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MiR-30a suppresses non-small cell lung cancer progression through AKT signaling pathway by targeting IGF1R

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Abstract

MicroRNAs play critical roles in the development and progression of human cancers. Although miR-30a has been suggested to function as a tumor repressor in several tumors, its role in non-small cell lung cancer (NSCLC) has not been investigated in detail. This study investigated the expression and role of miR-30a in human NSCLC. The expression of miR-30a is significantly decreased in clinical NSCLC tissues and cell lines. Overexpression of miR-30a inhibited NSCLC cell proliferation, G1/S and S/G2 transition in vitro, whereas suppression of miR-30a facilitated NSCLC cell proliferation, G1/S and S/G2 transition. Using a luciferase reporter assay, insulin-like growth factor 1 receptor (IGF1R) was determined to be a direct target of miR-30a. Furthermore, silencing IGF1R resulted in the same biologic effects of miR-30a overexpression in NSCLC cells, which included suppressed NSCLC cell proliferation and trigering cell cycle arrest through PI3K/AKT signaling pathway by inhibiting cell cycle regulators (CDK2, CDK4, Cyclin A2 , Cyclin D1). These results demonstrate that miR-30a influences NSCLC progression through PI3K/AKT signaling pathway by targeting IGF1R in A549 cells, which suggest miR-30a as a novel strategy for NSCLC diagnosis and treatment.

Key words: miR-30a, non-small cell lung cancer, proliferation, insulin-like growth factor 1 receptor, PI3K/AKT signaling pathway.

Introduction

Lung cancer is one of the most leading cause of cancer-related deaths in worldwide. Based on their distinct clinicopathological characteristics, lung cancer is divided into two groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is a predominant type of pulmonary carcinoma, which accounts for about 80% of all lung cancer cases (18,22). Despite advancements in treatments for NSCLC, including surgery, chemotherapy, radiotherapy, and recently developed molecular targeting therapies, such as inhibitors against vascular endothelial growth factor or epithelial growth factor receptor, the 5-year survival rate for the disease is still very poor. Therefore, further investigation of the molecular mechanisms underlying tumorigenesis and progression of NSCLC is essential for developing new prognostic biomarkers and effective therapeutic targets.

MicroRNAs (miRNAs) are a class of small non-coding RNAs with 20-23 nucleotides that regulate a wide range of genes in post-transcriptional level. MiRNAs bind to the 3′-untranslated region of mRNA, leading to translational repression or mRNA degradation. The genes regulated by miRNAs were involved in various physiological processes. Thus, miRNA play critical roles in cell growth, survival, differentiation, and apoptosis (16,38). The significance of miRNAs is underscored by the fact that they are usually deregulated in the process of cancer (14,28,33). Their dysregulation contributes to various aspects of carcinogenesis in nearly all kinds of cancer, negatively regulating either oncogenes or anti-oncogenes (11,31). Recently, miRNAs have emerged in NSCLC as both diagnostic and prognostic biomarkers (30). For example, MiR-198 suppresses proliferation and induces apoptosis of lung cancer cells via targeting FGFR1 (36). MicroRNA-34a inhibits the proliferation and promotes the apoptosis of NSCLC H1299 cell line by targeting TGFβR2 (22). MiR-545 suppresses cell proliferation, causes G0/G1 phase arrest, and induces cell apoptosis in lung cancer cells by targeting cyclin D1 and CDK4 genes (9). These reports indicate that miRNAs are involved in the progression of lung cancer, and that miRNAs represent a potential target in the treatment of lung cancer.

In our study, miR-30a is significantly downregulated in patients with lung cancer, which indicates its potential role as a tumor suppressor in cancer progression. The miR-30 family members includes miR-30a, b, c, d and e. The earlier studies proved that miR-30a is downregulated in prostate cancer (20) and hepatocellular carcinoma (24). In addition, miR-30a inhibits osteolysis by targeting RunX2 in giant cell tumor of bone (15). miR-30a inhibits tumorigenesis by targeting Insulin Receptor Substrate 2 in colorectal cancer (37). miR-30a regulates the growth of breast cancer cells (26). It is reported that miR-30b/c inhibits NSCLC cell proliferation by targeting Rab18 (39). miR-30d MicroRNA-30d-5p inhibits

tumour cell proliferation and motility by directly targeting CCNE2 in NSCLC (5). miR-30e promotes glioma cell invasion through EGFR stabilization by directly targeting CBL-B (21). However, miR-30a's roles and molecular mechanisms in lung cancer are unclear until now. Meanwhile, we found that insulin-like growth factor 1 receptor(IGF1R) had an overexpression in NSCLC compared with normal tissues. Using gene target prediction databases (TargetScan), we found that IGF1R might be a potential target of miR-30a. IGF1R is a transmembrane receptor tyrosine kinase mainly activated by IGF1 or IGF2 through autocrine and paracrine, which has kinase-independent biologic functions (17). The activated IGF1R binds to adaptor molecules such as insulin receptor substrates(IRSs) and Shc and then triggers downstream signaling cascades, such as phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways which regulate oncogenic transformation, growth and survival of cancer cells (6). The purpose of this study was to investigate the potential involvement of miR-30a in NSCLC. We demonstrated that overexpression of miR-30a potently inhibited NSCLC cell growth. More importantly, we provide evidence that IGF1R is a direct and functional target of miR-30a. Our data suggest that miR-30a may be a potential therapeutic target for treating NSCLC.

