

ARHGAP6 regulates the proliferation, migration and invasion of lung cancer cells

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Abstract. Lung cancer, a leading cause of cancer-related deaths, is frequently diagnosed in both males and females worldwide. In the present study, the Ras homologue GTPase activation protein 6 (ARHGAP6), which belongs to the Rho GTPase-activating protein (RhoGAP) family, was found to have low expression in tumor tissues from patients with lung cancer, accompanied by high expression of matrix metalloproteinase-9 (MMP9) and vascular endothelial growth factor (VEGF). In A549 and H1299 cells, upregulation of ARHGAP6 inhibited tumor growth and metastasis and reduced the levels of MMP9, VEGF and p-STAT3, while the levels STAT3 were unchanged, as demonstrated by CCK-8, migration and invasion assays as well as western blot analysis. In addition, interleukin 6 (IL-6)-induced migration, invasion and MMP9 and VEGF expression, and STAT3 signaling activity were suppressed by ARHGAP6 upregulation. Based on these data, we concluded that ARHGAP6 is critically important in lung cancer progression and that upregulation of ARHGAP6 benefits the treatment and prevention of lung cancer, possibly through the suppression of MMP9, VEGF and STAT3 signaling activation.

Introduction

Lung cancer, which is frequently diagnosed in both males and females, is the leading cause of cancer-related deaths worldwide (1,2). In 2008, there were more than 1.6 million lung cancer cases diagnosed, comprising ~12.7% of all new cancer cases (3). In general, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) are the two major types of lung cancer. Evidence suggests that tobacco smoking is the main cause of lung cancer, accounting for probably 90% of all lung cancer diagnoses (4,5). The risk of lung cancer

among active smokers is 10-fold higher than that among non-smokers (6). Despite improved survival intervals, the survival rate for lung cancer is still discouragingly low (7).

Migration and invasion are widely recognized as two major hallmarks of malignancy and are closely related to the biological behavior of cancer cells. Actin, a main component of the cytoskeleton, plays an important role in cell movement (8,9). The Ras homologue GTPase activation protein 6 (ARHGAP6) is a novel Rho GTPase-activating protein (RhoGAP) gene that plays a crucial role in regulating actin polymerization in several cellular processes, including tumor growth and metastasis (10,11). It was reported that ARHGAP6 inhibited the proliferation and metastasis of cervical carcinoma, caused cell cycle arrest, and induced cell apoptosis (12). ARHGAP6 isoform 1 variant, an Hb3 antigen, may be a novel biomarker of the progression of colorectal cancer (CRC); 8.3% of patients with CRC develop pulmonary metastases despite undergoing curative surgery (13). However, the role of ARHGAP6 expression in lung cancer remains unclear. Matrix metalloproteinase-9 (MMP9) and vascular endothelial growth factor (VEGF) are commonly highly expressed in lung cancer and play an important role in lung cancer progression (14-16). Studies have associated MMP9 expression to tumor growth, metastasis and angiogenesis (17,18). VEGF is a major growth factor involved in angiogenesis, and VEGF-mediated angiogenesis is a crucial process for tumor growth and metastasis (19,20). In addition, signal transducer and activator of transcription 3 (STAT3), a transcription factor, has been found to be activated in lung cancer (21). Indirectly or directly inhibiting STAT3 activity affects the transcription of several genes, including VEGF, somewhat inhibits angiogenesis and affects the formation of tumors (22,23). A previous study reported that interleukin 6 (IL-6) acted as a strong activator of STAT3 signaling in lung cancer (24). IL-6 is a key cytokine frequently upregulated in cancer and considered a mediator of malnutrition in lung cancer patients (25,26).

In this study, significantly reduced ARHGAP6 levels were observed in tumor tissues from patients with lung cancer, accompanied by high levels of MMP9 and VEGF, which indicated that ARHGAP6 was involved in lung cancer progression. *In vitro* upregulation of ARHGAP6 significantly inhibited the growth and metastasis of A549 and H1299 cells, accompanied by decreased protein levels of MMP9, VEGF and p-STAT3. Moreover, IL-6-induced migration, invasion and expression of MMP9, VEGF and p-STAT3 were suppressed by ARHGAP6

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upregulation. These results indicated that ARHGAP6 upregulation may benefit lung cancer patients through the suppression of MMP9, VEGF and STAT3 signaling.

Materials and methods

Lung cancer tissue and adjacent normal tissue. After written informed consent was obtained, twenty-five pairs of tumor and adjacent normal tissue from lung cancer patients (13 men and 12 women among the age of 50-80 years) treated at Liaoning Cancer Hospital and Institute (Liaoning, China) were collected from May to December 2016 and immediately frozen in liquid nitrogen. After pretreatment, the expression of ARHGAP6, MMP9 and VEGF in these samples was detected by real-time PCR. All experiments in this study were approved by the Ethics Committee of Liaoning Cancer Hospital and Institute.

