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miR-340 inhibits glioblastoma cell proliferation by suppressing CDK6, cyclin-D1 and cyclin-D2

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ABSTRACT

Glioblastoma development is often associated with alteration in the activity and expression of cell cycle regulators, such as cyclin-dependent kinases (CKDs) and cyclins, resulting in aberrant cell proliferation. Recent studies have highlighted the pivotal roles of miRNAs in controlling the development and growth of glioblastoma. Here, we provide evidence for a function of miR-340 in the inhibition of glioblastoma cell proliferation. We found that miR-340 is downregulated in human glioblastoma tissue samples and several established glioblastoma cell lines. Proliferation and neurosphere formation assays revealed that miR-340 plays an oncosuppressive role in glioblastoma, and that its ectopic expression causes significant defect in glioblastoma cell growth. Further, using bioinformatics, luciferase assay and western blot, we found that miR-340 specifically targets the 3′UTRs of CDK6, cyclin-D1 and cyclin-D2, leading to the arrest of glioblastoma cells in the G0/G1 cell cycle phase. Confirming these results, we found that re-introducing CDK6, cyclin-D1 or cyclin-D2 expression partially, but significantly, rescues cells from the suppression of cell proliferation and cell cycle arrest mediated by miR-340. Collectively, our results demonstrate that miR-340 plays a tumor-suppressive role in glioblastoma and may be useful as a diagnostic biomarker and/or a therapeutic avenue for glioblastoma.

1. Introduction

Glioblastoma is a prevalent and malignant form of tumor emanating from the brain with a 5 year survival rate of only 4–5 % and a median survival of approximately 15 months [1]. It is an incurable form of cancer and therapy is limited to tumor resection with subsequent radiation and chemotherapy. Despite progressive efforts to treat this form of cancer, pivotal clinical treatments and patient survival rate still needs significant improvement.

One of the hallmarks of glioblastoma, as with any other cancer, is uncontrolled cell proliferation, which has in many instances been found to be associated with dysregulated expression of various miRNAs in many cancers [2]. Characterizing such aberrant miRNA expression in glioblastoma and other cancers may yield biomarkers for early detection of disease and new therapeutic options. MicroRNAs (miRNAs) are non-coding RNAs that play critical regulatory roles in tumorigenesis. They function by complimentary binding to 3′UTRs of target mRNAs leading to degradation of the mRNA or inhibition of mRNA translation. Deregulation of miRNAs results in aberrant expression of target genes and ultimately leads to malignant development [3].

Expression of the miRNA miR-340 was previously reported to be inversely correlated with aggressiveness of breast cancer and that ectopic expression of miR-340 inhibits cancer cell invasion [4]. The oncosuppresive roles of miR-340 have been characterized in lung, colorectal, and osteosarcoma [5–7]. Genomic miRNA expression analysis revealed that miR-340 is dysregulated in glioblastoma [8–10]. However, the function of miR-340 in glioblastoma has not been explored previously.

Previous studies have highlighted the oncogenic nature of cell cycle regulators such as cyclins and cyclin-dependant kinase (CDK) family members in the tumorigenesis of glioblastoma. The cell cycle is driven mainly by CDKs, which are activated by forming specific complexes with cyclins in different cell cycle phases [11].

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Intermittent degradation of CDK-activating cyclins typically governs G1 to S phase periodicity through oscillation of CDK activity. Therefore, inhibition of CDK regulation is often implicated in aberrant cell proliferation. G1-specific cyclin-D1 was previously shown to be closely related to oncogenesis and progression of glioma and its knockdown has been shown to inhibit human glioblastoma cell proliferation [12]. Similarly, CDK6 is also overexpressed in glioblastoma [13]. Amplification of the CDK/cyclin pathway is mostly attributed to aberration in their ubiquitination and sumoylation status [14].

However, to date little has been reported on the role of miRNAs in regulating CDK/cyclin signaling in glioblastoma. Here, we show that miR-340 suppresses glioblastoma cell proliferation by exerting direct negative regulation of cyclin-D1, cyclin-D2 and CDK6 expression.

2. Materials and methods

2.1. Human samples

This research was approved by the Institutional Review Board of Harbin Medical University. Following informed consent, 35 samples with World Health Organization (WHO) grade II-IV glioma and five normal brain tissues were obtained from patients undergoing surgery at the Department of Neurosurgery, an affiliated hospital of Harbin Medical University, China. Collected tissues were immediately snap-frozen and stored at −80 °C.