Materials and methods

Human tissue specimens and cell lines

Human tissues were prepared from the First Affiliated Hospital, College of Medicine, Xi'an Jiaotong University. Informed consent was obtained before specimen collection. The experimental protocols were approved by the Ethics Committee of Xi'an Jiaotong University College of Medicine and followed the guidelines of the declaration of Helsinki. The specimen collection was conducted in accordance with the guidelines of the National Institutes of Health. The human bronchial epithelial cells (BEAS-2B) and human lung cancer cell lines (Sk-mes-1, NCl-H1299, NCl-H460, A549) were maintained in the Key Laboratory of Environment and Genes Related to Diseases at Xi'an Jiaotong University College of Medicine. These cells were cultured in RPMI-1640 (Gibco BRL, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL, USA) containing 2.0 mmol/L glutamine and 20 μg penicillin-streptomycin/mL at 37 °C in a humidified atmosphere containing 5% CO_2.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissues or cells using TRIzol reagent according to manufacturer's instructions (Invitrogen, USA). The RNA was reverse transcribed to cDNA from 1 mg of total RNA using a reverse PrimeScript™ RT reagent Kit (TaKaRa, USA). qRT-PCR analysis were conducted with Power SYBR Green. All protocols were performed according to the manufacturer's instructions. qRT-PCR reactions were carried out using an iQ Multicolor qRT-PCR Detection System (Bio-Rad, USA). Results were normalised to the expression of U6 or β-Actin. The primer sequences were as follows: miR-30a reverse-transcribed primer:5′-GTCGTATCC AGTGCGTGTCGTGGAGTCGGCAATTGCACTGG ATACGACCTTCCAG-3′; miR-30a forward: 5′-ATCCAGTGCGTGTCGTG-3′; miR-30a reverse: 5′-TGCTTGTAAACATCCTCGA-3′; U6 reverse-transcribed primer: 5′-CGCTTCACGAATTTGCGTGTCAT-3′; U6 forward: 5′-GCTTCGGCAGCACATATACTAAAAT-3′; U6 reverse: 5′-CGCTTCACGAATT TGCGTGTCAT-3′; IGF1R forward: 5′-TTTCCCACAGCAGTCCACCTC-3′; IGF1R reverse: 5′-AGCATCCTAGCCTTCTCACCC-3′; β-Actin forward: 5′-TGGCACC CAGCACAATGAA-3′; β-Actin reverse: 5′-CTAAGTCATAGTCCGCCTAGAAGC A-3′.

Luciferase reporter assay

The 3′ untranslated region (3′-UTR) of human IGF1R mRNA was constructed by synthetic oligonucletides and cloned in between the SacI and XhoI sites of the pmirGLO Dual-Luciferase miRNA target expression vector (Promega, USA). PmirGLO-IGF1R-3′UTR vector was co-transfected with miR-30a into HEK293 cell lines, with pmirGlO-vector as their control. Then, the cells were harvested and lysed for luciferase assays 24 h after transfection. Dual-Luciferase Assay System (Promega, USA) was utilized to measure the reporter activity according to the manufacturer's protocol.

Expression vector construction, inhibitor synthesis, siRNA synthesis and transfection.

The miR-30a expression vector (pre-miR-30a) and control vector were constructed with synthetic oligonucleotides and cloned in between the EcoRI and HindIII sites of the pcDNA6.2-GW/EmGFP vector (Invitrogen, USA). The 3′UTR of human IGF1R mRNA was constructed by synthetic oligonucleotides and cloned in between the SacI and XhoI sites of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA). The inhibitor of miR-30a and small interfering RNA (siRNA) targeting IGF1R were purchased from Gene-Pharma. The anti-miR-30a sequence was: 5′-AUCUGUGGCUUCACAGCUUCCAGU-3′. Scramble siRNA was used as negative control (named anti-control). The anti-control sequence was: 5′-CAGU ACUUUUGUGUAGUACAA-3′. Human IGF1R siRNA sense: 5′- GAAAAGCAGUCAAUGGAUUUU-3′, antisense: 5′- AAUCCAUUGACUGCUUUUCUU-3′; negative siRNA (NC-siRNA): sense 5′-UUCUCCGAACGUGUCACGUUU-3′, antisense: 5′-ACGUGACACGUUCGGAGAAUU-3′). siRNA oligonucleotide were synthesized and 1μg of siRNA mimic was transfected into cells by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's guidelines.

