

MiR-613 induces cell cycle arrest by targeting CDK4 in non-small cell lung cancer

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Abstract

Background Deregulation of microRNAs (miRNAs) has been associated with a variety of cancers, including non-small cell lung cancer (NSCLC). Here, we investigated anomalous miR-613 expression and its possible functional consequences in primary NSCLC samples and NSCLC-derived cell lines.

Methods The expression of miR-613 was measured by quantitative RT-PCR in 56 primary NSCLC tissues and adjacent non-tumor tissues. The effect of miR-613 up- or down-regulation in NSCLC-derived cells was evaluated in vitro by cell viability and colony formation assays and in vivo by growth assays in xenografted nude mice.

Results Using quantitative RT-PCR, we found that miR-613 was down-regulated in 76.8 % (43/56) of the primary NSCLC tissues tested when compared to the adjacent non-tumor tissues. We also found that the miR-613 mimic used reduced in

vitro cell viability and colony formation by inducing cell cycle arrest in NSCLC-derived cells, and inhibited in vivo tumor cell growth in xenografted nude mice. Inversely, we found that the miR-613 inhibitor used increased the viability and colony forming capacity of NSCLC-derived cells and tumor cell growth in xenografted nude mice. In addition, we identified CDK4 as a potential target of miR-613 using in silico Miranda predictions. Subsequent dual-luciferase reporter assays revealed that CDK4 acts as a direct target of miR-613. Concordantly, we found that both miR-613 mimics and inhibitors could decrease and increase CDK4 protein levels in NSCLC-derived cells, respectively.

Conclusions From our results we conclude that miR-613 may act as a tumor suppressor in NSCLC and may serve as a tool for miRNA-based NSCLC therapy.

Keywords Lung cancer · miR-613 · CDK4 · Cell cycle

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1 Introduction

Non-small cell lung cancer (NSCLC) is one of the most common cancers worldwide [1–4]. The initiation and progression of NSCLC is typically a multistage process, which is believed to involve the deregulation of multiple genes that are critical for various cellular processes, including cell cycle control, growth, apoptosis, migration and spreading. In the past decades, several studies have focused on the identification and functional assessment of genes and proteins that underlie the development of NSCLC [5–7]. In addition to these genes and proteins, also small regulatory RNA molecules, termed microRNAs (miRNAs), have recently been implicated in NSCLC development [8, 9].

MiRNAs are endogenous non-coding 20–22 nucleotide long RNAs that may act as post-transcriptional regulators of

gene expression [10]. It has been found that they can completely or incompletely bind to the 3'-UTRs of target mRNAs, thereby repressing their translation or promoting their degradation, respectively [11–13]. miRNA expression deregulation has been observed in a wide range of human diseases, including cancer [14], and it has been shown that this deregulation may play a pivotal role in processes such as cellular differentiation, proliferation, angiogenesis, apoptosis and invasion [15]. Based on these observations, it has been suggested that miRNAs may be applied in clinical cancer diagnosis, prognosis and therapy [16].

Very recently, we indentified several significantly deregulated miRNAs in NSCLC, including miR-27a#, miR-138, miR-206, miR-214, miR-498 and miR-613, using a miRNA expression microarray approach (unpublished data). Most of these miRNAs, except miR-613, have previously been reported to be involved in NSCLC or other types of cancer [17–21]. Here, we found that miR-613 is frequently down-regulated in primary human NSCLC tissues compared to adjacent non-tumor tissues. In addition, we found that miR-613 can induce in vitro cell cycle arrest and inhibit in vivo growth of NSCLC-derived cells, and that CDK4 may serve as a direct target of miR-613.

2 Materials and methods

2.1 Human tissue specimens

Fifty six pairs of human NSCLC and adjacent non-tumor tissue samples were obtained immediately after resection from patients undergoing primary surgical treatment in the First Affiliated Hospital of Sichuan Medical University, Si Chuan, China. None of the patients had undergone preoperative irradiation or chemotherapy. The tissue samples were frozen in liquid nitrogen and stored at -80°C until use. The tissue samples were obtained from 38 male and 18 female patients and their clinicopathological features are listed in Table 1. The average age of the patients was 56.5 years (range: 35 to 71 years). 21 patients (37.5 %) had tumors larger than 5 cm, 4 patients (7.1 %) had tumors smaller than 2 cm and 31 patients (55.4 %) had tumors 2–5 cm in size. 33 cases (58.9 %) were well differentiated (I + II), 21 cases (37.5 %) were moderately differentiated (III) and 2 cases (3.6 %) were poorly differentiated (IV). 19 patients (33.9 %) were diagnosed at TNM I, 17 patients (30.4 %) at TNM II, 16 patients (28.6 %) at TNM III and 4 patients (7.1 %) at TNM IV. Written consent for research purposes was obtained from the patients before tissue collection and the protocol was approved by the Institutional Review Board of First Affiliated Hospital of Sichuan Medical University and Sichuan Medical University.

