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Original Article

MicroRNA-940 inhibits glioma cells proliferation and cell cycle progression by targeting CKS1

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Abstract: Glioblastoma (GBM) is the most frequently occurred malignant human tumor that arise in brain with a poor prognosis. microRNAs (miRNAs) are vital small molecules during GBM initiation and progression. However, the expression of miR-940 and its potential function in GBM remain poor. Our study demonstrated that miR-940 was dramatically decreased in GBM cells and glioma tissues. Introduction of miR-940 significantly repressed proliferative ability of GBM cells. Notably, treatment of miR-940 dramatically suppressed tumor growth in an animal model, accompanied by decreased Ki67 expression. Functional experiments showed CKS1 as a target of miR-940, knockdown of CKS1 significantly induced the cell cycle arrest and restrained GBM cells proliferation, consistent with miR-940 treatment. Furthermore, reintroduction of CKS1 into glioma cells effectively rescued the tumor suppressive effect of miR-940. Correlation analysis indicated that miR-940 expression was inversely related to CKS1 mRNA levels in NBTs and gliomas. Together, miR-940/CKS1 signaling may be required for GBM progression and provide a new insight in diagnosis and prognosis of GBM patients.

Keywords: miR-940, CKS1, proliferation, glioma

Introduction

Among all the human malignant brain tumors, glioma accounts for the largest part, and glioblastoma multiforme (GBM, WHO grade IV) is its most malignant form [1, 2]. Although advanced treatments as surgical intervention, radiation and chemotherapy have been used for glioma patients, their prognosis were poor with its median survival less than 16 months [3, 4]. The fast growth of glioma cells is one typical feature of gliomas that results from genomic instability and gene deregulation [5]. Furthermore, incomplete understanding of glioma's pathogenesis leads to the limited therapeutic strategies for glioblastomas. Thus, studies are required to elucidate mechanisms underlying GBM development and find novel effective therapeutic strategies for this disease.

MicroRNAs (miRNAs) are noncoding RNAs which can regulate gene expression through binding to the 3'untranslated region (3'UTRs) of the target gene [6], thus play vital roles in regulating cell functions such as invasion, migration, proliferation and differentiation in different cancers [7, 8]. Moreover, miRNAs have been suggested to be connected with identifying disease type as diagnostic and prognostic indicators, and judging severity of disease [9, 10]. Many miRNAs were found deregulated in glioma cells and participate in glioma development [11-15]. Notably, miR-940 have been reported to inhibit tumor growth or invasion ability in many cancer including hepatocellular carcinoma [16, 17], pancreatic ductal adenocarcinoma [18], non-small cell lung cancer [19], breast cancer [20-22] and ovarian cancer [23]. Interestingly, it has been found tumor-promoting functions in gastric cancer [24, 25] and cervical cancer [26]. However, specific miR-940 functions during glioma initiation and progression have not been clearly clarified yet. Thus, researches of miR-940 expression and roles in glioma need to be further investigated.

Cyclin kinase subunit 1 (CKS1) is a small protein first characterized as one component of

Cyclin-CDK complexes, and related with transcriptional regulation, cell cycle regulation, as well as growth signaling pathways [27]. The aberrant CKS1 expression and its functions in various human cancers have been found previously, such as breast cancer [28-32], lung cancer [33], salivary cancer [34] and prostate cancer [35]. Our recent work found that CKS1 was upregulated in gliomas and was directly regulated by miR-936 [14]. However, specific mechanism of CKS1 underlying gliomas development still largely unknown.

Here, we showed that miR-940 expression was down-regulated in glioma cells and GBM tissues. Overexpression of miR-940 can restrain the proliferative ability of GBM cell and inhibited cell cycle progression by directly binding to CKS1 3'UTR region. Our data determined that miR-940 is a pivotal target for the diagnosis and prognosis of GBM patients and provide a novel insight into mechanisms governing GBM progression.

Materials and methods

Cell culture

U87, U251 and A172 human GBM cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). T98G, LN229, U118 and H4 cell were obtained from American Type Culture Collection ATCC. All the GBM cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Normal human astrocytes (NHAs) were gained from Lonza (Basel, Switzerland) and cultured in provided growth media supplemented with GA-1000, rhEGF, 5% FBS, ascorbic acid, insulin and L-glutamine.

Public datasets and clinical tissue specimens

Glioma miRNA data of 198 specimens were obtained from the Chinese Genome Atlas (CGGA) data portal (http://www.cgga.org.cn/portal.php). Clinical samples were provided by The First Affiliated Hospital of Nanjing Medical University. 14 low grade glioma tissues, 18 GBMs and 7 non-cancerous brain tissues were included in our study. The use and study of all the specimens were approved by the Research Ethics Committee of Nanjing Medical University, and written informed consents were obtained from all participants. The procedures were conducted according to the approved guidelines.

Plasmid construction, lentivirus packaging and stable cell lines generation

MiR-940 mimic or MiR-NC were purchased from Ribobio (Guangzhou, China). CKS1 plasmid was chemically synthesized and provided by Genechem (Shanghai, China). CKS1 cDNA was cloned to the pGL3 vector to construct pGL3-CKS1 recombinant plasmid according to the manufacturer's protocol. All plasmids were transfected into indicated cells accordingly. Premade lentiviral CKS1 short hairpin RNA (shRNA) constructs and a shCtrl construct were purchased from Open Biosystems. The lentiviral packaging kit was purchased from Genechem (Shanghai, China).

RNA extraction and quantitative real-time PCR

Total RNA from U87 and LN229 cells and human specimens were extracted using TRIzol Reagent (Invitrogen). cDNAs were synthesized and amplified by following the manufacturer's instructions. Quantitative real-time PCR was run using SYBR Premix Dimer Eraser (Takara, Dalian, China) on a 7900HT reaction system. Primers of miR-940 were purchased from Ribobio (Guangzhou, China) and U6 served as an internal control. 2-ΔΔCt method was used to calculate the fold changes.

Fluorescence in situ hybridization (FISH)

The expression of miR-940 in NBTs and GBM tissues was evaluated by FISH. The human miR-940 sequence is: 3'-CCCCUCGCCCCCGGGACGGAA-5'. The 5'-FAM labelled miR-940 probe sequence is: (LNA)-based probes against the miRNA-940 sequence is 5'-GGGGAGCGGGGGCCCTGCCTT-3' and was purchased from BioSense (Guangzhou, China). The procedure was performed according to the BioSense instructions. Briefly, frozen slices were fixed with 4% paraformaldehyde for 20 min, washed in PBS twice followed by treated with proteinase K at 37°C for 15 min and dehydrated in 70%, 85%, 100% ethanol for 5 min. probes were attached to the sections, which were denatured at 78°C for 5 mins. Hybridization procedure was performed overnight at 42°C in a moist chamber. Post-hybridization washes and nuclear staining with DAPI (Sigma) were performed the next day. A Zeiss LSM 510 META confocal

microscope system was used to evaluated the expression of miR-940.

