

Identification of crucial microRNAs and genes in hypoxia-induced human lung adenocarcinoma cells

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Background: Variations of microRNA (miRNA) expression profile in hypoxic lung cancer cells have not been studied so far. Therefore, using miRNA microarray technology, this study aimed to study the miRNA expression profile and investigate the potential crucial miRNAs and their target genes in hypoxia-induced human lung adenocarcinoma cells.

Materials and methods: Based on miRNA microarray, miRNA expression profiling of hypoxia-induced lung adenocarcinoma A549 cells was obtained. After identification of differentially expressed miRNAs (DE-miRNAs) in hypoxic cells, target genes of DE-miRNAs were predicted, and functional enrichment analysis of targets was conducted. Furthermore, the expression levels of DE-miRNAs and their target genes were validated by real-time quantitative polymerase chain reaction. In addition, using miRNA mimics, the effect of overexpressed DE-miRNAs on A549 cell behaviors (cell proliferation, cell cycle, and apoptosis) was evaluated.

Results: In total, 14 DE-miRNAs (nine upregulated miRNAs and five downregulated miRNAs) were identified in hypoxic cells, compared with normoxic cells. Target genes of both upregulated and downregulated miRNAs were enriched in the functions such as chromatin modification, and pathways such as Wnt signaling pathway and transforming growth factor (TGF)- β signaling pathway. The expression levels of several miRNAs and their target genes were confirmed, including hsa-miR-301b/*FOXF2*, hsa-miR-148b-3p/*WNT10B*, hsa-miR-769-5p/*SMAD2*, *ARID1A*, and hsa-miR-622. Among them, hsa-miR-301b was verified to regulate *FOXF2*, and hsa-miR-769-5p was verified to modulate *ARID1A*. In addition, the overexpression of hsa-miR-301b and hsa-miR-769-5p significantly affected the cell cycle of A549 cells, but not cell proliferation and apoptosis.

Conclusion: miRNA expression profile was changed in hypoxia-induced lung cancer cells. Those validated miRNAs and genes may play crucial roles in the response of lung cancer cells to hypoxia.

Keywords: hypoxia, lung cancer, A549, microarray, hsa-miR-301b, hsa-miR-769-5p

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Introduction

Lung cancer is a major cause of death worldwide, with 221,200 estimated deaths and 158,040 estimated new cases in the US in 2015.¹ Lung adenocarcinoma is the most common subtype and accounts for ~50% of lung cancer cases.² Despite improvement in medical technology, the mortality of lung cancer is still high.³

Hypoxia is a common feature in almost all of the solid tumors,⁴ and it plays a significant role in the progress of tumors.⁵ In the past years, the molecular variations in hypoxic lung cancer cells have been discovered. In hypoxic lung cancer cells, the truncated voltage-dependent anion channel 1 (VDAC1- Δ C) associated with chemoresistance is expressed, which protects cancer cells from apoptosis,⁶ and *VDAC1* has been identified as a potential mitochondrial marker for cancer therapy.⁷ Furthermore, the expression of

CXCR4 is dramatically enhanced in hypoxic non-small-cell lung cancer, which is modulated by the activation of hypoxia-inducible factor (HIF)-1 α and *PI3K/PTEN/AKT/mTOR* signal transduction pathway.⁸

Except for variations of protein expression, microRNAs (miRNAs) that play critical roles in the coordination of various cellular processes have also been discovered to express abnormally in hypoxic lung cancer cells. Previous studies have found that both miR-155 and miR-210 are highly expressed in hypoxic lung cancer cells, which radioprotect lung cancer cells.^{9,10} However, there is no study to explore the variation of miRNA expression profile in hypoxic lung cancer cells so far, and the regulatory relationships between varied miRNAs and genes in hypoxic lung cancer are still elusive.

In this study, to investigate the differentially expressed miRNAs (DE-miRNAs) in hypoxic lung cancer cells, miRNA expression profile analysis of hypoxia-induced lung adenocarcinoma A549 cells was performed based on miRNA microarray, which is a high-throughput technology to analyze miRNA expression profile.¹¹ Furthermore, to investigate the potential DE-miRNAs-related functions in hypoxic cells, target genes of DE-miRNAs were predicted and functional enrichment analysis of targets was conducted. In addition, expression levels of DE-miRNAs and their target genes were validated by real-time quantitative polymerase chain reaction (RT-qPCR), and the effect of DE-miRNAs on A549 cell behaviors (cell proliferation, cell cycle, and apoptosis) was evaluated. These results may contribute to the study of miRNA expression profile in hypoxia-induced lung cancer cells, and the validated miRNAs and genes may be candidate biomarkers in the clinical therapy of lung cancer.

Materials and methods

Cell culture and establishment of hypoxic cell model

Because a cell line was utilized in this study, the ethical permission obtained from a review board was unnecessary, as confirmed by the Ethic Committee for Use of Human Samples of the Harbin Medical University. The lung adenocarcinoma A549 cell line purchased from the Shanghai Cell Bank, Chinese Science Academy (Shanghai, People's Republic of China) was used in this study. The cells were divided into two groups: one group was cultured in a Biospherix C21 incubator (1% O₂, ProOx C21; Biospherix, NY, USA) at 37°C for 8 hours (hypoxia group) and another group was cultured in a normoxia condition at 37°C in 5% CO₂ for 8 hours (normoxia group). Two group cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's

Medium (DMEM) and Ham F12-medium (DMEM-F12) supplemented with 10% fetal bovine serum and 100 μ g/mL penicillin–streptomycin (1:1). Once cells had reached confluence, they were subcultured with 0.25% Trypsin–ethylenediaminetetraacetic acid.