Table 1 Patient data ($n = 56$)

| Characteristic | Value |
|--------------------------------|-----------------|
| Age (years) | 56.5 \pm 10.1 |
| Gender (male) | 38(67.8 %) |
| Tumor size* (cm) | 7.62 |
| Tumor grade | |
| Well-differentiated (G1-2) | 33 |
| Moderately-differentiated (G3) | 21 |
| Poorly-differentiated (G4) | 2 |
| TNM stage** | |
| I | 19 |
| II | 17 |
| III | 16 |
| IV | 4 |

* Diameter of the biggest nodule

** TNM: tumor-node-metastasis

2.2 Cell cultures

The human embryonic kidney-derived cell line HEK293T and the human NSCLC-derived cell lines A549 and SPCA1 were maintained in Dulbecco's modified eagle's medium (DMEM, Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 100 U penicillin/ml, 100 mg streptomycin/ml and 10 % fetal bovine serum (FBS, Gibco) at 37°C in a humidified atmosphere of 5 % CO_2 .

2.3 Transfections

The transfections were carried out using FuGene HD transfection reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. In brief, 2×10^4 A549 and SPCA1 cells or 5×10^4 HEK293T cells, seeded in 24-well plates, were transfected with the indicated plasmid DNAs, miRNA duplexes (GenePharma, Shanghai, China) or siRNAs (GenePharma) and collected 24–48 h after transfection for further assessment.

2.4 Quantitative real time PCR

cDNA synthesis and qRT-PCR-based miRNA expression analyses were carried out using TaqMan microRNA assay kits (Applied Biosystems) according to the manufacturer's protocol. Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen) from the clinical samples or the NSCLC-derived cell lines, and used to synthesize cDNA with gene-specific primers. Reverse transcription reactions were carried out using 100 ng RNA, 50 nM/L stem-loop RT primers, 1 \times RT buffer, 0.25 mM/L of each dNTP, 3.33 U/ μl MultiScribe reverse transcriptase and 0.25 U/ μl RNase inhibitor. 15 μl mixtures were incubated for 30 min at 16°C , 30 min at 42°C , 5 min at 85°C ,

and then held at 4 °C. The resulting cDNA product was subsequently used for qRT-PCR analysis. The PCR reaction mixtures (20 µl) included 1.33 µl RT product, 1 × TaqMan universal PCR master mix and 1 µl primers and probe mix from the TaqMan microRNA assay kit. The reaction mixtures were incubated in 96-well optical plates at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. The PCR reactions were run on a StepOne Plus real time PCR machine (Applied Biosystems) and the data were analyzed using SDS v2.3 software. The Ct value was defined as the fractional cycle number at which the fluorescence passed a fixed threshold. The fold change was calculated using the $2^{-\Delta\Delta C_t}$ method and presented as the fold-expression change in tumor tissues relative to their corresponding non-tumor tissues after normalization to the endogenous control.

2.5 Luciferase reporter constructs

The 3'-UTR fragment of CDK4 (GenBank accession no. NM_000075) containing the putative miR-613 binding sequence (1334-1344 nt) was amplified using the primers 5'-GCAATGGAGTGGCTGCCATGGAAG-3' (forward) and 5'-TGCCATTTAAAAATCTATATTCT-3' (reverse). The resulting PCR product was cloned into a *firefly* luciferase reporter vector (pGL3; Promega Corporation, Madison, WI, USA), and termed pGL3-CDK4-3'UTR. A plasmid that carried mutations in the complementary sites for the seed region of miR-613 was generated based on the pGL3-CDK4-3'UTR plasmid using a MutanBEST Kit (Takara Bio Inc., Shiga, JP), and termed pGL3-CDK4-3'UTR-mut. The correctness of the plasmids was confirmed by sequence analysis.

2.6 Cell viability and colony formation assays

Twenty four hours after transfection, 1000 A549 or SPCA1 cells were seeded in fresh 96-well plates in triplicate and maintained in DMEM containing 10 % FBS for 5 days. Next, the cells were tested for proliferation per 24 h using a Cell Titer-Blue cell viability assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions, and the fluorescence ratios were recorded using a multi-plate reader (Synergy 2, BioTek, Winooski, VT, USA). Additionally, 24 h after transfection, 2000 A549 or SPCA1 cells were seeded in fresh 6-well plates in triplicate and maintained in DMEM containing 10 % FBS for 2 weeks. Next, cell colonies were fixed in 20 % methanol and stained with 0.1 % Coomassie brilliant blue R250 at room temperature for 15 min. Finally, the colonies were counted using an ELISpot Bioreader 5000 (BIO-SYS, Karben, GE).

2.7 Cell cycle and apoptosis assays

Forty eight hours after transfection, 1×10^5 A549 and SPCA1 cells were harvested, washed once in phosphate buffer saline

(PBS), and fixed in 70 % ethanol at 4 °C overnight. Next, DNA staining was performed with 50 mg/ml propidium iodide and 1 mg/ml RNase A at room temperature for 30 min. The cell populations in the G0-G1, S and G2-M phases of the cell cycle were measured using Cell Lab Quanta SC flow cytometry (Beckman Coulter, Fullerton, CA, USA) and the data were analyzed using the FlowJo v7.6 software package.

