# Original Article MiR-4782-3p suppresses glioma migration and invasion by regulating p-cadherin

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Received December 31, 2016; Accepted January 27, 2017; Epub April 1, 2017; Published April 15, 2017

**Abstract:** MicroRNAs (miRNAs), a class of non-coding endogenous RNAs, were found to be involved in the regulation of tumor progression with post-transcriptional function. The aim of present study was to explore the role of miR-4782-3p in the development of glioma migration and invasion. Our findings indicated that the expression of miR-4782-3p was significantly repressed in glioma tissues compared to the adjacent normal tissues. In addition, our results demonstrated that miR-4782-3p could suppress the migration and invasion ability of U251 and U87 glioma cells. We also validated P-Cadherin (CDH3) as the potential target gene of miR-4782-3p using an enhanced green fluorescence protein (EGFP) reporter assay. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis were employed and confirmed that miR-4782-3p suppressed CDH3 mRNA and protein levels. The results suggested that miR-4782-3p could negatively regulate the expression of CDH3 by directly binding to the 3'-UTR of CDH3. We further found that CDH3-dependent cell mobility was associated with p120 catenin (p120ctn) transition from the membrane to the cytoplasm. In conclusion, our findings indicated that a miR-4782-3p *Sp/CDH3*/p120ctn pathway was strongly related to the regulation of glioma cell migration and invasion.

Keywords: MicroRNA, glioma, p-cadherin, migration, invasion

#### Introduction

A glioma, which is a highly invasive tumor, is characterized by a high rate of recurrence and high mortality and has become a major health burden. Glioma tumor cells that migrate from the primary site to metastasis inevitably cause a recurrence of the cancer, and tumor progression is seen clinically. Although aggressive surgery, chemotherapy, and radiation have been widely applied, the median survival time of a patient with a glioblastoma multiform is only 12-15 months [1]. Therefore, it is important to elucidate the molecular mechanism underlying the tumorigenesis of gliomas, and it may be helpful to explore new effective treatment strategies.

MicroRNAs are endogenously expressed, short, non-coding RNAs, which exert their function by regulating the expression of target genes by directly binding to the 3'untranslated regions (UTRs). MiRNAs function as tumor suppressors or oncogenes by targeting oncogenes or tumor suppressor genes, respectively [2]. Numerous studies have shown that miRNAs are associated with various human cancers [3-8], including gliomas [9, 10]. Our previous research also indicated that miR-10a regulated the migration and invasion ability of glioma cells [11]. Until now, rare research about the role of miR-4782-3p in cancers has been reported [12], while no research has been reported with respect to gliomas. Thus, we aim to investigate whether or not miR-4782-3p contributes to the progression of gliomas.

P-cadherin (CDH3) is a member of single-span transmembrane domain glycoproteins that contribute to cell-cell adhesion [13]. CDH3 is well established in mice placentas [14]; however, it is not found in human placentas. Although accumulating evidence has suggested that CDH3 is actually involved in regulating the migration and invasion of various cancers cells [15, 16], the exact molecular mechanism has not been well investigated.

p120 catenin (p120ctn) is a member of the catenin family and is the first identified substrate for src kinase [17]. Functionally, it has always associated with multiple oncogenes or tumor suppressors, including E-cadherin and RhoGTPase. Studies have demonstrated that the transition of p120ctn between membrane and cytoplasm could affect the migration and invasion ability of cancer cells [18, 19]. Furthermore, Taniuchi K et al [20] have reported that CDH3 could interact with p120ctn, promoting cell motility in pancreatic cancer. Thus, we hypothesized that CDH3/p120ctn interaction also plays a critical role in the regulation of glioma cell mobility.

In the present study, we found that the expression of miR-4782-3p was significantly repressed in glioma tissues, and miR-4782-3p could suppress the migration and invasion of glioma cells by directly targeting the 3'-UTR of CDH3. Moreover, we demonstrated that CDH3 affects glioma cell motility through the accumulation of p120ctn in the cytoplasm.

## Materials and methods

## Cell culture and transfection

U251 and U87 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA), which contains 10% of fetal bovine serum (FBS) and penicillin-streptomycin at 37°C with 5% of  $CO_2$ . Lipofectamine 2000 Reagent was used for the transfection of cells according to the manufacturer's protocol (Invitrogen, USA).

