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MicroRNA-490-3p inhibits proliferation of A549 lung cancer cells by targeting CCND1

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate the translation of messenger RNAs by binding their 3′-untranslated region (3′UTR). In this study, we found that miR-490-3p is significantly down-regulated in A549 lung cancer cells compared with the normal bronchial epithelial cell line. To better characterize the role of miR-490-3p in A549 cells, we performed a gain-of-function analysis by transfecting the A549 cells with chemically synthesized miR-490-3P mimics. Overexpression of miR-490-3P evidently inhibits cell proliferation via G1-phase arrest. We also found that forced expression of miR-490-3P decreased both mRNA and protein levels of CCND1, which plays a key role in G1/S phase transition. In addition, the dual-luciferase reporter assays indicated that miR-490-3P directly targets CCND1 through binding its 3′UTR. These findings indicated miR-490-3P could be a potential suppressor of cellular proliferation.

1. Introduction

CCND1 (cyclin D1) is a critical regulator of cell cycle, It promotes G1-S progression by forming active holoenzymes with CDK4 and CDK6, which sequentially phosphorylate the pRb complex. The pRb complex then release the E2F transcription factors, which trigger their target genes to regulate the G1/S phase transition [1]. As a key regulator of cell proliferation, the expression of CCND1 needs to be kept under strict control. It is subject to all aspects of regulation, including gene transcription, RNA processing, splicing, modification, protein stability and cellular localization [2]. Disregulation of any of these steps can induce overexpression of CCND1, which is known to promote tumorigenesis in many cell types [3].

MicroRNAs (miRNAs) are small (about 22 nucleotides), endogenous, non-coding RNAs that are involved in post-transcriptional gene regulation. They negatively regulate the stability of their target mRNAs at 3′UTR [4,5]. It is estimated that over 1000 microRNAs, which are encoded in human genome, target over 60% of mammalian genes [6]. Emerging evidence indicates that many microRNAs are in connection with cellular proliferation by targeting the particular and essential regulators of cell cycle [7–9]. Here, we demonstrate that miR-490-3P is markedly down-regulated in A549 lung cancer cells compared with the normal bronchial epithelial cell line (16HBE). Further studies reveal that miR-490-3P overexpression leads to inhibition of cell proliferation via G1-phase arrest, which could be partially rescued by forced expression of CCND1.

2. Materials and methods

2.1. Reagents

The miR-490-3P mimic (sense: 5′-CAACCUGGAGGACUCCAUG CUG-3′) and the negative control duplex lacking any significant homology to all human gene sequences (named as NC, sense: 5′-ACUACUGAGUGACAGUAGA-3′) were used in the transient gain-of-function study. A small interfering RNA (siRNA) targeting nucleotides 723–743 of human CCND1 mRNA was used for RNAi study (named as siCCND1, sense: 5′-GGAGAACAAACAGAUCA UCtt-3′) as described before [10]. All the RNA duplexes were synthesized by GenePharma (Shanghai, China).

2.2. Cell culture and transfection

The A549 cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (Hyclone, USA), penicillin (50 U/ml) and streptomycin (50 μg/ml) in humidified atmosphere contained 5% CO_2 at 37 °C. On the day before transfection, the cells were plated at 60% confluency in medium without antibiotics. The Lipofectamine 2000 Reagent (introvigen, USA) was used for transfection following the protocol provided by the manufacture. The normal bronchial epithelial cell line (16HBE) were cultured in DMEM medium, supplemented with the same concentration of fetal bovine serum and antibiotics as described above.