Cell culture. The A549 and H1299 human lung cancer cell lines and the 16HBE pulmonary epithelial cell line were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured in RPMI-1640 medium (product no. SH30809.01B; HyClone Laboratories; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% antibiotics (100X, a mixture of penicillin and streptomycin; product code: P1400-100; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) in a 5% CO₂ incubator (Thermo Forma 3111; Thermo Fisher Scientific, Inc.) at 37°C. The medium was replaced according to the growth demands of the cells during incubation.

Construction of the lentivirus. The mRNA sequence of the target gene was queried in NCBI, and primers and restriction sites were then designed for the coding sequence (CDS) and the selected vector. The 2925-bp full-length CDS of ARHGAP6 (NM_013427.2) synthesized by Genewiz Company (Shanghai, China) was inserted into the *EcoRI/BamHI* restriction sites of the pLVX-Puro plasmid, and the resulting plasmid was confirmed by DNA sequencing (Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China). The core plasmid pLVX-Puro-ARHGAP6 and the viral packaging plasmids psPAX2 and pMD2G (Addgene, Inc., Cambridge, MA, USA) were co-transfected into 293T cells with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of transfection, the virus particles in the medium were collected (15).

Experimental groups. To upregulate the expression of ARHGAP6 in the A549 and H1299 cell lines, lentivirus-mediated RNA overexpression was used. A549 and H1299 cells were divided into three groups: Infected with medium (control), infected with negative control lentivirus (vector) and infected with ARHGAP6 recombinant lentivirus (oe-ARHGAP6). After 48 h of infection, real-time PCR, western blot analysis and Transwell assays were carried out. In addition, proliferation assays were performed at 0, 24, 48 and 72 h.

To further investigate the effects of ARHGAP6 on lung cancer cells, A549 and H1299 cells were divided into three groups and then treated with medium (control), negative control lentivirus and 50 ng/ml IL-6 (Vector+IL-6),

or ARHGAP6 recombinant lentivirus and 50 ng/ml IL-6 (oe-ARHGAP6+IL-6). After 48 h of treatment, western blot analysis and Transwell assays were carried out.

Proliferation assay. A549 and H1299 cells in the logarithmic growth phase were digested with 0.25% trypsin (Beijing Solarbio Science & Technology Co., Ltd.), inoculated in triplicate in 96-well culture plates at a density of 3×10³ cells/well, and cultured in a humidified incubator overnight at 37°C with 5% CO₂. The following day, the cells were infected with medium, negative control lentivirus, or ARHGAP6 recombinant lentivirus, and after 0, 24, 48 and 72 h of infection, 100 µl of 10% Cell Counting Kit-8 (CCK-8; SAB, College Park, MD, USA) solution in serum-free medium was added to each well, and the plates were then placed in an incubator for 1 h. The absorbance at 450 nm was assessed by a microplate reader (Perlong Medical Equipment Co., Ltd., Beijing, China).

Real-time polymerase chain reaction (RT-PCR) assay. Total RNA was isolated from lentivirus-infected A549 and H1299 cells by TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), and RNA quality was confirmed by 1% agarose gel electrophoresis after quantification. A reverse transcriptase kit (Fermentas; Thermo Fisher Scientific, Inc.) was used to reverse transcribe RNA into cDNA. RT-PCR with cDNA as a template was conducted on an ABI PRISM 7300 instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a SYBR-Green PCR kit (Thermo Fisher Scientific, Inc.). The data were normalized to GAPDH, and the mRNA level of ARHGAP6 was calculated by the 2^{-ΔΔC_q} method (27). The primers were as follows: ARHGAP6, 5'-GAA TTTGACCGTGGGATTG-3' and 5'-CAGGGAGGTAGAAGG TATATG-3'; GAPDH, 5'-AATCCCATCACCATCTTC-3' and 5'-AGGCTGTTGTCATACTTC-3'. The RT-PCR procedure was as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 45 sec; one cycle at 95°C for 15 sec and 60°C for 1 min; and one cycle at 95°C for 15 sec and 60°C for 15 sec (28).

Western blot analysis. Treated A549 and H1299 cells were lysed in RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing protease and phosphatase inhibitors and incubated for ~30 min on ice. The proteins in the supernatant of the cell lysates were collected after centrifugation for 10 min at 12,000 × g at 4°C and quantified by a BCA quantification kit (Thermo Fisher Scientific, Inc.). After being separated by SDS-PAGE (JRDUN Biotechnology Co., Ltd, Shanghai, China), the proteins on the gel were transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA) by semidry electroblotting. The PVDF membranes were blocked in 5% skimmed milk (BD Biosciences, Franklin Lakes, NJ, USA) for 1 h at room temperature, followed by incubation with primary antibodies against ARHGAP6 (1:1,000; cat. no. NBPI-80837; Biocompare, San Francisco, CA, USA), MMP9 (1:1,000; cat. no. ab38898), VEGF (1:2,000; cat. no. ab69479), STAT3 (1:100; cat. no. ab50761), p-STAT3 (1:20,000; cat. no. ab76315; all from Abcam, Cambridge, MA, USA) or GAPDH (1:2,000; cat. no. 5174; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight with gentle shaking. After 5-6 washes with TBST, the membranes were incubated with secondary antibodies

