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ORIGINAL ARTICLE

IDH1 mutation promotes proliferation and migration of glioma cells via EMT induction

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Summary

Purpose: *To investigate whether IDH1 mutation or 2-hydroxyglutarate (2-HG), the oncometabolite produced by IDH mutations, is correlated to epithelial-mesenchymal transition (EMT)-like phenotype in glioma cells, so as to clarify how IDH1 mutation is good prognostic factor while 2-HG, being its oncometabolite, remains unknown.*

Methods: *U87 and T98 cell lines were treated with 10 mM exogenous 2-HG, and fresh 2-HG was replenished every 2-days intervals. Endogenously heterozygous mutation in IDH1 was generated via lentiviral transduction technology. Morphological analysis, wound healing assay and Boyden migration assay were used to detect the ability of migration of U87 and 2-HG-treated U87 cell lines, and immunoblotting was used to detect the EMT-related transcription factors in glioma cell line.*

Results: *Cellular morphology changed after IDH1 mutation and 2-HG stimulation. The Cell Counting Kit-8 assay, and wound healing assay showed that exogenous 2-HG promotes the proliferation and invasion of glioma cells. Western blot analysis showed that mesenchymal marker β-Catenin was increased in the exogenous 2-HG-treated and IDH1 mutated U87 cells, while epithelial markers E-cadherin and ZO1 were decreased.*

Conclusions: *Our study showed some evidence that both IDH1 mutation and 2-HG can lead to EMT-like phenotype and proliferation and migration in glioma cells. EMT-like biomarkers changed in IDH1 mutation cells which generated via lentiviral transduction technology or treated in 2-HG.*

Key words: *isocitrate dehydrogenase, glioma, 2-hydroxyglutarate, proliferation*

Introduction

Glioma, as the most common type of central nervous system tumor, can be classified into 4 clinicopathologic grades with histological similarities according to the 2016 World Health Organization Classification of Tumors of the Central Nervous System [1]. Grade II and III gliomas are described as lower-grade gliomas sometimes, and grade IV gliomas are described as glioblastoma multiforme (GBM) [1,2]. Thanks to the considerable progress in genomics, glioma subtypes are defined according to the genotype, such as IDH mutation and 1p/19q codeletion status and phenotype [3,4]. The primary treatments for gliomas include extended-margin tumor surgical resection followed by radiotherapy (RT) and/or temozolomide (TMZ). Nevertheless, the vast majority remain incurable and the prognosis of glioma patients remains dismal.

Analyzing the specific genetic characteristics of gliomas has improved the understanding of tumorigenesis and predictions of prognosis, and allows

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the use of individual target therapy [5,6]. Isocitrate dehydrogenase 1(IDH1) and isocitrate dehydrogenase 2(IDH2) mutations have been shown to be promising biomarkers for monitoring disease prognosis and predicting the response to treatment in glioma, especially IDH1. IDH1 mutations are found in 70-80% of grades II–III gliomas and 80-90% of secondary glioblastomas (GBMs) but in only 5-10% of primary GBMs [7,8]. These findings implicate IDH1 and IDH2 mutations as key points in early gliomagenesis. IDH1/2 mutations are also observed in acute myeloid leukemia (AML), cholangiocarcinoma, chondrosarcoma, and epithelial tumors of the prostate, lung, and colon [9,10].

The mutations in IDH1 confers glioma cells the ability on catalysis of isocitrate to the metabolite 2-hydroxyglutarate (HG) instead of α-ketoglutarate (α-KG) [11,12]. The heterozygous IDH1 mutation dominantly inhibited the enzyme's activity, which led to the increased level of 2-HG [13]. The accumulation of 2-HG promoted the transformation of human normal astrocytes, and multiple studies have shown that IDH1-mutant gliomas are associated with histone and cytosine-phosphate-guanine (CpG) island hypermethylation, which regulates cellular epigenetic status as well as block of cellular differentiation [14-17]. Nevertheless, 2-HG is essential for maintaining an oncogenic property of mutant IDH-containing cancer cells [18,19]. However, the precise mechanism of how IDH1 mutation and the resulting alterations in cellular metabolism contribute to tumor formation and behavior haven't been fully unravelled.

