Silencing of AP-4 inhibits proliferation, induces cell cycle arrest and promotes apoptosis in human lung cancer cells

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Abstract. Activating enhancer-binding protein (AP)-4 is a member of the basic helix-loop-helix transcription factors, and is involved in tumor biology. However, the role of AP-4 in human lung cancer remains to be fully elucidated. In the present study, the expression of AP-4 in human lung cancer tissues and cells was investigated by reverse transcription-quantitative polymerase chain reaction, and it was observed that the level of AP-4 was increased in tumor tissues and cells compared with their normal counterparts. AP-4 expression was knocked down by transfection with a specific small interfering RNA (siRNA) in lung cancer cells, and this indicated that siRNA-mediated silencing of AP-4 inhibited cell proliferation, arrested the cell cycle at the G0/G1 phase and induced apoptosis by modulating the expression of p21 and cyclin D1. The results of the present study suggest that AP-4 may be an oncoprotein that has a significant role in lung cancer, and that siRNA-mediated silencing of AP-4 may have therapeutic potential as a strategy for the treatment of lung cancer.

Introduction

Human lung cancer is one of the most commonly observed aggressive malignancies, with symptoms including coughing, weight loss, shortness of breath and chest pains (1). In 2012, 1.8 million cases of lung cancer occurred worldwide and the disease was responsible for ~1,590,000 mortalities (2), making lung cancer one of the most commonly diagnosed at an advanced stage (2). Despite great improvement in traditional treatments, including surgery, supplemented with radiotherapy and chemotherapy, the prognosis of these patients remains poor, with a low rate of 5-year survival (10-15%) (3). Therefore, the identification of potential molecular markers of lung cancer is required for the prediction of survival and the development of novel therapeutic targets.

Basic helix-loop-helix (bHLH) transcription factors are a family of transcriptional regulators that have significant roles in cell proliferation and differentiation, expression of intracellular genetic information and cell lineage determination, as well as other essential processes (4-7). Activating enhancer-binding protein (AP)-4 is a member of the bHLH leucine-zipper subgroup of bHLH proteins (8) and is involved in tumor biology (9-11). AP-4 is upregulated in gastric and hepatocellular carcinoma, and predicts poor prognosis (9,10). Activation of AP-4 induces epithelial-mesenchymal transition and enhances migration and invasion of colorectal cancer cells (11). AP-4 small interfering RNA (siRNA)-mediated gene silencing has an anticancer role in gastric carcinoma (12). However, the role of AP-4 in human lung cancer remains to be elucidated. Increased AP-4 in non-small cell lung cancer tissues compared with their normal counterparts (13) indicates that AP-4 may be a potential therapeutic target for the treatment of human lung cancer.

RNA interference is a process of sequence-specific post-transcriptional gene silencing mediated by double-stranded RNA, which is able to silence specific genes and provide a powerful approach for the analysis of gene functions in vitro and in vivo (14). At present, the most commonly utilized nucleic-acid-based sequence-specific gene silencing molecules are siRNAs, which consist of symmetrical duplexes of 19-21 base pairs (15). The siRNA method is able to inhibit target gene expression with specificity, potency and endurance, and has broad therapeutic potential for the treatment of various human diseases, including infections and cancer (16).

In the present study, to investigate the role of AP-4 in human lung cancer, the expression of AP-4 was measured in human lung cancer tissues and cells, and the function of AP-4 was investigated in human lung cancer cells by transfection with siRNAs. It was observed that the level of AP-4...
was increased in tumor tissues and cells compared with their normal counterparts, and the silencing of AP-4 mediated by siRNA inhibited cell proliferation, induced cell cycle arrest and promoted apoptosis by modulating the expression of p21 and cyclin D1 in lung cancer cells. The results of the present study imply that AP-4 may be a potential therapeutic target for the treatment of human lung cancer.

Materials and methods

Patients and tissue samples. Human lung cancer tissue samples and matched adjacent noncancerous lung tissue samples were obtained from 52 patients who underwent resection at the First Affiliated Hospital and Cancer Hospital Affiliated to Zhengzhou University (Zhengzhou, China) between March 2012 and September 2013. All patients had received a definite diagnosis by a clinical pathologist prior to surgery. No patients had received chemotherapy or radiotherapy prior to surgery. Each patient gave signed informed consent for the use of their tissues for research purposes. The study protocol was approved by the Institutional Ethics Committee of Zhengzhou University. The clinicopathological characteristics of the 52 patients are summarized in Table I. All lung cancer tissue specimens and adjacent noncancerous lung tissues were immediately frozen in liquid nitrogen and stored at -80°C until required.