Orthotopic glioma model and immunohistochemistry (IHC)

U87 glioma cells were transfected with lentivirus overexpressing miR-940 or the negative control sequences and carrying a luciferase reporter for 2 days. 6-week-old Nude mice were randomly assigned into two groups and a total of 5×10^5 U87 glioma cells were implanted intracranially using a 3D stereotactic instrument. Intracranial tumor growth volumes were assessed using Bioluminescence imaging. Before imaging, mice were anesthetized and injected with D-luciferin at 50 mg/mL intraperitoneally. The intensity of bioluminescence was detected in a IVIS Imaging System (Caliper Life Sciences) as well as the images were taken. All mice were observed until day 60 and survival time were analyzed. The remaining animals were exposed to CO_2 for euthanasia. All animal experiments were approved by the Nanjing Medical University Institutional Committee for Animal Research. IHC assay was used to determine the Ki67 expression of brain tissues.

Reporter assay

The wild-type (WT) and mutated putative miR-940 target on CKS1 were cloned into pGL3 Luciferase reporter vector (Invitrogen) to generate the pGL3-WT-CKS1-3'UTR and Pgl3-MUT-CKS1-3'UTR plasmids. Luciferase reporter assay was performed according to the manufacturer's protocol. Briefly, glioma cells were cultured in a 96-well plate and transfected with the vectors using Lipofectamine 2000 for 48 h. The Luciferase Assay System was used to measure the luciferase activity.

Cell cycle analysis

U87 and LN229 glioma cells transfected with lentivirus and/or plasmids were harvested and fixed with 70% ice-cold ethanol at -20°C overnight. The next day, cells were washed with PBS followed by centrifugation for 5 min at 1500 rpm. Then cells were resuspended using the Cell Cycle Staining Kit (Multi, Science, Hangzhou, China) and incubated for 30 minutes. FacsDiva (Becton Dickinson) was used to analyze the cell cycle phase distribution.

Western blotting and antibodies

Proteins were extracted from U87 and LN229 glioma cells, NBTs and glioma tissues. The concentration of protein was measured by Bio-Rad protein assay. Then, proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific, MA, USA). Membranes were blocked with 5% nonfat milk for 1 h and incubated in indicated primary antibodies against CKS1 (1:500; ab130529) and β-actin (1:5000; ab82-26) were purchased from Abcam (Cambridge, UK), CyclinE1 (1:1000; #4129), CDK2 (1:1000; #2546), CDC2 (1:1000; #9116) from Cell Signaling Technology (Massachusetts, USA) for 2 h at room temperature. After washed for three times with PBS, membranes were incubated in their relative horseradish peroxidase-conjugated antibody and signals were detected using an electrochemiluminescence detection system (Thermo Fisher Scientific).

Cell proliferation assay

U87 and LN229 glioma cells were harvested and seeded at a density of 300 cells/well in a six-well plate. Colony formation was observed 2 weeks later and colonies were washed twice, fixed with 4% methanol and stained with crystal violet (Sigma, USA) and counted. For CCK-8 assay, stabled transfected cells were seeded in a 96-well plate and cultured for 24, 48, 72 and 96 h. CCK-8 Cell Counting Kit (Dojindo, Japan) was used to detect the cell growth rate following the manufacturer's instructions. For EdU assay, cells were grown on coverslips in a 24-well dish. Cell-Light™ EdU Apollo®567 In Vitro Imaging Kit (100T) (Ribobio, Guangzhou, China) was used according to the manufacture's protocol. The EdU-positive cells were detected with a fluorescent microscope and were counted manually.

Statistics

All experiments were conducted three times, and data are presented as the means ± SD. Student's t test was used to analyze differences in pairwise comparison and ANOVA was performed for multivariate analysis. The correlation between miR-940 expression and CKS1 levels in NBTs and glioma tissues were analyzed by Kaplan-Meier analysis using GraphPad

Prism 7 software. $P < 0.05$ was considered statistically significant.

Results

Down-regulated miR-940 expression in glioma cell lines and glioma tissues

To evaluative the miR-940 expression profiles in glioma tissues, we analyzed 198 patients based on the Chinese Glioma Genome Atlas (CGGA) database. Results showed that high grade gliomas (HGG) exhibit significantly lower miR-940 expression than that of low grade gliomas (LGG) (**Figure 1A**). Kaplan-Meier survival analysis of these patients showed that groups of high miR-940 levels had much worse overall survival (OS) than those with low miR-940 expression levels (**Figure 1B**). Next, we analyzed the levels of miR-940 in normal human astrocytes (NHA) and 7 GBM cell lines (U87, U251, T98G, A172, LN229, H4 and LN118) using qPCR. All of these cells showed decreased levels of miR-940 with U87 and LN229 glioma cells most significant (**Figure 1C**). Moreover, we examined miR-940 expression in 7 non-cancerous brain tissues, 14 LGGs and 18 HGGs. NBTs were found to highly express miR-940 in comparison with gliomas and miR-940 expression was decreased in HGG as compared to that of LGG (**Figure 1D**). We also performed FISH assay with representative NBTs and gliomas, results were highly consistent with our previous work (**Figure 1E**). These results suggesting that miR-940 is downregulated in gliomas and indicating a potential marker for glioma patients.

MiR-940 inhibits glioma cells proliferation in vitro

Based on our finding that miR-940 was downregulated in glioma, we decided to investigate its roles in glioma. We stably transfected U87 and LN229 cells with miR-940, and qPCR analysis showed significantly increased level of miR-940 as compared to the negative control groups (**Figure 2A**). Then, we performed CCK8 assay to test the cell viability and results showed cell viability was strongly inhibited by miR-940 (**Figure 2B**, **2C**). Colony formation assay was conducted to determine long-term cell proliferation ability, results showed that colonies were significantly reduced after transfected with miR-940 in glioma cells compared with the negative control group (**Figure 2D-F**). To further evaluate our results, we performed EdU (5-ethynyl-2'-deoxyuridine) proliferation assay, and in line with our previous tests, miR-940 significantly decreased the EdU positive cells compared to the control group (**Figure 2G-I**). Since cell cycle progression play important roles in cell proliferation and miR-940 significantly inhibited proliferation of glioma cells, we wonder if miR-940 also affect glioma cell cycle progression. To verify our hypothesis, we conducted cell cycle analysis to test the glioma cell cycle distribution overexpressing miR-940. As we presumed, after transduced with miR-940 in glioma cells, we observed obviously upregulated percentage of G0/G1 phase and reduced S phase compared to the NC group (**Figure 2L**). We also analyzed the protein levels of several cell cycle related genes, CDK2, CDC2 and CyclinE1 by western blotting, results indicated that these proteins were dramatically downregulated after transfected with miR-940 as compared to the miR-NC group (**Figure 2J**). Together, these results indicated miR-940 paly an antitumor role in glioma cells.