2.2. Cell lines

Glioblastoma cell lines U8vIII, U87, T98G, LN229, U373 and U251 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and penicillin/streptomycin (Invitrogen). Normal human astrocyte (NHA) cell line was obtained from the Lonza group (Lonza, Basel, Switzerland) and cultured according to the manufacturer's instructions.

2.3. RNA isolation and real-time PCR (RT-PCR)

Total RNAs from cultured cells and frozen surgical glioblastoma tissues were extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. TaqMan microRNA assays kit (Applied Biosystems) was used to quantify expression of mature miR-340. U6 was used as the internal control for miRNA expression studies. Reverse transcription was performed using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's instructions. SYBR® Green Real-Time PCR Master Mix (Invitrogen) was used to quantify the expression of CDK6, cyclin-D1 and cyclin-D2 mRNAs. GAPDH was used as an endogenous control. Sequences of the primers used are as follows: *CDK6* forward, 5′-ATGAGCCGCCTGAGGTTG-3′; reverse, 5′- AGCTGTTCCGTCCCAGTAGATTA -3′; *cyclin-D1* forward, 5′-AGCTGTGCATCTACACCGAC-3′; reverse, 5′-TGTGAGGCGGTAGTAGGACA-3′; *cyclin-D2* forward, 5′-ATCACCAACACAGACGTGGA-3′; reverse, 5′- TGCAGGCTATTGAGGAGCA-3′; *GAPDH* forward, 5′-TGGACTCCACGACGTACTCAG-3′; reverse, 5′-CGGGAAGCTTGTCATCAATGGAA-3′. Quantitative miRNA expression was performed using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems) and the $2^{\Delta\Delta Ct}$ method.

2.4. Western blot

Cells were transfected with 50 nM negative control (NC) or miR-340 plasmid. Total protein was extracted 48 h later using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 5.0 mM EDTA pH 8.0, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride). Protein concentrations were determined using the BCA method (Thermo Scientific, Rockford, IL). Equal quantities of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked and then incubated with primary antibody (anti-CDK6, anti-cyclin-D1, anti-cyclin-D2, and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, membranes were washed and then incubated with secondary antibodies (Santa Cruz) for 1 h. Bound antibodies were detected using Enhanced Chemiluminescence (ECL) Super Signal West Pico (Pierce Biotech, Rockford, IL).

2.5. Cell transfections and luciferase assay

NC, miR-340, control, CDK6, cyclin-D1 and cyclin-D2 encoding plasmids were purchased from GenePharma (Shanghai, China). Wild-type full-length AKT1, BMPR1A, CDK6, cyclin-D1, cyclin-D2, SOX6, and Wnt11 3′UTR were amplified from the genomic DNA and ligated into the hRluc/TK luciferase reporter vector (Promega, Madison, WI). Mutant 3′UTR luciferase reporter vectors containing six mutated nucleotides on the predicted miR-340 binding sites

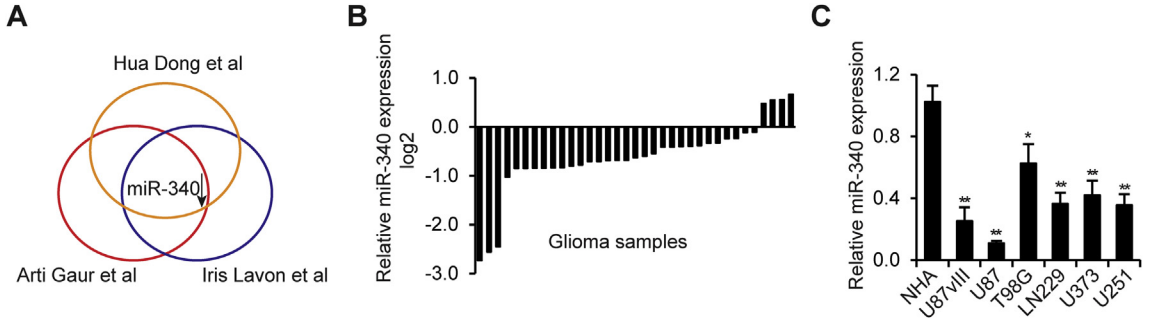

Fig. 1. miR-340 is downregulated in glioblastoma samples and cell lines. (A) Venn diagram showing miR-340 downregulation in glioblastoma by three published articles [8–10]. (B) miR-340 transcriptional levels were reduced in 31 of 35 glioblastoma samples. (C) miR-340 transcriptional levels were reduced in six glioblastoma cell lines. *$p < 0.05$, **$p < 0.01$ compared to NHA.

were constructed using the site-directed mutagenesis kit (Stratagene, La Jolla, CA). These plasmids were sequenced and then co-transfected with NC or miR-340 (50 nM) into glioblastoma cells using X-tremeGENE (Roche, Mannheim, Germany). Renilla luciferase plasmid (Promega) was co-transfected to normalize the relative luciferase values. Forty-eight hours post-transfection, cell were collected, lysed and their luciferase activity measured 48 h post-transfection using a dual-luciferase reporter assay system (Promega).