MTT assay

The A549 Cells were seeded into 96-well plates (5,000 cells/well in 100 μl medium) and incubated for 24 h. These cells were treated with control vector, miR-30a, anti-control, anti-miR-30a, NC-siRNA or IGF1R siRNA (70 nM) for 24, 48 and 72 h, respectively. At the end of culture, 20 μl of 5 mg/ml MTT (Sigma, USA) solution was added per well and the cells were incubated for another 4 h at 37 °C. Supernatants were removed and formazan crystals were dissolved in 150 μl of dimethylsulfoxide (Sigma, USA). Finally, optical density was determined at 490 nm using multi-microplate test system (BMG Labtechnologies, Germany). The results were collected as the mean of more than three

independent experiments.

Cell cycle analysis

The A549 Cells were cultured in 6-well plates for 24 h and treated by control vector, miR-30a, anti-control, anti-miR-30a, NC-siRNA or IGF1R siRNA (70 nM) for 48 h. At the end of culture, cells were fixed in 75 % ice-cold ethanol overnight at 4 °C. The fixed cells were stained with 50 μg/ml propidium iodide (PI) containing 50 μg/ml RNase A (DNase free) for 30 min at room temperature in the dark and analyzed by fluorescence-activated cell sorting (BD Biosciences, USA). The cell cycle distribution was evaluated by calculating the proportion of cells in G0/G1, S, and G2/M stages. The procedures were carried out in triplicate. Data obtained were presented as mean ± SEM.

Colony formation assay

Stably transfected cells were seeded at a density of 5,000 per 12-well plate, incubated for 2 weeks, and then stained with 0.5% crystal violet for 30 minutes. Excess dye was rinsed off three times with PBS. The number of colonies was counted by ImageJ.

Apoptosis analysis

Cells were incubated in 6-well plates for 24 h and treated by control vector, miR-30a, anti-control, anti-miR-30a, NC-siRNA or IGF1R siRNA (70 nM) for 48 h, then harvested and washed twice with PBS. The cells were stained by incubation with 5 μL FITC-Annexin V and 10 μL PI at 250 μg/ml for 15 min in the dark. Cells were washed with PBS and examined using flow cytometry. Quantification of apoptosis was determined by counting the number of cells stained by FITC-labeled Annexin V. Early apoptotic cells were identified with PI negative and FITC Annexin V positive; cells that were in late apoptosis or already dead were both FITC Annexin V and PI positive.

Western blot analysis

The A549 cells were treated by control vector, miR-30a, anti-control, anti-miR-30a, NC-siRNA or IGF1R siRNA (70 nM) for 48 h. Normal lung tissues, lung cancer tissues and the A549 cells were lysed in RIPA lysis buffer. Insoluble material was removed by centrifugation at 12,000 rpm for 15 min. Protein were subjected to electrophoresis using 10 % SDS–polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in 5 % non-fat dry milk in TBST. The membrane was incubated with primary antibodies overnight at 4 °C and then incubated with secondary antibody for 1 h at room temperature. The primary monoclonal antibodies included rabbit monoclonal anti-AKT (1:1,000, Santa Cruz, USA), rabbit monoclonal anti-p-AKT (1:1,000, Santa Cruz, USA), rabbit monoclonal anti-CDK2 (1:1,000, Santa Cruz, USA), rabbit monoclonal anti-CDK4 (1:1,000, Santa Cruz, USA), rabbit monoclonal anti-IGF1R (1:1,000, Santa Cruz, USA), rabbit monoclonal anti-cyclin A2 (1:1,000, Santa Cruz, USA), mouse monoclonal anti-cyclin D1 (1:1,000, Neomarker, USA) and mouse monoclonal anti-β-Actin (1:5,000, Santa Cruz, USA). The membranes were incubated in the dark with ECL (Amersham, USA) for chemiluminescence detection. The luminescent signal was detected by CCD camera, recorded and quantified with Syngene GBox (Syngene, UK). The results were collected as the mean of three independent experiments.

