IDH1^{R132H} decreases the proliferation of U87 glioma cells through upregulation of microRNA-128a

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Abstract. Mutations in isocitrate dehydrogenase 1 (*IDH1*) are found in >70% of secondary glioblastomas and lower-grade gliomas (grades II-III). Among the numerous phenotypic differences between *IDH1* mutant and wild-type glioma patients, the most salient is an improved survival rate for patients with a mutation. MicroRNAs (miRNAs) are a class of small, non-coding, single-stranded RNAs that can negatively regulate gene expression at the post-transcriptional level, predominantly by binding to the 3'-untranslated region of their target mRNAs. The dysregulated expression of several miRNAs has been reported to modulate glioma progression; however, it is unclear whether mutations in IDH1 regulate glioma cell proliferation through miRNA dysregulation. In the present study, stable overexpression of IDH1^{WT} or IDH1^{R132H} was established in the U87 glioma cell line. It was found that IDH1^{R132H} decreased cell proliferation of U87 glioma cells by inducing the expression of the miRNA miR-128a. This process was dependent on the transcription factor hypoxia inducible factor-1α (HIF-1α), which binds to a hypoxia response element in the promoter of miR-128a. Furthermore, miR-128a negatively regulated the expression of B-cell-specific Moloney murine leukemia virus integration site 1 protein (Bmi-1), which is involved in suppressing cell proliferation. These findings suggest that the IDH1^{R132H}-HIF-1α-miR-128a-Bmi-1 pathway is involved in glioma cell proliferation.

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Introduction

Glioblastoma multiforme (GBM; WHO grade IV glioma) is the most malignant brain tumor in adults. Even following treatment with surgical resection, radiotherapy and concomitant chemotherapy, the median survival time of patients with GBM is only 14.6 months (1). A number of molecular markers for GBM have been identified and are associated with diagnosis, prognosis and treatment. For example, somatic mutations in isocitrate dehydrogenase 1 (*IDH1*) have been identified in GBM patients, particularly in secondary GBM, which evolves from lower-grade gliomas (2). In 90% of IDH1 mutations in gliomas, the arginine at position 132 is replaced by a histidine (R132H mutation) (3). Patients with IDH1^{R132H} gliomas also have significantly improved survival rates (4). However, the mechanism responsible for this improved survival rate remains to be elucidated.

MicroRNAs (miRNAs) are a class of non-coding, single-stranded RNAs comprised of ~22 nucleotides (5). miRNAs are post-transcriptional regulators that bind complementary sequences on target mRNA transcripts. Target binding usually results in gene regulation by translational repression or mRNA degradation, thereby modulating a variety of biological processes (6). Several miRNAs are known to associate with clinical outcomes in GBM (5,7,8), including miR-128a, a crucial regulator in brain development (9-11). Aberrant expression of miR-128a has been detected in glioma (12) and is involved in cancer-associated biological processes, including cell proliferation, differentiation and apoptosis (13,14).

In order to evaluate the effect of the IDH1^{R132H} mutation and its association with miR-128a, a clonal U87 cell line overexpressing the IDH1^{R132H} mutant protein was generated. The present study also investigated the functional molecules upstream and downstream of miR-128a in IDH1^{R132H} overexpressing cells.

Materials and methods

Cell culture and reagents. U87 glioma cells and HEK 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA). SYBR Green PCR master mix and the TaqMan microRNA reverse transcription kit were purchased from Applied Biosystems (Foster City, CA, USA). YC-1 was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide.

Generation of constructs. The full-length human IDH1 coding sequence was amplified from HEK 293T cells (Sangon Biotech Co., Ltd., Shanghai, China). The cDNA was fused in-frame with a FLAG tag at the N-terminus using the following synthesized primers: Forward primer with *Mlu*I site: TTTCGTACGATGGATTACAAGGACGACGAT GACAA GTCCAAAAAAAT and reverse primer with *Bsi*WI site: TTTACGCGTGGTATGAACTTAAAGTTTGG. The amplified target was inserted into the *Mlu*I- and *Bsi*WI-linearized pHR-SIN vector. The IDH1^{R132H} mutation was generated in the vector described above by QuickChange (Stratagene, Santa Clara, CA, USA) with the following primer sequences: R132H, forward 5'-ACCTATCATCATAGGTCATCATGCTTA TGGG-3' and reverse 5'-TGACCTATGATGATAGGTT TTACCCATCCAC-3'.

