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MiR-15a expression analysis in non-small cell lung cancer A549 cells under local hypoxia microenvironment

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Abstract. – OBJECTIVE: Lung cancer is a common tumor in the clinic. Hypoxia is an important biological characteristic in the solid malignant tumor. MiRNA participates in cell proliferation, differentiation, and apoptosis. This study tested hypoxia-inducible factor 1-α (HIF-1α) in lung cancer patients and analyzed the microRNA-15a (miR-15a) expression in A549 cells under different local hypoxia microenvironments.

PATIENTS AND METHODS: A total of 40 non-small cell lung cancer (NSCLC) patients in First Affiliated Hospital of Zhengzhou University between Jan 2015 and Jan 2016 were involved in this study. The serum and tissue samples of lung cancer were collected. Serum HIF-1α level was tested by enzyme-linked immunosorbent assay (ELISA) assay. HIF-1α expression in tissue was evaluated by using the immunohistochemistry. A549 cells were cultured under normoxic, hypoxic, and anaerobic environment, respectively. HIF-1α mRNA and miR-15a levels were determined by RT-PCR.

RESULTS: HIF-1α levels were up-regulated in serum and tissue (*p*<0.05). HIF-1α mRNA increased, while miR-15a down-regulated in A549 from hypoxia and anaerobic groups compared with control (*p*<0.05). HIF-1α shRNA transfection significantly reduced HIF-1α and elevated miR-15a level (*p*<0.05). MiR-15a shRNA transfection exhibited no statistical impact on HIF-1α expression (*p*>0.05).

CONCLUSIONS: HIF-1α highly expressed in lung cancer patients. MiR-15a levels were down-regulated in A549 cells under hypoxia and anaerobic conditions.

Key Words

Lung cancer; A549; HIF-1α; miR-15a.

Introduction

Lung cancer shows the leading mortality among different malignant tumors. In spite of the continuous updating of lung cancer associated protein and gene research, early diagnosis and effective treatment for lung cancer still need further investigation, which is also the major cause of the rising incidence of lung cancer[1]. MiRNAs generate certain inhibition on gene transcription, thus involving in cell development and differentiation. MiRNAs exhibit tissue specificity. Several miRNAs show changes in lung cancer and may participate in the occurrence and development of lung cancer, such as Let-7, Let-34, Let-21, Let-143, Let-145, Let-31, and Let-146[2,3]. Hypoxia is a characteristic of solid tumor growth. Excessive growth of malignant tumor leads to insufficient blood and oxygen supply. It causes the change of cell structure and function. Moreover, hypoxia-induced physiological and pathological processes also influence miRNA expression[4-6]. This work tested HIF-1α expression in lung cancer tissue. Furthermore, A549 cells were cultured under the normoxic and hypoxic environments to detect miR-15a expression. HIF-1α mRNA and miR-15a plasmids were transfected with A549 cells to evaluate HIF-1α mRNA and miR-15a expressions. In addition, miR-15a expression in lung cancer under different local hypoxia microenvironments was also analyzed. Finally, the relationship between the miR-15a levels and HIF-1α mRNA was explored.

Patients and Methods

Patients

A total of 40 non-small cell lung cancer (NSCLC) patients in the First Affiliated Hospital of Zhengzhou University between Jan 2015 and Jan 2016 were selected. The patients were diagnosed as adenocarcinoma upon laboratory test, imaging, and pathology. Another 20 patients received pulmonary surgery due to trauma were chosen as control. No statistical difference was observed on gender and age between two groups (*p*>0.05).

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This study obtained the informed consent from objects or family member and was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Experimental Reagents and Materials

Human HIF-1α ELISA kit (Catalogue No. CSB-E12112h; CusaBio. Life Science, Wuhan, China), mouse anti-human HIF-1α monoclonal antibody (Catalogue No. ab16066; Abcam Biotech., Cambridge, Massachusetts, USA), rabbit anti-mouse polyclonal antibody (Catalogue No. ab19196; Abcam Biotech., Cambridge, MA, USA) were used in this study, The PCR primers were synthesized by the Sangon Bio. Ltd. (Shanghai, China). HIF-1α shRNA plasmid, and miR-15a shRNA plasmid were bought from TaKaRa (Dalian, China). Absolute ethyl alcohol, paraffin, hematoxylin, and neutral balsam were got from Changzhou Peaks Chemical co., Ltd (Changzhou, China). A549 cell line was obtained from the Basic Medical Institute, Chinese Academy of Medical Sciences (Beijing, China).