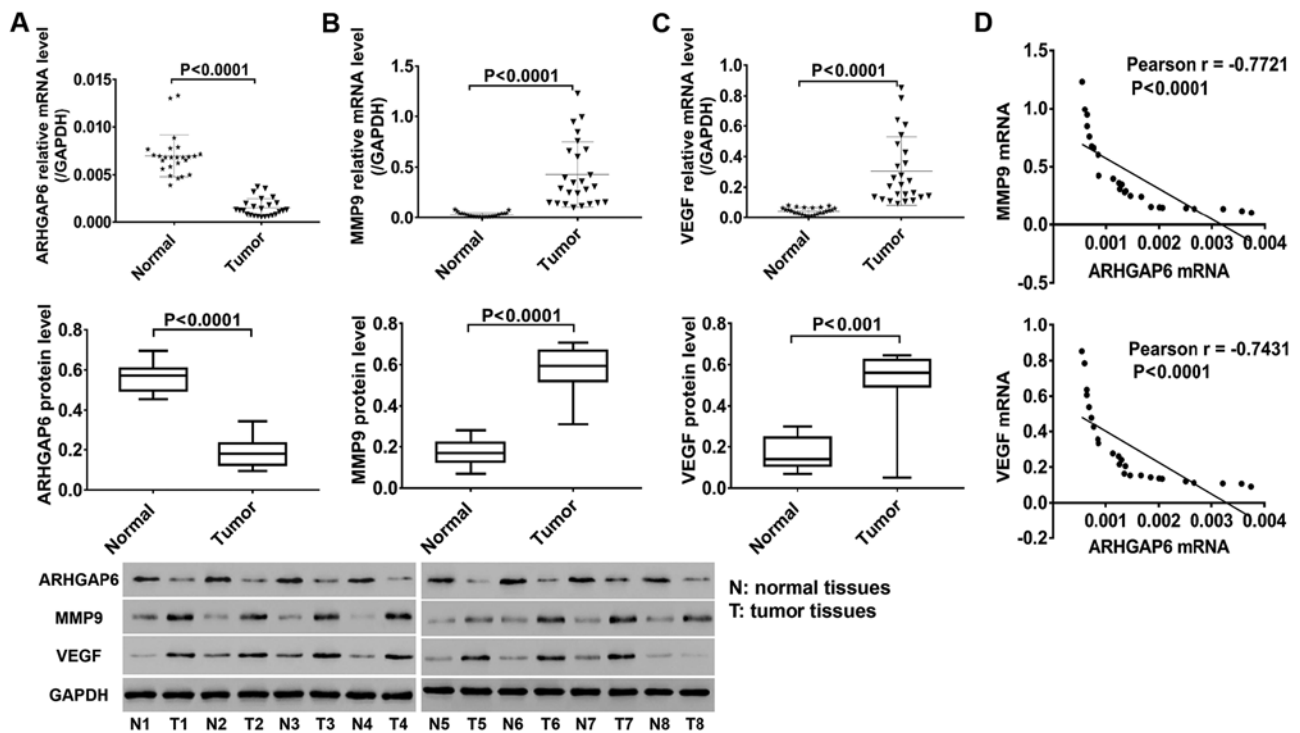


Figure 1. ARHGAP6 levels are significantly decreased in tumor tissues from lung cancer patients. Twenty-five pairs of tumor and adjacent normal tissues were collected from lung cancer patients. After RNA and protein extraction, the expression of (A) ARHGAP6, (B) MMP9, and (C) VEGF in these tissues was detected by RT-PCR and western blotting. (D) Pearson analysis was applied to determine correlations between ARHGAP6 and MMP9, and ARHGAP6 and VEGF ($P < 0.0001$). ARHGAP6, Ras homologue GTPase activation protein 6; MMP9, matrix metalloproteinase-9; VEGF, vascular endothelial growth factor.

(1:1,000; Beyotime Institute of Biotechnology, Haimen, China) of goat anti-rabbit (cat. no. A0208) and goat anti-mouse (cat. no. A0216) for 1 h at 37°C . Finally, the target protein bands were visualized on an ECL imaging system (Tanon-5200; Tanon Science and Technology Co., Ltd., Shanghai, China) after a 5-min incubation with chemiluminescent detection reagent (EMD Millipore) and the protein levels were calculated by ImageJ software 1.47v (National Institutes of Health, Bethesda, MD, USA).

