Original Article

Apatinib inhibits proliferation of different lung cancer cells in vitro and in vivo by promoting apoptosis and inducing cell cycle arrest

Mingtao Liu1,2, Xiuxiu Wang1, Lijun Jing1, Peng Jiang1,3, Yu Li1

1Department of Pulmonary Medicine, Qilu Hospital, Shandong University, Jinan, Shandong, China; 2Department of Pulmonary Medicine, Binzhou People’s Hospital, Binzhou, Shandong, China; 3Department of Pulmonary Medicine, Weihai Municipal Hospital, Weihai, Shandong, China

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Abstract: Apatinib is a specific VEGFR-2 inhibitor that blocks transmission of the VEGF/VEGFR-2 signaling pathway by competitively binding to the VEGFR-2 intracellular tyrosine ATP binding site and exerting biological effects. Different levels of VEGFR-2 expression in different lung cancer cells and the cell lines with low and high expression of VEGFR-2 were selected, and the effect of apatinib on their proliferation was observed in vitro and in vivo. In vitro studies showed that apatinib significantly inhibited the proliferation of VEGFR-2 high-expression lung cancer cell lines H1975 and H446 in a concentration-dependent manner, mainly by promoting apoptosis and cell cycle arrest. For the lung cancer cell line A549 with low VEGFR-2 expression, the same concentration of apatinib had no inhibitory effect. In vivo studies showed that both H1975 and H446 xenografts were significantly inhibited by low- and high-dose apatinib, while in A549 cells, low-dose apatinib had no significant inhibitory effect. Therefore, apatinib has different inhibitory effects on different lung cancer cells, and the inhibitory effect on lung cancer cells with high VEGFR-2 expression is more significant by promoting apoptosis and inducing cell cycle arrest in vitro and in vivo.

Keywords: Apatinib, expression level of VEGFR-2, proliferation, apoptosis, cell cycle arrest

Introduction

The incidence and mortality of lung cancer ranks first among all cancers [1], and because its early symptoms are not obvious, combined with the lack of effective early diagnoses, most lung cancer patients are diagnosed in advanced stages [2]. Currently, there is still no effective treatment for advanced lung cancer. Although new cytotoxic drugs and molecular targeted drugs are continuously developed, the 5-year survival rate of patients with advanced lung cancer is still low [3]. Improving the efficacy of treatments for patients with lung cancer and prolonging their survival is an urgent need for clinicians.

Folkman proposed that tumor growth depends on the formation of tumor blood vessels and thus proposed that anti-angiogenesis will be an important method for the treatment of tumors [4]. A variety of growth factors and their corresponding receptors are involved in tumor angiogenesis in which vascular endothelial growth factors (VEGFs) and their vascular endothelial growth factor receptors (VEGFRs) play major roles [5, 6]. The VEGFR family members comprise three types: VEGFR-1, VEGFR-2 and VEGFR-3 [7]. The VEGF/VEGFR-2 signaling pathway plays an important role in tumor angiogenesis, cell proliferation, invasion and migration. Therefore, blocking this signaling pathway and inhibiting tumor angiogenesis has become a new area of tumor-targeted therapy [8].

Apatinib is a small molecule tyrosine kinase inhibitor that selectively inhibits the phosphorylation of VEGFR-2 and its tyrosine by competitively binding to the VEGFR-2 intracellular tyrosine ATP binding site. Kinase activity blocks the transmission of the VEGF/VEGFR-2 signaling pathway and inhibits tumor angiogenesis, thereby inhibiting tumor growth [9, 10]. Currently, apatinib is mainly used in the treatment of advanced gastric cancer as third-line or above [11]. Research on the treatment of lung
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cancer with apatinib is mainly focused on the clinical study of patients with non-small cell lung cancer. LIU reported that a clinical trial of apatinib in patients with advanced non-small cell lung cancer found that patients with apatinib had a median PFS of 4 months and a disease control rate of 61.76% [12]. Cases of effective treatment of small cell lung cancer with apatinib have also been reported [13].

Apatinib has a therapeutic effect in patients with advanced lung cancer and can improve patient PFS and DCR to some extent. However, there are few basic studies on the effects of apatinib on lung cancer cells, especially the different types of lung cancer cells, and the mechanism of action has not been reported. For this study five lung cancer cell lines were selected with different expression levels of VEGFR-2 and determined the effect of apatinib on proliferation in vitro and in vivo and observed the safety of apatinib. This study may provide a theoretical basis for the more rational application of apatinib in patients with advanced lung cancer.

Materials and methods

Cell culture

The human lung cancer cell lines A549, HCC-827, H2228, H1975 and H446 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in RPMI-1640 (HyClone, USA) supplemented with 10% FBS (Gibco, NY), 100 U/mL penicillin (HyClone, USA), 50 mg/mL streptomycin (HyClone, USA), and 2 mmol/L glutamine in a humidified CO2 incubator at 37°C. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks obtained from the indicated sources.

