$IDH1^{R132H}$ Mutation Inhibits the Proliferation and Glycolysis of Glioma Cells by Regulating the HIF-1α/LDHA Pathway

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Research

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Abstract

Background: This study aims to explore the role and underlying mechanism of the $IDH1^{R132H}$ in the growth, migration, and glycolysis of glioma cells.

Methods: The alternation of IDH1, HIF-1α, and LDHA genes in 283 LGG sample (TCGA LGG database) was analyzed on cBioportal. The expression of these three genes in glioma tissues with $IDH1^{R132H}$ mutation or IDH1 wild type (IDH1-WT) and normal brain tissues was also assessed using immunohistochemistry assay. In addition, U521 glioma cells were transfected with IDH1-WT or $IDH1^{R132H}$ to explore the role of IDH1 in the proliferation and migration of glioma cells *in vitro*. Cell growth curve, Transwell mitigation assay, and assessment of glucose consumption and lactate production were conducted to evaluate the proliferation, migration, and glycolysis of glioma cells.

Results: The expression of HIF-1α and LDHA in $IDH1^{R132H}$ mutant was significantly lower than that in glioma cells with wild type IDH1 ($P<0.05$). $IDH1^{R132H}$ inhibited the proliferation and glycolysis of U521 glioma cells.

Conclusion: The IDH1 mutation $IDH1^{R132H}$ plays an important role in the occurrence and development of glioma through inhibiting the expression of HIF-1α and glycolysis.

1. Introduction

Due to aggressive infiltration and metastasis, glioma is a major cause of cancer deaths with a high rate of recurrence, and poor prognosis and clinical outcomes(1). There are two major types of glioma, astrocytoma and oligodendroglioma, which originates from astrocytes and oligodendrocytes, respectively(2). With intensive healthcare efforts, the five-year survival rate of glioma in the United States was only 5%(3). Therefore, understanding the molecular mechanisms underlying the development of glioma becomes a critical challenge to effective treatment of glioma worldwide(4).

A number of molecular markers, which are associated with the diagnosis, prognosis, and therapy of glioma have been identified and characterized. For example, mutations of the gene encoding isocitrate dehydrogenase 1 (IDH1) mutation have been detected in 70% of low-grade gliomas (WHO grading II-III) (4). In addition, approximately 90% of IDH1 mutations in glioma patients leads to the replacement of arginine by histidine at the 132nd amino acid ($IDH1^{R132H}$ mutation)(5). It has been reported that most gliomas patients with the $IDH1^{R132H}$ mutation exhibited significantly better response to treatment and longer survival compared with glioma patients with wild-type IDH1(6). However, the mechanism underlying the association between $IDH1^{R132H}$ mutation and improved survival rate has not been fully understood.

It is widely recognized that energy and nutrients are necessary for the proliferation of cells. Uncontrolled proliferation of solid tumor cells may cause severe hypoxia, especially in the center of tumors. In order to

survive under low oxygen conditions, tumor cells adapt metabolic patterns to the harsh microenvironment, which is known as metabolic reprogramming of tumor cells(7). Tumor metabolism has become a vital issue in tumor studies(8). Initially, Warburg *et al*. observed that rapidly proliferating cancer cells utilize glucose to produce lactic acid under aerobic conditions(9). While this metabolic pathway exhibits low efficiency in the production of adenosine triphosphate, it's important for cancer cells to synthesize macromolecules and generate sufficient energy for growth. This special metabolism of tumor cells is known as the Warburg effect(10, 11). Hypoxia-inducible factor 1α (HIF-1α) is an important regulator involved in glucose metabolism(12). In order to stimulate uncontrolled proliferation of cancer cells, HIF-1α reprograms the metabolic pathways of cancer cells in a different way compared with normal cells. HIF-1α altered the metabolism of amino acids and lipids through increasing the uptake of glucose and glutamine and the production of lactic acid(13). Therefore, HIF-1α is a potential target for cancer treatment, which may regulate cell proliferation, metabolism and carcinogenic stress. Biologically, HIF-1α is a transcription factor, which regulates chromatin structure to control several transcriptional processes.

The association between $IDH1^{R132H}$ mutation and HIF-1α in glioma has not been well understood (14–16). While HIF-1α-mediated anaerobic glycolysis plays an important role in the occurrence and development of a variety of tumors, only a few studies have shown that IDH1 mutations inhibit glycolysis. In order to evaluate the effect of $IDH1^{R132H}$ mutation on glioma growth and its association with glycolysis, we generated glioma U251 cell line with $IDH1^{R132H}$ overexpression. We also investigated the downstream molecules of the IDH1 pathway in U251 cell line overexpressing $IDH1^{R132H}$. Our study will provide new clues for the development of targeted therapy for glioma.

2. Materials And Methods

2.1 Alterations of IDH1, HIF-1α, and LDHA genes in glioma tissues from cBioPortal

The alterations including amplification, deep deletion, and mutations of IDH1, HIF-1α, and Lactate dehydrogenase A (LDHA) genes in glioma were analyzed on cBioPortal (http://www.cbioportal.org). OncoPrint was constructed in cBioPortal (TCGA provisional) to directly characterize the gene amplification, deep deletion, and mutation of IDH1, HIF-1α, and LDHA genes in glioma tissues. To comprehensively identify the alterations of IDH1, HIF-1α, and LDHA genes, we checked two data sets, the Brain Lower Grade Glioma (TCGA, Provisional) of 530 samples and the Merged Cohort of low-grade glioma(LGG) and glioblastoma(GBM) (TCGA, Cell 2016) with a total of 1102 samples. In addition, the Kaplan Meier survival curve of cBioportal was used to evaluate the association between IDH1, HIF-1α and LDHA gene alterations and overall disease-free survival of glioma patients.

