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# ARHGAP15 regulates lung cancer cell proliferation and metastasis via the STAT3 pathway

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**Abstract. – OBJECTIVE: Lung cancer, which is typically diagnosed at later stages, is a leading cause of cancer death among both males and females given its highly invasive and rapidly metastasizing nature. Rho GTPase activating protein 15 (ARHGAP15) is a member of the RhoGAP family and functions in multiple biological processes, such as cell proliferation and migration. However, the effect of ARHGAP15 in lung cancer and its underlying molecular mechanisms remain unclear.**

**PATIENTS AND METHODS: In this study, immunohistochemistry and Real Time PCR were performed to detect ARHGAP15 expression in lung cancer tissues and cells. Proliferation, transwell, and Western blot assays were further performed to explore the role and underlying mechanism of ARHGAP15 in lung cancer.**

**RESULTS: Reduced ARHGAP15 expression was observed in lung cancer tissues and cells. In vitro upregulation of ARHGAP15 in lung cancer cells strongly suppressed cell proliferation, migration, and invasion and was accompanied by reduced matrix metalloproteinase-2 (MMP2), MMP9, vascular endothelial growth factor (VEGF) expression, and the phosphorylation of the signal transducer and activator of transcription-3 (p-STAT3). In contrast, interleukin-6 (IL-6) had the opposite effect and the induction of IL-6 was counteracted by ARHGAP15 upregulation. In addition, the proliferation, migration, and cell invasion induced by ARHGAP15 silencing were potentially inhibited by the STAT3 inhibitor AG490 (100 μM), MMP2, MMP9, VEGF, and p-STAT3 levels decreased.**

**CONCLUSIONS: These results suggest that ARGFAP15 suppressed the proliferation and metastasis of lung cancer cells, which may occur through the inhibition of MMP2, MMP9, and VEGF expression via the STAT3 pathway inactivation.**

*Key Words:*
ARHGAP15, Proliferation, Migration, Invasion, STAT3 pathway, AG490, Interleukin-6.

### Abbreviations

ARHGAP15: Rho GTPase activating protein 15, MMP2: Matrix metalloproteinase-2; MMP9: Matrix metalloproteinase-9; VEGF: Vascular endothelial growth factor; STAT3: Signal transducer and activator of transcription-3; IL-6: Interleukin-6; CDS: coding DNA sequence; CCK-8: Cell Counting Kit-8.

## Introduction

Lung cancer, given its highly invasive and rapidly metastasizing nature, is a leading cause of cancer death among both males and females[1] and is typically diagnosed at later stages. Tobacco smoke is the main cause of lung cancer, accounting for approximately 85% of cases. The remaining 15% of lung cancers are attributed to



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other causes[2]. Compared with other cancers, the long-term prognosis of lung cancer is poor due to the lack of early stage observable symptoms[3-5] with a 5-year relative survival of approximately 6-14% in men and 7-18% in women[6]. Matrix metalloproteinases (MMPs), a group of zinc-dependent metalloenzymes, regulate a variety of cellular processes, including cell proliferation and metastasis in tumors[7]. MMP2 and MMP9 are two specific subsets of MMPs that have been intensively studied in cancers for several years. Previous studies[8,9] demonstrated that angiogenesis and tumor growth in MMP2-deficient mice was potently suppressed, and tumor metastasis was inhibited in MMP9-deficient mice. Vascular endothelial growth factor (VEGF), an angiogenic polypeptide that mediates tumor angiogenesis, it is crucial for the progression of a variety of cancers, including lung cancer[10-12]. Signal transducers and activators of transcription (STAT) family members are potential targets in various cancers, including lung cancer. Yu and Jove[13] have reported that STAT3, a member of the STAT family, is frequently activated in lung cancer cells. The inhibition of STAT3 affects the formation of tumors by inhibiting cell growth and tumor angiogenesis[14]. In addition, interleukin-6 (IL-6) is a multifunctional cytokine that acts as a strong activator of STAT3 signaling in lung cancer[15]. IL-6 is a major growth regulator in various tumors and is of key importance in carcinogenesis and cancer development[16,17]. Several studies[18-20] have demonstrated that IL-6 levels are elevated in certain neoplastic diseases, such as lung cancer, suggesting that elevated IL-6 levels are associated with poor clinical prognosis in cancer patients. Rho GTPase activating protein 15 (ARHGAP15) is a member of the RhoGAP family and functions in multiple biological processes, such as cell proliferation and migration. Takagi et al[21] demonstrated that ARHGAP15 is a potent tumor suppressor in human breast cancer. Furthermore, upregulation of ARHGAP15 expression inhibits the migration of glioma cells, suggesting that ARHGAP15 is a potential new target for glioma treatment[22]. However, the effect of ARHGAP15 in lung cancer and its underlying molecular mechanism remain unclear. In our current study, the low expression of ARHGAP15 was observed in tumor tissues in lung cancer patients and ARHGAP15 expression was correlated with overall survival rates. Biological function and signaling pathway assays demonstrated that ARHGAP15 might act as a tumor suppressor in lung cancer by inhibiting cell proliferation, migration, and invasion. Moreover, treatment with the STAT3 inhibitor AG490 exhibited an inhibitory effect on the proliferation and metastasis of lung cancer cells in a manner similar to ARHGAP15 upregulation. Therefore, we proposed that the regulation of ARHGAP15 may involve the STAT3 signaling.

## Patients and Methods

### Tumor and Adjacent Normal Tissues from Lung Cancer Patients

A total of seventy-six lung cancer patients treated in the Shandong Chest Hospital volunteered to participate in the current study. After obtaining written informed consents, seventy-six pairs of tumor and adjacent normal tissues were collected. Before the use, samples were frozen in liquid nitrogen. After the total RNA extraction from thirty pairs of tissues, ARHGAP15 expression levels in these tissues were quantified by real-time PCR. Moreover, immunohistochemistry was performed to detect the expression and location of ARHGAP15 in these tissues. The research Ethic Committee of Shandong Chest Hospital approved all experiments in this study.

### Cell Culture

Five human lung cancer cell lines, including A549, H1299, H1975, H358, and SKMES1, and the normal lung cell line 16HBE were purchased from Cell Bank of Chinese Academy of Science (Shanghai, China). The cells were cultured in a 5% $CO_2$ incubator (Thermo Fisher Scientific, Thermo Forma 3111, Rockford, IL, USA) at 37°C with Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, SH30809.01B, South-Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, 16000-044, Grand Island, NY, USA) and 1% antibiotic (100×, penicillin and streptomycin; Beijing Solarbio Science & Technology Co., Ltd., P1400-100, Beijing, China). The medium was replaced during incubation based on the cellular demand.

