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Original Article

EGFR/EGFRvIII partly regulates the tumourigenesis of glioblastoma through the SOX9-GLUT3 axis

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Abstract: EGFR/EGFR variant III (EGFRvIII) glioblastoma is seriously malignant, and the underlying mechanism remains unclear. In this study, EGFR and GLUT3 were found to be co-expressed in our collected tissues and associated with worse overall survival in glioblastoma via bioinformatics analysis. Functionally, *in vitro* and *in vivo* tests revealed that silencing GLUT3 substantially inhibited the viability of U87-EGFRvIII and LN229-EGFRvIII cells. Compared with wild-type U87 or LN229 cells, the expression level of SOX9 in U87-EGFRvIII or LN229-EGFRvIII cells (U87 and LN229 over-expressing EGFRvIII) was substantially increased. Chromatin immunoprecipitation and Dual-luciferase reporter assays revealed that SOX9 bound to the promoter of GLUT3 and promoted the expression of GLUT3. Collectively, our findings indicated that the EGFR/EGFRvIII-SOX9-GLUT3 axis mediated the tumourigenesis of glioblastoma and might be a potential target for glioblastoma therapy.

Keywords: EGFR, EGFRvIII, SOX9, GLUT3, glioblastoma

Introduction

Glioblastoma is one of the most common and fatal primary adult brain malignancies and seriously endangers human health [1]. Over the past two decades, the infiltrative growth and heterogeneity of glioblastoma have made it difficult to pinpoint an effective therapeutic strategy under recurrence-free status [2]. Even less optimistic, the overall survival time of patients with glioblastoma was generally less than 15 months [3]. Therefore, in-depth explorations of its infiltrative growth and heterogeneity are expected to be effective ways to solve this clinical dilemma.

Genome analysis revealed that EGFR was amplified and mutated in more than half of glioblastoma [4]. Upregulation of EGFR expression promoted the malignant characteristics of glioblastoma [5], including increased cell proliferation, cell invasion, and cell migration [6-8]. Among EGFR mutations, EGFR variant III (EGFRvIII) was the most active mutant in glioblastoma [9]. Moreover, EGFRvIII was found only in tumour tissues and tumour cells [10], and the elevated expression of EGFRvIII promoted cell proliferation, angiogenesis and invasion of glioblastoma [9]. Thus, EGFR/EGFRvIII is considered as a good candidate for targeted therapy in glioblastoma.

EGFRvIII participated in the regulation of cell function by downregulating the phosphorylation of GLUT3 in U87 cells [11]. However, the underlying mechanism by which EGFRvIII regulates GLUT3 is still unknown. SRY-box transcription factor 9 (SOX9) is a member of the SRY-related high mobility box transcription factor family [12], which contains super enhancers and regulates the expression of target genes [13]. Researches also revealed that SOX9 was promoted by EGFR in UroCa cells [14]. Therefore, we speculated that EGFR/EGFRvIII might regulate the expression of GLUT3 dependent on the super-enhancer function of SOX9 in glioblastoma.

In view of the above, our understanding of the interaction among EGFRvIII, SOX9 and GLUT3 in

Table 1. The primers for RT-PCR

Gene	Forward primer	Reverse primer
EGFR	5'-AAAGTTAAAATTCCCGTCGCTATCAAG-3'	5'-TCACGTAGGCTTCATCGAGGATTTC-3'
GLUT3	5'-GCCTTTGGCACTCTCAACCAG-3'	5'-AGTAGCAGCGGCCATAGCTC-3'
GAPDH	5'-TTCACCACCATGGAGAAGGC-3'	5'-GGCATGGACTGTGGTCATGA-3'

glioblastoma seemed to be insufficient. To address this issue, *in vitro* and *in vivo* experiments were performed to elucidate the latent mechanisms among EGFRvIII, SOX9 and GLUT3 in depth and to further reveal the infiltrative growth and heterogeneity of glioblastoma.

Material and methods

Glioblastoma specimens

Thirty glioblastoma specimens collected from the Affiliated Hospital of Hainan Medical College Institutional Clinical Care and Use Committee were tested by pathologists at Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. Informed consent was collected, and this research was approved by the ethics committee of the hospital (No. 2015-19-36).

Cell culture

U87 and LN229 were obtained from ATCC (Manassas, VA, the States). U87 and LN229 with stable ligand-independent activated EGFR mutant EGFRvIII (U87-EGFRvIII and LN229-EGFRvIII) were constructed in our previous study [15]. All cells were cultivated in DMEM (Invitrogen, Carlsbad, CA) including 10% FBS (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Beyotime, Shanghai, CN) and cultivated with 5% CO_2 at 37°C.

Cell transfection

The lentivirus-mediated short hairpin RNA (shRNA) knock-down of GLUT3 used in this study was accomplished with the following sequences: sh1-GLUT3: 5'-CTTGGTCTTTGTAGCCTTC-3' and sh2-GLUT3: 5'-CTTCCTCTTTCTAGCCTTC-3'. An empty lentivirus vector (sh-NC) was used as a negative control. The lentivirus that knock down SOX9 (sh-SOX9) and the plasmids that overexpress SOX9 (pcDNA-SOX9) were constructed in our previous study, and stable cells were made in the same way as described above [16]. The plasmid in the pLVX-EGFP (Clontech) vector was treated with virus packaged plasmids and transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen). Viral supernatant was transferred to the culture media of U87vIII and LN229vIII with 8 μg/ml polybrene (Sigma-Aldrich).