EMT is a critical reversible process of cell remodeling during embryonic development and carcinoma progression, which involves profound changes in cell morphology and behavior [20,21]. EMT is thought to be activated in cancer cells, linked to their dissociation from the primary tumor and their intravasation into blood vessels [20]. There are three major steps of malignant cancer progression, namely invasion, dissemination and metastasis, which are all closely related to classical EMT-associated features [22,23]. EMT plays a crucial role in generating and maintaining the malignant features of primary GBMs as well as of grades II–III gliomas. Studies have demonstrated that EMT promotes transcription factors (EMT-TFs) in the tumorigenesis and progression of primary GBMs. Precisely, TWIST1 enhances GBMs invasion along with mesenchymal changes, and the transcription factor Zinc finger E box-Binding homeobox 1 (ZEB1) promotes the primary GBMs tumor initiation, cell invasion and chemoresistance [24,25]. ZEB1 is proved to be overexpressed in IDH1/2-mutant grades II-III gliomas previously [26].

Herein, we generated clonal U87MG glioma and T98 glioma cell lines overexpressing the R132H mutant protein ($IDH1^{R132H}$). We also treated U87 glioma and T98 glioma cell lines with exogenous 2-HG at the same time, to verify that 2-HG has the same effect as IDH1 mutation in modifying glioma in transformation of cell phenotype and explore whether the EMT-like mechanism participates in IDH1 mutation glioma.

Methods

Cell culture

U87 and T98 cell lines were purchased from Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS), 100 U/ml penicillin sodium and 100 mg/ml streptomycin sulfate at 37°C in a humidified atmosphere containing 5% carbon dioxide. Exogenous 2-HG treatments were done at 10mM, and fresh 2-HG was replenished every 2-day intervals.

Generation of endogenously heterozygous IDH1 mutant cells

U87 or T98 cells with endogenously heterozygous mutations in IDH1 were generated via lentiviral transduction technology using a GV358 vector (GeneChem Co., Ltd, Shanghai, China). An empty vector was used as negative control. These cell lines were verified at Cancer Cell Line Encyclopedia (CCLE) that both the genomic and RNA levels do not contain the IDH1 mutation at the DNA level and thus was utilized as a nonexpressing control. The detailed information regarding the targeting and production of these cell lines is available upon request.

Morphological analysis

Two cell lines were grown to 70% confluence and visualized at 100×magnification with a Leica light microscope (DMI 3000B, Germany), and the digital images were captured with the matched Leica software. The two cell lines were compared for morphologic characteristics consistent with EMT [i.e., loss of polarity (spindle-shaped cells), increase in intercellular separation, and appearance of pseudopodia]. Blinded observers classified the images with regard to presence or absence of morphologic changes consistent with EMT. Representative images are shown, and scale bars represent 50 μm (Figure 1).

Cell viability and proliferation assay

Cell viability and proliferation were assayed with a Cell Counting Kit-8 kit (CCK-8; Dojindo, Japan) following the manufacturer's instructions. Briefly, 5×10^3 cells were seeded at equal densities into each well of a 96-well culture plate and incubated overnight. The medium used in cell culture was replaced with medium containing different concentrations of 2-HG (ranging from 5 μM to 40 μM) and cells were incubated for 48h. For cell prolifera-

tion assay, the media containing no 2-HG were replaced at a 3-day interval and the absorbance was measured at the same time each day for a total of 10 days continuously. For determining the absorbance, 10 µL of CCK-8 kit reagent was added to each well and incubated for 2h, and plates were read on a multimode microplate reader (SpectraMax M5, MDS, USA) at 450 nm. The percentage of cell viability was calculated relative to the untreated cells as the control and concentration-effect curves for 2-HG treatment were generated by nonlinear regression analysis. Each experiment was performed in triplicate and cell viability was based on absorbance.