MiR-940 targets CKS1 in glioma cells

As we know, miRNAs are noncoding RNAs composed of 18-23 nucleotides and exert their functions by binding to downstream target genes. Since we proved that miR-940 can restrain glioma cells proliferation, we next investigate its mechanism of downstream targets. Intersecting three prediction algorithms, including TargetScan, miRanda and miRWalk, we focused on CKS1 and examined its functions in glioma cells. To determine the relationship between CKS1 and miR-940, we conducted western blot assay to examine the protein levels of CKS1 in glioma tissues and non-cancerous brain tissues. The results showed that the expressions of CKS1 were dramatically increased in glioma tissues compared with that of the non-cancerous brain tissues. High grade gliomas also showed upregulated CKS1 expression than in low grade gliomas (**Figure 3A**, **3B**). next, we examined the correlation between CKS1 and miR-940 levels in each glioma specimen using Spearman's correlation analysis, results indicated that miR-940 and CKS1 levels were significantly negative correlated in each glioma tissue (**Figure 3C**). To further verify if miR-940 can directly binding to 3'-UTR region of CKS1, we performed dual luciferase reporter assays

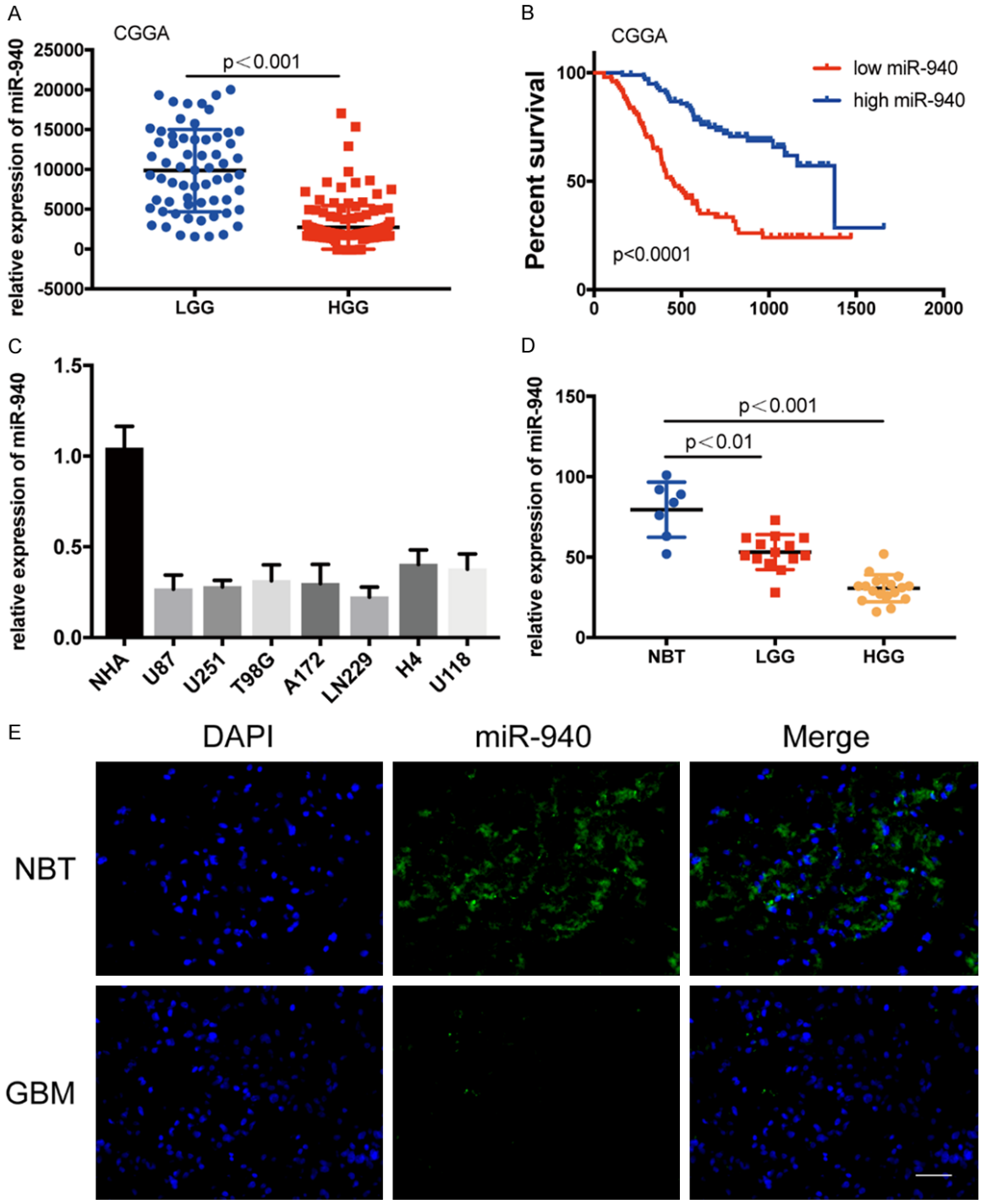


Figure 1. MiR-940 expression profiles in glioma cell lines and glioma tissues. A. CGGA database indicating reduced miR-940 expression and in high-grade glioma tissues compared with that in low-grade glioma tissues. B. Kaplan-Meier analysis of overall survival duration in GBM patients according to miR-940 expression using CGGA database. C. MiR-940 expression was detected in normal human astrocytes (NHAs) and seven glioma cell lines (U87, U251, T98G, A172, LN229, H4, H118). D. The expression of miR-940 in 7 non-cancerous brain tissues, 14 low-grade glioma tissues and 18 high-grade glioma tissues was measured by real-time PCR, miR-940 levels in normal brain tissues were significantly higher than in glioma specimens, and were indeed decreased with ascending pathological grade of tumor. E. The expression of miR-940 was determined by FISH in GBM specimens and normal brain tissues (scale bar, 50 μm).

