TNFAIP8 in Mesenchymal Glioblastoma Correlates with Metabolic Dysfunction and Poor Prognosis

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Abstract

TNFAIP8, a cytosolic TNF-α-inducible oncogenic molecule, plays pivotal roles in many types of cancers. However, the effect of TNFAIP8 on glioma remains elusive. In this study, we confirmed that TNFAIP8 promoted glioma viability and motility through alteration of nucleotide metabolism and tricarboxylic acid (TCA) cycle. Firstly, bioinformatic analysis indicated that TNFAIP8 expression elevated along with the WHO grade and predicted adverse prognosis in gliomas. Moreover, TNFAIP8 was enriched in gliomas with progressive genotypes, serving as an efficient indicator for mesenchymal glioma. To determine its function in glioblastoma progression, cell viability, migration and invasion were examined by loss/gain of function experiments. TNFAIP8 promoted glioma proliferation, migration, invasion and transition to mesenchymal phenotype. These findings were further confirmed *in vivo*. RNA sequencing and liquid chromatography-mass spectrometry (LC-MS) results showed impaired nucleotide metabolism and TCA cycle after TNFAIP8 knockdown, implying that TNFAIP8 enhanced glioblastoma progression *via* metabolic dysfunction. In summary, this study identified a novel oncogene in glioma with great potential for prognostic prediction and subtype identification. Alterations in metabolic process and transition towards mesenchymal subtype were supposed to be the underlying mechanisms for the oncogenic function of TNFAIP8.

Introduction

Glioma, the most common and lethal primary brain tumor, have distinct molecular alterations and marked heterogeneity in their histopathological appearances and clinical courses(1). Glioblastoma (GBM) with different biomarkers or by different profiling might even share totally different prognosis. Many endeavors were made to advance the integrative subtype profiling of GBM, on the attempt to improve the efficacy and efficiency for distinguishing subcategories among GBM. Molecular signatures including MGMT methylation, H3F3A mutation(2), and tumor-intrinsic transcriptional subtypes (proneural, mesenchymal, and classical) have been widely utilized in GBM classification schemes(3). Specific molecular events in gliomas like EGFR amplification, MYB alteration, MAPK pathway alteration, have shown their significance according to WHO CNS5(1). Our previous analysis found GBM with Olig2 or CD276 could share totally different prognosis, and a gene cluster featured with hub genes of Olig2 and CD276 could distinguish molecular subtypes in GBM (4). Interestingly, tumor necrosis factor-α-induced protein 8 (TNFAIP8) was superiorly defined as one of the independent factors for prognosis in GBM, which favored mesenchymal subtype (4).

TNFAIP8 family is a death effector domain (DED)-containing protein family, consisting of TNFAIP8, TIPE1, TIPE2 and TIPE3(5, 6). TNFAIP8 (aka SCC-S2, GG2-1, MDC-3.13) is a cytosolic antiapoptotic and oncogenic molecule(7, 8). TNFAIP8 expression could be upregulated by TNF-α stimulation and NF-κB activation, both of which are required to enhance cancer cell survival by reducing apoptosis through the inhibition of caspase-8 (9). In the meantime, translational products of P53 with a non-hotspot mutation was reported to upregulate TNFAIP8(10), which means that it might serve as a tumor-promoting molecule and a potential biomarker of tumor malignancy. It has been validated of its regulatory role in tumor

generation and progression (11, 12), as TNFAIP8 is highly expressed in neoplasms including cervical cancers(13), ovarian cancers(14, 15), breast cancers(7, 16), gastric cancers(17, 18), pancreatic cancers(19) and endometrial cancers(20). Although TNFAIP8 has been proved oncogenic in various types of malignant tumors, its function in glioma remained elusive yet.

In this study, the clinical significance and its function of TNFAIP8 in GBM were investigated. TNFAIP8 was highly expressed in GBM tissues and could be utilized as an indicator of mesenchymal subtype. In addition, we verified that abrogation of TNFAIP8 could suppress GBM cell viability and motility *in vitro* and hinder xenografted tumor growth *in vivo*. These alterations in malignant behavior were resulted from dysregulation of nucleotide metabolism and dysfunction of tricarboxylic acid (TCA) cycle. In general, these results suggested that TNFAIP8 could serve as a biomarker for mesenchymal GBM and a potential therapeutic target.

Materials And Methods

Patients

Paraffin-embedded glioma tissues (WHO grades 2-4) were collected from patients (n = 90) who received glioma resection surgery in Huashan Hospital, Fudan University. Normal brain tissue samples (n = 6) were taken from patients who underwent partial brain resection as decompression treatment. The informed consent was obtained from all patients and relatives.