Boyden migration assay

Cell migration was also assayed in transwell chambers (6.5 mm diameter, 8.0 µm pore size polycarbonate filters; Corning, New York, NY, USA). We seeded 10^4 cells in DMEM without FBS onto the upper chambers, and the chambers were then inserted into each well of a 24-well plate containing 600µL of DMEM with 10% FBS. The cells in the chamber were incubated at 37°C for 12h and then fixed with methanol. Cells that had not migrated were removed from the top of the inserts with a cotton swab. Cells that had migrated to the underside of the inserts were stained with DAPI. Migratory cells were counted in five randomly selected fields (200×) with an inverted light microscope.

Wound healing assay

U87 and 2-HG-treated U87 cells were placed in a 6-well plate and cultured at 37°C with 5% CO_2. After 48h, a straight-line scratch was made on an 80% confluent monolayer of cells using a sterile 20µL disposable serological pipette. The cells were washed with 1mL DMEM to remove debris and smooth the edge of the scratch and were also cultured in 2mL DMEM without FBS for 48h. Images of the cell proliferation were taken using a microscope at 0h, 24h, and 48h after the scratch. The percentage of migration area was calculated through ImageJ software (1.48v, National Institute of Health, USA).

Immunoblotting

Cells were lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) with phenylmethylsulfonyl fluoride (PMSF) (Solarbio Inc, Beijing, China). Lysates were spun at 16,000×g at 4°C for 30min and normalized for protein concentration by using the bicinchoninic acid (BCA) assay kit (Sigma-Aldrich). Western blot analysis was performed using a capillary-based automated system (Simon western blot analysis; ProteinSimple, California, USA, http://www.proteinsimple.com/simon.html). The western blot was performed using the standard manufacturer's protocol using a primary antibody dilution of 1:200 and the secondary antibodies from