Western blot assay (WB)

WB was conducted the same way as above [15]. The protein concentrations were tested using a BCA kit (Beyotime, Shanghai, CN). Fifty micrograms of protein were added into SDS–PAGE gels and transferred onto nitrocellulose membranes (GE healthcare). The antibodies we applied were β-actin (ab8229, 1:1000, Abcam), GLUT3 (G-5, 1:400, Santa Cruz), EGFR (1C6, 1:1000, Cell Signaling Technology) and anti-SOX9 (E-9, 1:1000, Santa Cruz). HRP-conjugated anti-rabbit (SC-2004, Santa Cruz) and anti-mouse (SC-2005, Santa Cruz) secondary antibodies were applied. The bands were visualized using the ECL Substrate Detection Kit (Bio-Rad) and were detected using Image Lab software v3.0.

Cell proliferation and colony formation assays

For the CCK-8 assay, cells were seeded in 96-well plates (1000 cells/well) including 10 μl of CCK-8 agent (Boster), and the absorbance at 450 nm was measured. For the colony formation, cells were plated in 6-well plates (1000 cells/well) and cultivated for 3 weeks. Cells were dyed with crystal violet and counted.

Real-time fluorescence quantitative PCR (RT-PCR)

Total RNA was obtained from cells using the TRIzol Plus RNA Purification Kit (Thermo Fisher) and was reverse transcribed into cDNA using the Reverse Transcription Kit (Takara). RT-PCR was conducted using Power SYBR Green Master Mix (Life Technologies) in the Applied Biosystems StepOne Plus Real-Time Thermal Cycling Block. The primers are shown in **Table 1**. GLUT3 was tested using the $2^{(-\Delta\Delta Ct)}$.

Luciferase assay

The promoter of GLUT3 was amplified with U87 and LN229 using the primer 5'-CTATTATTC-3' and was subcloned into the pGL3 vector (Promega). The pGL3-GLUT3 promoter was co-transfected with or without a SOX9 expression plasmid into U87-EGFRvIII and LN229-EGFRvIII cells using the Lipofectamine 2000 reagent (Thermo Fisher). pRL-TK (Renilla luciferase, Promega) was applied as a control. Luciferase and Renilla signals were tested using the Dual-Luciferase Reporter Assay Kit (Promega) according to a protocol (Berthold Technologies).

Chromatin immunoprecipitation (CHIP)

CHIP assay was conducted in the same way as described above [15]. A Chromatin Immunoprecipitation Kit (Millipore-Upstate) was applied to immunoprecipitate DNAs, and then the purified DNAs were tested using qPCR. Purified DNA was subjected to PCR using primers specific for a region in the GLUT3 promoter (440-448 nt), which spanned two putative SOX9-binding sites. The primer sequences: Forward: 5'-TAGCCCGGGCTCGAGGCGCCAGCAGTATCAATTTGAATTC-3' and Reverse: 5'-CGGAATGCCAAGCTTCCTTCTGTGTCCCCATCGCT-3'.

In vivo study

Six-week-old female athymic nude mice (BALB/c-nu/nu) were obtained from SLAC (Shanghai, China), with five mice in each group. In general, 5×10^5 U87-EGFRvIII or LN229-EGFRvIII cells transfected with sh-NC and sh1-GLUT3 were implanted into the mouse brain with stereotactic technology. Mice were euthanized at the onset of neuropathologic symptoms. Tumour volumes were determined using the largest tumour cross-section for each sample and were calculated by (W2 x L)/2. All tests were approved by the Animal Care and Use Committee (No. 2019-07-12).

Immunohistochemistry (IHC)

Tissue sections from paraffin-embedded de-identified human glioblastoma specimens were dyed with antibodies against GLUT3 (1:60, Abcam), EGFR (1:100, Abcam), Ki67 (1:60, Abcam), and SOX9 (1:100, Abcam), and non-specific IgG was applied as a negative control. The IHC staining procedure was explained in detail in a previous study [15].

Statistical analysis

GraphPad Prism 5.0 and SPSS 20.0 (International Business Machines Corp.) were applied to perform data analysis. The experimental results were represented as the mean ± SD. Statistical analysis was conducted using one-way ANOVA and Student's t-test. *P* value <0.05 indicates statistically significant difference.

Results

EGFR/EGFRvIII promoted GLUT3 and was associated with a worse overall survival in glioblastoma

In the 329 glioblastoma samples from the Rembrandt tumour database (http://Rembrandt.nic.nih.gov/), high expression levels of EGFR and GLUT3 were associated with worse overall survival (**Figure 1A**). Spearman correlation analysis showed that EGFR and GLUT3 were positively co-expressed at the mRNA level according to the Rembrandt tumour database (**Figure 1B**). In our collected glioblastoma samples, high-EGFR and high-GLUT3 expressions were associated with worse overall survival (**Figure 1C**). And EGFR and GLUT3 were positively co-expressed at the mRNA level (**Figure 1D**). Further IHC results indicated that in EGFR-negative tissues (n=3), the expression of GLUT3 was lower compared to that of EGFR-positive tissues (n=3) (**Figure 1E**). In conclusion, EGFR co-expressed with GLUT3, and was associated with worse overall survival in glioblastoma.

Compared with wild-type U87 and LN229, EGFR and GLUT3 were substantially overexpressed in U87-EGFRvIII and LN229-EGFRvIII at the mRNA level (**Figure 2A**), and the protein levels of EGFR and GLUT3 were confirmed by WB (**Figure 2B**). Additionally, after adding the EGFR agonist NSC228115 (HY-101084; MedChemExpress, 0.36 μM for 24 h) to U87-EGFRvIII cells, the expression of GLUT3 increased substantially, and after adding the EGFR inhibitor osimertinib (AZD-9291; MedChemExpress, 1 nM for 24 h) to U87-EGFRvIII cells, the expression of GLUT3 decreased substantially (**Figure 2C**). Compared with the osimertinib group, when NSC228115 and osimertinib were co-administrated, the expression of GLUT3 increased substantially. Moreover, compared with the NSC228115 group, GLUT3 in the NSC228115+osimertinib group was substantially reduced (**Figure 2C**).