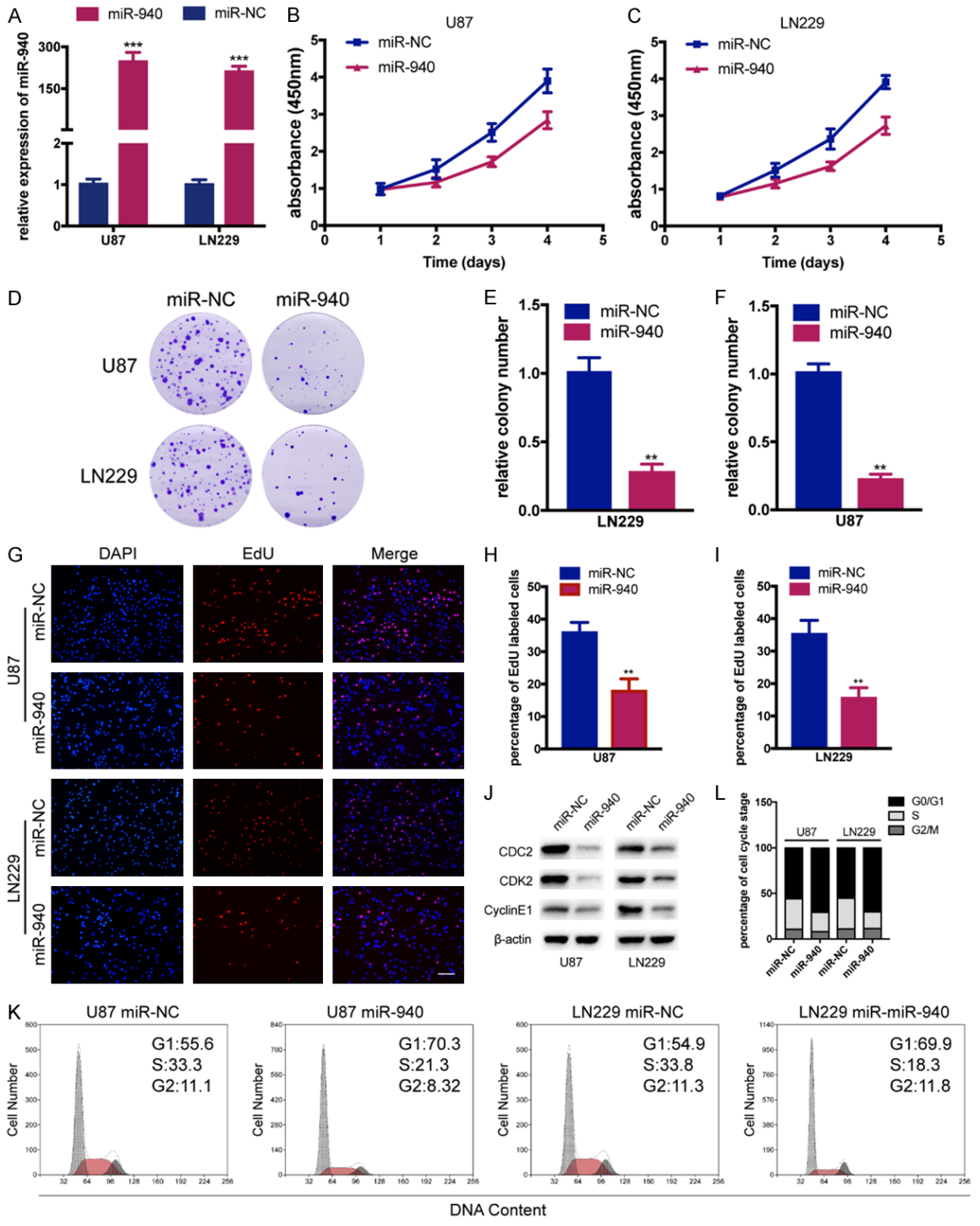


Figure 2. Overexpression of miR-940 inhibits glioma cell proliferation in vitro. A. The expression of miR-940 in U87 and LN229 cells transfected with miR-940 mimic and miR-ctrl were analized by qRT-PCR. (**P < 0.01). B, C. CCK-8 assay showing the proliferation rate of U87 and LN229 cells following culture for 96 h. Data are means of three independent experiments ± SD. D-F. Colony formation assay was used to evaluate the long-term cell viability. Data are means of three independent experiments ± SD. G-I. EdU assay was used to examine the proliferating cells. Representative images are shown (scale bar: 100 μm). J. Western blot analysis of CDC2, CDK2, and cyclinE1 expression in U87 and LN229 cells in U251 cells after transfection. β-actin was used as the loading control. K, L. Flow cytometry was used to detect the cell cycle distribution of U87 and LN229 glioma cells transfected with miR-940 mimic and miR-NC respectively.

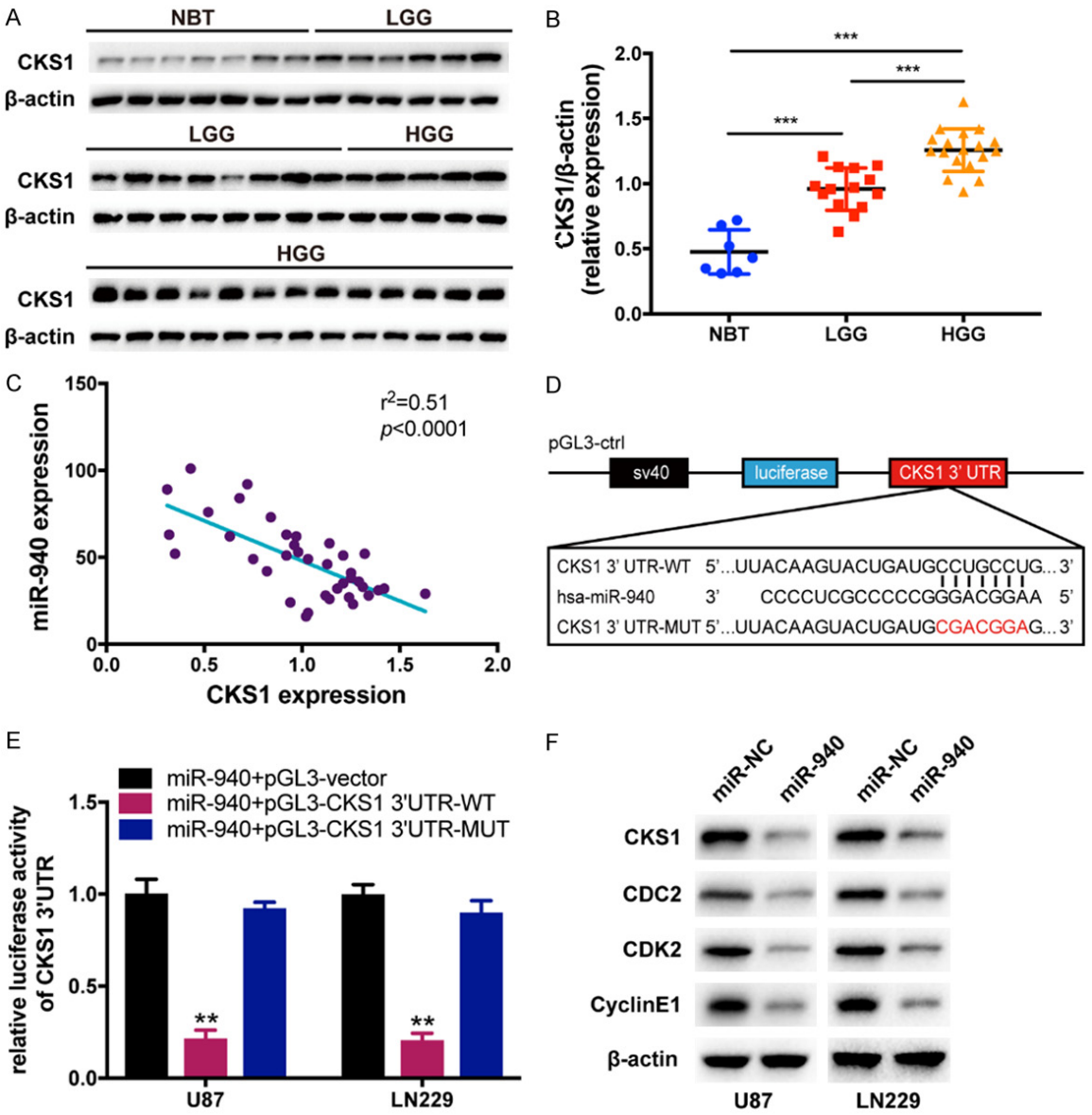


Figure 3. CKS1 is a direct target of miR-940 in U87 and LN229 cells. A, B. The expression levels of CKS1 in NBTS and glioma specimens were evaluated by western blotting; β-actin was used to normalize the fold changes. The non-neoplastic brain tissues (n = 7) were collected from brain trauma surgery. The low-grade (n = 14) represents samples derived from grades I and II glioma tissues, high-grade (n = 18) represents grades III and IV glioma tissues. Data are means of three independent experiments ± SD. (***P < 0.001). C. Pearson's correlation analysis of the relative expression levels of miR-940 and the relative protein levels of CKS1. D. Predicted binding sites of wild-type (WT) and mutated sequences of miR-940 in the 3'-UTR of CKS1 mRNA. E. Luciferase reporter assays were performed in U87 and LN229 cells transfected with pGL3-Vector, pGL3-CKS1-3'UTR-WT, or pGL3-CKS1-UTR-MUT reporter with miR-940 mimic. Data are means of three independent experiments ± SD. (**P < 0.01). F. CKS1, CDC2, CDK2 and CyclinE1 expression levels in indicated cells were determined by western blotting.

