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ORIGINAL ARTICLE

MicroRNA-9 regulates the proliferation, migration and invasion of human glioma cells by targeting CDH1

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Summary

Purpose: *Glioma causes significant mortality worldwide. The currently available treatment strategies are flawed and the therapeutic targets are limited. Accumulating evidence suggests that microRNAs (miRs) are involved in the development and progression of different cancers. Herein, the therapeutic potential of miR-9 was explored in human U87 glioma cells.*

Methods: *qRT-PCR was used for expression analysis. WST-1 assay was used for determination of cell viability. Acridine orange (AO) / ethidium bromide (EB) and annexin V/propidium iodide (PI) were used for the detection of apoptosis. Flow cytometry was used for cell cycle analysis. Wound healing and transwell assays were used to monitor cell migration and invasion. Protein expression was determined by western blot analysis.*

Results: *The results showed that miR-9 is significantly downregulated in U87 glioma cells. Overexpression of miR-9 caused significant inhibition in the proliferation of U87 glioma cells. The miR-9-triggered growth inhibition was mainly due to the induction of apoptosis which was concomitant with increase in the Bax/Bcl-2 ratio. Overexpression of miR-9 also induced arrest of U87 glioma cells at G2/M checkpoint of the cell cycle. Additionally, transwell and wound healing assays showed that miR-9 caused significant decrease in the migration and invasion of U87 glioma cells. Bioinformatics analysis showed that miR-9 exerts its effects by inhibiting Cadherin-1 (CDH1). However, overexpression of CDH1 could nullify the effects of miR-9 on the growth, migration and invasion of glioma cells.*

Conclusion: *Taken together, miR-9 may exhibit therapeutic implications in the treatment of glioma.*

Key words: *glioma, microRNA, cell cycle arrest, CDH1, migration, invasion*

Introduction

Over the last few years, huge research endeavours have been directed to explore the roles of microRNAs (miRs) in human cells which regulate the expression of target genes via post transcriptional regulation and are around 19-23 nucleotides in length [1]. It is now believed that miRs control the majority of the human genes and are thus involved in vital cellular functions [2] and therefore are considered to exhibit therapeutic implications in treating human diseases such as cancer [3].miR-9 has been shown to control a diverse array of molecular processes [4] and has been reported to cause suppression of cancer cell proliferation via inhibition of NF-κB1 expression [5]. In another study, miR-9 has been observed to regulate the motility and metastasis of human colorectal cancer cells [6]. The involvement of miR-9 has also been reported in the regulation of gastric cancer cell growth and metas-

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tasis via targeting of cyclin D1 [7]. In yet another study, miR-9 has been shown to target the matrix metalloproteinase 14 to suppress metastasis of neuroblastoma cells [8]. Nonetheless, there is no report on the role and therapeutic implication of miR-9 in glioma and therefore, this study was undertaken to investigate the role and therapeutic implications of miR-9 in glioma. Accounting for about 77% of all the primary tumors of brain, glioma includes all tumors of glial origin [9]. Considered to be among the most destructive human cancers, gliomas cause tremendous mortality worldwide [10]. Surgery followed by chemo- and radiotherapy is generally employed for the management of these tumors [11]. Despite improvements in treatment, the average survival still remains 16 months for grade 4 gliomas [12]. Therefore, there is need for the identification of biomarkers for early detection and exploration of novel therapeutic targets for efficient treatment of gliomas [13]. The present study revealed that miR-9 is significantly suppressed in glioma cells and overexpression of miR-9 suppresses the growth of glioma cells inducing apoptosis. Additionally, miR-9 was also shown to cause a remarkable inhibition of the cell migration and invasion of the U87 cells and therefore miR-9 may exhibit therapeutic implications in glioma and may prove useful in glioma treatment.