### Construction of the Lentivirus

ShRNA constructs targeted to the ARHGAP15 (AY219338.1) gene were synthesized and inserted into the Agel I/Ecol I restriction sites of a pLKO.1-puro vector. The full-length coding DNA sequence (CDS) region of ARHGAP15 is 1428 bp and the EcoR I/BamH I restriction sites were selected. The CDS sequence of ARHGAP15

containing restriction sites was synthesized by Genewiz Company (Shanghai, China) and then inserted into EcoR I/BamH I restriction sites of a pLVX-Puro vector. Through DNA sequencing (Majorbio, Shanghai, China), pLKO.1-shARHGAP15 and pLVX-Puro-ARHGAP15 plasmids were confirmed and cotransfected into 293 cells with viral packaging plasmids psPAX2 and pMD2G (Addgene, Inc.; Cambridge, MA, USA) using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The virus particles in the medium were collected by ultracentrifugation at 48 h after transfection[23].

### Experimental Grouping

ARHGAP15 expression in human lung cancer cells (A549, H1299, and H358) was regulated by ARHGAP15 interference or overexpression lentiviruses. A549 and H1299 cells were respectively divided into groups for infection with medium (Control), Vector, or ARHGAP15 overexpression lentivirus (oeARHGAP15). In addition, H358 cells were infected with medium (Control), negative control lentivirus (shNC), or shARHGAP15 lentivirus (shARHGAP15-1, shARHGAP15-2, shARHGAP15-3). The efficiency of ARHGAP15 lentivirus was evaluated at 48 h after transfection by eal-time PCR and Western blot.

Further, A549 and H1299 cells were respectively divided into five groups to treat with medium, Vector, oeARHGAP15, Vector + 50 ng/ml IL-6 (PEPROTECH, 200-06, Rocky Hill, NJ, USA), or oeARHGAP15 + 50 ng/ml IL-6. H358 cells were divided into four groups to treat with shNC, shNC + 100 μM AG490 (STAT3 inhibitor; Selleck, S1143, Houston, TX, USA), shARHGAP15, or shARHGAP15 + 100 μM AG490. Proliferation assays were conducted at 0, 24, 48, and 72 h after treatment. Cell migration and invasion and the expression of relative proteins were detected at 48 h.

### Immunohistochemical Detection

After paraffin embedding, the tissues were cut into 4- to 7-μm thick slices and then incubated in a 65°C oven for 30 min. The slices were deparaffinized by xylene I and xylene II (Shanghai Sinopharm, Shanghai, China); rehydrated in 100%, 95%, 85%, and 75% ethanol; and subjected to antigen retrieval by heating 0.01 M sodium citrate buffer (pH 6.0) for 10-15 min. Following blocking in 0.3% $H_2O_2$ for 10 min in a wet box, the slices were incubated with ARHGAP15 antibody (Abcam, ab102580, Cambridge, MA, USA) overnight at 4°C. The next day, the slices were incubated in horseradish peroxidase (HRP)-labeled broad-spectrum secondary antibody (Long Island, D-3004, Shanghai, China) for 1 h at room temperature. After diaminobenzidine (DAB) staining (Long island, FL-6001), the slices were counterstained with hematoxylin (BASO, 714094, Zhuhai, China) for 3 min and 1% hydrochloric acid for alcohol differentiation. The images were captured by a microscopy (NIKON, ECLIPSE Ni) and analyzed using an IMS image analysis system (NIKON, DS-Ri2).

### Real Time-Polymerase Chain Reaction (RT-PCR) Assay

Total RNA in cells was extracted using TRIzol reagent (Invitrogen, 1596-026, Carlsbad, CA, USA) according the manufacturer's recommendations. After quantification and RNA integrity were confirmed, the extracted RNA was reverse transcribed into cDNA using Reverse Transcription Kit (Fermentas, #K1622, Vilnius, Lithuania). Then, using cDNA as templates, RT-PCR was conducted on ABI 7300 Real-Time PCR system (Applied Biosystems, ABI-7300, Foster City, CA, USA) with a SYBR Green PCR Kit (Thermo Fisher Scientific, Inc., #K0223, Waltham, MA, USA). The primers sequences were as follows: ARHGAP15, F: 5' GACGACAGCCAGTGGGAGG 3' and R: 5' GCTTTGCGTGGACATGAGG 3'; GAPDH, F: 5' AATCCCATCACCATCTTC 3' and R: 5' AGGCTGTTGTCATACTTC 3'. The RT-PCR procedure was as follows: 95°C for 10 min; (95°C for 15 Sec; 60°C for 45 Sec) × 40, 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, 60°C for 15 s[24]. Reactions were performed in triplicate and were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). ARHGAP15 mRNA level was calculated by the method[25] of $2^{-\Delta\Delta Cq}$.

### Western Blot Analysis

Total proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer (Solarbio, R0010, Beijing, China) containing protease and phosphatase inhibitors and quantified using a BCA quantification kit (Thermo Fisher Scientific, PICPI23223, Waltham, MA, USA). Approximately 25 μg protein were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; JRDUN Biotechnology Co., Ltd, Shanghai, China) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, HATF00010, Billerica, MA, USA). After blocking in 5% skim milk (BD Bioscienc-

es, BYL40422, Franklin Lakes, NJ, USA) for 1 h at room temperature, the blots were probed with primary antibodies against ARHGAP15 (1:1000, Abcam, ab102580), MMP2 (1:1000, Abcam, ab37150), MMP9 (1:2000, ABGENT, AP6214a, San Diego, CA, USA), VEGF (1:1000, Cell Signaling Technology [CST], #2479, Danvers, MA, USA), STAT3 (1:1000, CST, #9139), p-STAT3 (1:2000, CST, #9145), and GAPDH (1:2000, CST, #5174) overnight at 4°C with gentle shaking. The next day, the blots were washed by TBST 5-6 times followed by 1-h incubation in secondary antibodies labeled with horseradish peroxidase (HRP; 1:1000, Beyotime, Shanghai, China) at room temperature. Later, the blots were washed with Tris-Buffered Saline and Tween-20 (TBST) for 5-6 times and developed by 5-min incubation with chemiluminescent reagent (Millipore, Billerica, MA, USA, WBKLS0100). The blots were visualized on an enhanced chemiluminescence (ECL) imaging system (Tanon, Tanon-5200, Shanghai, China), and GAPDH served as an endogenous reference. Relative protein levels were calculated using ImageJ software (http://rsb.info.nih.gov/ij/, Bethesda, MD, USA).