2.8 Tumorigenicity assays in nude mice

Male BALB/c nude mice (5–6 weeks old) were obtained from the Shanghai Experimental Animal Center (Shanghai, China). All animal handling and experimental procedures were approved by the Animal Experiments Ethics Committee of the Sichuan Medical University. For the in vivo tumorigenicity assays, all pyrimidine nucleotides in the miR-613 mimic, the miR-613 inhibitor or the NC duplex were substituted by their 2'-O-methyl analogues to improve the RNA stability. MiR-613 mimic or miR-613 inhibitor transfected A549 cells (1×10^6) were suspended in 100 µl PBS and injected subcutaneously into the left side of the posterior flank of 6 BALB/c nude mice, respectively. Nonrelated control (NC) transfected or non-transfected A549 cells (1×10^5) were injected subcutaneously into the right side of same 12 mice. Tumor growth was examined daily and the tumor volumes were calculated every week using the formula for hemi-ellipsoids: $V = \text{length (cm)} \times \text{width (cm)} \times \text{height (cm)} \times 0.5236$. After 5 weeks, the mice were sacrificed and the tumors were dissected and photographed.

2.9 Dual-luciferase reporter assay

HEK293T cells, seeded in 24-well plates in triplicate, were co-transfected with pGL3-CDK4-3'UTR or pGL3-CDK4-3'UTR-mut and the miR-613 mimic or nonrelated control RNA duplex (NC duplex, GenePharma) using the FuGene HD transfection reagent. The pRL-TK construct (Promega Corporation, Madison, WI, USA) was also transfected as a normalization control. The cells were collected 48 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay kit (Promega Corporation) and recorded using a multi-plate reader (Synergy 2, BioTek).

2.10 Western blotting

Total protein was extracted using a modified RIPA buffer with 0.5 % sodium dodecyl sulfate (SDS) and a proteinase inhibitor cocktail (Complete Mini, Roche). Equal amounts of protein from NSCLC tissues and their adjacent non-tumor tissues were electrophoresed in 10 % SDS-PAGE mini gels and transferred to PVDF membranes (Immobilon P[®] SQ, Millipore, Billerica, MA, USA). After blocking with 5 % nonfat milk, the membranes were incubated with a rabbit anti-CDK4 antibody

(1:1000 dilution, Epitomics, Inc., Burlingame, CA, USA) or a mouse anti-GAPDH antibody (1:5000 dilution, Epitomics, Inc.) at 4 °C overnight, followed by incubation with a HRP-conjugated goat anti-rabbit or goat anti-mouse antibody (1:10,000 dilution, KPL, Gaithersburg, MA, USA) for 1 h at room temperature. Finally, signals were developed using Super Signal West Pico chemoluminescent substrate (Pierce, Rockford, Ill, USA), visualized by the Gene Gnome HR Image Capture System (Syngene, Frederick, MD, USA) and analyzed by Gene tools (Syngene).

2.11 miRNA target prediction

Miranda (<http://www.microrna.org/microrna/home.do>) was used to in silico predict miRNA targets and conserved sites bound by the seed region of miR-613.

2.12 Statistical analysis

Data are presented as mean \pm SD. Comparisons were made by using a two-tailed t test or one-way ANOVA for experiments with more than two subgroups. Correlation analyses were performed using Spearman correlation coefficient. $P < 0.05$ was considered statistically significant. Associations between miR-613 expression and cancer-specific survival rates were assessed using the Kaplan-Meier method.

3 Results

3.1 MiR-613 is down-regulated in NSCLC

To investigate the relevance of miR-613 in NSCLC, we first set out to measure its expression in 18 pairs of tumor versus adjacent non-tumor tissues by qRT-PCR. MiR-613 was found to be down-regulated in more than 70 % of the tumor tissues compared to the non-tumor tissues tested (Fig. 1a). To validate these initial qRT-PCR data, the expression of miR-613 was measured in another series of 38 paired NSCLC tissues. Again, miR-613 showed a significant down-regulation in the tumor tissues compared to the adjacent non-tumor tissues (56 pairs in total, Fig. 1b). In addition, we found that patients whose primary tumors did not exhibit miR-613 down-regulation showed a trend towards a better survival (Fig. 1c). The mean survival in the poor survival group was 68.4 months (low miR-613 expression, $n = 42$), whereas the mean survival in the better survival group was 99.8 months (high miR-613 expression, $n = 14$). No statistical significant correlations were observed between miR-613 expression and the other clinicopathological features tested (age, sex, stage and tumor size; data not shown).