Cell migration and invasion assays. The migration and invasion activities of A549 and H1299 cells were measured by Transwell assays using a modified Boyden chamber (Transwell Costar; cat. no. 342; Corning Inc., Corning, NY, USA) as previously described (29). The 24-well plates and Transwell chambers were soaked in 1X PBS for 5 min before inoculation (one more step for the invasion assay: 80 μl of Matrigel was placed in small chambers and clotted in a 37°C incubator for 30 min). After 24 h of serum starvation, infected and treated cells were digested by trypsin, washed, resuspended in serum-free medium, and inoculated in the upper chamber at a density of 5×10^4 cells/well. Then, 0.7 ml of RPMI-1640 medium containing 10% FBS as a chemoattractant was added to the lower chamber. After 24 h of incubation in a 37°C incubator, the migrating and invading cells on the lower side of the membrane were fixed with 1 ml of 4% formaldehyde for 10 min. After removing the fixative and washing once with 1X PBS, the cells were incubated in 1 ml of 0.5% crystal violet for 30 min, washed once with 1X PBS and dried. The number of cells that migrated and invaded from the upper chamber to the lower surface was counted with a microscope at $\times 200$.

Statistical analysis. GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analyses. Quantitative data comparisons between two groups were analyzed by paired Student's t-test (for parametric data), while differences among multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. Pearson analysis was used to determine correlations between two groups. The data are presented as the mean \pm SD of three independent experiments, and a P-value of < 0.05 was considered to indicate a statistically significant difference.

Results

ARHGAP6 levels are significantly decreased in tumor tissues from lung cancer patients. Twenty-five pairs of tumor and adjacent normal tissues from lung cancer patients were collected, and the expression of ARHGAP6, MMP9 and VEGF in these tissues was quantified by RT-PCR and western blotting. As shown in Fig. 1, ARHGAP6 mRNA (Fig. 1A, upper image) and protein (Fig. 1A, lower image) levels were significantly reduced in tumor tissues from lung cancer patients compared to adjacent normal tissues, accompanied by significantly increased levels of MMP9 (Fig. 1B) and VEGF (Fig. 1C). Pearson analysis revealed negative correlations between ARHGAP6 and MMP9 (Fig. 1D, upper image) and between ARHGAP6 and VEGF (Fig. 1D, lower image). These data indicated that ARHGAP6 may be implicated in the progression of lung cancer.

The expression of ARHGAP6 in the 16HBE, A549 and H1299 cell lines. The expression of ARHGAP6 in three cell lines

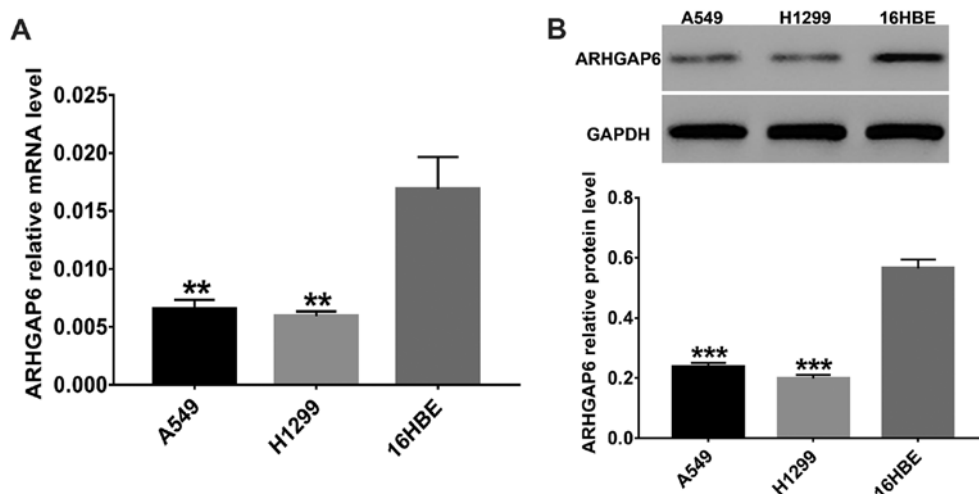


Figure 2. The expression of ARHGAP6 in the 16HBE, A549 and H1299 cell lines. In 16HBE pulmonary epithelial cells and A549 and H1299 lung cancer cell lines, the levels of (A) ARHGAP6 mRNA and (B) protein were detected by RT-PCR and western blot analysis, respectively. All data are presented as the mean \pm SD of three independent experiments (** $P < 0.01$, *** $P < 0.001$ compared to 16HBE cells). ARHGAP6, Ras homologue GTPase activation protein 6.