### Proliferation Assay

Cell Counting Kit-8 (CCK-8; Signalway Antibody [SAB], CP002, Pierland, TX, USA) solution was applied to assess cell proliferation. A549, H1299, and H358 cells seeded in 96-well plates (TRUELINE, TR4001, USA) at a density of $3 \times 10^3$ cells/well and cultured overnight. The cells were then divided for lentivirus infection (oeARHGAP15/Vector or shARHGAP15/shNC) and then treated with IL-6 or AG490. After incubation for 0, 24, 48, and 72 h, 100 µl mixture of CCK-8 and serum-free medium at a volume ratio of 1:10 was added and incubated for 1 h at 37°C. The absorbance value (OD) at 450 nm was measured on a microplate reader (Perlong, DNM-9602, Beijing, China).

### Transwell Assay

Transwell assays using a modified Boyden chamber (Transwell, Costar, 3422, Cambridge, MA, USA) were employed to detect cell migration and invasion as described[26]. Before inoculation, the 24-well plates and transwell chambers (COSTAR, 3422, USA) were soaked with phosphate-buffered saline (PBS) for 5 min (one more step for invasion: chambers were coated with 80 µl of Matrigel and clotted in a 37°C incubator for 30 min). Following starvation in serum-free medium overnight, the infected and treated cells were digested by trypsin and inoculated in the upper chamber ($5 \times 10^4$ cells/well), and RPMI-1640 medium with 10% FBS was added to the lower chamber. After 24 h of incubation, the non-migrated and non-invaded cells on the upper chamber were scraped carefully, and the migrated and invaded cells on the lower chamber were fixed with 4% formaldehyde (Shanghai Sinopharm, Shanghai, China) for 10 min followed by 30 min incubation of 0.5% crystal violet (Solarbio, C8470, Shanghai, China). The numbers of migrated and invaded cells were counted under microscope from at least 3 random fields at a magnification of 200×.

### Statistical Analysis

All experiments in this study were conducted thrice independently and data were presented as the mean ± standard deviation (SD). Statistical testing was performed using GraphPad prism 7.0 software (GraphPad Software, La Jolla, CA, USA). The differences between the two groups were compared using Student's *t*-test, whereas one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc test was applied for multiple groups. The differences between the groups of Kaplan-Meier survival curves were determined by the log-rank test. $p < 0.05$ indicated statistical significance.

## Results

### ARHGAP15 Expression was Decreased in Tumor Tissues of Lung Cancer Patients

The analysis of the available expression data of the tumor samples (488) and paracancer samples (58) of lung cancer patients obtained from The Cancer Genome Atlas project (TCGA) revealed that ARHGAP15 expression was considerably reduced in lung tumor tissues compared with paracancer tissues (Figure 1A). Consistently, our findings demonstrate that ARHGAP15 levels in the tumors of lung cancer patients were significantly decreased (Figure 1B). Immunohistochemistry was performed on tissues, further revealing the reduced level of ARHGAP15 in the tumors of lung cancer patients (Figure 1C). In addition, the correlation between ARHGAP15 expression and clinical pathological features was analyzed by Kaplan-Meier survival curves of 76 patients produced from ARHGAP15 expression and fol-

