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MicroRNA-202 induces cell cycle arrest and apoptosis in lung cancer cells through targeting cyclin D1

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Abstract. – **OBJECTIVE:** MicroRNAs are critical regulators in tumorigenesis. This study is aimed at investigating the function of miR-202 in the proliferation of human lung cancer cells.

PATIENTS AND METHODS: Lung cancer tissues and paired adjacent normal tissues were collected from 20 patients; the expression of miR-202 was tested by Realtime PCR; cell proliferation and cell cycle distribution were determined by CCK-8 assay and flow cytometry, respectively; apoptosis was determined by TUNEL assay and Western blot analysis of cleaved-PARP; the relationship between miR-202 and cyclin D1 mRNA 3'UTR was determined by luciferase activity assay.

RESULTS: MiR-202 was significantly reduced in lung cancer tissues compared with the adjacent normal tissues. Overexpression of miR-202 inhibited cell proliferation and induced a G0/G1 cell cycle arrest and apoptosis. Luciferase activity assay and Western blot analysis together showed that miR-202 can bind to the 3'UTR of cyclin D1 mRNA directly and inhibits cyclin D1 expression at the protein level. In addition, restoration of cyclin D1 expression partially abolished cell cycle arrest and apoptosis induced by miR-202.

CONCLUSIONS: MiR-202 is constantly downregulated in lung cancer and functions as a tumor suppressive gene via targeting cyclin D1. Modulating the level of miR-202 may be a novel therapeutic method for lung cancer.

Key Words:
Lung neoplasms, MicroRNAs, Cell cycle, Apoptosis.

Introduction

Lung cancer is one of the most common and aggressive cancers over the world. It is estimated that about 1 million people are dying of lung cancer each year[1]. The conventional treatment strategy for lung cancer, including surgery, chemotherapy and radiotherapy, did not guarantee a decent prognosis in lung cancer patients; recent reports showed that the five-year survival rate of lung cancer is only 16%[2]. Thus, developing new drugs beyond the traditional treatment methods is becoming an urgent need.

Uncontrolled cell proliferation is one the most important hallmarks of cancer cells, and the aberrant cell cycle progression accounts for the dysregulated cell growth, which ultimately leads to the formation of tumors. The cell cycle is properly controlled under the coordination of several cyclins and cell cycle inhibitors[3], the cyclins function to drive cell cycle progression by activating Cyclin-Dependent Kinases (CDKs)[4]. Different cyclins are involved in different cell cycle stages[4,5]. When forms a complex with CDK4, cyclin D1 activates its enzyme activity and drives the G1/S transition of the cell cycle. Previous studies have reported the overexpression of cyclin D1 in cancers such as bladder cancer[6] and lung cancer[7], and high cyclin D1 expression was identified to be associated with a higher risk of relapse and death in breast cancers[8], which indicated the pivotal role of cyclin D1 in tumor biology. A growing body of evidence has shown that a class of small non-coding RNAs, which has been referred to as microRNAs (miRs), profoundly influences the development of progression of cancers[9, 10]. By posttranscriptional repression of a network of genes, microRNAs are implicated in several cellular processes including cell cycle regulation[11]. Gene encoding miR-202 is located in the chromosome site 10q26, and miR-202 is reported to be a tumor

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suppressor in several cancers such as gastric cancer[12], hepatocellular carcinoma[13] and osteosarcoma[14]. However, the function of miR-202 and its possible regulatory mechanism in lung cancer is still undefined.

As such, the main goal of this study was to determine whether miR-202 is differentially expressed in lung cancer and to analyze its role in cancer cell growth. Our results showed that miR-202 level was significantly reduced in tumor tissues, and miR-202 overexpression in lung cancer cells inhibited cell growth by inducing cell cycle arrest and apoptosis. Moreover, we report cyclin D1 as a novel target of miR-202 for the first time. Therefore, our study preliminarily defines the tumor suppressive role of miR-202 in lung cancer.

Patients and Methods

Tissue Collection

The surgically removed tumor tissues and the paired adjacent normal tissues of human lung cancer were obtained by Wuhan Central Hospital under the policies of the Ethics Review Board. Informed consent was obtained by each individual. The tissues were stored in liquid nitrogen before experiments.