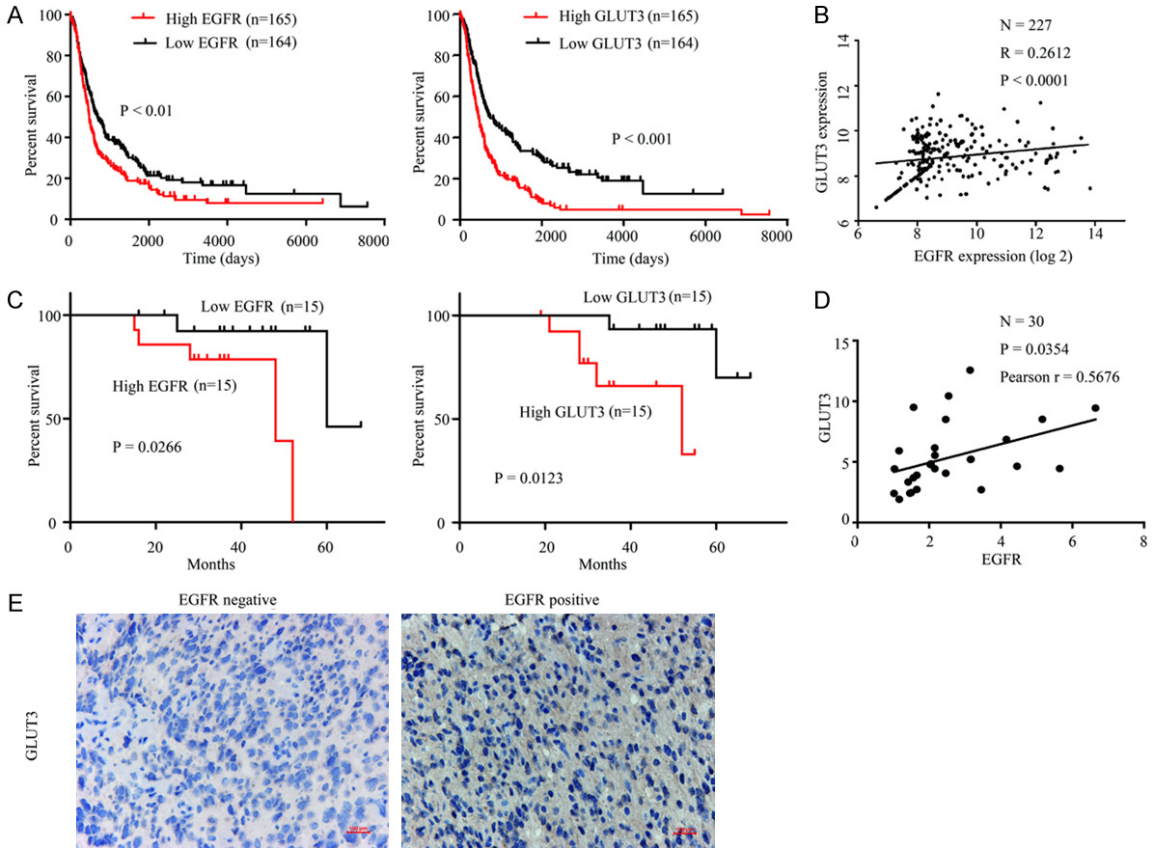

Figure 1. EGFR and GLUT3 were co-expressed in glioblastoma and were correlated with worse overall survival. (A) Elevated expression of EGFR and GLUT3 was related to worse overall survival in glioblastoma according to Rembrandt database analysis. (B) EGFR and GLUT3 were positively co-expressed at the mRNA level according to Rembrandt database analysis. (C) High levels of EGFR and GLUT3 were associated with poorer overall survival, and (D) EGFR and GLUT3 were co-expressed at the mRNA level via RT-PCR detection in glioblastoma in our collected samples. (E) IHC results showed that in EGFR-positive tissues (n=3), the expression of GLUT3 was relatively higher than that in EGFR-negative tissues (n=3). Original magnification: 40×; scale bar: 100 μm.

In conclusion, EGFR/EGFRvIII promoted the expression of GLUT3 in glioblastoma.

GLUT3 was required for the tumourigenesis of EGFRvIII-driven glioblastoma

The knockdown efficiency of GLUT3 was detected by WB in U87-EGFRvIII cells and LN229-EGFRvIII cells (**Figure 3A**). Clone formation assay (**Figure 3B**) showed that compared with the sh-NC group, the proliferation of U87-EGFRvIII and LN229-EGFRvIII cells in the sh1-GLUT3 and sh2-GLUT3 groups was substantially inhibited, and this result was further confirmed by CCK-8 assay in U87-EGFRvIII cells and LN229-EGFRvIII (**Figure 3C**). To further investigate whether GLUT3 was required for the tumourigenesis of EGFRvIII-driven glioblastoma, an orthotopic model was constructed *in vivo*. The H&E results showed that after silencing GLUT3, the tumour area of U87-EGFRvIII and LN229-EGFRvIII cells was substantially reduced (**Figure 3D**), and IHC detecting Ki67 was used to further verify our hypothesis (**Figure 3E**). These data supported that GLUT3 was critical for cell proliferation and tumour growth in EGFRvIII-driven glioblastoma *in vitro* and *in vivo*.