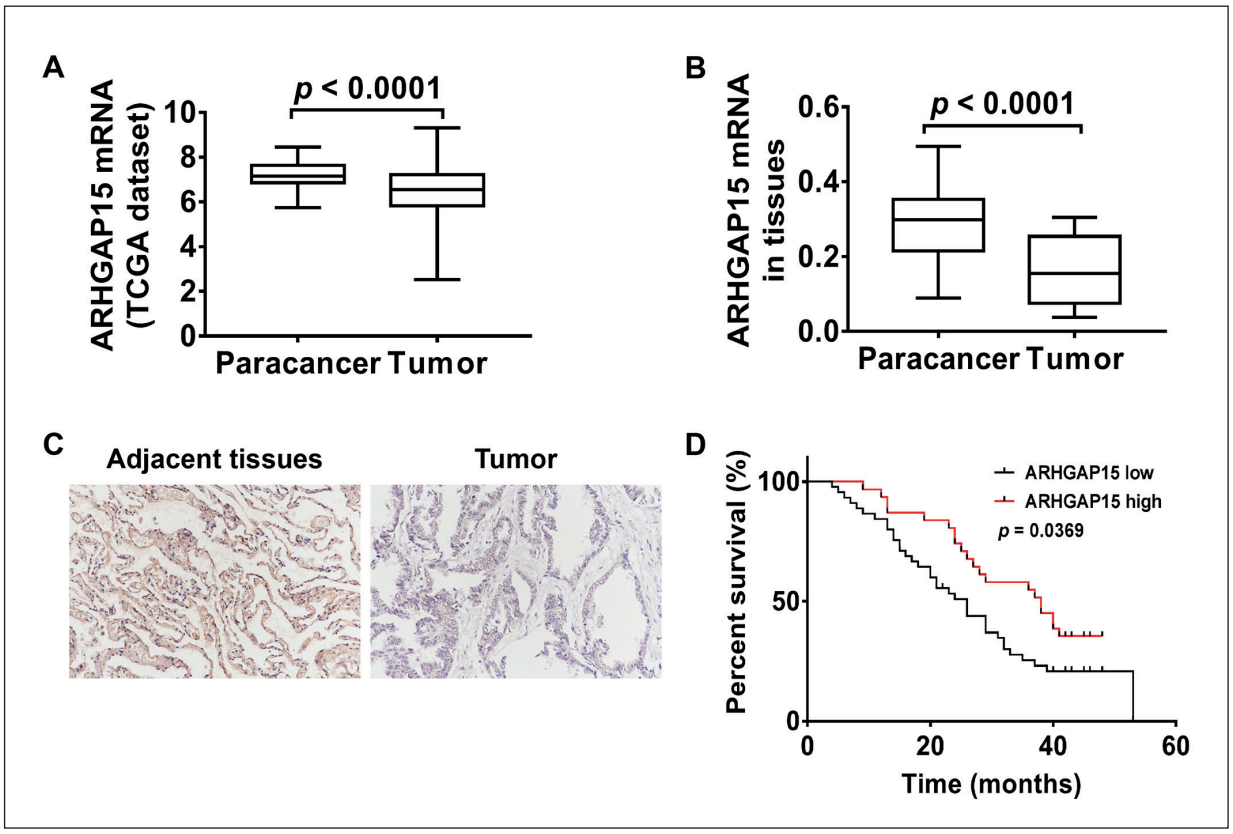


**Figure 1.** ARHGAP15 expression was reduced in tumor tissues of lung cancer patients. **A,** ARHGAP15 mRNA expression analysis in 488 lung tumor tissues and 58 paracancer tissues from TCGA dataset. **B**, Analysis of ARHGAP15 mRNA expression in thirty pairs of tumors and paracancer tissue samples. ***C,*** ARHGAP15 expression and localization in tumor and adjacent tissues was determined by immunohistochemical staining. Magnification: 200×. ***D,*** Survival rate of 76 lung cancer patients was assessed by Kaplan-Meier survival analyses. Compared with low ARHGAP15 tumors (n = 45), tumors with high ARHGAP15 (n = 31) had a favorable prognosis ($p < 0.01$).

low-up records. Using log-rank tests, we found that patients with low ARHGAP15 levels had a worse survival rate compared with high ARHGAP15 levels (Figure 1D). These findings indicate that ARHGAP15 expression may be involved in lung cancer progression.

### *ARHGAP15 Expression is Reduced in Lung Cancer Cells*

ARHGAP15 expression was detected by RT-PCR and Western blot in lung cancer cell lines (A549, H1299, H1975, H358, and SKMES1) and normal lung (16HBE). As shown in Figure 2, compared with normal lung cells, ARHGAP15 mRNA (Figure 2A) and protein (Figure 2B) levels in lung cancer cells were significantly decreased. In A549 and H1299 cells, ARHGAP15 levels were considerably reduced compared with other lung cancer cells but were relatively increased in H358. These results further verified the close correlation between ARHGAP15 expression and lung cancer progression. A549, H1299 and H358 cell lines were used in the following experiments.

### *Up- and Downregulation of ARHGAP15 in Lung Cancer Cells by Lentivirus Infection In Vitro*

To study the regulating effect of ARHGAP15 in lung cancer *in vitro*, Vector/oeARHGAP15 and shNC/shARHGAP15 (shARHGAP15-1, shARHGAP15-2, and shARHGAP15-3) lentiviruses were utilized to regulate ARHGAP15 levels in A549, H1299 and H358 cells. As shown in Figure 3, oeARHGAP15 lentiviruses strongly upregulated the expression of ARHGAP15 mRNA and protein in A549 (Figure 3A) and H1299 (Figure 3B) cells. All three shARHGAP15 len-

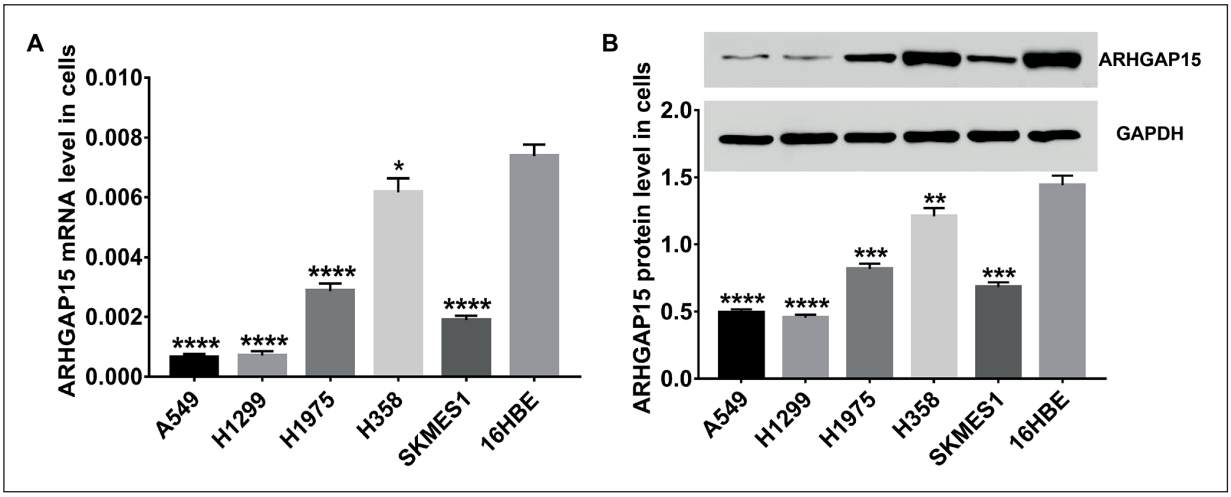


**Figure 2.** ARHGAP15 expression in lung cancer cells was reduced. ***A***, ***B***, ARHGAP15 mRNA and protein expression in five lung cancer cell lines and normal lung cells were analyzed by RT-PCR ***(A)*** and Western blot ***(B)***, respectively. Data are presented as the mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared with 16HBE.

tiviruses exhibited a strong inhibitory effect on ARHGAP15 expression in H358 (Figure 3C). shARHGAP15-3, which exhibited better down-regulated efficacy compared with the other two lentiviruses, was selected for the follow-up experiments.