RNA isolation, purification, and array procedures

The total RNA for miRNA microarray preparation was isolated using mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA), and then purified by miRNeasy Mini Kit (Qiagen, Hilden, Germany). The integrity of RNA was inspected by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

The RNA was reverse transcribed into cDNA after purification. Afterward, according to the manufacturer's instructions, the cDNA was labeled and homogenized. The labeling was performed by miRNA Complete Labeling and Hyb Kit (Agilent Technologies), and hybridization was performed on the human miRNA (8*60K) v19.0 array in a hybridization oven (Agilent Technologies) at 55°C for 20 hours. Subsequently, slides were washed using Gene Expression Wash Buffer Kit (Agilent Technologies). Then, slides were scanned by Feature Extraction software 10.7 (Agilent Technologies) and Agilent Microarray Scanner (Agilent Technologies). Raw microarray data were normalized by a quantile algorithm in Gene Spring Software 11.0 (Agilent Technologies).

Identification of DE-miRNAs

The fold change (FC) model¹² was used to identify miRNAs that were differentially expressed between hypoxia group and normoxia group. The FC value of miRNA expression between two groups was calculated, and miRNAs with FC value >2 were selected as DE-miRNAs.

Prediction of DE-miRNA target genes

Target genes of DE-miRNAs were predicted based on the TargetScan database,¹³ which is a commonly used tool for the prediction of miRNA targets by searching the presence of conserved sites (7mer and 8mer) in the seed region of each miRNA.¹³

Enrichment analysis of DE-miRNA targets

The gene ontology (GO) database¹⁴ and Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY database¹⁵ were used to perform the GO functional and pathway enrichment analyses, respectively, of DE-miRNA target genes.

The *P*-value of each term was calculated by Fisher's exact test¹⁶ and adjusted using the Benjamini–Hochberg method.¹⁷ Only the functional terms with adjusted *P*-value <0.05 were considered significant.

RT-qPCR assay

The total RNA for mRNA expression detection was extracted from A549 cells by TriZol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Afterward, total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Corporation, Fitchburg, WI, USA). After cDNA synthesis, mRNA expression levels were tested using SYBR green qPCR supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA). Primer sequences of genes are listed in Table S1.

Furthermore, the total RNA for miRNA expression detection was isolated using mirVana™ miRNA Isolation Kit and then purified by miRNeasy Mini Kit. miRNA expression levels were determined using miRcute miRNA qPCR Detection Kit (Tiangen, Beijing, People's Republic of China). Primer sequences of miRNAs are listed in Table S2. Expression levels of both mRNA and miRNA were analyzed by a CFX96™ real-time detection system (Bio-Rad Laboratories Inc.). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method,¹⁸ and relative FC of miRNAs was normalized to endogenous 18S rRNA.

Western blot analysis

Approximately 1×10^7 A549 cells from hypoxia group and normoxia group were harvested and lysed in radioimmuno-precipitation assay lysis buffer (1% Triton-100, 0.1% sodium dodecyl sulfate [SDS], 1 mmol/L ethylenediaminetetraacetic acid, 150 mmol/L NaCl, and 10 mmol/L Tris–HCl, pH 7.5) for 30 minutes in ice. Subsequently, the lysate was transferred to 1.5 mL Eppendorf tubes, and then centrifuged at 12,000 rpm for 30 minutes at 4°C. The supernatant was transferred to a fresh tube and mixed with equal volumes of 2× SDS and boiled for 20 minutes. Afterward, an equal volume of sample (containing 50 µg of protein) was fractionated by 10% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membrane for 2 hours. After blocking nonspecific binding sites with 5% skimmed milk in 1× Tris-buffered saline containing 0.1% Tween-20 (TBST, 0.1% Tween 20, 50 mmol/L of Tris, 150 mmol/L of NaCl, pH 7.6) for 1 hour, membranes were probed with anti-HIFα antibody (dilution 1:1,000, product code: 3716; Cell Signaling Technology, Boston, MA, USA) at 4°C overnight, washed five times

with 1× TBST, and incubated with horseradish-peroxidase-conjugated secondary antibody (dilution 1:10,000, product code: 1464325; Hoffman-La Roche Ltd., Basel, Switzerland) for 1 hour at room temperature. The membrane was visualized with the enhanced chemiluminescence Western blotting analysis system (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Then, the protein expression level was quantitated by image densitometry, and the ratio of HIFα/glyceraldehyde-3-phosphate dehydrogenase signal was statistically analyzed, respectively.

Luciferase assay

Based on the 3' untranslated region (3' UTR) sequences of genes in the miRBase database¹⁹ and the University of California Santa Cruz (UCSC) database²⁰, gene primers were designed using the Primer5 software (PRIMER-E Ltd, Plymouth, UK). Primer sequences of 3' UTR in genes are listed in Table S3.

A549 cells were plated in a 12-well plate and transfected with a dual luciferase reporter plasmid containing the 3' UTR sequence of miRNA target gene using Lipofectamine 2000 (Thermo Fisher Scientific). A control empty plasmid was utilized as a negative control for reporter plasmids. Subsequently, miRNA mimics or scrambled controlled miRNA mimics (Shanghai GenePharma Co., Ltd., Shanghai, People's Republic of China) were cotransfected with cells. After transfection for 30 hours, Dual-Luciferase® Reporter Assay (product code: E1910; Promega Corporation) was performed according to the manufacturer's instructions. The “Renilla” luciferase activity was detected using a FLUOstar OPTIMA fluorescence microplate reader (BMG LABTECH, Offenburg, Germany) and normalized to the firefly luciferase activity.