in U87 glioma cells co-transfected with miR-940 mimic and vectors harboring wild-type or mutant CKS1 3'-UTRs (**Figure 3D**). As shown in **Figure 3E**, luciferase activity was strongly repressed by miR-940 but not altered obviously with mutated 3'UTR. Parallel experiments were conducted in LN229 cells and similar results were found. To test whether miR-940 can regulate the CKS1 protein expression level, we performed western blotting and results showed CKS1 expression was strongly downregulated by overexpressing miR-940 both in U87 and

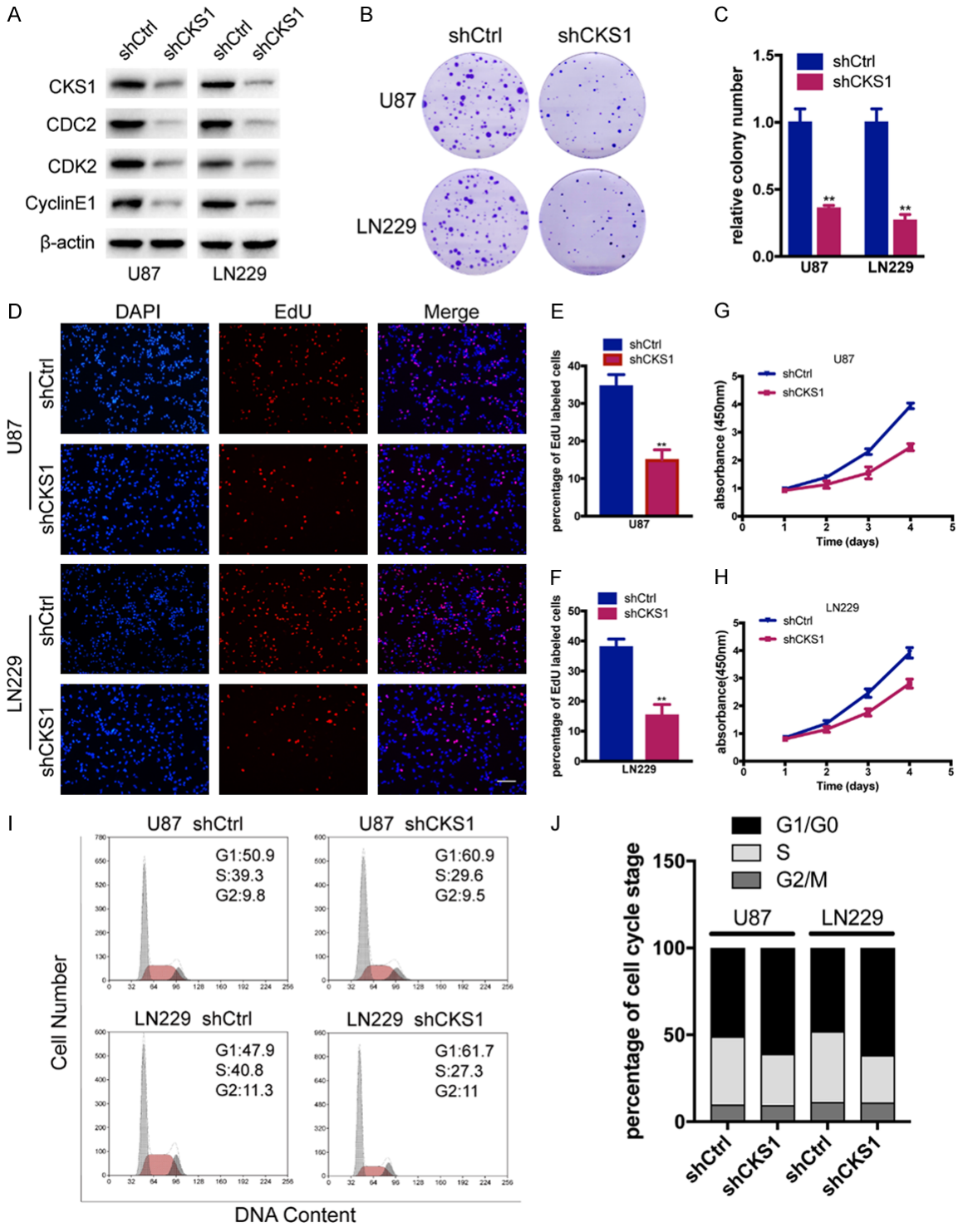


Figure 4. CKS1 knockdown suppresses GBM cells proliferation and cell cycle progression in vitro. A. Western blot analysis of CKS1, CDC2, CDK2 and CyclinE1 expression in U87 and LN229 cells after knockdown of CKS1. B, C. Colony formation assay was used to evaluate the long-term cell viability of indicated cells. Data are means of three independent experiments ± SD. (**P < 0.01). D-F. EdU assay was used to determine the proliferating cells. Data are means of three independent experiments ± SD. (**P < 0.01). G, H. Proliferation ability was determined using the CCK-8 assay following culture for 96 h. Data are means of three independent experiments ± SD. (**P < 0.01). I, J. Flow cytometry was used to analyze the cell cycle distribution of U87 and LN229 glioma cells after knockdown of CKS1.

LN229 cell (**Figure 3F**). Together, our data suggest miR-940 directly targeting CKS1 through binding to 3'-UTR region of CKS1.

CKS1 knockdown exhibited a tumor suppressive function in glioma cells

Previous work have demonstrated CKS1 knockdown can suppress tumor proliferation in several cancers including GBM. Here, we wonder where CKS1 is a direct target of miR-940. To asses our hypothesis, we transduced shCtrl and shCKS1 into LN229 and U87 glioma cells to inhibit CKS1 expression. Western blotting indicated that the protein level of CKS1, as well as CDC2, CDK2 and CyclinE1, were significantly inhibited by shCKS1 (**Figure 4A**). The effects of CKS1 knockdown on cell growth and viability were tested in LN229 and U87 glioma cells using colony formation, CCK-8 and EdU assays. Similar results were detected in all these experiments that knockdown of CKS1 strongly dampened the proliferation ability of GBM cells (**Figure 4B-H**). Flow cytometric analysis showed that CKS1 knockdown significantly increased the percentage of G0/G1 cell cycle phase and decreased the percentage of S phase, which was consistent with the effect of miR-940 overexpression (**Figure 4I**, **4J**). Together, these data suggested that CKS1 knockdown exhibited a tumor suppressive function in glioma cells.

Reintroduction of CKS1 restored the tumor malignant phenotype on glioma cells

As shown in our previous experiments, miR-940 overexpression restrained the proliferation ability of glioma cells by directly targeting CKS1. We wondered whether these phenotype changes caused by overexpressing miR-940 in glioma cells were induced by the expression changes of CKS1 as well. To test this thought, we transfected U87 and LN229 cells with CKS1 plasmids which were stably expressing miR-940 or miR-NC. Results indicated that the downregulated protein levels of CKS1, CDC2, CDK2 and CyclinE1 due to miR-940 overexpression were significantly rescued by reintroduction of CKS1 (**Figure 5A**). To investigate whether CKS1 was a directly regulated by miR-940 that cause the malignant phenotype changes in glioma cells, we conducted colony formation, CCK-8, and EdU assays. Results showed repressed proliferation prompted by miR-940 in glioma cells were dramatically rescued by miR-940 overexpression (**Figure 5B-H**), as well as the cell cycle phase arresting (**Figure 5I**, **5J**). Taken together, these results indicated CKS1 function as a direct target of miR-940, contributing to enhanced proliferative ability in GBM cells.