2.6. MTS assay

Cells were plated in 96-well plates (2000 cells/well) and transfected with 50 nM NC or miR-340 and incubated for two days. Subsequently, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) (Promega) was added into each well containing 100 μl medium after cells had been cultured for an additional zero, one, two or three days and incubated at 37 °C for 2 h in a 5% CO_2

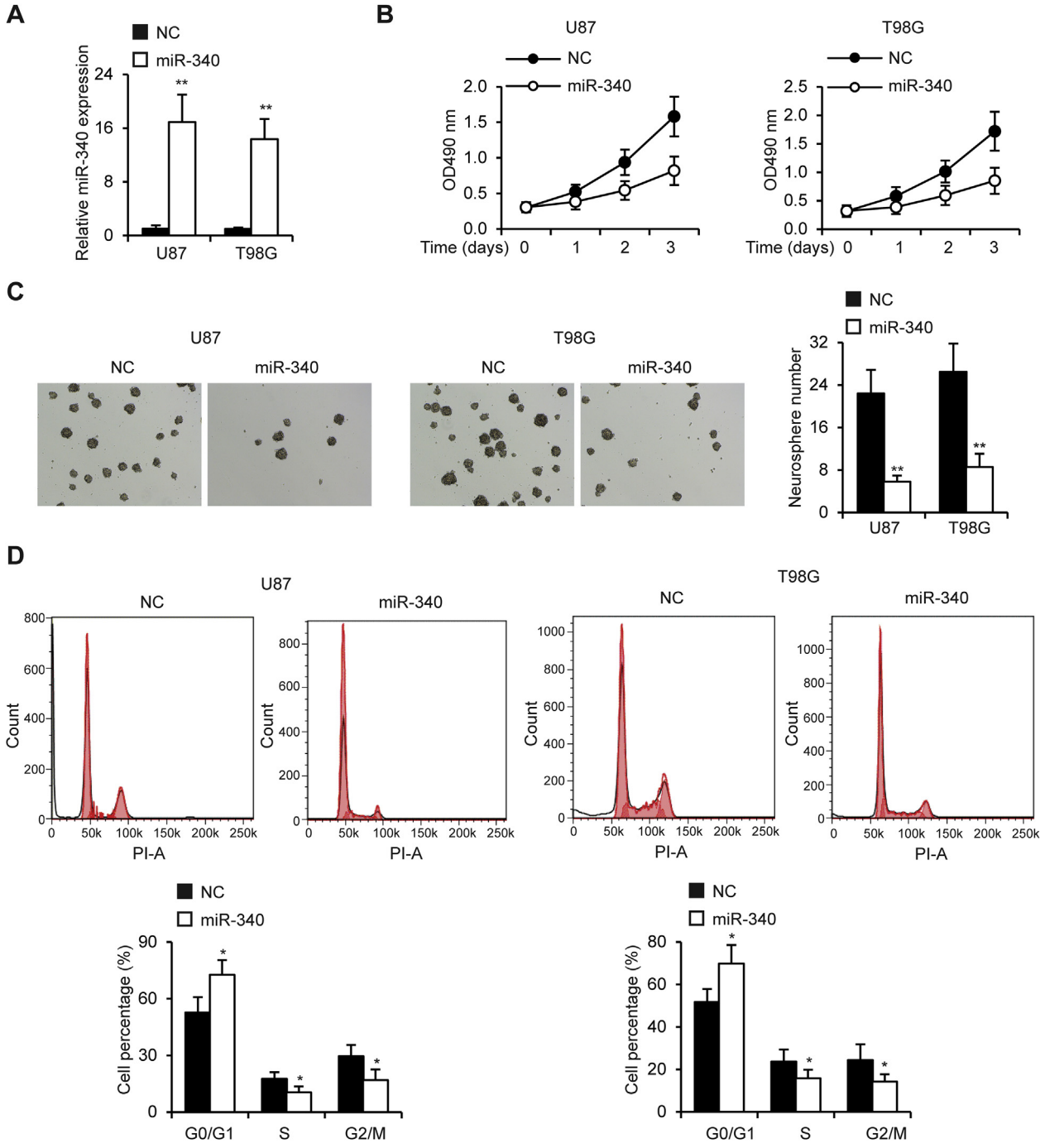

Fig. 2. miR-340 inhibits glioblastoma cell proliferation. (A) miR-340 expression in U87 and T98G cells transfected with miR-340 or NC. (B) MTS assay in U87 and T98G cells transfected with miR-340 or NC. (C) Neurosphere formation assay and quantification of neurospheres in U87 and T98G cells transfected with miR-340 or NC. (D) Cell cycle analysis in U87 and T98G cells transfected with miR-340 (top) and quantitation of cell in G0/G1, S and G2/M phases (bottom). *$p < 0.05$, **$p < 0.01$ compared to NC transfected glioblastoma cells.

incubator. Absorbance was detected at 490 nm with a microplate reader (Bio-Rad).

2.7. Cell cycle analysis

Cells (5×10^5) were transfected with 50 nM NC or miR-340 on 6 well plate. Forty-eight hours later, the cells were collected and washed twice with PBS, and then fixed with ice-cold 70% ethanol. After fixation, cells were incubated with RNase I (1 μg/ml) at 37 °C for 1 h. Propidium iodide (20 μg/ml) was added and samples were analyzed by flow cytometry (LSRII, BD Biosciences, San Jose, CA)

2.8. Neurosphere formation assay

Cells (5×10^3 per well) were seeded into 24-well plates in triplicate and then transfected with NC or miR-340. The DMEM medium was replaced with neural stem cell medium (Invitrogen) containing 20 ng/ml EGF, 20 ng/ml FGF and 2 μg/ml heparin supplement (Invitrogen) 48 h later and changed every two days. Numbers of neurospheres with a diameter larger than 50 μm were counted using NIH Image J software 10 days later.

2.9. Statistical analysis

Data is expressed as mean ± SEM of three independent experiments. Differences among groups were analyzed using one-way ANOVA, followed by Bonferroni post-hoc analyses. A p-value < 0.05 was considered significant. All statistical analyses were conducted using SPSS 20.0.