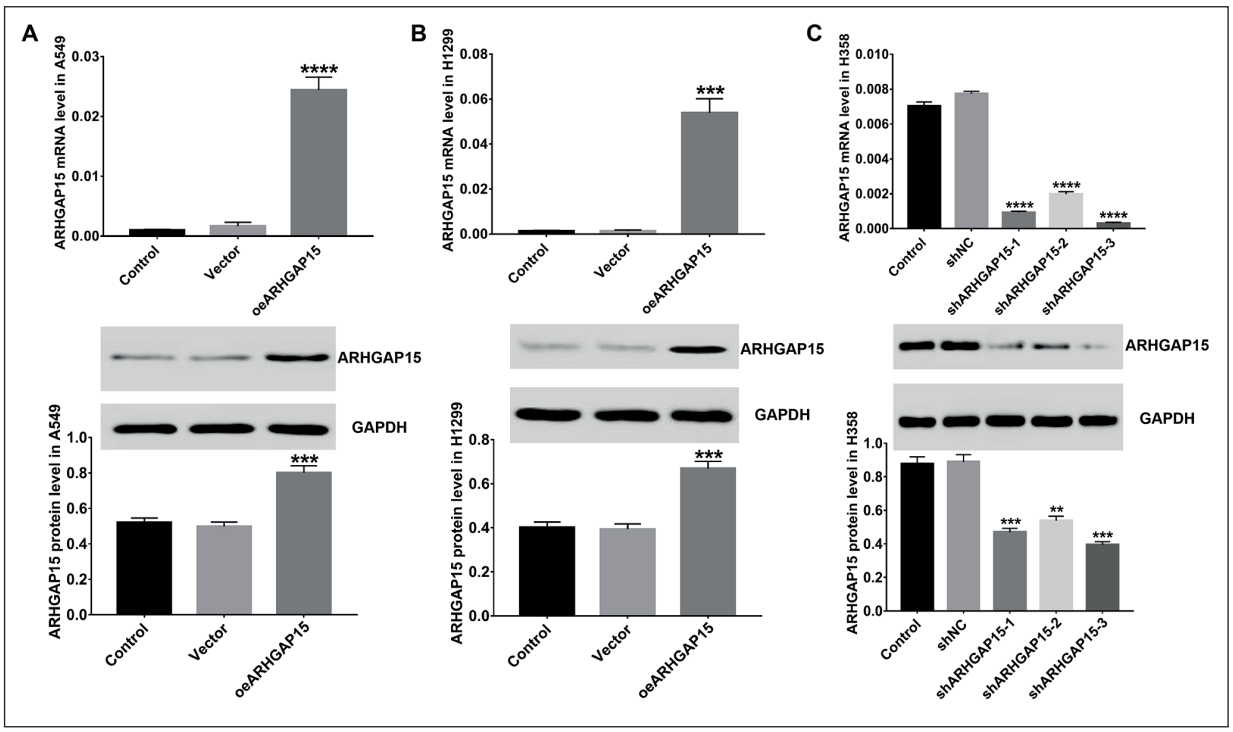


**Figure 3.** Up- and downregulation of ARHGAP15 in lung cancer cells by lentivirus infection *in vitro*. A549, H1299, and H358 cells were infected with shNC/shARHGAP15 or Vector/oeARHGAP15 lentivirus for 48 h, whereas medium-treated cells served as control. ***A***, ***B***, Efficiency of oeARHGAP15 in upregulating ARHGAP15 expression in A549 ***(A)*** and H1299 ***(B)*** cells was evaluated by RT-PCR and Western blot. ***C***, Downregulating efficiency of three shARHAGP15 in H358 cells was evaluated. All data are presented as the mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared with shNC or Vector.

### Upregulation of ARHGAP15 Inhibited IL-6-Induced Lung Cancer Cell Proliferation and Metastasis In Vitro

IL-6 is a strong activator of STAT3 signaling in lung cancer, regulates the growth of a variety of tumors and plays a crucial role in carcinogenesis[16,17,27]. Here, after lentivirus infection and 50 ng/ml IL-6 treatment, the cell proliferation (Figures 4A and 4D), migration, and invasion (Figures 4B and 4E) of A549 and H1299 cells infected with oeARHGAP15 was significantly inhibited concurrent with a decrease in MMP2, MMP9, and VEGF protein levels and STAT3 phosphorylation, whereas STAT3 levels were unchanged (Figures 4C and 4F). In contrast, IL-6 had an opposite effect on lung cancer cells and the induction of IL-6 was counteracted by oeARHGAP15 infection. Studies have related MMP2, MMP9 and VEGF to tumor angiogenesis, growth and metastasis [8-12]. STAT3 mediates transcription of gene, such as VEGF and is critically important for the progression of human lung cancer[28]. These results demonstrated that the ARHGAP15 upregulation strongly inhibited lung cancer cell proliferation and metastasis *in vitro*, which may occur through inhibition of MMP2, MMP9, and VEGF expression via the STAT3 pathway inactivation.

### Downregulation of ARHGAP15 Induced Lung Cancer Cell Proliferation and Metastasis Via STAT3 Pathway Activation

Numerous reports have used AG490, an inhibitor of JAK2-STAT3, *in vitro* assays to study signal transduction and regulation of JAK2-STAT3[29]. To further investigate the mechanism of ARHGAP15 in lung cancer cells, shARHGAP15 lentivirus and AG490 (100 μM) were applied. As shown in Figure 5, ARHGAP15 silencing-induced lung cancer cell proliferation (Figure 5A), migration and invasion (Figure 5B) were significantly inhibited by AG490 treatment. Similarly, elevated levels of MMP2, MMP9, VEGF and p-STAT3 in ARHGAP15-silenced lung cancer cells were also reduced (Figure 5C). These results further demonstrated that ARHGAP15 suppressed lung cancer cell proliferation, migration, and invasion likely by inhibiting MMP2, MMP9, and VEGF expression via STAT3 pathway inactivation.

## Discussion

Compared with other cancers, such as colorectal cancer, lung cancer causes more death and its incidence and mortality are increasing worldwide[30]. In the present study, we found that ARHGAP15 expression was low in lung cancer tissues and cells. In addition, the upregulation of ARHGAP15 suppressed lung cancer cell proliferation and metastasis potentially through the inhibition of MMP2, MMP9, and VEGF expression via STAT3 pathway inactivation. Recently, several members of the RhoGAP family have been observed to function in lung cancer. As a tumor suppressor in lung cancer, ARHGAP10 affects cell proliferation, migration, and invasion and is a potential attractive target for lung cancer treatment[31]. The downregulation of the proto-oncogene ARHGAP5 effectively inhibits lung cancer cell metastasis[32]. In the current study, low expression of ARHGAP15 was observed in both lung cancer tissues and cells. We conjectured that ARHGAP15 has the potential to be an attractive target for the diagnosis and therapy of lung cancer and its positive immunostaining may have some prognostic values. *In vitro*, the upregulation of ARHGAP15 significantly inhibited lung cancer cell proliferation and metastasis with a concurrent reduction in MMP2, MMP9, VEGF, and p-STAT3 levels. Previous studies[23,27] have reported that MMP2, MMP9, and VEGF are typically highly expressed in lung cancer and are essential for tumor growth and metastasis. For instance, the downregulation of MMP2 can inhibit endometrial cancer cell proliferation and invasion[33]. MicroRNA 5692a potentially promotes hepatocellular carcinoma (HCC) cell invasion and metastasis by regulating MMP9 expression[34]. Moreover, VEGF-mediated angiogenesis is a key process that potentially contributes to cancer cell extravasations and metastasis[35]. Our research results are consistent with these reports. In contrast, IL-6 had an opposite effect as that of ARHGAP15 on lung cancer cell proliferation and metastasis and IL-6 induction was counteracted by the ARHGAP15 upregulation. IL-6 is a mediator of malnutrition in lung cancer patients and drives the STAT3 pathway in lung cancer[20,36]. These findings indicated that ARHGAP15 functioned as a suppressor in lung cancer potentially through the inactivation of the STAT3 pathway. VEGF transcription is regulated by the STAT3 pathway, which is activated in lung cancer[36]. Green tea catechin treatment inhibits angiogenesis and tumor formation through the suppression of STAT3 pathway activation[14]. These findings are consistent with our observation that ARHGAP15 silencing-induced lung cancer cell proliferation,