ELISA

Fasting venous blood was extracted from the patients. The sample was centrifuged to obtain the supernatant and stored at -80°C. Next, the standard substance was added to the plate. After sampling, washing, developing, and stopping, the plate was read at 450 nm wavelength.

Immunohistochemistry

The tumor tissue and para-carcinoma tissue was fixed in formalin. After embedding and sectioning, the slice was further dewaxed and repaired. Next, the slice was blocked and incubated in primary antibody for 1 h. After incubated in secondary antibody for 10 min, the slice was stained by DAB and stopped. After re-dyeing and sealing, the slice was photographed by computer image system.

Routine Cell Culture

A549 cell line was cultured in RPMI-1640 medium Gibco BRL. Co. Ltd. (Grand Island, N Y, USA) and maintained at 37°C and 5% CO_2.

Hypoxia Cell Culture

A549 cell line was cultured under 37°C, 1% O_2, and 5% CO_2.

Anaerobic cell culture

$CoCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) was used to induce chemical hypoxia condition. The final concentration of $CoCl_2$ was 600 μmol/L. A549 cells were cultured for 6 h to observe cell morphology.

Real-time PCR

Total RNA was extracted from the cells and reverse transcribed to cDNA. PCR reaction was performed at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. The primers used were listed in Table I.

Cell Transfection

The cells were seeded on 96-well plate and cultured for 60% fusion rate. Lipofectamine 2000 was adopted for transient transfection according to the manual. Lipo2000 was added to Opti-MEM for 5 min, while plasmid was added to Opti-MEM for 5 min. They were mixed and added to the plate at 2 ml Opti-MEM and 500 μl mixture per well. HIF-1α and miR-15a expressions were observed.

Statistical Analysis

All data analysis was performed by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Measurement data was depicted as mean ± standard deviation. Enumeration data was compared by ANOVA, while measurement data was compared by Student's *t*-test. $p<0.05$ was considered as statistical significance.

Table I. Primer sequences.

	Gene	Temperature (°C)	Length (bp)
HIF-1α	5'-CACCATGAAGCCTACACTGTGTTTCC-3'	60	561
	5'-TTAAACCATTCGGCAGCAGCGG-3'		
miR-15a	5'-GGGGTAGCTTATCAGACTG-3'	60	486
	5'-AGTGCGTGTCGTGGAGTC-3'		
GAPDH	5'-GCCAAGGTCATCCATGACAA CTTTGG-3'	60	314
	5'-GCCTGCTTCACCACCTTCTTG ATGTC-3'		

Table II. HIF-1α content in the blood.

Group	Cases	HIF-1α (ng/ml)
Experimental group	40	1.47±0.07*
Control	20	0.02±0.01

*$p<0.05$, compared with control.

Results

ELISA Detection of Serum HIF-1α Content

Peripheral venous blood was extracted to test serum HIF-1α content. It was showed that HIF-1α level in the experimental group was significantly higher than that of the control ($p<0.05$, Table II).

Immunohistochemistry Detection of HIF-1α Expression in the Tissue

It was exhibited that the positive rate of HIF-1α in lung cancer tissue reached 75%, which was higher than the para-carcinoma tissue and control ($p<0.05$, Table III, Figure 1).

HIF-1α mRNA and miR-15a expressions in A549 cells under normoxia, hypoxia, and anaerobic conditions

Real-time PCR was applied to test HIF-1α mRNA and miR-15a expressions in A549 cells under normoxic, hypoxic, and anaerobic conditions. HIF-1α mRNA was obviously upregulated, while miR-15a markedly reduced in A549 cells from hypoxia group and anaerobic group compared with normoxia group ($p<0.05$). HIF-1α mRNA gradually elevated, whereas miR-15a gradually declined in A549 cells at 6 h, 12 h, and 24 h ($p<0.05$, Figure 2).

HIF-1α shRNA Transfection Affected HIF-1α mRNA and miR-15a Expressions

HIF-1α shRNA transfection significantly reduced HIF-1α expression and increased miR-15a level compared with control ($p<0.05$, Table IV, Figure 3).

MiR-15a shRNA Transfection Affected HIF-1α and miR-15a Expression

MiR-15a shRNA transfection significantly enhanced miR-15a expression while showed no obvious impact on HIF-1α ($p<0.05$, Table V, Figure 4).