Cell Culture

Cell line for human lung cancer A549 was purchased by American Type Culture Collections (ATCC, Manassas, VA, USA). F-12K Medium supplemented with 10% Fetal Bovine Serum (FBS, GIBCO, Life Technologies, Grand Island, NY, USA) and the appropriate amount of penicillin-streptomycin solution (Gibco). Cells were cultured in a humidified CO2 incubator (Thermo Scientific, Waltham, MA, USA).

Transfection

The synthesized oligos for miR-202 mimics or negative control (NC) were all purchased from Guangzhou RiboBio Technology (Guangzhou, China). The construct for cyclin D1 overexpression, pCMV-cyclin D1 (Plasmid #19927) and its empty vector control (Plasmid #32530) were all obtained from Addgene (Cambridge, MA, USA). To deliver the microRNA or/and plasmid into cells, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used as per manufacture's recommended dosage. MiR-202 or NC was transfected at the molar concentration of 100nM, and plasmids were transfected at the concentration of 1μg/ml. For mock transfection control, Lipofectamine 2000 was added to the culture medium alone.

Real-time PCR

MiR-202 levels were quantified using the SYBR green-based fluorescent quantitative real-time PCR system. Briefly, after the RNAs were isolated from the cells with Trizol Reagent (Invitrogen), the RNA templates were reverse transcribed into cDNA using the GoScript Reverse Transcription System from Promega (Madison, WI, USA). For reverse transcription, the stem-loop primer for miR-202 (RiboBio) was used. The cDNA templates were amplified on a BioRad CFX96 thermocycler (Richmond, CA, USA). The expression of U6 snRNA was used to normalize miR-202 level in each group.

Proliferation Assay

A549 cells were seeded in 96-well plates at 10^4 cells/well, cells grown at the 70-80% confluence were transfected with miR-202 or NC. The living cells at different time points (0, 24, 48 and 72h) were determined using the Cell Counting Kit-8 (CCK-8, Beyotime Biotechnology, Shanghai, China) according to the manufacture's protocol.

Western Blot

After 72h post-transfection, cells were harvested in RIPA buffer (Beyotime, Shanghai, China) and the whole cell lysates were mixed with 5x SDS-loading buffer (Beyotime), the mixture was then heated at 95°C for 5min. The protein samples were then loaded on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrophoresis was applied at 120v for 90 min. Protein was, then, electro-transferred to polyvinylidene fluoride (PVDF) membranes and followed by blocking with 5% non-fat milk in phospho-buffered saline (PBS) at room temperature for 2h. The antibodies were diluted in blocking buffer and incubated with membranes overnight at 4°C. On the second day, membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 2h. The membranes were then visualized using the ECLplus kit (Beyotime). Quantification of the Western blot bands was performed by the Image Pro Plus software (Media Cybernetics). The antibodies for cleaved-PARP (cPARP) and cyclin D1 were purchased by Cell Signaling Technology (Beverley, MA, USA), the antibody for β-actin was purchased by CWBio (Beijing, China), the antibody for Ki67 was purchased by Abcam (Cambridge, MA, USA).

Cell Cycle Analysis

Cell cycle distribution was determined using the flow cytometry technique. A commercial kit based on propidium iodide (PI) staining was obtained by Beyotime. The procedures were in accordance with the instruction provided by the manufacturer. After transfection for 72h, cells were fixed in 70% ethanol overnight, On the following day, incubation with RNase A was performed followed by staining with PI for 25 min, cells were sorted and analyzed on a BD FACSCalibur™ Flow Cytometer (Becton Dickinson, San Jose, CA, USA).

Luciferase Reporter Assay

The luciferase reporter was constructed by RibBio, the CCND1 3'UTR were inserted into the pmiR-RB-Report™ construct between *hRluc* and *hluc* gene (Figure 4A). For mutated 3'UTR construct, the base pairs in the seed sequence binding region were modified to GCGAACA (5' to 3'). The luciferase reporters were then transfected with miR-202 or NC into A549 cells. The luciferase activity was detected 48h after transfection using the Dual-Glo luciferase assay system (Promega, Madison, WI, USA).