Methyl thiazolyl tetrazolium assay

A549 cells were cotransfected with miRNA mimics or scrambled controlled miRNA mimics in a 96-well plate. After transfection for 24 hours, 48 hours, and 72 hours, methyl thiazolyl tetrazolium at 20 µL was added to each well and incubated with cells for 4 hours. Following 200 µL dimethyl sulfoxide being added in each well, the optical density was measured at 570 nm using a microplate reader (BioTek, Winooski, VT, USA).

Flow cytometry analysis

A549 cells cotransfected with miRNA mimics or scrambled controlled miRNA mimics for 72 hours were washed two times with precooled phosphate-buffered saline and then

incubated with 20 µg/mL RNase A for 30 minutes at 37°C, followed by staining with 10 mg/mL propidium iodide for 30 minutes in the dark at 37°C. Afterward, the samples were evaluated by a flow cytometry (BD Biosciences, San Jose, CA, USA), and the data were analyzed using FlowJo software (Tree Star, San Carlos, CA, USA).

Statistical methods

Statistical analysis was performed using the SPSS 19.0 software (IBM Corporation, Armonk, NY, USA). One-way ANOVA followed by Duncan's test was used to compare the group means.

Results

DE-miRNAs in hypoxic A549 cells

Based on the Western blot analysis, HIFα was expressed in the A549 cells cultured in the hypoxia incubator (1% O₂), but not in cells cultured in the normoxic condition (Figure 1), indicating that the hypoxic A549 cell model was successfully established.

Based on the analysis of miRNA microarray data, a total of 14 miRNAs were differentially expressed between hypoxic cells and normoxic cells, including nine upregulated miRNAs

Table 1 DE-miRNAs in hypoxic A549 cells compared with normoxic cells

Category	Human miRNA name	FC
Upregulated miRNAs	hsa-miR-301b	5.520343
	hsa-miR-148b-3p	4.894202
	hsa-miR-30d-3p	4.533417
	hsa-miR-769-5p	4.265323
	hsa-miR-190a	4.106842
	hsa-miR-1180	4.027596
	hsa-miR-19b-1-5p	3.745773
	hsa-miR-596	3.717442
	hsa-miR-10b-3p	3.554867
Downregulated miRNAs	hsa-miR-202-3p	-3.746923
	hsa-miR-125b-5p	-3.992389
	hsa-miR-424-3p	-4.042714
	hsa-miR-181a-3p	-4.174865
	hsa-miR-622	-5.794464

Abbreviations: DE, differentially expressed; FC, fold change; miRNA, microRNA.

(eg, hsa-miR-301b and hsa-miR-769-5p) and five downregulated miRNAs (eg, hsa-miR-622 and hsa-miR-181a-3p) in hypoxic cells (Table 1).

Target genes of DE-miRNAs

To investigate the genes that were regulated by the DE-miRNAs in hypoxic cells, target genes of DE-miRNAs were predicted using the TargetScan database. Four upregulated miRNAs (hsa-miR-301b, hsa-miR-148b-3p, hsa-miR-769-5p, and hsa-miR-1180) and two downregulated miRNAs (hsa-miR-622 and hsa-miR-202-3p) were predicted to target a set of genes, eg, hsa-miR-301b regulated genes such as *FOXF2* and *CREB5*; hsa-miR-769-5p modulated genes such as *ARID1A* and *SMAD2*; hsa-miR-622 regulated genes such as *G3BP1* and *CELF2*; and hsa-miR-202-3p targeted genes such as *TFAP2B* and *CLCN5* (Table 2).

Functional analysis of DE-miRNAs targets

To further reveal what biological functions the DE-miRNAs were involved in, the GO functional and pathway enrichment analyses of DE-miRNA target genes were performed. The targets of upregulated miRNAs were mainly enriched in the functions such as "transcription", "multicellular organismal development", "chromatin modification", "ion transport", and "cell cycle", which were also enriched by a set of target genes of downregulated miRNAs (Table 3).

Furthermore, a set of pathways was enriched by target genes of upregulated miRNAs such as "Wnt signaling pathway", "TGF-β signaling pathway", "MAPK signaling pathway", and "focal adhesion". These pathways were also enriched by target genes of downregulated miRNAs (Table 4).

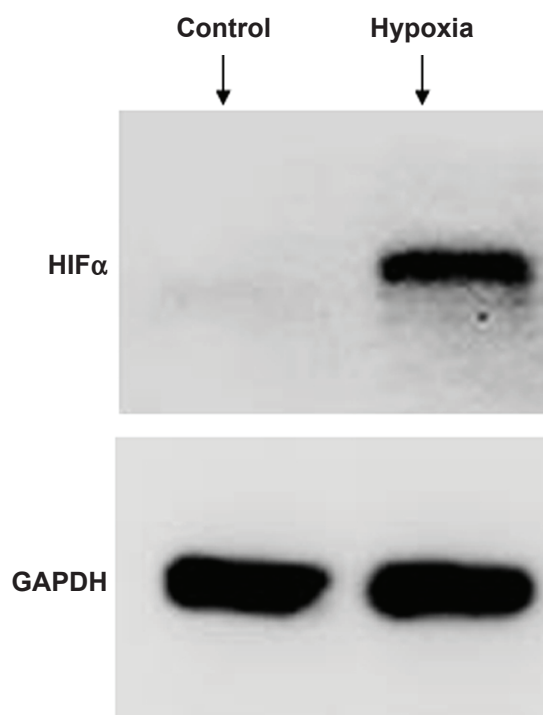


Figure 1 Expression of HIFα in A549 cells detected by Western blot analysis.
Notes: "Control" represents the group of A549 cells cultured in the normoxic condition, and "hypoxia" represents the group of A549 cells cultured in a hypoxia incubator (1% O₂).
Abbreviations: HIFα, hypoxia-inducible factor α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Target genes of DE-miRNAs

