

Regulation of angiogenic factors by the PI3K/Akt pathway in A549 lung cancer cells under hypoxic conditions

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Abstract. The aim of the present study was to investigate the influence of hypoxia and PI3K inhibition on angiogenic factors in A549 lung cancer cells. A549 cells were treated with the PI3K inhibitor LY294002 under normoxic and hypoxic conditions. Untreated cells were used as the control group and those treated by the inhibitor, as the suppression group. The cells were further divided based on normoxic or hypoxic conditions and named: Normoxic control group, normoxic suppression group, hypoxic control group and hypoxic suppression group. Expression levels of hypoxia-inducible factor (HIF)-1 α and AKT1 mRNA in all groups were determined by reverse transcriptase-quantitative polymerase chain reaction and concentrations of vascular endothelial growth factor (VEGF), angiotensin II (ANG-II), transforming growth factor (TGF)- α/β 1, and tumor necrosis factor (TNF)- α in the culture supernatant were measured by enzyme-linked immunosorbent assay. The expression levels of HIF-1 α and AKT1 mRNA in the hypoxic control group were higher than those in the normoxic control group and the expression levels of HIF-1 α and AKT1 mRNA in the hypoxic control group were higher than those in the hypoxic suppression group. Compared to the normoxic control and normoxic suppression groups, the concentrations of VEGF and TNF- α in supernatant were higher in the hypoxic control and hypoxic suppression groups,

respectively. However, TGF- α and TGF- β 1 demonstrated the opposite trend of the aforementioned factors. The concentration of ANG-II in the hypoxic suppression group was higher than that in the normoxic suppression group. In addition, compared to the normoxic control group and hypoxic control group, the concentrations of VEGF and TGF- β 1 in supernatant were lower in the normoxic suppression group and in the hypoxic suppression group, respectively. In conclusion, the results of the present study suggest that hypoxia can stimulate A549 lung cancer cells to secrete VEGF and TNF- α and inhibit TGF- α and TGF- β 1. The ability of A549 cells to secrete VEGF and TGF- β 1 is regulated by PI3K/Akt, and ANG-II expression may be regulated by the PI3K/Akt pathway under hypoxic condition.

Introduction

Angiogenesis plays a vital role in the growth and metastasis of lung cancer. It has been reported that vascular endothelial growth factor (VEGF), as the strongest angiogenic factor, not only acts on the proliferation and differentiation of endothelial cells but also as a chemotactic factor for directional movement of activated monocytes to a site of inflammation and tumor growth (1,2).

It is clear that hypoxia-inducible factor (HIF)-1 α regulates VEGF protein synthesis through the PI3K pathway and the hypoxia-activated PI3K/Akt/mTOR pathway (3). Transforming growth factor (TGF)- β 1 can induce EMT and enhance tumor metastasis (4). There are many reports on the PI3K/Akt signaling pathway and angiogenesis in A549 lung cancer cells, whereas reports on regulation of angiogenic factors [i.e., angiotensin II (ANG-II), TGF- β 1 or tumor necrosis factor (TNF)- α] by PI3K/Akt signaling and the effect of PI3K inhibition is limited. Thus, in the present study, we used the PI3K inhibitor LY294002 on A549 cells under normoxic and hypoxic conditions. The migratory ability of A549 cells was determined by scratch assay. The levels of HIF-1 α and AKT1 mRNA were determined by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and concentrations of VEGF, ANG-II, TGF- α/β 1 and TNF- α in the culture supernatant were measured by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). The findings of the present

