Overexpression of isocitrate dehydrogenase-1^{R132H} enhances the proliferation of A172 glioma cells via aerobic glycolysis

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Abstract. Gliomas are the most common type of primary malignancy of the central nervous system. The identification of mutations in the gene encoding isocitrate dehydrogenase-1 (*IDH1*) represents a key area of investigation in studies on glioma. The IDH1^{R132H} mutation is a heterozygous point mutation, which affects the amino acid arginine at position 132, however, the metabolic importance of this mutation in tumor cell growth remains to be elucidated. In the present study, A172 glioma cell lines stably overexpressing either wild-type IDH1 or IDH1^{R132H} were produced. The results demonstrated that the IDH1^{R132H} mutation enhanced the proliferation of the A172 glioma cells *in vitro*. Furthermore, IDH1^{R132H} performed this function by elevating the expression levels of hypoxia inducible factor-1α, leading to an increase in the expression levels of the key glycolytic enzymes, glucose transporter 1 and hexokinase 2. Therefore, the metabolism was shifted towards aerobic glycolysis, leading to an increase in glucose uptake and lactate production. These findings demonstrated that the IDH1^{R132H} molecular target was involved in orchestrating the Warburg effect in mutant IDH1^{R132H} glioma cells.

Introduction

Gliomas, the most common type of primary brain tumor, are classified into four grades (I-IV) according to the pathological and clinical criteria established by the World Health Organization (1). These include specific histological subtypes, the majority of which are astrocytomas, oligodendrogliomas and ependymomas.

The majority of normal cells produce energy via the oxidation of pyruvate in the mitochondria. Cancer cells metabolize more glucose than their normal counterparts, which is achieved predominantly via aerobic glycolysis in the cytosol, producing high levels of lactate (2). This phenomenon is known as the Warburg effect. The persistent activation of aerobic glycolysis in cancer cells can promote the progression of cancer (3) and mutations in metabolic enzymes can predispose cells to neoplasia, either by activating oncogenes or by eliminating tumor-suppressor genes (4).

The human genome has five isocitrate dehydrogenase (*IDH*) genes encoding three distinct IDH enzymes, the activities of which are dependent on either nicotinamide adenine dinucleotide phosphate or nicotinamide adenine dinucleotide (5). IDH enzymes catalyze the oxidative decarboxylation of isocitrate to produce α-ketoglutarate (α-KG), thus, IDH is involved in the metabolism and energy production required for cell survival (6). Mutations in the *IDH1* gene are detected in >70% of secondary glioblastomas and lower-grade gliomas (grades II-III) (7). The predominant *IDH1* mutation in glioma involves an amino acid substitution at arginine 132 (IDH1^{R132}), which resides in the enzyme's active site (8-10). This mutation causes IDH1 to lose its normal catalytic activity and gain the ability to catalyze the reduction of α-KG to produce 2-hydroxyglutarate (2-HG), leading to the accumulation of 2-HG and altering cell metabolism (4,11). As 2-HG is a competitive inhibitor of multiple α-KG-dependent dioxygenases, this results in genome-wide changes in histone and DNA methylation (12), which are associated with tumorigenesis (6).

Although IDH1^{R132H} is the most common *IDH1* mutation, the role of IDH1^{R132H} in glioma remains to be fully elucidated. Clinical studies have revealed that patients with gliomas containing *IDH1* mutations have increased survival rates (7,13), which may be correlated to increased rates of response to chemotherapy or radiotherapy (14,15).

The present study investigated the functional impact of the IDH1^{R132H} mutation by establishing a clonal A172 cell line

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overexpressing IDH1^{R132H}, and evaluating its association with aerobic glycolysis.

Materials and methods

Cell culture and inhibitors. The A172 glioma cells (American Type Culture Collection, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA), 4.5 g/L glucose and 100 U/ml penicillin/streptomycin (both from Sangon Biotech Co., Ltd., Shanghai, China). The cells were dissociated using enzyme-free cell dissociation solution (EMD Millipore, Bedford, MA, USA) and cultured at 37˚C in a humidified atmosphere of 5% CO_2. The YC-1 (Sigma-Aldrich, St. Louis, MO, USA) and 2-deoxyglucose (2-DG; Sigma-Aldrich) inhibitors were dissolved in dimethyl sulfoxide (DMSO; Sangon Biotech Co., Ltd.) and sterilized water, respectively, prior to use.