# Clinical glioma samples and RNA preparation

Human glioma tissues were collected from the Department of Neurosurgery of Tianjin Huan Hu Hospital of China. The study procedure was approved by the Tissue Committee and Research Ethics Board of Huan Hu Hospital, and written informed consent was received from all of the patients. After resection, samples were frozen immediately in liquid nitrogen, stored at -80°C, and histologically classified and graded by clinical pathologists according to World Health Organization (WHO) guidelines. There were 8 tumors from WHO grades I and II and 12 tumors from WHO grades III and IV. Total RNA and miRNAs were isolated with mir-Vana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol.

# Plasmid construction and oligonucleotides

The following vectors were constructed in this study: pcDNA3-pri-4782-3p, pcDNA3/EGFP-CDH3-3'UTR, pcDNA3/EGFP-CDH3-3'UTR-mut, pSilencer/shR-CDH3, pA3M1-CDH3. miR-4782-3p inhibitor, ASO-miR-4782-3p and the corresponding control ASO-NC were purchased from GenePharma (Shanghai, China).

# miRNA target prediction

In order to investigate the role of miR-4782-3p, we predicted the targets of miR-4782-3p with Targetscan (http://www.targetscan.org), miRanda (http://www.microrna.org), and PicTar (http://www.pictar.bio.nyu.edu).

# EGFP reporter assay

The EGFP coding region was cloned into pcDNA3 to construct a pcDNA3-EGFP vector. Then, the wild-type or mutant-type CDH3 3'-UTR was cloned into the pcDNA3-EGFP vector to construct pcDNA3/EGFP-CDH3-3'UTR or pc-DNA3/EGFP-CDH3-3'UTR mutant type. EGFP reporter assays were performed according to the instructions of the previous study [11]. Fluorescence intensities were detected with as F-4500 fluorescence spectrophotometer (Hi-tachi, Tokyo, Japan). RFP expression vector was used as the internal control.

## Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot assay

mRNAs were reverse transcribed to generate cDNA using oligo-dT primers or stem-loop reverse transcriptase (RT) primers, respectively. Then the cDNA was used as template for the amplification reaction. RT-qPCR was performed using SYBR Premix Ex Taq (TakaRa, Dalian, China), and the reaction conditions was shown as follows:  $94^{\circ}$ C for 4 min followed by 40 cycles for  $94^{\circ}$ C for 1 min,  $56^{\circ}$ C for 1 min and  $72^{\circ}$ C for 1 min. Relative expression levels of genes were calculated by the  $2^{\Delta\Delta Ct}$  method. All of the primers were synthesized by AuGCT Inc (Beijing, China). miR-4782-3p was detected using the following primers. RT primer: GTCGTATCCAGT-



GCAGGGTCCGAGGTGCACTGGATACGACGTTCT-AG. And forward primer for miR-4782-3p was 5'-TGCGGTGATTGTCTTCATATC-3'. The reverse primer was 5'-CCAGTGCAGGGTCCGAGGT-3'. Forward primers for CDH3 were 5'-GCTGGGG-AAAGTATTCAT-3' and the reverse primer was 5'-CACCCAATCTCTCTTGTGT-3'.

After transfection, cells were lysed with RIPA lysis buffer to generate the cell lysates. Cell lysates were then fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes and blocked with 5% skimmed milk. The membrane were incubated with rabbit anti-human monoclonal primary antibodies (anti-CDH3, anti-E-cadherin, anti-Vimentin, anti-N-cadherin, anti-Fibronectin) overnight at 4°C. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the endogenous control. Then HRP-labeled corresponding IgG was added to incubate with membranes at room temperature for 2 hours. Finally, the protein expression levels were assessed by enhanced chemilumescence and exposure to film (Fujifilm, Tokyo, Japan). Antibodies used in this study were purchased from Amyjet Scientific Inc (Abcam, Cambridge, MA, USA).

## Migration and invasion assays

Using a DMEM medium without serum, 5×10<sup>4</sup> U251 or U87 cells were cultured in the upper Transwell chamber (Corning, Cambridge, MA, USA), which was coated with 40 µl of matrigel (Clontech, Mountain View, CA, USA) for invasion assay. The lower Transwell chamber was added with the DMEM medium containing 20% of FBS. The incubation time in the migration or invasion assays was 24 hours or 48 hours, respectively. After incubation, cells on the upper chamber were wiped with cotton swabs, whereas the migrative and invasive cells on the opposite side of the membrane were stained with crystal violet. Cell numbers were calculated at high magnification (100×) in five random visual fields to calculate the mean value, and data were presented as means ± standard deviation (SD).