Methods

Cell lines and culture conditions

The normal astrocytes and glioma cell lines (U87, U118, M059K and Hs683) were procured from American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Waltham, MA, USA), antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 2 mM glutamine. The cells were cultured in an incubator (Thermo Fisher Scientific, Inc.) at 37°C with 98% humidity and 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

The total RNA was extracted from glioma and the normal astrocytes with the assistance of RNeasy kits (Qiagen, Inc., Valencia, CA, USA). To reverse transcribe the cDNA, the Omniscript RT (Qiagen, Inc.) was employed using 1 µg of the extracted RNA. The cDNA was then used as a template for RT-qPCR analysis with the assistance of the *Taq* PCR Master Mix kit (Qiagen, Inc.) according to the manufacturer's protocol. The reaction mixture consisted of 20 µl containing 1.5 mM $MgCl_2$, 2.5 units *Taq* DNA Polymerase, 200 µM dNTP, 0.2 µM of each primer and 0.5 µg DNA. The cycling conditions were as follows: 95°C for 20 sec, followed by 40 cycles of 95°C for 15 sec, and 58°C for 1 min. GAPDH was used as internal control and the relative quantification ($2^{-\Delta\Delta Cq}$) method was used to evaluate the quantitative variation between the samples.

Cell transfection

The miR-9 mimics and negative control (NC) were synthesized by RiboBio (Guangzhou, China). The transfection was then carried out by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. As the U87 cells reached 80% confluence, the appropriate concentrations of miR-9 mimics or NC were transfected into these cells.

Cell proliferation assay

The proliferation rate of U87 cells was monitored by WST-1 assay. In brief, U87 cells were cultured in 96-well plates at a density of 2×10^5 cells/well. The cells were then transfected with miR-negative control (miR-NC) or miR-9 mimics and again incubated for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for 4 h. The optical density (OD) 450 nm was taken using a victor 3 microplate reader to determine the proliferation rate at 0, 12, 24, 48 and 96 h time intervals.

Colony formation assay

For assessment of the colony formation potential of the U87 cells, the cells were collected at the exponential phase of growth and were then counted using a hemocytometer. The platting of the transfected cells was performed at 200 cells/well. The plates were then maintained at 37°C for 6 days. Following incubation of the cells for 6 days, they were subjected to washing with phosphate buffered saline (PBS) and fixation with methanol. The U87 cells were then stained with crystal violet, and analyzed by light microscopy (Olympus Corporation, Tokyo, Japan).

Flow cytometric cell cycle analysis

After transfection, the U87 cells were cultured for 24 h at 37°C. The U87 cells were harvested and PBS-washed. Afterwards, the U87 cells were stained with PI and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Dual-luciferase reporter assay

The miR-9 target was identified by TargetScan online software (http://www.targetscan.org). The miR-9 mimics or NC were co-transfected with Plasmid pGL3-CDH1´-UTR-WT or pGL3-CDH1´-UTR-MUT into U87 cells. Dual-luciferase reporter assay (Promega) was carried out 48 h after transfection. *Renilla* luciferase was used for normalization.

AO/EB assay

After transfection with miR-NC or miR-9 mimics, the U87 cells were cultured in 6-well plates for 24 h at 37°C. The cells were then collected by centrifugation and washed with PBS. After this, the cells were stained in a solution of AO/EB for 20 min. Then, the cells were again PBS-washed and finally assessed by a fluorescence microscope.

Annexin V/PI assay

In brief, miR-NC or miR-9 mimics-transfected U87 cells (5×10^5 cells per well) were incubated for 24 h. This was followed by staining of these cells with annexin V-FITC/PI. The percentage of apoptotic U87 cells was determined by flow cytometry.

Wound healing assay

In brief, miR-NC or miR-9 mimics-transfected U87 cells were cultured in 6-well plates for 24 h. Then, after making a scratch line on the cells using a 200 µl sterile pipette tip, the plates were incubated at 37°C in 5% CO_2. Wound healing was observed at 0 and 24 h using an inverted microscope.

Cell invasion assay

The effects of miR-9 overexpression on the invasion ability of U87 cells was determined by transwell assay with Matrigel. The U87 cells were transfected with miR-9 mimics and around 200 ml cell culture were placed onto the upper chambers and only DMEM was placed in the bottom wells. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded via the chambers were fixed with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200x magnification.