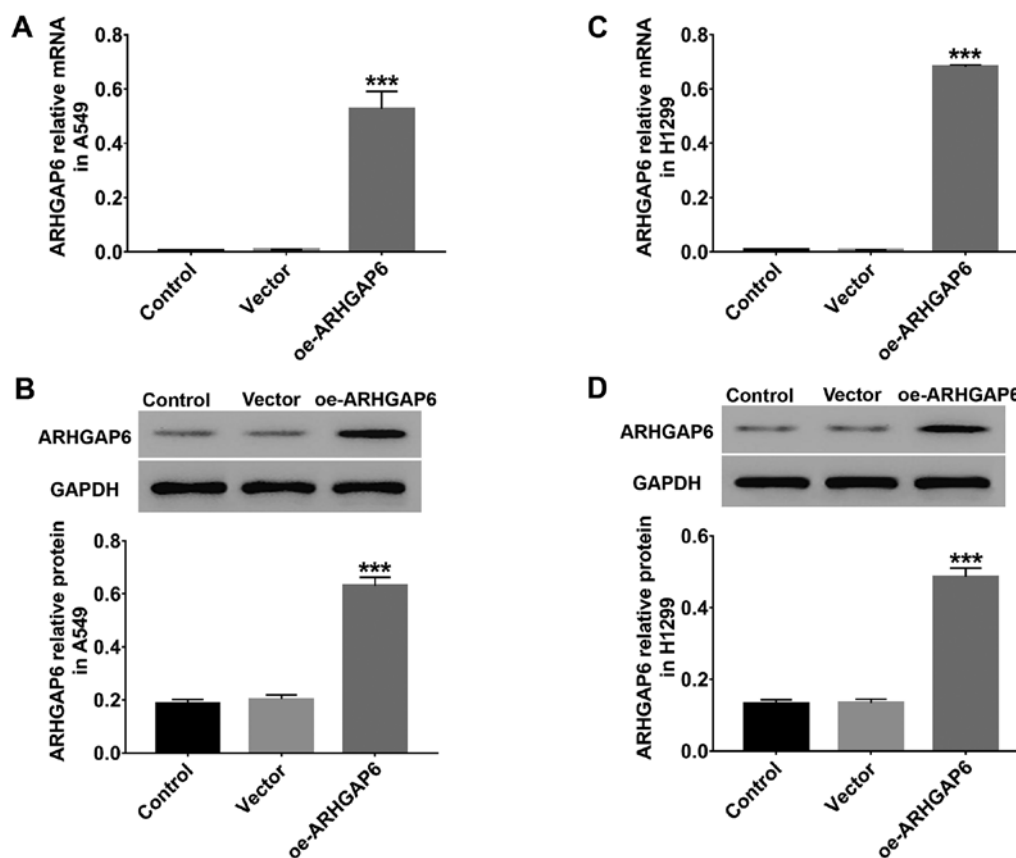


Figure 3. Upregulation of ARHGAP6 in the A549 and H1299 cell lines. (A and B) A549 and (C and D) H1299 cells were infected with ARHGAP6 recombinant lentivirus to upregulate ARHGAP6 expression. After 48 h of infection, the levels of ARHGAP6 mRNA and protein were quantified by RT-PCR and western blot analysis, respectively. Data are presented as the mean \pm SD of three independent experiments (*** $P < 0.001$ compared to the vector). ARHGAP6, Ras homologue GTPase activation protein 6.

(16HBE, A549 and H1299) was detected *in vitro*. As shown in Fig. 2, the levels of ARHGAP6 mRNA (Fig. 2A) and protein (Fig. 2B) in A549 and H1299 cells were much lower than those in the 16HBE cells, which indicated that ARHGAP6 expression was closely associated with lung cancer. The A549 and H1299 cell lines were used for further study.

Upregulation of ARHGAP6 in the A549 and H1299 cell lines. For further study, ARHGAP6 recombinant lentivirus was used to upregulate the expression of ARHGAP6 in A549 and H1299 cells. After 48 h of infection, the levels of ARHGAP6 mRNA and protein were quantified by RT-PCR and western blotting, respectively. The results revealed in Fig. 3 indicated