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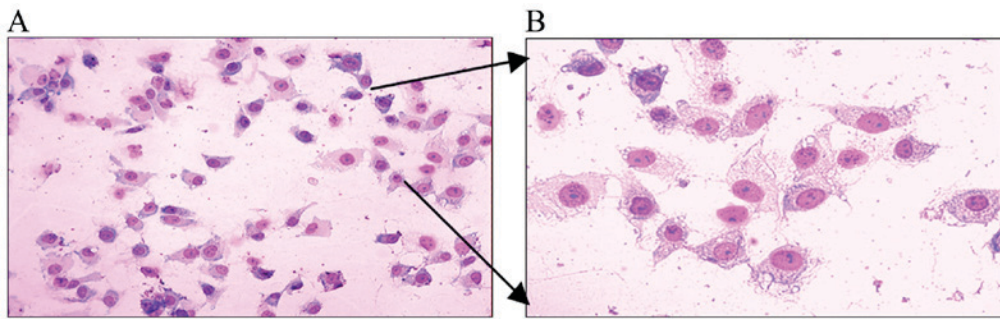


Figure 1. A549 cells stained with Wright-Giemsa. Cells demonstrate epithelial cell-like adherent growth. Magnification, (A) x40, (B) x100.

study provide a theoretical basis for the prevention and treatment of lung cancer in the hypoxic environment.

Materials and methods

Cell lines and cell culture. The human non-small cell lung cancer A549 cell line, was a gift from the Central Laboratory of Basic Medical Sciences, Fourth Military Medical University (Xi'an, China). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Sijiqing Biotechnology Co., Hangzhou, China) and penicillin/streptomycin (Ybiotech, Shanghai, China). The cells were maintained at 37°C in an incubator with 5% CO₂ and medium was changed every 2-3 days, then divided into 2-3 culture flasks. Logarithmic growth phase cells were used for subsequent experiments. For hypoxic exposure, the cells were placed in an incubator chamber that was tightly sealed and thoroughly flushed with 1% O₂/5% CO₂, and balance N₂ and incubated at 37°C. PI3K inhibitor, LY294002 (Beyotime Biotech, Jiangsu, China) was added to the medium at a final concentration of 30 μM (5). Untreated cells were taken as the control group and cells treated by the inhibitor as the suppression group. The cells were then cultured under normoxic or hypoxic conditions and termed normoxic control group, normoxic suppression group, hypoxic control group and hypoxic suppression group, respectively. The cells were collected after 48 h culture in the incubator. Experiments were repeated 3 times.

Observation of morphology and migration. A549 cell morphology was observed by Wright-Giemsa stain. The migratory ability of A549 cells was determined by scratch assay at the 0, 6 and 20 h time-points, when cultured under normoxic and hypoxic conditions, under an inverted fluorescence microscope (Olympus, Tokyo, Japan).

RNA extraction, reverse transcription and quantitative PCR. Total RNA was extracted from cell samples with TRIzol reagent (Ambion Life Technologies, Carlsbad, CA, USA) and quantified with a NanoDrop 2000 Spectrophotometer (Thermo Scientific Inc., Bremen, Germany). The first strand cDNA was synthesized by M-MLV reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The target mRNAs in cultured A549 cells were quantified by RT-qPCR using TransScript First-Strand cDNA Synthesis Super Mix (TransGen Biotech Co., Ltd., Beijing, China) using the AB/Applied Biosystems 7500

Real-Time PCR Detection System (Applied Biosystems Life Technologies, Foster City, CA, USA). Each PCR reaction was performed in triplex tubes, and GAPDH was used as an endogenous control to standardize the amount of sample mRNA. The total reaction volume was 20 μl and thermal profile was as follows; two-step PCR amplification, pre-denaturing: 95°C for 30 sec; 95°C for 5 sec, and 60°C annealing for 31 sec, for a total of 40 cycles. The raw data were analyzed with iQ5 software (Bio-Rad, Berkeley, CA, USA) (6). The primers [Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China)] used for qPCR were: HIF-1α forward, 5'-ATACATGGTACCCACGAAGTG TTCCTTTG-3' and reverse, 5'-ATACATCTCGAGAAA GAG ACAAGTCCA-3'; AKT1 forward, 5'-ATGAGCGACGTGGCT ATTGT-3' and reverse, 5'-TGAAGGTGCCATCATTCTTG-3'; GAPDH forward, 5'-ATCAAGAAGGTGGTGAAGCA-3' and reverse, 5'-CAAAGGTGGAGGAGTGGGT-3'.