2.2 Glioma specimens

Glioma tissues were obtained from patients undergone surgical tumor removal in the Department of Neurosurgery of Sixth Medical Center, Chinese PLA General Hospital. All the patients who participated in

this study have signed informed consent, and the protocol involving human specimens has been approved by the hospital Ethics Committee. The glioma specimens were rapidly frozen in liquid nitrogen after removed from patients and stored at − 80 ℃ for subsequent analyses.

2.3 Cell lines and cell culture

Human glioma cell lines U251 was purchased from the American type culture collection (ATCC, USA) and have been checked to ensure they are free of contamination. U251 cells were cultured in Dulbecco's modified eagle medium/F12 mixed medium supplemented with 10% FBS at 37°C in a humidified incubator containing 5% of CO_2.

2.4 Immunohistochemical analysis

The antibodies against HIF-1α and LDHA were purchased from (Abcam, UK). Immunohistochemical assays were conducted using Goat anti-Rabbit (ZSGB-BIO) or Goat anti-Mouse (ZSGB-BIO) secondary antibodies. Cell nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) and examined under Olympus light microscope. Representative images were captured and analyzed using the Image ProPlus v.6.0 software.

2.5 RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from U253 cells using the TRIzol reagent (Invitrogen, USA), and reversely transcribed into cDNA using the Reverse Transcription cDNA Kit (Osaka, Japan) according to the manufacture's protocols. RT-qPCR was conducted on the ABI Prism 7500 real time PCR platform (Applied Biosystems, Life Technologies, Carlsbad, CA) using the SYBR Premix EX Taq (Takara Dalian, Dalian, China). Human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control in RT-PCR assay. Primers used for stem-loop reverse-transcription PCR of IDH1: IDH1-human-F: GGTGACATACCTGGTACATAACTTTG, IDH1-human-R: GTGTGCAAAATCTTCAATTGACT, GAPDH-F: TCATTGACCTCAACTACATGG, GAPDH-R: TCGCTCCTGGAAGATGGTG. After RT-PCR reactions, the PCR products were collected and analyzed by 1% agarose gel electrophoresis.

2.6 Cell transfection with the IDH1 mutant ($IDH1^{R132H}$-mut) and wild-type IDH1 (IDH1-wt)

$IDH1^{R132H}$ mutant and IDH1-wt lentivirus were purchased from the Gene Pharma company. A total of 10,000 cells were seeded into 12-well plates and incubated at 37°C for 2 h prior to transfection with lentivirus in the medium containing 5 μg/mL polybrene. Lentivirus vector-treated or not treated cells were used as controls.

2.7 Cell proliferation assay

Primary or treated cells were harvested and mechanically separated by pipetting. Then the cells were seeded into 24-well plates (5×10^4 cells/well), and incubated for 7 days. The cells were counted every day

using a hemocytometer. The experiment was performed in triplicate.

2.8 Transwell invasion assay

Transwell invasion assay was performed using 24-well Transwell inserts (diameter 8 mm, Corning, NY, USA) precoated with Matrigel (60–80 μL, Corning, NY, USA) on the top surface of the polycarbonic membrane (pore size 8 μm). Briefly, the dissociated cells were seeded into the upper chambers of Transwell inserts (5 x 10^4 cells / insert) in 200 μL medium. The lower compartments were filled with 500 μL 10% of FBSD/F-12 medium according to the protocol. After incubation at 37°Cfor 48 h, the cells, which migrated into lower chambers, were harvested and counted under a microscope (Nikon, Tokyo, Japan).

2.9 Western blot analysis

U251 cells were harvested and lysed in RIPA buffer on ice for 30 min. Total protein was isolated from the cells the Total Protein Extraction Kit according to the manufacture's protocol (Millipore, USA). The protein concentration was determined using the BCA Protein Assay Kit (Solarbio, China). Equal quantity of proteins was loaded and separated by gel electrophoresis, and transferred to PVDF membrane (Millipore, USA), The membranes were incubated with the primary antibodies at 4°C overnight. The primary antibodies were anti-IDH1 (Abcam, USA) and anti-GAPDH (Zhongshan Golden Bridge Biotechnology, China). Then horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (1:2000, Zhongshan Golden Bridge Biotechnology, China) were added and incubated for additional 2 h. The immunocomplex on the membrane was examined under an Image Quant LAS 4000 mini (GE, USA) with HRP substrate luminol reagent and peroxide solution.

2.10 Glucose uptake and lactate production assay

U251cells were cultured in sugar-free DMEM (dulbecco's modified eagle medium) for 16 h, and then cultured in high-sugar DMEM (4.5g/L glucose) for 24 h under non-ionic conditions. The medium was then removed and the glucose level in U251 cells was determined using the fluorescence-based Glucose Assay kit (BioVision, Milpitas, California, USA) according to the manufacturer's instructions. Lactic acid levels were measured using the colorimetric method (Beotim, Wuxi, China) based on lactate oxidase reading at 540nm according to the manufacturer's instructions, and standardized to cell numbers.

2.11 Statistical analysis

All experiments were conducted in triplicate and experimental data were presented as mean ± SD. Independent 2-tailed student's *t*-test, or two-way analysis of variance (ANOVA) was used to identify statistical significance (*: $P < 0.05$ and **: $P < 0.01$).