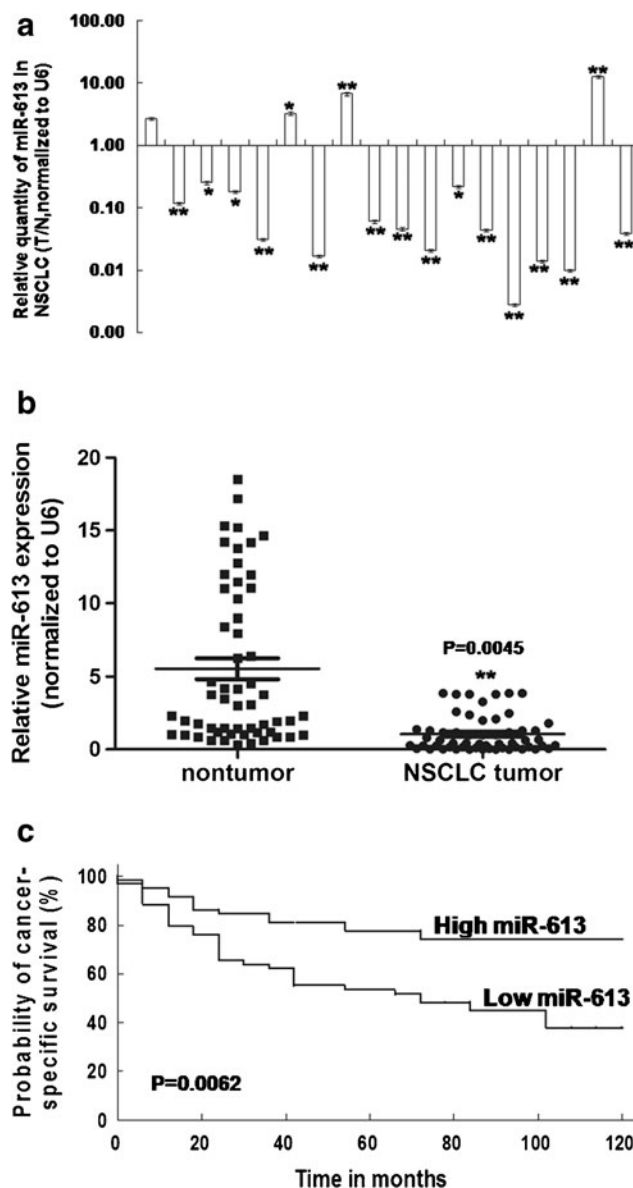


Fig. 1 MiR-613 is downregulated in NSCLC. **a** Expression of miR-613 in 18 NSCLC tumor tissues (T) and adjacent non-tumor (N) tissues. **b** Expression of miR-613 in 56 NSCLC tumor tissues (T) and adjacent non-tumor (N) tissues. U6 was used as an internal control. * $P < 0.05$; ** $P < 0.01$. **c** Probability of cancer-specific survival by miR-613 expression levels in NSCLC

3.2 MiR-613 induces cell cycle arrest

The significant reduction in miR-613 expression observed in the primary tumor tissues versus the non-tumor tissues suggests a possible role in NSCLC development. To test this hypothesis, we first set out to assess the effect of miR-613 on the growth of A549 and SPCA1 NSCLC-derived cells transfected or not with the miR-613 mimic, miR-613 inhibitor or NC duplex. We found that the expression of miR-613 was increased 25-fold (A549) and 22-fold (SPCA1) in cells transfected with 20 nM miR-613 mimic, and was decreased 30-fold (A549) and 43-fold

(SPCA1) in cells transfected with 20 nM miR-613 inhibitor (Fig. 2a). From day 2 (A549) or day 3 (SPCA1) onwards after transfection, the viability of the cells transfected with the miR-613 mimic significantly decreased compared to that of the NC duplex transfected or non-transfected cells, whereas the viability of the cells transfected with the miR-613 inhibitor significantly increased (Fig. 2b). These results indicate miR-613 can inhibit the growth of NSCLC-derived cells.

To validate the inhibitory effect of miR-613 on cellular growth, colony forming assays were performed on A549 and SPCA1 cells transfected or not with the miR-613 mimic, miR-613 inhibitor or NC duplex. As shown in Fig. 2c, A549 and SPCA1 cells transfected with 20 nM miR-613 mimic exhibited fewer and smaller colonies (218 and 161 colonies, respectively) compared to the NC duplex transfected (783 and 729 colonies, respectively) and non-transfected cells (756 and 692 colonies, respectively), whereas cells transfected with 20 nM miR-613 inhibitor exhibited more and larger colonies (1238 and 1349 colonies, respectively).

To further confirm the above findings, an in vivo mouse model was used (see materials and methods). After treatment

with the miR-613 mimic or miR-613 inhibitor for 5 weeks, the tumor volume curves revealed significant decreases in growth rates during the 3rd, 4th and 5th weeks after treatment with the miR-613 mimic and significant increases in growth rates during the 4th and 5th weeks after treatment with the miR-613 inhibitor, whereas no significant differences in growth rates were observed in the NC group and the non-transfected control group (Fig. 2d). These results indicate that miR-613 significantly inhibits the growth of NSCLC-derived A549 cells in the xenograft mouse model.