Western blotting

The transfected U87 cells were cultured at 37°C for 24 h and then centrifuged at 1200 rpm. The cell pellet was washed with PBS and then resuspended in RIPA lysis buffer. Thereafter, the concentrations of the proteins were determined and equal concentrations of the proteins were loaded on SDS-PAGE gel (15%). The samples were transferred to polyvinylidene fluoride membranes and blocking was done using 5% skimmed milk powder. This was followed by membrane incubation with primary antibodies at 4°C for 24 h. Next, the membranes were incubated with horseradish peroxidase-linked secondary biotinylated antibodies for 2 h. The membranes were then washed and immunoreactive bands were observed by ECL-PLUS/Kit as per the manufacturer's guidelines.

Statistics

The experiments were performed in triplicate and the values presented as the mean±standard deviation. Student's t-test (for comparison between two samples) and one way analysis of variance (ANOVA) followed by Tukey's test (for comparison between more than two samples) were used for statistical analysis using GraphPad Prism software (version 7; GraphPad Software, Inc., La Jolla, CA, USA. P<0.05 was considered to indicate a statistically significant difference.

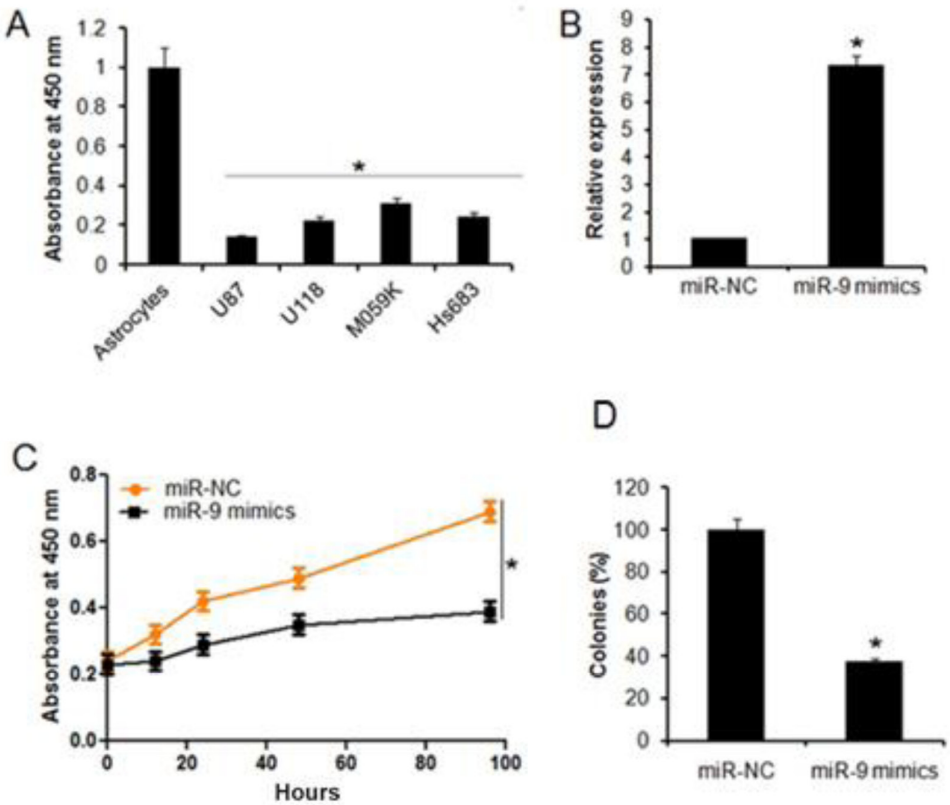

Figure 1. A: Expression of miR-9 in different glioma and normal astrocytes as determined by qRT-PCR. **B:** Expression of miR-9 in miR-NC or miR-9 mimics transfected U87 cells. **C:** Cell viability of miR-NC or miR-9 mimics transfected U87 cells as determined by WST-1 assay; and **D:** colony formation assay showing the effect of miR-NC or miR-9 mimics transfection on the colony formation of U87 cells. The experiments were performed in triplicate and the values are expressed as mean ± SD (*p<0.05).