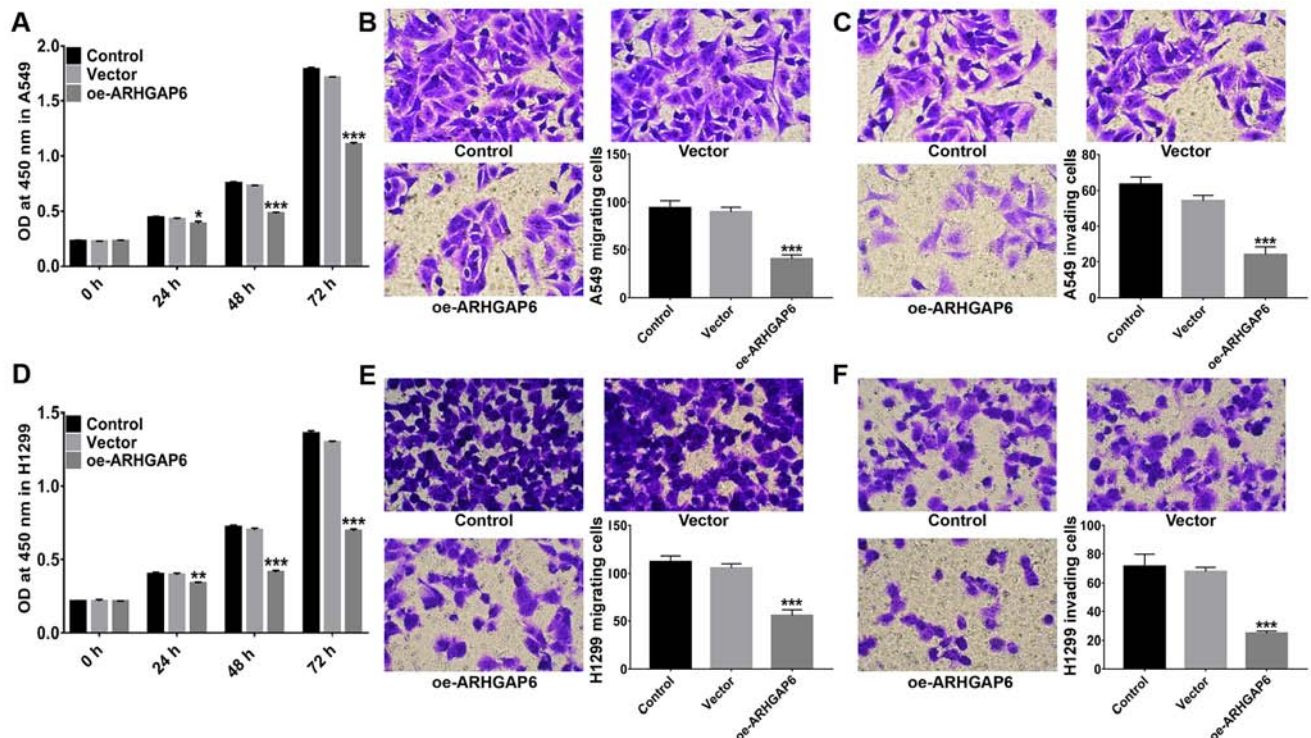


Figure 4. Effects of ARHGAP6 upregulation on the proliferation, migration and invasion of A549 and H1299 cells. After upregulating ARHGAP6 in A549 and H1299 cells, (A and D) cell proliferation was evaluated by CCK-8 assays, while the numbers of (B and E) migrating and (C and F) invading cells were determined by Transwell assays. Data are presented as the mean \pm SD of three independent experiments (* P <0.05, ** P <0.01, and *** P <0.001 compared to the vector). ARHGAP6, Ras homologue GTPase activation protein 6.

that in both A549 (Fig. 3A and B) and H1299 (Fig. 3C and D) cells, the expression of ARHGAP6 was markedly upregulated by infection with ARHGAP6 recombinant lentivirus. Therefore, the ARHGAP6 lentivirus was used in the following experiments due to its effective upregulation of ARHGAP6 expression.

Effect of ARHGAP6 upregulation on the proliferation, migration and invasion of A549 and H1299 cells. Following the upregulation of ARHGAP6 expression, the proliferation of A549 and H1299 cells was evaluated by CCK-8 assays. As revealed in Fig. 4, in both A549 (Fig. 4A) and H1299 cells (Fig. 4D), proliferation was significantly inhibited when ARHGAP6 was upregulated. In addition, the migration activities of A549 (Fig. 4B) and H1299 cells (Fig. 4E) were significantly suppressed after ARHGAP6 upregulation. Similarly, ARHGAP6-overexpressing A549 (Fig. 4C) and H1299 (Fig. 4F) cells were less invasive. These findings demonstrated that ARHGAP6 upregulation had an inhibitory effect on the growth and metastasis of lung cancer cells. Targeting ARHGAP6 may be a potential strategy for the treatment and prevention of lung cancer.

The altered expression of several associated genes after ARHGAP6 upregulation. Several cancer-associated genes (MMP9, VEGF and STAT3/p-STAT3) were analyzed to further study the effects of ARHGAP6 upregulation on lung cancer cells. After ARHGAP6 was upregulated in A549 and H1299 cells, the levels of these genes were analyzed by western blotting. As revealed in Fig. 5, in both A549 (Fig. 5A)

and H1299 (Fig. 5B) cells, the protein levels of MMP9, VEGF, and p-STAT3 were markedly reduced after ARHGAP6 upregulation, while the levels of STAT3 were unchanged. Activation of MMP9 and VEGF transcription has been revealed to enhance tumor migration and the invasion of lung cancer (30,31). STAT3, which mediates the transcription of several genes, including VEGF, is critically important for the progression of human lung cancer (32). Thus, we concluded that ARHGAP6 upregulation may inhibit the growth and metastasis of lung cancer cells by inhibiting MMP9, VEGF, and STAT3 signaling.

ARHGAP6 upregulation significantly suppresses IL-6-induced migration, invasion, and MMP9, VEGF and p-STAT3 expression. It has been reported that IL-6 is an important multifunctional cytokine that regulates the growth of a variety of tumors and plays a crucial role in carcinogenesis (16,33,34). Here, after lentivirus infection and treatment with 50 ng/ml IL-6, the migration and invasion activities of H1299 cells and the protein levels of MMP9, VEGF, STAT3 and p-STAT3 were detected. As shown in Fig. 6, IL-6 induced the migration (Fig. 6A) and invasion (Fig. 6B) of H1299 cells, and ARHGAP6 upregulation significantly inhibited these changes induced by IL-6. Moreover, the IL-6-induced expression of MMP9, VEGF and p-STAT3 was significantly reduced by ARHGAP6 upregulation, while the level of STAT3 was unchanged (Fig. 6C). All the data demonstrated that the upregulation of ARHGAP6 inhibited lung cancer metastasis through the suppression of MMP9, VEGF, and STAT3 signaling.