ELISA. The concentrations of VEGF, ANG-II, TGF-α/β1 and TNF-α in the culture supernatant were determined by ELISA according to the human ELISA kit instructions (Xinbosheng Biotechnology Co., Ltd., Beijing, China).

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation and were analyzed with Student's t-test (two-tailed). P<0.05 was considered to indicate a statistically significant difference.

Results

Cell morphology and the effect of hypoxia and LY294002 on the migration of A549 cells. A549 cells were stained with Wright-Giemsa and appeared as having epithelial cell-like adherent growth [Fig. 1A (x40 magnification) and B (x100 magnification)]. Whether under normoxic or hypoxic conditions, scratch wounds were completely filled after 20 h in untreated cells. By contrast, the scratch wounds were not completely filled after 20 h when the cells were treated with LY294002 (Fig. 2).

Effect of hypoxia and LY294002 on HIF-1α and AKT1 mRNA expression. Compared to the normoxic control group, the levels of HIF-1α and AKT1 mRNA were higher in the hypoxic control group. However, compared to the hypoxic suppression group, the levels of HIF-1α and AKT1 mRNA were higher than in the hypoxic control group (Table I and Fig. 3).

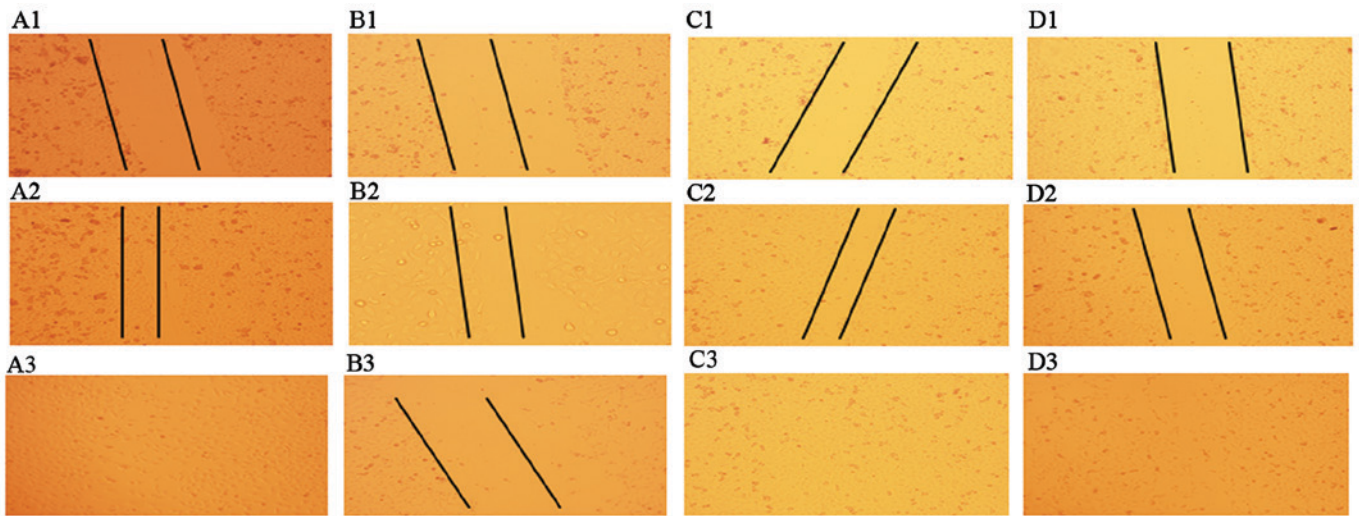


Figure 2. The hypoxia and PI3K inhibitor LY294002 effects in the migration of A549 cells (magnification, x10). (A1-A3) Normoxic control group, (B1-B3) normoxic suppression group, (C1-C3) hypoxic control group, and (D1-D3) hypoxic suppression group at 0, 6 and 20 h, respectively. Whether under normoxic or hypoxic condition, the scratch wound was completely filled by cells after 20 h when untreated. Scratch wounds were incomplete after 20 h when treated with LY294002.