Reagents and antibodies

Apatinib (Selleck, s2221, Shanghai, China) was dissolved in 0.1% DMSO (Sigma Aldrich) at a concentration of 10, 20 mmol/L and stored at -20°C for vitro testing. Apatinib mesylate (Heng Rui, Jiangsu, China) were ground into powder and dissolved 0.5% CMC (Sorbo, Shanghai, China) for vivo testing. Primary antibodies against AKT (ab8805), phosphor-AKT (ab8932), ERK (ab54230), phospho-ERK (ab201015), el-F4E (ab205824), phosphor-elF4E (ab2008-58); HIF-1α (ab51608), VEGF (ab46154), CD31 (ab28364), β-actin (ab8227) were purchased from abcam (Cambridge, MA, USA). Primary antibodies against PARP (9532), caspase3 (9662), cleaved-caspase3 (9664), cyclinB1 (4138), P53 (9282), VEGFR-2 (2472), Ki67 (9449) and anti-rabbit or anti-mouse IgG horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Cell Signaling Technology (Boston, MA, USA).

Cell proliferation

Five lung cancer cells were plated into 96-well plates (2000~4000/well) in 100 μl of complete medium, and six parallel wells were assigned to each group, as well as a negative control (without cells). After overnight incubation, cells were exposed to various concentrations (0, 1, 10, 50, 100, 250 μM) of apatinib for a further 72 hours. Cell proliferation was evaluated by Cell Counting Kit 8 (CCK8, BestBio, Shanghai, China). Briefly, the inhibitory rates of each cell line with different concentration of apatinib were calculated by comparing the OD values of the experimental groups to that of the empty group. The half maximal inhibitory concentration (IC50) of apatinib that could inhibit growth by 50% were calculated by concentration-response cure fitting using GraphPad Prism 5.0 software. Each IC50 value was expressed as the mean ± SD.

Colony formation assays

For the clonogenic assays, five tumor cell lines in the logarithmic growth phase were seeded in 6-well plates in complete culture medium, and six parallel wells were assigned to each group, as well as a negative control (without cells). After overnight incubation, cells were exposed to various concentrations (0, 1, 10, 50, 100, 250 μM) of apatinib for a further 72 hours. Cell proliferation was evaluated by Cell Counting Kit 8 (CCK8, BestBio, Shanghai, China). Briefly, the inhibitory rates of each cell line with different concentration of apatinib were calculated by comparing the OD values of the experimental groups to that of the empty group. The half maximal inhibitory concentration (IC50) of apatinib that could inhibit growth by 50% were calculated by concentration-response cure fitting using GraphPad Prism 5.0 software. Each IC50 value was expressed as the mean ± SD.

Cell cycle analysis

For the cell cycle assays, three tumor cell lines include A549, H1975 and H446 were harvested and washed twice with cold PBS and then were fixed with pre-chilled 70% ethanol for 24 h at 4°C. The fixed cells were washed and resuspended in 200 μL of PBS plus 200 μL of RNase
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A in a water bath at 37°C for 30 min. Next, the cells were filtered and incubated with 300 μL of propidium iodide at 4°C for 30 min in the dark. The stained cells were detected by flow cytometry and were analyzed using ModFit LT 4.1 software.

Assessment of apoptosis

The treated cells were washed twice with PBS, resuspended in binding buffer at a density of 1 × 10^7 cells/mL and then were stained using an annexin V-FITC-PI apoptosis detection kit (BD, USA) for 20 min at room temperature in the dark according to the manufacturer’s protocols. Thereafter, the labeled cells were detected by flow cytometry and were analyzed using FlowJo 7.6 software. Next, tunel apoptosis detection kit was used to detect apoptosis of three kind cells treated by different concentration apatinib according to the manufacturer’s manual (Yeasen, China).

Immunofluorescence assay

Cells were seeded on coverslips and were fixed with 4% paraformaldehyde for 30 min at room temperature. After permeabilization with 0.2% TX-100 (Sigma, × 100) for 10 minutes at room temperature, cells were incubated with cleaved caspase 3 antibody at 4°C for 12 h. After washing with PBS, cells were incubated with Fluorescein-Conjugated Goat anti-Rabbit IgG (H+L) for 1 h. Immunofluorescence images were viewed under fluorescence microscope (Olympus I × 81, Japan). Cleaved caspase 3 were quantified with the Image J software. Mean fluorescence of the nuclear staining was obtained by selecting the “measure” option. After deducting any background staining, the mean fluorescence of at least 50 cells from each treatment was used for statistical analysis. Data was represented as mean ± SD.