Stable overexpression of IDH1^{WT} and IDH1^{R132H} constructs in U87 cells. HEK 293T cells were seeded in 60 mm plates ($4x10^6$ cells/plate) in DMEM with 10% heat-inactivated FBS 1 day prior to transfection. Cells were transfected with 5.2 μg of either pHR-SIN-IDH1WT or pHR-SIN-IDH1R132H, along with 2.36 μg of pSPAX2 and 0.8 μg of pMD2 G plasmids using Lipofectamine 2000 in DMEM. After 6 h, the transfection media was removed and replaced with antibiotic-free DMEM with 10% heat-inactivated FBS. Lentiviral particles were harvested at 72 h post-transfection. U87 glioma cells were plated 1 day prior to transduction, at 20% confluency, in 60 mm plates with DMEM containing 10% phosphate-buffered saline (PBS). To transduce cells, 3 ml of conditioned media containing viral particles and 6 μg/ml polybrene (Sigma-Aldrich) were added to each culture. After 16 h, the conditioned media was removed and replaced with DMEM containing 15% FBS.

Protein extraction and western blotting. Cells prepared for protein extraction were immediately placed on ice and washed with ice-cold PBS solution. Total protein extraction was achieved using RIPA lysis buffer containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) and PMSF. Proteins were resolved on 8-12% SDS-polyacrylamide gels (Sangon Biotech Co. Ltd., Shanghai, China) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked in Tris-buffered saline and Tween 20 (TBST) with 5% non-fat dry milk for 1 h and incubated overnight at 4˚C with the following respective primary antibodies in TBST with 5% bovine serum albumin: Rabbit monoclonal anti-human IDH1 (1:1,000; cat. no. 8137; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse monoclonal anti-human IDH1R132H (1:200; cat. no. SAB4200548; Sigma-Aldrich), mouse monoclonal anti-FLAG M2 (1:500; cat. no. F3165; Sigma-Aldrich) or mouse monoclonal anti-human HIF-1α (1:1,000; cat. no. AH339; Beyotime Institute of Biotechnology, Shanghai, China). Immunospecific bands were detected using an ECL substrate (Sigma-Aldrich). β-actin immunoblotting was performed as a loading control.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from cells using TRIzol reagent (Invitrogen Life Technologies). Total RNA (1 μg) was used as a template for a Moloney murine leukemia virus-RT reverse transcriptase reaction, which was performed according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). qPCR was performed using SYBR Green Master mix (Applied Biosystems). β-actin amplification was used as an internal control. To quantitate the expression level of miR-128a, 1 μg of total RNA was reverse transcribed in 50 μl reaction using TaqMan Reverse Transcription Reagents (Applied Biosystems) with the stem loop primer. Reverse transcribed cDNA (2 μl) was subjected to SYBR Green mix and the results were normalized to RNU48.

The following primers were used: RNU48, forward 5'-TGATGATGACCCCAGGTAACTC-3' and reverse 5'-GAG CGCTGCGGTGATG-3'; Bmi-1, forward 5'-CTGCAGCTC GCTTCAAGATG-3' and reverse 5'-CACACACATCAGGT GGGGAT-3'; β-actin, forward 5'-ATGACTTAGTTGCGTTAC ACC-3' and reverse 5'-TGCTGTCACCTTCACCGTTC-3'; miR-128a stem loop primer: 5'-GTCGTATCCAGTGCAGGG TC CGAGGTATTCGCACTGGATACGACAAAGAG-3'; miR-128a, forward 5'-TCGCGTTCACAGTGAA-3' and reverse 5'-GTGCAGGGTCCGAGG-3'.