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2.3. RNA isolation and quantative real-time PCR

Total RNA was isolated from cultured cells by using RNAiso plus (TaKaRa, Japan) and reverse transcribed into cDNAs with PrimeScript RT reagent kit (TaKaRa, Japan). Small RNA was extracted from cultured cells by RNAiso for small RNA (TaKaRa, Japan) and reverse transcribed into cDNAs using One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa, Japan). IQ SYBR Green Supermix (BIO-RAD, USA) was used for real-time PCR, which was performed to quantify the expression of miR-490-3P and CCND1 by the $2^{-\Delta\Delta CT}$ method with U6 and GAPDH as internal control respectively. All the qPCR primers were synthesized by Sangon Biotech (Shanghai, China) and the sequences are listed as follows: CCND1 (forward: 5′-GCTGCGAAGTGGAAACCATC-3′; reverse: 5′-CCTCCTTCTGCACA CATTTGAA-3′), GAPDH (forward: 5′-AAGGTGAAGGTCGGAGTCA-3′; reverse: 5′-GGAAGATGGTGATGGGATTT-3′), miR-490-3P (forward: 5′-CAACCTGGAGGACTCCATGCTG-3′) and U6 (forward: 5′-TGCGGGTGCTCGCTTCGGCAGC-3′).

2.4. Western blotting analysis

The cultured cells were harvested and lysed directly for 45 min. The protein concentration was measured and equilibrated by using Piece BCA Protein Assay Kit (Thermo Scientific, USA). The equivalent amounts of protein were separated by 10% SDS–polyacrylamide gels and transfected to pre-wetted PVDF membranes. The menbranes were then blocked in 5% non-fat-milk for 1 h and incubated overnight with primary antibody. On the following day, the membranes were washed in TBS-T for three times and incubated with HRP-conjugated secondary antibody in TBS-T for 1 h. The enhanced chemiluminescence (ECL) was used to detect the secondary antibody. The CCND1 expression levels were analyzed by using rabbit polyclonal anti-CCND1 antibody (Epitomics, USA), and the anti-GAPDH antibody (Sangon Biotech, China) was used to detect the expression of GAPDH, which was used as endogenous control to normalize the expression level of CCND1.

2.5. Cell cycle analysis by flow cytometry

Forty-eight hours after transfection, the cells were harvested, washed with pre-chilled PBS and fixed with 75% ethanol at −20 °C for 24 h. The Propidium iodide staining was processed by using the Cell Cycle and Apoptosis Analysis Kit (Beyotime, China). The cell cycle analysis was performed by BD LSRII Flow cytometry system with FACSDiva software (BD Bioscience, USA) and the data was analyzed by ModFit LT software.

2.6. Colony formation assay

The assay was performed as previously described [11]. Twenty-four hours after transfection, cells were harvested and the single-cell suspension was made. Five hundreds of transfected cells were then seeded into 6-well plate and incubated for 14 days without any disturbance. After incubation, the cells were washed with PBS twice and fixed with absolute methanol for 15 min, then stained with 0.1% crystal violet. The rate of colony formation was calculated by following formula: colony formation rate = (number of colonies/number of seeded cells) × 100%.

2.7. Cell growth/cell viability assay

Cells were seeded into 96-well plates at the density of 6000 per well. After overnight incubation, the cells were transfected with RNA duplexes (miR-490-3P mimics, siCCND1, NC) at the progressive concentration for 48 or 72 h. Sequentially, the medium was removed and the cell counting solution (WST-8, Dojindo Laboratories, Tokyo, Japan) was added to each well and incubated at 37 °C for another 1 h. The absorbance of the solution was measured spectrophotometrically at 450 nm with MRX II absorbance reader (Dynex Technologies, Chantilly, VA, USA).