Construct generation. The full-length human wild-type (WT) IDH1 coding sequence was amplified from the HEK293T cells (Sangon Biotech Co., Ltd.). The following synthesized primers (Invitrogen Life Technologies) were used to fuse the cDNA in-frame with a FLAG tag at the N-terminus: Forward 5'-TTT CGT ACG ATG GAT TAC AAG GAC GAC GAT GAC AAG TCC AAA AAA AT-3' containing the *Mlu*I site and reverse 5'-TTT ACG CGT GGT ATG AAC TTA AAG TTT GG-3' containing the *Bsi*WI site. This was inserted into the *Mlu*I- and *Bsi*WI-linearized pHR-SIN vector (Open Biosystems, Huntsville, AL, USA). The IDH1^{R132H} mutation was generated in the pHR-SIN-IDH1^{WT} using a QuikChange Site-Directed Mutagenesis kit according to the manufacturer's instructions (Stratagene, Santa Clara, CA, USA) with the following primer sequences: 5'-R132H, 5'-ACC TAT CAT CAT AGG TCA TCA TGC TTA TGG G-3' and 3'-R132H, 5'-TGA CCT ATG ATG ATA GGT TTT ACC CAT CCA C-3'.

Stable overexpression of the IDH1^{WT} and IDH1^{R132H} constructs in the A172 cells. The HEK293T cells ($4x10^6$) were seeded into 60 mm plates in DMEM cell culture medium with 100 U/ml penicillin/streptomycin 1 day prior to transfection. The cells were transfected with either 5.2 μg pHR-SIN-IDH1^{WT} or pHR-SIN-IDH1^{R132H}, 2.36 μg pSPAX2 and 0.8 μg pMD2G plasmids using Lipofectamine 2000 (Invitrogen Life Technologies) in DMEM. After 6 h, the transfection media was replaced with DMEM cell culture medium without penicillin/streptomycin and the lentiviral particles were harvested 72 h post-transfection. The A172 glioma cells (total number: $2x10^5$) were plated in 60 mm plates in DMEM with 10% phosphate-buffered saline (PBS) at a confluence of 20% 1 day prior to transduction. The cells were transduced by adding 3 ml media containing viral particles and 6 μg/ml polybrene (Sigma-Aldrich). After 16 h, the conditioned media was replaced with DMEM containing 15% FBS.