Transferase-mediated Deoxyuridine Triphosphate-Biotin nick end Labeling (TUNEL) staining

TUNEL assay was conducted using an In situ cell death detection kit (Roche, Mannheim, Germany). Cells were seeded on coverslips in a 12-well plate; after transfection for 72h, cells were fixed with 4% paraformaldehyde and processed as the protocol recommended by the manufacturer. The TUNEL positive cells were analyzed under a fluorescent microscope, 5 fields of each slide were analyzed. And the experiments were repeated 3 times.

Statistical Analysis

All data were expressed as mean ± SEM, the differential expression of miR-202 in tumor samples was determined using Wilcoxon test, and the expression level of miR-202 in each pair (tumor tissue and normal tissue) was transformed to log2 fold change. One way analysis of variance (ANOVA) was applied to the rest of the comparisons, followed by Bonferroni test, $p<0.05$ was considered significant. All experiments were repeated 3 times.

Results

MiR-202 Expression is Decreased in Lung Cancer Tissues

To evaluate the possible role of miR-202 in lung cancer, we tested its expression in tumor tissues and paired adjacent normal tissues. We found that miR-202 expression was down-regulated in 75% of the patients (Figure 1A). Moreover, the average expression of miR-202 was significantly down-regulated as well in these tumor tissues (Figure 1B). These results indicated the potential anti-tumor function of miR-202 in lung cancer cells.

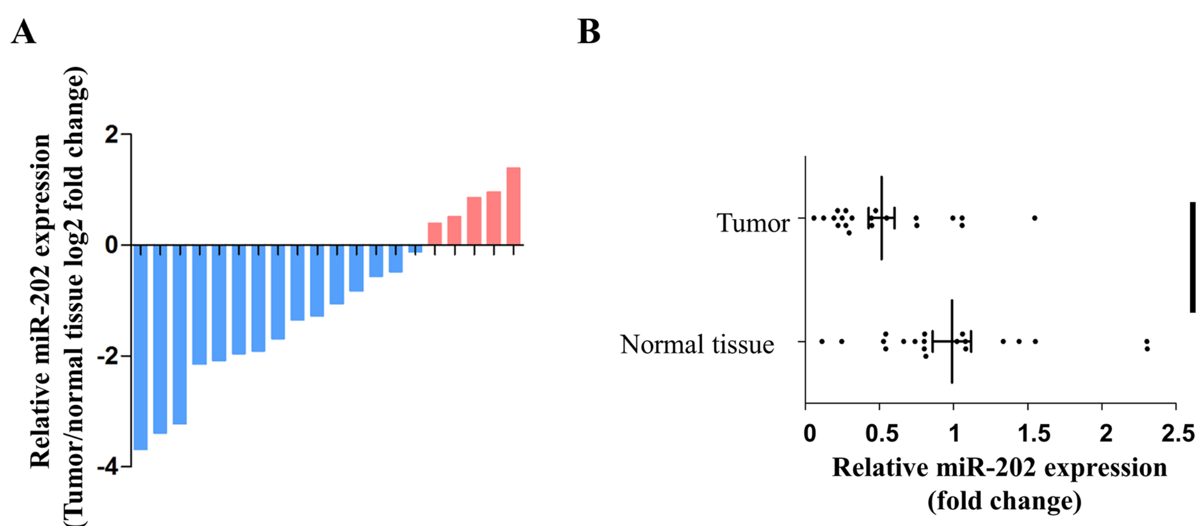

Figure 1. Downregulation of miR-202 in lung cancer tissues. ***(A)*** Relative expression of miR-202 in each pair of the tissue specimens, the relative expression was transformed to the log2 fold change. ***(B)*** The relative expression of miR-202 in lung cancer tissues and adjacent normal tissues, $^*p<0.05$.