Category	Human miRNA name	Target genes
Upregulated miRNAs	hsa-miR-301b	FOXF2, ESRI, MARK3, MYB, CREB5, etc
	hsa-miR-148b-3p	NOG, WNT10B, PTEN, SMAD5, SERBP1, etc
	hsa-miR-769-5p	ARID1A, SMAD2, CPEB1, PTCH1, GRAMD3, etc
	hsa-miR-1180	SCN5A, ISOC2, FOXF4, BAD, NRXN2, etc
Downregulated miRNAs	hsa-miR-622	G3BP1, CELF2, SORBS1, SRSF1, CBX5, etc
	hsa-miR-202-3p	TFAP2B, CLCN5, ATP2B4, LIN28B, SNX30, etc

Abbreviations: DE, differentially expressed; miRNA, microRNA.

Table 3 The top ten enriched GO terms of target genes of upregulated and downregulated miRNAs

Category	GO ID	GO name	Adjusted P-value	Gene count	Genes
Target genes of upregulated miRNAs	GO:0006351	Transcription, DNA dependent	6.22E-66	6	THRB, CREB1, NR3C1, ZNF217, AR, ESRI
	GO:0007275	Multicellular organismal development	2.36E-50	180	NDRG2, WNT10B, BMP3, NUMBL, GAP43, etc
	GO:0006811	Ion transport	1.68E-33	70	KCNQ4, KCNH8, ATP7A, KCNQ3, SLC24A4, etc
	GO:0016568	Chromatin modification	1.23E-31	56	DNMT1, SUV420H1, ARID1A, SETD7, SUZ12, etc
	GO:0007399	Nervous system development	6.69E-25	92	STAT3, GAP43, ATXN3, NOG, ARID1A, etc
	GO:0008152	Metabolic process	3.97E-24	29	RPP14, LPGAT1, GFPT1, INSIG1, NAT14, etc
	GO:0010467	Gene expression	6.47E-20	1	EDA
	GO:0007049	Cell cycle	5.98E-18	86	CD2AP, PAPD5, ERBB2IP, ING1, SETDB2, etc
	GO:0006412	Translation	6.00E-14	7	QRSL1, CYLD, AARSD1, MRPS25, EIF5A2, etc
	GO:0006508	Proteolysis	1.56E-12	39	ADAMTS5, ADAMTS4, CTSA, RNPEPL1, LONRF1, etc
Target genes of downregulated miRNAs	GO:0006351	Transcription, DNA dependent	5.84E-35	1	NR3C1
	GO:0006811	Ion transport	2.19E-22	44	SLCO4C1, CACNA1I, MRS2, ATP5F1, CACNG2, etc
	GO:0007275	Multicellular organismal development	5.21E-21	88	EFNB3, WNT2B, LRP1, FLVCR1, DLL4, etc
	GO:0016568	Chromatin modification	4.05E-13	28	ATXN7L3, TLK2, ING5, NCOR1, EYA3, etc
	GO:0007399	Nervous system development	2.48E-08	42	FGF11, TFAP2B, JHDM1D, TMOD2, NLGN1, etc
	GO:0006417	Regulation of translation	7.58E-07	16	EIF2C3, IREB2, EIF2C4, CPEB2, DNAJC1, etc
	GO:0006412	Translation	9.56E-07	3	MRPS25, EIF5A, IGF2BP3
	GO:0007596	Blood coagulation	9.93E-07	1	ITGB3
	GO:0006333	Chromatin assembly or disassembly	1.91E-06	6	CDYL, MPHOSPH8, CHD9, CBX5, CHD7, ARID4A
	GO:0008152	Metabolic process	2.13E-06	12	AGPAT1, IREB2, SMUG1, NAA30, RPP14, etc

Note: GO terms are ordered from small to large P-values.

Abbreviations: GO, gene ontology; miRNA, microRNA.

Table 4 The top ten enriched pathways of target genes of upregulated and downregulated miRNAs