Immunofluorescence. The cells ($5x10^5$) were seeded onto glass coverslips for 24 h, fixed with 4% paraformaldehyde and permeabilized using 0.4% Triton X-100 in PBS (all from Sangon Biotech Co., Ltd.) for 10 min. Non-specific binding was blocked using PBS containing 5% FBS and the cells were incubated with primary antibodies against FLAG (mouse monoclonal anti-FLAG M2; 1:500; F3165; Sigma-Aldrich) or IDH1^{R132H} (mouse monoclonal anti-human; 1:500; DIA-H09; Dianova GmbH, Hamburg, Germany) at 4˚C overnight. As a negative control, cells were treated with immunoglobulin G under the same conditions. The cells were washed three times in PBS prior to incubation with the respective Alexa Fluor 594-conjuated secondary antibody (goat anti-mouse; 1:1,000; 8890; Cell Signaling Technology, Inc., Danvers, MA, USA). Following staining, the cells were imaged using a DM2500 Leica microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Protein extraction and western blotting. The transfected A172 cells were immediately placed on ice and washed with ice-cold PBS. The total protein was prepared using radioimmunoprecipitation lysis buffer containing a protease inhibitor cocktail (1:1,000; 04693124001; Roche Diagnostics GmbH, Mannheim, Germany) and phenylmethanesulfonylfluoride (Sigma-Aldrich). The proteins were resolved on 8-12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) by electroblotting. The membranes were blocked using Tris-buffered saline with 0.1% Tween-20 (TBST; Sangon Biotech Co., Ltd.) containing 5% non-fat dry milk for 1 h and incubated at 4˚C overnight with their respective primary antibodies against IDH1 (rabbit monoclonal anti-human; 1:1,000; 8137; Cell Signaling Technology, Inc.), IDH1R^{132H} (mouse monoclonal anti-human; 1:1,000; DIA-H09; Dianova GmbH) or FLAG (mouse monoclonal anti-FLAG M2; 1:1,000; F3165; Sigma-Aldrich). Immunolabeling was detected using enhanced chemiluminescence reagents (Sigma-Aldrich) and visualized using an Amersham Imager 600 (GE Healthcare, Uppsala, Sweden). β-actin was used as a loading control.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. The total RNA was isolated from the cells using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. Subsequently, 1 μg total RNA was used as a template for RT in a Moloney Murine Leukemia Virus Reverse Transcriptase reaction (Fermentas, Burlington, Ontario, Canada). RT-qPCR was performed using SYBR Green Master mix (Applied Biosystems Life Technologies, Carlsbad, CA, USA) and β-actin was used as an internal control. The following primers were used: Glucose transporter 1 (Glut1), forward 5'-CTTTGTGGCCTTCTTTGAAGT-3' and reverse 5'-CCACACAGTTGCTCCACAT-3'; hexokinase 2 (HK2), forward 5'-GATTGTCCGTAACATTCTCATCGA-3' and reverse 5'-TGTCTTGAGCCGCTCTGAGAT-3' and β-actin, forward 5'-GGCGGCACCACCATGTACCCT-3' and reverse 5'-AGGGGCCGGACTCGTCATACT-3'. The PCR thermal cycling conditions were as follows: Cycling began with 2 min at 50˚C and 10 min at 95˚C. Thermal cycling proceeded with 40cycles of 95˚C for 0.5 min and 60˚C for 2 min. All reactions were performed in the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The comparative Ct method was used to calculate the expression of mRNA relative to that of β-actin.