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed using a ChIP kit (cat. no. 17-371; EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Cells were crosslinked with 1% formaldehyde for 20 min at 37˚C and quenched in 0.125 M glycine. DNA was immunoprecipitated from sonicated cell lysates and quantified using SYBR Green Real-time PCR analysis. The following primer sequences were used: Forward: 5'-GTAATAGAATTTTCATATTG-3' and reverse: 5'-ATTTTGCCATGTTGAAGAAC-3'. Fold enrichment was calculated based on Ct as $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{IP} - Ct_{Input}$ and $\Delta(\Delta Ct) = \Delta Ct_{antibody} - \Delta Ct_{IgG}$.

Cell proliferation. Briefly, 5,000 cells per well were plated in 96-well plates and cultured for 96 h. Following incubation, CCK-8 (10 μl) was added to each well and allowed to incubate at 37˚C for 2 h. Cell proliferation was then quantified by measuring the optical density at a wavelength of 450 nm (SpectraMax M2 Multimode Microplate Reader; Molecular Devices, LLC, Sunnyvale, CA, USA). Each experiment was performed in triplicate.

Statistical analysis. The data are presented as the mean ± standard deviation. The samples were analyzed using a two-tailed unpaired Student's t-test, unless otherwise noted. $P<0.05$ was considered to indicate a statistically significant difference.

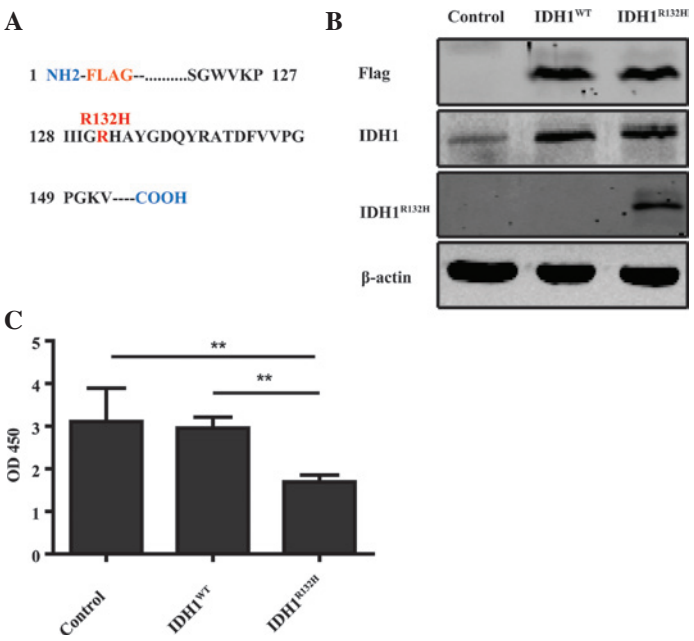

Figure 1. Overexpression of IDH1^{R132H} decreases U87 cell proliferation. (A) Schematic diagram of the FLAG-IDH1 fusion protein amino acid sequence. The FLAG tag was linked to the N-terminus of IDH1. Red indicates the FLAG tag and mutation at amino acid residue 132 of IDH1. (B) Representative western blot analysis for FLAG-tagged IDH1^{WT} and IDH1^{R132H} in stably transfected U87 cells. (C) CCK-8 proliferation assays were performed after a 4 day incubation of U87 cells containing IDH1^{WT}, IDH1^{R132H} or control expression vectors. **P<0.01. IDH1, isocitrate dehydrogenase 1; OD, optical density.

Results

Overexpression of IDH1^{R132H} decreases U87 cell proliferation. To determine the effects of glioma-associated IDH1^{R132H}, N-terminal FLAG-tagged IDH1^{WT}, IDH1^{R132H} and control expression constructs were generated for stable expression in U87 cells (Fig. 1A). Western blot analysis with FLAG, IDH1 and IDH1^{R132H} specific antibodies confirmed the expression of the appropriate IDH1 protein products in U87 cell lines (Fig. 1B). Previous clinical studies demonstrated that patients with IDH1^{R132H}-positive tumors have a significant survival advantage (4,15). However, the role of IDH1^{R132H} in glioma cell proliferation is not fully understood. To assess the role of IDH1^{R132H} in U87 cell proliferation, CCK-8 proliferation assays were performed. The proliferation of U87 cells overexpressing IDH1^{R132H} was significantly decreased compared with the control and IDH1^{WT}-expressing cells over a 4-day incubation period (Fig. 1C; P<0.01).