3. Results

3.1. The alternations of IDH1, HIF-1α, and LDHA genes in glioma tissues

Alterations of IDH1, HIF-1α, and LDHA genes were identified in 77%, 0.4%, and 1.4% of the sequenced cases, respectively, in the LGG data obtained from the OncoPrint schematic of cBioPortal (Fig. 1A), and 51%, 0.1%, and 0.6% of the sequenced cases, respectively, in the LGG and GBM data. In addition, 90% of IDH1 mutation led to the replacement of arginine at position 132 by histidine ($IDH1^{R132H}$ mutation). The cancer types were listed in the chart (Fig. 1B, 1C, 1D). Regarding the LGG dataset, only one patient harbored deep deletion of the HIF-1α gene, and 4 patients had alterations of the LDHA gene, including two cases of gene amplification and two cases of deep deletion. Correlation analyses between gene alterations and patient survival showed that the overall survival rate (OS) and progression-free survival rate (PFS) of LGG patients were associated with IDH1 mutation (Fig. 2A, 2B) ($P < 0.05$). The correlation of the gene alternations and the clinical features of glioma were shown in Table 1 and Table 2.

Table 1
The correlation of the level of IDH1 expression with the clinical features of glioma

		IDH1		
Characteristics	NO. of patients	Mutation	No alteration	p-Value
Gender				0.612
Male	10	8	2	
Female	10	7	3	
Age(years)				0.541
> 60	4	3	1	
< 60	12	9	3	
Sample type				
primary	20	10	10	
recurrent	0			
Cancer types				
Oligoastrocytoma	3	2	1	
Anaplastic Astrocytoma	4	1	3	
Oligodendroglioma	6	3	3	
Anaplastic Oligoastrocytoma	2	1	1	
Astrocytoma	5	3	2	
Overall survival				0.025
Deceased	6	2	6	
Living	14	8	4	
Total	20	10	10	
IDH1: isocitrate dehydrogenase 1.				

Table 2
The correlation of the level of IDH1, HIF1-α and LDHA expression of glioma sample

	HIF1-α			p-Value	LDHA			p-Value	
Characteristics	High	Low	NA	0.046	High	Low	NA	0.031	Total
$IDH1^{R132H}$-mut	0	6	4		1	6	3		10
IDH1wt	5	3	2		4	3	3		10

$IDH1^{R132H}$-mut: isocitrate dehydrogenase 1 mutated with the replacement of arginine by histidine at the 132nd amino acid. IDH1wt: isocitrate dehydrogenase 1 wild type. LDHA: Lactate Dehydrogenase A.

3.2. Low-expression of HIF-1a and LDHA in glioma tissues with $IDH1^{R132H}$-mut

We first identified the expression of HIF-1α and LDHA in 10 $IDH1^{R132H}$ and 10 IDH1-wt glioma specimens using immunohistochemical staining. The results showed significantly reduced expression of HIF-1α and LDHA in glioma tissues with $IDH1^{R132H}$ mutation compared with that in glioma samples with IDH1-wt (Fig. 3, $P < 0.001$). The clinical characteristics of the patients who donated the tumor specimens were list in Table 3.

Table 3
Glioma tissues clinical characters

		IDH1		
Characteristics	NO. of patients	Mutation	No alteration	p-Value
Gender				0.612
Male	10	8	2	
Female	10	7	3	
Age(years)				0.541
> 60	4	3	1	
< 60	12	9	3	
Sample type				
primary	20	10	10	
recurrent	0			
Cancer types				
Oligoastrocytoma	3	2	1	
Anaplastic Astrocytoma	4	1	3	
Oligodendroglioma	6	3	3	
Anaplastic Oligoastrocytoma	2	1	1	
Astrocytoma	5	3	2	
Overall survival				0.025
Deceased	6	2	6	
Living	14	8	4	
Total	20	10	10	
IDH1: isocitrate dehydrogenase 1.				

3.3. IDH1^{R132H} inhibited the proliferation and migration of glioma cells *in vitro*

To explore the effects of IDH1^{R132H} mutation on glioma growth and infiltration, U251 glioma cells were transfected with IDH1^{R132H} mutation or IDH1-wt lentivirus. The transfection efficiency was assessed by

fluorescence microscope, and then quantified by RT-qPCR. Both U251 cells transfected with IDH1^{R132H} mutation (U251- IDH1^{R132H}-mut) and IDH1-wt (U251-IDH1-wt) exhibited significantly higher levels of expression of LDHA1 than U251 cells and U251 cells transfected with empty vector (U251-vector), respectively (Fig. 4A, $P < 0.001$). As shown in Fig. 4B, the proliferation of U251-IDH1^{R132H}-mut was suppressed compared with U251 cells transfected with U251-IDH1-wt, U251-vector, or U251 cells without any transfection ($P < 0.001$). In addition, IDH1^{R132H} mutation also inhibited the migration of U251 glioma cells compared with other groups (Fig. 4C).

3.4. IDH1^{R132H} mutation suppressed HIF-1α-mediated glycolysis in glioma cells

IDH1^{R132H} mutation decreased the expression of HIF-1α and LDHA at both mRNA and protein levels (Fig. 5A, 5B). The effects of IDH1 on glucose consumption and lactate production in U251-IDH1^{R132H}-mut and U251-IDH1-wt cells were also confirmed. As shown in Fig. 5C and Table 4, IDH1^{R132H}-mut inhibited glucose consumption and lactate production in U251 glioma cells ($P < 0.001$). However, glucose consumption and lactate production were increased in the U251-vector and U251-IDH1-wt groups ($P < 0.001$).