2.8. Dual-luciferase reporter assay

Based on the human CCND1 mRNA sequence in Genbank, a pair of annealing oligonucleotide, which contained the putative binding site for miR-490-3P, were designed (forward: 5′-cGGCCCTGCAGC CAGCTCACGTCCAGGTTCAACCCACAGCTACTTGGTTg-3′, reverse: 5′-tcgacAACCAAGTAGCTGTGGGTTGAACCTGGACGTGAGCTGGCTGCAG GGCCgagct-3′) and synthesized by Sangon Biotech (Shanghai, China). The double-stranded annealing products were inserted into the down-stream of the firefly luciferase reporter in the pmirGLO Dual-luciferase miRNA target expression vector (Promega, USA). In addition, a pair of mutant annealing oligonucleotide containing a mutant sequence in the seeding region of miR-490-3P (forward: 5′-cGGCCCTGCAGCCAGCTCACGAGGTCCAACAACCCACAGCTACTTGG TTg-3′, reverse: 5′-tcgacAACCAAGTAGCTGTGGGTTGTTGGACCTCG TGAGCTGGCTGCAGGGCCgagct-3′) was also cloned into the same region of the vector. HEK 293T cells were plated in 24-well plates for 24 h, and then co-transfected with 50 nM of miRNA-490-3P mimics or negative control and 100 ng dual-luciferase vector, which contained either wildtype or mutant 3′-UTR. 48 h after transfection, The luciferase activity was measured by Dual-luciferase Reporter Assay System (Promega, USA).

2.9. CCND1 rescue experiments

The coding sequence of human CCND1 excluding its 3′UTR was inserted into the pTarget vector (GeneCopoeia, USA). The A549 cells were co-transfected with negative control oligos or miR-490-3P mimics and with empty pTarget vector or pTarget-CCND1. Forty-eight hours after transfection, the cells were harvested and analyzed using cell cycle assay by flow cytometry, which was described above. Western blotting was also used to signify the expression of CCND1.

2.10. Statistical analysis

All the data were presented as means ± SD (standard deviation). Each experiment was repeated at least three times. Differences between samples were analyzed by *t*-tests using Graphpad prism version 5 software. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. miR-490-3P is down-regulated in A549 lung cancer cells compared with the normal bronchial epithelial cell line

It has been pointed out by recent studies that miR-490-3P acts as a modulator of cell proliferation through different pathways in different cell lines [12,13]. We wondered whether it could regulate cell proliferation in other ways. Using microRNA target gene prediction database, we found that CCND1, which was widely accepted as a critical regulator of cell proliferation, was simultaneously predicted as a target gene of miR-490-3P by TargetScan, miRanda and miRwalk. We therefore hypothesized that miR-490-3P might be a potential regulator of cell cycle through directly targeting CCND1. Considering the different growth rate of tumor cells and normal cells, We used A549 lung cancer cells and normal bronchial epithelial cell line (16HBE) for pre-experiment. Real-time RT-PCR was used to quantify and compare

the expression levels of miR-490-3P in the two bronchiolar epithelial cell lines. We found that miR-490-3P expression levels were significantly decreased in A549 cells compared with 16HBE cells (Fig. 1A). On the contrary, the expression levels of CCND1 were relatively increased in A549 cells (Fig. 1B).

3.2. miR-490-3P suppresses cell proliferation, inhibits clonogenicity, and induces G1-phase arrest in A549 lung cancer cells

The low expression level of miR-490-3P in A549 cells led us to elucidate whether miR-490-3P could function as a suppressor of cell proliferation. The cells were therefore transfected with miR-490-3P mimics for gain-of-function analysis. Although there was no significant dosage effect, different concentration of miR-490-3P mimics demonstrated potent inhibitory effects (Fig. 1C). In parallel, miR-490-3P also obviously impaired the colony forming ability of A549 cells. The colony formation capacity of miR-490-3P transfected cells was much worse than those transfected with NC oligos (Fig. 1D). To figure out the underlying mechanisms for suppressed cell proliferation and clonogenicity by miR-490-3P, We used flow cytometry to observe the distribution of the cell cycle after transfected with miR-490-3P mimics at a concentration of 50 nM. Apparently, forced expression of miR-490-3P, as well as the overexpression of siCCND1, induced significant G1-phase arrests in A549 cells (Figs. 1E and 3A). These results indicated that miR-490-3P was a negative regulator of cell proliferation in A549 lung cancer cells.