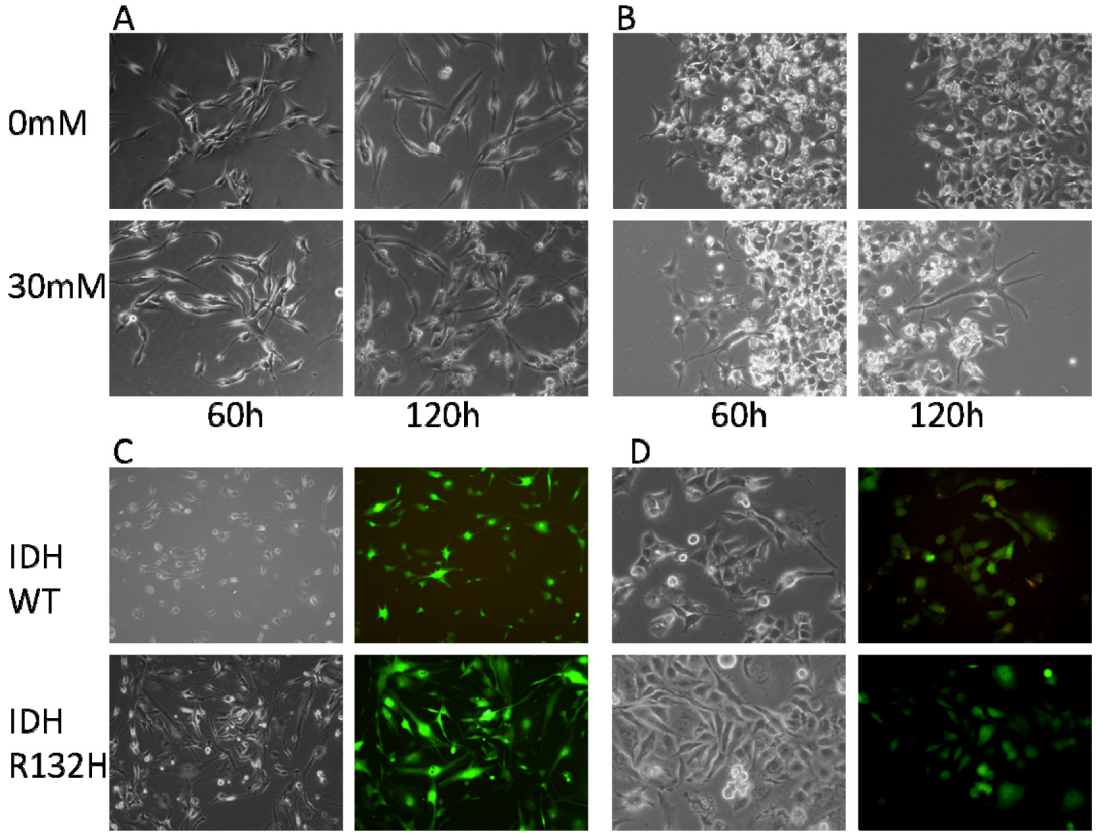

Figure 1. IDH mutation and 2-HG correlates with EMT-like phenotypes. **A:** representative microscopy images of the U87 cells. Scale bar, 50 µm. **B:** representative microscopy images of the T98 cells cells. Scale bar, 50 µm. **C:** Phase contrast (left panels) and fluorescent micrographs (right panels) of U87 cells 5 days after infection with lentivirus containing IDH WT-GFP or IDH1R132H-GFP. **D:** Phase contrast (left panels) and fluorescent micrographs (right panels) of T98 cells. Scale bar=50 µm in lower right panel.

ProteinSimple were used neat. The following antibodies were used: IDH1^{R132H} (MABC171, Merck-Millipore), IDH1(HPA036248, Sigma-Aldrich), ZO1 (Cell Signaling Technology, 8193), E-cadherin (Cell Signaling Technology, 3195), β-Catenin (Cell Signaling Technology, 8480), GAPDH (Millipore, MAB374).

Statistics

All results are presented as mean±standard error of the mean (SEM). P values were calculated using a two-tailed t test. Statistics for gene expression analysis are described above. P<0.05 was considered to indicate a statistically significant difference.

Results

IDH1mutation and 2-HG stimulation changed the morphology of glioma cells

U87 and T98 cells were treated with 2-HG every 48h for a week. IDH1 mutated glioma cells were generated and observed with a fluorescence microscope. The bodies of cells turned round and became much larger in 2-HG-treated cells and the protrusions were significantly shorter or faded away. After one-week treatment, cell size and nuclei were much larger compared to those of IDH1 wild type and cell body protrusions were significantly longer (Figure 1). These changes in cell morphology indicated that IDH1 mutation and 2-HG induced reorganization of the cell cytoskeleton.

Exogenous 2-HG promoted glioma cells proliferation

Cell proliferation was analyzed using Cell Counting Kit-8 (CCK-8; Dojindo, Japan) as the manufacturer's protocols recommended. Briefly, 1,000 cells/well were seeded in a 96-well plate. Forty-eight h after incubation with exogenous 2-HG in concentrations varying from 5 to 30mM, 10 μl CCK-8 were added to each well, followed by