Cell culture and transfection. The lung cancer cell lines, A549 and H1299 (American Type Culture Collection, Manassas, VA, USA), were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) medium supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), 100 μ/ml streptomycin and penicillin (Ameresco, Inc., Framingham, MA, USA) at 37°C in a humidified incubator with an atmosphere containing 5% CO₂. The human bronchial epithelial (HBE) cell line was obtained from Xiangfu Bio (Shanghai, China), and originated from the American Type Culture Collection. HBE cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS; Gibco; Thermo Fisher Scientific, Inc.), 2 μM glutamine (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Ameresco, Inc.) at 37°C in a humidified incubator with an atmosphere containing 5% CO₂. The human bronchial epithelial (HBE) cell line was obtained from Xiangfu Bio (Shanghai, China), and originated from the American Type Culture Collection. HBE cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS; Gibco; Thermo Fisher Scientific, Inc.), 2 μM glutamine (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Ameresco, Inc.) at 37°C in a humidified incubator with an atmosphere containing 5% CO₂. The siRNA sequences used for silencing of human AP-4 were designed by GenScript siRNA Target Finder online software 2015 (GenScript, Piscataway, NJ, USA) and synthesized by GenScript. The nucleotide sequences were as follows: Forward, 5' -CGGGAUUCAGUCCUCUATATT-3' and reverse, 5' -UGAGGUGACUGAAUCCCGCG-3'. The negative control siRNA (scrambled siRNAs) was supplied by GenScript and the sequences were as follows: Forward, 5' -UGAGGUGACUGAAUCCCGCG-3'. The siRNAs were transfected into the cultured cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from lung cancer tissues and matched adjacent noncancerous lung tissues using RNAiso Reagent (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. RNA (500 ng) was reverse-transcribed to complementary DNA using a PrimeScript RT Reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. The RT reaction mixture was prepared on ice, and contained 2 μl 5X PrimeScript Buffer, 0.5 μl 1X PrimeScript RT Enzyme Mix I, 0.5 μl Oligo dT Primer (50 μM) and 0.5 μl Random 6 mers (100 μM; Takara Bio, Inc.). After dispensing aliquots of the mix into microtubes (Corning Incorporated, Corning, NY, USA), 500 ng RNA sample and RNase Free dH₂O was added to total 10 μl. The reaction mixture was subsequently incubated at 37°C for 5 min, followed by 85°C for 5 sec and 4°C for a minimum of 10 min. No reverse transcriptase was used as a negative control. Expression of AP-4 messenger RNA (mRNA) was evaluated by RT-qPCR on a 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with Premix Ex Taq (Probe qPCR) Master Mix and a ROX reference Dyel (50x) (Takara Bio, Inc.). The level of expression was calculated based on the PCR cycle number, and the relative gene expression level was determined using the 2-ΔΔCq method as previously described (17). The ratios of AP-4, p21 and cyclin D1 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalize the relative levels of AP-4 expression. The primers and probes used were as follows: AP-4 forward primer, 5'-GCGGATCCTATGAGATT-3', AP-4 reverse primer, 5'-TTAGTGGAATGTGGCAGAGG-3' and AP-4 probe, 5'-6-carboxyfluorescein (FAM)-TGCCCACTCAGAGGTGCCC-tetramethylrhodamine (TAMRA) 3'; GAPDH forward primer, 5'-CATCAATGGAATCCCATCA-3', GAPDH reverse primer, 5'-TTCTCAATGTTGTGGAAGAC-3' and GAPDH probe, 5'-FAM-TACTCAAGCGCCA GCATCGCC-TAMRA 3'.

Western blot analysis. Cells were lysed with a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology, Haimen, China). Protein (100 μg) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked for 1 h in Tris buffered saline with Tween-20 (TBST; Beyotime Institute of Biotechnology) containing 5% non-fat dried milk (Nestlé, Beijing, China) at room temperature, and subsequently incubated with polyclonal rabbit primary antibodies against AP-4 (dilution, 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), p21 (dilution, 1:1,000; Santa Cruz Biotechnology, Inc.), cyclin D1 (dilution, 1:1,000; Santa Cruz Biotechnology, Inc.) and GAPDH (dilution, 1:1,000; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Following washing three times with TBST, the membranes were incubated with horseradish-peroxidase-conjugated goat secondary antibody (dilution, 1:3,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Protein bands were detected using enhanced chemiluminescence reagents (GE Healthcare Life Science, Chalfont, UK), and quantified using Adobe Photoshop CS4 software (Adobe Systems, Inc., San Jose, CA, USA).