Category	Pathway ID	Pathway name	Adjusted P-value	Gene count	Genes
Target genes of upregulated miRNAs	4310	Wnt signaling pathway	2.60E-14	47	CCND3, SMAD2, FOSL1, CER1, WNT10B, etc
	4144	Endocytosis	9.39E-13	53	SMAD2, FLT1, ADRBK2, PSD3, EPN2, etc
	4350	TGF- β signaling pathway	6.16E-11	30	SMAD2, TNF, NOG, ROCK2, BMPRI1B, etc
	4010	MAPK signaling pathway	7.26E-11	59	FGFR1, RELA, FGF2, TNF, MAP3K5, etc
	4916	Melanogenesis	2.96E-10	32	CALM2, CREB3L2, WNT10B, KRAS, FZD6, etc
	4510	Focal adhesion	6.59E-10	47	PAK6, FLT1, ITGA9, TNXB, XIAP, etc
	4810	Regulation of actin cytoskeleton	1.46E-09	48	ARHGEF4, FGFR1, KRAS, FGF2, ARHGEF7, etc
	4360	Axon guidance	2.49E-09	35	PAK6, DPYSL5, UNC5D, UNC5C, EPHB4, etc
	4722	Neurotrophin signaling pathway	6.18E-09	34	KRAS, SH2B3, SOS1, MAP3K5, PIK3R1, etc
	4910	Insulin signaling pathway	1.56E-08	35	CALM2, KRAS, PRKACB, SOS1, PIK3R1, etc
Target genes of downregulated miRNAs	4010	MAPK signaling pathway	6.97E-12	43	PLA2G3, TRAF6, MAP3K1, MEF2C, HRAS, etc
	4722	Neurotrophin signaling pathway	6.28E-09	25	MAP3K1, FOXO3, TRAF6, YWHAZ, FASLG, etc
	4360	Axon guidance	3.77E-08	24	SRGAP3, SRGAP1, HRAS, NFAT5, ROBO2, etc
	4115	p53 signaling pathway	5.46E-07	16	BBC3, IGF1, PMAIP1, CDK6, FAS, etc
	4310	Wnt signaling pathway	5.71E-07	24	WIF1, LRP6, DVL3, NFAT5, PPP2R1B, etc
	4510	Focal adhesion	2.54E-06	27	LAMA1, COL4A1, IGF1, HRAS, COL3A1, etc
	5220	Chronic myeloid leukemia	1.91E-04	13	PTPN11, CDK6, BCL2L1, RBL1, TGFBRI, etc
	4512	ECM-receptor interaction	2.16E-04	14	COL4A2, LAMA1, COL4A1, COL3A1, COL6A3, etc
	4920	Adipocytokine signaling pathway	4.56E-04	12	PPARGC1A, CAMKK1, LEP, PPARA, ADIPOR2, etc
	4350	TGF- β signaling pathway	6.70E-04	13	PPP2R1B, TGFBRI, ACVR1C, ACVR2A, ACVR2B, etc

Note: Pathway terms are ordered from small to large P-values.

Abbreviations: ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; miRNA, microRNA; TGF, transforming growth factor.

Expression of miRNAs and target genes in established hypoxic A549 cell model

To verify the expression levels of DE-miRNAs and their targets in hypoxic A549 cells, RT-qPCR was utilized to determine the expression levels of three DE-miRNAs with a higher FC and a set of their targets (hsa-miR-301b/[*CREB5*, *FOXF2*], hsa-miR-148b-3p/[*NOG*, *WNT10B*], hsa-miR-769-5p/[*SMAD2*, *ARID1A*], hsa-miR-622/[*G3BP1*], and hsa-miR-202-3p/[*TFAP2B*]). According to the results of RT-qPCR, the downregulation of genes *ARID1A*, *FOXF2*, *SMAD2*, and *WNT10B* was consistent with the results of bioinformatic analysis, whereas upregulated *CREB5* and *NOG*, as well as downregulated *G3BP1* and *TFAP2B*, in hypoxic A549 cells was inconsistent with the predicted results (Figure 2A). Meanwhile, hsa-miR-301b, hsa-miR-148b-3p,

and hsa-miR-769-5p were upregulated, and hsa-miR-622 was downregulated in hypoxic A549 cells, comparing with normoxic cells, which was consistent with the predicted results (Figure 2B).

Regulatory relationships of DE-miRNAs and genes

To further confirm the regulatory relationships of DE-miRNAs and their target genes, dual-luciferase reporter assay was performed. In this assay, the validated hsa-miR-301b and its target *FOXF2*, as well as hsa-miR-769-5p and its target *ARID1A*, were selected for the confirmation.

First, the cloning vectors containing the 3' UTR sequence of *FOXF2* and *ARID1A* was successfully established (Figure 3A and Figure S1). According to the luciferase assay,