## Immunofluorescence staining

Transfected glioma cells (4,000/well) were placed into 14-well plates and fixed with paraformaldehyde at 4°C for 30 minutes. Then, the cells were blocked with 10% of normal donkey serum for 30 minutes. The primary antibody (rabbit anti-p120ctn, 1:100 dilution) was added to the well and incubated at 4°C overnight. Next, the cells were rinsed with phosphate buffered saline (PBS) and incubated with a fluorescein isothiocyanate-conjugated secondary antibody. Nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent staining was visualized using a fluorescence microscope (model Eclipse 660, Nikon, Japan).

# Statistical analysis

Results were acquired from at least three independent experiments. Data were analyzed with a two-tailed Student's t-test and presented as means  $\pm$  SD. *P*<0.05 was considered to be statistically significant.

# Results

MiR-4782-3p was downregulated in glioma tissues and associated with glioma cell migration and invasion

To determine whether or not miR-4782-3p attributed to the tumorigenesis of gliomas, we first detected the expression of miR-4782-3p in glioma tissues and paired adjacent noncancerous tissues. Compared to the noncancerous tissues, the miR-4782-3p expression was significantly decreased in glioma tissues, and the expression was associated with the grades of the tumor. The miR-4782-3p expression was much lower in tumors of grade III and grade IV (Figure 1A). This finding indicates that miR-4782-3p levels are markedly decreased in glioma tissues and that the levels may be involved in glioma progression. Furthermore, to better understand its biological function, an expression plasmid pcDNA3-pri-4782-3p (pri-4782-3p) and an antisense oligonucleotide of miR-4782-3p (ASO-miR-4782-3p) were constructed and their transfection efficiency was validated (data not shown). Then, these constructs were transfected into U251 and U87 glioma cells. The following cell migration and invasion analysis suggested that the overexpression of miR-4782-3p significantly suppressed U251 cell migration or invasion by almost 74% or 78%, whereas these capacities were increased by approximately 1.8- or 2.5-fold when endogenous miR-4782-3p was silenced by the antisense oligonucleotide (Figure 1B and 1C). The results in U87 cells were consistent with U251 cells (Figure 1D and 1E). All of the data suggest



**Figure 2.** MiR-4782-3p decreased the CDH3 expression by directly targeting its 3'-UTR. A. The predicted site of miR-4782-3p binding to the 3'-UTR of CDH3 and the mutations in the binding sites. B. The EGFP reporter assay of U251 cells transfected with wild-type CDH3 3'-UTR or mutant-type CDH3 3'-UTR with pri-miR-4782-3p or ASO-miR-4782-3p. C. qRT-PCR analysis of CDH3 expression in U251 cells when miR-4782-3p was over-expressed or inhibited. D. Western blot analysis of CDH3 protein expression in U251 cells when the expression of miR-4782-3p was changed. E. qRT-PCR analysis of CDH3 mRNA levels in glioma tissues normalized to  $\beta$ -actin. F. Correlation between the CDH3 and miR-4782-3p expression level in glioma tissues. \*\*, *P*<0.01.

that miR-4782-3p may be a suppressor in gliomas by inhibiting cell migration and invasion capabilities.

#### CDH3 is a direct target of miR-4782-3p

The results above demonstrated that miR-4782-3p could affect glioma cell mobility. Hence, we tried to explore the mechanism through which miR-4782-3p exerts its function. Combined with bioinformatics analysis and gene functions, which were related to cell mobility, the *CDH3* gene was chosen for further study.

The miR-4782-3p binding site was located at nucleotides 588-594 of CDH3 3'-UTR. To further determine whether or not miR-4782-3p directly targets CDH3, an EGFP reporter assay was executed. Vectors carrying wild-type or mutant-type CDH3 3'-UTR were constructed (Figure 2A) and then co-transfected with the



**Figure 3.** miR-4782-3p down-regulates CDH3 expression by targeting its 3'-UTR. A. The EGFP reporter assay was performed in U87 cells cotransfected with pcDNA3/EGFP-CDH3 3'-UTR wild type or pcDNA3/EGFP-CDH3 3'-UTR mutant with pri-4782-3p or ASO-miR-4782-3p. B. qRT-PCR analysis was used to detect the CDH3 mRNA level in U87 cells transfected with pcDNA3-pri-4782-3p, ASO-miR-4782-3p or the corresponding controls. C. Western blot analysis was used to detect the protein level of CDH3 in transiently transfected U87 cells. \*\*, P<0.01.

pri-4782-3p or ASO-miR-4782-3p constructs into U251 cells. As shown in **Figure 2B**, the EGFP intensities with the wild-type 3'-UTR decreased by almost 75% in the pri-miR-4782-3p group or increased approximately 1.8-fold in the ASO-miR-4782-3p group. However, the EGFP intensity of the CDH3 3'-UTRmutant-type group was not affected whether miR-4782-3p was overexpressed or inhibited. These findings indicate that miR-4782-3p could directly target CDH3 by binding to its 3'-UTR.