Overexpression of IDH1^{R132H} in U87 cells induces the expression of miR-128a. Several miRNAs associated with *IDH1* mutations have been revealed via miRNA expression profiling and the miRNA expression signature has been identified as a prognostic biomarker for GBM patients (7,11). However, whether IDH1^{R132H} induces a specific miRNA that controls cell proliferation remains to be elucidated. According to the *IDH1* mutation-specific 23-miRNA signature analyzed by Wang *et al* (11), miR-128a was significantly overexpressed in *IDH1* mutant patient samples compared with wild-type samples. miRNA expression data was analyzed using The Cancer Genome Atlas (TCGA) portal (https://tcga-data.nci.nih.gov/tcga/), which contains data from seven IDH1^{R132H} and 133 wild-type *IDH1* patients. In this dataset, miR-128a was overexpressed in IDH1^{R132H} compared with wild-type *IDH1* patients (Fig. 2A; P=0.0078). To confirm this expression pattern, qPCR was performed to detect miR-128a levels in IDH1-expressing U87 cells. In agreement with the clinical samples, U87 cells overexpressing IDH1^{R132H} had significantly higher levels of miR-128a compared with control and IDH1^{WT} cells (Fig. 2B; P<0.01).

HIF-1α is required for miR-128a expression induced by IDH1^{R132H}. The ectopic expression of mutant IDH1 in cultured cells also increases the levels of HIF-1α (16). It was hypothesized that increased HIF-1α expression may therefore control IDH1^{R132H}-induced miR-128a expression. In agreement with this hypothesis, HIF-1α protein levels were found to be significantly higher in U87 cells overexpressing IDH1^{R132H} compared with the control and IDH1^{WT} cells (Fig. 3A). In order to assess whether HIF-1α is required for miR-128a overexpression in IDH1^{R132H} cells, cells were treated with YC-1, an inhibitor of HIF-1α activity (17). YC-1 significantly decreased the miR-128a level in IDH1^{R132H} cells, however, similar effects were not observed in the control and IDH1^{WT} cells (Fig. 3B; P<0.01). Notably, there is a conserved HIF-1α binding sequence GCGTG (hypoxia response element; HRE) ~2.5 kilobases upstream of the gene encoding pri-miR-128a (Fig. 3C). Therefore, HIF-1α could potentially regulate miR-128a expression by binding to this HRE. To investigate whether HIF-1α binds this element in the promoter of the miR-128a gene, ChIP assays were performed. In IDH1^{R132H} overexpressing U87 cells, HIF-1α was in fact enriched at this HRE suggesting that it regulates miR-128a expression (Fig. 3D; P<0.01). Taken together, the data demonstrated that HIF-1α binds the HRE in the pri-miR-128a promoter and is required for IDH1^{R132}-induced miR-128a expression.

miR-128a regulates the decreased proliferation rate induced by IDH1^{R132H}. Previous studies have reported that miR-128a

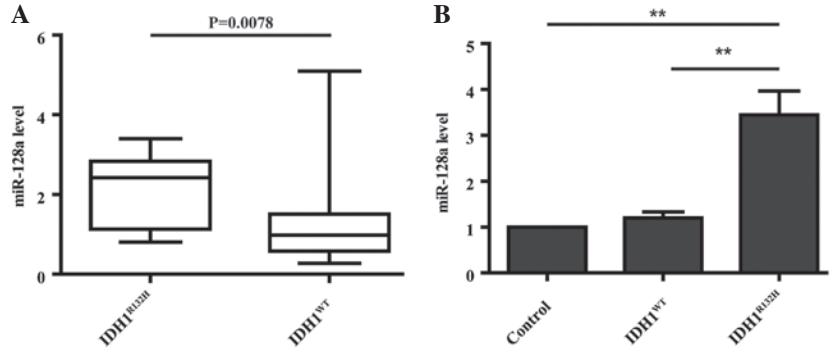

Figure 2. Overexpression of IDH1^{R132H} in U87 cells induces the expression of miR-128a. (A) Analysis of miR-128a expression from The Cancer Genome Atlas in IDH1^{R132H} patients compared with wild-type *IDH1* patients. (B) Quantitative polymerase chain reaction analysis of miR-128a expression in IDH1^{WT}, IDH1^{R132H} and control overexpressing U87 cells. **P<0.01. IDH1, isocitrate dehydrogenase 1; miR-128a, microRNA-128a.