Cell proliferation assay. Cell proliferation following transfection with siRNA was assessed by Cell Counting Kit-8 (CCK-8)
Table I. Expression of activating enhancer-binding protein-4 messenger RNA in lung cancer cases.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tumor</th>
<th>Non-tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Expression level</td>
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<tr>
<td>Gender</td>
<td>0.343</td>
<td>0.499</td>
</tr>
<tr>
<td>Male</td>
<td>31</td>
<td>1.147±0.3486</td>
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<tr>
<td>Female</td>
<td>21</td>
<td>1.248±0.4115</td>
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<td>Age, years</td>
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<td>0.204</td>
</tr>
<tr>
<td>&lt;60</td>
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<td>1.224±0.3393</td>
</tr>
<tr>
<td>≥60</td>
<td>34</td>
<td>1.168±0.3957</td>
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<tr>
<td>Tumor size, cm</td>
<td>0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.850</td>
</tr>
<tr>
<td>&lt;3</td>
<td>27</td>
<td>1.340±0.4117</td>
</tr>
<tr>
<td>≥3</td>
<td>25</td>
<td>1.023±0.2460</td>
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<tr>
<td>Differentiation</td>
<td>0.828</td>
<td>0.747</td>
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<tr>
<td>Well</td>
<td>22</td>
<td>1.178±0.3472</td>
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<tr>
<td>Moderate</td>
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<td>1.220±0.4231</td>
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<tr>
<td>Poor</td>
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<td>1.126±0.3413</td>
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<td>Histology</td>
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<td>Squamous carcinoma</td>
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<tr>
<td>Adenocarcinoma</td>
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<tr>
<td>Other</td>
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<tr>
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<td>19</td>
<td>1.421±0.4413</td>
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<tr>
<td>II &amp; III</td>
<td>33</td>
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<tr>
<td>Lymph node status</td>
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<tr>
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<tr>
<td>Positive</td>
<td>28</td>
<td>0.991±0.2275</td>
</tr>
</tbody>
</table>

<sup>a</sup>Statistically significant (P<0.05).

(A Beyotime Institute of Biotechnology). A549 and H1299 cells were seeded into 96-well plates (Corning Incorporated) at 3x10<sup>3</sup> cells/well. The cells were cultured at 37°C for 0, 24, 48 and 72 h. CCK-8 reagent (10 µl) was added to each well. Following incubation for 4 h at 37°C, the absorbance was measured at 450 nm with a multi-detection microplate reader (Synergy HT; Bio-Tek Instruments, Inc., Winooski, VT, USA).

**Apoptosis assay.** Cells were collected in tubes 48 h subsequent to transfection with siRNA, washed once using cold phosphate-buffered saline (PBS; Beijing Dingguo Changsheng Biotechnology Co., Ltd.), suspended in binding buffer (eBioScience, Inc., San Diego, CA, USA) and stained with Annexin V-fluorescein isothiocyanate (eBioscience, Inc.) in the dark at room temperature for 15 min. In total, 10 µl propidium iodide (PI; 25 µg/ml; eBioscience, Inc.) was added to each tube and cells were incubated at room temperature for 10 min. Within 1 h of staining, the cells underwent flow cytometry, which was performed a FACSCantoII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and cells were analyzed using BD FACSDiva Software 6.0 (BD Biosciences). Viable cells were Annexin V/PI<sup>-</sup>, early apoptotic cells were Annexin<sup>+</sup>/PI<sup>-</sup> and late apoptotic/dead cells were Annexin<sup>+</sup>/PI<sup>+</sup>. Nonviable cells that had undergone necrosis were Annexin/PI<sup>+</sup>.

**Cell cycle assay.** Cells were harvested 48 h subsequent to transfection with siRNA, fixed in 70% ice-cold ethanol (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) overnight, washed with 1X PBS and stained by PI (50 µg/ml) with RNase (50 µg/ml) in the dark at room temperature for 30 min. Cell cycle stage analysis was performed by flow cytometry (FACSCantoII) using ModFit software version 3.0 (Verity Software House, Topsham, ME, USA).

**Statistical analysis.** All data are presented as the mean ± standard deviation. Means were compared two groups by Student’s t-test, while means of three or more groups were compared with one-way analysis of variance using SPSS for Windows version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**AP-4 expression is significantly increased in lung cancer tissue.** Expression of AP-4 in 52 human lung cancer tissue samples and matched adjacent noncancerous lung tissue samples was assessed using RT-qPCR. The level of AP-4 mRNA in lung cancer tissues was three times higher compared with that in the noncancerous tissue samples.
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(P<0.0005; Fig. 1A). The association between AP-4 mRNA expression and clinicopathological characteristics of the lung cancer patients was additionally analyzed (Table 1). The levels of AP-4 mRNA were significantly associated with tumor size (P=0.002), lymph node status (P<0.001) and TNM stage (P=0.0006), but not with sex (P=0.343), age (P=0.615), differentiation degree (P=0.828) or histology (P=0.240) in tumor tissue samples. In addition, western blotting indicated that the levels of AP-4 protein in A549 and H1299 human lung cancer cells were significantly increased compared with those in human bronchial epithelial (HBE) cells (P=0.006; Fig. 1B).