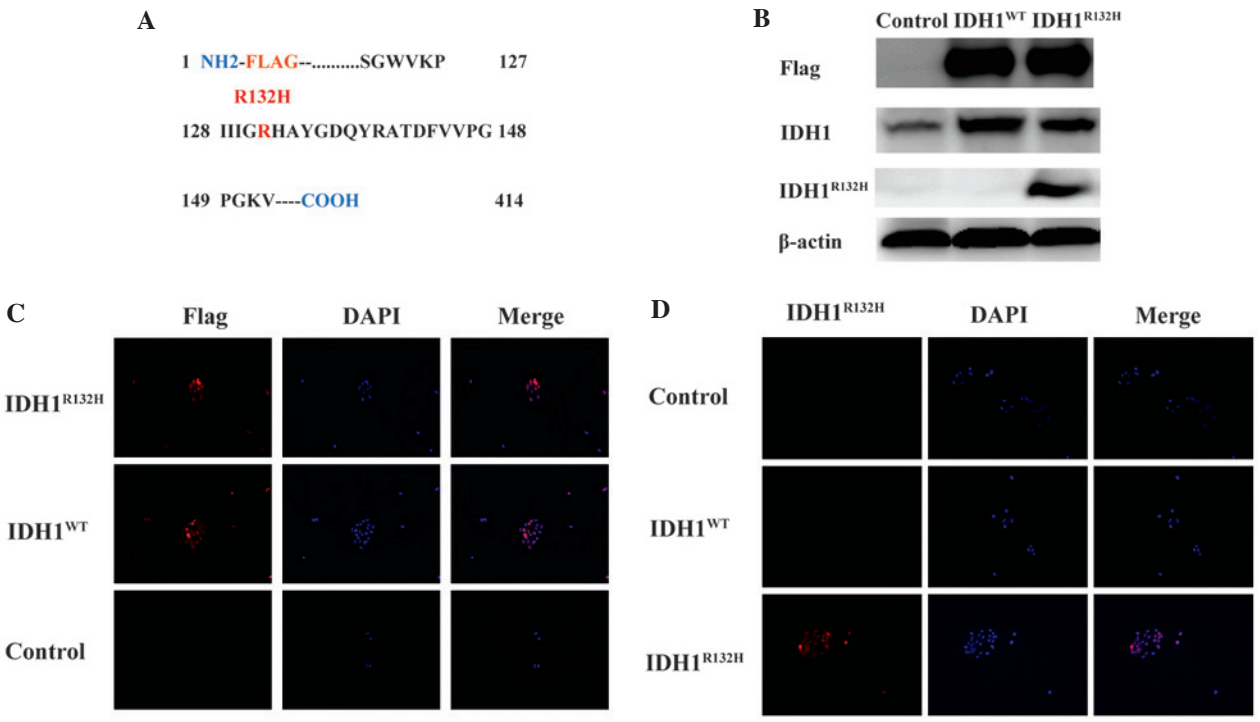

Figure 1. Establishment of A172 glioma cell lines overexpressing IDH1^{WT} or IDH1^{R132H}. (A) Amino acid sequence of the FLAG-IDH1 protein used to fuse the FLAG tag to the N-terminus of IDH1. The FLAG and IDH1^{R132} residue is highlighted in red. (B) Representative western blot analysis of the FLAG-tagged IDH1^{WT} and IDH1^{R132H} proteins in the transfected A172 cells. (C and D) Immunofluorescence images of the diffuse distribution of IDH1^{WT} and IDH1^{R132H} proteins stained with anti-FLAG or anti-IDH1^{R132H} antibodies, respectively (magnification, x20). IDH1, isocitrate dehydrogenase-1; WT, wild-type, R132H, mutation in arginine 132; DAPI, 4',6-diamidino-2-phenylindole.

3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cell proliferation assay. The cells ($5x10^3$) were seeded into 96-well plates and cultured for 24, 48, 72 or 96 h, respectively. Following the incubation period, MTT was added to each well at a final concentration of 5 mg/ml and the cells were incubated at 37˚C for a further 4 h. The reaction was terminated by adding 150 μL DMSO and the absorbance was measured using a BioTek ELISA reader (BioTek Instruments Inc., Winooski, VT, USA) at a wavelength of 490 nm. Each experiment was performed in triplicate.

Colony formation assay. Cells were trypsinized (trypsin from Sigma-Aldrich) to generate a single-cell suspension and seeded into three parallel 60-mm dishes with 500 cells in each dish. Fourteen days following seeding, the colonies were stained with 0.5% crystal violet (Sangon Biotech Co., Ltd.). The number of colonies containing ≥50 cells was determined, and the results were reported as a percentage of the number of colonies in untreated cultures of each corresponding clone.

Measurement of glucose uptake. The cells were subjected to serum starvation for 12 h prior to being cultured in DMEM containing 25 mM glucose. The cells were washed three times with PBS and incubated for 3 h in DMEM containing 1 mCi/ml 2-Deoxy-D-(1,2-3H) glucose (PerkinElmer, Inc., Boston, MA, USA). Following incubation, the cells were washed three times using ice-cold PBS and solubilized in 1% SDS. The radioactivity of each aliquot was determined in a scintillation counter (LS6500 Multipurpose Scintillation Counter; Beckman Coulter, Fullerton, CA, USA). Each assay was performed in triplicate.

Measurement of extracellular lactate. The cells ($5x10^5$) were seeded into 60 mm dishes and incubated in DMEM with 10% FBS at 37˚C overnight. The media was replaced with DMEM without FBS and the cells were incubated for 1 or 2 h. The supernatant was collected and the lactate levels were quantified by colorimetric assay using a Lactate Assay kit (BioVision, Inc., Milpitas, CA, USA), according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was determined using Student's t-test. The data are expressed as the mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Stable expression of IDH1^{WT} and IDH1^{R132H} constructs in the A172 glioma cells. To determine the effects of overexpression of human glioma-associated IDH1 mutant proteins within the context of an isogenic glioma cell, N-terminus FLAG-tagged IDH1^{WT}, IDH1^{R132H} and control constructs were produced and stably expressed in the A172 glioma cells (Fig. 1A). Western blot analysis using anti-FLAG, anti-IDH1 and mutant-specific anti-IDH1^{R132H} antibodies confirmed the expression of their respective IDH1 proteins (Fig. 1B). Subsequent