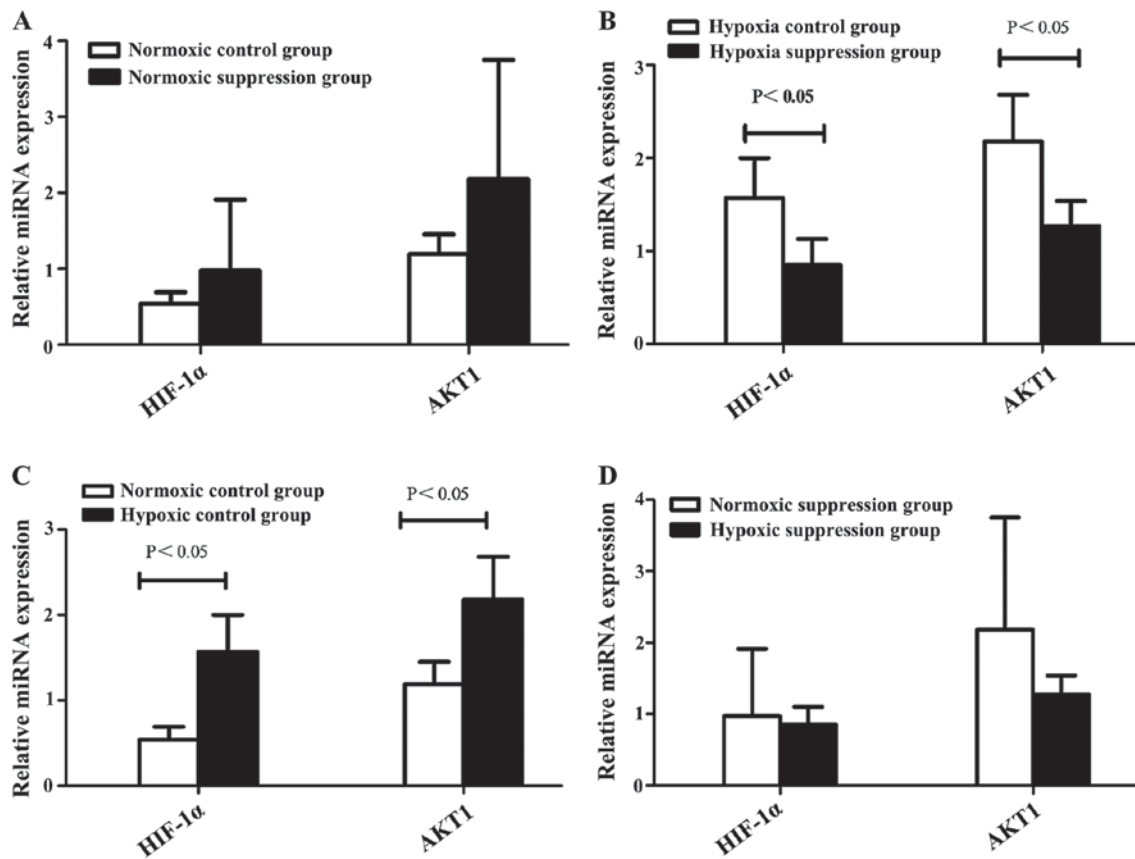


Figure 3. The effect of hypoxia and LY294002 on the expression of hypoxia-inducible factor (HIF)-1 α and AKT1 mRNA. (A) The expression levels of HIF-1 α and AKT1 mRNA in the normoxic control group and normoxic suppression group had no significant differences ($P > 0.05$). (B) The expression levels of HIF-1 α and AKT1 mRNA in the hypoxic suppression group were significantly lower than in the hypoxic control group ($P < 0.05$). (C) The expression levels of HIF-1 α and AKT1 mRNA in the hypoxic control group were significantly higher than in the normoxic control group ($P < 0.05$). (D) The expression levels of HIF-1 α and AKT1 mRNA in the normoxic suppression group and hypoxic suppression group were not significantly different ($P > 0.05$).