EGFRvIII promoted the expression of GLUT3 in a manner dependent on the combination of SOX9 and the GLUT3 promoter

In the 329 clinical glioblastoma samples from the Rembrandt tumour database, as shown in **Figure 4A**, high SOX9 was associated with poor overall survival, and this result was confirmed by our collected samples (**Figure 4B**). Spearman correlation analysis showed that EGFR, SOX9 and GLUT3 were positively co-expressed at the

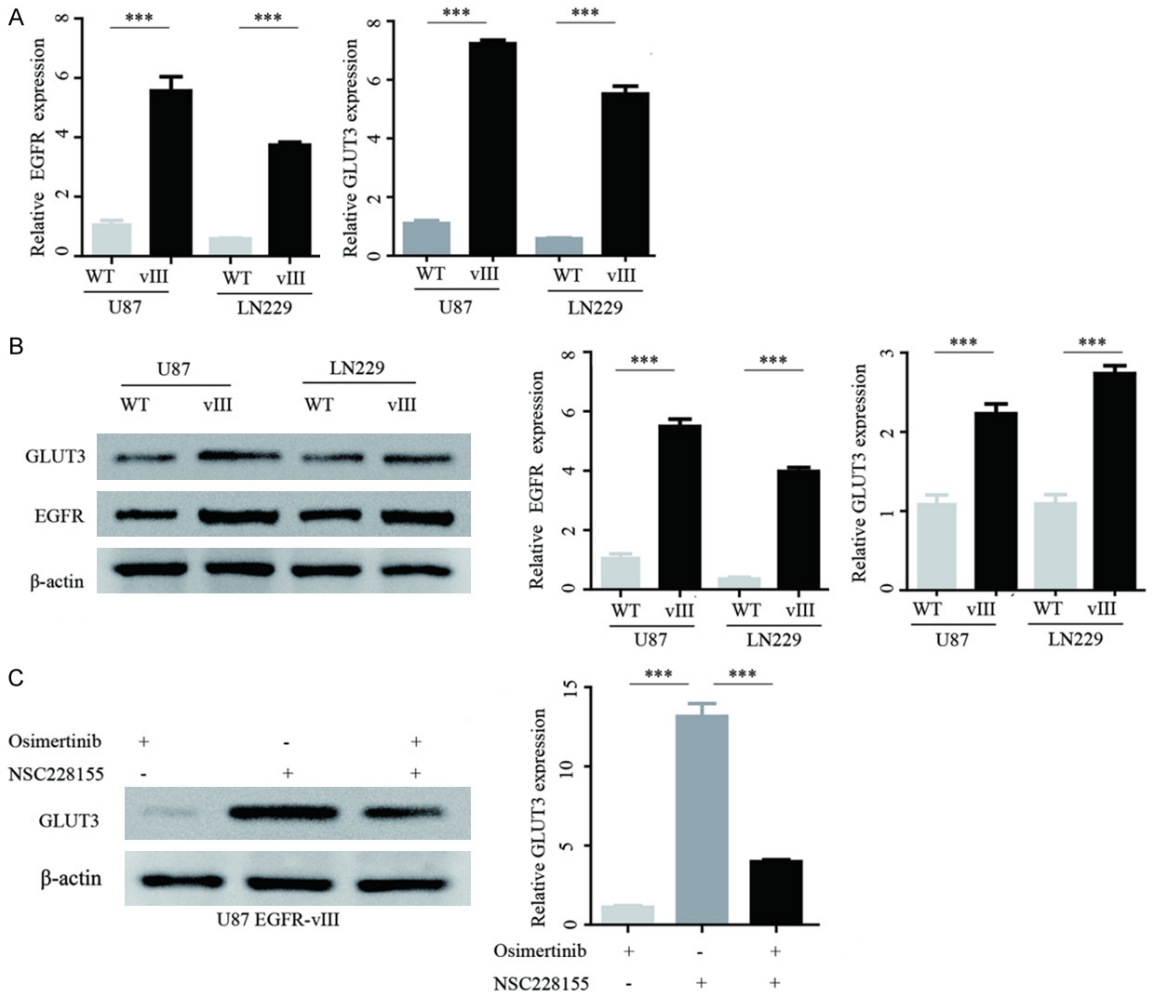

Figure 2. EGFR/EGFRvIII upregulated GLUT3 expression in glioblastoma. (A and B) EGFR and GLUT3 were substantially overexpressed in U87-EGFRvIII and LN229-EGFRvIII cells at the mRNA (A) and protein (B) levels compared with those in wild-type U87 and LN229 cells. (C) Through treatment of U87-EGFRvIII cells with osimertinib and NSC228115, we found that EGFR promoted the expression of GLUT3. Mean ± SD. ***P<0.001. P values were calculated using two-tailed Student's t-tests and one-way ANOVA.

mRNA level (**Figure 4C** and **4D**). Further IHC results indicated that the expressions of EGFR and GLUT3 were reduced in SOX9-positive tissues (n=3) compared with those in SOX9-negative tissues (n=3). In addition, according to the Rembrandt tumour database, compared with $EGFR^{low}/GLUT3^{low}$ or $GLUT3^{low}/SOX9^{low}$ tissues, the overall survival was poorer in $EGFR^{high}/GLUT3^{high}$ or $GLUT3^{high}/SOX9^{high}$ tissues, respectively (**Figure 4C**). Together, EGFR, SOX9 and GLUT3 were positively co-expressed with each other, and associated with poorer overall survival.

RT-PCR assay revealed that SOX9 was substantially elevated in U87-EGFRvIII and LN229-EGFRvIII cells compared with that in wild-type U87 and LN229 cells (**Figure 5A**). We constructed a shRNA that silenced SOX9 (**Figure 5B**) and a plasmid that overexpressed SOX9 (**Figure 5C**), and RT-PCR assay indicated that silencing SOX9 inhibited the expression of GLUT3 (**Figure 5D**), while overexpression of SOX9 promoted the expression of GLUT3 (**Figure 5E**) in U87-EGFRvIII and LN229-EGFRvIII.