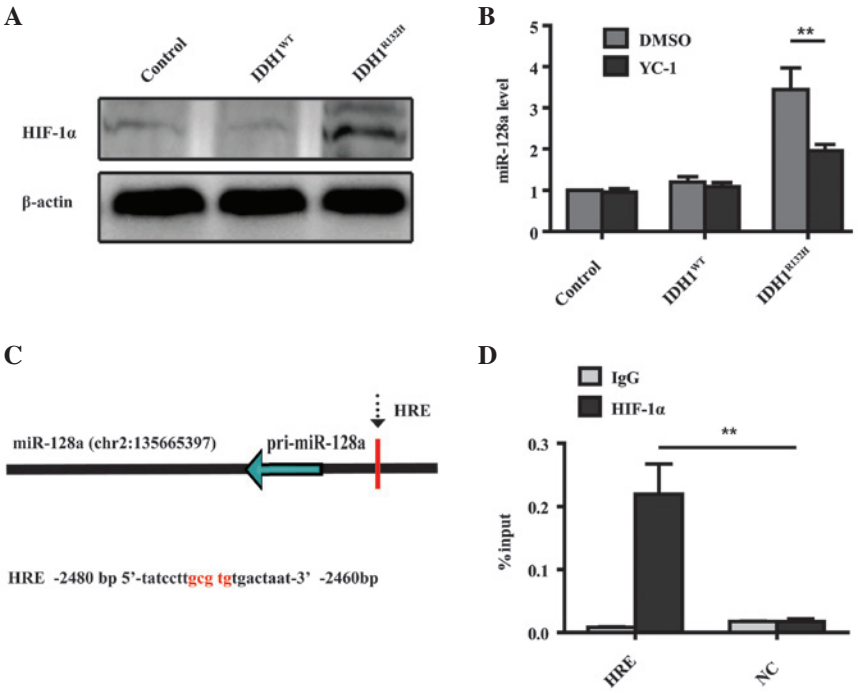

Figure 3. HIF-1α is required for miR-128a expression induced by IDH1^{R132H}. (A) Western blot analysis of HIF-1α expression in IDH1^{WT}, IDH1^{R132H} and control overexpressing U87 cells. (B) U87 cells were treated with 5 μM YC-1, a HIF-1α inhibitor, and the level of miR-128a was determined by quantitative polymerase chain reaction. (C) Diagram of the putative HRE site in the promoter of miR-128a. (D) Chromatin immunoprecipitation was performed in IDH1^{R132H}-overexpressing U87 cells with an HIF-1α-specific antibody. **P<0.01. IDH1, isocitrate dehydrogenase 1; HIF-1α, hypoxia inducible factor-1α; HRE, hypoxia response element; miR-128a, microRNA-128a; DMSO, dimethyl sulfoxide; IgG, immunoglobulin G; NC, negative control.

is downregulated in human glioblastoma (18) and its expression inhibits cell growth *in vitro* and *in vivo* (19). To examine the biological function of miR-128a expression, U87 cells were transfected with miR-128a mimics or the miR-128a inhibitor and CCK-8 proliferation assays were performed. Overexpression of miR-128a decreased the cell proliferation rate, whereas, knockdown of miR-128a with the miR-128a inhibitor enhanced cell proliferation (Fig. 4A; P<0.01). IDH1^{R132H} overexpressing cells were also treated with the miR-128a inhibitor, which rescued the decreased proliferation rate induced by IDH1^{R132H} (Fig. 4B; P<0.01). miR-128a causes a marked decrease in the expression of the oncogene Bmi-1 in U87 cells by direct regulation of the Bmi-1 mRNA 3'-untranslated region (19). Therefore, it was hypothesized that Bmi-1 may be involved in the decreased proliferation of IDH1^{R132H} cells. qPCR was performed to detect the Bmi-1 level in IDH1^{R132H} overexpressing cells. The results demonstrated that it was decreased compared with the control and IDH1^{WT} cells (Fig. 4C; P<0.01). Furthermore, overexpression of miR-128a decreased the mRNA expression of Bmi-1, whereas inhibition of miR-128a enhanced Bmi-1 expression (Fig. 4D; P<0.01). Taken together, it can be concluded that IDH1^{R132H} restricts cell proliferation by inducing the expression of miR-128a, which downregulates Bmi-1 expression.

