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Actin Filament-Associated Protein 1-Like 1 Mediates Proliferation and Survival in Non-Small Cell Lung Cancer Cells

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The actin filament-associated protein (AFAP) family consists of 3 novel adaptor proteins: AFAP1, AFAP1L1, and AFAP1L2/XB130. Although evidence shows that AFAP1 and AFAP1L2 play an oncogenic role, the effect of AFAP1L1 on tumor cell behavior has not been fully elucidated, and it remains unknown whether AFAP1L1 could be a prognostic marker and/or therapeutic target of lung cancer.

Material/Methods: Human A549 non-small cell lung cancer (NSCLC) cells were used in this study. AFAP1L1 gene was knocked down by AFAP1L1 short hairpin RNA (shRNA) transfection. Cell proliferation was analyzed using Celigo image cytometry and MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay, cell cycle progression was assessed with flow cytometry, and cell apoptosis was determined by flow cytometry after annexin-v staining. The PathScan intracellular signaling array was used to investigate cancer-related signaling proteins influenced by knocking down AFAP1L1 in A549.

Results: AFAP1L1 gene expression was successfully inhibited by the AFAP1L1-shRNA transfection. Cell proliferation was inhibited and cell proportions in G1 and G2/M phases were increased, and cell apoptosis was increased in the AFAP1L1-shRNA transfected cells as compared with negative control shRNA transfected cells. Using the PathScan intracellular signaling array, we found that downregulation of AFAP1L1 significantly activated P38 and caspase 3, and inhibited PRAS40 activation.

Conclusions: Our data show that AFAP1L1 promotes cell proliferation, accelerates cell cycle progression, and prevents cell apoptosis in lung cancer cells. Therefore, AFAP1L1 might play an oncogenic role in NSCLC.

MeSH Keywords: Apoptosis Inducing Factor • Carcinoma, Non-Small-Cell Lung • Cell Proliferation • p38 Mitogen-Activated Protein Kinases

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Background

Lung cancer continues to be the leading cause of cancer death in many countries, and over 75% of lung cancers are non-small cell lung cancer. Due to the aging of the global population, it is predicted that lung cancer will be the sixth most common cause of death by 2030 [1]. Despite the improvements in survival for many other types of cancer in recent years, there have been only slight improvements in the early diagnosis and survival of lung cancer, mainly due to limited advances in screening and treatment [1–3]. To address these issues, further studies that focus on the underlying molecular mechanisms of lung cancer progression, such as cell survival, proliferation, and cell death, are necessary.

The actin filament-associated protein (AFAP) family consists of 3 new adaptor proteins – actin filament-associated protein 1 (AFAP1), actin filament-associated protein 1-like 1 (AFAP1L1), and actin filament associated protein 1-like 2 (AFAP1L2)/XB130 – which have been demonstrated to participate in the regulation of various signaling pathways involved in tumorigenesis by forming protein-protein and/or protein-lipid complexes, and might have the potential to be used as prognosis markers or therapeutic targets [4–7]. AFAP1, the most intensively investigated member of the family, has been described in multiple cell types, including fibroblasts, epithelial, and endothelial cells [8,9], and was reported to be involved in the tumor cell growth, cell adhesion, and invasion in cells of several cancers [10–12]. AFAP1L2, also termed XB130, has been demonstrated to mediate tumor cell survival, proliferation, and migration under certain circumstances [13–15]. In contrast to AFAP1 and AFAP1L2, little is known about AFAP1L1, especially its role in lung cancer. Therefore, in the present study, we selected lung cancer cell line A549, and by knocking down AFAP1L1 *in vitro*, aimed to investigate the role of AFAP1L1 in the proliferation and survival of NSCLC. We also used the high-throughput PathScan® Antibody Array in a preliminary study to determine which proteins were most influenced by AFAP1L1 downregulation.

Material and Methods

Cell lines and agents

Human lung cancer cell lines A549 (A549-CRM*CCL-185), H1299 (CRL-5803™), and H1688 (CCL-257™) and human lung normal cells, human fetal lung fibroblast cell line MRC-5 (CCL-171™), and human bronchial epithelial cell line BEAS-2B (CRL-9609™) were purchased from the American Type Culture Collection (ATCC, USA). The human high-metastasis lung cancer cell line 95-D was purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% glutamine, in 5% CO_2 at 37°C. All the cell lines were used to evaluate which one had relatively higher mRNA level of AFAP1L1.

Real-time polymerase chain reaction

Total RNA was extracted with Trizol (Pufei, Shanghai, China), and cDNA was synthesized from total RNA using the M-MLV Reverse Transcriptase kit (Promega). Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using SYBR Master Mixture (Takara, Japan) on a MX3000P real-time PCR system (Stratagene, CA). Primers for AFAP1L1 (Gene Bank accession, NM_152406) are: 5'-CGTGGATGTGGAGACCTTAAC-3', 5'-CTCGGTTCTTGCCATACTTGT-3'; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are: 5'-TGACTT CAACAGCGACACCCA-3', 5'-CACCCTGTTGCTGTAG CCAAA-3'. All assays were performed in triplicate. AFAP1L1 gene expression levels were normalized to the level of GAPDH and calculated with the $2^{-\Delta\Delta Ct}$ method.