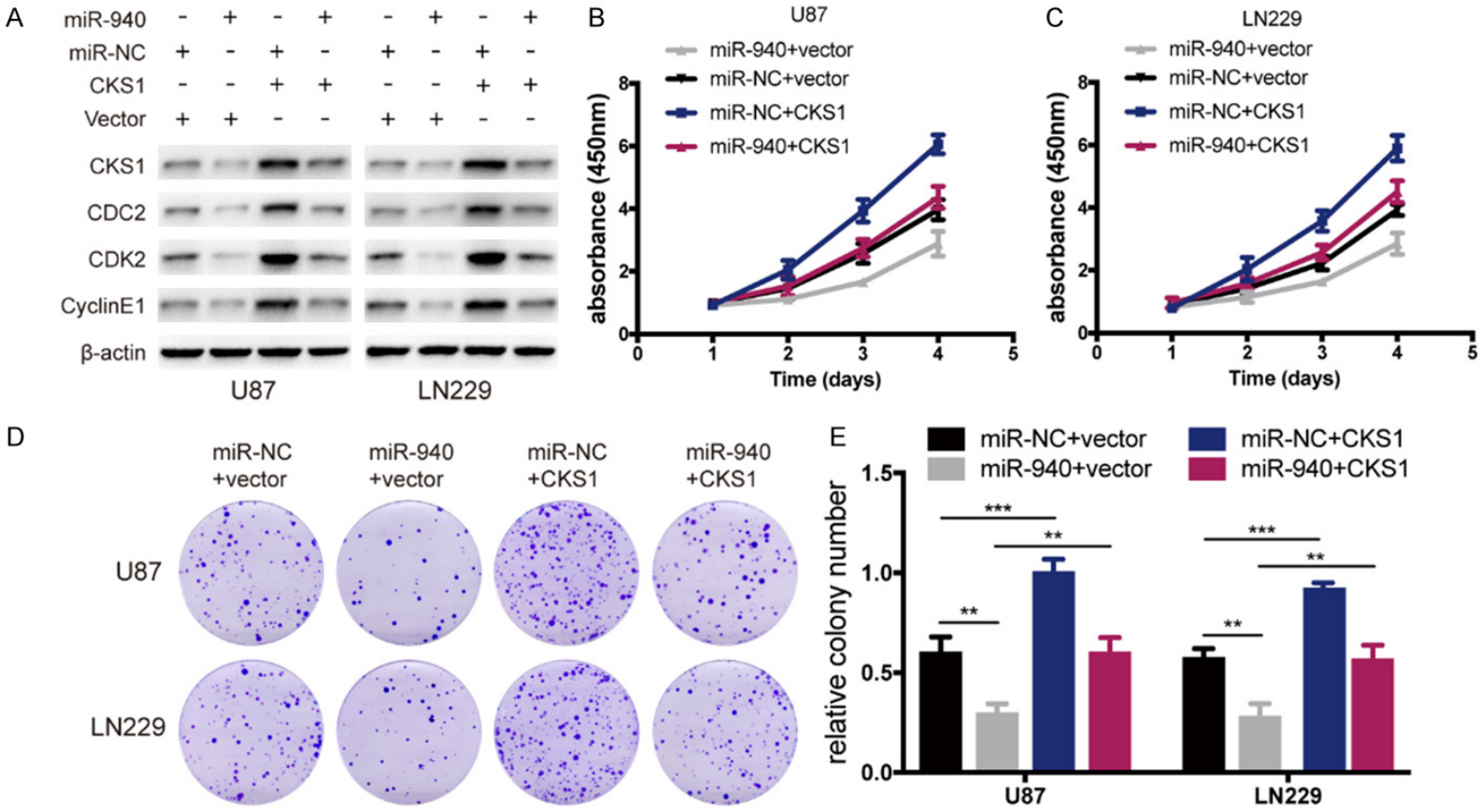
miR-940 overexpression repressed tumor growth in nude mice model

To determine the inhibitory effects of miR-940 of GBM cell proliferation in vivo, we employed an intracranial nude mice xenograft model. We injected U87 cells transfected with lentiviruses stably expressing luciferase with miR-940 or miR-NC on nude mice brains. On days 7, 14, 21 and 28 after implantation, the tumor volumes of the miR-940 groups were dramatically decreased as compared with the miR-NC groups (**Figure 6A**). To compare the survival of different groups, we used Kaplan-Meier survival curves and noted that mice treated with miR-940 injections showed significantly prolonged survival days as compared to that of the miR-NC injected groups (**Figure 6B**). Moreover, HE staining showed that the tumor volumes of the miR-940 groups were significantly smaller than that of the control groups. In addition, we performed immunohistochemical assay to assess the expression of Ki67, a classical biomarker for proliferation. Results showed that the expressions of Ki67 were significantly reduced in the miR-940 groups (**Figure 6C**). Together, our data indicated the tumor-suppressive functions of miR-940 in vivo.

Discussion

Fast proliferative ability has been characterized as one typical feature of GBM, leading to the difficulties of treatments and dismal prognoses. Molecular mechanisms of GBM generation remain largely unknown. Thus, finding key genes and understanding their functional process in regulating GBM development are pivotal to successfully cure GBM patients [5]. Accumulating evidence indicates miRNAs, small noncoding molecules that exert vital roles in human cancers progression and serve as biomarkers for the diagnosis, prognosis of cancers [10]. Here, we investigated miR-940 expression and its potential role, as well as its target gene CKS1 in GBM proliferation.

MiR-940 has been reported to exert inverse functions as tumor-suppressor or tumor-promoter in different cancers. Previous studies