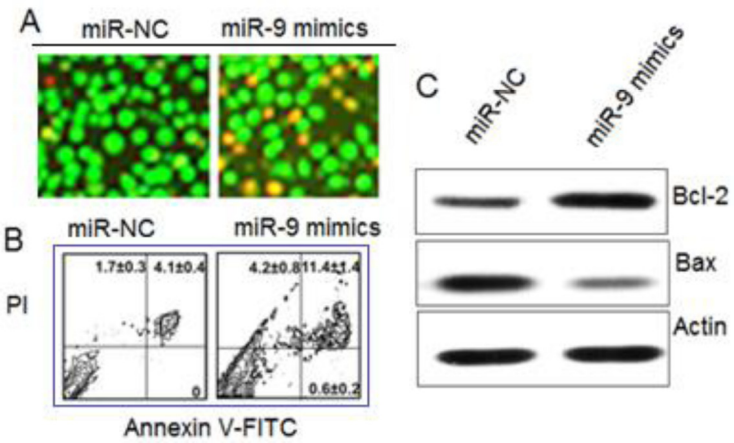

Figure 2. A: AO/EB staining showing induction of apoptosis in miR-NC or miR-9 mimics transfected U87 cells. **B:** Annexin V/PI staining assay showing the percentage of apoptosis in miR-NC or miR-9 mimics transfected U87 cells. **C:** Effect of miR-NC or miR-9 mimics on the expression of Bax and Bcl-2. The experiments were performed in triplicate.

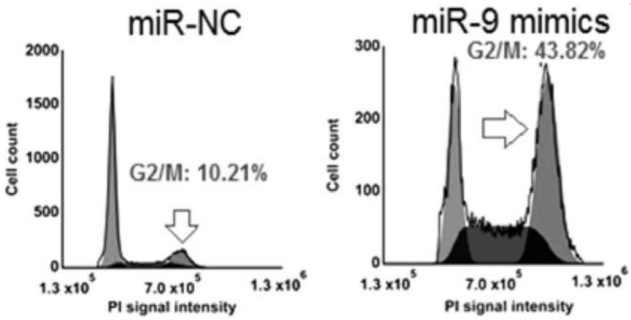

Figure 3. Flow cytometry showing the distribution of the miR-NC and miR-9 mimics transfected U87 cells in different cell cycle phases. The Figure shows that miR-9 overexpression induces G2/M arrest of the U87 glioma cells. The experiments were performed in triplicate.

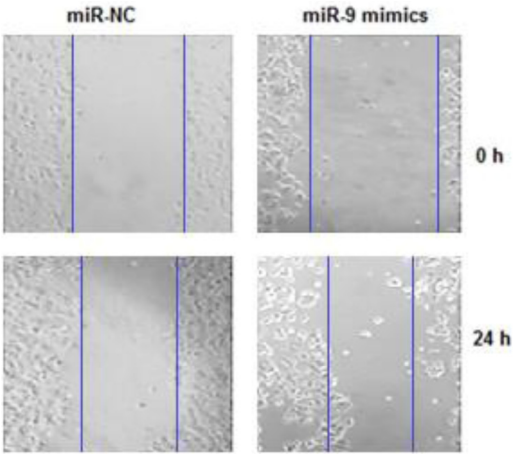

Figure 4. Wound healing assay showing the effects of miR-NC or miR-9 mimics on the migration of the U87 cells. The Figure shows that miR-9 overexpression inhibits the migration of the human U87 glioma cells. The experiments were performed in triplicate.