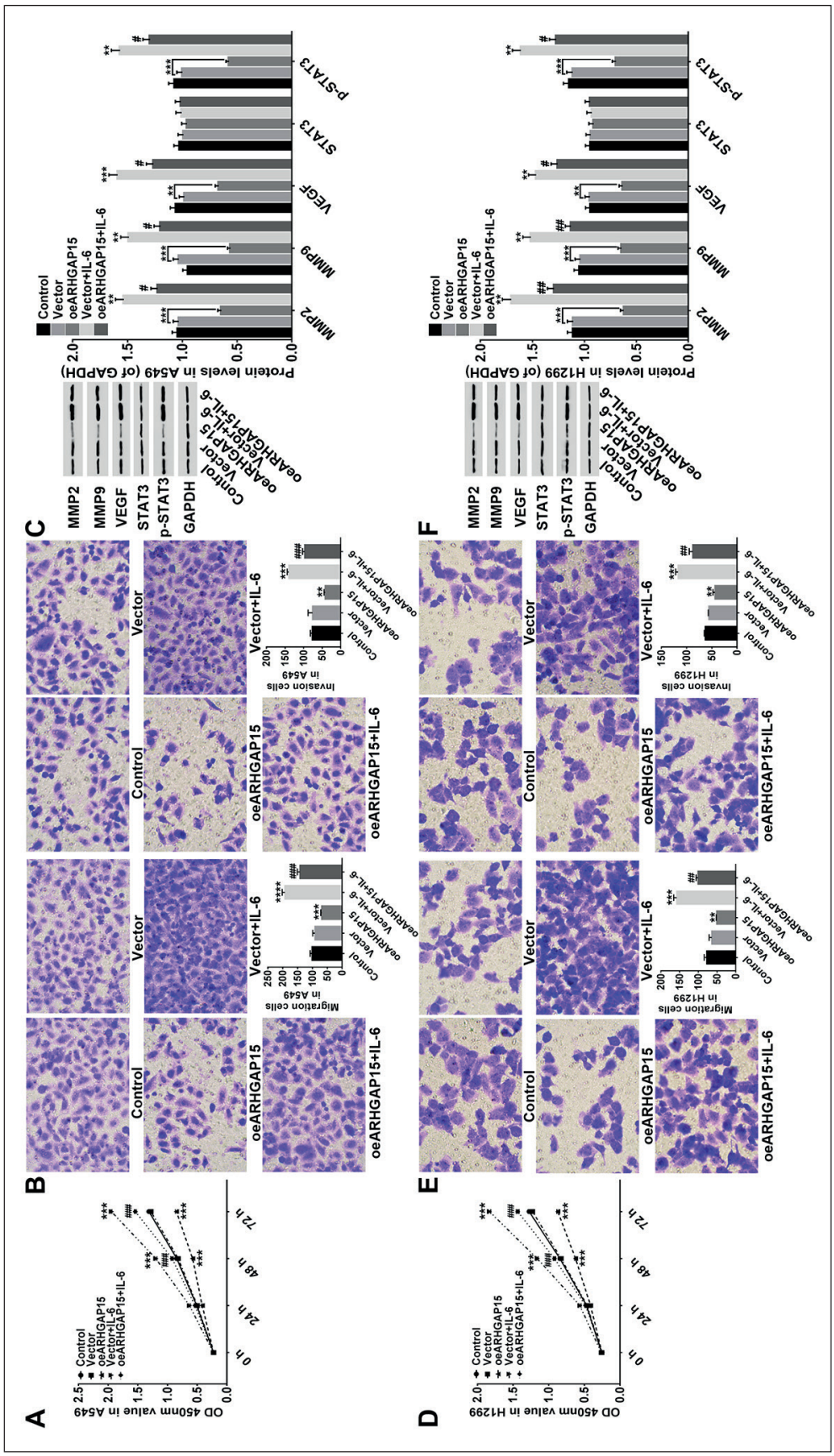


**Figure 4.** Upregulation of ARHGAP15 inhibited IL-6-induced lung cancer cell proliferation and metastasis *in vitro*. A549 and H1299 cells were infected with Vector/oeARHGAP15 and treated with IL-6 (50 ng/ml), whereas medium-treated cells served as control. ***(A, D)*** A549 ***(A)*** and H1299 ***(D)*** cell proliferation was evaluated at 0, 24, 48 and 72 h using CCK-8 assays. ***(B, E)*** A549 ***(B)*** and H1299 ***(E)*** cell migration and invasion were assessed at 48 h by transwell. ***C***, ***F***, MMP2, MMP9, VEGF, and p-STAT3 expression in A549 ***(C)*** and H1299 ***(F)*** cells was detected by Western blot. The results are expressed as the mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared with Vector, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with oeARHGAP15.