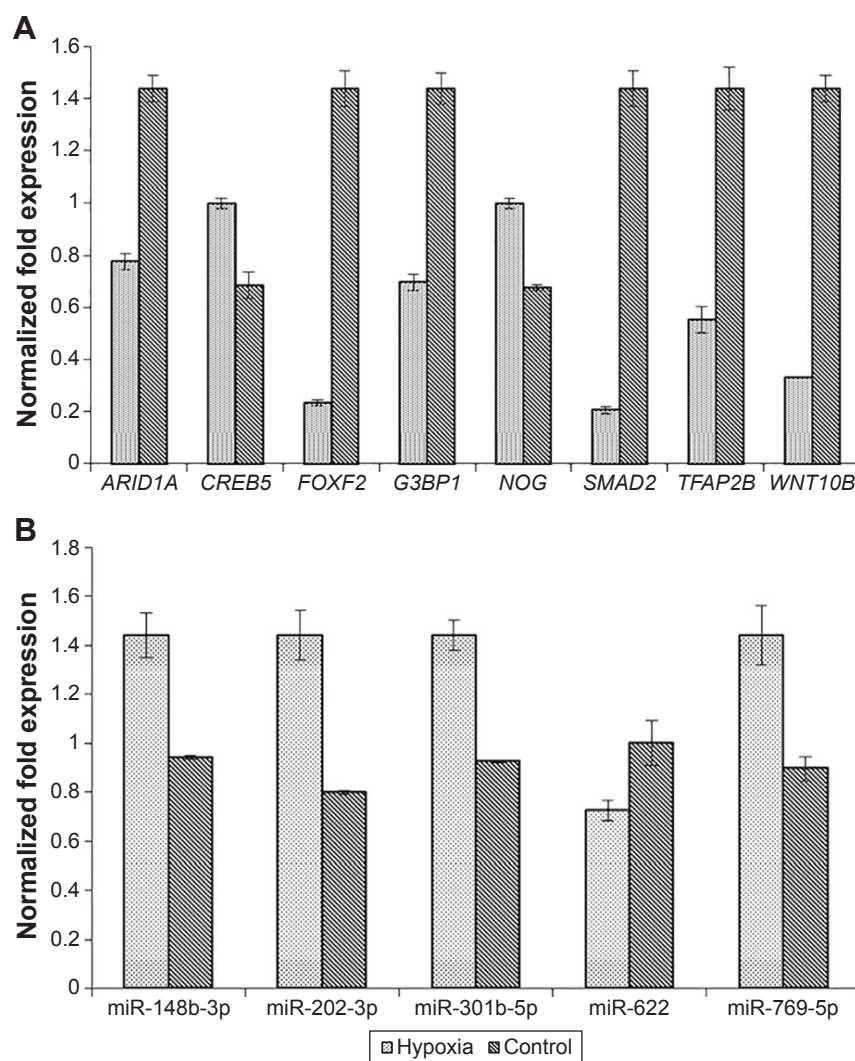


Figure 2 Expression levels of DE-miRNAs and target genes in hypoxic and normoxic A549 cells.

Notes: (A) Expression levels of miRNA target genes. (B) Expression levels of miRNAs. "Control" represents the group of A549 cells cultured in the normoxic condition, and "hypoxia" represents the group of A549 cells cultured in a hypoxia incubator (1% O₂).

Abbreviations: DE, differentially expressed; miRNA, microRNA.

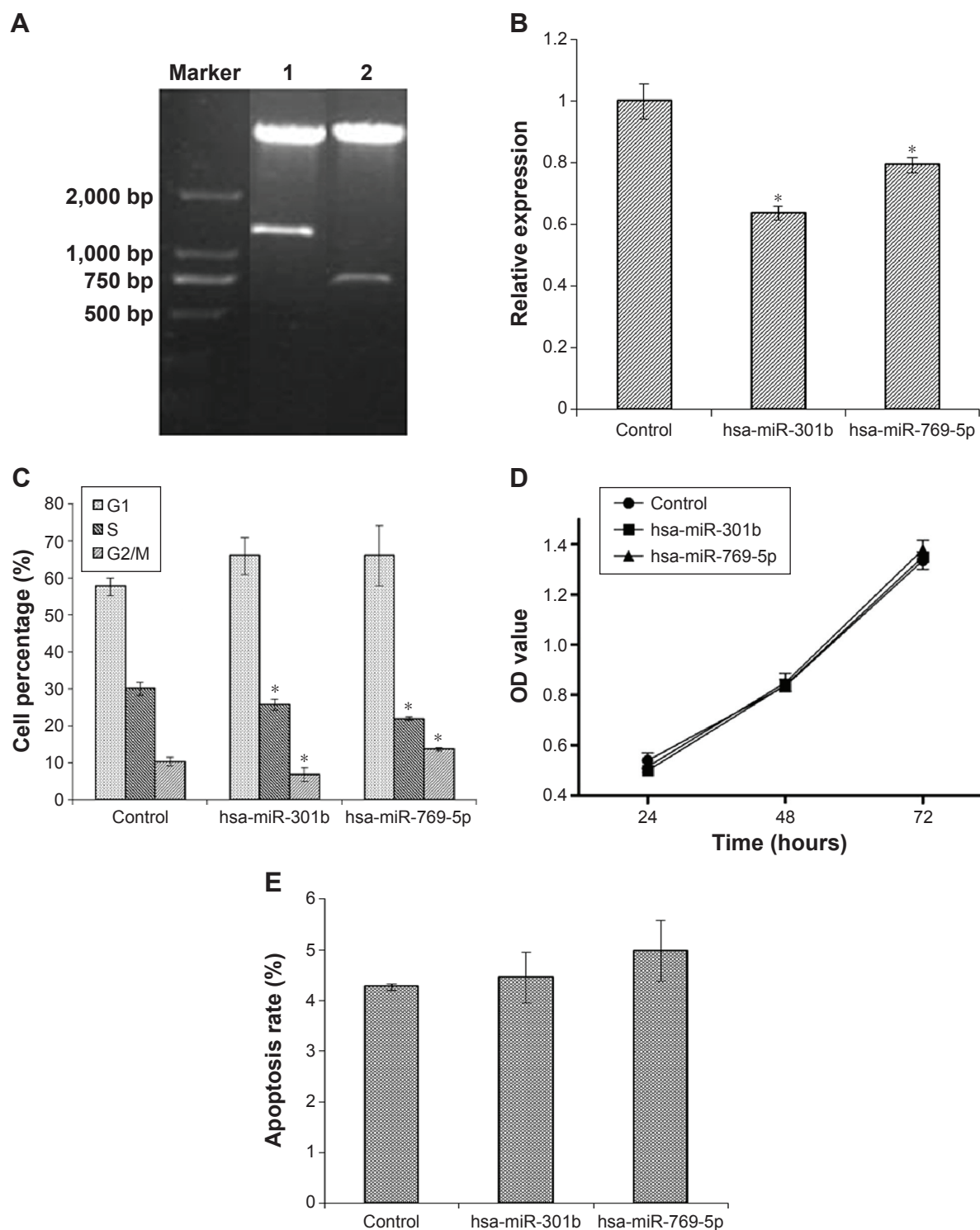


Figure 3 Validation of regulatory relationships of hsa-miR-301b/hsa-miR-769-5p and their targets, and effect of miRNA overexpression on A549 cells.