Results

miR-9 suppresses the proliferation and colony formation of glioma cells

To unveil the role of miR-9 in glioma, the expression prolife of miR-9 was determined in 4 different glioma cell lines as well as in normal astrocytes by qRT-PCR. The results showed that miR-9 was significantly suppressed in the glioma cells relative to its expression in normal astrocytes (Figure 1A). The expression of miR-9 was 7-fold lower in glioma cells. The U87 exhibited the lowest expression of miR-9 out of all the glioma cell lines (U87, U118, M059K and Hs683). To investigate the effects of miR-9 on the growth of the glioma U87 cells, the U87 cells were transfected with miR-9 mimics. The overexpression of miR-9 in U87 cells was validated by qRT-PCR which showed 7.3-fold increase of miR-9 expression (Figure 1B). Next, the proliferation rate of miR-9 overexpressing U87 cells was examined at different time intervals (90, 12, 24, 48 and 96 h). The results showed that miR-9 overexpression resulted in remarkable decrease in the proliferation rate of the U87 cells (Figure 1C). The colony formation assay showed that miR-9 overexpression caused around 63% decrease in the colony formation of the U87 cells (Figure 1D).

miR-9triggers apoptosis and G2/M arrest of U87 glioma cells

The AO/EB staining of the miR-9-overexpressing U87 cells was performed to assess whether miR-9 overexpression triggers apoptosis in these cells. Interestingly, it was found that miR-9 caused nuclear fragmentation and membrane blebbing of the U87 cells, indicative of apoptotic cell death (Figure 2A). The annexin V/PI staining showed that the percentage of apoptosis was 1.2% in miR-NC

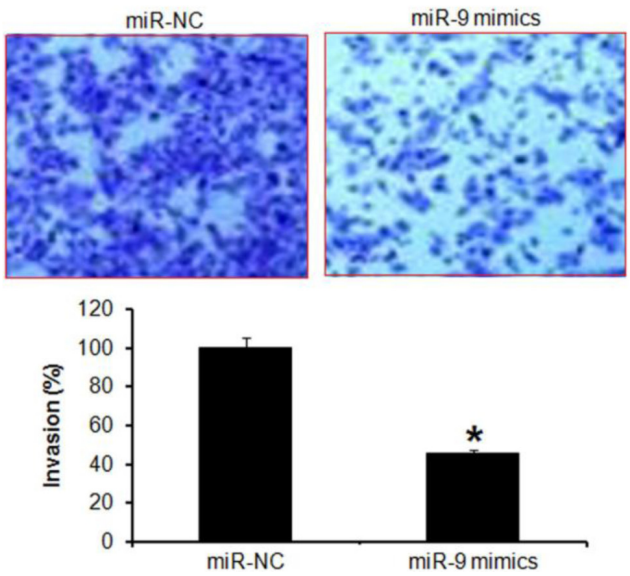

Figure 5. Transwell assay showing the effects of miR-NC or miR-9 mimics on the invasion of the U87 cells. The Figure shows that miR-9 overexpression inhibits the invasion of the U87 glioma cells (*p<0.05). The experiments were performed in triplicate.

transfected U87 cells as compared to 23.4% in the miR-9 mimics-transfected U87 cells (Figure 2B). Additionally, the expression of Bax was increased and that of Bcl-2 was decreased upon miR-9 overexpression (Figure 2C).

Next, the miR-9 mimics-transfected U87 cells were subjected to cell cycle analysis by flow cytometry. The results showed that miR-9 mimics-transfected also caused a considerable enhancement in the G2/M phase cells (Figure 3). The percentage of G2/M phase cells was 10.21% in miR-NC-transfected U87 cells in comparison to 43.82% in miR-9-mimics-transfected U87 cells, indicative of the G2/M arrest (Figure 3).

miR-9 suppresses the migration and invasion of U87 cells

The effects of miR-9 on the migration of U87 cells were determined by wound healing assay. The results showed that miR-35 caused significant decrease in the migration of the U87 cells,