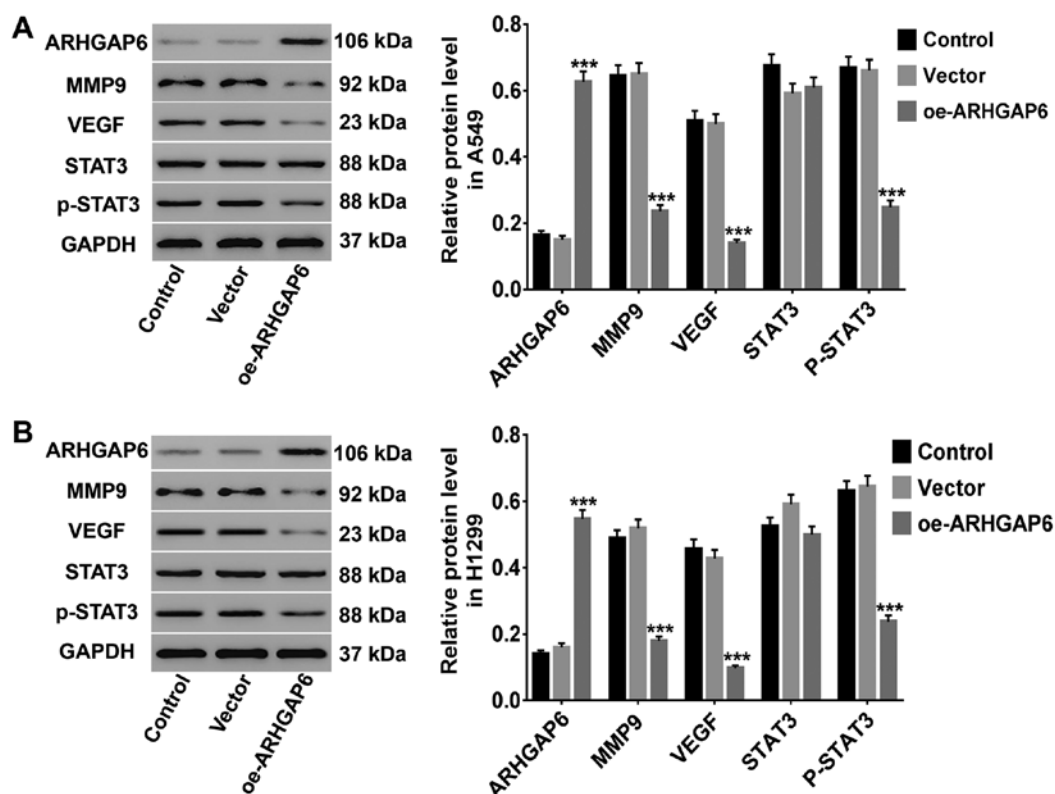


Figure 5. The altered expression of several associated genes after ARHGAP6 upregulation. After ARHGAP6 upregulation, the protein levels of MMP9, VEGF, STAT3 and p-STAT3 in (A) A549 and (B) H1299 cells were detected by western blot analysis. All data are presented as the mean \pm SD of three independent experiments (*** P <0.001 compared to the vector). ARHGAP6, Ras homologue GTPase activation protein 6; MMP9, matrix metalloproteinase-9; VEGF, vascular endothelial growth factor.

