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# MicroRNA-383 inhibits anchorage-independent growth and induces cell cycle arrest of glioma cells by targeting CCND1

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ABSTRACT

In recent years, microRNAs (miRNAs) have been proved to be closely related to the tumorigenesis and progression. An increasing number of researches have shown that microRNAs function as oncogenes or tumor suppressor genes in human malignant tumors. This study aims to explore the effects of microRNA-383 (miR-383) on malignant biological function of human gliomas. We detected the expression of miR-383 in glioma tissues and normal brain tissues by quantitative real-time PCR. Anchorage-independent growth assays, and flow cytometry were used to evaluate the functions of miR-383 that involves in cell growth and cell cycle. Western blotting assay was used to examine protein expression levels of Cyclin D1 (CCND1), a cell cycle-associated oncogene which has a predicted binding site of miR-383 within its 3′-untranslated region (3′-UTR), and luciferase activity assay was used to evaluate the 3′-UTR activity of CCND1. In this study, we found that miR-383 expression level was lower in gliomas than normal brain tissues. Overexpression of miR-383 in U251 and U87 cells showed a significant inhibitory effect on cell growth, which accompanied with cell cycle G0/G1 arrest as well as downregulation of CCND1 expression. Moreover, CCND1 was verified to be one of the direct targets of miR-383. In summary, this study suggested that miR-383 plays the role of tumor suppressor by targeting CCND1 in glioma cells, and may be useful for developing a new therapeutic strategy for gliomas.



## 1. Introduction

Gliomas are the most common primary brain tumors, which represent more than 30% of the central nervous system tumors, and accounted for 80% of the intracranial malignant tumors [1]. Glioma characterized by its high mortality, of which the median survival time of glioblastoma is less than 12 months [2]. Although great progress has been made in early diagnosis and comprehensive treatment for gliomas, the survival period of gliomas has no obvious improvement. Therefore, it is necessary to investigate more reasonable and effective treatment for gliomas.

MicroRNAs are recognized as a class of endogenous, single-stranded and noncoding small RNAs, about 20–24 nucleotides in length, and act as post-transcriptional regulators of gene expression by base pairing to partially complementary sequences in the 3′-UTR of multiple target mRNAs, resulting in silencing of the mRNA [3]. They play important roles in many normal biological processes, such as cell growth, proliferation, apoptosis, and so on. Recent studies have suggested that the abnormal expression of miRNAs contribute to the formation and growth of cancers [4].

Recently, a few of authors have found that miR-383 was associated with some human cancers. It has been reported that miR-383 functions as a negative regulator of cell proliferation by directly targeting interferon regulatory factor-1 in testicular germ cell tumor [5]. Another research demonstrated that miR-383 is down regulated in medulloblastoma, and acts as a regulator controlling cell growth of medulloblastoma through targeting PRDX3 [6]. Furthermore, Liang et al. [7] reported that normal brain tissue displayed the highest expression level of miR-383 among all normal tissues, suggesting that miR-383 may play an important role in brain. However, the expression and function of miR-383 remain unclear in glioma, the most common neuroepithelial cancer of brain. Therefore, in our current study, we first compared the expression level of miR-383 in normal brain tissues and glioma tissues, and then we explored the functions of miR-383 that involves cell growth, and cell cycle in glioma cells. Furthermore, we identified that miR-383 directly targeted and negatively regulated the cell cycle-associated oncogene CCND1 in glioma cells. Which elucidates a novel molecular mechanism involved in glioma progression and may suggest new therapeutic strategy for gliomas.

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## 2. Materials and methods

### 2.1. Clinical samples

Glioma tissue samples were collected from Renmin Hospital of Wuhan University, and the pathological grades of tumor were determined by neuropathologist according to the WHO criteria for gliomas. This study was approved by the Ethical Committee of Renmin Hospital of Wuhan University, and each patient had written informed consent. A total of 16 glioma tissue samples and 8 normal brain tissues (obtained from tissues adjacent to brain contusion and laceration of brain trauma patients), as well as 6 pairs of glioma tissues and normal brain tissues were collected and processed for extraction of RNA and protein.

### 2.2. Cell culture, plasmid construction and transfection

Glioma cell lines (U251 and U87) were obtained from the Cell Bank of the Chinese Academy of Sciences, and cultured in Dulbecco's Modified Eagle's Medium (Invitrogen), supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were maintained in a humidified incubator at 37 °C and supplemented with 5% $CO_2$.