3. Results

3.1. miR-340 is downregulated in glioblastoma samples and cell lines

Global miRNA expression analysis consistently showed that miR-340 expression is downregulated in glioblastoma [8–10] (Fig. 1A). To further confirm whether miR-340 is downregulated in glioblastoma samples, we performed RT-PCR and found that transcriptional levels of miR-340 were decreased in 88.5% (31 out of 35) of the clinical glioblastoma samples (Fig. 1B). To evaluate miR-340 expression in glioblastoma cell lines, RT-PCR was performed on six established glioblastoma cell lines. The results showed that miR-340 transcriptional levels were downregulated in all six glioblastoma cell lines (Fig. 1C). Since miR-340 expression was highest in T98G and lowest in U87 cells, we selected these two cell lines for subsequent experiments to account for expression variability.

3.2. miR-340 inhibits glioblastoma cell proliferation

To characterize the biological significance of miR-340 in glioblastoma development, we performed a series of cell proliferation assays. U87 and T98G cells were transfected with miR-340 and 48 h later analyzed for miR-340 expression. RT-PCR results showed that miR-340 expression was approximately 16 fold more after transfection compared to NC (Fig. 2A). These cells were then subjected to proliferation assays. The MTS assay results showed that miR-340 overexpression significantly reduced the OD490 value compared to control (Fig. 2B). To further explore the role of miR-340 in glioblastoma pathophysiology, neurosphere formation assay was performed in U87 and T98G cells. We found the number of neurosphere decreased by 3 fold in U87 and T98G cell lines expressing miR-340 compared to controls (Fig. 2C). We further performed cell cycle analysis and observed that more cells were arrested in G0/G1 phase in glioblastoma cells transfected with miR-340. The percentage of cells in G0/G1 stage was increased from 50% to 70% (Fig. 2D).