Statistical Analysis

Each experiment was repeated at least 3 times independently. Statistical analysis was performed with SPSS 15.0 software. The Student's t test was used for comparisons of 2 independent groups. The relationship between the expression of miR-30a and clinicopathologic characteristics was conducted with Chi-square test. Spearman's correlation was used to explore the association between miR-30a and IGF1R expression. The data were presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

miR-30a is frequently reduced in human lung cancer tissues and lung cancer cell lines

To explore the role of miR-30a in lung cancer, we analyzed the expression of miR-30a in 45 pairs of lung cancer and adjacent normal tissues by real-time PCR. There were 86.7% (39/45) of lung cancer samples with downregulation of miR-30a compared with the matched normal tissues, the expression of miR-30a decreased to 52.1% in cancer tissues (Fig. 1A). Table 1 showed the associations between various clinicopathologic variables and miR-30a expression levels in tumor specimens. The

Table 1. Patient characteristics and clinicopathologic correlation of miR-30a expression.

Characteristics	Number of cases	miR-30a expression		P-value
		High	Low	
Age (years)				0.321
≥60	21	2	19	
<60	24	4	20	
Gender				0.221
Male	31	4	27	
Female	14	2	12	
Smoking status				0.315
Never	17	2	15	
Current	20	3	17	
Former	8	1	7	
TNM Stage				0.008
I-II	18	5	13	
III-IV	27	1	26	

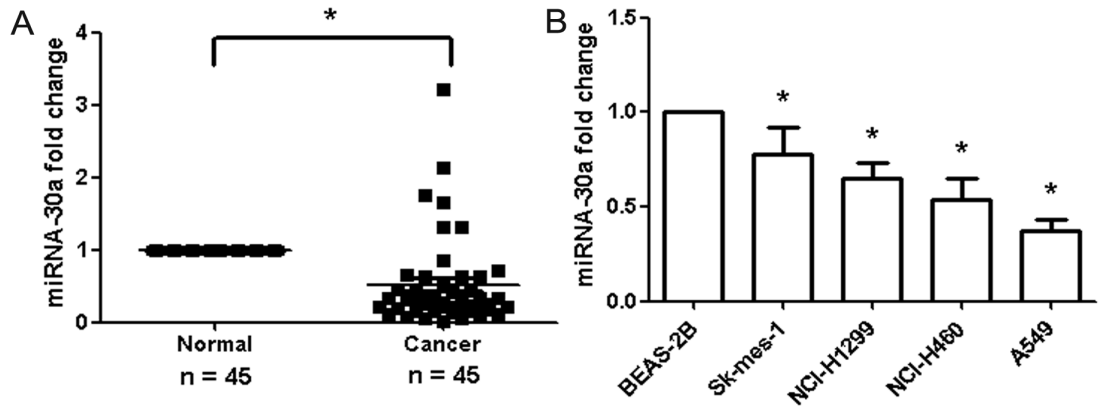

Figure 1. miR-30a is downregulated in lung cancer tissues and cell lines. (A) qRT-PCR was performed to examine miR-30a expression in 45 paired human lung cancer and adjacent normal tissues. (B) qRT-PCR was used to analysis of miR-30a expression in normal lung and lung cancer cells (* $P < 0.01$).

NSCLC tissues with advanced TNM stage (III-IV) more frequently showed low miR-30a expression than those with low TNM stage (I-II, $P < 0.001$, Table 1). However, miR-30a expression was not associated with other clinicopathologic variables, including age, gender and smoking (all P > 0.05). Next, we analyzed the expression of miR-30a in 4 lung cancer cell lines (Sk-mes-1, NCl-H1299, NCl-H460, A549) and human bronchial epithelial cells (BEAS-2B). The results showed that miR-30a was downregulated in tumor cell lines as well, and the expression of miR-30a was lowest in A549 cells (Fig. 1B; $P < 0.01$). Therefore, A549 cells were chosen to be used in the following experiments. These data suggested that miR-30a might act as a tumor suppressor in lung cancer.