The 3′-UTR luciferase reporter constructs was made by cloning CCND1 3′-UTR sequence which was predicted to interact with miR-383 and the mutant sequence into pMIR-report vector (Ambion) at the XhoI and HindIII sites respectively, the constructed vectors were named pMIR-CCND1 3′-UTR-wt and 3′-UTR-mut. To construct the CCND1 ectopic expression vector, the complete coding sequence of CCND1 was amplified and cloned into the RcCMV vector (Invitrogen) at the HindIII and XbaI sites, the constructed vector was named RcCMV-CCND1.

MiR-383 mimics and negative control miRNA mimics (Ambion) and the constructed plasmids were transfected into U251 and U87 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Oligonucleotides or plasmids were used at a final concentration of 50 nM. The transfection efficiency was 62%–74%.

### 2.3. RNA Isolation and quantitative real time PCR

Total miRNAs from glioma tissue samples and glioma cell lines were extracted using mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. Quantitative reverse transcription was performed to synthesize cDNA using miScript II RT Kit (Qiagen), and quantitative PCR was performed using miScript PCR Starter Kit (Qiagen) according to the manufacturer's protocol. The primer sequence of hsa-miR-383 was as follows: 5′-TCGGTGTTAGTGGAAGACTAGA-3′. U6 snRNA was used as the endogenous normalizer. Cycle threshold (Ct) values was determined, and the relative expression of miRNA was calculated using the $2^{-\Delta\Delta Ct}$.

### 2.4. Western blotting

Total protein was extracted from the established cells or tissue samples then quantified using protein assay kit (Bio-Rad). The proteins were resolved by SDS–PAGE, and then transferred to PVDF membrane, blocked in 5% dry milk at room temperature for one hour and probed with antibodies at 4 °C overnight using anti-CCND1 (1:200, Abcam) and anti-β-actin (1:5000, Abcam). All results were detected using an enhanced chemifluorescence Western blot system (Typhoon FLA 9500, GE).

### 2.5. Anchorage-independent growth assay

3 ml of basal modified Eagle's medium supplemented with 0.5% agar, 10% FBS was added into each well of the 6-well plates to form a layer of base gel. $1 \times 10^4$ U251 or U87 cells were suspended in 1 ml of basal modified Eagle's medium supplemented with 0.33% agar and 10%, then added into each well on the top of base gel. Plates were incubated at 37 °C in 5% $CO_2$ for about one week. Colonies were observed and counted under microscope, the colony with more than 32 cells were scored and the results were presented as colony number per $10^4$ cells.

### 2.6. Flow cytometry for cell cycle analysis

48 h of post-transfection, the glioma cells were harvested and fixed in 70% ethanol at −20 °C overnight. Then the cells were suspended in propidium iodide (PI) working solution (0.1% Triton X-100, 0.2 mg/ml RNase A, and 50 mg/ml propidium iodide) at 4 °C, and then cell cycle distribution was analyzed by Beckman Coulter flow cytometer.

### 2.7. Luciferase activity assay

U251 and U87 cells were transfected with the 3′-UTR luciferase reporter vectors together with negative control miRNA mimics or miR-383 mimics, and Renilla expression vector pRL-TK (Promega) was co-transfected in all cases for normalization. 48 h of post-transfection, luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

### 2.8. Statistical analysis

Data's shown in the graphs were presented as mean ± SD. Student's *t* test was used to determine the significance of differences between different groups of each experiment. $P \leqslant 0.05$ were considered statistically significant differences.

## 3. Results

### 3.1. The expression of miR-383 was down-regulated in glioma tissues, while CCND1 was up-regulated

To investigate whether miR-383 plays a role in glioma tumorigenesis and progression, we conducted quantitative real-time PCR to quantify the expression level of miR-383 in different grades of glioma tissues, and normal brain tissues. The results indicated that the expression levels of miR-383 were significantly lower in glioma tissues especially in high-grade gliomas than normal brain tissues ($P < 0.01$; Fig. 1a). Meanwhile, we examined the protein expression level of CCND1 in paired glioma tissues and normal brain tissues, and found that it was up-regulated in glioma tissues (Fig. 1b). Furthermore, we confirmed the decrease of miR-383 in glioma tissues using paired glioma tissues and normal brain tissues ($P < 0.01$; Fig. 1c). Therefore, we speculate that miR-383 functions as a tumor-suppressor in gliomas.