Western blot analysis

Cells and tissues were lysed by radio immunoprecipitation assay (RIPA, Beyotime, China) buffer containing phenyl methane sulfonyl fluoride (PMSF, Solarbio, China) with mild sonication. The concentrations of total proteins were measured by the BCA Protein Assay Kit (Beyotime, China). Equal amounts of protein were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on polyvinylidene fluoride (PVDF, Millipore, Billerica, USA). Protein bands were visualized via enhanced chemiluminescence (ECL, Millipore, USA) and were analyzed using the western blot imaging system (Al600 images, GE, USA), followed by measurement of the density of each band using Image J software.

RNA preparation and RT–PCR analysis

Total RNA were isolated with Trizol reagent (Invitrogen, USA). The reverse transcription reactions were performed by use of Reverse Transcription Reagent kits (Takara, Japan). Real-time PCR was conducted with SYBR Green mix (Takara, Japan). The primer for VEGFR-2 was designed as 5'-ACCCCTTGAGTCCAATCACACA-3' (forward), 5'-CTTCCTCCTCAATGCGCAA TAACA-3' (reverse); The primer for GAPDH was designed as 5'-GGAAGCT TGTCATCAATGGAAATC-3' (forward), 5'-TGATGACCCTTTTGGCTCCC-3' (reverse). The expression of VEGFR-2 mRNA was calculated and normalized using the 2^ΔΔCt method relative to GAPDH.

Tumor xenograft mouse models

Five to six week old female BALB/c nude mice were purchased from Nanjing University Biomedical Research Institute (Nanjing, China). All animal experiments were performed in the animal research center of Shandong Medical Academy in accordance with the National Institutes of Health guide for the care and use of laboratory animals. A549, H1975, and H446 cell lines were selected to construct three nude mouse xenograft models. Approximately 2 × 10^6 cells were suspended in 0.2 ml of PBS, subcutaneously injected into the right chest wall of each nude mouse, and tumors formed in approximately 10 days. The tumor volume was calculated as L × W^2/2 (L and W are the length and width of the tumor, respectively). The nude mice with transplanted tumor volumes of 50-150 mm^3 were selected for intervention grouping. There was no significant difference in the average volume of transplanted tumors between the nude mice before intervention. Fifteen tumor-bearing nude mice from each of the three cell lines were randomly divided into three groups according to a random number table. The animals were treated for 21 consecutive days once daily by oral gavage with apatinib (80, 120 mg/kg) or vehicle. The dose of apatinib refers to previously published litera-
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The body weight and the tumor volume were recorded every 3 days by the same person. At harvest, the mice were sacrificed under anesthesia. The tumor tissues were fixed in 4% paraformaldehyde for immunohistochemistry or stored at -80°C for western blotting, and blood was harvested for ELISA assays. The heart, liver, kidney, thyroid and pancreas were fixed in 4% paraformaldehyde and electron microscopy fixative for H&E staining and electron microscopy specimen preparation respectively. All animal experiments were performed with the approval of Shandong University Animal Care and Use Committee.

Immunohistochemical staining

For immunohistochemical staining, the transplanted tumor tissues were fixed in 4% paraformaldehyde, and paraffin blocks were prepared after dehydrating and embedding. A series of 3 mm sections were obtained from each paraffin block. Each slice was baked at 65°C for 1 hour and dewaxed by xylene. Then, the slides were dehydrated using ethanol and antigens were repaired by EDTA. The peroxidase was removed using 3% H₂O₂ and blocked using 5% BSA for 30 minutes. Incubation with the primary antibody was conducted overnight at 4°C. The sections were incubated with a biotin secondary antibody for 20 minutes at room temperature. Targeted proteins were visualized using the peroxidase substrate diaminobenzidine. Staining intensities were estimated in five random fields per section by three independent observers individually.

Toxicological study

Hematoxylin and eosin staining: For hematoxylin and eosin (H&E) staining, the heart, liver, kidney, thyroid, and pancreas tissues of the mice were fixed in 4% paraformaldehyde and embedded in paraffin and then cut into 4 μm sections. The sections were stained with both hematoxylin and eosin and then photographed using a light microscope.

Electron microscope specimen production

The heart, liver, kidney, thyroid, and pancreas tissues of the mice were fixed in 3% glutaraldehyde at 4°C for 4 hours and then washed with 0.1 M sodium dicarboxylate buffer and soaked in 1% citric acid at 4°C for 2 hours. Next, the tissues were washed with 0.1 M sodium diformate buffer twice and were dehydrated by graded ethanol. The tissues were permeated with propylene oxide, completely embedded in an embedding solution, and placed in a 40°C incubator for 12 hours. Then, the slides were transferred to an embedding plate and incubated at 60°C for 48 hours. After the fabrication, the ultrastructure of the organs were observed by transmission electron microscopy (JEOL, Japan).