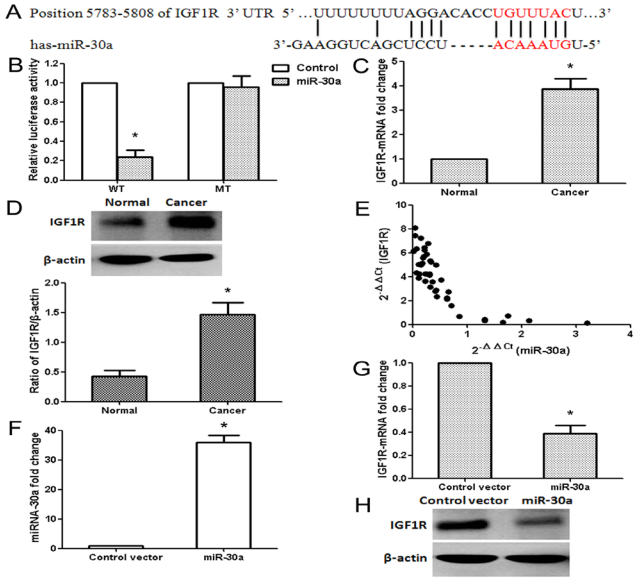

Figure 2. miR-30a directly targets the IGF1R gene. (A) Bioinformatics predicted interactions of miR-30a and their binding sites at the 3′-UTR of IGF1R. (B) The luciferase reporter plasmid containing wild-type or mutant IGF1R 3′-UTR was cotransfected into HEK293 cells with miR-30a or control. Luciferase activity was determined by the dual luciferase assay. (C) IGF1R mRNA expression in lung cancer and normal tissues. Data were related to normal tissues to show fold change. (D) IGF1R protein in lung cancer and normal tissues were measured by Western blotting. (E) The miR-30a and IGF1R expression levels were inversely correlated. $2^{-\Delta\Delta Ct}$ values of miR-30a and IGF1R mRNA were subjected to a Pearson correlation analysis (r = -0.766, p = 0.004, Pearson's correlation). (F) The expression of miR-30a was determined after transfected cells with pre-miR-30a vector. (G) The level of IGF1R mRNA was examined after miR-30a treatments. (H) The expression of IGF1R protein was analysed. β-actin was used as a control (* $P < 0.01$, n = 3).

IGF1R is a target gene of miR-30a in lung cancer

Through the miRBase and TargetScan databases, we found matching bases between the 3'UTR of IGF1R and miR-30a (Fig. 2A). In order to determine whether miR-30a regulates IGF1R, we performed a luciferase assay to evaluate the relationship between miR-30a and IGF1R. We transfected the wild-type IGF1R-3′UTR construct into HEK293 cells in combination with miR-30a or control (miR-negative). miR-30a led to a reduction of luciferase activity of IGF1R-3′UTR construct compared to control ($P < 0.01$). After the conserved targeting regions for miR-30a recognition were mutated, the relative luciferase activity of the reporter gene was restored (Fig. 2B). It indicates that miR-30a may suppress IGF1R expression through its binding sequences at the 3′UTR. The results also showed that IGF1R mRNA was obviously up-regulated in lung cancer tissues compared with their normal tissues control and IGF1R protein increased to 3.42 folds (Fig. 2C, D; P = 0.006, n = 21). We assessed the effect of miR-30a on IGF1R using data obtained from qRT-PCR. A significantly inverse correlation was observed between IGF1R mRNA and miR-30a (Fig. 2E; n = 45, r = -0.766, p = 0.004, Pearson's correlation). These data showed the reciprocal regulation of miR-30a and IGF1R. After transfected in A549 cells with miR-30a mimics, the miR-30a expression was up-regulated compared with cells transfected with the control vector (Fig. 2F; P = 0.002). A reduction of the IGF1R mRNA and protein expression levels was observed in transfected cells with pre-miR-30a vector compared with control vector (Fig. 2G, H; P = 0.007). These results indicate that miR-30a directly recognizes the 3′UTR of IGF1R mRNA and inhibits IGF1R translation.

Overexpression of miR-30a decreases lung cancer cell proliferation through suppressing AKT signaling pathway in vitro.

To examine the role of miR-30a in lung cancer cell proliferation, MTT assays, cell cycle analysis, and colony formation assay were performed. The results showed that the overexpression of miR-30a inhibited the proliferation of lung cancer A549 cells at 24, 48, and 72 h after transfection (Fig. 3A; P = 0.005). We examined the cell

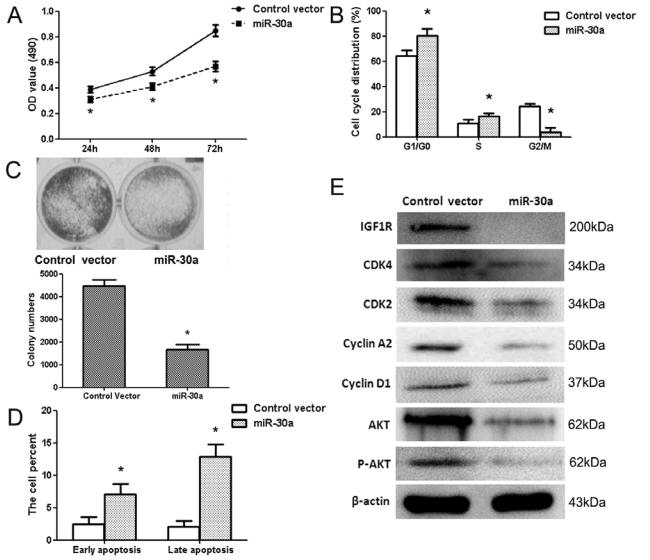

Figure 3. Effect of miR-30a on lung cancer A549 cell progression in vitro. (A) Proliferation of A549 cells was determined by an MTT assay at 24, 48 and 72 h after transfection with miR-30a or control vector, respectively. (B) The the cell cycle was visualized via PI staining. The data show the percentage of cells in the G1/G0, S and G2/M phases. (C) miR-30a inhibited colony formation. (D) Apoptosis was visualized using Annexin-V/PI staining. The data showed the percentage of early apoptosis and late apoptosis. (E) The expression analysis for IGF1R/AKT signaling pathway regulation proteins at 48 hours after transfection with pre-miR-30a or control vector by Western blot analysis (* $P < 0.01$, n = 3).