### 3.2. MiR-383 inhibits the anchorage-independent growth and induces cell cycle G0/G1 arrest of glioma cells

In order to explore the tumor-suppressor role of miR-383 in glioma cells, we increased the miR-383 level in U251 and U87 cells (Fig. 2a), and detected the influence of miR-383 on cell growth and cell cycle of gliomas. Data presented in Fig. 2 demonstrated that miR-383 significantly inhibits anchorage-independent growth

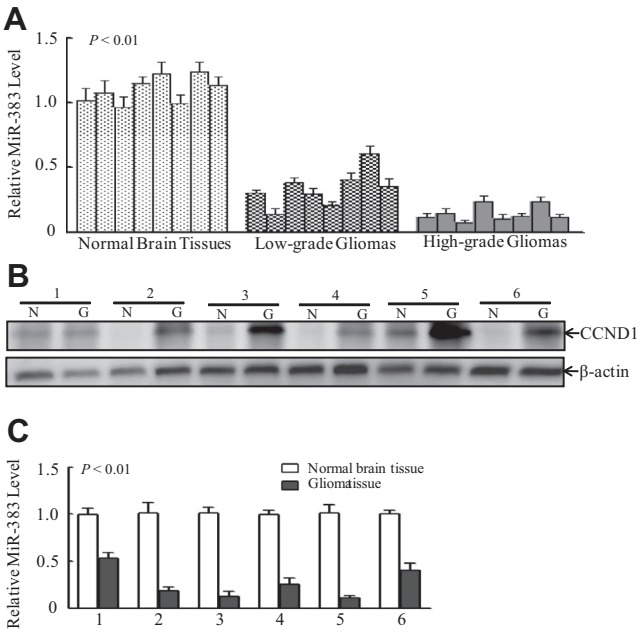


**Fig. 1.** MiR-383 was downregulated in gliomas tissues, while CCND1 was upregulated. (A) MiR-383 relative expression levels in eight normal brain tissues, eight low-grade glioma tissues and eight high-grade glioma tissues were measured using quantitative real-time PCR. U6 was used as the endogenous normalizer. The *P* value indicates the comparison of the three groups. (B) CCND1 protein expression levels in the 6 pairs of normal brain tissues (N) and glioma tissues (G) were detected by Western blotting assay, and β-actin was used as the endogenous normalizer. (C) MiR-383 relative expression levels in 6 pairs of normal brain tissues and glioma tissues were detected using quantitative real-time PCR. The *P* value indicates the comparison of the two in each pair.

of both U251 and U87 cells ($P < 0.01$; Fig. 2b and c). Flow cytometry analysis showed that the inhibition of anchorage-independent growth was accompanied by the induction of cell cycle arrest at G0/G1 phase (Fig. 2d).

### 3.3. CCND1 is identified as a direct target of miR-383 in glioma cells

We examined the protein expression levels of the cell cycle-associated oncogene CCND1, which showed inverse correlation with miR-383 (Fig. 3a). TargetScan showed that the 3′-UTR of CCND1 has a predicted binding site of miR-383. To investigate whether CCND1 was directly targeted by miR-383, a luciferase activity assay was performed to validate interaction between miR-383 and the predicted binding site. The wild type reporter vector includes the sequence of the potential binding site, while the mutant reporter vector was point mutated in the predicted binding site (Fig. 3b). It was demonstrated that miR-383 can reduce the fluorescent intensity of the wild type reporter vector in both U251 and U87 cells, while no effect was observed in both glioma cell lines transfected with the point mutation reporter vector ($P < 0.01$; Fig. 3c).

### 3.4. MiR-383 regulates glioma cell growth and cell cycle via down-regulating CCND1

To evaluate the contribution of CCND1 down-regulation by miR-383 to anchorage-independent growth and cell cycle regulation, ectopic expression vector RcCMV-CCND1 (without any UTR sequence) and miR-383 mimics were transfected together or separately into U251 cells (Fig. 4a). The results indicated that overexpression of CCND1 in glioma cells promotes anchorage-independent growth and cell cycle progression. More importantly,