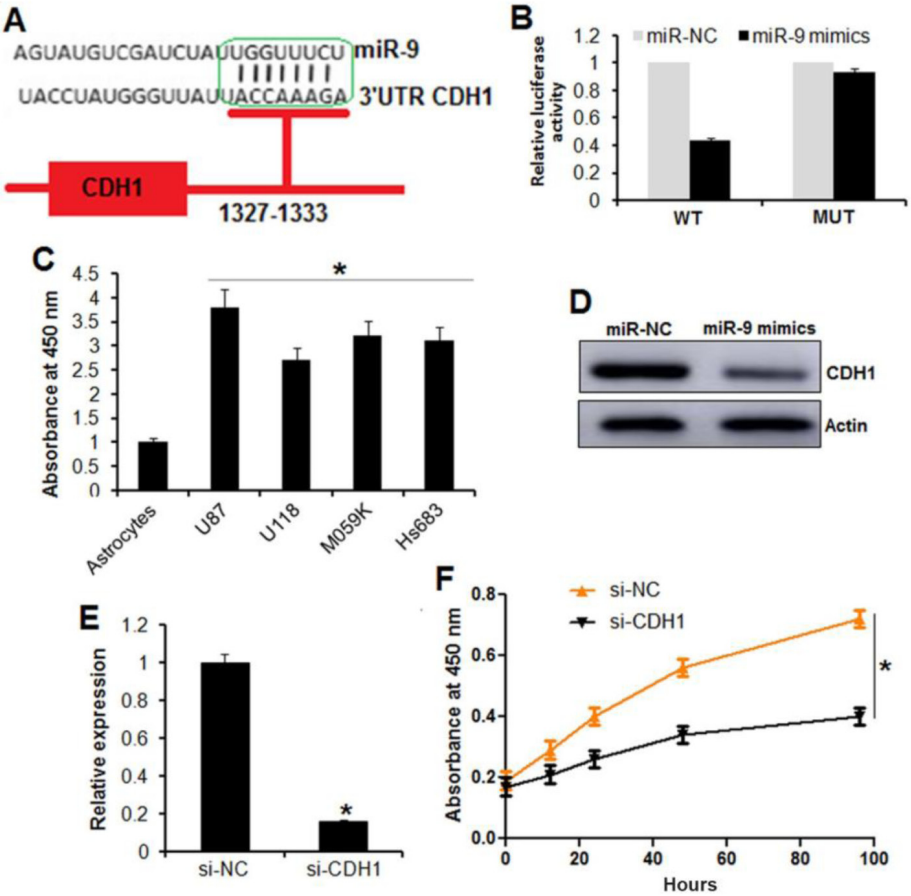

Figure 6. A: TargetScan analysis showing CDH1 as the target of miR-9. **B:** Dual luciferase assay interaction between miR-9 and CDH1. **C:** Expression of CDH1 in different glioma and normal astrocytes (*p<0.05). **D:** Western blot analysis expression of CDH1 in miR-NC and miR-9 mimics transfected U87 cells showing miR-9 overexpression inhibits the expression of CDH1. **E:** Expression of CDH1 in si-NC or si-CDH1 U87 cells showing suppressed expression of CDH1. **F:** Proliferation rate of si-NC or si-CDH1 transfected U87 cells as depicted by CCK8 assay. The Figure shows that silencing of CDH1 inhibits the proliferation of U87 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

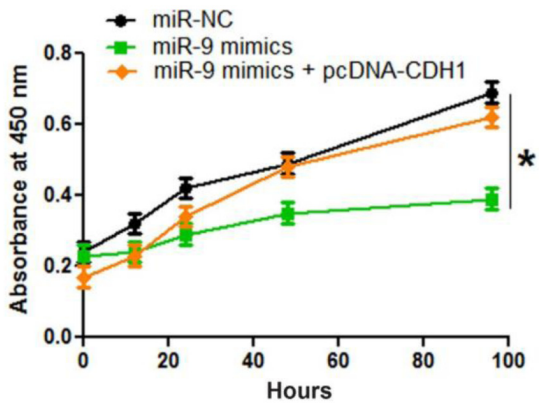

Figure 7. Rescue assay showing the effect of CDH1 overexpression on growth inhibitory effects of miR-9 mimics on U87 glioma cells. The Figure shows that CDH1 overexpression abolishes the inhibitory effects of miR-9 mimics. The experiments were performed in triplicate (*p<0.05 for miR-9 mimics Vs miR-9 mimics + pcDNA-CDH1).