Lentivirus vectors for AFAP1L1 small interfering RNA and cell transfection

Lentiviral pGCSIL-GFP vectors for human AFAP1L1 short hairpin RNA (shRNA) or negative control were constructed by GeneChem Biotech, Shanghai, China. The recombinant virus was packaged in A549 cells using a Lentivector Expression system (GeneChem Biotech). The target sequences for AFAP1L1 and negative control gene were: 5'-TGCCAATCAATACAAGTAT-3' and 5'-TTCTCCGAACGTGTCACGT-3' respectively. For cellular infection, A549 cells at a density of 2×10^5 cells/well in 6-well culture plates and infected with lentivirus-mediated AFAP1L1-shRNA or NC-shRNA with a multiplicity of infection (MOI) of 10 in the presence of polybrene (5 μg/mL). The GFP expression level was detected by fluorescence microscopy (Olympus, Japan) to determine the infection efficiency and harvested at 72 h after infection. AFAP1L1 mRNA expression was determined by real-time PCR, and protein expression of AFAP1L1 was measured by Western blotting. Briefly, cell lysate was prepared by RIPA buffer and bicinchoninic acid assay (BCA) was used to determine protein concentration. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%, 25 μg/hole), then transferred to a PVDF membrane (Millipore Life Science, Boston, MA, USA). The membrane was cultured overnight at 4°C in skimmed milk. The 1: 300 diluted Rabbit anti-human AFAP1L1 monoclonal antibody (Abcam, CA, USA) or 1: 2000 dilution mouse anti-human β-actin monoclonal antibody (Santa Cruz, CA, USA) was added, cultured for 1 h at room temperature, then fluorescent dye labeled 1: 5000 secondary antibodies were added and cultured for 2 h at room temperature. The Odyssey® imaging system (LI-COR Biosciences, Lincoln, NE, USA) was used

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for semi-quantitative analysis (Quantity One image analysis system (BioRad Laboratories, Inc., Hercules, CA, USA).

Cell count assay

A549 cells were seeded into 96-well plates (1500 cells/well) and incubated at 37°C in a 5% CO_2 environment for 3 days. Cell count assay was evaluated using the Celigo image cytometer (Nexcelom Bioscience, USA). The captured cell images were analyzed using Celigo software (Nexcelom Bioscience).

MTT assay

A549 cells were seeded into 96-well plates (2000 cells/well), and incubated at 37°C in a 5% CO_2 environment for 1, 2, 3, 4, and 5 days. At each time point, after addition of 5 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 20 µL/well), cells were incubated for another 4 h and the supernatant was removed. Then, 100 µL DMSO was added to each well and cells were incubated for another 5 min with constant shaking. The absorbance (A) at 490 nm was measured using a spectrophotometric plate reader (TECAN infinite M200, TECAN, Switzerland) and cell growth curves were plotted.

Cell cycle analysis

Cell cycle distribution was analyzed with flow cytometry. The logarithmically growing A549 cells were trypsinized and re-suspended in DMEM with 10% FBS, then rinsed with pre-cold D-Hanks. After centrifugation, the supernatant was removed and cells were fixed in 70% cold ethanol for 1 h. Fixed cells were rinsed with D-Hanks solution and permeabilized with 0.1% Triton X-100 and 2 mg/ml RNase A in D-Hanks solution for 30 min. The cells were then rinsed with D-Hanks solution and stained with 50 mg/ml propidium iodide (PI) (Sigma-Aldrich, USA). Stained cells were analyzed with the Guava easyCyte HT flow cytometry system (Millipore, USA).

Cell apoptosis analysis

At the fifth day after lentivirus transfection, A549 cells were stained with 200 µl cell suspension containing 10 µl Annexin V-APC (Cat. 88-8007, eBioscience, USA) at room temperature in the dark for 10–15 min, then flow cytometry analysis was performed on the Guava easyCyte HT flow cytometry system (Millipore).

Path Scan intracellular signaling array

To detect the activation of intracellular signaling, the PathScan intracellular signaling array was used. Briefly, 5 days after lentivirus infection, A549 cells were collected and lysed. Intracellular signaling was detected using a PathScan intracellular signaling array kit (Cell Signaling Technology) following the manufacturer's instructions.

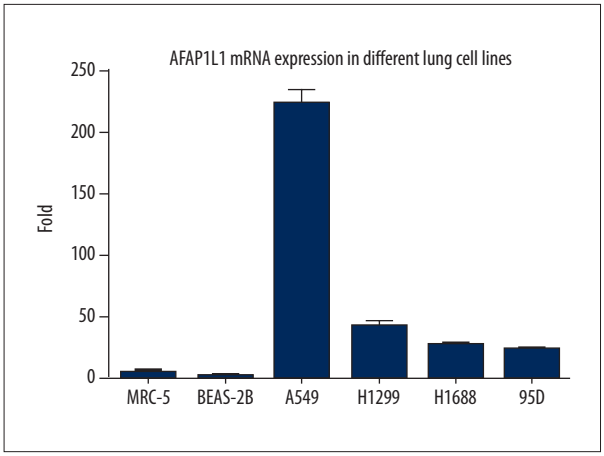

Figure 1. AFAP1L1 mRNA expression in different lung cancer cell lines and lung normal cell lines. ΔCt=Ct (AFAP1L1)–Ct (GAPDH). The fold number was calculated by $2^{\Delta Ct}$.