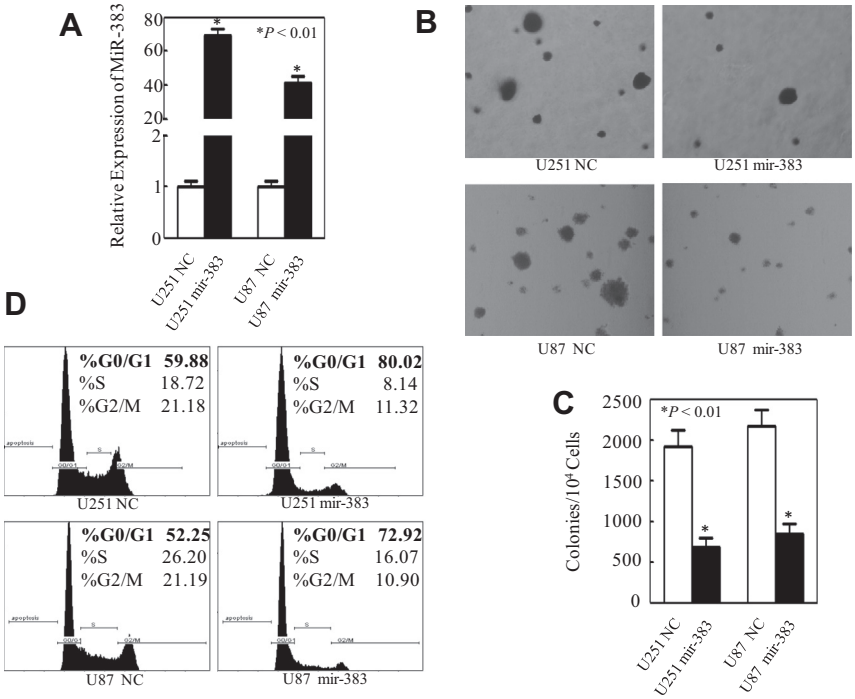


**Fig. 2.** The influence on cell growth and cell cycle progression of glioma cells by transfected with miR-383 mimics. (A) Relative expression of miR-383 in U251 and U87 cells transfected with miR-383 or negative control miRNA (NC). (B and C) The colony formation activities of U251 and U87 cells transfected with miR-383 or NC were detected using anchorage-independent growth assays. (D) The cell cycle distribution of U251 and U87 cells transfected with miR-383 or NC were measured using propidium iodide staining and flow cytometry analyses.

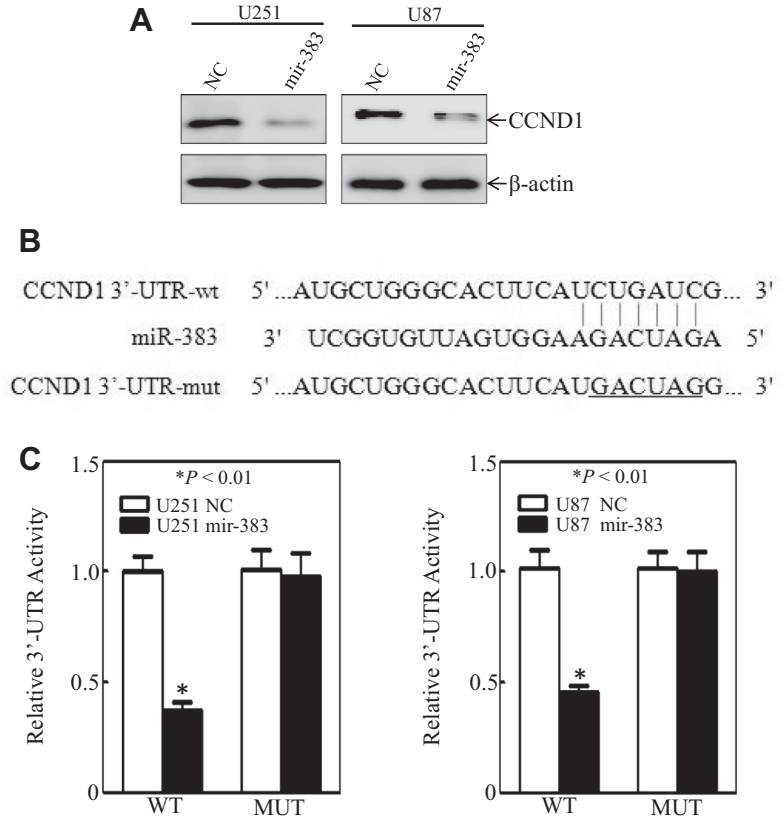


**Fig. 3.** MiR-383 directly and negatively regulates CCND1. (A) U251 and U87 cells were transfected with miR-383 or NC, and the endogenous CCND1 protein expression levels were detected by Western blotting assay. (B) The sequence of miR-383 binding sites in CCND1 3′-UTR predicted by TargetScan database, the wild type CCND1 3′-UTR (wt) and the mutated CCND1 3′-UTR (mut) sequences were shown. (C) The luciferase reporter vectors including either wild type (WT) or mutated (MUT) binding sites were co-transfected with miR-383 into U251 and U87 cells, and the luciferase activity assay was used to evaluate the 3′-UTR activity of CCND1. The normalized fluorescent intensity histograms were shown.