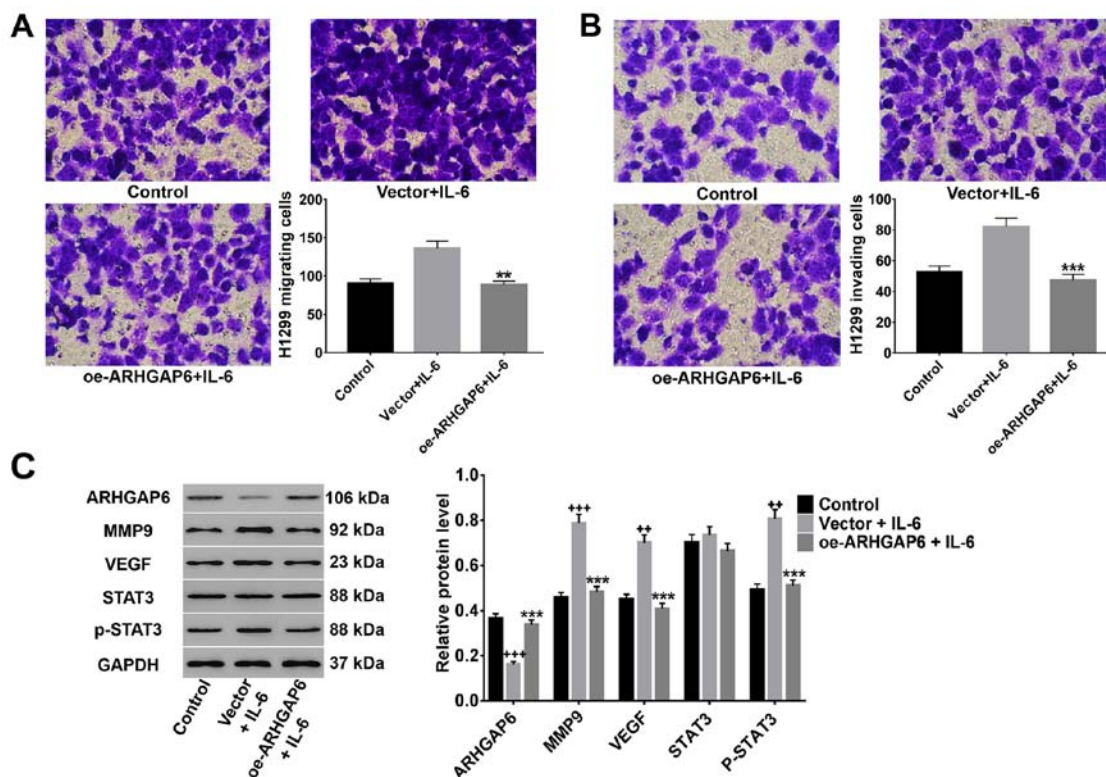


Figure 6. ARHGAP6 upregulation significantly suppresses IL-6-induced migration and invasion, as well as MMP9, VEGF and p-STAT3 expression. After treatment with ARHGAP6 lentivirus and 50 ng/ml IL-6, the (A) migrated and (B) invaded A549 and H1299 cells were determined by Transwell assays. (C) In addition, the expression of MMP9, VEGF, STAT3 and p-STAT3 was detected by western blot analysis. All data are presented as the mean \pm SD of three independent experiments (** P <0.01, *** P <0.001 compared to the control, *** P <0.001 compared to the vector + IL-6 group). ARHGAP6, Ras homologue GTPase activation protein 6; MMP9, matrix metalloproteinase-9; VEGF, vascular endothelial growth factor.

Discussion

Lung cancer, due to its typical diagnosis at later stages and poor long-term prognosis (35), causes more deaths than other types of cancer, such as breast and colorectal cancer (6). Thus, new potential treatments or prevention methods to reduce the incidence and mortality of lung cancer are urgently needed. Several members of the RhoGAP family have been discovered to function in lung cancer. For example, a study has reported that ARHGAP10 acts as a tumor suppressor in lung cancer (36), and the metastasis of lung cancer cells was effectively suppressed by downregulation of the proto-oncogenic protein ARHGAP5 (37). In this study, we found that ARHGAP6 upregulation was beneficial for lung cancer treatment in that it suppressed cell proliferation, migration and invasion via inhibition of STAT3 signaling activation and the expression of MMP9 and VEGF.

Multiple studies have observed a significant increase in MMP9 in lung cancer, and MMP9 performs important roles in tumor growth and metastasis (14,15). In addition to lung cancer, other cancers such as breast cancer exhibit high expression of MMP9 (38-40). MMP9 is associated with pathological type, and positive immunostaining for MMP9 has prognostic value for the distant metastasis or local recurrence of lung cancer (17,41,42). VEGF has been reported to induce tumor blood vessel proliferation and possibly contributes to the extravasation of cancer cells and the formation of metastasis (43). These previous findings are consistent with our discovery that ARHGAP6 was decreased and that MMP9 and VEGF were elevated in tumors from lung cancer patients. Similar to ARHGAP10 and ARHGAP5, we concluded that ARHGAP6 is likely a potentially attractive target for lung cancer diagnosis and treatment. ARHGAP6 upregulation had inhibitory effects on the growth and metastasis of A549 and H1299 cells; the high levels of MMP9, VEGF, and p-STAT3 were markedly reduced, while STAT3 was unchanged. These data indicated that ARHGAP6 was critically important for the growth and metastasis of lung cancer cells, and MMP9 and VEGF, which play a previously described crucial role in lung cancer (18,43), were negatively regulated by ARHGAP6 expression. STAT3 signaling, which is activated in NSCLC cells, was reported to regulate VEGF transcription, and disruption of its activity inhibits tumor angiogenesis (22,23). Therefore, we speculated that the inhibitory effects of ARHGAP6 upregulation on the growth and metastasis of lung cancer cells possibly occurs through the suppression of MMP9, VEGF, and STAT3 signaling. This hypothesis was further supported by the finding that ARHGAP6 upregulation significantly suppressed the effects of IL-6 treatment on lung cancer cells. There are still some limitations in our research such as the small sample size. Although a small sample size may lead to false positives in statistical analysis, our results are still of clinical value. In the future, we will expand the sample size to more fully ascertain our findings.

In conclusion, we found that upregulation of ARHGAP6 had an inhibitory effect on the cell growth and metastasis of lung cancer, possibly through the suppression of MMP9, VEGF, and STAT3 signaling. Targeting ARHGAP6 may be beneficial for the treatment and prevention for lung cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YW and YM conceived and designed the study. YW, MX, RH and KX performed the experiments. YW and YM wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experiments in this study were approved by the Ethics Committee of Liaoning Cancer Hospital and Institute. Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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