Serum biochemistry

Blood samples were obtained from the vein of the inner canthus while the mice were under anesthesia from a 1% pentobarbital sodium intraperitoneal injection. All of the nude mice were fasted for more than 8 hours before taking blood. Blood was allowed to coagulate at room temperature for 30 minutes and was centrifuged at 3,000 rpm for 10 minutes to separate the serum. Biochemical tests for the level of ALT, AST, BUN, Cr, LDH-L, CK and glucose in serum were performed. ELISA kits (cloud clone, USA) were used to detect the concentration of FT₃ and FT₄ in the serum. The specific procedure followed the steps provided by the ELISA kit. All tests were repeated 3 times, and the data are represented as the mean ± SD.

Statistical analysis

All the experiments in our research were conducted at least three times individually. The results are presented as mean ± standard deviation (SD).

Data were analyzed using GraphPad Prism software (version 5.0). The Two-way ANOVA was used to value the overall statistical significance for the means of multiple samples. A P-value of less than 0.05 was considered statistically significant (*represent P values<0.05).

Results

Apatinib exerts anti-proliferative activity against lung cancer cells in vitro

CCK8 assay was performed on a panel of five lung cancer cell lines (A549, HCC827, H2228, H1975, ad H446). The results show that as the concentration of apatinib increased, the inhibition of proliferation of the five lung cancer cell lines were increased. In five lung cancer cells, the inhibition of proliferation of H1975 and H446 were more obvious. When the concentration of apatinib reached 50 μM, the inhibition
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rate of H1975 and H446 were 54.6 ± 5.48% and 65.74 ± 2.05%, respectively, while the inhibition rates of A549, HCC827 and H2228 were 11.36 ± 0.89%, 12.80 ± 1.26%, and 19 ± 0.75%, significantly lower than H1975 and H446. There were similar results at 48 and 72 hours (Figure 1A-C). The results showed that the IC\(_{50}\) values of apatinib for the five lung cancer cell lines at 24 hours were as follows: A549: 186.5 ± 4.86 μM, HCC827: 181.86 ± 4.86 μM, H2228: 223.85 ± 11.7 μM, H1975: 31.12 ± 1.38 μM, and H446: 13.94 ± 1.88 μM. The IC\(_{50}\) values of H1975 and H446 were significantly lower than that of the other three lung cancer cells (P<0.05). Over time, the IC\(_{50}\) values of apatinib for the five lung cancer cell lines decreased to some extent, but the IC\(_{50}\) values of H1975 and H446 were still significantly lower than that of other lung cancer cell lines (P<0.05) (Table 1).
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Table 1. IC\textsubscript{50} values for apatinib of five cells at different times (μM)

<table>
<thead>
<tr>
<th>Cells</th>
<th>Times</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td></td>
<td>186.6 ± 5.9</td>
<td>161.1 ± 5.4</td>
<td>155.3 ± 4.8</td>
</tr>
<tr>
<td>HCC827</td>
<td></td>
<td>157.4 ± 5.7</td>
<td>144.2 ± 7.5</td>
<td>139.3 ± 6.2</td>
</tr>
<tr>
<td>H2228</td>
<td></td>
<td>233.8 ± 14.3</td>
<td>210.2 ± 18.4</td>
<td>192.4 ± 9.9</td>
</tr>
<tr>
<td>H1975</td>
<td></td>
<td>31.2 ± 1.8*</td>
<td>18.7 ± 2.4*</td>
<td>14.6 ± 1.2*</td>
</tr>
<tr>
<td>H446</td>
<td></td>
<td>13.9 ± 2.3*</td>
<td>11.4 ± 2.1*</td>
<td>9.9 ± 0.7*</td>
</tr>
</tbody>
</table>

The IC\textsubscript{50} values of apatinib for five lung cancer cell lines at different times, *p<0.05.

Relative VEGFR-2 protein and mRNA expression levels

The CCK8 results confirmed that in these five lung cancer cell lines, the inhibitory effects of apatinib on H1975 and H446 were more obvious. Apatinib specifically binds to VEGFR-2 to exert its biological effects, and the expression of VEGFR-2 in the five cell lines was different, leading to different inhibition effects of apatinib. Expression of VEGFR-2 protein and mRNA was observed by Western blot and qPCR. Western blot analysis showed that the expression levels of VEGFR-2 protein in H1975 and H446 cell lines were significantly higher than those in the other three cell lines (Figure 1D and 1E). Similar results were observed at the RNA level (Figure 1F). The qPCR results showed that the relative expression levels of VEGFR-2 mRNA in H1975 and H446 were significantly higher than that of the other three lung cancer cells.

Colony formation assay

To further observe the effects of different concentrations of apatinib on the proliferation of different lung cancer cells, colony formation assay was performed. A549, HCC827 and H2228 cells were selected to represent VEGFR-2 low expressing cell lines, and H1975 and H446 cells were selected to represent VEGFR-2 high expressing cell lines. A difference in the proliferation of lung cancer cell lines with different expression levels of VEGFR-2 after apatinib treatment was observed. Following 2 weeks of culture, apatinib inhibited the number of the colonies formed by H1975 and H446 cells in a dose-dependent manner. However, apatinib did not inhibit the formation of colonies in A549, HCC827 and H2228 cells (Figure 1G and 1H). Collectively, these results revealed that apatinib can inhibit the formation of colonies in VEGFR-2 high-expression lung cancer cell lines.