CHIP assay revealed that the protein of SOX9 was substantially elevated in U87-EGFRvIII compared with wild-type U87 cells (**Figure 6A**). Through bioinformatics analysis (http://jaspar.genereg.net/), we found that there were binding sites between SOX9 and the promoter area

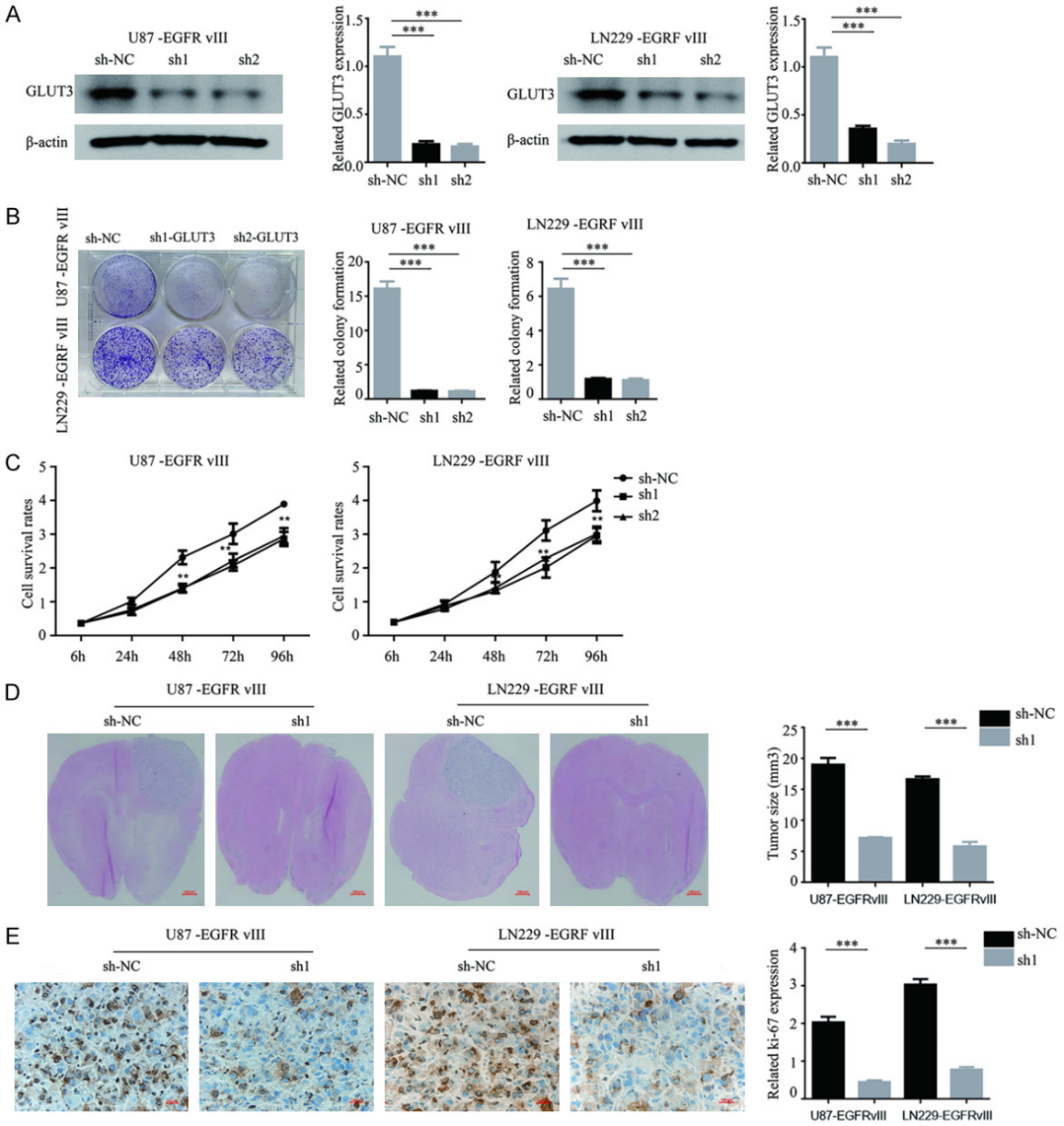

Figure 3. GLUT3 was crucial for the cell viability of EGFRvIII-driven glioblastoma. A. The efficiency of GLUT3 depletion using two different shRNAs (sh1-GLUT3 and sh2-GLUT3) in U87-EGFRvIII and LN229-EGFRvIII cells was tested by WB. B. Silencing of GLUT3 inhibited the proliferation of U87-EGFRvIII and LN229-EGFRvIII by clone formation experiment. C. CCK-8 results showed that inhibition of GLUT3 reduced the proliferation of U87-EGFRvIII and LN229-EGFRvIII cells. D. Representative images of H&E staining after GLUT3 knockdown in U87-EGFRvIII cells were listed and the tumor mass was calculated as (W2 x L)/2. Original magnification: 40×; scale bar: 100 μm. E. IHC analysis of Ki67 indicated that knockdown of GLUT3 inhibited the proliferation of U87-EGFRvIII cells. Original magnification: 40×; scale bar: 100 μm. Mean ± SD. Data represented two or three independent experiments with similar results. **P<0.01 and ***P<0.001, paired Student's t-test, compared with parental or EGFRvIII cells or tumours treated with sh-NC.

of GLUT3 (**Figure 6B**). Further CHIP assay indicated that inhibition of SOX9 reduced the protein of GLUT3 (**Figure 6C**), while the up-regulation of SOX9 promoted the protein of GLUT3 (**Figure 6D**) in U87-EGFRvIII and LN229-EGFRvIII. In addition, the promoter sequence of GLUT3 was further constructed, and the luciferase reporter assay revealed that after transfection of U87-EGFRvIII and LN229-EGFRvIII cells with si-SOX9, the promoter fluo-