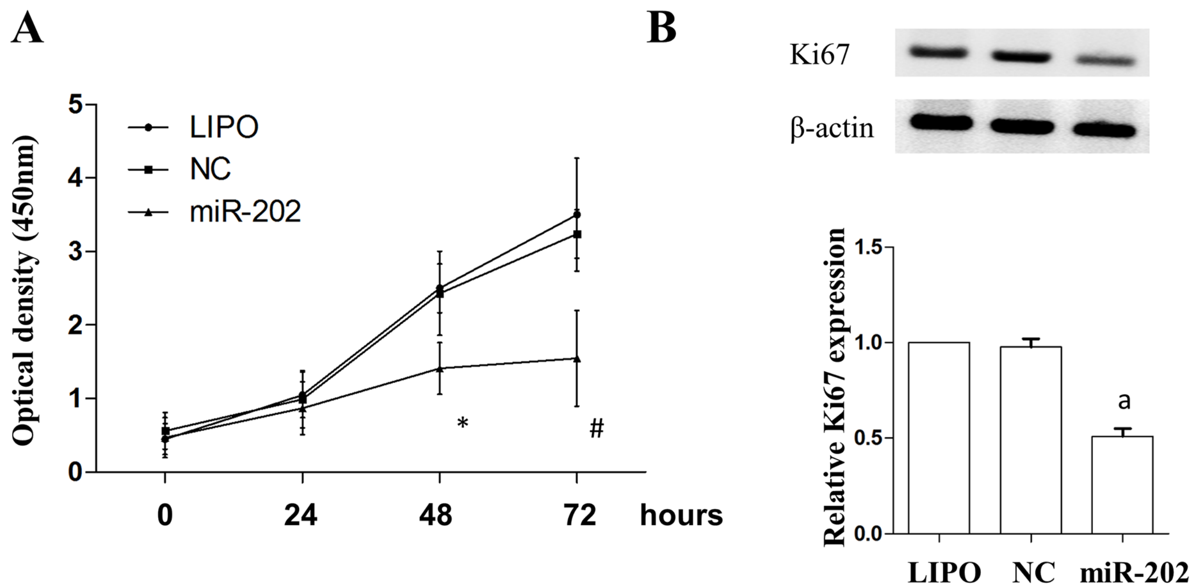

Figure 2. The effect of miR-202 on cell proliferation. ***(A)*** The growth curve of cells after transfection. The living cells were measured by CCK-8 assay, and the optical density at 450nm in each time point was plotted, *p<0.05 *vs.* NC at 48h, #p<0.05 *vs.* NC at 72h. ***(B)*** The expression of proliferation marker Ki67 after LIPO treatment or transfection of NC or miR-202 for 72h, [a]p<0.05 *vs.* NC. LIPO, lipofectamine 2000; NC, negative control.

MiR-202 Inhibits Proliferation of Lung Cancer Cells

We, then, conducted cell proliferation assay to examine the role of miR-202 in the proliferation of lung cancer cells. MiR-202 or its negative control (NC) was transfected, and Lipofectamine 2000 transfection reagent (LIPO) was given alone as the mock control group. MiR-202 transfection resulted in a significant inhibition of cell growth (Figure 2A), and Western blot analysis also revealed a decreased protein level of the proliferation marker Ki67 (Figure 2B).

MiR-202 Induces Cell Cycle Arrest and Apoptosis

We also analyzed the cell cycle distribution in each group after transfection, as shown in Figure 3A. There were approximately 50% cells in the G0/G1 phase in mock transfected cells (LIPO) or NC transfected cells. This proportion was significantly increased in miR-202 transfected cells (Figure 3A), which suggested that miR-202 overexpression resulted in cell cycle arrested in the G0/G1 phase. We also analyzed the extent of apoptosis in transfected cells, miR-202 overexpression increased the expression of apoptosis indicator cleaved-PARP (cPARP) (Figure 3B), and TUNEL staining also confirmed that more cells underwent apoptosis after miR-202 transfection (Figure 3C).