Discussion

Clinical data demonstrate that patients with the IDH1^{R132H} mutation have an improved outcome compared with those with the wild-type sequence. However, the mechanistic role

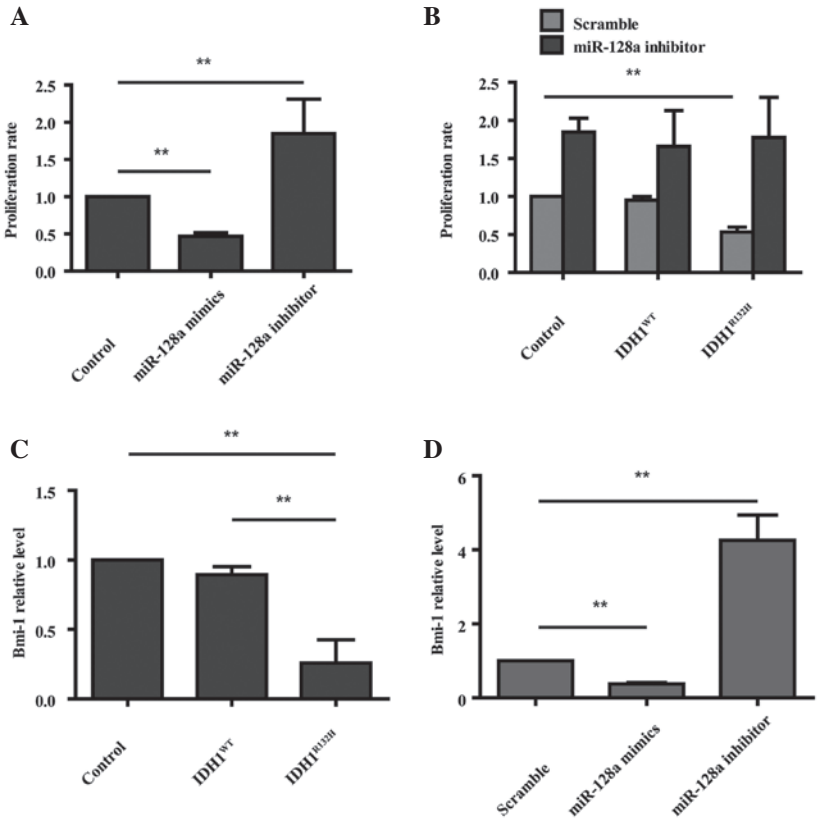

Figure 4. miR-128a regulates the decreased proliferation rate induced by IDH1^{R132H}. (A) CCK-8 proliferation assay of U87 cells treated with 50 nM miR-128a mimics or the miR-128a inhibitor. (B) CCK-8 proliferation assay of IDH1^{WT}, IDH1^{R132H} and control overexpressing U87 cells treated with the miR-128a inhibitor or scramble control. (C) qPCR analysis of Bmi-1 expression in IDH1^{WT}, IDH1^{R132H} and control overexpressing U87 cells. (D) qPCR analysis of Bmi-1 expression in U87 cells treated with miR-128a mimics, the miR-128a inhibitor or scramble control. **P<0.01. IDH1, isocitrate dehydrogenase 1; miR-128a, microRNA-128a; qPCR, quantitative polymerase chain reaction; Bmi-1, B-cell-specific Moloney murine leukemia virus integration site 1 protein.

by which IDH1^{R321H} regulates tumor development is not fully understood. In the present study, the functional impact of the tumor-associated IDH1^{R132H} mutation was examined in U87 glioma cells *in vitro*. Following establishing stable expression of IDH1^{R132H} in U87 cells, it was found that it reduced cell proliferation, which is consistent with the findings of Bralten *et al* (20). This lends support to the theory that *IDH1* mutations protect against tumor proliferation and may explain why IDH1^{R132H} is an independent favorable prognostic marker in glioma patients (15,21).