3.3. miR-490-3P down-regulates the expression of CCND1 via its 3′-UTR

As the effect of miR-490-3P was similar to that of siCCND1 (Fig. 3B and C), We investigated whether CCND1 was a direct target of miR-490-3P. We used quantative real-time PCR and western blot to observe the expression of CCND1 in both mRNA and protein level in A549 cells transfected with miR-490-3P mimics. The results demonstrated that both of the expression levels were decreased (Fig. 2A and B). Further more, the target analysis

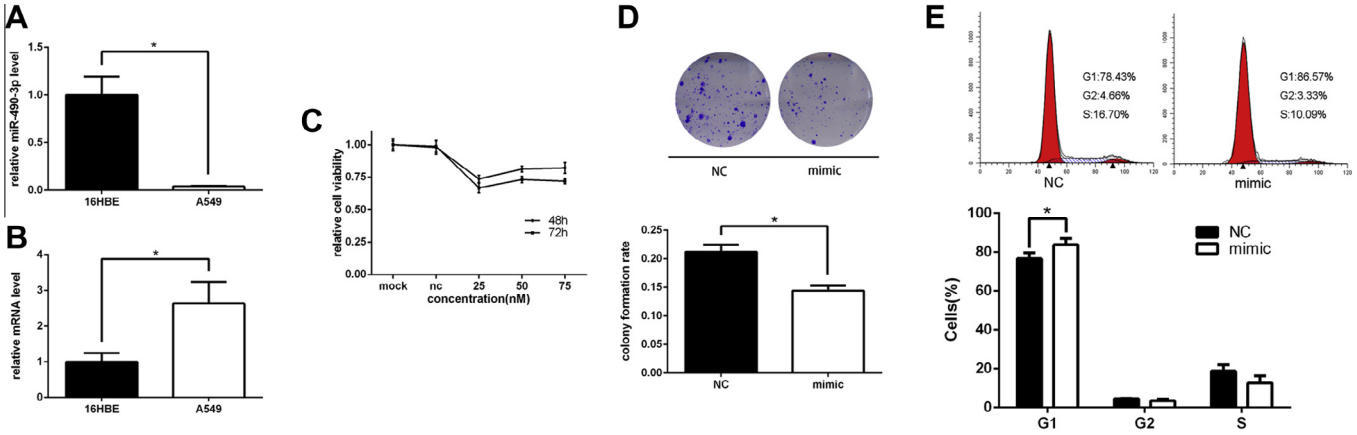

Fig. 1. The altered expression of miR-490-3P and CCND1 in A549 lung cancer cells compared with normal bronchial epithelial cell line and over-expression of miR-490-3P inhibited cell proliferation. Expression levels of miR-490-3P and CCND1 by real-time PCR analysis were normalized with U6 and GAPDH respectively. (A and B) The miR-490-3P and CCND1 levels in A549 cells were detected and compared with normal bronchial epithelial cell line (16HBE) (*$P < 0.05$). (C) Cell growth/viability assay. The relative cell viability of miR-490-3P mimics treated groups was lower than that of NC oligos treated. Cell viability of 0 Nm (mock) was regarded as 1.0. (D) The colony formation rate of miR-490-3P mimics transfected groups was lower compared with NC oligos transfected groups (*$P<0.05$). (E) Over-expression of miR-490-3P induced significant G1-phase arrest (representative histograms are shown above. The indicated percentages are the average of triplicate experiments) (*$P < 0.05$).