Suppression of Cyclin D1 by miR-202 Confers its Anti-tumor Activity

To gain insights into the molecular mechanism of the anti-tumor role of miR-202, we searched the online target prediction database www.microrna.org, we noticed that the gene encodes for cyclin D1, *CCND1*, was a potential target of miR-202. The predicted pairing sequence with miR-202 that locates in the 3'UTR was shown in Figure 4A. We, therefore, constructed luciferase reporter to examine whether cyclin D1 is a direct target of miR-202. As shown in Figure 4B, miR-202 inhibited the luciferase activity in wild type (WT) 3'UTR reporter, whereas co-transfection with mutant (MUT) 3'UTR reporter abolished this effect. Moreover, miR-202 transfection resulted in a decrease in cyclin D1 expression at the protein level (Figure 4C). Importantly, co-transfection of a cyclin D1 expressing plasmid reduced the cleavage of PARP (Figure 4D). In addition, co-transfection of cyclin D1 expressing plasmid partially relieved the G0/G1 cell cycle arrest induced by miR-202 (Figure 4E). These results indicated that the anti-tumor role of miR-202 is highly dependent on its direct suppression on cyclin D1.

Discussion

In our present study we revealed miR-202 as a novel tumor suppressor, which could be potentially developed as a therapeutic agent for lung

cancer. We observed multiple effects including cell cycle arrest and apoptosis after replenishment of miR-202 in lung cancer cells. We further revealed for the first time that miR-202 regulates the cell cycle progression by inhibiting the critical promotive factor of G1-S transition, cyclin D1. Therefore, miR-202 might be an important regulator in lung cancer tumor genesis.

Increasing evidence has demonstrated the critical involvement of microRNA in the regulation of multiple cellular events that controls the tumorgenic behavior of lung cancer cells[15-17]. For example, the miR-17-92 cluster has been well established as a set of oncogenic microRNAs[18] and reversely, several other microRNAs such as let-7 and miR-26b have been identified as tumor suppressors in lung cancer[19, 20]. Emerging evidence suggested that microRNAs not only participate in the pathogenesis of cancers but also can be potentially applied to the clinical practice, since microRNAs are widely implicated in the early detection, prognosis prediction and therapeutics of lung cancer[21]. To broaden our understandings on the microRNA-regulated tumor cell behavior, we focused our efforts to some novel microRNAs that have been rarely studied. A previous study has shown that miR-202 was underexpressed in asbestos-related lung cancer tissues[22]. Wang et al[23] reported the downregulation of miR-202 in the metastatic loci of lung cancer compared with its primary loci. These results suggested that miR-202 might play a role in the development and progression of lung cancer. Our data also revealed the decreased expression of miR-202 in tumor tissues compared with that in adjacent normal tissues, which is in consistent with these two previous findings.

The role of miR-202 in other cancers has been previously reported. Sun et al[14] reported that Gli2 was targeted by miR-202 to confer its pro-apoptotic role in osteosarcoma. Likewise, in hepatocellular carcinoma, the important component of Wnt/β-catenin signaling pathway LRP6 is suppressed by miR-202, which leads to a decrease in cell proliferation[13]. In addition, the similar tumor suppressive role of this microRNA in colorectal carcinoma has been discovered although a different target gene has been identified[24]. In the present study, ectopic expression of miR-202 significantly reduced the proliferation activity and induced both cell cycle arrest and apoptosis. These results showed a good consistency with the evidences in other types of cancer cells, further confirming the critical anti-tumor role of miR-202. Importantly, we identified cyclin D1 as a novel target gene of miR-202. Zhang et al[13] evidenced that miR-202 mediated suppression of Wnt/β-catenin contributes to cyclin D1 down-regulation, the results are in accordance with ours. Moreover, we propose that there also might also be a direct regulating mechanism between miR-202 and cyclin D1. Our study implied the complex regulatory network between multiple genes and microRNAs.