our results revealed the inhibition of anchorage-independent growth of glioma cells by miR-383, and this effect can be reversed by ectopic expression of CCND1 ($P < 0.01$; Fig. 4b and c). Furthermore, the induction of G0/G1 growth arrest in glioma cells by miR-383 was also reversed by ectopic expression of CCND1 (Fig. 4d). These data suggested that miR-383 can inhibit the anchorage-independent growth, and induce G0/G1 growth arrest of glioma cells by down-regulating CCND1.

## 4. Discussion

In recent years, increasing numbers of microRNAs have been found to play an important role in tumor development and progression of gliomas. The abnormal expression of miRNAs were usually associated with the malignant biological behaviors of gliomas, such as malignant growth, proliferation, invasion, apoptosis inhibition, chemotherapy resistance, and so on. It has been reported that miR-15b, miR-23b, miR-137, miR-152 and miR-195 were downregulated and function as tumor suppressors in gliomas [8–11]. On the contrary, miR-10b, miR-21 and miR-221/222 were reported overexpression in gliomas, which has been suggested to promote the malignant progression of gliomas, and function as oncogenes [12–14]. In this study, we first detected the expression level of miR-383 in glioma tissues, and found that miR-383 was significantly downregulated in glioma tissues than normal brain tissues. Furthermore, our data demonstrated that the expression level of miR-383 was negatively related to the malignant degree of gliomas, the expression of miR-383 in high-grade gliomas was significantly lower than low-grade gliomas. Therefore, we speculate that miR-383 plays the role of tumor suppressor genes in gliomas.

Anchorage-independent growth is a characteristic of cancer cells exists in almost all stages of tumorigenesis and progression. This feature of malignant growth is believed to be particularly important in gliomas, because rapid growth of brain tumor directly contributed to high mortality [15]. In order to investigate the function of miR-383 in glioma growth, we overexpressed the miR-383 in glioma cells by transfecting with miR-383 mimics. The number of the colony formation in soft agar was significantly less than the control group, which transfected with negative control miRNA mimics. This suggests that miR-383 has the function of inhibiting anchorage-independent growth of glioma cells. Increasing evidence had indicated that deregulation of the cell cycle is associated with aberrant cell growth and cancer [16,17]. Thus, we further detected whether miR-383 is involved in the cell cycle regulation. Our data showed that there's a blockage of the transition from G0/G1 phase to S phase, which due to the up-regulation of miR-383. That is miR-383 can induce cell cycle G0/G1 arrest of glioma cells and may explain the inhibition of cell growth caused by miR-383.

The oncogene CCND1 is a key regulator in the control of cell cycle progression from G0/G1 phase to S phase. It can promote G0/G1 to S phase transition, accelerate cell cycle, and consequently promote cell growth. Abnormal expression of CCND1 has been reported in a variety of human tumors, including breast cancer [18,19], bladder cancer [20,21], colon cancer [22,23], and glioma also included [24,25]. Growing evidence demonstrated that CCND1 is a potential therapeutic target in cancer [26,27]. In this study, we