Table 4
The concentration of Glu and Lac

	Glu OD(505nm)	Gluconcentration(mg/ml)	Lac OD(530nm)	Lac concentration(mg/ml)
U251	1.05	3.04	2.03	2.51
U251-Vector	1.1	3.19	2.1	2.62
U251-IDHwt	0.75	2.17	3.6	4.46
U251-IDHmut	1.7	4.93*	1.43	1.77*

Glu: glucose. Lac: lactate. OD: optical density. IDH1^{R132H}-mut: isocitrate dehydrogenase 1 mutated with the replacement of arginine by histidine at the 132nd amino acid. IDH1wt: isocitrate dehydrogenase 1 wild type. *p < 0.05

4. Discussion

Glioma is the most common malignant tumors in central nervous system (CNS), however, the pathogenesis of glioma remains unclear. Currently, most clinical data demonstrated that glioma patients with the IDH1^{R132H} mutation had a preferable outcome compared with glioma patients with IDH1 wild-type. Paradoxically, IDH mutation was proven to be a triggering event in gliomagenesis. For example, the 2-hydroxybutyrate (2-HG), which is the vice-product of IDH1 mutant, can promote glioma growth

through its capacity to competitively inhibit a-KG-dependen enzymes. Interestingly, we identified that there is an alterations of IDH1, HIF-1α, and LDHA genes in the glioma data obtained from the OncoPrint schematic of cBioPortal as showing in Fig. 1A. Therefore, we investigated the role of $IDH1^{R132H}$ mutation in the development of glioma in this study.

Notably, the expression of HIF-1α was significantly decreased in U521 glioma cells tranfected with $IDH1^{R132H}$ mutation in our study. As we know, HIF-1α was an important transcription factor, and could promote tumor growth under hypoxia condition(17). The stability of HIF-1α is regulated by alpha ketoglutaric acid (α-KG), an important enzyme product of IDH1(18). This decreased trendency of HIF-1α was consistent with better clinical prognosis in gliomas, and alterations of HIF-1α in the glioma database obtained from the OncoPrint schematic of cBioPorta. However, Zhao et al.(19) demonstrated that $IDH1^{R132H}$ over-expression increased the protein level of HIF 1α in U87 glioma cells in the previous study. This difference may attribute to application of two different cell lines in these two experiments.

Our experiment also showed that the $IDH1^{R132H}$ mutation inhibited the proliferation and migration of glioma U251 cells *in vitro*. However, whether this result was correlted with $IDH1^{R132H}$ mutation, and the underlying mechanism was the next step that we wanted to explore. Generally, HIF-1α played important roles in glycolysis. In order to reach proliferate rapidly, cancer cells need more energy through glycolysis than normal cells. Meanwhile, Lactate dehydrogenase A (LDHA) plays a principal role in cancer metabolism(7, 20, 21). It has been shown that LDHA correlated with a number of clinicopathological features and the survival outcomes of a variety of tumors(22, 23). And many previous studies demonstrated that inactivation of HIF-1α/LDHA axis in cancer could inhibit the Warburg effect and tumor progression(23–25).

Therefore, to explore the role of $IDH1^{R132H}$ mutation in HIF-1α-mediated glycolysis in glioma, we examined the expression of LDHA levels in $IDH1^{R132H}$ mutated glioma U251 cells. As seen in Fig. 4C and Table 4, $IDH1^{R132H}$-mut inhibited glucose consumption and lactate production in U251 glioma cells ($P < 0.001$). However, glucose consumption and lactate production were increased in the U251-vector and U251-IDH1-wt groups ($P < 0.001$). Meanwhile, $IDH1^{R132H}$ mutation decreased the expression of HIF-1α and LDHA at both mRNA and protein levels (Fig. 4A, B). These down-regulation of LDHA in transfected U251- $IDH1^{R132H}$-mut cell lines indicated that $IDH1^{R132H}$ mutation inhibited the glycolysis in U251 glioma cells through regulating the HIF-1α/LDHA pathway. This result is in line with the other studies. Charles et al.(26) firstly demonstrated the downregulation of LDHA in IDH(mt) derived BTSCs, and concluded that silencing of LDHA was associated with increased methylation of the LDHA promoter. In recent research, Victor et al.(27) disclosed that the aggressive glioma models had lost DNA mythylation in the promoters of glycolytic enzymes, especially LDHA, and have increased mRNA metabolite levels compared with the indolent model. Our results suggest that the $IDH1^{R132H}$ mutation reduced the expression of HIF-1α, which then inhibited the glycolysis and proliferation of glioma cells probably through interaction with LDHA. However, the underlying mechanism needs further investigation.

5. Conclusions

This study investigated the role of the $IDH1^{R132H}$ mutation in the proliferation and migration of glioma cells and potential underlying mechanisms. Our research indicated that $IDH1^{R132H}$ mutated gliomas have decreased glycolytic capacity, which may lead to their slow growth pattern and better clinical prognosis compared with IDH1-wt gliomas. Therefore, targeting the HIF-1α/LDHA pathway may be a potential therapeutic approach for the treatment of gliomas.