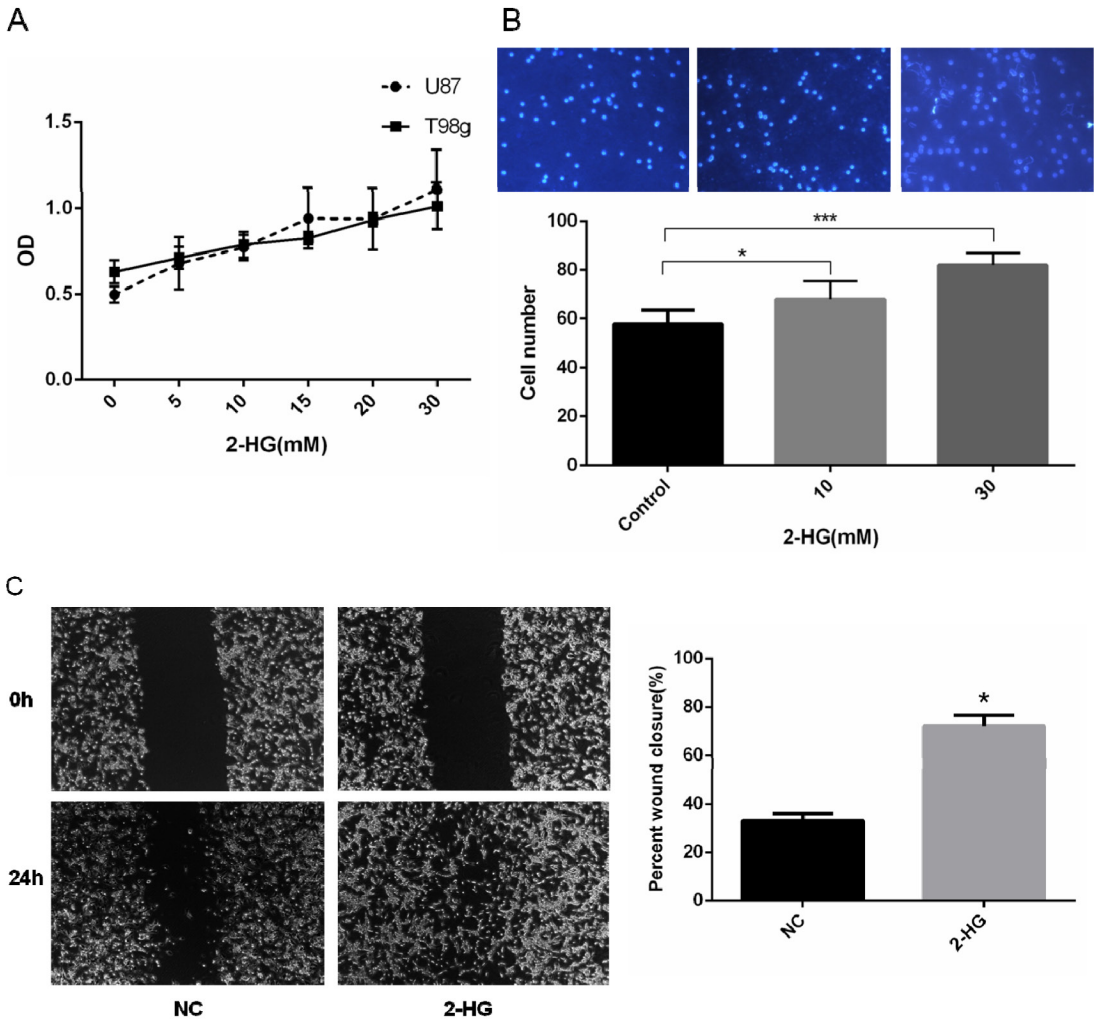

Figure 2. 2-HG increases proliferation of U87 cells. Cells were treated with Exogenous 2-HG (5 and 30 mM) during 5 days. **A:** Cell proliferation was measured via CCK-8 kit. The data were obtained from three independent experiments and are expressed as mean ± S.E.M. versus vehicle (p<0.05). **B:** Representative images of U87 cells after exposure to 10 or 30 mM 2-HG of Boyden assay. **C:** Representative images of U87 cells after exposure to 30 mM 2-HG of wound healing assay. Scale bar, 50 μm. (*p<0.05, ***p<0.001).

incubation for 2h in the dark. The absorbance (Ab) was recorded at a wavelength of 450 nm by a microplate reader (Thermo Multiskan MK3; Thermo Fisher Scientific, Inc.) and cell viability was calculated using the following equation: Cell viability (%)=[(Absample- Abblank) / (Abcontrol- Abblank)] x 100%, (where Absample, Abcontrol and Abblank are the Ab values of each sample, the cells cultured in culture medium without any additional substances and the culture medium without cells in wells, respectively) (Figure 2A).