Statistical analysis

Statistical analysis was performed with SPSS version 16.0 (SPSS Inc., USA). Qualitative data are expressed as mean ± standard deviation (SD) and comparisons between groups were made using *t* tests. A value of $P<0.05$ was considered statistically significant.

Results

AFAP1L1 gene expression in lung cancer cell lines

As shown in Figure 1, real-time PCR results showed that AFAP1L1 mRNA levels in the human lung cancer cell lines were significantly higher than in human normal cell line BEAS-2B and MRC-5. The A549 cell line had the relatively highest mRNA expression among 4 human lung cancer cells, so we selected A549 cells to perform the following *in vitro* study.

Knockdown of AFAP1L1 expression using AFAP1L1 shRNA

To investigate the role of AFAP1L1 in lung cancer cell line A549, gene knockdown experiments using AFAP1L1 shRNA were performed. Results showed that AFAP1L1 shRNA successfully knocked down AFAP1L1 expression at the mRNA and protein levels in A549 cells. Real-time PCR results showed that AFAP1L1 shRNA vector inhibited AFAP1L1 mRNA expression compared to control vectors, and Western blot analysis results also showed that AFAP1L1 protein level was significantly reduced in AFAP1L1 shRNA-infected cells than in the control-transfected A549 cells (all $P<0.01$, Figures 2A, 2B).

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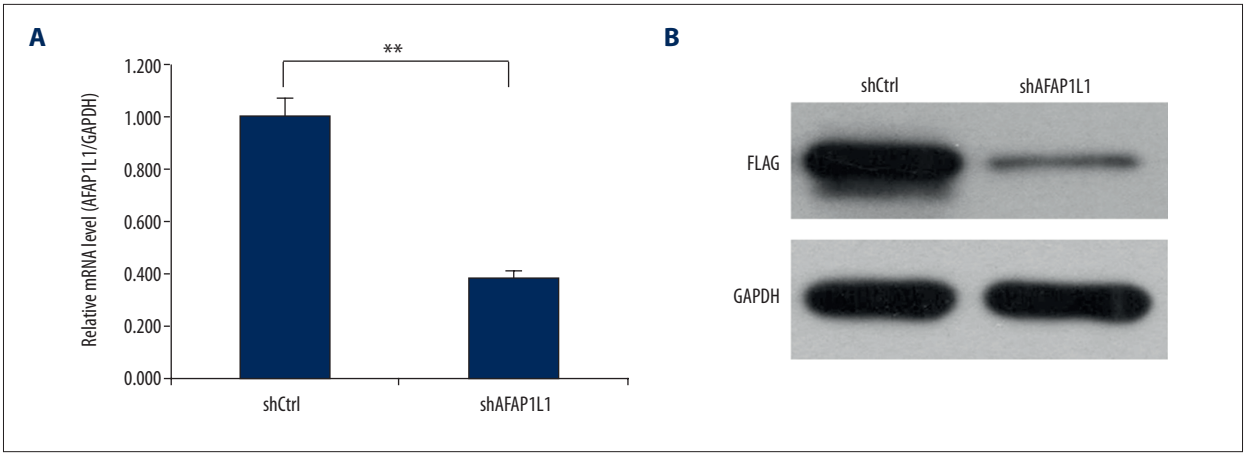

Figure 2. Knockdown of AFAP1L1 expression using AFAP1L1 shRNA. (**A**) AFAP1L1 mRNA expression in A549 cells transfected with AFAP1L1 shRNA or control shRNA. (**B**) AFAP1L1 protein expression in A549 cells transfected with AFAP1L1 shRNA or control shRNA. * P<0.01 *vs.* shCtrl. sh AFAP1L1 – cells transfected with AFAP1L1- shRNA; shCtrl – cells transfected with control shRNA.