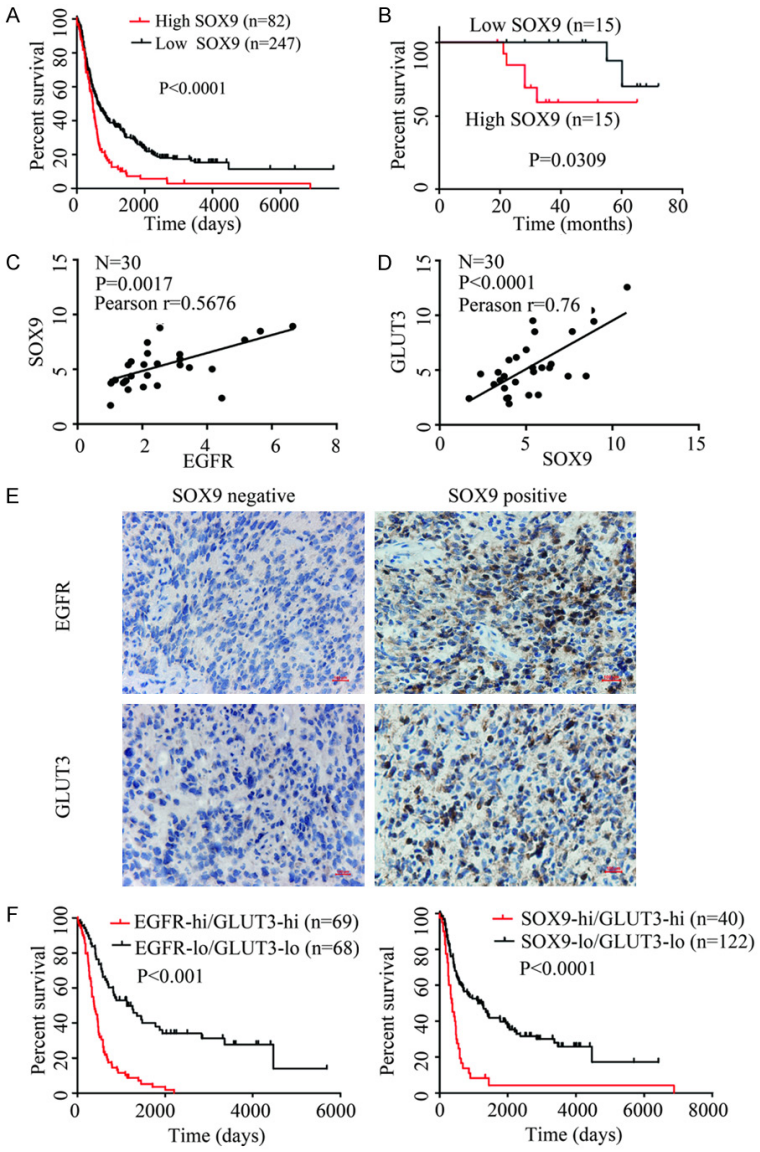

Figure 4. SOX9 was positively co-expressed with EGFR and GLUT3, and was correlated with worse overall survival. (A and B) Elevated expression of SOX9 was correlated with worse overall survival in glioblastoma according to Rembrandt database analysis (A) and our collected samples (B). (C and D) RT-PCR results showed that SOX9 was positively co-expressed with EGFR and GLUT3 at mRNA level. (E) Compared with SOX9-negative tissues (n=3), the expression of EGFR and GLUT3 was higher than that in SOX9-positive tissues (n=3). Original magnification: 40×; scale bar: 100 μm. (F) When EGFR/GLUT3 or GLUT3/SOX9 were simultaneously highly expressed, the prognosis for patients with glioblastoma was worsened.

rescence of GLUT3 was decreased (**Figure 6E**), and after transfection of U87-EGFRvIII and LN229-EGFRvIII cells with different concentrations of plasmids overexpressing SOX9, the promoter fluorescence of GLUT3 was increased in a dose-dependent manner (**Figure 6F**). In conclusion, EGFR/EGFRvIII partly promoted the expression of GLUT3 by the binding of SOX9 to the promoter of GLUT3 (**Figure 6G**).

Discussion

In this work, we identified the crucial role of GLUT3 in the tumourigenesis of EGFRvIII-driven glioblastoma *in vitro* and *in vivo*. Mechanistically, the sensitization of SOX9 was triggered by EGFRvIII, and SOX9 further promoted GLUT3 expression by binding to the GLUT3 promoter.

It has been reported that compared with normal glial cells, the expression of GLUT3 is increased, which is one of the malignant features in glioblastoma cells [17]. To investigate whether EGFRvIII-driven glioblastoma is depended on GLUT3, we explored the regulation of GLUT3 by EGFRvIII and investigated the importance of GLUT3 on the progression of EGFRvIII glioblastoma through *in vitro* and *in vivo* experiments. Our results revealed increased GLUT3 expression in U87-EGFRvIII and LN229-EGFRvIII compared to controls. After silencing GLUT3 in U87-EGFRvIII cells, cell growth decreased substantially. However, the potential mechanism by which EGFGvIII regulates GLUT3 is still unknown, so we further explored it.