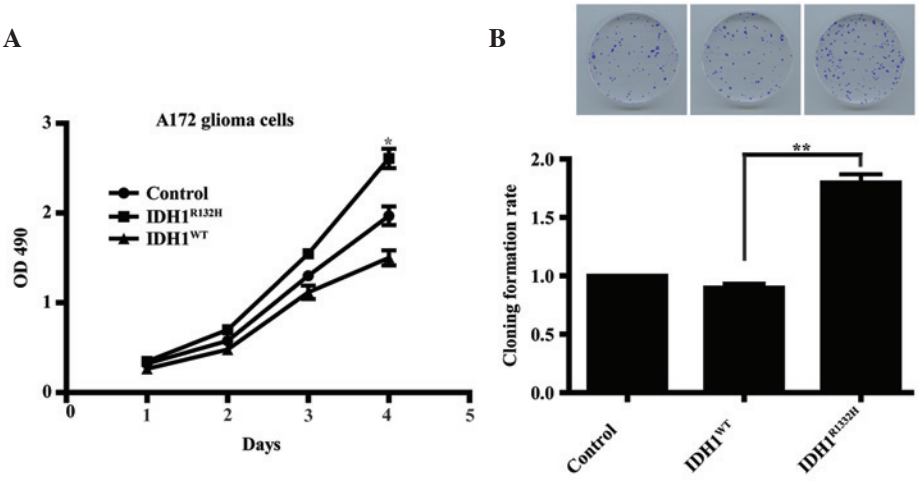

Figure 2. Overexpression of IDH1^{R132H} increases A172 cell growth. (A) 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide growth curves revealed an increase in cell growth following the ectopic expression of IDH1^{R132H} compared with the IDH1^{WT} and control cells (*P<0.05). (B) A corresponding colony formation assay demonstrated that the colony sizes were larger (top) and the quantification (bottom) revealed that the cell growth rate was significantly increased in the IDH1^{R132H}-expressing cells compared with the IDH1^{WT} (**P<0.01) and the control cells. The data are expressed as the mean ± standard deviation based on three independent experiments . IDH1, isocitrate dehydrogenase-1; WT, wild-type; R132H, mutation in arginine 132; OD, optical density.