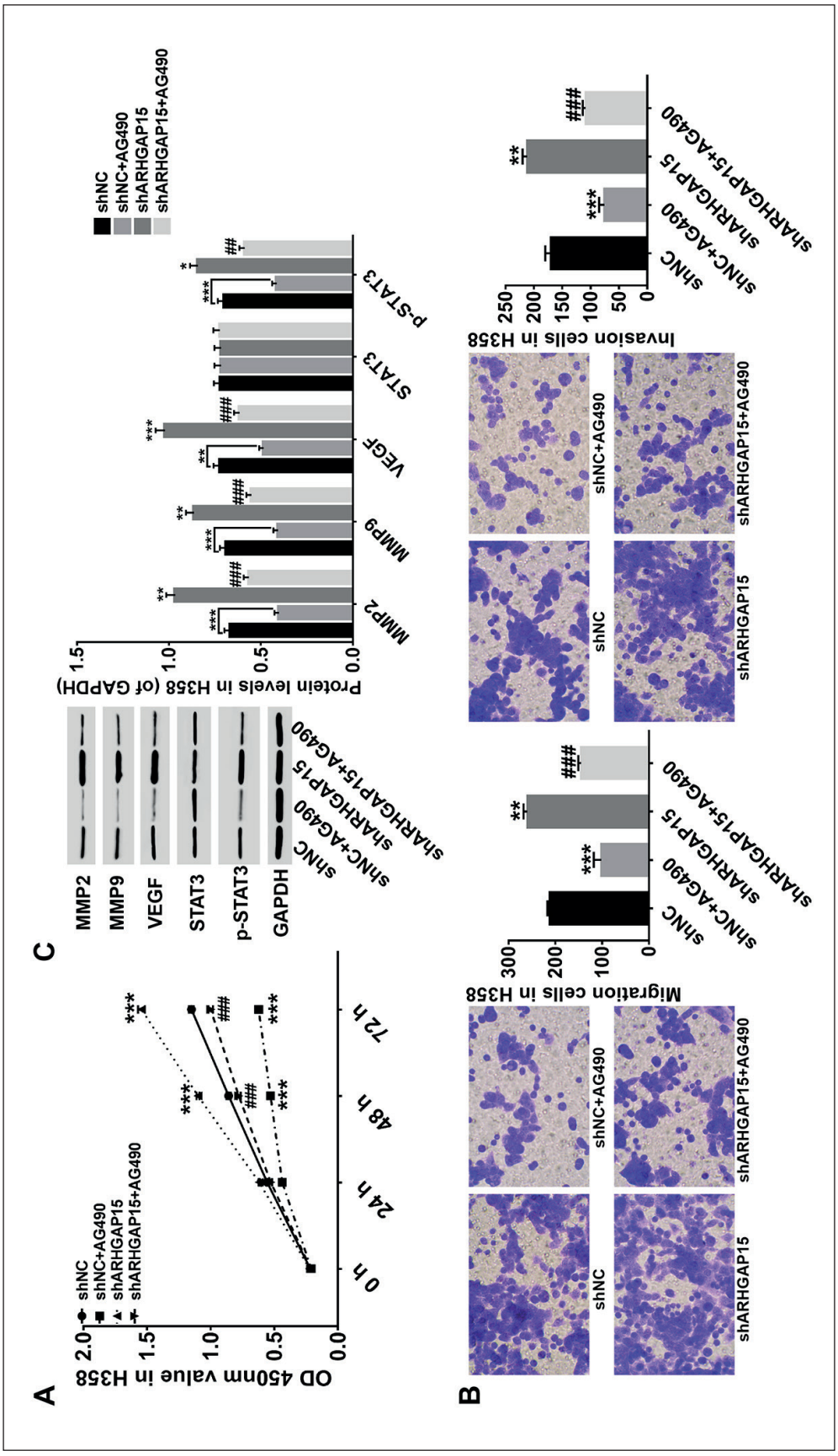


**Figure 5.** Downregulation of ARHGAP15 induced lung cancer cell proliferation and metastasis via STAT3 pathway activation. H358 cells were infected with shNC/shARHGAP15 and treated with AG490 (STAT3 inhibitor, 100 μM). ***A***, H358 cell proliferation was evaluated at 0, 24, 48 and 72 h using CCK-8 assays. ***B***, Migration and invasion ability of H358 cells were assessed by transwell. ***C***, MMP2, MMP9, VEGF, and p-STAT3 expression in H358 cells was detected by Western blot. The results are presented as the mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with shNC, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with shARHGAP15.

migration, and invasion were potently suppressed by the STAT3 inhibitor AG490, whereas MMP2, MMP9, and VEGF protein levels were reduced. These findings further indicated that ARHGAP15 inhibited lung cancer cell proliferation and metastasis potentially through the inhibition of MMP2, MMP9, and VEGF expression via the STAT3 pathway inactivation.

## Conclusions

We demonstrated that ARHGAP15 upregulation significantly suppressed lung cancer cell proliferation and metastasis likely by inhibiting MMP2, MMP9, and VEGF expression via STAT3 pathway inactivation. ARHGAP15 targeting may prove to be a potential approach for the lung cancer treatment.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