A
miR-940
miR-NC
CKS1
Vector
CKS1
CDC2
CDK2
CyclinE1
β-actin
U87
LN229
B
U87
miR-940+vector
miR-NC+vector
miR-NC+CKS1
miR-940+CKS1
absorbance (450nm)
Time (days)
C
LN229
miR-940+vector
miR-NC+vector
miR-NC+CKS1
miR-940+CKS1
absorbance (450nm)
Time (days)
D
miR-NC +vector
miR-940 +vector
miR-NC +CKS1
miR-940 +CKS1
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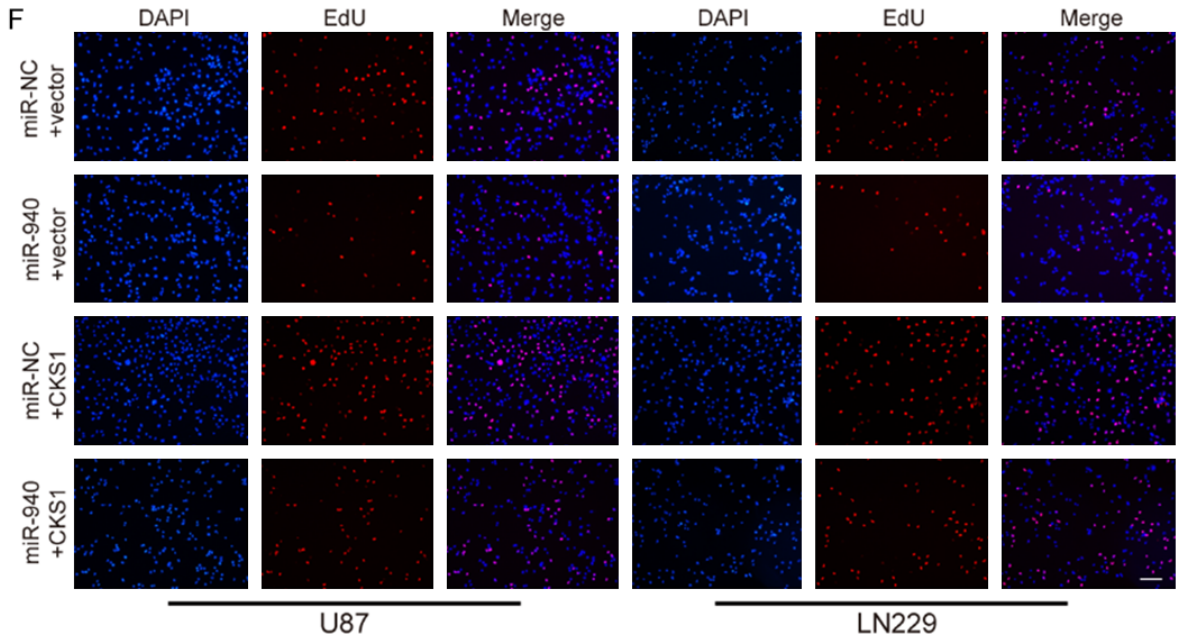
miR-940+CKS1
relative colony number
U87
LN229


F
DAPI
EdU
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DAPI
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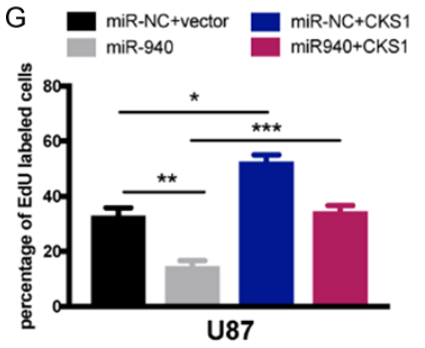
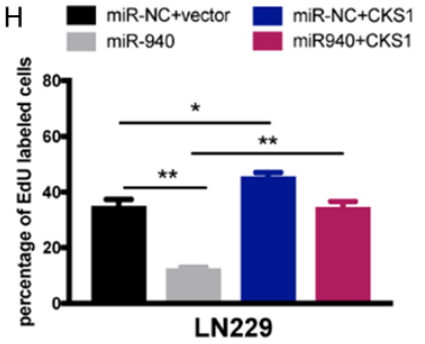
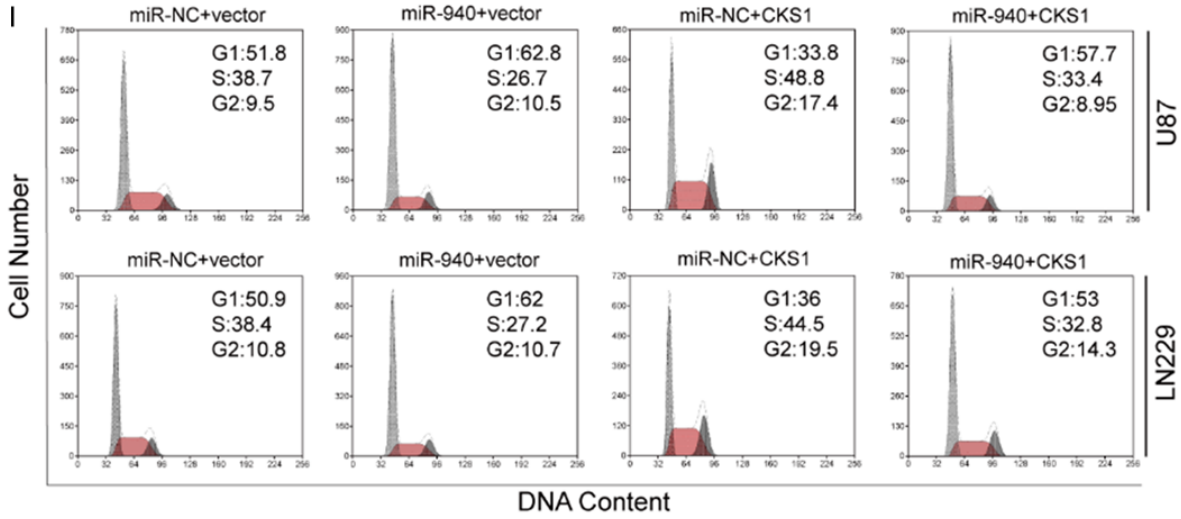
miR-NC +vector
miR-940 +vector
miR-NC +CKS1
miR-940 +CKS1
U87
LN229


G
miR-NC+vector
miR-940
miR-NC+CKS1
miR940+CKS1
percentage of EdU labeled cells
U87


H
miR-NC+vector
miR-940
miR-NC+CKS1
miR940+CKS1
percentage of EdU labeled cells
LN229


I
miR-NC+vector
G1:51.8
S:38.7
G2:9.5
miR-940+vector
G1:62.8
S:26.7
G2:10.5
miR-NC+CKS1
G1:33.8
S:48.8
G2:17.4
miR-940+CKS1
G1:57.7
S:33.4
G2:8.95
U87
miR-NC+vector
G1:50.9
S:38.4
G2:10.8
miR-940+vector
G1:62
S:27.2
G2:10.7
miR-NC+CKS1
G1:36
S:44.5
G2:19.5
miR-940+CKS1
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LN229
Cell Number
DNA Content

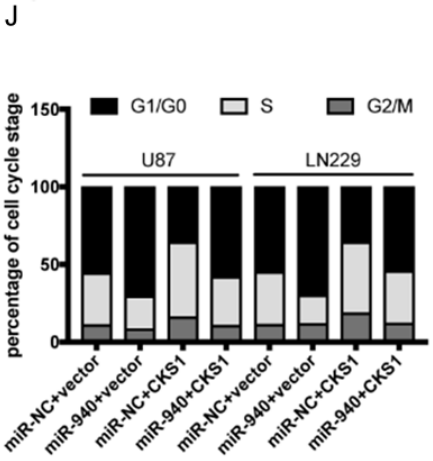
J
G1/G0
S
G2/M
U87
LN229
percentage of cell cycle stage
miR-NC+vector
miR-940+vector
miR-NC+CKS1
miR-940+CKS1
miR-NC+vector
miR-940+vector
miR-NC+CKS1
miR-940+CKS1

Figure 5. Reintroduction of CKS1 abrogated the inhibitory effects of miR-940 on GBM cell proliferation and cell cycle transition. A. Western blot analysis of CKS1, CDC2, CDK2 and CyclinE1 in U87 and LN229 glioma cells transfected with vector or CKS1 in the presence of miR-NC or miR-940. β-actin served as the loading control. B-E. Cell viability of glioma cells was detected using the CCK-8 and colony formation assays. Data are means of three independent experiments ± SD. (**P < 0.01). F-H. Cell proliferative potential was evaluated using the EDU assay 48 h after co-transfection. I, J. Cell cycle distribution of indicated cells was measured using flow cytometry.