Cell cycle regulation

The capacity of apatinib to induce cell cycle arrest was demonstrated in H1975 and H446 cells, but not in the A549 cell line. Apatinib induced a dose-dependent G2 phase arrest in H1975 and H446 cells. At 0, 10 and 20 μM apatinib, the G2 phase ratio of the H1975 and H446 cells increased significantly (H1975: 7.45 ± 1.13%, 19.2 ± 2.12%, and 41.2 ± 0.98%, respectively; H446: 10.09 ± 0.80, 20.33 ± 1.31%, and 45.47 ± 1.02%, respectively). There was a significant difference in the comparison (P<0.05) (Figure 2B and 2C). However, the effect on A549 cells was minor, as there was no significant difference compared with the control group (Figure 2A). Western blot was used to detect the expression of cyclin B1 and p53, which were G2 phase related proteins. The results showed that with the increase of apatinib concentration, the expression of cyclin B1 were decreased while p53 increased in H1975 and H446 cell lines. There was a significant difference compared with the control group (P<0.05) (Figure 2E and 2F). Changes in the expression levels of the two proteins led to an increase in the proportion of cells staying in the G2 phase, inhibiting their conversion to the M phase, thereby inhibiting the proliferation of both cell lines. For A549 cells, the expression levels of cyclin B1 and p53 were not significantly different from those in the control group (P>0.05) (Figure 2D). Therefore, apatinib can induce dose-dependent G2 phase arrest in H1975 and H446 cell lines with high expression of VEGFR-2.

Effects on apoptosis

The effects of different concentrations of apatinib on the apoptosis of A549, H1975 and H446 cells were detected by flow cytometry and a TUNEL assay. The flow cytometry results showed that the apoptosis rates of H1975 and H446 cells in the 0, 10 and 20 μM groups were as follows: H1975: 1.9 ± 1.47%, 7.8 ± 1.50%, and 15.9 ± 1.92%; H446: 0.98 ± 0.42%, 15.2 ± 2.65%, and 32.6 ± 2.68%. As the concentration of apatinib increased, the apoptosis rate of H1975 and H446 cells increased significantly in a dose-dependent manner compared to the control group (P<0.05). In the A549 cells, the percentage of apoptosis was lower after treatment with apatinib, and there was no statistically significant difference from the control group (P>0.05) (Figure 3A).
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The TUNEL test results were similar to the flow results. The results showed that the apoptosis rates of H1975 and H446 cells in the 0, 10, and 20 μM apatinib groups were as follows: H1975: 0.53 ± 0.22%, 18.6 ± 2.31%, and 34.5 ± 2.91%; H446: 0.88 ± 0.57%, 28.1 ± 3.9%, and 55.9 ± 6.2%. There was a statistically significant difference (P<0.05) for the H197 and H446 cells, but there was no significant difference in the percentage of apoptosis between the three groups in A549 cells (P>0.05) (Figure 3B).

Western blot results showed that the relative expression of c-caspase3 and c-PARP in H1975 and H446 cells increased significantly with the increase of apatinib concentration (P<0.05). However, there was no significant difference in the relative expression of c-caspase3 and c-PARP in A549 cells (P>0.05) (Figure 4B). The results of an immunofluorescence assay showed that the fluorescence intensity of c-caspase3 in H1975 and H446 cells was significantly higher than in the control group after

Figure 2. Apatinib induced cell cycle arrest in three cell lines. A-C. A549, H1975 and H446 cells were treated with 0, 10 or 20 μM apatinib for 24 hours and the cell cycle was analyzed by flow cytometry. D-F. Effect of apatinib on the expression of cyclin B1 and p53 was tested by western blotting in A549, H1975 and H446 cells, respectively. The cells were treated with 0, 10 or 20 μM apatinib for 24 hours. Representative results are shown, and similar results were obtained in three other independent trials.
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Treatment with apatinib (P<0.05), while no increase in the fluorescence intensity of c-caspase3 was observed in A549 cells (Figure 4A). It was further confirmed that apatinib could induce apoptosis by activating the caspase3 pathway in a dose-dependent manner in H1975 and H446 cell lines with high VEGFR-2 expression.