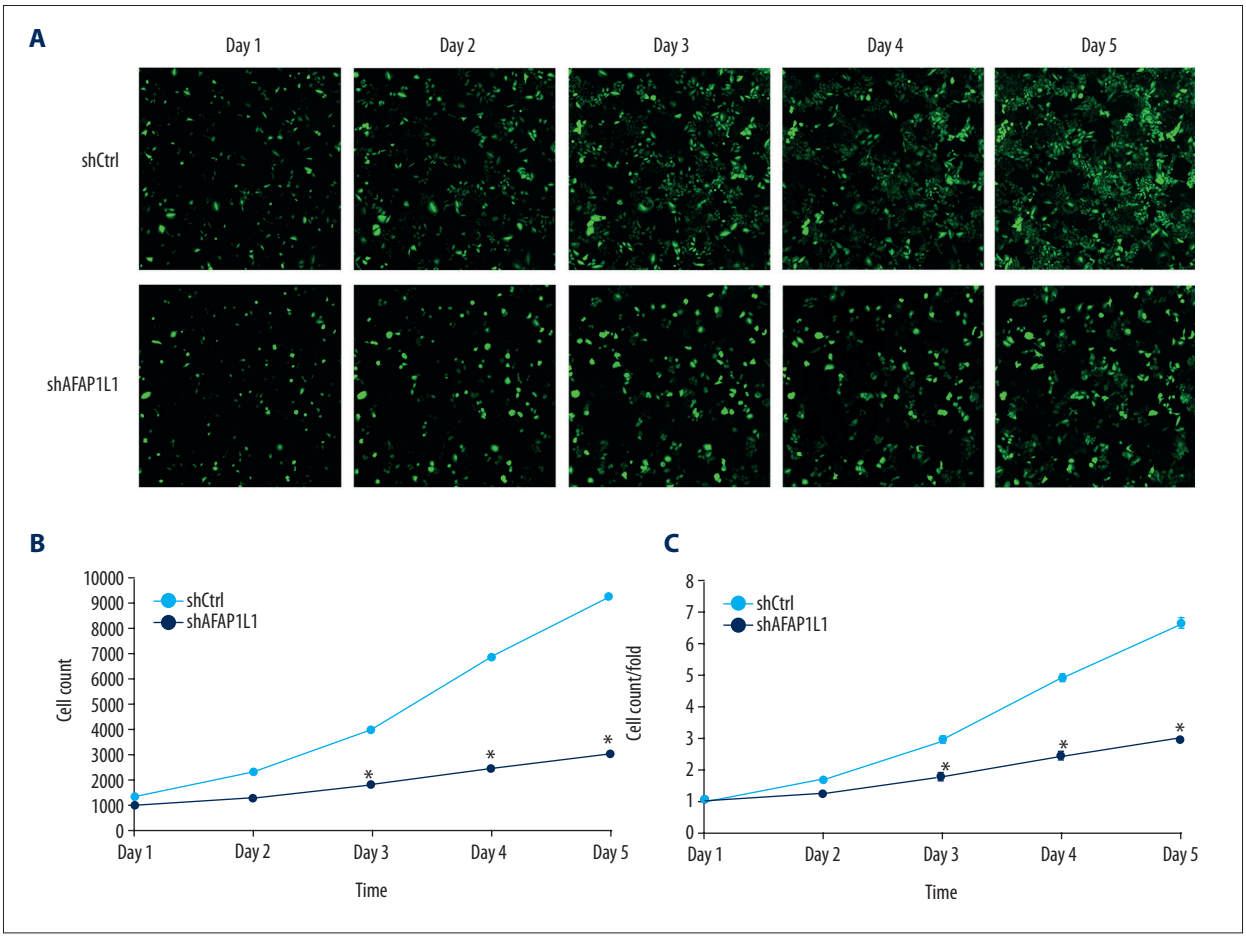

Figure 3. Effects of AFAP1L1 knockdown on A549 cell proliferation. (**A, B**) Representative images and corresponding line chart of Celigo image cytometry analysis. (**C**) MTT assay results. *P<0.001 *vs.* shCtrl. sh AFAP1L1 – cells transfected with AFAP1L- shRNA; shCtrl – cells transfected with control shRNA.