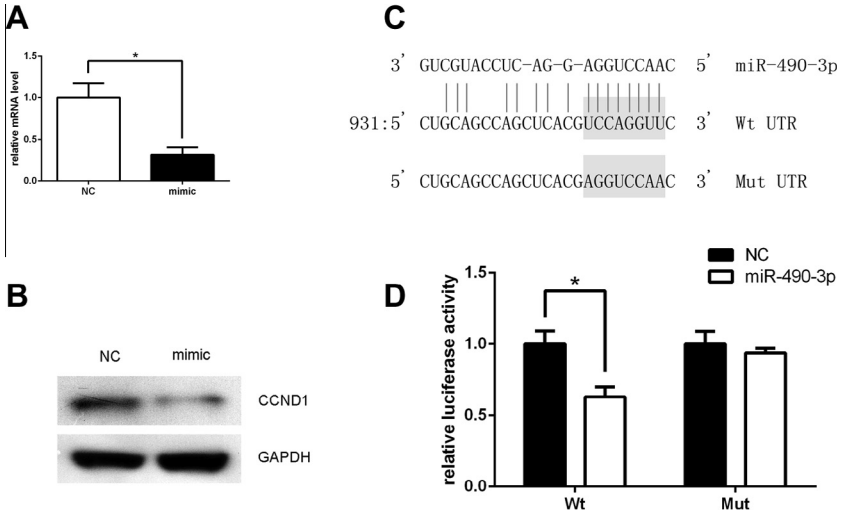

Fig. 2. CCND1 was a direct target of miR-490-3P. (A) The miR-490-3P mimics reduced the expression of CCND1 at the level of mRNA significantly (*$P < 0.05$). (B) Western blot analysis was performed to detect the expression of CCND1 and GAPDH following transfected with miR-490-3P mimics. (C) Starting from the No.931 base in the 3′UTR of CCND1, a predicted binding site of miR-490-3P was shown. The mutated sequence was highlighted in grey (bottom). (D) 293T cells were co-transfected with 50 nM of either miR-490-3P mimics or NC oligos and 200 ng pmirGLO Dual-luciferase miRNA Target Expression vector containing either the Wt or Mut 3′UTR of CCND1. The relative firefly luciferase activity normalized with renilla luciferase was measured 48 h after transfection (*$P < 0.05$).

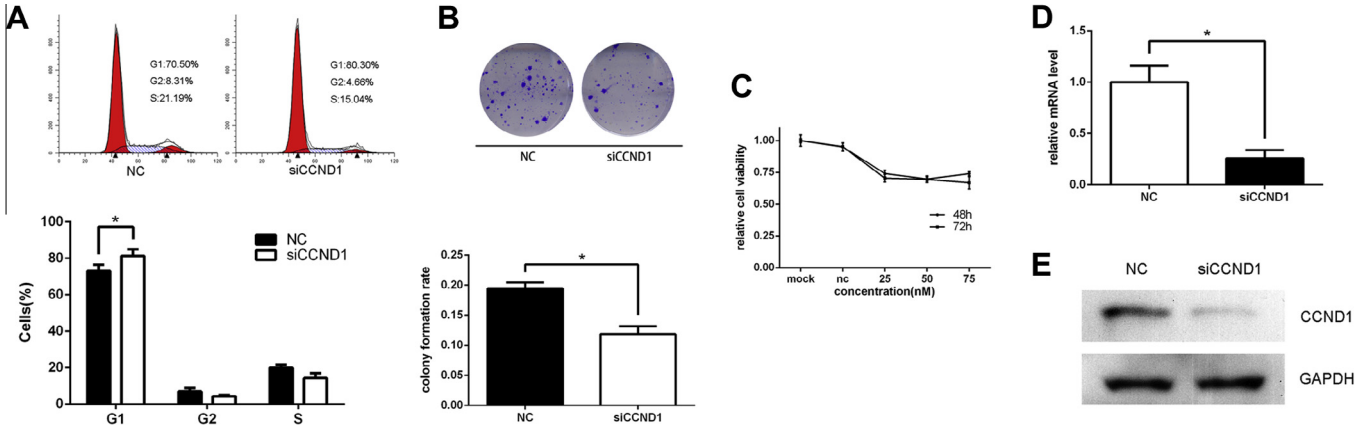

Fig. 3. Knock-down of CCND1 represented the effect of miR-490-3P. (A) Down-regulation of CCND1 by siCCND1 induced significant accumulation of cells in G1 phase. Representative histograms were shown above. The indicated percentages are the average of triplicate experiments (*$P < 0.05$). (B) SiCCND1 reduced the colony formation rate. Representative wells were presented) (*$P < 0.05$). (C) SiCCND1 caused significant cell viability reduction, respectively 48 h and 72 h after transfection. (D and E) Knock-down of CCND1 reduced the expression of CCND1 both at the mRNA and protein level (*$P < 0.05$).