Silencing of AP-4 inhibits the proliferation of human lung cancer cells. In order to investigate the involvement of AP-4 in the proliferation of human lung cancer cells, A549 and H1299 cells were transfected with scrambled siRNA or AP-4-specific siRNA, and cell proliferation was analyzed using CCK-8. The proliferation of A549 and H1299 cells was decreased following transfection with AP-4-specific transfection for 24 h (P=0.0276, P=0.0322, respectively). Following 72 h of transfection, the proliferation of the two cell lines was significantly decreased (A549, P=0.0017; H1299, P=0.0035) (Fig. 2). These results demonstrated that silencing of AP-4 expression was able to inhibit the proliferation of human lung cancer cells.

Silencing of AP-4-induced cell cycle arrest at G0/G1 phase. AP-4 silencing inhibited the proliferation of lung cancer cells; therefore, the present study aimed to establish whether this effect was caused by cell cycle arrest. Following transfection with scrambled siRNA or AP-4-specific siRNA for 48 h, the cell cycles of A549 and H1299 cells were analyzed using flow cytometry. There was an increased proportion of AP-4 siRNA-transfected cells in the G0/G1 phase (A549, P=0.0083; H1299, P=0.012) and a reduced proportion of these cells in the S phase compared with the G0/G1 and S phase proportions observed in untransfected and scrambled

Figure 1. Expression of AP-4 is increased in lung cancer tissues and cell lines. (A) AP-4 mRNA was analyzed by reverse transcription-quantitative polymerase chain reaction in lung cancer tissue samples and matched adjacent noncancerous lung tissue samples. **P<0.01. (B) AP-4 protein in lung cancer cells and HBE cells was measured by western blotting. GAPDH was used as an internal control. Data are presented as the mean a standard deviation. "P<0.01 compared with HBE cells. AP-4, activating enhancer-binding protein-4; mRNA, messenger RNA; HBE, human bronchial epithelial. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 2. Silencing of AP-4 expression suppresses the proliferation of lung cancer cells. (A) Proliferation of A549 cells was determined by CCK-8 following siRNA transfection for 0, 24, 48 and 72 h. (B) Proliferation of H1299 cells was determined by CCK-8 following siRNA transfection for 0, 24, 48 and 72 h. OD values among A459 and H1299 cells transfected with scrambled siRNA or AP-4-specific siRNA and cells without transfection were compared by one-way analysis of variance. **P<0.01. AP-4, activating enhancer-binding protein-4; CCK, cell counting kit; siRNA, small interfering RNA; OD, optical density.
Figure 3. Transfection with AP-4-specific siRNA arrests the cell cycle at the G0/G1 phase in human lung cancer cells. (A) The proportion of cells in each phase of the cell cycle was investigated by flow cytometry. (B) Data were aggregated and presented as a graph. AP-4, activating enhancer-binding protein-4; siRNA, small interfering RNA.

Figure 4. AP-4 silencing promotes apoptosis of lung cancer cells. (A) A549 cells transfected with siRNA, or not, were double stained with Annexin V/FITC and PI and analyzed by flow cytometry. (B) H1299 cells transfected with siRNA, or not, were double stained with Annexin V/FITC and PI and analyzed by flow cytometry. The percentages of apoptotic cells with various treatments are presented in the bar graphs. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.01. AP-4, activating enhancer-binding protein-4; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate; PI, propidium iodide.
siRNA-transfected cells (Fig. 3A and B). Therefore, silencing of AP-4 may be capable of inducing cell cycle arrest at the G0/G1 phase.

**Silencing of AP-4 promotes apoptosis in human lung cancer cells.** The present study measured the effects of AP-4 silencing on the apoptosis of A549 and H1299 cells. As demonstrated in Fig. 4, the percentages of apoptotic cells in non-transfected or scramble siRNA-transfected A549 and H1299 cells were 4.5% and 2.8%, respectively. Following transfection with AP-4-specific siRNA, the percentages of apoptotic A549 and H1299 cells significantly increased to 15% (P=0.005) and 10% (P=0.006), respectively. These findings indicated that silencing of AP-4 may promote the apoptosis of lung cancer cells.