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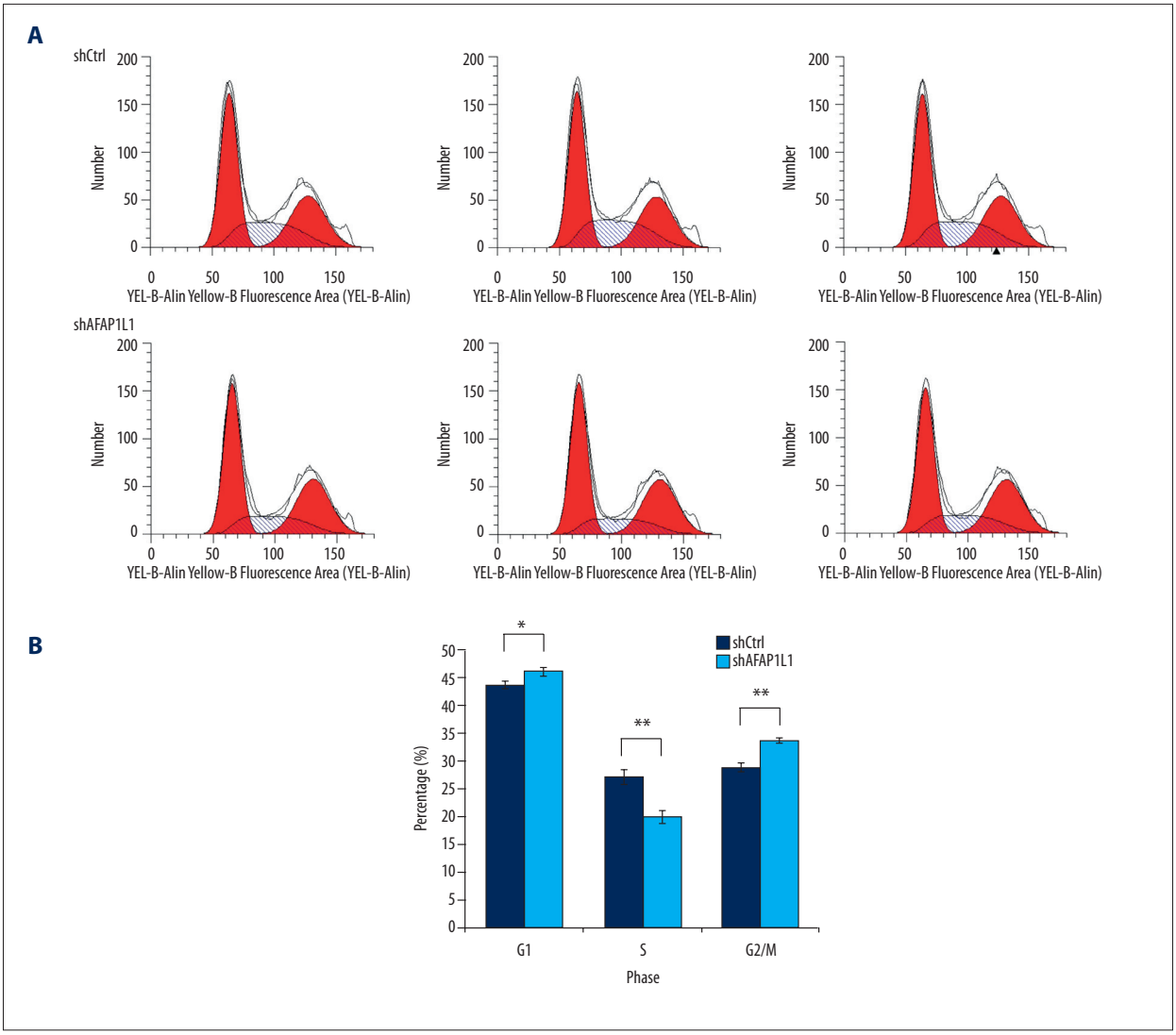

Figure 4. Effects of AFAP1L1 knockdown on A549 cell cycle progression. (**A**) Histograms of cell cycle distribution was analyzed with flow cytometry. (**B**) Bar graph of cell cycle distribution analysis. * P<0.05, ** P<0.01 *vs.* shCtrl. sh AFAP1L1 – cells transfected with AFAP1L- shRNA; shCtrl – cells transfected with control shRNA.

Knockdown of AFAP1L1 leads to a decline in cell proliferation

Celigo image cytometry was used to evaluate cell proliferation. When compared with that in the control group, the cell growth was significantly inhibited in the AFAP1L1 shRNA group. A significant reduction in cell count was observed in AFAP1L1 shRNA group at 3 days after transfection, and the inhibitory effect became more evident at 4 days and 5 days (all P<0.001, Figure3 A, 3B). Furthermore, MTT assay was utilized for verifying the effect of AFAP1L1 shRNA on cell proliferation, and results were the same as in the Celigo analysis (Figure 3C).

Knockdown of AFAP1L1 inhibits cell cycle progression

When compared with the control group, the proportions of cells in G1 and G2/M phases increased significantly, whereas that in S phase reduced markedly in the AFAP1L1 shRNA group (all P<0.05). This result indicates that AFAP1L1 plays an important role in cell cycle modulation (Figure 4).

Knockdown of AFAP1L1 promotes cell apoptosis

To investigate whether the AFAP1L1 expression affected lung cancer cell apoptosis, we evaluated the apoptosis ratio between shAFAP1L1 A549 and control cells by annexin-v staining and flow cytometry assay. Results showed that cell apoptosis was significantly increased in the AFAP1L1-shRNA groups