cycles using a flow cytometer 48 h after transfection. The population of G1/G0 and S stage increased, and the population of G2/M stage decreased significantly in the miR-30a overexpression group (Fig. 3B; P = 0.008). The colony formation assay results showed that cell colony numbers decreased to 0.37 folds in miR-30a group compared to the control vector (Fig. 3C; P = 0.003). To examine the possible effects of miR-30a on cell death, we observed the measurement of apoptosis by Annexin-V/PI staining. The results showed that the proportion of early apoptosis and late apoptosis increased to 2.84 and 6.13 folds in the miR-30a overexpression group, respectively (Fig. 3D; P = 0.004). These findings suggested that miR-30a could suppress the proliferation of lung cancer A549 cells in vitro. To further investigate the possible molecular mechanisms of miR-30a inhibiting cell proliferation, we detected the protein expression of IGF1R and its downstream pathway regulators by Western blotting after transfection with pre-miR-30a. Our results showed that miR-30a reduce the expression of IGF1R (0 folds) and AKT (0.15 folds) protein, and the phosphorylation of p-AKT (0.18 folds). We also investigated the underlying mechanisms of cell-cycle regulation. miR-30a could reduce the expression of CDK2 (0.22 folds), CDK4 (0.19 folds), Cyclin A2 (0.26 folds) and Cyclin D1 (0.31 folds) (Fig. 3E; $P < 0.01$). These results demonstrate that miR-30a affects lung cancer cell proliferation and cell cycle by controlling IGF1R/AKT pathways.

Inhibition of miR-30a promotes cell growth of lung cancer A549 cells

Human lung cancer A549 cells were transfected with miR-30a antisense oligonucleotides (anti-miR-30a) or control (anti-control). The results of MTT assay showed that anti-miR-30a promoted the proliferation of lung cancer A549 cells (Fig. 4A; P = 0.005). The number of the S and G2/M phase cells significantly increased in the anti-miR-30a group compared to the anti-control group, meanwhile the number of the G1/G0 phases cells remarkably decreased (Fig. 4B; P = 0.007). In addition, cell colony numbers increased to 2.06 folds in anti-miR-30a group compared to the anti-control (Fig. 4C; P = 0.004). There was no significant difference among the proportion of early apoptosis and late apoptosis of A549 cells in different treatment groups after anti-miR-30a treatments or anti-control (Fig. 4D). Furthermore, the knockdown of miR-30a increased the expression of IGF1R (3.03 folds), AKT (5.31 folds), p-AKT (2.14 folds), CDK2 (3.76 folds), CDK4 (2.42 folds), Cyclin A2 (3.85 folds) and Cyclin D1 (5.73 folds) (Fig. 4E; $P < 0.01$). Together with the above experiments, these results suggested an essential contribution of endogenous miR-30a to the anticarcinogenesis of A549 cells in lung cancer progression.

Knockdown of IGF1R produces similar effects to that of miR-30a overexpression in lung cancer cells

Next, we silenced IGF1R expression by RNAi method to test whether IGF1R is involved in the antitumor effects of miR-30a. From mRNA and protein expression levels, IGF1R can be specifically knocked-down by siRNA in A549 cells, IGF1R mRNA and protein decreased to 0.30 folds and 0.15 folds, respectively (Fig. 5A and F; P = 0.006). As shown in Fig. 5B-E, silencing of IGF1R resulted in suppressed cell proliferation and clone formation (0.46 folds, P = 0.008), induced G1/G0 and S phases arrest, cell apoptosis which followed the same trend as miR-30a in A549 cells. Furthermore, this was confirmed by an analysis of protein expression in the AKT pathway. As shown