Figure 3. Effects of apatinib on the apoptosis rates of A549, H1975 and H446 cells were detected by flow cytometry and TUNEL assay. A. Apoptosis rates for A549, H1975, and H446 cells treated with 0, 10, or 20 μM apatinib for 24 hours were detected by flow cytometry. B. The apoptosis rates of A549, H1975 and H446 cells were detected by TUNEL assay.
Tumor growth and immunohistochemistry

The in vivo antitumor efficacy of apatinib was evaluated in A549, H1975 and H446 cancer cells by utilizing BALB/c nude mouse xenograft models. The animals were repeatedly administered vehicle or apatinib once daily via oral gavage (80 mg/kg/day and 120 mg/kg/day) for 21 consecutive days. In the nude mouse xenograft model using A549 cells, there was no significant difference in the volume of the 80 mg/kg apatinib group compared with the control group (P>0.05) while the volumes of the xenografted tumors in the 120 mg/kg group were significantly smaller than that of the control group (P<0.05). Therefore, apatinib exhibited dose-dependent antitumor efficacy in the mice with xenografted H1975 and H446 lung cancer cells (Figure 5B and 5C).

Immunohistochemical staining was performed to determine the expression of CD31, Ki67 and c-caspase3 in different groups of xenografted tumor tissues. In the xenografted tumor tissues formed by A549 cells, there was no significant difference in the expression of CD31, Ki67 or c-caspase3 of the apatinib 80 mg/kg group compared with the control group (P>0.05), while the number of CD31-positive micro-vessels...
were significantly reduced, the number of Ki67-positive cells were significantly decreased, and the expression of c-caspase3 was significantly increased in both the 80 mg/kg and 120 mg/kg groups in a dose-dependent manner (P<0.05) (Figure 5E and 5F).

**Related signaling pathways of xenograft tumors**

Apatinib exhibited dose-dependent suppression of VEGFR-2 activation and downstream sig-
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Figure 6. Effect of apatinib on related signaling pathways in A549, H1975, and H446 cell xenograft models. A. Expression of p-AKT, p-Erk, p-eIF4E1, HIF-1α, and VEGF was measured by western blot. Blots are representative of three independent experiments. B. The quantifications of the associated gray protein intensities of p-AKT, p-Erk, p-eIF4E1, HIF-1α and VEGF in each group are presented.
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In A549 tumor tissues, the expression levels of p-AKT, p-Erk, p-eIF4E1, HIF-1α, and VEGF of downstream signaling pathways were significantly decreased in the 120 mg/kg group (P<0.05), but there was no difference in their expression between the 80 mg/kg group and the control group (P>0.05) (Figure 6A). In the H1975 and H446 tumor tissues, the expression levels of p-AKT, p-Erk, p-eIF4E1, HIF-1α, and VEGF protein in the 80 mg/kg and 120 mg/kg groups were significantly lower than control group, and with a dose-dependent efficacy (Figure 6B and 6C). All assays were repeated three times, and the images in the article were the most representative.

Toxicological study

Apatinib were well tolerated in all of the tested groups, with no mortality or significant loss of body weight (<5% relative to the vehicle-ma-
tched controls) observed during treatment (Figure 7A). There was no statistically significant difference in ALT, AST, BUN, Cr, CK, or LDH-L in serum between the different groups (P>0.05), while the blood glucose levels of the high dose apatinib group were significantly higher than that of control group (P<0.05). The levels of FT3 and FT4 were significantly lower than in the control group (P<0.05) (Figure 7B). No typical pathological damage was found in HE staining of various organs, but the ultrastructure of various organs, such as the endoplasmic reticulum and mitochondria, under electron microscope had different degrees of damage, and the damage in the high dose group were more obvious (Figure 7C and 7D).

Discussion

Inhibition of tumor neovascularization provides a new therapeutic approach for patients with advanced lung cancer [14, 15]. Since the first anti-angiogenic targeting drug bevacizumab was used clinically, numerous clinical studies have confirmed that bevacizumab combined with chemotherapy or targeted therapy can significantly improve the survival of patients with advanced non-small cell lung cancer [16, 17]. For example, studies have shown that TP chemotherapy combined with bevacizumab can significantly prolong PFS and OS in patients with advanced non-squamous NSCLC [18]. Apatinib is a specific VEGFR-2 antagonist that competitively binds to VEGFR-2, blocks VEGF-mediated signaling, inhibits tumor angiogenesis, and thus controls tumor growth [19]. There have been more than 30 clinical studies on the treatment of advanced lung cancer with apatinib [20]. Most patients had advanced progressive non-small cell lung cancer, and a few patients had small cell lung cancer. Most of the treatments were concentrated in the third line or above treatment. In all of the clinical studies, the median PFS was more than 5 months, and the disease control rate reached 60-85% [21-23]. The clinical results of apatinib confirmed that patients can benefit in the short term, but resistance developed rapidly, and improved long-term OS was not obvious [24]. There is a desire to improve the efficacy of apatinib in clinical practice.

