may be a potential target for anti-tumour drugs as well as gene therapy for treating lung cancer.

Acknowledgements

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