Exogenous 2-HG promoted the invasion of glioma cells

The migratory capacity of U87 and exogenous 2-HG-treated U87 cells were measured by both wound healing assay and Boyden migration assay. Greater numbers of exogenous 2-HG-treated cells migrated through the collagen membrane compared to untreated cells in Boyden migration assay ($p<0.05$) (Figure 2B), and wound healing assay showed that exogenous 2-HG-treated cells had a faster healing speed than that of untreated cells ($p<0.05$) (Figure 2C).

IDH1 mutation correlates with EMT-like phenotypes

To determine whether the generation of IDH1 mutation and treatment of 2-HG could create specific molecular changes consistent with EMT in glioma cells, western blotting was done on cell lysates from U87 and exogenous 2-HG-treated U87 cells. The expression of the epithelial adhesion molecules E-cadherin and ZO1 was decreased in 2-HG-treated U87 cells, and a concurrent marked increase in the expression of the mesenchymal markers β-Catenin was observed (Figure 3).

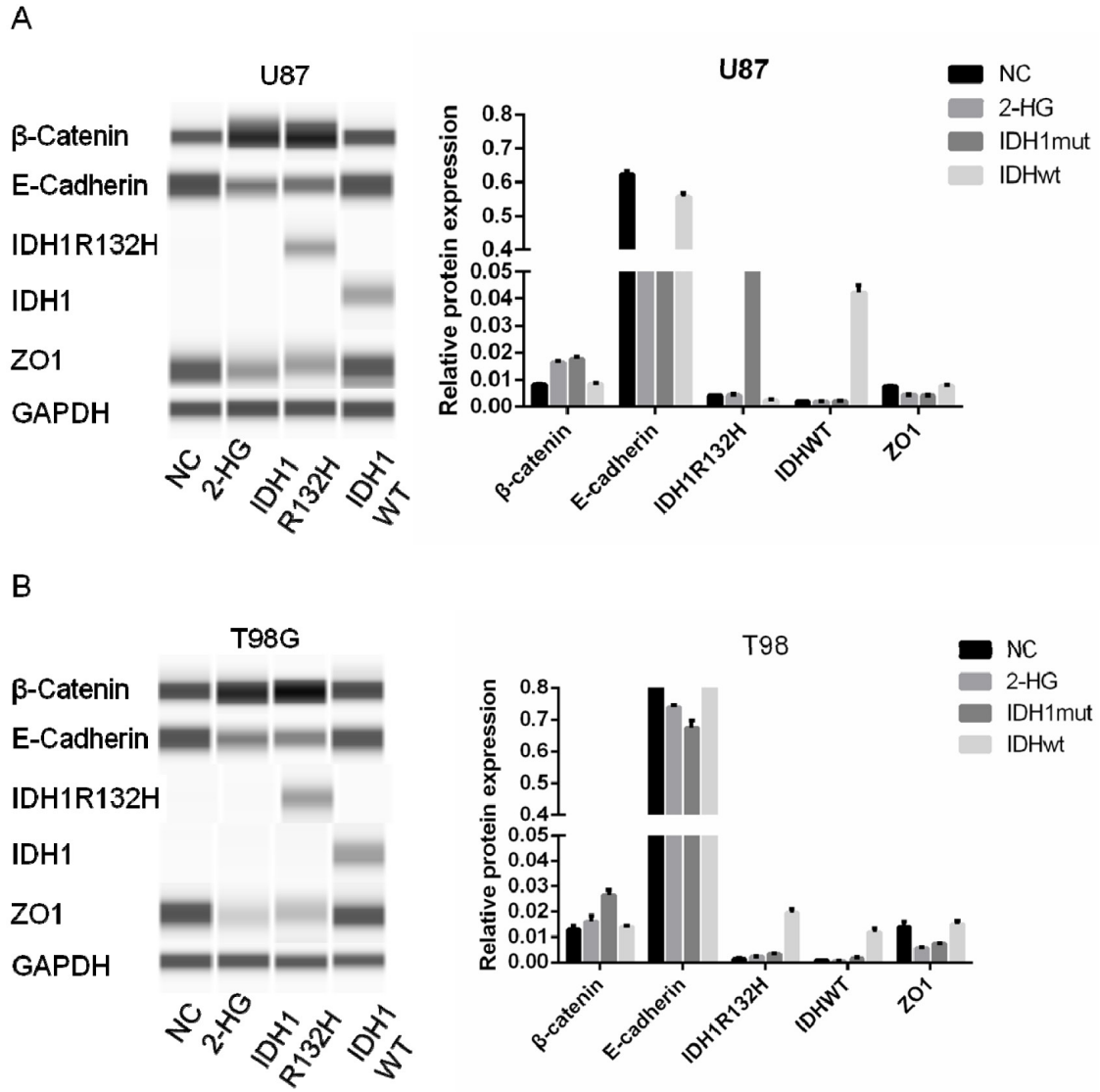

Figure 3. IDH mutation and 2-HG correlates with increased β-Catenin and decreased E-cadherin or ZO1 expression. **A:** western blot of U87 parental and IDH1 mutant clones analyzed by immunoblotting for the indicated proteins. **B:** western blot of T98 cell line ($p<0.05$).

Discussion

Our study has shown that both transfection of $IDH1^{R132H}$ lentiviral and exogenous treatment of 2-HG can lead to morphological changes in glioma cells. 2-HG can also promote the proliferation and migration of glioma cells, and epithelial protein like ZO1 and E-cadherin are increased in glioma cells transfected with $IDH1^{R132H}$ lentiviral compared with those in glioma cells transfected with $IDH1^{WT}$ lentiviral. These indicate that EMT played a crucial role in the proliferation and migration of IDH mutated glioma cells.

Morphologic evidence of EMT has been found at the invasive front of human tumors. The synaptic number and size of glioma cells changed after treatment of exogenous 2-HG or transfection of $IDH1^{R132H}$ lentiviral. As known, EMT, which is considered as an essential early step and a critical process in tumor metastasis [27], it is a process notably characterized by morphological changes of cancer stem cell traits [20,28]. This finding indicated that there might be some correlation between IDH1 mutation and invasive status of glioma cells. 