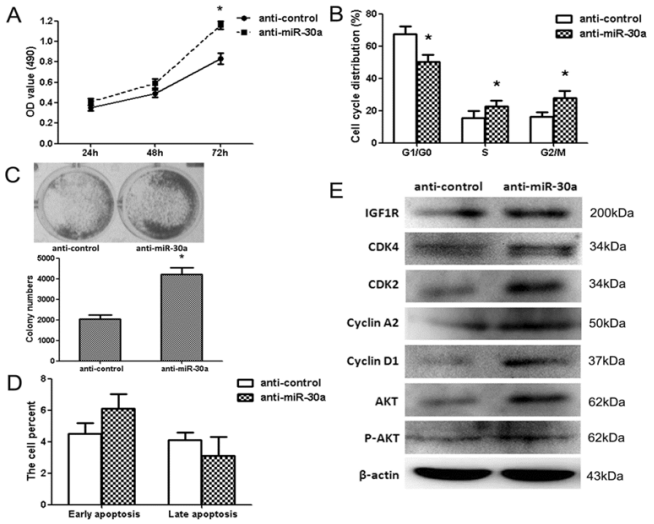

Figure 4. Inhibition of miR-30a promotes the proliferation of lung cancer A549 cells. (A) MTT assay showed that anti-miR-30a increased the activity of A549 cells at 72 h. (B) Flow cytometry analysis showed the percentage of cells in the G1/G0, S and G2/M phases. (C) The inhibitor of miR-30a promoted colony formation. (D) The data showed the percentage of early apoptosis and late apoptosis. (E) The expression analysis for IGF1R/AKT signaling pathway regulation proteins after transfection with the inhibitor of miR-30a or anti-control (* $P < 0.01$, n = 3).

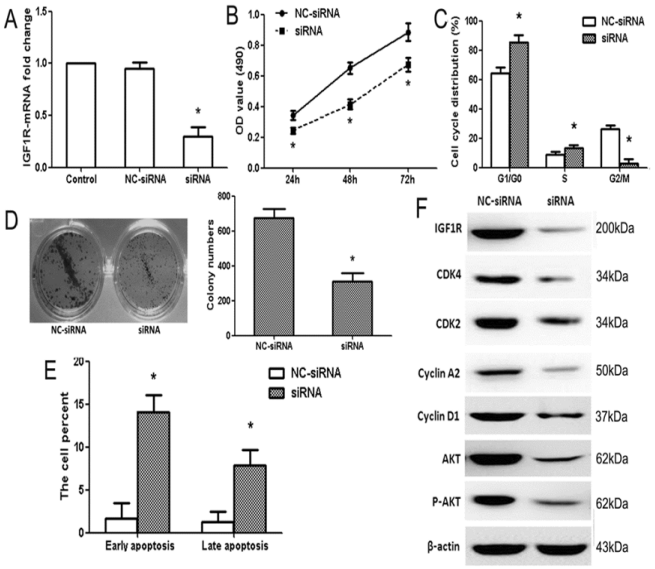

Figure 5. Silencing of IGF1R could suppress lung cancer cell proliferation and induces cell apoptosis in accordance with miR-30a. (A) qRT-PCR was performed to determine the IGF1R mRNA expression after transfection with IGF1R-siRNA. (B) MTT assay showed that IGF1R-siRNA decreased the activity of A549 cells at 24, 48 and 72 h. (C) Flow cytometry analysis showed the percentage of cells in the G1/G0, S and G2/M phases. (D) IGF1R-siRNA inhibited colony formation. (E) The data showed the percentage of early apoptosis and late apoptosis. (F) The expression analysis for IGF1R/AKT signaling pathway regulation proteins in A549 cells at 48 h after transfection with IGF1R-siRNA (* $P < 0.01$, n = 3).

in Fig. 5F, the expression of AKT (0.17 folds) protein and the phosphorylation of p-AKT (0.13 folds) were suppressed by IGF1R-siRNA. For cell-cycle regulation, IGF1R-siRNA could reduce the expression of CDK2 (0.34 folds), CDK4 (0.20 folds), Cyclin A2 (0.09 folds) and Cyclin D1 (0.39 folds). These results demonstrate that miR-30a and its target gene IGF1R share similar cellular and molecular effects in lung cancer A549 cells.

Discussion

In the last decade, dysregulation of miRNAs has been shown to be a common event that can control cell survival, proliferation, migration and apoptosis in NSCLC development and progression (9,22,36). Thus, miRNAs are increasingly viewed as a potential diagnostic and therapeutic tool (29). Up to now, only a small proportion of identified miRNAs have been investigated to elucidate their critical roles in NSCLC. Identifying miRNAs specifically involved in NSCLC will help in developing new targets for diagnosis and therapy. The aim of the present work is to elucidate the biological functions of miR-30a in NSCLC. The results showed that miR-30a was frequently downregulated in both lung cancer tissues and cell lines. Especifically, the expression of miR-30a was lowest in advanced TNM stage (III-IV) and A549 cells. Therefore, miR-30a might locate in primary tissue of lung cancer and paly more important in advanced TNM stage. In addition, overexpression of miR-30a inhibited NSCLC cell proliferation and cell viability by blocking cell-cycle transition in vitro. But Patnaik et al. reported that miR-30a overexpression did not lead to an alteration in cell cycle and proliferation in A549 cell line (27). Their results showed that the expression of miR-30a increased to 2 folds after transfection pre-miR-30a vector, but our results showed that miR-30a increased to 35.4 folds after transfection. The two different results might due to distinct transfection rate. The higher expression level of miR-30a may lead to significant effects by silencing completely target gene. We investigated potential targets of miR-30a. IGF1R was postulated to be a target of miR-30a using different databases. As indicated on reporter assaying, miR-30a repressed the construct with the IGF1R 3′-UTR. Overexpression of miR-30a inhibited the expression of I IGF1R mRNA and protein; miR-30a overexpression suppressed IGF1R 3′UTR luciferase report activity and this effect was abolished by mutation of the miR-30a seed binding site. The results suggest that miR-30a may play the critical role of a negative regulator or tumor suppressor for cell proliferation partly mediated by repressing IGF1R expression in NSCLC. It is reported that miR-198, miR-30b/c, miR-34a and miR-545 can inhibit lung cancer cell proliferation, but the effect of miR-30a inhibition lung cancer cell proliferation is obviously better than them. This will help expand our understanding of NSCLC and aid in developing miR-30a for new targets of diagnosis and therapy.