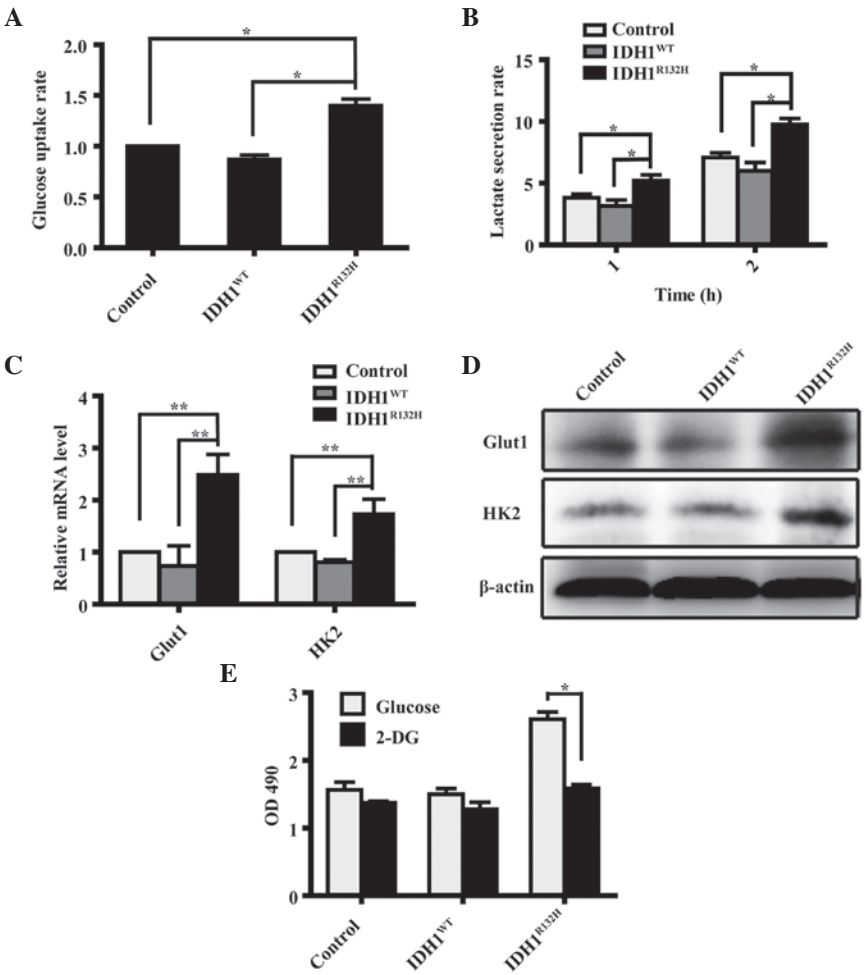

Figure 3. Overexpression of IDH1^{R132H} increases aerobic glycolysis in the A172 glioma cells. Quantification revealed that the rates of (A) glucose uptake and (B) extracellular lactate secretion were significantly increased in the IDH1^{R132H}-expressing cells compared with the IDH1^{WT} and control cells. (C) Reverse transcription quantitative polymerase chain reaction demonstrated that the mRNA levels of Glut1 and HK2 were increased in the IDH1^{R132H}-expressing cells compared with the IDH1^{WT} and control cells (**P<0.01). (D) Western blot analysis confirmed that the corresponding protein levels of Glut1 and HK2 were also increased in the IDH1^{R132H} cells. (E) 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay revealed that the IDH^{R132H}-induced increase in cell proliferation was inhibited by 2-DG, an inhibitor of glycolysis (*P<0.05). The data are expressed as the mean ± standard deviation based on three independent experiments. IDH1, isocitrate dehydrogenase-1; WT, wild-type; R132H, mutation in arginine 132; Glut1, glucose transporter 1; HK2, hexokinase 2; 2-DG, 2-deoxyglucose; OD, optical density.