## References


1) Alberg AJ, Ford JG, Samet JM; American College of Chest Physicians. Epidemiology of lung cancer: accp evidence-based clinical practice guidelines (2nd Edition). Chest 2007; 132 (3 Suppl): 29S-55S.
2) Fucito LM, Czabafy S, Hendricks PS, Kotsen C, Richardson D, Toll BA; Association for the Treatment of Tobacco Use and Dependence/Society for Research on Nicotine and Tobacco Synergy Committee. Pairing smoking-cessation services with lung cancer screening: a clinical guideline from the Association for the Treatment of Tobacco Use and Dependence and the Society for Research on Nicotine and Tobacco. Cancer 2016; 122: 1150-1159.
3) Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H. SEER Cancer Statistics Review, 1975–2009 (Vintage 2009 Populations). National Cancer Institute. Lung Cancer 2012;
4) Schwartz AG, Prysak GM, Bock CH, Cote ML. The molecular epidemiology of lung cancer. Carcinogenesis 2007; 28: 507-518.
5) Spiro SG, Silvestri GA. One hundred years of lung cancer. Am J Respir Crit Care Med 2005; 172: 523-529.
6) Youlden DR, Cramb SM, Baade PD. The international epidemiology of lung cancer: geographical distribution and secular trends. J Thorac Oncol 2008; 3: 819-831.
7) Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002; 2: 161-174.
8) Itoh T, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H, Itohara S. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer Res 1998; 58: 1048-1051.
9) Itoh T, Tanioka M, Matsuda H, Nishimoto H, Yoshioka T, Suzuki R, Uehira M. Experimental metastasis is suppressed in MMP-9-deficient mice. Clin Exp Metastasis 1999; 17: 177-181.
10) Ustuner Z, Saip P, Yasasever V, Vural B, Yazar A, Bal C, Ozturk B, Ozbek U, Topuz E. Prognostic and predictive value of vascular endothelial growth factor and its soluble receptors, VEGFR-1 and VEGFR-2 levels in the sera of small cell lung cancer patients. Med Oncol 2008; 25: 394-399.
11) Lucchi M, Mussi A, Fontanini G, Faviana P, Ribechini A, Angeletti CA. Small cell lung carcinoma (SCLC): the angiogenic phenomenon. Eur J Cardiothorac Surg 2002; 21: 1105-1110.
12) Brattström D, Bergqvist M, Hesselius P, Larsson A, Lamberg K, Wernlund J, Brodin O, Wagenius G. Elevated preoperative serum levels of angiogenic cytokines correlate to larger primary tumours and poorer survival in non-small cell lung cancer patients. Lung Cancer 2002; 37: 57-63.
13) Yu H, Jove R. The STATs of cancer — new molecular targets come of age. Nat Rev Cancer 2004; 4: 97-105.
14) Leong H, Mathur PS, Greene GL. Green tea catechins inhibit angiogenesis through suppression of STAT3 activation. Breast Cancer Res Treat 2009; 117: 505-515.
15) Yeh HH, Lai WW, Chen HH, Liu HS, Su WC. Autocrine IL-6-induced Stat3 activation contributes to the pathogenesis of lung adenocarcinoma and malignant pleural effusion. Oncogene 2006; 25: 4300-4309.
16) Heinrich PC, Behrmann I, Serge H, Hermanns HM, Gerhard M-N, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 2003; 374: 1-20.
17) Schafer ZT, Brugge JS. IL-6 involvement in epithelial cancers. J Clin Invest 2007; 117: 3660-3663.
18) Deans C, Wigmore SJ. Systemic inflammation, cachexia and prognosis in patients with cancer. Curr Opin Clin Nutr Metab Care 2005; 8: 265-269.
19) Katsumata N, Eguchi K, Fukuda M, Yamamoto N, Ohe Y, Oshita F, Tamura T, Shinkai T, Saijo N. Serum levels of cytokines in patients with untreated primary lung cancer. Clin Cancer Res 1996; 2: 553-559.
20) Martín F, Santolaria F, Batista N, Milena A, González-Reimers E, Brito MJ, Oramas J. Cytokine levels (IL-6 and IFN-γ), acute phase response and nutritional status as prognostic factors in lung cancer. Cytokine 1999; 11: 80-86.
21) Takagi K, Miki Y, Onodera Y, Ishida T, Watanabe M, Sasano H, Suzuki T. ARHGAP15 in human breast carcinoma: a potent tumor suppressor regulated by androgens. Int J Mol Sci 2018; 19. pii: E804.

22) Sun Z, Zhang B, Wang C, Fu T, Li L, Wu Q, Cai Y, Wang J. Forkhead box P3 regulatesARHGAP15 expression and affects migration of glioma cells through the Rac1 signaling pathway. Cancer Sci 2017; 108: 61-72.

23) Xu X, Cao L, Zhang Y, Yin Y, Hu X, Cui Y. Network analysis of DEGs and verification experiments reveal the notable roles of PTTG1 and MMP9 in lung cancer. Oncol Lett 2018; 15: 257-263.

24) Hong J, Kang B, Kim A, Hwang S, Ahn J, Lee S, Kim J, Park JH, Cheon DS. Development of a highly sensitive real-time one step RT-PCR combined complementary locked primer technology and conjugated minor groove binder probe. Virol J 2011; 8: 330.

25) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2–ΔΔCT method. Methods 2001; 25: 402-408.

26) Kobayashi T, Hino SI, Oue N, Asahara T, Zollo M, Yasui W, Kikuchi A. Glycogen synthase kinase 3 and h-prune regulate cell migration by modulating focal adhesions. Mol Cell Biol 2006; 26: 898-911.

27) Wójcik E, Jakubowicz J, Skotnicki P, SaskorczyÐska B, Kulpa JK. IL-6 and VEGF in small cell lung cancer patients. Anticancer Res 2010; 30: 1773-1778.

28) Zhang ZH, Hu BD, Yu M, Zhang YB. Expressions of leptin, STAT3, p-STAT3 and bcl-2 in lung cancer and their clinical significance. Tumor 2010; 30: 529-534.

29) Huang C, Yang G, Jiang T, Huang K, Cao J, Qiu Z. Effects of IL-6 and AG490 on regulation of Stat3 signaling pathway and invasion of human pancreatic cancer cells in vitro. J Exp Clin Cancer Res 2010; 29: 51.

30) Nana-Sinkam SP, Powell CA. Molecular biology of lung cancer: diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. Chest 2013; 143: E30S-E39S.

31) Teng JP, Yang ZY, Zhu YM, Ni D, Zhu ZJ, Li XQ. The roles of ARHGAP10 in the proliferation, migration and invasion of lung cancer cells. Oncol Lett 2017; 14: 4613-4618.

32) Wang J, Tian X, Han R, Zhang X, Wang X, Shen H, Xue L, Liu Y, Yan X, Shen J, Mannoor K, Deepak J, Donahue JM, Stass SA, Xing L, Jiang F. Downregulation of miR-486-5p contributes to tumor progression and metastasis by targeting protumorigenic ARHGAP5 in lung cancer. Oncogene 2014; 33: 1181-1189.

33) Ofek P, Ben-Meir D, Kariv-Inbal Z, Oren M, Lavi S. Cell cycle regulation and p53 activation by protein phosphatase 2C alpha. J Biol Chem 2003; 278: 14299-14305.

34) Dai F, Duan X, Liang YY, Lin X, Feng XH. Coupling of dephosphorylation and nuclear export of Smads in TGF-beta signaling. Methods Mol Biol 2010; 647: 125-137.

35) Matsuyama W, Hashiguchi T, Mizoguchi A, Iwami F, Kawabata M, Arimura K, Osame M. Serum levels of vascular endothelial growth factor dependent on the stage progression of lung cancer. Chest 2000; 118: 948-951.

36) Song L, Rawal B, Nemeth JA, Haura EB. JAK1 activates STAT3 activity in non-small-cell lung cancer cells and IL-6 neutralizing antibodies can suppress JAK1-STAT3 signaling. Mol Cancer Ther 2011; 10: 481-494.