Notes: (A) Electrophoretogram of enzyme-digested product of the cloning vector containing the 3' UTR sequence of *FOXF2* and/or *ARID1A*. "1" represents the cloning vector containing the 3' UTR sequence of *ARID1A* and "2" represents the cloning vector containing the 3' UTR sequence of *FOXF2*. (B) Luciferase activity of the 3' UTR sequence of *FOXF2* and *ARID1A* in A549 cells transfected by hsa-miR-301b and hsa-miR-769-5p mimics. "Control" represents the group of A549 cells transfected by a scrambled miRNA sequence; "hsa-miR-301b" represents the group of A549 cells transfected by hsa-miR-301b mimic; "hsa-miR-769-5p" represents the group of A549 cells transfected by hsa-miR-769-5p mimic. (C) Cell percentage of A549 cells in the G1, S, and G2/M phases. "Control" represents the group of A549 cells transfected by a scrambled miRNA sequence; "hsa-miR-301b" represents the group of A549 cells transfected by hsa-miR-301b mimic; "hsa-miR-769-5p" represents the group of A549 cells transfected by hsa-miR-769-5p mimic. * $P < 0.05$, compared with the control. (D) The mean OD value of A549 cells. "Control" represents the group of A549 cells transfected by a scrambled miRNA sequence; "hsa-miR-301b" represents the group of A549 cells transfected by hsa-miR-301b mimic; "hsa-miR-769-5p" represents the group of A549 cells transfected by hsa-miR-769-5p mimic. (E) Apoptosis rate of the A549 cells transfected by hsa-miR-301b and hsa-miR-769-5p mimics. "Control" represents the group of A549 cells transfected by a scrambled miRNA sequence; "hsa-miR-301b" represents the group of A549 cells transfected by hsa-miR-301b mimic; "hsa-miR-769-5p" represents the group of A549 cells transfected by hsa-miR-769-5p mimic.

Abbreviations: miRNA, microRNA; OD, optical density; UTR, untranslated region.

the luciferase activity of the 3' UTR sequence of *FOXF2* was significantly weaker in A549 cells transfected by hsa-miR-301b mimic than the controls. Similarly, the luciferase activity of the 3' UTR sequence of *ARID1A* was weaker in A549 cells transfected by hsa-miR-769-5p mimic than the controls (Figure 3B).

Effect of DE-miRNAs on A549 cell behaviors

To further investigate whether the overexpression of hsa-miR-301b and hsa-miR-769-5p affected cell behaviors of A549 cells or not, cell proliferation, cell cycle, and apoptosis of A549 cells transfected by hsa-miR-301b and hsa-miR-769-5p mimics were determined.

After transfection of miRNA mimics, according to the flow cytometry analysis, the percentage of A549 cells transfected by hsa-miR-301b and hsa-miR-769-5p mimics in the G1 phase was increasing, whereas the cell percentage was significantly reduced in the S phase, comparing with the controls (Figure 3C). However, the cell proliferation of A549 cells did not change significantly over time, comparing with the controls (Figure 3D). In addition, the apoptosis rate of A549 cells transfected by miRNA mimics did not significantly vary, comparing with the controls (Figure 3E).

Discussion

Hypoxia is common in almost all solid tumors, and it plays an important role in the development of tumors.^{4,5} In the current study, based on miRNA microarray, the miRNA expression profile in hypoxia-induced lung adenocarcinoma A549 cells was analyzed. In total, 14 DE-miRNAs (nine upregulated miRNAs and five downregulated miRNAs) were identified in hypoxic cells, comparing with normoxic cells. Among them, four upregulated miRNAs and two downregulated miRNAs were predicted to regulate a set of genes. The regulatory relationships of hsa-miR-301b and its target *FOXF2*, as well as hsa-miR-769-5p and its target *ARID1A*, were confirmed, and their expression levels in hypoxic A549 cells were also verified.

In this study, hsa-miR-301b was confirmed to be upregulated, and its target gene *FOXF2* was downregulated in hypoxic A549 cells. The expression of miR-301b has been found to be increased in lung tissue of hypoxia-treated mice.²¹ In hypoxia-induced pulmonary hypertension, miR-130 promotes vasoconstriction by regulating the peroxisome proliferator-activated receptor γ and vasoactive factors.²² A previous study has discovered the regulatory

relationship of miR-301b and *FOXF2*.²³ *FOXF2* encodes FOX F2, which is a transcription factor and is expressed in lung and placenta.²⁴ In hypoxia-induced lung tissue, the DNA-binding activity of FoxF2 is significantly increased.²⁵ Besides, in lung tissue, Foxf2 mRNA exhibits a reduction after butylated hydroxytoluene injury.²⁶ These results suggest the important role of *FOXF2* in lung. Furthermore, in this study, the percentage of A549 cells transfected by hsa-miR-301b mimics in the G1 phase was increasing, whereas the cell percentage was significantly reduced in the S and G2/M phases, compared with the controls, indicating that A549 cells were blocked in the G1 phase. However, the cell proliferation and apoptosis rate of A549 cells transfected by hsa-miR-301b mimics were not significantly affected. The results suggest that the overexpression of hsa-miR-301b has no obvious influence on the cell proliferation and apoptosis of A549 cells. There is no other evidence to prove the significant roles of miR-301b and *FOXF2* in hypoxia-induced lung cancer; thus, dysregulated hsa-miR-301b and *FOXF2* may play pivotal roles in hypoxia-induced lung cancer cells via mediating transcription of other genes.