VEGFR-2 is mainly expressed on the surface of vascular endothelial cells and promotes the formation of new blood vessels after binding to VEGF [25]. The increase of tumor neovascularization is closely related to tumor proliferation, invasion and metastasis. Blocking tumor angiogenesis can control tumor growth and metastasis to some extent. Apatinib competitively binds to VEGFR-2 on tumor vascular endothelial cells, blocks the binding of VEGF/VEGFR-2, reduces tumor angiogenesis, and starves tumors, thereby inhibiting tumor growth indirectly. However, whether apatinib as a targeted drug can directly act on tumor cells and inhibit their growth is unknown. For apatinib to have a direct inhibitory effect on lung cancer cells, the surface of lung cancer cells must express VEGFR-2. If lung cancer cells express high VEGFR-2, such lung cancer cells can be targeted for treatment with apatinib. Apatinib is less effective on cells that do not express VEGFR-2. Among the common lung cancer cell lines are those that highly express VEGFR-2, and the inhibitory effect of apatinib on lung cancer cells with high or low expression of VEGFR-2 is unknown. This study demonstrates that apatinib has different inhibitory effects on the proliferation of lung cancer cell lines with different VEGFR-2 expression levels in vitro and in vivo, thus providing a theoretical basis for further optimizing the treatment strategy of apatinib in clinical practice.

This study selected common lung cancer cell lines A549, HCC827, H2228, H1975, and H446, the first four of which are lung adenocarcinoma cells, which represent wild type, EGFR mutant, ALK mutant, and EGFR mutant with a T790M mutation cell lines, and H446 is a small cell lung cancer cell line. By detecting the expression of VEGFR-2 at both mRNA and protein expression levels, we determined that H1975 and H446 lung cancer cell lines highly expressed VEGFR-2, and the other three cell lines expressed VEGFR-2 at a lower level. A CCK-8 test confirmed that the same concentration of apatinib had the most obvious inhibitory effect on the H1975 and H446 cell lines, and the IC_{50} level was significantly lower than that of the other three lung cancer cells. Using a colony formation assay, cell cycle detection and apoptosis assay, we determined that apatinib can effectively inhibit the proliferation of H1975 and H446 cells and promote apoptosis in a dose-dependent manner. However, in the other three lung cancer cell lines with low expression of VEGFR-2, low and high concen-
trations of apatinib could not effectively inhibit proliferation and the cell cycle arrest was not obvious, and no obvious apoptosis was observed. Apatinib has obvious inhibitory effects on H1975 and H446 cells in vitro, and no neovascularization was tested in vitro. Therefore, apatinib does not inhibit vasculogenesis in this instance but inhibits the proliferation of lung cancer cells with high VEGFR-2 expression by directly acting on VEGFR-2 on the surface of these two lung cancer cells and inhibiting the activation of downstream signaling pathways. Additionally, studies have confirmed that there is cross-talk between the VEGF pathway and the downstream effectors of the EGFR pathway [26]. Once the downstream proteins of the VEGF pathway are affected, they can also affect proliferation via the EGFR signaling pathway. Therefore, for lung cancer cells that highly express VEGFR-2, apatinib can significantly inhibit proliferation.

From the results of in vivo studies, apatinib has an inhibitory effect on transplanted tumors of A549, H1975 and H446 lung cancer cells. However, for the transplanted A549 tumors, 80 mg/kg apatinib could not effectively inhibit tumor growth while 120 mg/kg apatinib significantly inhibited tumor growth. In the nude mice transplanted with H1975 and H446 cells, 80 and 120 mg/kg administration groups showed significant anti-tumor effects in a dose-dependent manner. The inhibitory effect of apatinib on nude mice xenografts formed by high VEGFR-2 expressing lung cancer cells is superior to that of low expression lung cancer cell lines. However, unlike the in vitro results, apatinib showed almost no inhibition in vitro in A549 cells. In nude mouse xenograft models, however, there are numerous vascular endothelial cells, which highly express VEGFR-2, due to the presence of tumor blood vessels. Therefore, apatinib can indirectly inhibit tumor growth, though this inhibition was not observed in the 80 mg/kg group. In the high-dose group, apatinib’s indirect inhibition of tumor growth was observed. In transplanted tumors formed by H1975 and H446 cells with high VEGFR-2 expression, apatinib not only inhibited tumor growth by inhibiting tumor angiogenesis but also directly acted on tumor cells to directly inhibit tumor growth. At low doses, it can significantly inhibit xenograft growth, and the antitumor effect was significantly better than in A549 cells with low VEGFR-2 expression. The results of the study partially reveal why there is a difference in the efficacy of the clinical application of apatinib in the treatment of lung cancer patients, which may be related to the differential expression of VEGFR-2 in lung cancer cells.