**AP-4 silencing alters the expression of p21 and cyclin D1.** In order to investigate the mechanisms underlying the effect of AP-4 silencing on the cell cycle of human lung cancer cells, the expression of certain cell-cycle-associated regulators, cyclin-dependent kinase inhibitor p21 and cyclin D1, were examined using western blotting in siRNA-transfected A549 and H1299 cells. Transfection with AP-4-specific siRNA in A549 and H1299 cells reduced the expression of AP-4 (P=0.0009 and P=0.0002, respectively) and cyclin D1 protein (P=0.0023 and P=0.0007, respectively), but increased the levels of p21 expression (P=0.0062 and P=0.0039, respectively) (Fig. 5A and B). This suggested that AP-4 silencing may be capable of suppressing the proliferation of human lung cancer cells and inducing cell cycle arrest at the G0/G1 phase by modulating the expression of certain cell cycle regulators.

**Discussion**

Despite numerous advances in diagnosis and therapy for lung cancer, it still possesses one of the highest mortality rates worldwide, and has a poor patient prognosis and quality of life (1). Given the frequent failure of conventional treatment strategies, a number of cancer-associated molecules have been investigated with the aim of developing novel anticancer therapies (18).

A number of studies have demonstrated that transcription factor AP-4 is upregulated in several human cancers, including gastric, hepatocellular, colorectal and breast cancer, and that it may be associated with prognosis (9,10,19,20). These observations suggest that AP-4 may have a significant role in the tumorigenesis or progression of a number of types of cancer (11,12,21,22).

In the present study, AP-4 expression was significantly increased in lung cancer tissue compared with normal tissue, as described previously (13). However, AP-4 mRNA levels were negatively correlated with tumor size (P=0.002), lymph node status (P<0.001) and Tumor-Node-Metastasis stage (23) (P<0.001), which contrasts with the results of a number of previous studies (9,10,13). These differences may have been due to the use of RT-qPCR in the present study, compared with the use of immunohistochemical staining in these previous studies (9,10,13).

Transcription factors form a transcription initiation complex with RNA polymerase II, which participates in transcription initiation in order to regulate gene expression (24). As an ubiquitously-expressed transcription factor, AP-4 was initially identified to activate viral late gene transcription by binding the simian virus 40 enhancer (25). AP-4 is
a direct target gene of c-Myc, which has crucial involvement in cell proliferation, cell cycle progression, apoptosis and differentiation (26). In addition, AP-4 binds the recognition motifs located in the vicinity of the p21 promoter and is able to mediate the transcriptional repression of p21, which is a key cell cycle inhibitor (27). Ge et al (22) reported that c-Myc activated by the Wnt signaling pathway may promote hepatocellular carcinoma progression, through a direct inhibitory effect of AP-4 on p21. The present study identified that silencing of AP-4 was able to inhibit the proliferation of human lung cancer cells and induce cell cycle arrest at the G0/G1 phase. In order to investigate the potential mechanisms underlying the effect of AP-4 silencing on human lung cancer cells, several cell-cycle-associated regulators, cyclin D1 and p21, were examined. Cyclin D1 promotes progression through the G1/S phase of the cell cycle (28), while p21 has been hypothesized to be a negative regulator of the cell cycle and proliferation (29). In the present study, the expression of cyclin D1 and AP-4 was reduced following transfection with siRNA, but levels of p21 were upregulated. These results implied that AP-4 silencing suppressed the proliferation of human lung cancer cells and induced cell cycle arrest at the G0/G1 phase by modulating the expression of certain cellular regulators. Furthermore, the present study identified that silencing of AP-4 expression promoted apoptosis. AP-4 silencing is able to increase the levels of caspase-9 and downregulates expression of B-cell lymphoma (Bcl)-2 and Bcl-extra large expression in human gastric cancer cells (12), suggesting that knockdown of AP-4 may be able to activate mitochondrial (intrinsic) and death receptor (extrinsic) signaling pathways of apoptosis in cancer cells (30,31).

In conclusion, the present study demonstrated that expression of transcription factor AP-4 was significantly increased in lung cancer tissues and cells, and siRNA-mediated silencing of AP-4 inhibited cell proliferation, induced cell cycle arrest and promoted apoptosis of human lung cancer cells, as well as causing decreased expression of cyclin D1 and increased expression of p21. The results of the present study suggest that AP-4 may be an oncprotein that has a significant role in lung cancer. Although additional investigation is required in order to investigate the precise mechanisms underlying this role, AP-4-specific-siRNAs may be worth investigating as a novel therapeutic agent for the treatment of human lung cancer.

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References