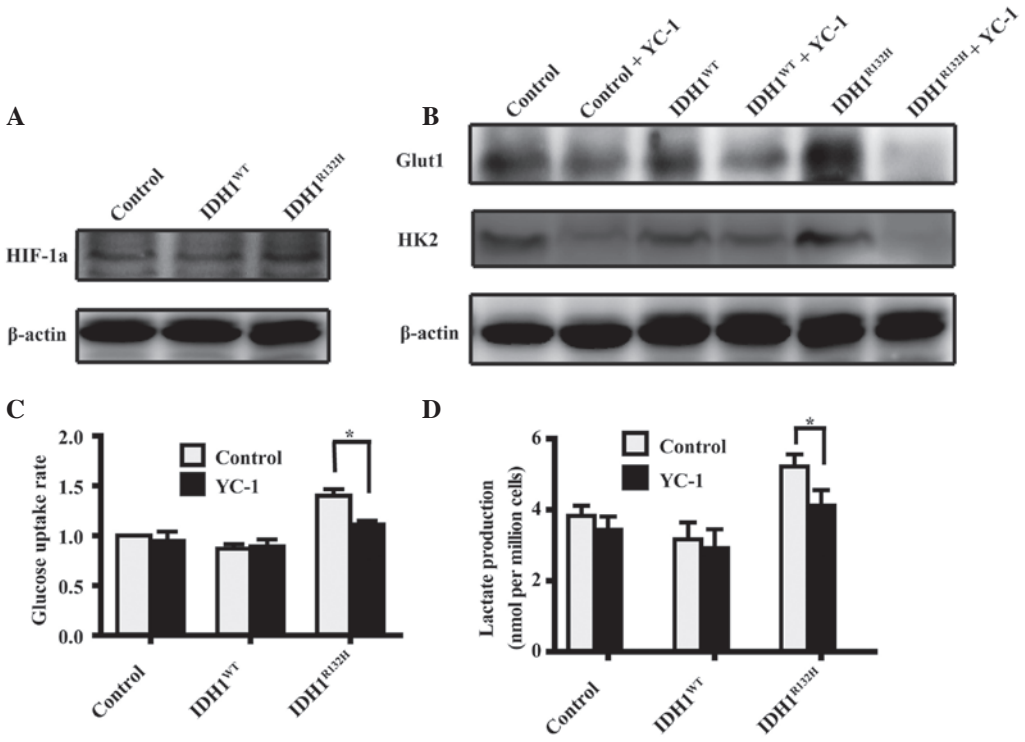

Figure 4. HIF-1α is required for the enhanced glycolysis induced by IDH1^{R132H}. (A) Western blotting revealed that the expression of HIF-1α was upregulated in the IDH1^{R132H}-expressing cells compared with the IDH1^{WT} and control cells. (B) Western blotting revealed that the levels of Glut1 and HK2 were elevated in the IDH1^{R132H}-expressing cells and that this can be inhibited by blocking HIF-1α activity using YC-1. Inhibition of HIF-1α activity using YC-1 inhibited the increase in (C) glucose uptake and (D) lactate secretion in the IDH1^{R132H}-expressing cells. The data are expressed as the mean ± standard deviation based on three independent experiments (*P<0.05). IDH1, isocitrate dehydrogenase-1; HIF, hypoxia inducible factor; Glut1, glucose transporter 1, HK2, hexokinase 2; WT, wild-type; R132H, mutation in arginine 132.

immunofluorescence analysis revealed that the steady-state cellular localization of the IDH1^{R132H} mutant protein was indistinguishable from that of the IDH1^{WT} protein, indicating that the mutation caused no significant alteration to the targeting of IDH1 (Fig. 1C and D). The high specificity of the IDH1^{R132H} antibody was also indicated.

Overexpression of IDH1^{R132H} enhances A172 cell proliferation. The role of IDH1^{R132H} in glioma remains to be fully elucidated. To assess the importance of IDH1^{R132H} in A172 glioma cell growth, an MTT assay was used to compare the proliferation rates between the two types of IDH1-transfected cells and the control cells. The results revealed that following incubation for 4 days, the growth curve for the IDH1^{R132H} cells were significantly higher compared with those for the IDH1^{WT} and the control cells (Fig. 2A; P<0.05). This observation was supported by colony formation assays, which revealed that clonogenicity was significantly increased in the mutant IDH1^{R132H} cells compared with the IDH1^{WT} and the control cells (Fig. 2B; P<0.01).

IDH1^{R132H} cells exhibit increased levels of glycolysis. Increased glucose uptake and lactate secretion are characteristics of proliferating cells undergoing glycolysis (2,16). As IDH1 is important in metabolism, the present study hypothesized that impaired IDH1 activity may alter cell metabolism. Therefore, the levels of glucose uptake and extracellular lactate were measured to investigate whether the IDH1^{R132H} mutation was responsible for a metabolic shift in the IDH1^{R132H} A172 cells. The results demonstrated that the levels of glucose uptake and extracellular lactate in the IDH1^{R132H} cells were significantly higher compared with the IDH1^{WT} and control cells (Fig. 3A and B, respectively; P<0.05).

Glut1 and *HK2* are important genes involved in cell glycolysis (17-19), therefore their expression levels were assessed in IDH1-transfected cells using RT-qPCR and western blot analysis. The results revealed that the mRNA and protein expression levels of Glut1 and HK2 were increased significantly (Fig. 3C and D; P<0.01) in the IDH1^{R132H} cells compared with the IDH1^{WT} or control cells.

To elucidate whether the metabolic shift triggered by IDH1^{R132H} was involved in cell proliferation, the A172 cells overexpressing IDH1 were treated with 2-DG, an inhibitor of glycolysis, at a final concentration of 12 mM (20) and the effects on cell growth were analyzed using an MTT assay. The results revealed that 2-DG abrogated the IDH1^{R132H}-induced cell proliferation (Fig. 3E; P<0.05).

These findings suggested that IDH1^{R132H}-induced glycolysis promoted cell proliferation in the A172 glioma cells.