To investigate the mechanism underlying the growth inhibitory effect of miR-613, flow cytometry was carried out. We found that the percentages of the miR-613 mimic transfected A549 and SPCA1 cells in the G0-G1 phase were 18 % (A549) and 11 % (SPCA1) higher than that of the NC duplex transfected or non-transfected cells, which coincided with a 44 % (A549) and a 38 % (SPCA1) decrease in S phase cells (Fig. 3a). In the miR-613 inhibitor transfected cells, the percentages of cells in the G0-G1 phase were 8 % (A549) and 6 % (SPCA1) lower than that of the NC duplex transfected or non-transfected cells, which coincided with a 14 % (A549)

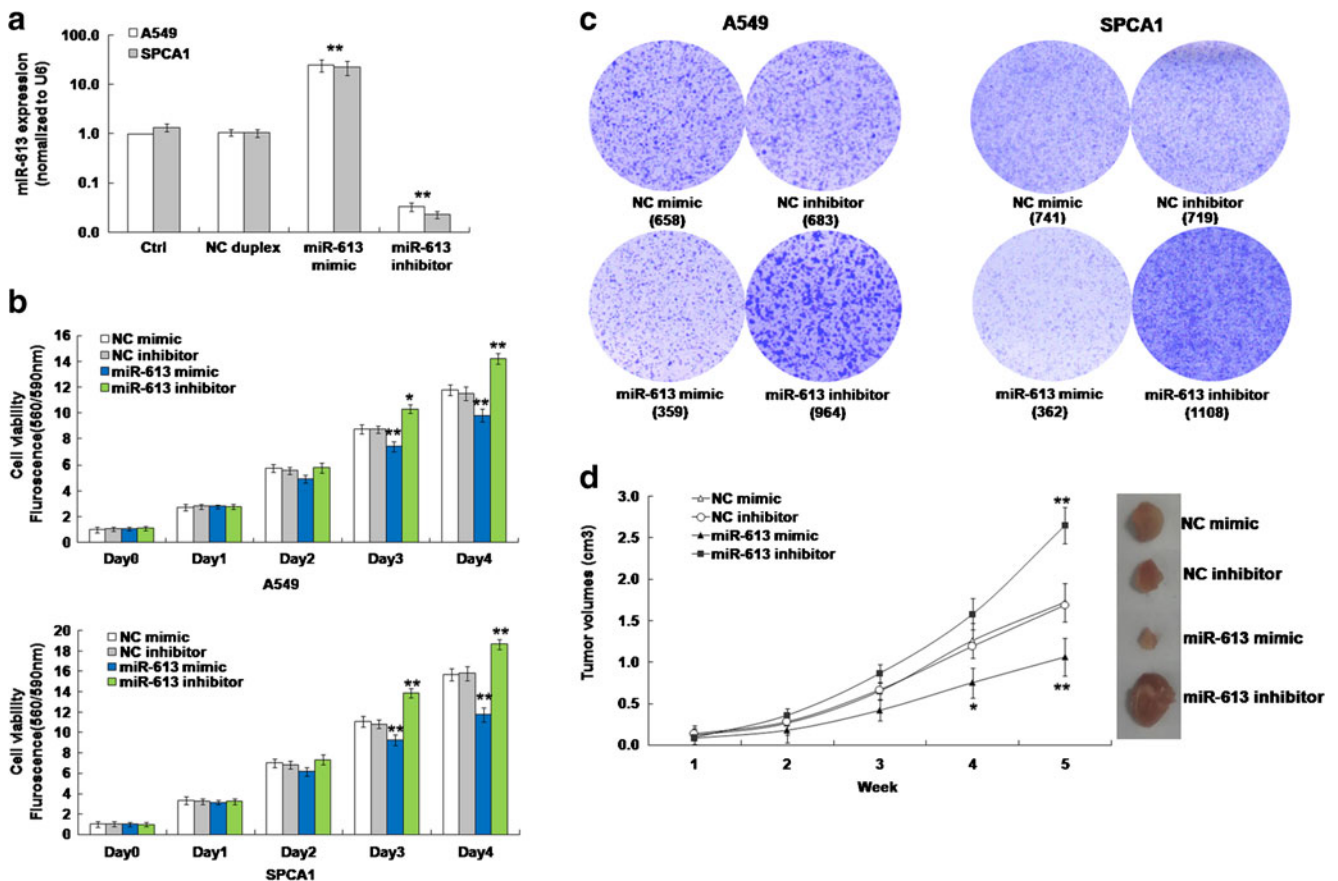


Fig. 2 MiR-613 suppresses cellular growth. **a** Expression of miR-613 in A549 and SPCA1 cells after transfection with miR-613 mimic, miR-613 inhibitor or NC duplex. **b** Effect of miR-613 on the viability of NSCLC-derived cells. **c** Effect of miR-613 on colony formation of NSCLC-derived cells. **d** Effect of miR-613 on tumor growth in xenografted nude

mice. Representative results (**b-d**) of A549 and SPCA1 cells transfected with miR-613 mimic, miR-613 inhibitor, NC mimic or NC inhibitor. Columns, means of three independent experiments; bars, \pm SD; * $P < 0.01$; ** $P < 0.001$