3.3. miR-340 directly targets CDK6, cyclin-D1, and cyclin-D2

Based on the results obtained using three miRNA target prediction algorithms (microRNA.org, RNA22, and DIANA), we discovered seven predicted miR-340 target genes (AKT1, BMPR1A, CDK6, cyclin-D1, cyclin-D2, SOX6 and Wnt11). These genes have been previously shown to be involved in cancer pathophysiology [15–19] (Fig. 3A). The 3′UTR sequences containing the putative miR-340 binding sites of these genes were ligated into the hRluc/TK luciferase reporter vector and cotransfected with miR-340 expressing plasmid into U87 cells. Two days later, luciferase activity was measured and the results showed that only CDK6, cyclin-D1 and cyclin-D2 expression was downregulated by miR-340 (Fig. 3B). Comparing the sequences of miR-340 with that of CDK6, cyclin-D1 and cyclin-D2 3′UTR, we found multiple potential miR-340 binding sites (Fig. 3C). Therefore, we generated 3′UTR luciferase reporter plasmids for CDK6, Cyclin-D1 and Cyclin-D2 genes with the six common nucleotides on each predicting binding site of miR-340 mutated (Fig. 3D) and performed luciferase assay. As shown in Fig. 3E, the inhibition of luciferase activity induced by miR-340 on wild type 3′UTR reporter vectors of these three genes was fully rescued when the binding sites were mutated, which indicates CDK6, Cyclin-D1 and Cyclin-D2 genes are regulated by miR-340 specifically. To further confirm that CDK6, cyclin-D1 and cyclin-D2 are miR-340 targets, we transfected miR-340 into U87 and T98G cells and performed western blot. The results revealed that the expression of CDK6, cyclin-D1 and cyclin-D2 was less in miR-340 overexpression cells than in NC cells (Fig. 3F). We next analyzed the correlation between miR-340 and CDK6, cyclin-D1 or cyclin-D2 using spearman correlation analysis and found a reverse correlation between miR-340 and its putative targets (Fig. 3G–I).

3.4. miR-340 regulates glioblastoma cell proliferation via CDK6, cyclin-D1 and cyclin-D2

To investigate whether miR-340 affects glioblastoma cell growth via direct regulation of CDK6, Cyclin-D1 and Cyclin-D2, we rescued the expression of CDK6, cyclin-D1 and cyclin-D2 in miR-340 overexpressing cells and measured cell proliferation and performed cell cycle analysis. Consistent with results in Fig. 3D, our western blot showed downregulation of CDK6, cyclin-D1 and cyclin-D2 in miR-340 expressing cells and ectopic expression of plasmids expressing these cell cycle regulators rescued their protein levels (Fig. 4A). These cells were then used to assess the effect of miR-340 on proliferation. MTS assay revealed that miR-340 overexpression inhibits cell proliferation and that exogenous expression of CDK6, cyclin-D1 or cyclin-D2 partly rescues proliferative ability (Fig. 4B). Furthermore, we performed cell cycle analysis and found that miR-340 arrests more U87 cells in G0/G1 phase, whereas reintroducing CDK6, cyclin-D1 or cyclin-D2 partly rescues cell cycle progression towards S- and G2/M-phase (Fig. 4C).

4. Discussion

MiRNAs are well known regulators of cell growth and proliferation. Most of these RNA molecules exert their effect by directly regulating components of the cell cycle machinery, and deregulation in their levels is known to contribute to tumor development [20]. For example, miR-34a is downregulated in tumors and its forced expression causes G1 cell cycle arrest through inhibition of CDK/cyclin axis [21]. Similarly, the miR-221 family regulates cancer cell growth by targeting regulators of $p27^{Kip1}$ and $p57^{Kip2}$ [22]. In