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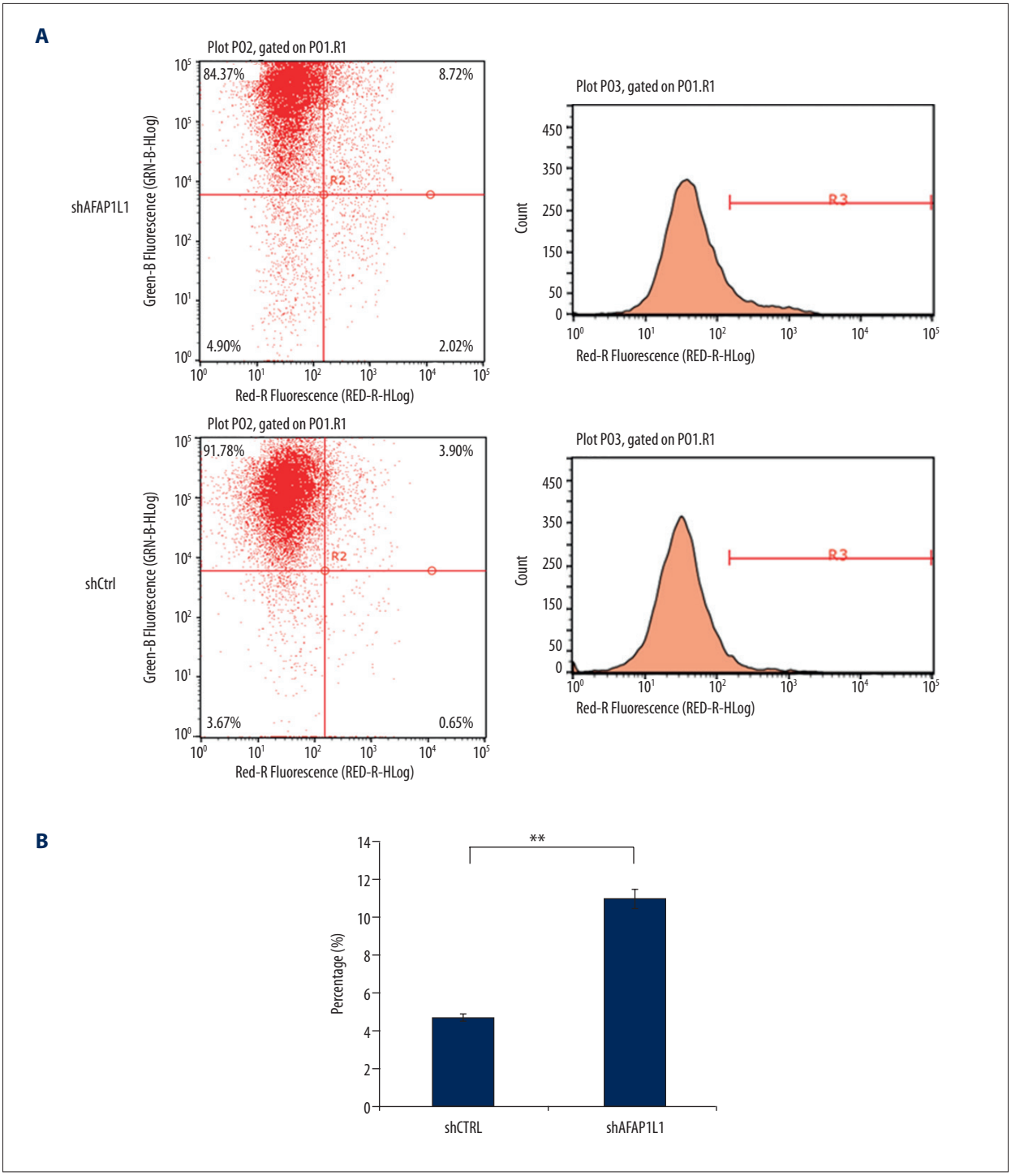

Figure 5. Effects of AFAP1L1 knockdown on A549 cell apoptosis. (**A**) Histograms of cell apoptosis analyzed with flow cytometry after annexin-v staining. (**B**) Bar graph of cell apoptosis analysis. * P<0.01 *vs.* shCtrl. sh AFAP1L1 – cells transfected with AFAP1L-shRNA; shCtrl – cells transfected with control shRNA.

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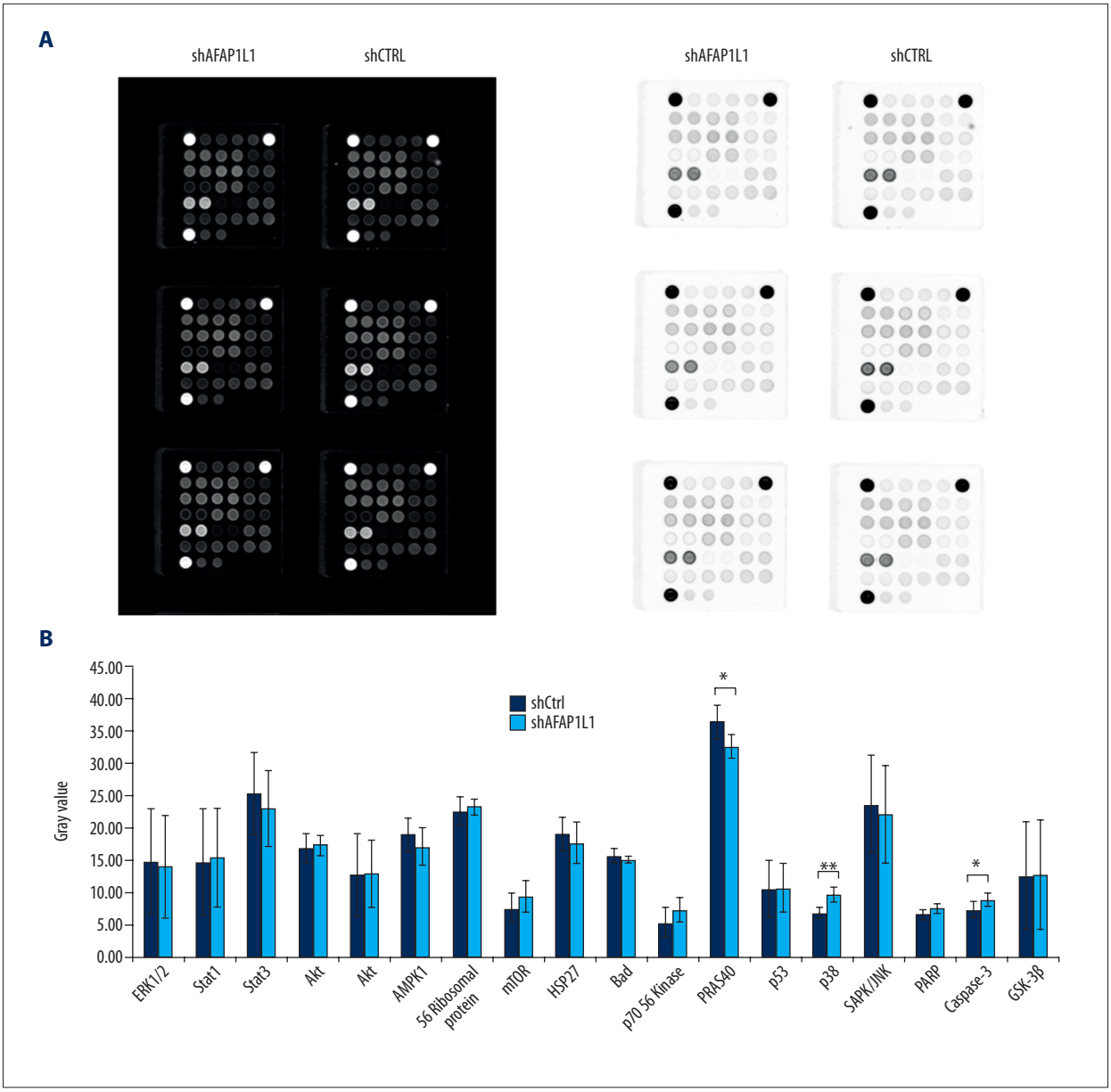