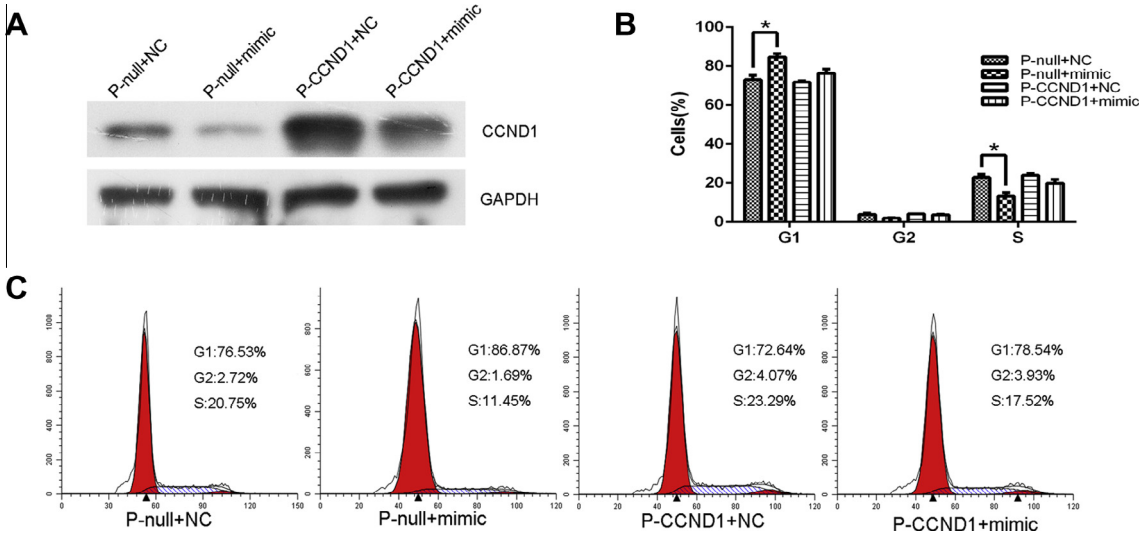

Fig. 4. Forced expression of CCND1 rescued miR-490-3P-dependent G1-phase arrest. (A) Either miR-490-3P mimics or NC oligos were co-transfected with pTarget-CCND1 or empty pTarget vector into A549 cells. Western blot analysis was then performed to detect the expression of CCND1 and GAPDH. (B and C) Forced expression of CCND1 circumvented the G1-phase arrest induced by miR-490-3P mimics (*$P < 0.05$).

identified a single predicted miR-490-3P binding site in the 3′-UTR of CCND1 mRNA (Fig. 2C). A part of the 3′-UTR of CCND1, which contained the putative miR-490-3P binding site, was cloned in the downstream of the firefly luciferase of pmirGLO Dual-Luciferase miRNA Target Expression vector. In addition, We also constructed the mutant vector, which means it contained the mutant binding site (Fig. 2C). As expected, forced expression of miR-490-3P in HEK 293T cells significantly reduced the relative luciferase activity compared with negative control. Nevertheless, the luciferase activity of the vector with mutated 3′-UTR was unaffected by the same treatment (Fig. 2D). These data suggested that CCND1 was a direct target of miR-490-3P.