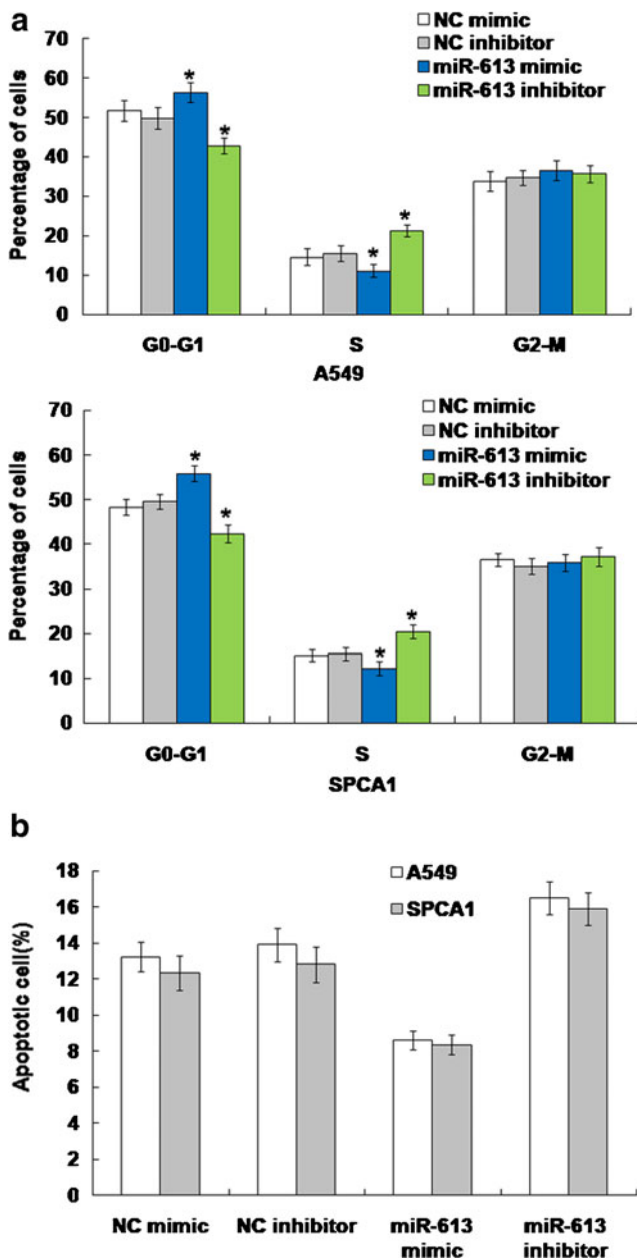


Fig. 3 MiR-613 induces cell cycle arrest. **a** Effect of miR-613 on the cell cycle. **b** Effect of miR-613 on apoptosis. Representative results of A549 and SPCA1 cells transfected with miR-613 mimic, miR-613 inhibitor, NC mimic or NC inhibitor. Columns, means of three independent experiments; bars, \pm SD; * $P < 0.01$; ** $P < 0.001$

and a 19 % (SPCA1) increase in S phase cells (Fig. 3b). These results indicate that miR-613 may inhibit NSCLC-derived A549 and SPCA1 proliferation by inducing cell cycle arrest in the G1/S phase.

3.3 CDK4 is a direct target of miR-613

It is generally accepted that miRNAs may exert their functions through regulating the expression of downstream target genes. CDK4 was predicted to be a

potential target of miR-613 by Miranda, since the 3'-UTR of the CDK4 mRNA contains a complementary site for the seed region of miR-613 (Fig. 4a). To validate whether CDK4 acts as a direct target of miR-613, human CDK4 3'-UTR fragments containing wild-type or mutant miR-613 binding sequences (Fig. 4a) were cloned downstream of the firefly luciferase reporter gene in pGL3. In HEK293 cells co-transfected with the reporter plasmids and the miR-613 mimic or NC duplex, the luciferase activity of the reporter that contained the wild-type 3'-UTR was found to be significantly suppressed by the miR-613 mimic, whereas the luciferase