Figure 6. Mechanism study of AFAP1L1 silencing in lung cancer cells. (**A**) Intracellular signaling array after Lv-shAFAP1L1 infection. (**B**) Plot histogram of AFAP1L1-related signaling molecules in lung cancer cells by SignaLink 2.0 analysis.

as compared to the negative control groups, and the apoptosis rate increased by more than 2-fold ($P<0.01$) (Figure 5).

Mechanism study of AFAP1L1 silencing in A549 cells

To investigate the regulatory mechanism of AFAP1L1 in the tumorigenesis of lung cancer, multiple signaling pathways were analyzed in A549 cells after AFAP1L1 knockdown. AFAP1L1-triggered signal transduction was determined using the PathScan intracellular signaling array kit. Knockdown of AFAP1L1 obviously inhibited the activation of PRAS40 and induced the activation of P38 by influencing their phosphorylation status (Figure 6A, 6B, Table 1). Moreover, depletion of AFAP1L1 significantly induced the cleavage of caspase 3, indicating that AFAP1L1 affected apoptosis of lung cancer cells. Further studies are needed to clarify the mechanisms of AFAP1L1 in lung cancer progression.

Discussions

Adaptor proteins are non-enzymatic proteins that have the ability to link different cellular signaling molecules through protein-binding motifs. Based on their similar structures, 3

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Table 1. SignaLink 2.0 analysis of AFAP1L1-related signaling molecules in A549 cells by PathScan intracellular signaling array.

	Target	Phosphorylation site	Modification	Average gray value		STDEV		P value	Up/down
				shCtrl	shAFAP1L1	shCtrl	shAFAP1L1		
1	Positive control	N/A	N/A	\	\	\	\	\	\
2	Negative control	N/A	N/A	\	\	\	\	\	\
3	ERK1/2	Thr202/Try204	Phosphorylation	14.73	14.05	8.10	7.88	0.8852	–4.64%
4	Stat1	Tyr701	Phosphorylation	14.70	15.37	8.21	7.59	0.8868	4.54%
5	Stat3	Tyr705	Phosphorylation	25.20	22.92	6.20	5.82	0.5255	–9.06%
6	Akt	Thr308	Phosphorylation	16.75	17.35	2.20	1.55	0.5971	3.58%
7	Akt	Ser473	Phosphorylation	12.78	12.92	6.44	5.14	0.9692	1.04%
8	AMPKA	Thr172	Phosphorylation	19.03	17.17	2.52	2.99	0.2699	–9.81%
9	S6 Ribosomal protein	Ser235/236	Phosphorylation	22.57	23.33	2.16	1.28	0.4708	3.40%
10	mTOR	Ser2448	Phosphorylation	7.60	9.47	2.29	2.58	0.2147	24.56%
11	HSP27	Ser78	Phosphorylation	18.93	17.68	2.67	3.25	0.4827	–6.60%
12	Bad	Ser112	Phosphorylation	15.72	15.05	1.13	0.43	0.2053	–4.24%
13	p70 S6 Kinase	Thr389	Phosphorylation	5.50	7.37	2.28	1.84	0.1494	33.94%
14	PRAS40	Thr246	Phosphorylation	36.25	32.35	2.59	1.82	0.0129	–10.76%
15	p53	Ser15	Phosphorylation	10.52	10.68	4.34	3.74	0.9446	1.58%
16	p38	Thr180/Tyr182	Phosphorylation	6.90	9.65	0.83	1.06	0.0005	39.86%
17	SAPK/JNK	Thr183/Tyr185	Phosphorylation	23.53	22.03	7.56	7.50	0.7374	–6.37%
18	PARP	Asp214	Cleavage	6.67	7.60	0.66	0.85	0.0589	14.00%
19	Caspase-3	Asp175	Cleavage	7.40	8.93	1.25	0.93	0.0364	20.72%
20	GSK-3β	Ser9	Phosphorylation	12.60	12.77	8.30	8.39	0.9731	1.32%