Effect of hypoxia and LY294002 on levels of angiogenic factors. Compared to the normoxic control group and normoxic suppression group, the concentrations of VEGF and TNF- α in culture supernatant were higher in the hypoxic

control group and hypoxic suppression group. However, TGF- α and TGF- β 1 showed an opposite trend of expression. The concentration of ANG-II in the hypoxic suppression group was higher than in the normoxic suppression group.

Table I. Effect of hypoxia and PI3K inhibitor LY294002 on HIF-1 α and AKT1 mRNA expression (mean \pm SD, n=9).

Group	Normoxic		Hypoxic	
	HIF-1 α	AKT1	HIF-1 α	AKT1
Control	0.54 \pm 0.15	1.19 \pm 0.26	1.57 \pm 0.43 ^a	2.18 \pm 0.50 ^a
Suppression	0.97 \pm 0.94	2.18 \pm 1.57	0.85 \pm 0.28	1.27 \pm 0.27
t-test	-1.050	-1.469	0.3716	5.183
P-value	0.353	0.216	0.021	0.007

^aCompared to normoxic control group P<0.05. SD, standard deviation; HIF-1 α , hypoxia-inducible factor-1 α .

Table II. Effect of hypoxia and PI3K inhibitor LY294002 on the concentrations of angiogenic factors (mean \pm SD, n=9).

Group	Items	Control	Suppression	t-test	P-value
Normoxic	VEGF (pg/ml)	468.26 \pm 9.93	360.59 \pm 24.50	13.670	0.000
	TNF- α (pg/ml)	10.34 \pm 2.89	9.22 \pm 1.94	1.148	0.281
	TGF- α (ng/ml)	520.93 \pm 90.74	541.49 \pm 60.64	-1.208	0.258
	ANG-II (ng/ml)	12.66 \pm 4.39	14.67 \pm 7.29	-1.666	0.130
	TGF- β 1 (pg/ml)	242.07 \pm 40.31	54.49 \pm 19.28	14.425	0.000
Hypoxic	VEGF (pg/ml)	502.90 \pm 23.90 ^a	457.83 \pm 44.82 ^a	3.565	0.007
	TNF- α (pg/ml)	16.88 \pm 3.84 ^a	17.40 \pm 3.49 ^a	-0.321	0.756
	TGF- α (ng/ml)	471.21 \pm 62.82 ^a	504.04 \pm 52.58 ^a	-2.091	0.066
	ANG-II (ng/ml)	14.66 \pm 5.25	16.47 \pm 6.67 ^a	-1.715	0.121
	TGF- β 1 (pg/ml)	32.32 \pm 2.58 ^a			

^aCompared to normoxic group P<0.05 (TGF- β 1 was lower than the minimal detection concentration indicated by the kit). SD, standard deviation, VEGF, vascular endothelial growth factor; TNF- α , tumor necrosis factor- α ; TGF, transforming growth factor; ANG-II, angiotensin II.

In addition, compared to the normoxic control group and hypoxic control group, the concentrations of VEGF and TGF- β 1 in supernatant were lower in the normoxic suppression group and in the hypoxic suppression group, respectively (Table II and Fig. 4).

Discussion

Lung cancer ranks as the primary cause of cancer death worldwide, and is the most commonly diagnosed cancer worldwide. In 2012, there were 1.8 million lung cancer diagnoses representing 13% of the total (7). The most common cause of death in 2012 was lung cancer. The number of deaths from lung cancer were 1.6 million and this represented 19.4% of total deaths in 2012 (7). Previous studies have demonstrated that the biological behavior of solid tumor growth includes invasion and metastasis as well as tumor-related angiogenesis and remodeling. The PI3Ks are a family of lipid kinases whose primary biochemical function is to generate second messengers by phosphorylating the 3-hydroxyl group of phosphatidylinositols (8). Akt (protein kinase B) is a serine/threonine kinase activated downstream of PI3K- α , that is involved in promoting cell differentiation, inhibition of cell death and other important biological functions (8). Studies have shown that the overexpression