As reported by a previous study, EGFRvIII promoted the tumourigenesis of glioblastoma through SOX9- and FOXG1-dependent transcriptional regulatory networks [18]. We also concluded that EGFRvIII elevated SOX9. Generally, EGFRvIII regulates the cellular functions of glioblastoma through RTK pathways, such as the phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) pathways [9]. In oesophageal cancer, SOX9 activated the

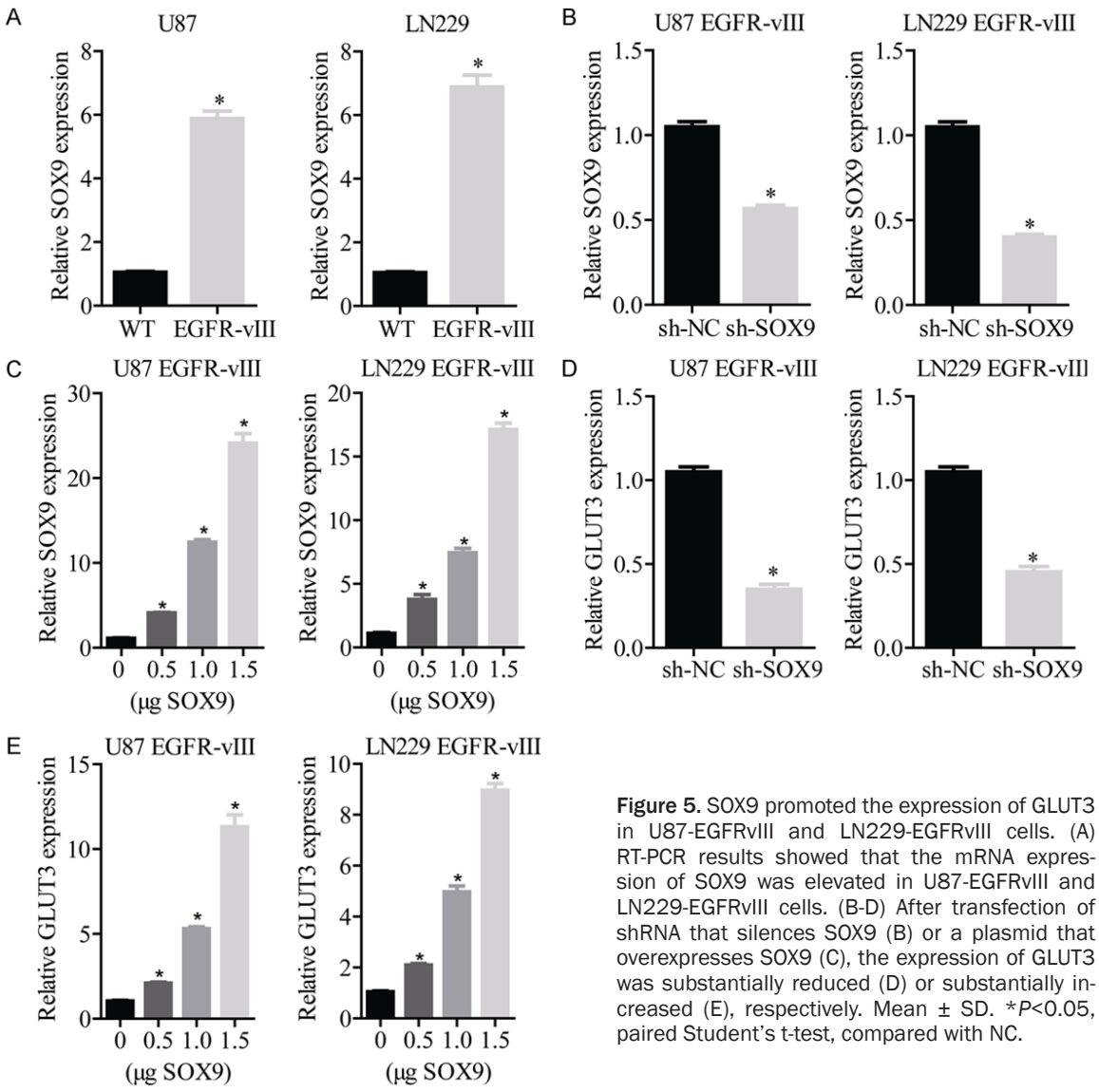

Figure 5. SOX9 promoted the expression of GLUT3 in U87-EGFRvIII and LN229-EGFRvIII cells. (A) RT-PCR results showed that the mRNA expression of SOX9 was elevated in U87-EGFRvIII and LN229-EGFRvIII cells. (B-D) After transfection of shRNA that silences SOX9 (B) or a plasmid that overexpresses SOX9 (C), the expression of GLUT3 was substantially reduced (D) or substantially increased (E), respectively. Mean ± SD. **P*<0.05, paired Student's t-test, compared with NC.

PI3K/AKT through the miR-203a axis [19]. Consistently, inhibition of the PI3K/Akt by miR-605 depended on the downregulation of SOX9 expression in glioblastoma [20]. Therefore, by promoting the expression of SOX9, EGFRvIII further activated the PI3K/AKT. Inhibition of the PI3K signalling pathway led to decreased expression of SOX9 in fibroblasts [21]. In addition, the hinder of the PI3K/AKT could also inhibit the expression of SOX9 in NP cells [22]. Therefore, we speculate that there is a positive feedback loop among the EGFRvIII, SOX9 and PIAK/AKT pathways in glioblastoma, and these meaningful mechanisms need to be further confirmed.

It is well known that as a member of the transcription factor family [12], SOX9 contains super enhancers and regulates the expression of target genes [13]. Therefore, we speculated that SOX9 might be essential for the regulation of GLUT3 by EGFRvIII. On this basis, RT-PCR, luciferase reporter and CHIP assays were conducted, and the results showed that SOX9 bound to the promoter and promoted the expression of GLUT3. As shown in **Figure 2A** and **2B**, EGFR was substantially up-regulated in U87-EGFRvIII and LN229-EGFRvIII compared with wild-type U87 and LN229 cells. A previous study reported that EGFR was activated in wild-type hair follicle stem cells labelled with SOX9 [23]. It is suggested that EGFRvIII might promote the expression of EGFR by activating SOX9, which requires further verification in glioblastoma. In dedifferentiated mouse acinar