new adaptor proteins – AFAP1, AFAP1L1, and AFAP1L2 – were defined in the AFAP family. They all contain 2 pleckstrin homology (PH) domains flanking a serine/threonine-rich region, 2 Src homology (SH) 2-binding motifs, and 1 or 2 SH3-binding motifs [16–18]. AFAP1 and AFAP1L2/XB130 associate with Src via their SH3-binding motifs [17,19], whereas recent evidence suggests that AFAP1L1 does not bind strongly to Src and is hypothesized to have unique functions [20]. Studies have shown that AFAP1 and AFAP1L2 play roles in many aspects of tumor cell behavior [10–15]; however, few reports are available concerning the function or involvement of AFAP1L1 in cancer. One of the key characteristics of malignant tumor is the uncontrollable autonomous growth resulting in sustained division and proliferation. Recent reports have revealed that AFAP1L1 can contribute to the tumor growth of spindle cell sarcomas and colorectal cancer and have the potential to serve as prognostic marker and/or therapeutic target [16,21]; nevertheless, the role of AFAP1L1 in the lung cancer has not been investigated.

In the present study, we compared the AFAP1L1 mRNA level in human lung cancer cell lines with human lung normal fibroblasts and bronchial epithelial cells. Our results demonstrated that AFAP1L1 mRNA level in lung cancer cells was clearly higher than in lung normal cells, which suggests that AFAP1L1 plays an oncogenic role in lung cancer. Among these 4 cell lines, A549 has relatively higher AFAP1L1 mRNA level, so A549 was selected for the following knockdown experiment.

We constructed recombinant lentivirus containing shRNA targeting AFAP1L1, and transfected them into human lung cancer A549 cells. Cell proliferation, cell cycle distribution, and apoptosis were investigated after the knocking down of AFAP1L1. Real-time PCR and Western blots results showed the mRNA and protein expression of AFAP1L1 was markedly reduced in the AFAP1L1-shRNA group compared to the negative control group, which demonstrated the knockdown efficacy was high. Celigo analysis and MTT assay revealed that cell count/proliferation was markedly reduced in the AFAP1L1 shRNA

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group compared to the negative control group. Flow cytometry results showed that, as compared to the negative control group, more cells were arrested in G1 and G2/M phases and those in S phase were significantly reduced in the AFAP1L1-shRNA group. In addition, cell apoptosis was significantly increased in the AFAP1L1-shRNA groups as compared to the negative control groups. One report showed that, unlike AFAP1, AFAP1L1 is not predicted to be a strong binding partner for cSrc, whereas it does interact with cortactin [20]; nevertheless, there is limited information regarding the underlying signaling pathways of the biological effects of AFAP1L1. Research has shown that AFAP1L2 regulates cancer cell cycle progression and survival through multiple Akt downstream molecules, such as p27Kip1, p21Cip1/WAF1, FOXO3a, GSK3b, caspase 8, and caspase 9 [13,22,23]. As Akt plays a crucial role in regulating cell proliferation, cell cycle progression, and survival in multiple types of cancer [24], AFAP1L2 might be an upstream regulator of the phosphatidyl-inositol-3-kinase (PI3K)/Akt pathway, and AFAP1L2 also acts as a regulator of several tumor-related microRNAs [25]. We speculate that these signaling molecules/pathways might be also involved in the regulation mechanisms of AFAP1L1 in lung cancer cells; however, this hypothesis needs further investigation.

Intracellular signaling array showed that AFAP1L1 depletion induces the activation of P38 and inhibits the activation of PRAS40. PRAS40 knockdown reduced the ability of tumor necrosis factor (TNF)-α and cycloheximide to induce apoptosis in several cancers, including HeLa cells [26] and colorectal cancer [27]. P38 activation has been reported to be capable suppressing the cell cycle in prostate cancer [28] and hepatocellular carcinoma [29], and in the current study, similar cell cycle blocking was also observed by down-regulating AFAP1L1. We hypothesized that inhibition of P38 might be one of the molecular mechanisms involved. More importantly, cleavage of caspase 3, one of most important apoptosis triggers, was significantly inhibited by silencing the AFAP1L1, which has been demonstrated by PathScan® Intracellular Signaling Array in the present study. Therefore, knockdown of AFAP1L1 induced apoptosis, potentially due to the inhibition of PRAS40 and induction of caspase 3cleavage.

Conclusions

Results of the present study conclusively demonstrate that knocking down of AFAP1L1 leads to significantly inhibited cell cycle progression, increased apoptotic cells, and attenuated cell growth in lung cancer cells. All these findings indicate that AFAP1L1 is as an oncogenic factor in lung cancer, and therefore might be serve as a prognostic marker and/or therapeutic target.

Conflict of interest

None.

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