rate of PI3K/Akt pathway was 84.75% in non-small cell lung cancer and was related with high proliferative activity of tumors (9). The results of the present study demonstrate that A549 cell migration was not significantly affected by hypoxia, while migration after treatment with LY294002 significantly decreased. Although hypoxia had no effect on the migration of A549 cells, RT-qPCR showed that hypoxia increased levels of HIF-1 α and AKT1 mRNA and treatment with LY294002 reduced the levels of HIF-1 α and AKT1 under hypoxic conditions. However, there were no such changes under normoxic conditions. These findings suggest that hypoxia can activate PI3K/Akt signaling in A549 cells and the migratory ability of these cells is related to the PI3K/Akt pathway (3).

More significant is the observation that hypoxia stimulated A549 cells to secrete VEGF and TNF- α and reduce the expression of TGF- α and TGF- β 1. ANG-II displayed a trend of increasing in the hypoxic control group compared to the normoxic control group, but there was no statistically significant difference. Hypoxia stimulated A549 cells treated by LY294002 to secrete VEGF and TNF- α and to reduce expression of TGF- α and TGF- β 1, while increasing the secretion of ANG-II. This indicates that hypoxia can stimulate A549 cells to secrete VEGF and TNF- α and to inhibit TGF- α and TGF- β 1. The ability of A549 cells to secrete VEGF