6. Abbreviations And Si Units

Isocitrate dehydrogenas, IDH; Hypoxia-inducible factor 1α, HIF-1α; Lactate dehydrogenase A, LDHA; Low-grade glioma, LGG; Glioblastoma, GBM; Glyceraldehyde-3-phosphate dehydrogenase, GAPDH; Dulbecco's modified eagle medium, DMEM. h, hour; μg, microgram; mL, milliliter; μL, microliter; g, gram; L, liter.

7. Declarations

7.1 Ethics approval and consent to participat

This study was approved by ethics committee of the sixth medical center of PLA general hospital (HZKY-PJ-2020-56). And this study was performed in accordance with the Declaration of Helsinki and the rules of Good Clinical Practice. Informed consent was waived because of the retrospective study design.

7.2 Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

7.3 Conflict of interest

The authors declare no potential conflicts of interest.

7.4 Funding

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7.5 Author contributions

Yonggang Wang planned experiments; Shuwei Wang performed experiments and analysed data; Hailong Li wrote the paper and revised it for important intellectual content.

7.6 Consent for publication

Not applicable.

7.7 Acknowledgements

Not applicable.

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Figures

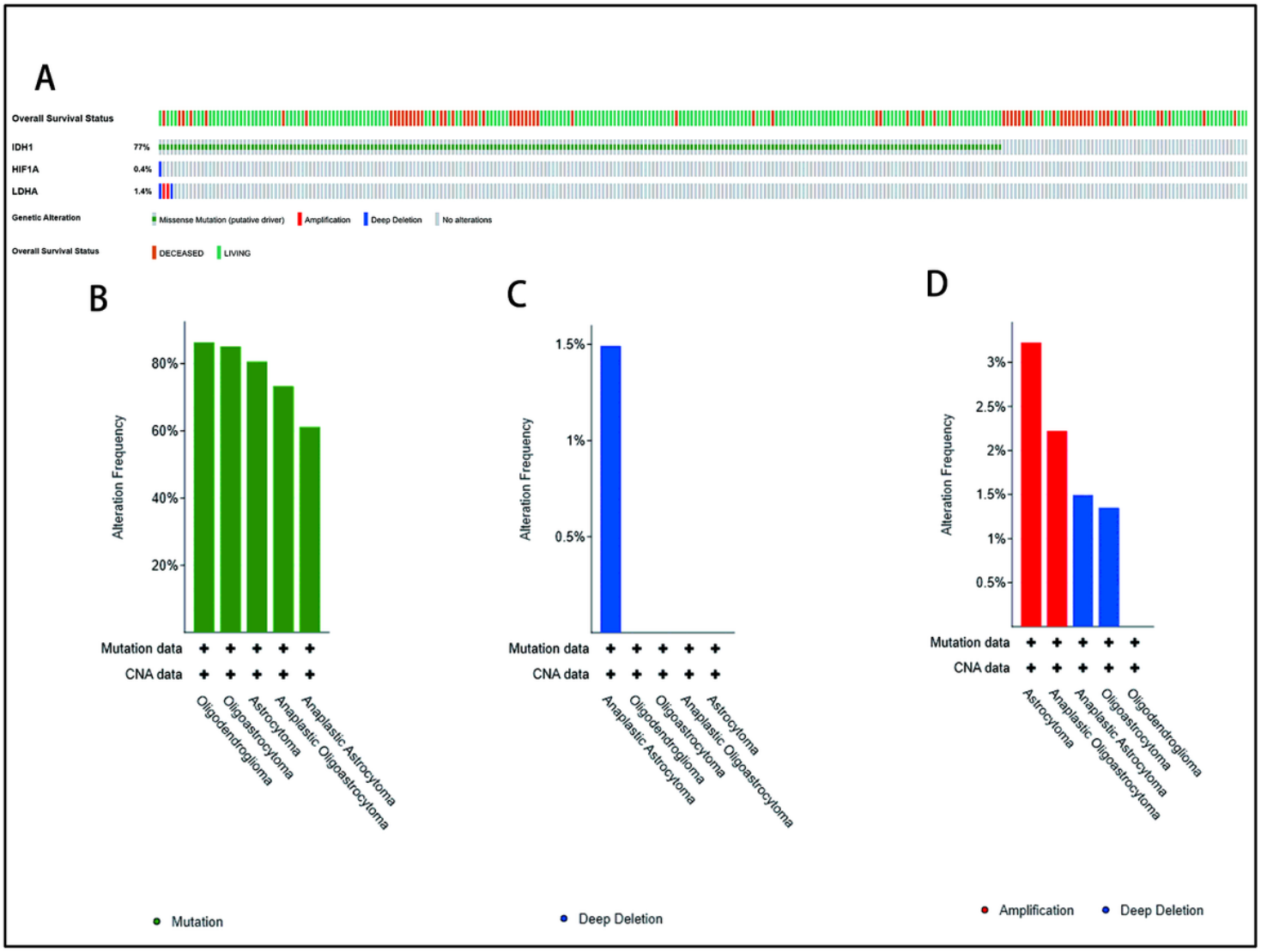

Figure 1

The levels of IDH1, HIF-1α and LDHA mRNA in glioma tissues on the Oncomine and TCGA databases. (A) Gene alterations in the LGG dataset. (B, C, D) The cancer types. Gene alteration analysis based on the TCGA database in different subtypes. From left to right: IDH1, HIF-1α, and LDHA.