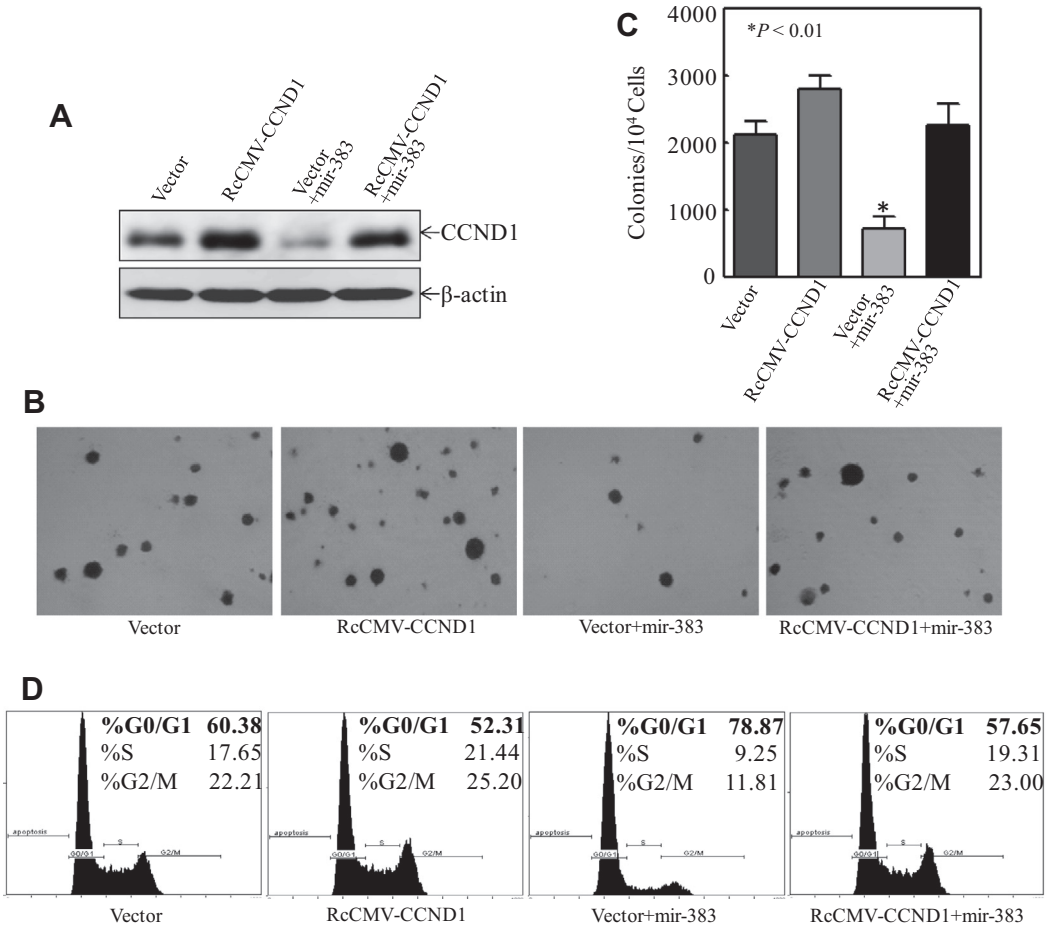


**Fig. 4.** MiR-383 inhibits glioma cells growth and cell cycle progression by targeting CCND1. (A) CCND1 expression vector (RcCMV-CCND1) and miR-383 mimics were transfected together or separately into U251 cells, and CCND1 protein expression level was detected by Western blotting assay. (B and C) The colony formation activities of glioma cells transfected with RcCMV-CCND1 and miR-383 separately or together were detected using anchorage-independent growth assays. (D) The cell cycle distribution of glioma cells transfected with RcCMV-CCND1 and miR-383 separately or together were measured using propidium iodide staining and flow cytometry analyses.

identified that CCND1 was up-regulated in glioma tissues, and found that the increased miR-383 level in glioma cells resulted in down-regulation of CCND1 protein expression. Meanwhile, we searched TargetScan database and found that CCND1 has a predicted binding site of miR-383 within its 3′-UTR. To verify whether CCND1 is a direct target of miR-383 in glioma cells, the luciferase reporter assay was applied and the data indicated that miR-383 could bind efficiently to the predicted miR-383 binding site within the CCND1 3′-UTR. Which is an important evidence for the direct interaction between miR-383 and CCND1. And we further found that the inhibition of cell anchorage-independent growth and the induction of cell cycle G0/G1 arrest which caused by miR-383 mimics in glioma cells were reversed by ectopic expression of CCND1, accompanied with the restoration of CCND1 expression level. Therefore, we conclude that miR-383 plays a role of tumor suppressor in glioma cells by negative regulation of CCND1.

In conclusion, our study demonstrated that miR-383 is significantly down-regulated in gliomas compared with normal brain tissues. CCND1 is a direct target of miR-383, and miR-383 functions as a negative regulator of CCND1. MiR-383 might be considered as a tumor suppressor to inhibit cell anchorage-independent growth and induce cell cycle G0/G1 arrest via direct target CCND1 in glioma cells, which may provide suggestions for new therapeutic strategies for gliomas.

## Conflict of interest

The authors declare that they have no conflict of interest.

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