Discussion

The mortality of lung cancer tops the list of a malignant tumor. NSCCL accounts for more than 80% of all lung cancer. In recent years, researchers focused on the investigation about hypoxic

Table III. HIF-1α expression in the tissue.

Group	Cases	HIF-1α expression intensity			
		–	+–++	+++	Positive rate (%)
Experimental group					
Cancer tissue	40	10	19	11	75*#
Para-carcinoma tissue	40	30	10	0	25#
Control	20	17	3	0	15

*$p<0.05$, compared with para-carcinoma tissue.
#$p<0.05$, compared with control.

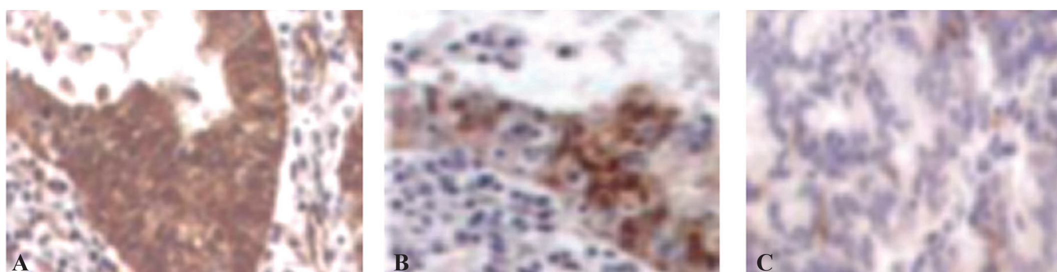

Figure 1. HIF-1α expression in tissue (×400). **A**, HIF-1α expression in cancer tissue. **B**, HIF-1α expression in para-carcinoma tissue. **C**, HIF-1α expression in control.

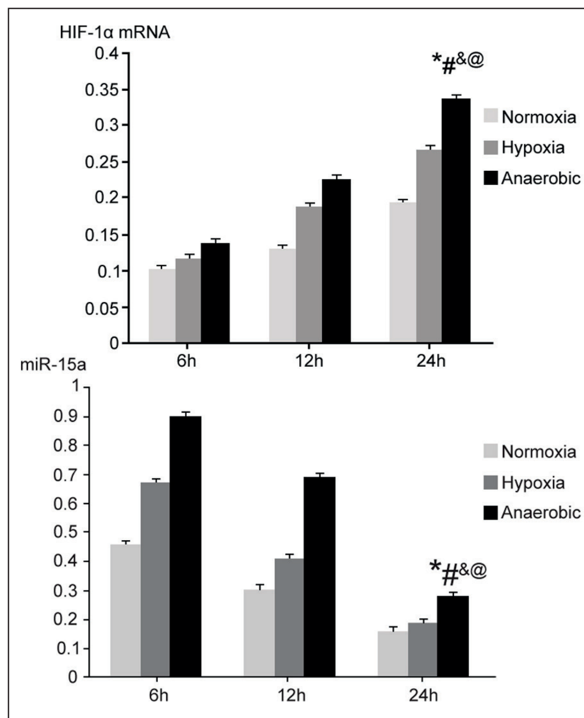

Figure 2. HIF-1α mRNA and miR-15a expressions in A549 cells under normoxia, hypoxia, and anaerobic conditions. * $p < 0.05$, compared with normoxia group. # $p < 0.05$, compared with hypoxia group. & $p < 0.05$, compared with 6 h. @ $p < 0.05$, compared with 12 h.

microenvironment and its impact on lung cancer. However, its specific molecular mechanism, relevant miRNA changes, and signaling pathway have not been fully elucidated[7]. Uncontrollable malignant proliferation is a particular feature of malignant tumor cells, which may generate hypoxia condition. The tumor cells may make corresponding stress, leading to a series of gene transcription and expression participated by HIF-1α[8]. It was pointed out that HIF-1α existed in the form of a heterodimer, whose biological activity was regulated by α subunit[9,10]. In this study, lung cancer serum and tissue samples were collected from our hospital. Serum HIF-1α level was tested. A549 cells were cultured under normoxic, hypoxic, and anaerobic environment.

Figure 3. HIF-1α shRNA transfection affected HIF-1α and miR-15a expression.

HIF-1α mRNA and miR-15a were determined to analyze miR-15a and HIF-1α expressions in A549 cells under different local hypoxia microenvironments and their relationship.