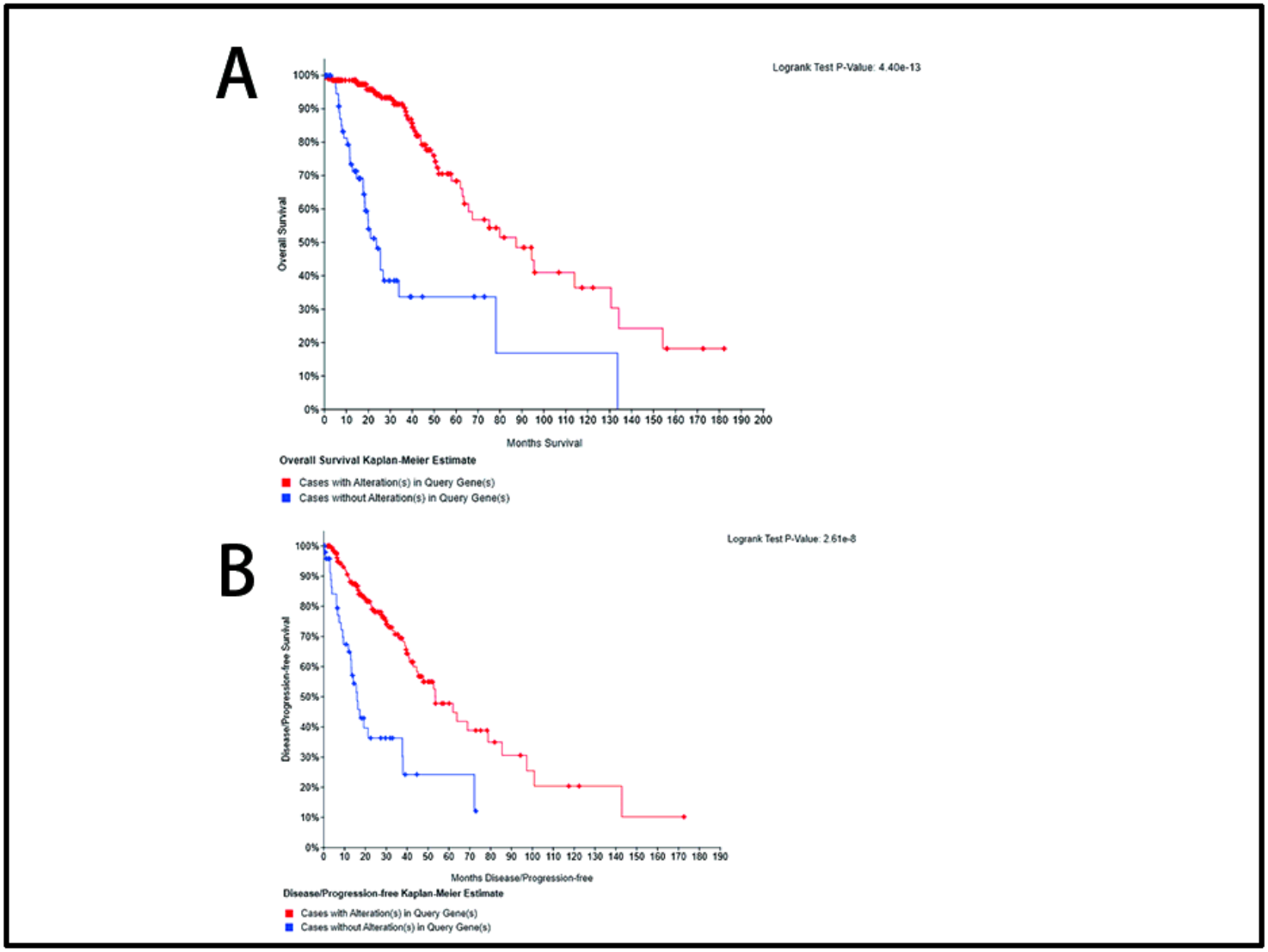

Figure 2

The overall survival (OS) and the progress-free survival (PFS) of LGG patients with (A) and without (B) IDH1 mutation.