3.4. restoration of CCND1 rescues miR-490-3P-induced G1-phase arrest

To determine the functional relevance of CCND1 targeting by miR-490-3P, we asked if CCND1 overexpression could hold back the inhibitory effects of miR-490-3P on cell cycle arrest. For this purpose, we inserted the human CCND1 coding sequence into the pTarget vector and CCND1 expression was regained after transfected with this pTarget-CCND1 vector (Fig. 4A). Flow cytometry was used to evaluate the cell cycle of A549 cells co-transfected with either miR-490-3P mimics or negative control and with either pTarget-CCND1 or empty pTarget vector. We observed that forced expression of CCND1 rescued G1-phase arrest in the presence of miR-490-3P mimics (Fig. 4B and C). Therefore, CCND1 is a functionally relevant target downstream of miR-490-3P that modulates the cell cycle in A549 cells.

4. Discussion

The cell proliferation is driven by cycle machinery operating in the cell nucleus. A group of proteins called cyclins, bind and activate cyclin-dependent kinases (CDKs). The cyclin-CDK complexes then phosphorylate the retinoblastoma proteins (pRb). That causes pRb to release the E2F transcription factors, which then activate their downstream genes essential to G1/S phase transition, thus drives cell cycle progression [14,15]. Proper progression through the cell cycle is monitored by checkpoints that sense possible defects during DNA synthesis and chromosome segregation [14]. As an essential step in carcinogenesis, the deregulation of cell cycle

underlies the aberrant cell proliferation that characterizes cancer and loss of cell cycle checkpoint control promotes genetic instability [16–18].

As a critical regulator of cell proliferation, CCND1 expression needs to be kept under tight control [2]. In different cellular contexts, different pathways play a dominant role in regulating the expression of CCND1, whereas their disregulation can contribute to overexpression of CCND1 in tumorigenesis [3,19]. Recently, an increasing number of studies have demonstrated that CCND1 is frequently overexpressed in cancers and its overexpression can be attributed to many factors including increased transcription, translation, and protein stability [20]. As for lung cancers, experimental evidences have shown that the tumor cell proliferation can be promoted or inhibited through up or down-regulating the expression of CCND1 [21–23].

During the past decades, the regulative function of microRNAs in cell proliferation has been well validated in accumulating studies [7–9,11,24] and the alteration of microRNAs was a common events in tumor tissues [25,26]. The discrepancy of microRNA expression may contribute to tumor development by disrupting the regulation of cell cycle [27–29]. An increasing number of studies reveal that microRNAs have been turned out to be promising diagnostic and prognostic molecular biomarkers as well as therapeutic targets in cancers [30–32]. Until now, miR-490-3P has been validated to act as a regulator of cell proliferation in hepatocellular carcinoma cells and vascular smooth muscle cells [12,13], but as a negative regulator of cell proliferation by directly targeting CCND1, it has not been reported yet. This is the first study to determine the function of miR-490-3P in A549 lung cancer cells.

In this study, we demonstrated the relative expression of miR-490-3P was significantly decreased in A549 lung cancer cells compared with normal bronchial epithelial cell line. In order to find out the roles of miR-490-3P, we conducted gain-of-function studies. When transfected with miR-490-3P mimics, the A549 cells showed significant longer G1 phase. The decrease of colony formation and cell viability may be due to the mechanism of miR-490-3P-induced cell cycle arrest. More importantly, we found that overexpression of miR-490-3P down-regulated both mRNA and protein levels of CCND1. Immediately after that, using dual-luciferase reporter assay, we demonstrated that miR-490-3P inhibited CCND1 expression by directly targeting its 3′UTR. Finally, the rescue experiment indicated that forced expression of CCND1 could rescue the effects of miR-490-3P on cell cycle.

In summary, our study indicated the down-regulated expression of miR-490-3P in A549 lung cancer cells, and for the first time, we demonstrated that CCND1 may be one of the direct targets of miR-490-3P involved in cell proliferation. Although further studies are needed to validate the other targets and determine the expression of miR-490-3P in lung cancer tissues or other cell lines, our experimental data suggests that miR-490-3P should be noticed to be a potential suppressor of cell proliferation.

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