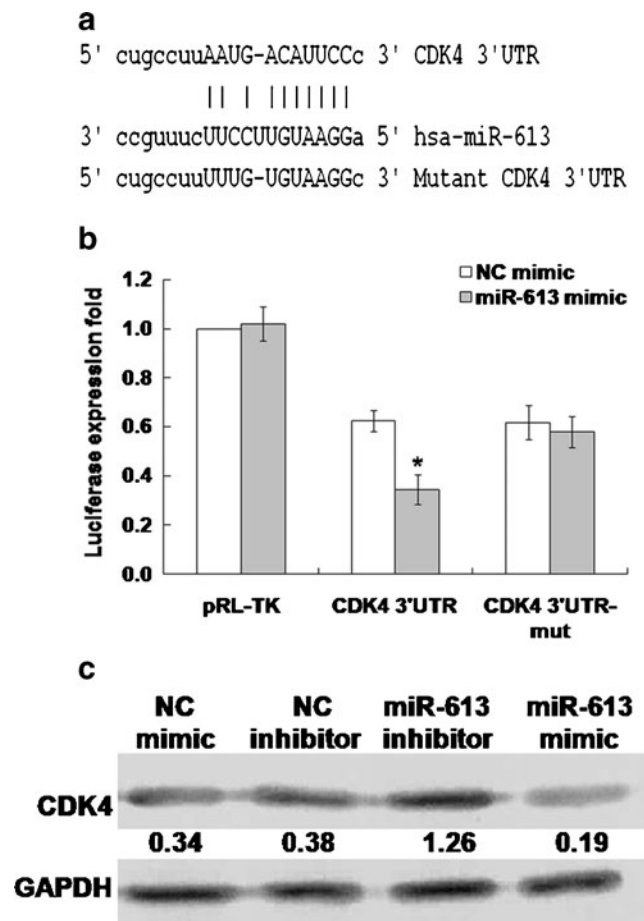


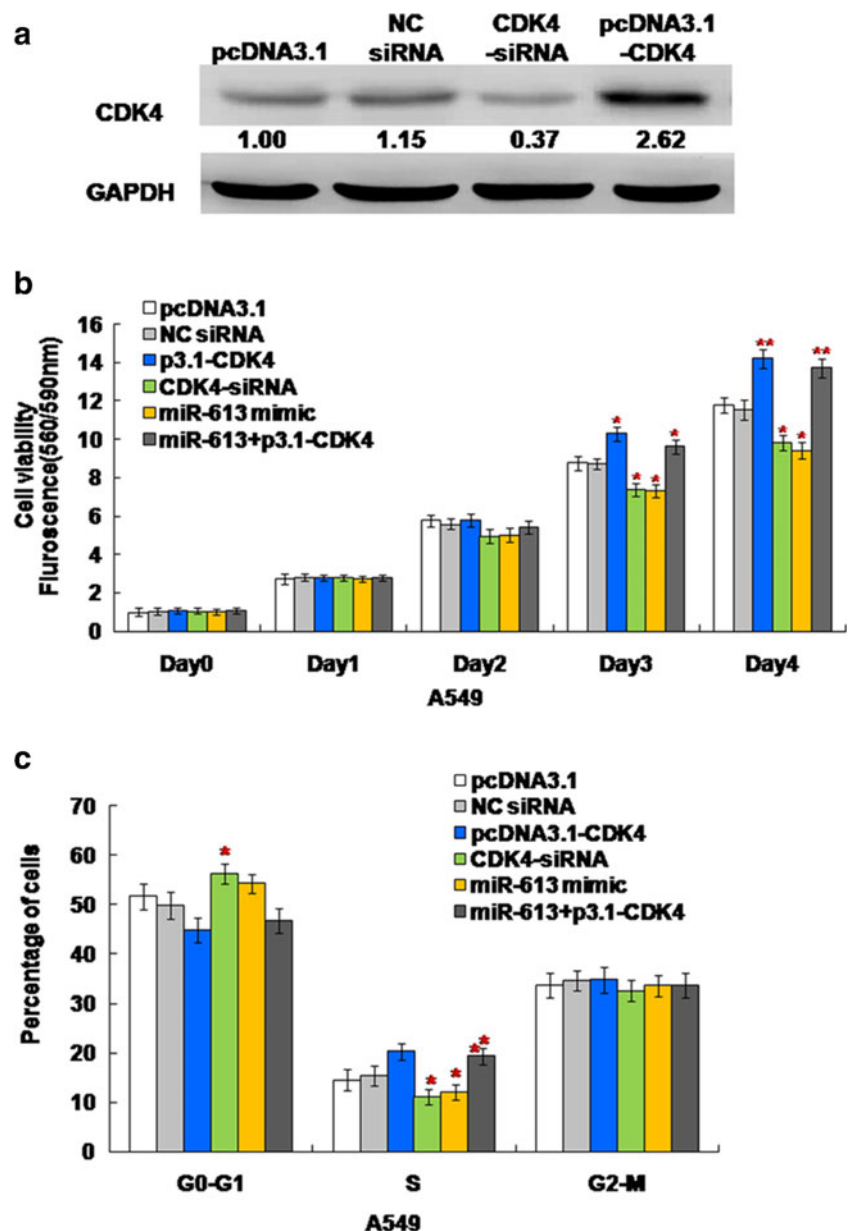
Fig. 4 CDK4 is a direct target of miR-613. **a** Putative miR-613 binding sequence in the 3'-UTR of CDK4 mRNA. A mutation was generated in the CDK4 3'-UTR sequence in the complementary site of the miR-613 seed region. **b** Suppressed luciferase activity of wild-type CDK4 3'UTR by miR-613 mimic. HEK293T cells were co-transfected with pGL3-CDK4-3'UTR or pGL3-CDK4-3'UTR-mut, and miR-613 mimic or NC duplex. Firefly luciferase activity of each sample was measured 48 h after transfection and normalized to Renilla luciferase activity. **c** Expression of endogenous CDK4 is regulated by miR-613. The expression level of endogenous CDK4 in A549 cells was assessed 48 h after transfection with miR-613 mimic, miR-613 inhibitor or NC duplex by Western blotting. GAPDH was used as an internal control. Columns, means of three independent experiments; bars, \pm SD; * $P < 0.01$

activity of the mutant reporter was unaffected (Fig. 4b), indicating that miR-613 may suppress CDK4 expression through the miR-613 binding sequence in its 3'-UTR. Furthermore, we found that transfection of the miR-613 mimic decreased CDK4 expression and that transfection of the miR-613 inhibitor increased CDK4 expression in A549 cells at the protein (Fig. 4c), but not the mRNA, level (data not shown) suggesting that CDK4 expression may be inhibited by miR-613 at the posttranscriptional level. Together, these results show that miR-613 may regulate the expression of endogenous CDK4 by directly targeting the 3'-UTR of its mRNA and, thus, that human CDK4 may be a new target of miR-613.