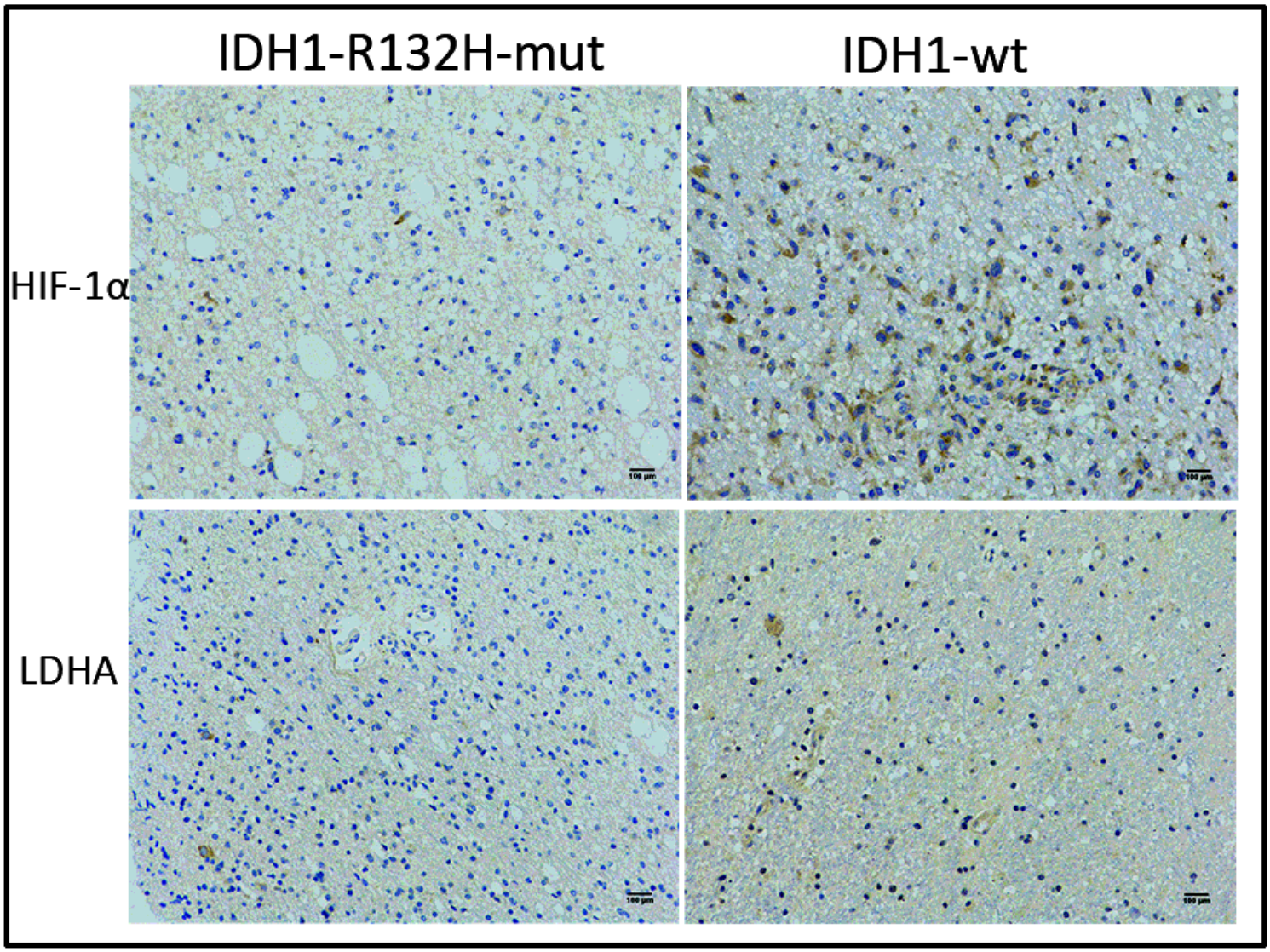

Figure 3

Immunohistochemistry assay showed that the expression of HIF1−α and LDHA in IDH1R132H glioma tissues was significantly lower than that in IDH1 wild type glioma tissues (Scale Bar=100um). *P<0.05.