The IGF1R is frequently overexpressed in human gastric cancer (40), colorectal cancer (32), and breast cancer (19), suggesting that aberrant IGF1R expression may contribute to initiation and progression of malignancies. Few reports have evaluated expression of IGF1R in NSCLC patients (23). We analyzed expression of IGF1R mRNA and protein in NSCLC samples using quantitative real-time PCR and western blot. Interestingly, IGF1R expression was significantly upregulated in NSCLC samples as compared with normal tissues, which was consistent with Nurwidya et al.'s findings (23). But little is known about the mechanisms underlying these positive effects mediated by IGF1R in NSCLC. IGF1R is a tyrosine kinase receptor that is activated by IGFs, the activated IGF1R not only activates the PI3K-AKT signaling pathways, which stimulates cell proliferation and protects from apoptosis (1,7).

Although IGF1R plays potent proliferative role in different steps of oncogenesis, there still exist controversies about regulatory mechanism. It is reported that processes of cancer cells could be suppressed by metformin or dietary protein restriction through insulin-independent and insulin-dependent mechanisms (8,35). Recent studies by Fontana et al. indicated that siRNA directed against IGF1R in the pGBMcell lines resulted in inhibition of p-AKT and induced an accumulation of cells in G1 phase in contrast to a reduction of S and G2 phases (3,10). Urbanska et al. reported that IGF1R inhibitor (NVP-AEW541) in BsB8 cells significantly downregulated GSK3b-mediated phosphorylation and N-Myc to induce a G1-phase arrest (34). Our results showed that miR-30a induce G1- phase and S-phase arrest by targeting IGF1R in NSCLC A549 cells. To further reveal the functions of IGF1R in NSCLC, we silenced IGF1R by synthetic IGF1R siRNA and observed that IGF1R siRNA-treated A549 cells displayed significant reduction of IGF1R mRNA and protein level, consequently resulting in inhibited cell proliferation and colony formation, blocked G1/S and S/G2 transition in NSCLC cells.

Gao et al. reported that IGF1R was involved in the PI3K/AKT signaling pathway in oral squamous cell carcinomas (12). This signaling pathway has been implicated in promoting tumor cell survival, proliferation, migration and invasion (2,4). In the present study, our results showed that miR-30a and IGF1R silencing suppressed NSCLC cell proliferation through inhibiting PI3K/AKT signaling pathway. Further experiments revealed that IGF1R expression was positively correlated with cell cycle-related protein CDK2, CDK4, Cyclin A2 and Cyclin D1 in NSCLC cells, suggesting that IGF1R can upregulate expression of CDK4/Cyclin D1 and CDK2/Cyclin A2 complex via Akt signaling pathway in the proliferative progression of NSCLC. Gao et al. reported that IGF1R silencing blocked S/G2 transition throught AKT signaling pathway inhibiting the expression of CDK2/Cyclin A2 complex (12). It was reported that the inhibition of AKT signaling pathway could block G1/S transition throught suppressing the expression of CDK4/Cyclin D1 complex (13,25). These findings are concordance with our results. Our findings suggested that increased IGF1R can upregulate expression of CDK2/Cyclin A2 and CDK4/Cyclin D1 complex via AKT signaling pathway in the proliferative progression of NSCLC.

In summary, we investigated the role of miR-30a, its targeted gene IGF1R, and their potential implication in pathologic processes of NSCLC. Our findings suggest that miR-30a may be a novel tumor suppressor that inhibites the proliferation of NSCLC cells through PI3K/AKT signaling pathway related cell-cycle progression by targeting IGF1R. Our findings provide evidence that miR-30a might be a effective tool for future diagnosis and therapy of NSCLC. However, more work is needed to uncover the roles and mechanisms of miR-30a in NSCLC in vitro, and explore the effects of miR-30a in normal cells.

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