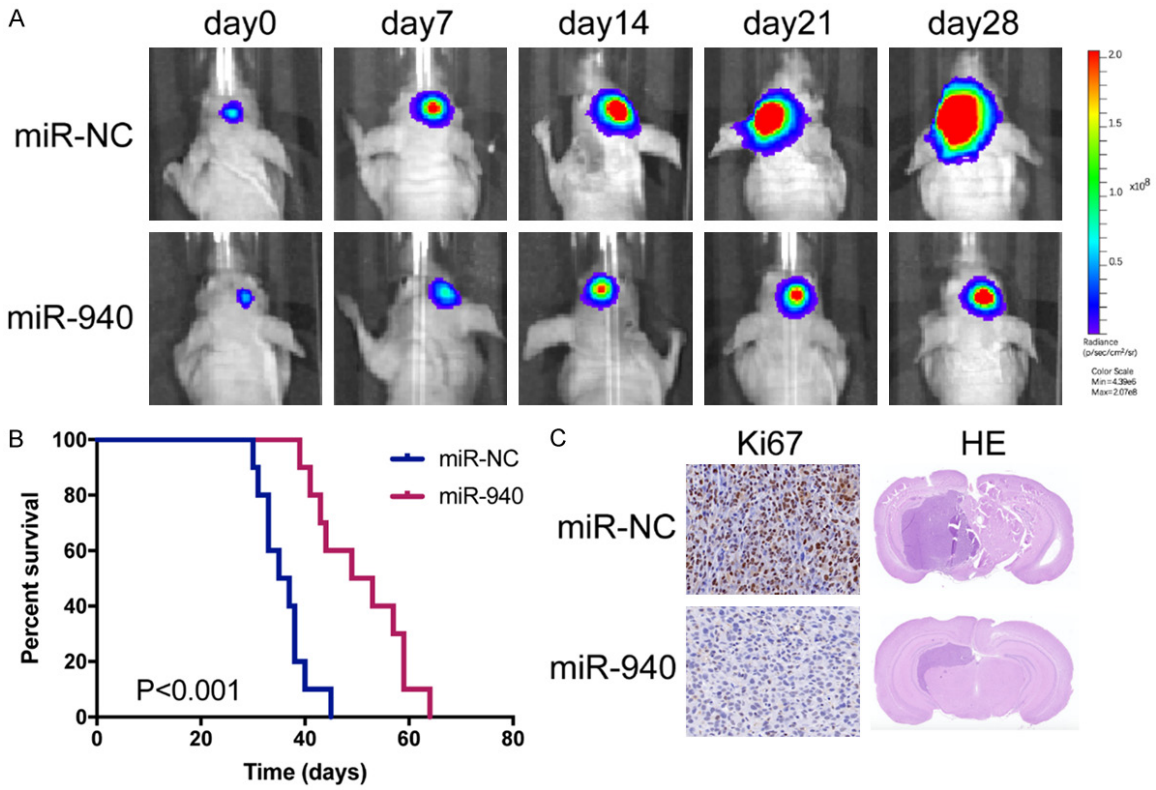

Figure 6. MiR-940 suppresses tumor growth in an intracranial xenograft model. A. U87 cells pretreated with a miR-NC or miR-940 lentivirus and a lentivirus containing luciferase were implanted in the brains of nude mice, and tumor formation was assessed by bioluminescence imaging. Bioluminescent images were measured at days 7, 14, 21 and 28 after implantation. B. Overall survival was determined by Kaplan-Meier survival curves, and a log-rank test was used to assess the statistical significance of the differences. C. After sacrifice, mouse brain tissues were harvested, embedded, and cut into paraffin sections for immunohistochemistry and HE histology analysis. The expression of Ki67 in the LV-miRNA-940 group was dramatically reduced.

showed that miRNA-940 suppressed cell invasion in hepatocellular carcinoma [16, 17], and this tumor-suppressive ability have also been found in pancreatic ductal adenocarcinoma [18], non-small cell lung cancer [19], breast cancer [20-22] and ovarian cancer [23]. Inversely, recent studies indicated that miR-940 increases the migration and proliferative ability of gastric cancer cells through up-regulation of PD-L1 expression [24], and miR-940 induces cervical cancer progression by the inhibition of PTEN and p27 [26]. Here, we found miR-940 was decreased in CGGA dataset, glioma cells and glioma tissues. Moreover, patients with high miR-940 levels had worse overall survival (OS) than those with low miR-940 levels. These results indicated that miR-940 function as a potential diagnostic and prognostic biomarker for glioma patients.

Accumulating evidence have suggested miRNAs play vital roles in regulating a variety of cell functions such as invasion, migration, proliferation, differentiation and development in various cancers [7, 8]. To further explore the specific miR-940 biological function in glioma, we treated U87 and LN229 glioma cells with lentivirus carrying miR-940 mimics and performed CCK-8, EdU and Colony formation assays. Results showed restoration of miR-940

leads to repressed glioma cell proliferation and induces cell cycle progression. Notably, animal studies showed a remarkably reduced tumor volume in an intracranial xenograft model following miR-940 overexpression treatment, suggesting its potential therapeutic biomarker for glioma patients.

Since miRNAs are noncoding RNAs that exert their biological functions by binding to the 3'untranslated region (3'UTRs) of the target genes [6], we next investigated its potential downstream targets. Using prediction algorithms, we focused on Cyclin kinase subunit 1 (CKS1), a protein which exert important roles in transcriptional regulation, cell cycle progression and growth signaling pathways [27]. The aberrant expression of CKS1 were also found in various human cancers such as breast cancer [28-32], lung cancer [33], salivary cancer [34] and prostate cancer [35]. Our previous work found that CKS1 was highly expressed in glioma cells and was a direct target of miR-936 [14]. By testing, we showed miR-940 suppressed GBM cells proliferation and induces cell cycle progression through directly targeting CKS1 using luciferase reporter assays. Furthermore, we found CKS1 is upregulated and inversely correlated with miR-940 levels in gliomas. Together, we conclude upregulated CKS1 level may result from the decreased miR-940 level in gliomas. To verify if CKS1 is functionally regulated by miR-940 in glioma cells, we knocked down CKS1 in U87 and LN229 cells using shRNA. Results showed knockdown of CKS1 significantly dampened GBM cell proliferation and induced cell cycle arrest, which were consistent with the results of overexpression of miR-940. Moreover, overexpression of CKS1 antagonized the effects of miR-940 overexpression. Together, we provide the first insight into miR-940 functions as a GBM-suppressor by directly targeting CKS1 in glioma.

In summary, our findings showed that miR-940 is dramatically downregulated and functions as a tumor suppresser by directly targeting CKS1 in gliomas. Though studies on miRNAs are still on early stages and mechanisms underlying their biological functions are largely unknown, our work are encouraging and indicate that miR-940/CKS1 axis has diagnostic, prognostic and therapeutic potentials for glioma patients. However, more efforts should be made to investigate mechanisms of reduced miR-940 level in GBM as well as more possible downstream targets of miR-940. Furthermore, studies of CKS1 and its mechanisms during GBM initiation and progression should also be carried out in the future.

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Disclosure of conflict of interest

None.

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