as evidenced from the scratch width (Figure 4). The transwell assay also showed that miR-9 significantly suppressed the invasion of the U87 cells. The migration of the U87 cells was suppressed by 65% upon miR-9 overexpression (Figure 5).

miR-9 targets CDH1 in U87 glioma cells

The TargetScan revealed that CDH1acts as the target of miR-9 in U87 glioma cells (Figure 6A). CDH1 was further confirmed as the target of miR-9 by dual luciferase assay (Figure 6B). The qRT-PCR results further revealed that the CDH1 was significantly overexpressed in the U87 glioma cells with expression upto 3.8-fold higher as compared to the normal astrocytes (Figure 6C). However, overexpression of miR-9 resulted in suppression of CDH1 in U87 cells (Figure 6D). The effects of CDH1 silencing were also examined on the U87 cell proliferation and it was observed that silencing of CDH1 in U87 cells resulted in decrease of the U87 cell proliferation (Figure 6E). However, it was found that overexpression of CDH1 in U87 cells could abolish the growth inhibitory effects of miR-9 overexpression on U87 cell proliferation (Figure 7).

Discussion

Gliomas cause tremendous mortality and are among the most aggressive malignant tumors. The limited availability of reliable and efficient therapeutic targets/agents hurdles the treatment of gliomas [14]. The wide array of roles that miRs play out in humans by regulating the expression of genes suggests that miRs may prove useful therapeutic targets for treating human diseases, including cancer [15]. In this study, we deciphered the role of miR-9 in glioma. The results of the qRT-PCR revealed that the expression of miR-9 is remarkably downregulated in glioma cells. These findings are in agreement with previous investigations wherein miR-9 had been shown to be downregulated in human hepatocellular carcinoma [16].

miR-9 was overexpressed in U87 glioma cells. The results showed that miR-9 overexpression caused decline in the proliferation rate of the U87 cells. AO/EB staining showed that miR-9 overexpression resulted in nuclear fragmentation of the U87 cells. This was also accompanied with increase in Bax and decrease in the Bcl-2 expression. These results are in concordance with previous studies wherein miRs have been shown to regulate apoptosis in cancer cells. For example, miR-15 and miR-16 have been shown to promote apoptotic cell death of cancer cells by suppressing the expression of Bcl-2 [17]. Similarly, miR-34a induced apoptosis in neuroblastoma cells [18]. The cell cycle analysis of the miR-9 overexpressing U87 cells was also performed and it was found that miR-9 overexpression caused increase in the percentage of the G2/M phase U87 cells. The percentage of apoptosis in the miR-NC-transfected cells was 10.21% as compared to 43.82% in the miR-9 overexpressing U87 cells. Previous studies have also shown that miRs regulate the cell cycle related process in cancer cells [19,20]. For example miR-106b induces cell cycle arrest of human prostate cancer cells [20]. The miR-9 has also been shown to regulate the migration and invasion of cancer cells, for instance, miR-9 has been shown to cause suppression of the cell migration of colorectal cancer cells and herein we also observed that miR-9 suppresses the migration and invasion of U87 glioma cells. The miRs exert their effects by suppressing the expression of their target genes [21] and herein we found that miR-9 exerted its effects on the U87 glioma cells by targeting CDH1. The miR-9 has also been previously shown to target CDH1 in ovarian cancer cells, further confirming our results [22].

Conclusion

The findings of the present study revealed that miR-9 is downregulated in glioma cells. Overexpression of miR-9 in U87 glioma cells inhibited their proliferation via induction of apoptosis and cell cycle arrest. Overexpression of miR-9 also suppressed the migration and invasion of the U87 glioma cells, indicative of the therapeutic implications of miR-9 in glioma treatment.

Conflict of interests

The authors declare no conflict of interests.