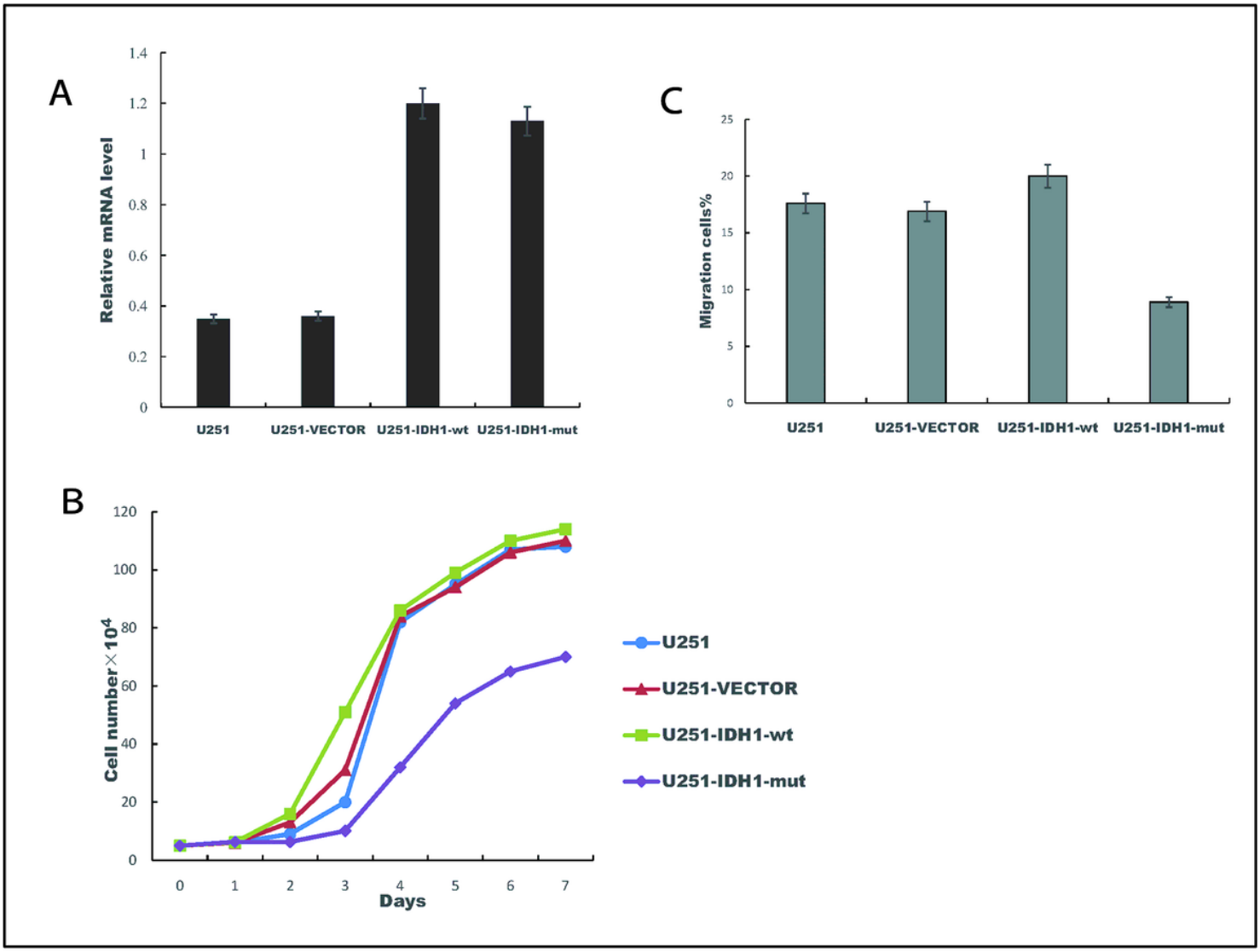

Figure 4

IDH1R132H inhibited the proliferation and migration of glioma cells in vitro. A. IDH1 expression based on RT-qPCR assay. B. IDH1R132H inhibited glioma cell proliferation. *P<0.05. C. IDH1R132H suppressed glioma cell migration. *P<0.05.

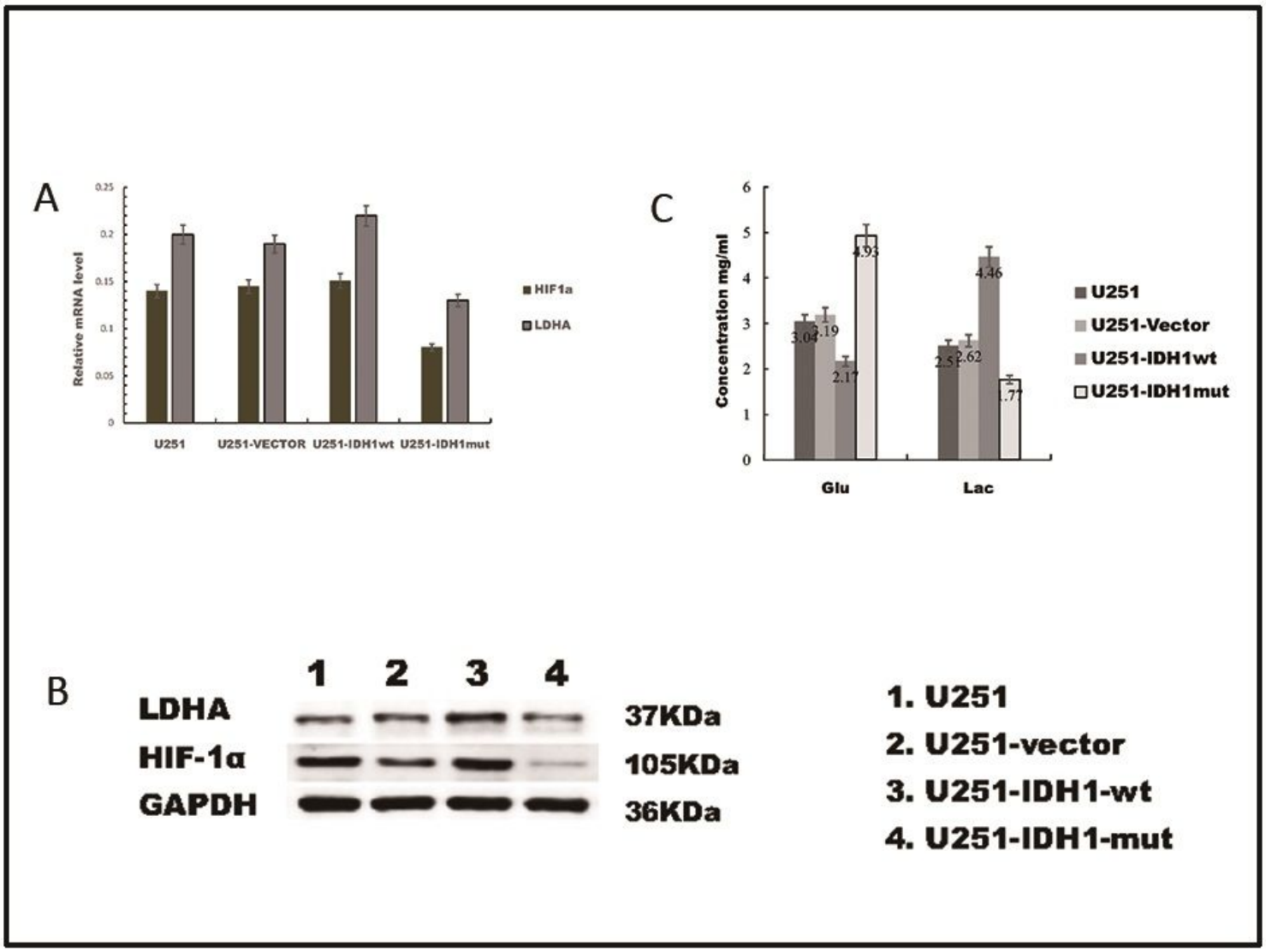

Figure 5

IDH1R132H suppressed HIF-1α-mediated glycolysis in glioma cells. IDH1R132H decreased the level of HIF-1α and LDHA (A) mRNA and (B) protein. C. IDH1R132H inhibited glycolysis in glioma cells.