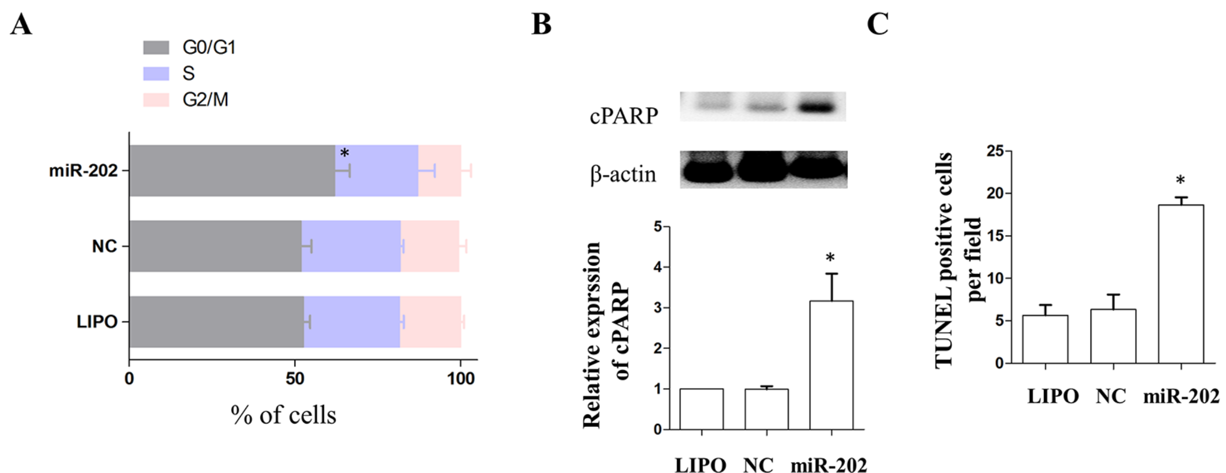

Figure 3. The effect of miR-202 on cell cycle distribution and apoptosis. ***(A)*** The effect of miR-202 on cell cycle distribution. Cell cycle analyzed by flow cytometry after LIPO treatment or transfection of NC or miR-202 for 72h, *$p<0.05$ *vs.* NC regarding the cell proportion at G0/G1 phase. ***(B)*** The effect of miR-202 on cPARP expression. β-actin is an internal control, *$p<0.05$ *vs.* NC. ***(C)*** The effect of miR-202 on cell apoptosis. Apoptosis was measured by TUNEL staining, the average TUNEL positive cells per 200x- microscopic field was evaluated. *$p<0.05$ *vs.* NC. LIPO, lipofectamine 2000; NC, negative control; cPARP, cleaved PARP.