Since miR-4782-3p can target CD-H3, we wanted to know whether or not miR-4782-3p affects the expression of CDH3 in cells. In U251 cells, gRT-PCR and Western blot analysis were performed and showed that miR-4782-3p significantly suppressed the expression of CDH3 by 79% and 81%, respectively. In contrast, the CDH3 expression level was increased by almost 2.6- and 3.2fold when miR-4782-3p was inhibited (Figure 2C and 2D). Similar results were obtained in U87 cells (Figure 3). Furthermore, the expression of CDH3 was examined in clinical specimens. Compared to noncancerous tissues, the CDH3 expression in glioma tissues was obviously increased (Figure 2E). More interestingly, the CDH3 expression was inversely correlated with the miR-4782-3p expression in tissues (Figure 2F). Taken together, these data suggest that miR-4782-3p can negatively regulate the expression of CDH3 by targeting its 3'-UTR.

#### MiR-4782-3p reduces glioma cell migration and invasion by suppressing CDH3 expression

To determine the exact role of CDH3 in gliomas, we used short-hairpin RNA-targeting CDH3 mRNA to specifically suppress the expression of CDH3. Protein levels were assessed by Western blot analysis (**Figure 4A**). As a result, a knockdown of endogenous CDH3 protein expression led to a significant inhibition of migration in U251 cells and U87 cells by 81%

and 82%, respectively (**Figure 4B**) and an inhibition of invasion of these same cells by almost 79% or 80%, respectively (**Figure 4C**). This observation is consistent with the effect of miR-4782-3p over-expression, which further supports the concept that decreased CDH3



3p-induced decrease in the CD-H3 level (**Figure 5A**). The following data showed that restoration of CDH3 offset the inhibition effect of migration and invasion induced by miR-4782-3p in U251 cells (**Figure 5B**). Similar results were also seen in U87 cells (**Figure 5C**). In brief, this evidences demonstrates that miR-4782-3p exerts the inhibitory effect on glioma cell motility by regulating CDH3 directly.

CDH3 contributes to cell mobility by interacting with p120ctn, but not epithelial-mesenchymal transition (EMT)

To further explore the pathway by which CDH3 regulates the cell motility of glioma cells, we focused on studying the EMT process which is related to cell motility in various cancers [21]. To explore whether or not EMT participates in regulating the metastatic phenotype of glioma cells, we detected the expression of EMT-related genes when CDH3 mRNA was inhibited (Figure 6A and 6B). Finally, the protein expression of mesenchymal markers (vimentin, N-cadherin, and fibronectin) did not significantly changed, even though the protein expression of the epithelial marker E-cadherin was increased. This finding suggests that the EMT process may not be involved in regulating the CDH3-dependent cell motility in gliomas.

expression may be the main mechanism by which miR-4782-3p regulates glioma development.

To determine whether or not miR-4782-3p exerts its biological function on gliomas via direct regulation of CDH3, we performed a rescue experiment. First, we co-transfected miR-4782-3p with CDH3 expression plasmid without the 3'-UTR and confirmed that over-expression of CDH3 essentially neutralized the miR-4782Previous studies have demonstrated that the accumulated expression of p120ctn in cytoplasm could promote cell motility [22, 23]. Studies have also reported that CDH3 could interact with p120ctn and promote cell motility in pancreatic cancer [20]. Therefore, we assumed that this regulation pathway was also involved in gliomas. Immunofluorescence staining analysis was used to detect p120ctn trafficking after CDH3 was blocked and showed that p120ctn obviously relocalized at the plas-



**Figure 5.** Over-expression of CDH3 neutralizes the effect on cell motility induced by miR-4782-3p. A. Western blot analysis of the CDH3 protein expression in glioma cells. B. Restoration of CDH3 promotes cell motility influenced by miR-4782-3p in U251 cells. C. Restoration of CDH3 counteracts the effect on cell motility caused by miR-4782-3p in U87 cells. \*\*, *P*<0.01.

ma membrane (**Figure 6C**). In contrast, the p120ct of the control group localized mostly in the cytoplasm. This result strongly indicates that CDH3 expression can induce cytoplasmic accumulation of p120ctn in glioma cells to further regulate cell motility.

