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

YOUNG BANG XIE1,2*, YALI QI2, YANMIAO ZHANG1,2, JIAIYI CHEN1,2, TIANYI WU3 and YUHAI GU2*

1Research Center for High Altitude Medicine, Medical College of Qinghai University, Xining, Qinghai 810001; 2Department of Respiratory Medicine, Qinghai Provincial People's Hospital, Xining, Qinghai 810007; 3National Key Laboratory of High Altitude Medicine, High Altitude Medical Research Institute, Xining, Qinghai 810000, P.R. China

Received May 5, 2016; Accepted November 2, 2016

DOI: 10.3892/ol.2017.5811

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. The cells were further divided based on normoxic or hypoxic conditions and named: Normoxic control group, normoxic hypoxia suppression group, hypoxic control group and hypoxic hypoxia suppression group. Expression levels of hypoxia-inducible factor (HIF)-1α 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/β1, and tumor necrosis factor (TNF)-α in the culture supernatant were measured by enzyme-linked immunosorbent assay. The expression levels of HIF-1α and AKT1 mRNA in the hypoxic control group were higher than those in the normoxic control group and the expression levels of HIF-1α and AKT1 mRNA in the hypoxic control group were higher than those in the hypoxic suppression group. Compared to the normoxic control and normoxic hypoxia suppression groups, the concentrations of VEGF and TNF-α in supernatant were higher in the hypoxic control and hypoxic hypoxia suppression groups, respectively. However, TGF-α and TGF-β1 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-β1 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-β1. The ability of A549 cells to secrete VEGF and TGF-β1 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)-1α regulates VEGF protein synthesis through the PI3K pathway and the hypoxia-activated PI3K/Akt/mTOR pathway (3). Transforming growth factor (TGF)-β1 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-β1 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-1α and AKT1 mRNA were determined by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and concentrations of VEGF, ANG-II, TGF-α/β1 and TNF-α in the culture supernatant were measured by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). The findings of the present
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 or 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'-ATACATGGTACCCACGAAAGTG TTCCTTTG-3' and reverse, 5'-ATACATCTCGAGAAAG GAG ACAAGTCCA-3'; AKT1 forward, 5'-ATGAGCGACGTGGCT ATTGT-3' and reverse, 5'-TGAAGGTGCCATCATTTCTTG-3'; GAPDH forward, 5'-ATCAAAGAAGTGGTGAAGCA-3' and reverse, 5'-CAAAGGTGAGAGGTGTTG-3'..

ELISA. The concentrations of VEGF, ANG-II, TGF-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).
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-β1 showed an opposite trend of expression. The concentration of ANG-II in the hypoxic suppression group was higher than in the normoxic suppression group.
In addition, compared to the normoxic control group and hypoxic control group, the concentrations of VEGF and TGF-β 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-1α and AKT1 mRNA and treatment with LY294002 reduced the levels of HIF-1α 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-β1. 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-β1, while increasing the secretion of ANG-II. This indicates that hypoxia can stimulate A549 cells to secrete VEGF and inhibit TGF-α and TGF-β1. The ability of A549 cells to secrete VEGF

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

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

| Group | Items | Control | Suppression | t-test | P-value |
|-------|-------|---------|-------------|--------|---------|
| Normoxic | VEGF (pg/ml) | 468.26±9.93 | 360.59±24.50 | 13.670 | 0.000 |
| | TNF-α (pg/ml) | 10.34±2.89 | 9.22±1.94 | 1.148 | 0.281 |
| | TGF-α (ng/ml) | 520.93±90.74 | 541.49±60.64 | -1.050 | 0.258 |
| | ANG-II (ng/ml) | 12.66±4.39 | 14.67±7.29 | -1.208 | 0.130 |
| | TGF-β1 (pg/ml) | 242.07±40.31 | 54.49±19.28 | -1.208 | 0.066 |
| Hypoxic | VEGF (pg/ml) | 502.90±23.90 | 457.83±44.82 | 3.565 | 0.007 |
| | TNF-α (pg/ml) | 16.88±3.84 | 17.40±3.49 | -0.321 | 0.756 |
| | TGF-α (ng/ml) | 471.21±62.82 | 504.04±52.58 | -2.091 | 0.066 |
| | ANG-II (ng/ml) | 14.66±5.25 | 16.47±6.67 | -1.715 | 0.121 |
| | TGF-β1 (pg/ml) | 32.33±2.58 |          |        |        |

*Compared to normoxic group P<0.05 (TGF-β1 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.
and TGF-β1 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-β1, and it was more pronounced under normoxic conditions. The levels of TGF-β1 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-β1. 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 vascular 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.

Acknowledgements

The present study was funded by Foundation Research Project of Qinghai Provincial Health and Family Planning Commission and Qinghai Province Key Specialty (respiratory).

References

1. Nourse MB, Halpin DE, Scatena M, et al: VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering. Arterioscler Thromb Vasc Biol 30: 80‑89, 2010.

2. Avraham ‑Davidi I, Yona S, Grunewald L, Landsman L, Cochain C, Silvestre JS, Mizrahi H, Faroja M, Strauss‑Ayali D, Mack M, et al: On‑site education of VEGF‑recruited monocytes improves their performance as angiogenic and arteriogenic accessory cells. J Exp Med, 210: 2611‑2625, 2013.

3. Kim YJ, Choi WI, Jeon BN, Choi KC, Kim K, Kim TJ, Ham J, Jang HJ, Kang KS and Ko H: Stereospecific effects of ginsenoside 20‑Rg3 inhibits TGF‑β1‑induced epithelial‑mesenchymal transition and suppresses lung cancer migration, invasion and anoikis resistance. Toxicology 322: 23‑33, 2014.

4. Fang Z: Study on PI3K as an anti‑cancer angiogenesis and vascular mimicry common targets (unpublished PhD thesis). Huazhong University of Science and Technology, 2010.

5. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real‑time quantitative PCR and the 2(‑Delta Delta C(T)) method. Methods 25: 402‑408, 2001.

6. World Health Organization: Latest world cancer statistics: Global cancer burden rises to 14.1 million new cases in 2012: Marked increase in breast cancers must be addressed. WHO, Geneva, 2013.

7. Cantley LC: The phosphoinositide 3‑kinase pathway. Science 296: 1655‑1657, 2002.

8. Liao DW, Wang L, Zhang XG and Liu MQ: Expression and significance of PTEN/PI3K signal transduction‑related proteins in non‑small cell lung cancer. Ai Zheng 25: 1238‑1242, 2006 (In Chinese).