To further investigate the mechanism by which apatinib inhibits xenografts, changes were observed in the levels of the relevant signaling pathway proteins. Apatinib can reduce neovascularization, inhibit proliferation, promote apoptosis and effectively control the growth rate of xenografts in nude mice through multiple cell pathways. Studies have reported that the most important signaling pathways are AKT and ERK [27]. Apatinib can effectively reduce the phosphorylation level of key proteins in the above signaling pathways, thereby further reducing the phosphorylation of eukaryotic initiation factor 4E1, which plays an important role in promoting tumor cell proliferation and inhibiting apoptosis and neovascularization [28]. Apatinib reduces the phosphorylation level of 4E1, thereby effectively inhibiting tumor proliferation and promoting apoptosis. Additionally, the phosphorylation levels of key proteins in the above signaling pathways were reduced, which can reduce the expression of hypoxia-inducible factor 1α. When the level of HIF1α is decreased, the level of VEGF secreted by tumor cells decreases, effectively inhibiting the formation of new blood vessels [29]. The results confirm that apatinib can inhibit tumor growth through multiple signaling pathways, and key proteins on these pathways can become targets for new anti-tumor drugs.

This study found that apatinib has obvious effects on H1975 and H446 both in vitro and in vivo. Therefore, patients with EGFR expression accompanied by L858R and T790M mutations and small cell lung cancer may benefit from small molecule anti-angiogenic drugs acting on VEGFR such as apatinib. At present, in patients with EGFR accompanied by L858R and T790M mutations, osimertinib is the first choice [30]. Apatinib was more effective for the H1975 cell line, but whether osimertinib combined with apatinib would be more effective remains to be confirmed in clinical studies. Significantly, for patients with resistance to osimertinib with L858R/T790M/C797S mutations [31], apatinib may be beneficial, which will require more clinical studies to confirm.
Effect of apatinib on lung cancer cells

Small cell lung cancer accounts for approximately 15% of lung cancer [32]. Compared with non-small-cell lung cancer, the biological behavior of small cell lung cancer is more aggressive. There are many driving genes in non-small-cell lung cancer, and targeted therapy can be selected under the guidance of molecular markers [33]. However, the pathogenesis and driving genes of small cell lung cancer are not clear yet, and targeted therapy is not applicable [34]. At present, relevant basic research via genome sequencing has determined that small cell lung cancer has changes in signaling pathway genes, but it is not clear whether these are driving genes. Treating small cell lung cancer remains difficult and improving the survival of patients with small cell lung cancer is urgently needed in clinical practice. Currently, the main first-line chemotherapy for small cell lung cancer is etoposide combined with platinum. Although early efficacy is obvious, the long-term effect is poor, and the overall median survival time of patients is shorter [35]. Can anti-angiogenic drugs be applied to the treatment of small cell lung cancer? From the results of our study, small cell lung cancer cells express high levels of VEGFR-2, and the results confirm that apatinib had a good inhibitory effect on small cell lung cancer in vitro and in vivo. Clinical cases have reported that apatinib has an effect on small cell lung cancer [36]. In ongoing clinical research, apatinib monotherapy or combination chemotherapy and radiotherapy for small cell lung cancer have gradually been extended to second-line or first-line clinical research, seeking a new breakthrough for the treatment of small cell lung cancer patients.

Apatinib has an inhibitory effect on lung cancer cells with high VEGFR-2 expression, but its safety and damage to major organs have not been reported. The main side effects of apatinib are hypertension, proteinuria, fatigue, and hand-foot syndrome in clinical trials [37]. The overall tolerance of the animals was acceptable, and there was no significant difference in body weight. HE staining of the heart, liver, and kidney tissues showed no obvious pathological damage under a light microscope. However, under transmission electron microscopy, the ultrastructure of these organs was damaged to some extent, and the damage was more obvious in the high dose group, including mitochondria of cardiomyocytes, hepatocytes, and renal tubular epithelial cells beginning to vacuolize, mitochondrial mites becoming destroyed, part of the endoplasmic reticulum expanding, and the nucleus appearing pyknotic. The results indicated that the application of apatinib resulted in damage to some organs, but under the current dose and times, there was no significant pathological damage under light microscope or changes in liver or kidney function or myocardial enzymes. However, attention should be paid to the damage of these organs after long-term treatment. For endocrine glands, the pancreas and thyroid were chosen for observation. There was still no obvious pathological damage under light microscope, but under the electron microscope, the endoplasmic reticulum system showed obvious expansion, some mitochondrial damage was obvious, and blood glucose and thyroid hormone levels also changed significantly in the high dose group. These findings indicated that endocrine gland damage by apatinib is more obvious and worthy of clinical attention. Studies have found that anti-angiogenic therapy drugs cause different degrees of damage to different organs, which may be related to the expression of VEGFR in different vascular endothelial cells. Endocrine glands express higher levels of VEGFR and are more likely to be damaged [38]. Clinical treatments must focus on the function of these organs.

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Address correspondence to: Yu Li, Department of Pulmonary Medicine, Qilu Hospital, Shandong University, 107 West Wenhua Road, Jinan 250012, Shandong, China. Tel: 86-15854385306; Fax: 86-053182169114; E-mail: 842764917@qq.com

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