## Discussion

Cancer development is a highly orchestrated process that requires a complex transcriptional and post-transcriptional regulation of gene expression [24]. MiRNAs target multiple genes and play important roles in the progression of various cancers [25]. Recent reports show that miRNAs have an important impact on the development of gliomas [26-28]. However, the effect of miR-4782-3p on the malignant phenotype of cancers, including gliomas, has not been fully explored.

A glioma is a highly invasive and aggressive primary brain tumor, and invasion into surrounding tissue is the most characteristic biological phenotype of a glioblastoma, which is also the major reason underlying its poor prognosis. Thus, developing novel approaches that focus on controlling cell motility of this malignancy may be an effective therapeutic strategy. Our findings here showed that the expression of miR-4782-3p was significantly repressed in gliomas. and miR-4782-3p could suppress the motility of U251 and U87 cells. To determine the exact mechanism of miR-4782-3p, miRanda, Targetscan and PicTar bioinformatics were used to predict the potential target genes. Combined with the analysis results and gene functions, we finally identified CDH3 as the direct target. First, we found that miR-4782-3p significantly reduces the expression of CDH3 mRNA and protein in U251 and U87 cells. Second, the EGFP re-



porter assay validated that miR-4782-3p directly targets the 3'-UTR of CDH3. Third, the CDH3 expression was found to be increased in glioma tissues compared to noncancerous tissues, and the CDH3 expression was inversely correlated with the miR-4782-3p expression in tis-



**Figure 7.** Proposed model depicting mechanism for function of miR-4782-3p in glioma. "+" represents activation, "-" represents inhibition.

sues. Fourth, a knockdown of endogenous CDH3 led to a significant inhibition of cell motility; however, restoration of CDH3 neutralizes the inhibition effect induced by miR-4782-3p in glioma cell motility. These findings further support the assertion that miR-4782-3p regulates the cell metastatic phenotype by directly targeting CDH3 mRNA, thus decreasing the expression of CDH3 protein in gliomas.

CDH3 has received much interest in the last few years. CDH3 behaves differently in different types of cancer; therefore, its exact role in the generation and development in cancer remains mostly unknown [29]. For instance, CDH3 has been reported to suppress invasion in colorectal cancer and melanoma [30, 31]. However, CDH3 appears to behave as an oncogene, which could increase cell motility in other cancers [32, 33]. In this study, we first identified that CDH3, as an oncogene, promotes glioma cell motility, which was the opposite effect of miR-4782-3p. Meanwhile, the increased expression of CDH3 counteracted the inhibition of motility induced by miR-4782-3p in glioma cells.

Cadherins have been demonstrated to play critical roles in signal transduction, adhesion, and migration [34]. To further reveal the way in which CDH3 affects cell invasion and migration, we focused on studying EMT, which is reported to be involved in regulating cell motility. However, we found only a slight increase in E-Cadherin when CDH3 was knocked down in U251 and U87 cells. The expression of mesenchymal markers did not alter significantly. Thus, CDH3-dependent cell motility in gliomas may not involve the EMT process.

Studies have found two forms of p120ctn. One form of which is bound to cadherins under the plasma membrane, while the other form is present in the cytoplasm. The level of p120ctn in the cytoplasm can be regulated by cadherins. Accumulating evidence revealed that shifting p120ctn from the membrane to the cytoplasm or the alteration of total p120ctn in cancer cells can alter the cell migratory activity to affect disease progression, including bladder, colon, prostate, esophagus, lung, and breast cancers [35-38]. Moreover, evidence suggested that CDH3 could promote cell motility in pancreatic cancer by interacting with p120ctn [36]. Based on these findings, we speculated that the CDH3/p120ctn interaction also regulates cell motility in gliomas. Subsequently, we detected p120ctn trafficking after the inhibition of CDH3 and observed an obvious p120ctn relocalization at the plasma membrane when CDH3 was silenced.

In conclusion, we demonstrated that miR-4782-3p can regulate the migration and invasion of glioma cells by directly targeting CDH3 mRNA. CDH3 regulates the cell motility of gliomas by interacting with p120ctn. Our study suggests that the miR-4782-3p/CDH3/p120ctn signaling pathway modulates cell motility in gliomas (**Figure 7**). All of these findings may help to elucidate the mechanism of glioma pathogenesis and may contribute to the diagnosis and therapy of gliomas in the future.

## Acknowledgements

This work was supported by the National Nature Science Foundation of China (No. 81301967) and the Science and Technology Foundation of Tianjin Health Bureau (No. 2014KY18).

## Disclosure of conflict of interest

## None.

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