3.4 CDK4 knockdown induces cell cycle arrest

To assess whether CDK4 expression down-regulation, like miR-613 expression up-regulation, results in NSCLC growth cessation, the effect CDK4 expression knockdown on cellular growth was examined. First, A549 cells were transfected or not with the CDK4 siRNA or control siRNA. After 72 h a dose-dependent knockdown of CDK4 was observed in the siRNA transfected cells (Fig. 5a). Through cell viability and cell cycle analyses, we subsequently found that the in vitro knockdown of CDK4 resulted in a repression of cell viability (Fig. 5b) and an induction of cell cycle arrest in A549 cells (Fig. 5c). In order to confirm the effect of miR-613 on CDK4 expression, rescue experiments were performed for the cell

Fig. 5 CDK4 induces cell cycle arrest. **a** Efficient siRNA-mediated inhibition of CDK4 expression. The expression of endogenous CDK4 was assessed 48 h after transfection with CDK4 siRNA or NC siRNA by Western blotting. GAPDH was used as an internal control. **b** CDK4 knockdown reduces the viability of NSCLC-derived cells. Representative cell viability results are shown of A549 cells transfected with CDK4 siRNA and NC siRNA, or not transfected cells. **c** CDK4 knockdown induces cell cycle arrest in NSCLC-derived cells. Representative cell cycle results are shown of A549 cells transfected with CDK4 siRNA, NC siRNA and, or not transfected cells. Columns, means of three independent experiments; bars, \pm SD; * $P < 0.01$; ** $P < 0.001$



viability and cell cycle assays. We found that exogenous overexpression of CDK4 in miR-613 mimic-transfected A549 cells reversed the effect of the miR-613 mimic (Fig. 5b and 5c). Similar data were obtained with CDK4 siRNA transfected SPCA1 cells (data not shown). These results indicate that CDK4 is most likely involved in the induction of NSCLC-derived cell cycle arrest by miR-613.

4 Discussion

In the past, miRNAs have been found to be frequently deregulated in NSCLC, and some miRNAs were found to be associated with particular clinicopathological features of NSCLC, such as metastasis, recurrence and prognosis [22–24]. Moreover, compelling evidence indicates that miRNAs may play important roles in NSCLC progression and may directly contribute to the proliferation, avoidance of apoptosis and metastasis of NSCLC. Identifying these miRNAs and the corresponding targets that are essential for NSCLC progression may provide promising therapeutic opportunities.

Previously, miR-613 was reported to regulate lipid homeostasis by targeting the liver X receptor α (LXR α), suggesting that miR-613 may serve as a target for regulating lipid homeostasis [25–27]. In addition, miR-613 was found to play an inhibitory role in repressing the Wnt signaling pathway and, as such, might serve as a diagnostic or therapeutic tool in Wnt-dependent diseases, such as cancer [28]. Here, we found that miR-613 is frequently down-regulated in primary human NSCLC tissues, and that miR-613 can suppress cell viability and induce cell cycle arrest in NSCLC-derived cell lines, and can inhibit in vivo tumor growth in a xenograft nude mouse model. These results suggest that miR-613 may play an important role in NSCLC tumorigenesis.

Based on an in silico Miranda search, CDK4 was predicted to be a target of miR-613. CDK4 is known to be expressed in nearly all proliferating cells and to be able to promote cell cycle progression [36]. CDK4 has been reported to be regulated by several miRNAs, including miR-206, miR-506, miR-138, miR-124, miR-545 and miR-302 [29–35]. We, for the first time, identified CDK4 as a target of miR-613 in NSCLC, which may provide new insights into the mechanisms underlying its tumorigenesis. In addition, cell cycle progression is known to be regulated by a family of cyclin-dependent kinases (CDKs) and their activating partners (Cyclins). The G1/S phase cell cycle transition is regulated primarily by D-type Cyclins (D1, D2 or D3) in complex with CDK4/CDK6, and E-type Cyclins (E1 or E2) in complex with CDK2. These complexes cooperate in phosphorylating and in preventing Rb binding to E2F, thus activating E2F-mediated transcription and driving cells from the G1 into the S phase of the cell cycle [36]. Whether the CDK/pRb/E2F pathway is also involved in the

miR-613-mediated induction of cell cycle arrest in NSCLC cells requires further investigation.

In conclusion, we here provide evidence that low miR-613 expression contributes to cell viability and cell cycle progression in NSCLC via direct binding to the CDK4 3'-UTR. As such, miR-613 may act as a tumor suppressor and as a miRNA-based NSCLC therapeutic agent.

Authors' contributions Li Duo performed the research and drafted the article. Li Dong-Qiong and Liu Dan performed the research. Tang Xiaojun designed the research and revised the article. All the authors have read and approved the final version to be published.

Compliance with ethical standards

Competing interest The authors declare that they have no competing interests.

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