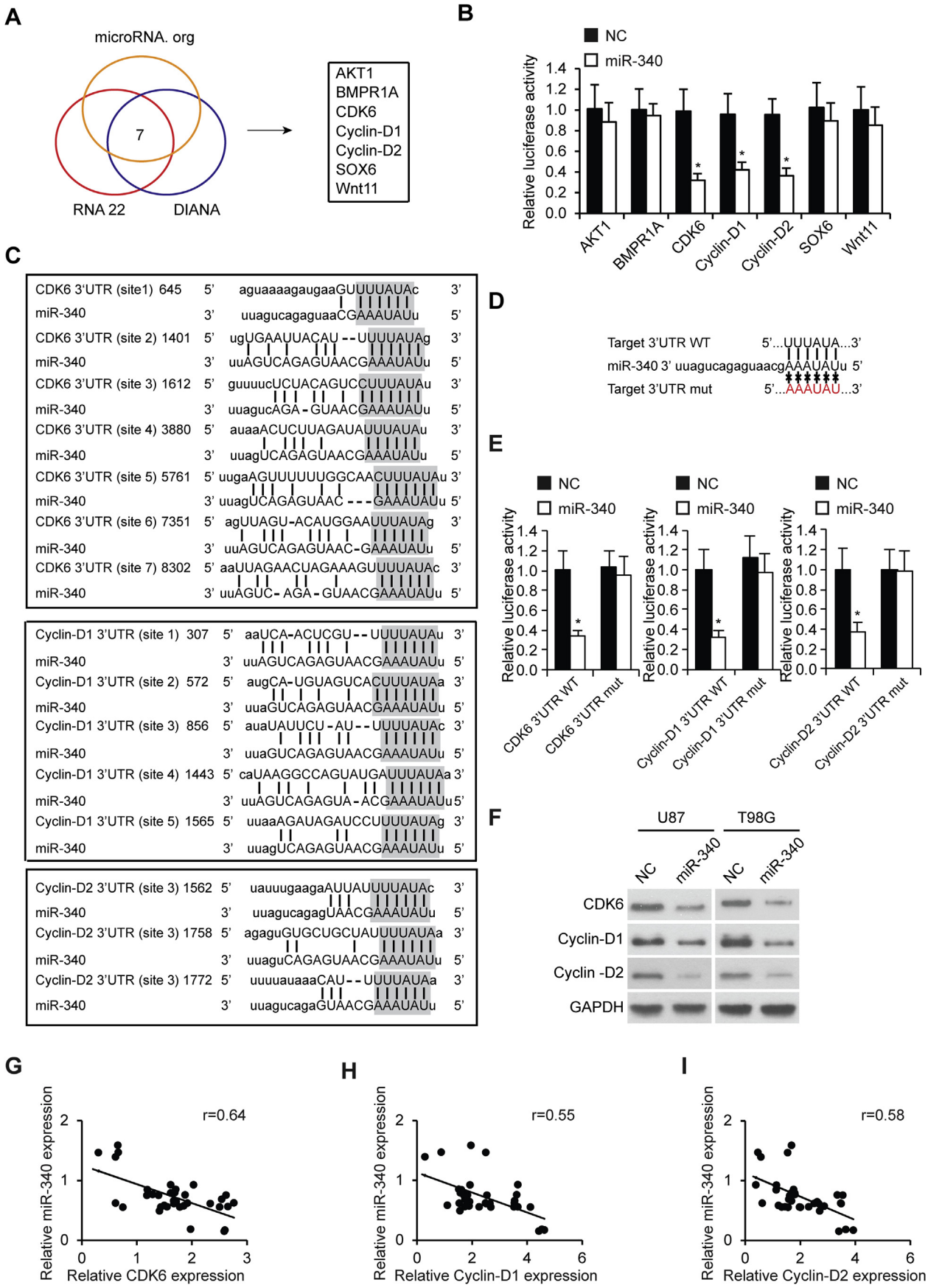

Fig. 3. CDk6, cyclin-D1, cyclin-D2 are miR-340 targets. (A) Venn diagram showing miR-340 targets (AKT1, BMPR1A, CDK6, cyclin-D1, cyclin-D2, SOX6 and Wnt11) predicted with microRNA.org, RNA22 and DIANA algorithms. (B) Luciferase assay in U87 cells co-transfected miR-340 with AKT1, BMPR1A, CDK6, cyclin-D1, cyclin-D2, SOX6 and Wnt11 wild type