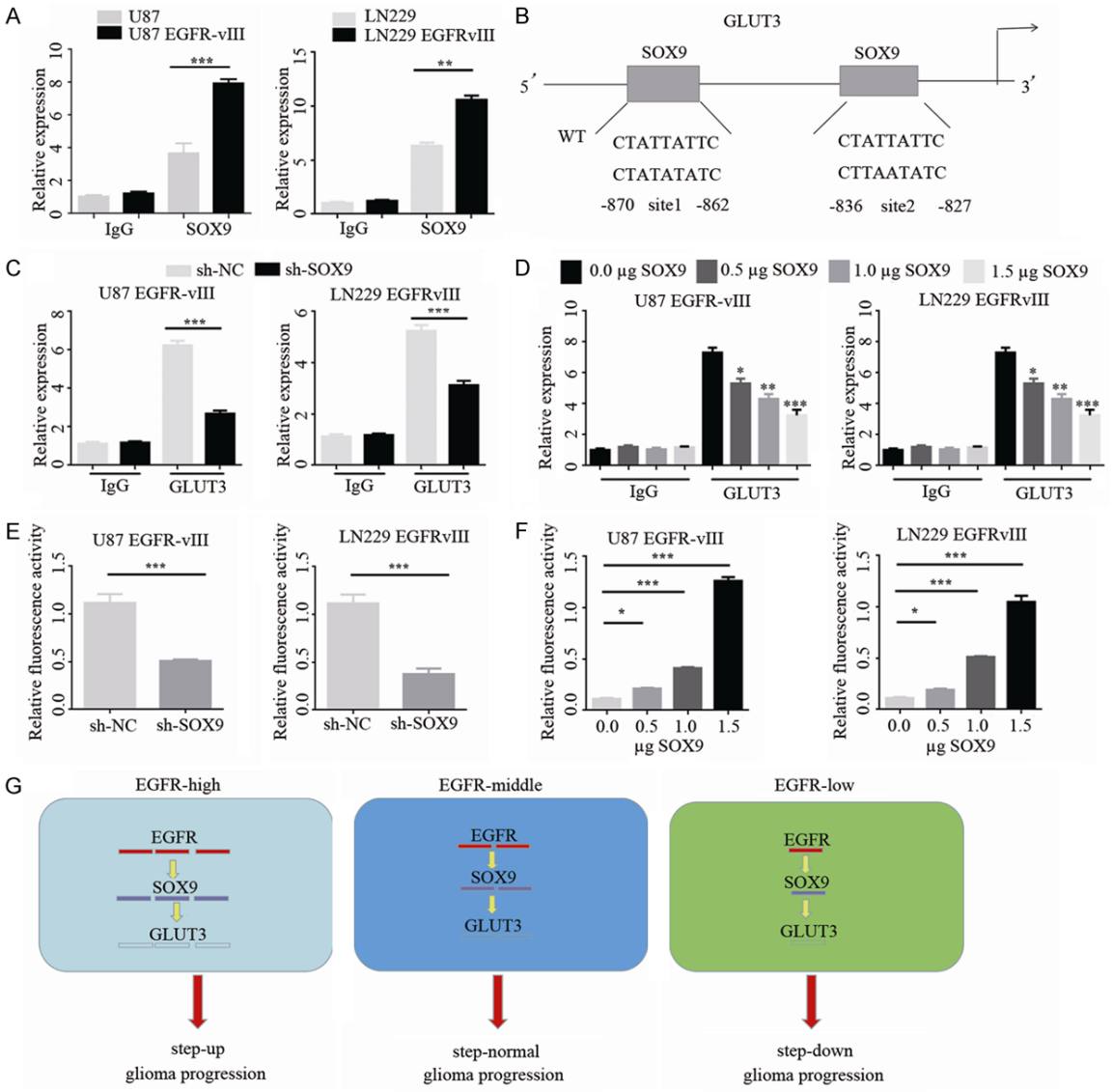

Figure 6. EGFR/EGFRvIII promoted the expression of GLUT3 through the combination of SOX9 and the GLUT3 promoter. (A) EGFR/EGFRvIII promoted the expression of SOX9, as detected by CHIP (D) assay. (B) There were binding sites between SOX9 and the promoter area of GLUT3 through bioinformatics analysis. (C and D) Inhibition of SOX9 reduced the expression of GLUT3 (C), while up-regulation of SOX9 promoted the expression of GLUT3 (D), as detected by CHIP assays. (E and F) Silencing SOX9 reduced the expression of GLUT3 (E), while overexpression of SOX9 promoted the expression of GLUT3, as detected by luciferase reporter assays. (G) The potential workflow of EGFR/EGFRvIII in glioblastoma was listed. Mean ± SD. ***P<0.001. P values were calculated using one-way ANOVA. $EGFR^{high}$ represents EGFR/EGFRvIII high-expression and $EGFR^{low}$ represents EGFR/EGFRvIII low-expression.

cells, EGFR promoted the formation of the NFATc1 and C-JUN complex, which led to SOX9 transcriptional activation [24]. EGFR, GLUT3 and SOX9 were positively expressed in our collected thirty glioblastoma specimens, suggesting that EGFR might upregulate GLUT3 through SOX9. Based on the above, we speculated that EGFRvIII promoted the transcriptional activation of EGFR through SOX9 and promoted the expression of GLUT3 by forming a positive feedback loop (EGFRvIII-SOX9-EGFR-SOX9). However, there are some limitations in our research. We have not studied the epithelial-mesenchymal transition, metastasis and drug resistance of tumor cells. We hope to solve the above problems in our follow-up research to improve our research conclusions.

In conclusion, this study elucidated that the SOX9-GLUT3 axis is a potential mechanism by

which EGFR/EGFRvIII promotes glioblastoma cell vitality. However, due to the limitations of experimental funds and conditions, whether a positive feedback loop EGFRvIII-SOX9-EGFR-SOX9 exists to regulate the cell vitality of glioblastoma remains to be further explored.

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Disclosure of conflict of interest

None.

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