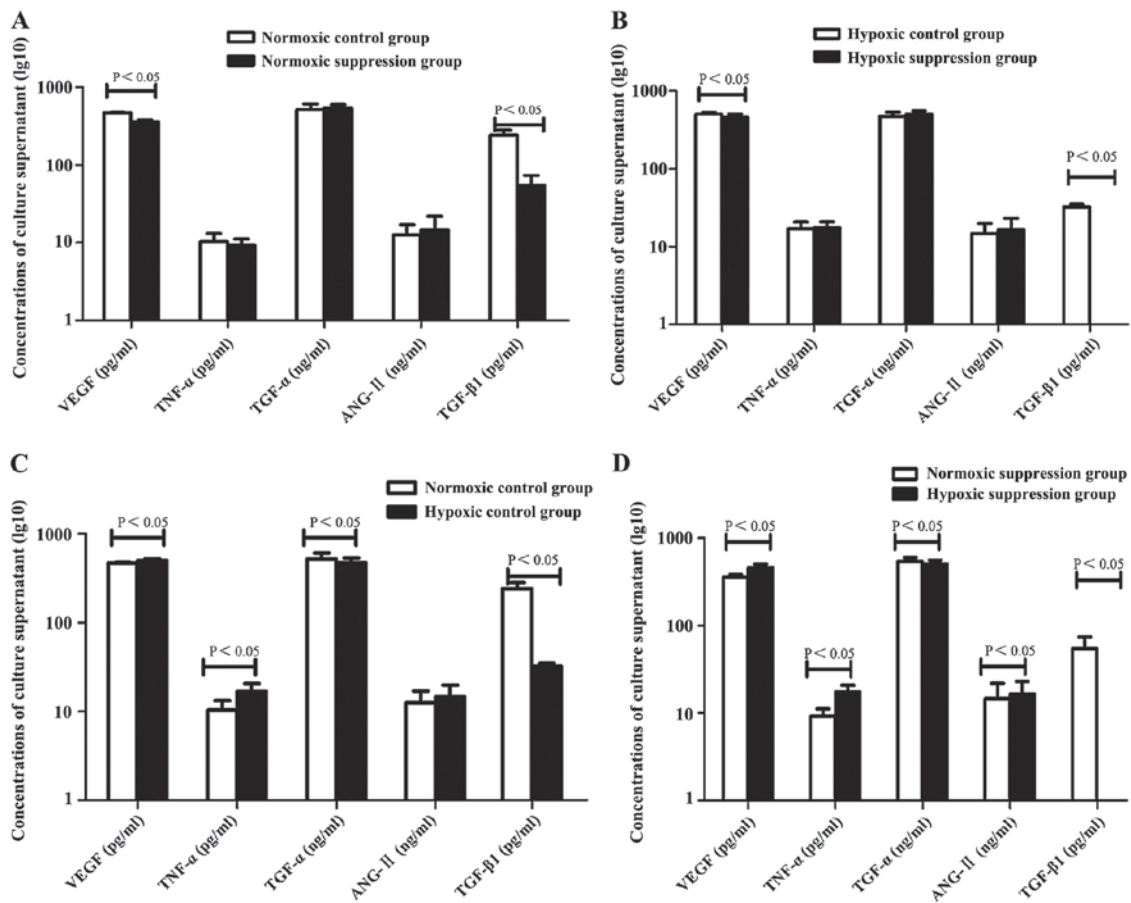


Figure 4. Effect of hypoxia and LY294002 on the concentration of angiogenic factors. (A) The concentrations of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)- β 1 in the normoxic suppression group were significantly lower than in the normoxic control group ($P < 0.05$). (B) The concentrations of VEGF and TGF- β 1 in the hypoxic suppression group were significantly lower than in the hypoxic control group ($P < 0.05$). (C) The concentrations of VEGF and TNF- α in the normoxic control group were significantly lower than in the hypoxic control group ($P < 0.05$), whereas TGF- α and TGF- β 1 showed opposite trends of expression. (D) The concentrations of VEGF, TNF- α and angiotensin II (ANG-II) in the normoxic suppression group were lower than in the hypoxic suppression group, but TGF- α and TGF- β 1 showed the opposite trend of the afore-mentioned factors ($P < 0.05$).

and TGF- β 1 is partially regulated by PI3K/Akt and ANG-II expression may be dependent on the PI3K/Akt pathway under hypoxic conditions. The present study shows that the PI3K/Akt signaling pathway is related to invasion and metastasis of lung cancer cells (3,10,11), and VEGF plays an important role in angiogenesis and invasion.

A549 cells treated with the PI3K/AKT inhibitor, LY294002 *in vitro*, under normoxic or hypoxic conditions, were significantly inhibited in their ability to secrete VEGF and TGF- β 1, and it was more pronounced under normoxic conditions. The levels of TGF- β 1 in A549 cell supernatant after treatment with LY294002 were below the lower detection limit of the ELISA under hypoxic conditions. This indicates that the PI3K/Akt signaling pathway affected more than the levels of VEGF and TGF- β 1. Studies have shown that VEGF also activates PI3K/Akt/Forkhead signaling to inhibit apoptosis, promote DNA synthesis and transition from G1 to S phase in endothelial cells (12). In addition to angiogenesis, research suggests that the phenomenon of vascular mimicry was a part of cancer pathogenesis in lung tissue (13,14). This was related to patient prognosis. Together with high expression of matrix metalloproteinases, degradation of the extracellular matrix in highly malignant tumor cells in a hypoxic microenvironment

formed a vessel-like structure. PI3K inhibitors also inhibited the ability to form pipeline tumor cells connected to each other by inhibiting matrix metalloproteinase (MMP)-2 and MMP-9 and extracellular matrix degradation, which inhibited vasculogenic mimicry (15,16). These observations suggested that tumor angiogenesis was related to a number of factors. There is insufficient evidence that targeting VEGF or the VEGF receptor has a therapeutic effect related to the PI3K/Akt pathway (17-19).

However, other studies reported that large doses of LY294002 did not completely block VEGF transcription, suggesting that other factors are involved in the regulation of VEGF expression. Multiple signaling pathways communicate with each other, thus forming a signaling network. This phenomenon is limiting in regards to the efficacy of a single target drug to have an effect. Previous findings have also shown that when microvascular lung endothelial cells and squamous or adenocarcinoma lung cancer cells are co-cultured *in vitro*, this increased the blood supply to each other, suggesting that non-angiogenic factors cannot be ignored in tumor therapy (20). Thus, the effect of single target tumor therapy has limitations. In practice, we need to consider both tumor molecular biology and pathology in order

to select targeted drugs to achieve individualized treatment and improve efficacy.

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