In the current study, hsa-miR-769-5p and its target *ARID1A* were also validated to be dysregulated in hypoxia-induced A549 cells. *ARID1A* encodes a protein belonging to the switch/sucrose non-fermentable (SWI/SNF) family; members of which have helicase and ATPase activities and modulate transcription of certain genes by changing the chromatin structure around those genes.²⁷ In this study, *ARID1A* was distinctly enriched in the function of chromatin modification. A previous study has discovered that the SWI/SNF chromatin-remodeling complex directly targets HIF1 α in the cellular response to hypoxia, and the damage of SWI/SNF function makes cells resistant to hypoxia-induced cell cycle arrest.²⁸ In this study, another target gene of hsa-miR-769-5p, *SMAD2*, was also verified to be downregulated in hypoxic A549 cells. *SMAD2* encodes a SMAD protein, which is a signal transducer that mediates multiple signaling pathways.²⁹ In this study, *SMAD2* was enriched in the Wnt signaling pathway and TGF- β signaling pathway. There is evidence that beta-catenin in the Wnt signaling pathway is able to enhance HIF1-mediated transcription, thus promoting cancer cell adaptation to hypoxia and survival.³⁰ Besides, HIF-1 α inactivates the Wnt signaling by binding to beta-catenin, which contributes to the hypoxia-induced growth arrest of tumor cells.³¹ Furthermore, a study has reported that hypoxia enhances the expression of TGF- β 1 and the phosphorylation status of Smad2 in hepatic stellate cells.³² The mRNA expression level of TGF- β receptor is distinctly

increased in hypoxia-induced gastric cancer cells, and hypoxia stimulates the epithelial mesenchymal transition of gastric cancer cells via the TGF- β /TGF- β R signaling.³³ These findings indicate the crucial roles of the Wnt signaling pathway and TGF- β signaling pathway in the cellular response to hypoxia. Overall, hsa-miR-769-5p is likely to exhibit its functions in hypoxia-induced lung cancer cells via regulating the chromatin modification-related gene *ARID1A* and signaling pathway-related gene *SMAD2*, which needs to be confirmed by further experiments.

Despite the aforementioned results, this study has some limitations. In this study, the effects of overexpression of DE-miRNAs cell behavior in the hypoxia condition have not been studied, which will be investigated in our further study. Besides, we would verify the expression of DE-miRNAs and their targets in lung adenocarcinoma tissue from patients in our future study.

Conclusion

A set of miRNAs and their target genes was validated to be differentially expressed in hypoxic lung cancer cells, compared with normoxic cells. Among them, hsa-miR-301b and its target *FOXF2*, as well as hsa-miR-769-5p and its targets *ARID1A* and *SMAD2*, may play crucial roles in the response of lung cancer cells to hypoxia. These results provide new information for the study of hypoxia-induced lung cancer cells, and the DE-miRNAs and their targets are expected to be selected as candidate biomarkers for the clinical therapy of lung cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Primer sequences of genes

Gene	Primer sequences
<i>FOXF2</i>	Forward: CCGTTACCAGCATCACTCTACT Reverse: CGCAGGGCTTAATATCCTGACA
<i>CREB5</i>	Forward: CCCTGCCCAACCCTACAATG Reverse: GGACCTTGCATCCCCATGAT
<i>NOG</i>	Forward: CCATGCCGAGCGAGATCAAA Reverse: TCGGAAATGATGGGGTACTGG
<i>WNT10B</i>	Forward: CATCCAGGCACGAATGCGA Reverse: CGGTTGTGGGTATCAATGAAGA
<i>SMAD2</i>	Forward: TCATAGCTTGGATTACAGCCAG Reverse: TTCTACCGTGGCATTTCGGTT
<i>G3BP1</i>	Forward: CCAGCAGAACTCTCACGACC Reverse: CTGAGCGAAGGACGAAGACG
<i>TFAP2B</i>	Forward: CCATCCCGGAATGGAAGACG Reverse: TCACCGATTTGGGAGGAACTG
<i>GAPDH</i>	Forward: GGAGCGAGATCCCTCCAAAAT Reverse: GGCTGTTGTCATACTTCTCATGG
<i>β-actin</i>	Forward: CTCCATCCTGGCCTCGCTGT Reverse: GCTGTCACCTTCACCGTTCC
<i>18S rRNA</i>	Forward: GGACACGGACAGGATTGACA Reverse: GACATCTAAGGGCATCACAG

Table S2 Primer sequences of miRNAs

miRNA	Primer sequences
miR-301b-5p	GCTCTGACGAGGTTGCACTACT
miR-148b-3p	TCAGTGCATCACAGAACTTTGT
miR-769-5p	TGAGACCTCTGGGTTCTGAGCT
miR-622	ACAGTCTGCTGAGGTTGGAGC
miR-202-3p	AGAGGTATAGGGCATGGGAA
U6-Forward	CTCGCTTCGGCAGCAC
U6-Reverse	AACGCTTCACGAATTGCGT

Abbreviation: miRNA, microRNA.

Table S3 Primer sequences of 3' UTR in genes *FOXF2* and *ARID1A*

Gene 3' UTR	Primer sequences
<i>FOXF2</i> 3' UTR	EcoRI Forward: GGAATTCACGGAAAGAGGCCAAGCGATG
<i>FOXF2</i> 3' UTR	XhoI Reverse: CCGCTCGAGTGTTATATACATTTTATTGAAAAA
<i>ARID1A</i> 3' UTR	EcoRI Forward: GGAATTCAGCCGTGGGACACCTCC
<i>ARID1A</i> 3' UTR	XhoI Reverse: CCGCTCGAGAAAGTCTTACCAAGATTTAATGTAC

Abbreviation: UTR, untranslated region.