In our study, lung cancer patients were selected as an experimental group, while traumatic patients received pulmonary surgery were chosen as control. Peripheral venous blood was extracted to test serum HIF-1α content. The HIF-1α content was 1.47 ± 0.07 ng/ml, which was higher than the control. Lung tissue was obtained to detect HIF-1α expression in tissue. It was exhibited that the positive rate of HIF-1α in lung cancer tissue reached 75%, which was higher than the para-carcinoma tissue and control. More and more evidences revealed that hypoxia was common in the malignant tumor[11]. HIF-1α keeps expression under hypoxic condition to play its biological activity. It specific regulates oxygen homeostasis. The previous studies reported that HIF-1α widely exited in hypoxic tumor cells that can perform early cell response[12,13]. Our results demonstrated that HIF-1α highly expressed in the serum and tissue of lung cancer patients.

MiRNA can bind with the untranslated region of target mRNA to degrade mRNA[14]. Researches indicated that HIF can regulate a variety of miRNAs. For instance, it can regulate miR-210, miR-199a, and miR-424 expression in the head and neck cancer and pancreatic cancer[15,16]. In this study, A549 cells were cultured under normoxic, hypoxic, and anaerobic conditions. HIF-1α mRNA elevated, while miR-15a downregulated in A549 cells under hypoxia group and anaerobic

Table IV. HIF-1α shRNA transfection affected HIF-1Symbol and miR-15a expression

Group	HIF-1α (ng/ml)	miR-15a
Transfection group	0.082±0. 008*	1.306±0. 024*
Control	0.323±0.004	0.156±0. 015

*$p<0.05$, compared with control.

Table V. MiR-15a shRNA transfection affected HIF-1α and miR-15a expression

Group	HIF-1α (ng/ml)	miR-15a
Transfection group	0.306±0. 019*	1.016±0. 034*
Control	0.331±0.012	0.247±0. 021

*$p<0.05$, compared with control.

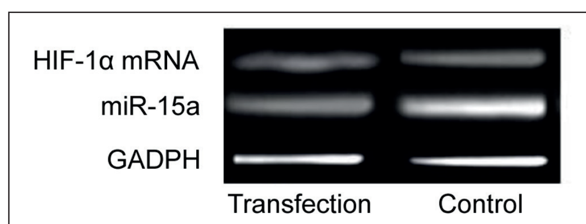

Figure 4. MiR-15a shRNA transfection affected HIF-1α and miR-15a expression.

group. HIF-1α gradually increased, whereas miR-15a gradually declined following time extension. The HIF-1α level was closely associated with oxygen supply. HIF-1α was at a relative low level under sufficient oxygen supply. HIF-1α level increased under hypoxic microenvironment, resulting in the regulation of target gene expression[17]. MiR-15a is a type of miRNA with antitumor effect. MiR-15a downregulates about 68% in chronic lymphocytic leukemia[18-20]. It was suggested that miR-15a downregulation was related to tumor size, TNM stage, relapse, metastasis, and mortality. On the contrary, it exhibited no correlation with gender, tumor site, and differentiation. MiR-15a could be treated as an indicator for the early diagnosis, early treatment, and prognosis of lung cancer. Our results indicated that HIF-1α mRNA increased, while miR-15a reduced in A549 cells under hypoxia and anaerobic conditions with time extension.

HIF-1α shRNA transfection significantly reduced HIF-1α expression and increased miR-15a level compared with control. MiR-15a shRNA transfection significantly enhanced miR-15a expression while showed no impact on HIF-1α. It suggested that HIF-1α can regulate miR-15a, whereas miR-15a cannot regulate HIF-1α. HIF-1α can regulate miR-15a expression in A549 cells.

Conclusions

HIF-1α highly expressed in the serum and tissue of lung cancer patients. HIF-1α mRNA increased, while miR-15a reduced in A549 cells under hypoxia and anaerobic conditions with time extension. MiR-15a was regulated by HIF-1α, while HIF-1α was not affected by miR-15a. HIF-1α can regulate miR-15a expression in A549 cells. MiRNA has a specific advantage in gene regulation, especially in various diseases. Investigations focused on miRNA may provide new prospect on malignant tumor classification and prognosis. HIF-1α and miR-15a can provide new thought and strategy for lung cancer targeted therapy, which shows important reference value for lung cancer early diagnosis and treatment.

Conflict of interest

The Authors declare that they have no conflict of interests.

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