2-HG has shown to be essential for maintaining the oncogenic property of mutant IDH-containing cancer cells [19]. IDH1 mutations are initial events that define major clinical and prognostic classes of gliomas. IDH mutation can be sufficient to establish the glioma hypermethylator phenotype [29], which impairs histone demethylation and results in a block to cell differentiation [17]. The Flavahan's study suggested that IDH mutations promoted gliomagenesis by disrupting chromosomal topology and allowing aberrant regulatory interactions that induced oncogene expression. Disruption of chromosomal topology and oncogene insulation may be more generally relevant to methylator phenotypes observed in colorectal and renal cell carcinomas, leukemia and other malignancies [30]. The effects of IDH mutations in glioma tumorigenesis have not yet fully revealed, despite these tremendous strides in our knowledge regarding mutant IDH.

EMT is viewed as an essential early step and a critical process in tumor metastasis [27]. TWIST1 enhances GBM invasion in concert with mesenchymal changes. The present findings demonstrated the potential role of applying carcinoma EMT as a framework to enhance our understanding of GBM invasion and further unravel that a neural form of mesenchymal change, analogous to carcinoma EMT, may contribute broadly to glioma malignancy. On the other hand, ZEB1 has been specifically detected in poorly differentiated pancreatic carcinomas, and it is thought to suppress the expression of stemness-inhibiting miRNAs to maintain a stem-like phenotype. Based on these findings, we propose targeting Twist1/E-Cadherin/EMT and/or Zeb1/E-Cadherin/EMT mediated mesenchymal change as a therapeutic strategy, with the potential to inhibit GBM invasion and tumor growth, and enhance treatment responses.

Although 2HG has been suggested as a source of malignant progression, which was also verified in our study, IDH-mutant gliomas are associated with better outcomes [31]. It is possible that the positive effect of IDH mutations could be disrupted by stress conditions during radiotherapy and chemotherapy, allowing the negative effect of 2HG to prevail. Further clinical evidence should be searched to solve these controversial results.

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Conflict of interests

The authors declare no conflict of interests.

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