References

1. Ramassone A, Pagotto S, Veronese A, Visone R. Epigenetics and microRNAs in cancer. Int J Molec Sci 2018;19:459.
2. Pichler M, Calin GA. MicroRNAs in cancer: from developmental genes in worms to their clinical application in patients. Br J Cancer 2015;113:569.
3. Ji W, Sun B, Su C. Targeting microRNAs in cancer gene therapy. Genes 2017;8:21.
4. Baroni S, Romero-Cordoba S, Plantamura I et al. Exosome-mediated delivery of miR-9 induces cancer-associated fibroblast-like properties in human breast fibroblasts. Cell Death Disease 2016;7:e2312.
5. Guo LM, Pu Y, Han Z et al. MicroRNA-9 inhibits ovarian cancer cell growth through regulation of NF-κB1. FEBS J 2009;276:5537-46.
6. Zhu L, Chen H, Zhou D et al. MicroRNA-9 up-regulation is involved in colorectal cancer metastasis via promoting cell motility. Med Oncol 2012;29:1037-43.
7. Zheng L, Qi T, Yang D et al. microRNA-9 suppresses the proliferation, invasion and metastasis of gastric cancer cells through targeting cyclin D1 and Ets1. PLoS One 2013;8:e55719.
8. Zhang H, Qi M, Li S et al. microRNA-9 targets matrix metalloproteinase 14 to inhibit invasion, metastasis, and angiogenesis of neuroblastoma cells. Molec Cancer Ther 2012;11:1454-66.
9. Ohgaki H, Kleihues P. Epidemiology and etiology of gliomas. Acta Neuropathologica 2005;109:93-108.
10. Ostrom QT, Bauchet L, Davis FG et al. The epidemiology of glioma in adults: a "state of the science" review. Neuro-Oncology 2014;16:896-913.
11. Malmer B, Iselius L, Holmberg E, Collins A, Henriksson R, Grönberg H. Genetic epidemiology of glioma. Br J Cancer 2001;84:429.
12. Bondy ML, Scheurer ME, Malmer B et al. Brain tumor epidemiology: consensus from the Brain Tumor Epidemiology Consortium. Cancer 2008;113:1953-68.
13. Schwartzbaum JA, Fisher JL, Aldape KD, Wrensch M. Epidemiology and molecular pathology of glioma. Nat Rev Neurol 2006;2:494.
14. Peckham-Gregory EC, Montenegro RE, Stevenson DA et al. Evaluation of racial disparities in pediatric optic pathway glioma incidence: Results from the Surveillance, Epidemiology, and End Results Program, 2000–2014. Cancer Epidemiol 2018;54:90-4.
15. Vannini I, Fanini F, Fabbri M. Emerging roles of microRNAs in cancer. Curr Opin Genetics Develop 2018;48:128-33.
16. Zhang J, Cheng J, Zeng Z et al. Comprehensive profiling of novel microRNA-9 targets and a tumor suppressor role of microRNA-9 via targeting IGF2BP1 in hepatocellular carcinoma. Oncotarget 2015;6:42040.
17. Cimmino A, Calin GA, Fabbri M et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proceedings of the National Academy of Sciences 2005;102:13944-9.
18. Welch C, Chen Y, Stallings RL. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. Oncogene 2007;26:5017.
19. Ivanovska I, Ball AS, Diaz RL et al. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Molec Cell Biol 2008;28:2167-74.
20. Li B, Shi XB, Nori D et al. Down-regulation of microRNA 106b is involved in p21-mediated cell cycle arrest in response to radiation in prostate cancer cells. Prostate 2011;71:567-74.
21. Park YR, Lee ST, Kim SL et al. MicroRNA-9 suppresses cell migration and invasion through downregulation of TM4SF1 in colorectal cancer. Int J Oncol 2016;48:2135-43.
22. Yanaihara N, Noguchi Y, Saito M et al. microRNA gene expression signature driven by miR-9 overexpression in ovarian clear cell carcinoma. PLoS One 2016;11:e0162584.