Wang *et al* reported that the *IDH1* mutation-specific microRNA signature predicted a favorable prognosis in glioblastoma patients with the *IDH1* wild type (11). However, it remained to be determined whether the IDH1^{R132H} mutation regulated cell growth through microRNAs. miR-128a expression is markedly reduced in human glioblastoma specimens compared with adjacent brain samples without tumors (18,19,22). A previous study suggested that loss of miR-128 expression is an early event in gliomagenesis (23). By analyzing the TCGA data, miR-128a was found to be overexpressed in human IDH1^{R132H} GBM patients compared with wild-type *IDH1* patients. In addition, miR-128a expression was increased in IDH1^{R132H} overexpressing U87 glioma cells. The result demonstrating that miR-128a is upregulated in IDH1^{R132H} gliomas is noteworthy given that miR-128a is downregulated in IDH1^{WT} glioblastomas (11). This demonstrates that increased miR-128a expression is an important characteristic of mutant IDH1^{R132H} gliomas.

miR-128a is downregulated in GBM, suggesting that it may function as a tumor suppressor in brain tumor progression (24) and may be associated with the survival of GBM patients (11). In U87 cells, overexpression of miR-128a suppressed cell proliferation. By contrast, knockdown of miR-128a increased cell proliferation. Our findings are consistent with a study on miR-128a in medulloblastoma cells (25). Furthermore, the present study illustrated that inhibition of miR-128a in IDH1^{R132H} overexpressing U87 cells reversed the cell proliferation induced by IDH1^{R132H}, suggesting that miR-128a is responsible for IDH1^{R132H}-induced cell proliferation.

HIF-1α, an important transcription factor of the cellular response to hypoxia, is known to facilitate tumor growth when oxygen is low (26). The stability of HIF-1α is regulated by α-ketoglutaric acid, which is an important enzyme product of IDH1 (27). The results demonstrated that overexpression of the IDH1^{R132H} mutant increased HIF-1α protein levels in U87 glioma cells, which is consistent with a former study by Zhao *et al* (16). In the present study, miR-128a expression was significantly decreased when the activity of HIF-1α was inhibited, which indicated that HIF-1α may regulate miR-128a in IDH1^{R132H} cells. In addition, an HRE located in the promoter upstream of the gene encoding pri-miR-128a was identified and HIF-1α was found to specifically bind the site in IDH1^{R132H} overexpressing U87 cells. This suggests that the transcription factor HIF-1α regulates miR-128a expression. Currently, to the best of our knowledge, the present study is the first to demonstrate

that HIF-1α acts as a regulator of IDH1^{R132}-dependent miR-128a expression by directly binding an HRE within its promoter.

Bmi-1, a direct target of miR-128a, is upregulated in several types of cancer, including gliomas and is a positive regulator of neural stem cell renewal (19,28-31). The present study demonstrated that Bmi-1 was downregulated by miR-128a in IDH1^{R132H} overexpressing cells. Bmi-1 is an oncogene involved in regulating cell proliferation as a transcriptional repressor (19), and studies in transgenic mice revealed a critical role for Bmi-1 in driving glioma growth (32). The present data indicated that inhibition of Bmi-1 may be a mechanism of miR-128a-mediated growth restriction in IDH1^{R132H} overexpressing cells.

In conclusion, the present study described the function and mechanism of action of miR-128a in IDH1^{R132H} glioma cells. The results of the present study demonstrated that the IDH1^{R132H} mutation leads to an increase in the levels of HIF-1α protein, which binds to an HRE in the miR-128a promoter to induce its expression. The enhanced expression of miR-128a subsequently inhibits Bmi-1 expression, thereby decreasing cell proliferation in IDH1^{R132H} glioma cells. The present study provides novel insight into the pathophysiology of miR-128a in *IDH1* mutant glioma. Finally, our characterization of the IDH1^{R132H}-HIF-1α-miR-128a-Bmi-1 pathway may have therapeutic implications for the treatment of glioma.

Acknowledgements

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