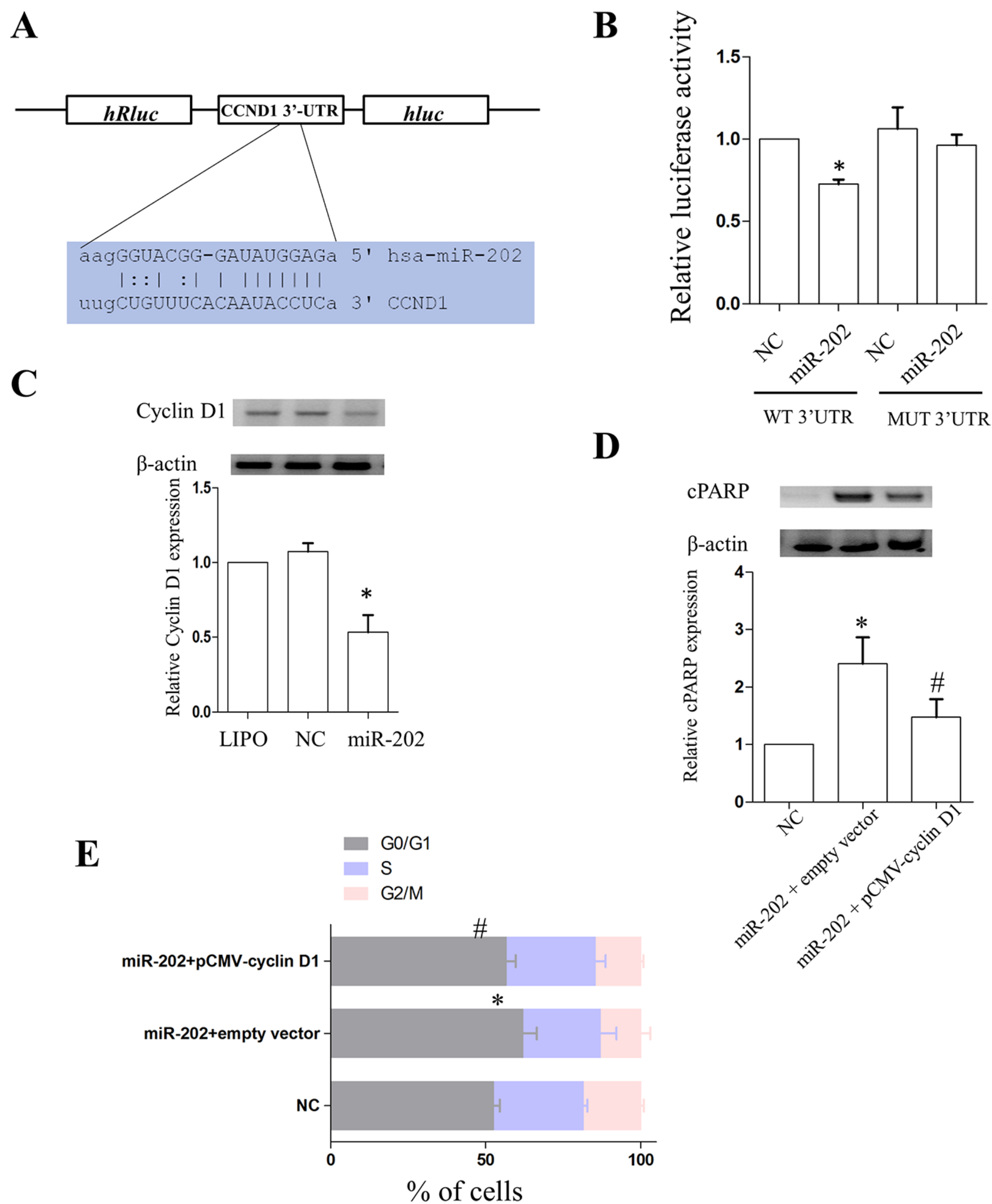

Figure 4. Cyclin D1 as a direct mediator of the anti-tumor effects of miR-202. ***(A)*** The scheme of luciferase reporter construction, the 3'UTR sequence was inserted into the backbone between *hRluc* and *hluc* gene, the predicted binding site was shown in the blue box. ***(B)*** The effect of miR-202 on luciferase activity. *$p<0.05$ *vs.* NC WT 3'UTR ***(C)*** The effect of miR-202 on the protein level of cyclin D1. *$p<0.05$ *vs.* NC. ***(D)*** The effect of cyclin D1 restoration on cPARP expression. Cells were co-transfected with miR-202 and plasmids for 72h and the whole cell lysates were collected for Western blot, *$p<0.05$ *vs.* NC, #$p<0.05$ *vs.* miR-202+empty vector. ***(E)*** The effect of cyclin D1 restoration on cell cycle distribution. Cells were transfected in the same way as described above, *$p<0.05$ *vs.* NC and #$p<0.05$ *vs.* miR-202+empty vector regarding the cell proportion at G0/G1 phase. NC, negative control; WT, wild type; MUT, mutant; cPARP, cleaved PARP.

Cyclin D1 has been demonstrated to be a prognostic marker of lung cancer[25]. Studies have shown that cyclin D1is abundantly expressed in lung cancers[7]. It has also been demonstrated that cyclin D1 drives the malignant transformation in lung[5], suggesting that inhibition of cyclin D1 might be feasible to treat patients with lung cancer. Given the multiple targets of miR-202 revealed by other studies in addition to cyclin D1, we speculated that miR-202 might exert strong and diverse antitumor effects. The *in vivo* experiment for the anti-tumor role of miR-202 is worth of further investigation.

Conclusions

In summary, miR-202 is underexpressed in lung cancer tissues compared with the adjacent normal tissue, and replenishment of miR-202 in lung cancer cells can induce cell cycle arrest and apoptosis, which is through targeting the cyclin D1. Therefore, miR-202 might be a potential tool for lung cancer treatment. More *in vivo* data are needed to validate our results.

Conflicts of interest

The authors declare no conflicts of interest.

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