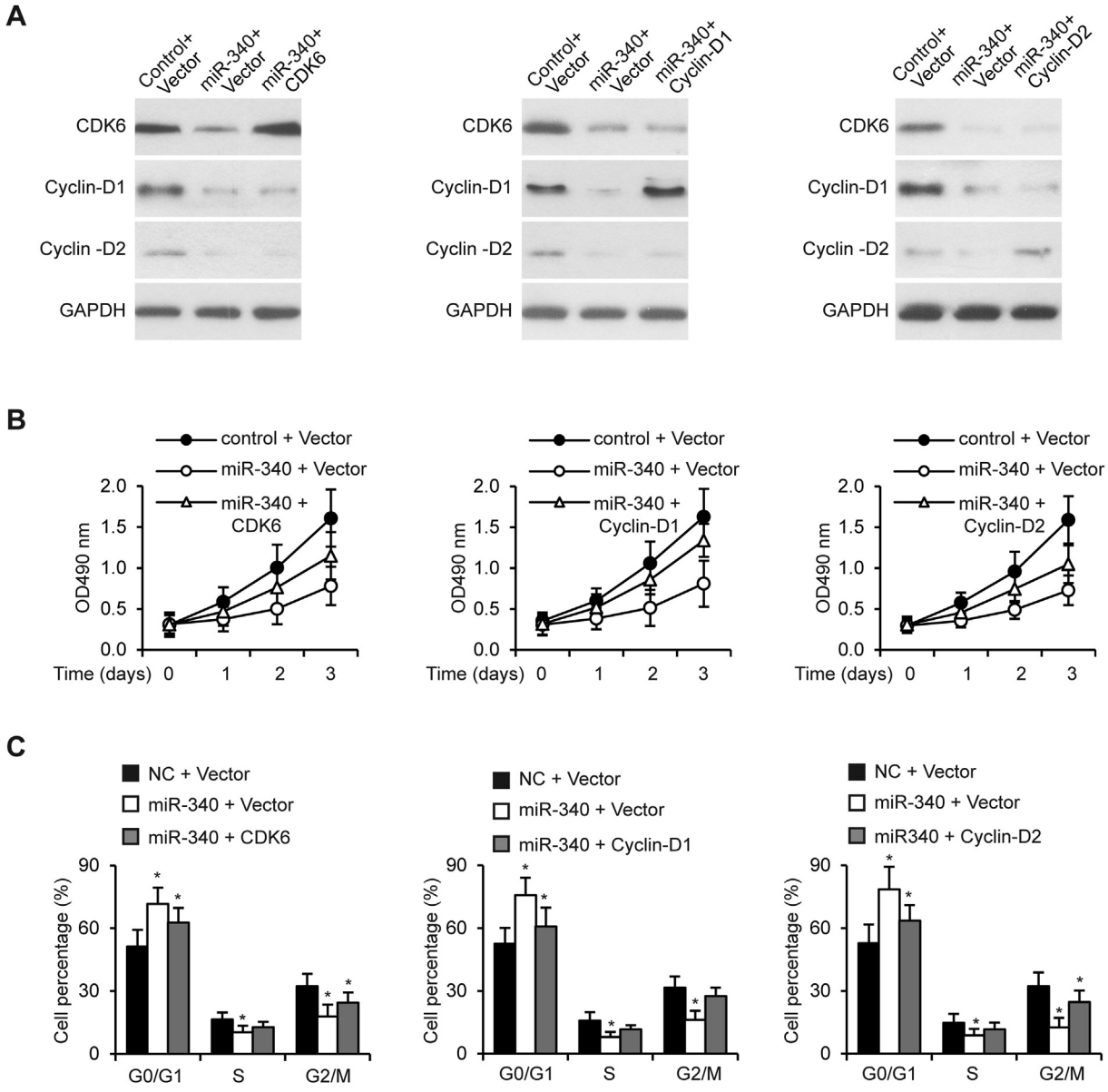

Fig. 4. miR-340 regulates glioblastoma cell proliferation via CDK6, cyclin-D1 and cyclin-D2. (A) Western blot of CDK6, cyclin-D1, and cyclin-D2 levels in U87 cells either transfected with miR-340 or co-transfected with miR-340 and CDK6, cyclin-D1 or cyclin-D2, compared to control. (B) MTS assay in U87 cells either transfected with miR-340 or co-transfected with miR-340 and CDK6, cyclin-D1 or cyclin-D2, compared to control. (C) Cell cycle analysis in U87 cells co-transfected with miR-340 and CDK6, cyclin-D1 or cyclin-D2, and quantitation of G0/G1, S and G2/M phases. $^{*}p < 0.05$ compared to control cells.

concert with these tumor-suppressive miRNAs, our study now shows that miR-340 is downregulated in ~89% of glioblastoma tumor samples examined and 100% of examined glioblastoma cell lines. We found that ectopic expression of miR-340 in glioblastoma cells causes cell cycle arrest at G0/G1 leading to inhibition of cell proliferation and neurosphere formation. These results demonstrate a putative role of miR-340 as a tumor-suppressor, which has also been found in several other cancers previously. Wu et al. [4] showed that miR-340 inhibited breast cancer cell growth and migration by targeting oncoprotein c-Met. Sun et al. [6] showed oncosuppresive role of miR-340 in colorectal cancer by inhibiting pyruvate kinase isozymes, PKM. Recently, Fernandez et al. [5] reported that miR-340 induces apoptosis and inhibits non-small cell lung cancer cell proliferation by modulating regulators of p27. It is important to acknowledge that Fernandez et al. [5] also reported an anti-proliferative role of miR-340 in glioblastoma. However, the mode of miRNA regulation was reported to be through induction of cellular apoptosis by stabilizing the CDK inhibitor $p27^{Kip}$.