Hypoxia inducible factor (HIF)-1α is required for the enhanced glycolysis induced by IDH1^{R132H}. The overexpression of mutant IDH1 has been demonstrated to reduce the levels of α-KG and increase the levels of HIF-1α (11). Therefore, the present study hypothesized that the IDH1^{R132H} mutation may be involved in promoting or stabilizing HIF-1α, thereby enhancing aerobic glycolysis. Western blot analysis confirmed that the protein expression of HIF-1α was

increased in the IDH1^{R132H} cells compared with the IDH1^{WT} and the control cells (Fig. 4A).

To investigate whether HIF-1α is required in IDH1^{R132H}-induced glycolysis, the activity of HIF-1α was inhibited in the IDH1-tranfected cells by treating the A172 cells with YC-1 at a final concentration of 5 μM (21). Western blot analysis revealed that the levels of Glut1 and HK2 were reduced in the IDH1^{R132H} cells following exposure to YC-1, whereas no effects were observed in the IDH1^{WT} or control cells (Fig. 4B). Consistent with these findings, YC-1 treatment significantly decreased the rate of glucose uptake and extracellular lactate secretion in the mutant IDH1^{R132H} cells compared with the IDH1^{WT} or control cells (Fig. 4C and D, respectively; $P<0.05$). These results demonstrated that HIF-1α was important in modulating aerobic glycolysis in the IDH1^{R132H} A172 glioma cells.

Discussion

The association between the *IDH1* mutation and the development of glioma remains to be elucidated. The present study demonstrated that the overexpression of IDH1^{R132H} increased cell proliferation in the A172 glioma cells via glycolysis. Previous studies on the effect of the IDH1^{R132H} mutation in cell proliferation have been contradictory (15,22). Zhu *et al* (22) reported that U87 cells stably expressing IDH1^{R132H} exhibit a higher proliferation rate and degree of cell growth compared with wild-type U87 cells. By contrast, Bralten *et al* (23) demonstrated that U87MG-IDH1^{R132H} cells exhibit decreased cell proliferation and that mice injected with U87 IDH1^{R132H}-expressing cells have significantly higher survival rates compared with those injected with IDH1^{WT}-expressing cells. Based on results from the present study, it was suggested that these conflicting effects may be due to cell heterogeneity. In addition, the improved survival rate of patients with the IDH1^{R132H} mutant tumors may be attributed to the enhanced sensitivity of IDH1^{R132H} glioma cells to radiation (15). This suggests that an IDH1^{R132H} mutation induced alternative mechanism may be involved in tumor growth and its response to therapy.

Compared with normal cells, glioma cells have a high rate of aerobic glycolysis (24), which is fundamental in cell proliferation (25,26). To demonstrate that the IDH1^{R132H} mutation leads to enhanced aerobic glycolysis, thereby promoting cell proliferation in glioma cells, the levels of glycolytic enzymes were measured. The results demonstrated that the expression levels of Glut1 and HK2 increased in the IDH1^{R132H} cells and, furthermore, the increase in cell proliferation was abrogated by the inhibition of aerobic glycolysis, suggesting that IDH1^{R132H}-induced glycolysis was responsible for cell proliferation.

HIF-1α is important in the glycolytic metabolism of cancer cells (27,28). Furthermore, *Glut1* and *HK2* are target genes of HIF-1α (11,29,30). The present study revealed that the protein expression of HIF-1α was elevated in the IDH1^{R132H}-expressing A172 cells and inhibiting HIF-1α activity not only reduced the levels of Glut1 and HK2, but also significantly decreased the rate of glucose uptake and secretion of lactate in the mutant IDH1^{R132H} cells. This indicated that HIF-1α may act as an upstream signaling molecule in the initiation of IDH1^{R132H}-induced glycolysis.

In conclusion, the results of the present study suggested that the IDH1^{R132H} mutation leads to increased protein expression of HIF-1α, prompting a metabolic shift to aerobic glycolysis via increase in the expression of glycolytic enzymes, Glut1 and HK2, thereby enhancing glioma cell proliferation *in vitro*. These results may provide insight into the mechanisms underlying the development of glioma. Furthermore, by identifying IDH1^{R132H} as a potential chemotherapeutic target, these findings may have broader implications in glioma therapy, with potentially favorable outcomes in the treatment of glioma from combined therapy involving the anti-HIF pathway.

Acknowledgments

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