3′UTR luciferase reporter plasmids. (C) Putative miR-340 binding sites within CDK6, cyclin-D1 and cyclin-D2. (D) Six common nucleotides (red) in the predicted binding sites of miR-340 on 3′UTR of CDK6, cyclin-D1, cyclin-D2 genes were mutated. (E) Luciferase activity in U87 cells co-transfected with miR-340 and wild type (WT) or mutant 3′UTR luciferase reporter plasmids of CDK6, cyclin-D1 and cyclin-D2 genes. (F) Western blot of CDK6, cyclin-D1 and cyclin-D2 levels in U87 and T98G cells transfected with miR-340 compared to NC. GAPDH was used as a loading control. Spearman correlation analysis between miR-340 and CDK6 (G), cyclin-D1 (H), or cyclin-D2 (I) in 35 clinical glioblastoma samples. $^{*}p < 0.05$ compared to NC transfected glioblastoma cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cell cycle defects associated with tumors are frequently caused by alteration of CDK activity, causing aberrant cell proliferation and genetic/chromosomal instability. Currently, it is believed that CDKs are essential players in each cell cycle phase, and therefore general CDK activity blocking therapeutics are unlikely to only affect tumor cells [11]. Cyclin-D forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition [23]. However miRNA therapeutics may circumvent this problem as miRNAs appear to have specificity for smaller subsets of CDKs and cyclins, rather than broad specificity. Recent studies have highlighted the role of CDK/cyclin signaling in cell cycle progression of cancer stem cells (CSC). Koyama-Nasu et al. [24] showed that cyclin-D2 was overexpressed in glioblastoma stem cells (GSC) and its gene interference caused G1 arrest and suppression of GSC growth in transplanted mice. Similarly, several studies have shown upregulation of CDKs and dysregulation of CDK inhibitors in CSC, leading to tumor formation [11]. Our report shows that miR-340 overexpression in U87 and T98G significantly reduced the number of neurospheres that formed. Neurospheres are comprised of neural stem cells and progenitor cells, and several studies have shown distinct CSC populations in U87 and T98G cell lines [25,26]. Therefore, it is possible that endogenous miR-340 might be downregulated in the CSC population of glioblastoma cells and in the absence of the negative regulation of CDK and cyclins, glioblastoma CSCs might undergo continual renewal, proliferation, and differentiate into cancer cells. This is a plausible hypothesis supported by several studies that report the role of miRNAs in CSC pathophysiology. Gal et al. [27] reported that miR-451 inhibits tumor growth through regulation of CSC. Gangemi et al. [28] reported that knockdown of SOX2 expression in glioblastoma-initiating cells inhibits proliferation and results in loss of CSC tumorigenicity. In addition, miR-128 has been shown to specifically inhibit the self-renewal capacity of CSCs by directly targeting *BMI-1*, a polycomb family transcriptional repressor required for maintenance of neural stem cells in the peripheral and central nervous system [29]. Furthermore, miR-199b-5p and miR-34a are important for the self-renewal potential of GSCs via the Notch signaling pathway [30]. In our study, we found that glioblastoma cells are arrested in the G0/G1 phase when miR-340 is overexpressed with concomitant reduced S and G2/M cell population. Further, we found that rescuing CDK/cyclin expression reverses this effect and leads to normal cell cycle progression. It would be interesting to see if CSCs have aberrations in miR-340 expression and whether this could be the underlying reason behind amplification of CDKs in glioblastoma. Finally, CSCs in glioblastoma are known to be resistant to radiation and chemotherapy, which eventually results in tumor recurrence. Targeting CSCs is an important avenue in the treatment of glioblastoma, therefore selectively inhibiting the CDK/cyclin axis in CSC might impede development and proliferation of cancer cells. However, our experiment has the limitation that we did not determine the origin (CSCs or mature glioblastoma cells) of aberrant expressed miR-340 in our study.

In summary, we found that miR-340 is frequently downregulated in glioblastoma clinical samples and glioblastoma cells. Our study provides *in vitro* experimental evidence that supports the role of miR-340 in regulating cell cycle progression in glioblastoma by negatively regulating the expression of CDK6, cyclin-D1 and cyclin-D2. This new insight may prove useful for novel diagnostic methods and as a therapeutic avenue for glioblastoma.

Conflict of interest

The authors have declared that no conflict of interest exists.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.088.

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