Dataset and bioinformatics analysis

Clinical information and gene expression data for glioma patients in the TCGA, CGGA databases were downloaded from GlioVis website (http://gliovis.bioinfo.cnio.es/)(21). The nomogram and calibration plots were constructed using the RMS package of R software. Gene ontology (GO) analyses were performed to verify the biological processes using the R package of enrichplot and clusterProfiler(22). Metabolite enrichment was performed by MetaboAnalyst5.0 (https://www.metaboanalyst.ca/home.xhtml). The related biological processes were visualized by Cytoscape 3.7.2.

Cell culture and lentivirus transfection

The glioma cell lines U251, U87 were purchased from the Chinese Academy of Sciences Cell Bank. Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (HyClone, GE Healthcare Life Sciences) in 5% CO2 in a humidified incubator at 37°C. Short hairpins sh-TNFAIP8#1 and sh-TNFAIP8#2 (5'-CCATCGCCACCACCTTAAT-3' and 5'- GGCCATTCTTTATAGGAAT-3') were ligated to the lentiviral vector pLKD-LUC-Puro (ObiO, Shanghai, China). U87 cells were infected with the shRNA lentiviruses. After 48h, the medium was replaced with fresh medium containing 2 μg/mL puromycin (Thermo Fisher Scientific) for stable cells selection. Plasmid construction of GV141-TNFAIP8 was performed and U251 glioma cells

were transfected with GV141-TNFAIP8 to induce the overexpression of TNFAIP8 and with empty GV141 vector (GV141) as a control. Western blot assay was performed to evaluate transfection efficiency.

Western blot analysis

Cells and glioma tissues were lysed with RIPA lysis buffer (Thermo Fisher Scientific, MA, USA) supplemented with the protease and phosphatase inhibitor cocktail (Sigma-Aldrich, MO, USA). Protein lysates (equal amounts) were electrophoresed on SDS-PAGE and transferred onto PVDF membranes (Merck Millipore, MA, USA). The membrane was blocked with skimmed milk for 1 h, and then incubated with primary antibodies overnight at 4℃. In the second day, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (ZSGB-BIO; Beijing, China) for 1 h at room temperature. The membranes were visualized with ECL (Merck Millipore, MA, USA), and β-actin was used as a loading control. The primary antibodies were used as follows: TNFAIP8 (abcam; ab195810); E-Cadherin (CST; #3195); N-Cadherin (CST; #13116); Vimentin (CST; #5741); β-Actin (CST; #3700).

Cell viability assay

The Cell Counting Kit-8 (CCK-8) was performed to assess the cell viability following the manufacturer's instructions (YEASEN, Shanghai, 40203ES60). Briefly, U87 or U251 cells were incubated in 96-well plates for 24, 48, and 72 h. The CCK-8 solution (10 μL) was added to each well and the absorbance at 450 nm wavelength (OD450) was measured in a Microplate Reader (Bio-Rad) after incubation for 1 h at 37 °C.

Luciferase-based ATP assay

Luminescent ATP detection assay kit (Promega; G7572) was used to measure ATP production. In brief, cultured glioma cells and ATP standard dilution series were prepared. Then add D-Luciferin and firefly luciferase reagents into the reaction mix and incubate for 10 min. Luminescence signal was quantified using the Glomax multi-detection system (Promega, Wisconsin, USA). The luminescence was proportional to the ATP production as an index of cell number.

Transwell assay

Glioma cells were added to the top chamber in serum-free media. For invasion assays, the membrane was precoated with Matrigel. The bottom chamber was filled with DMEM containing 10% FBS. After incubation, the membrane was fixed in 4% paraformaldehyde for 15 min after removing the cells in the top chamber and stained with crystal violet for 15 min. Photographs of three randomly selected fields of adherent cells were taken and cell number were counted.

Metabolite Extraction and LC-MS/MS quantification for metabolites participating in nucleotide metabolism and TCA cycle

Intracellular level of metabolites participating in nucleotide metabolism and TCA cycle were measured by liquid chromatography-mass spectrometry as described before(23). Briefly, cells were washed with ice-

cold PBS twice and harvested in 80% pre-cold (−80℃) methanol and centrifuged at 4℃ at 14,000 rpm for 30 min. The supernatant was lyophilized and then was resuspended in reconstitution fluid (ACN: H2O = 5:95), mixed well and centrifuged for 15 min at 14,000 rpm at 4℃. The supernatant was then subjected to LC-MS/MS analysis (Waters ACQUITY UPLC R I-Class system coupled with Xevo R TQXS mass spectrometry). Separation of the metabolite was conducted in Waters ACQUITY UPLC HSS T3 column. The Analyst Software MassLynx v4.2 was used for analysis.

Xenograft models

To establish the intracranial gliomas, U87 cells (1×10^6) transfected with lenti-pLKD-LUC-Puro-shTNFAIP8#1 or lenti-control virus were stereotactically implanted into the brains of 4-week-old nude mice (SLAC Laboratory Animal Center, Shanghai, China). Bioluminescence imaging was performed to measure intracranial tumor growth. Kaplan-Meier survival curves were plotted according to the survival time. When the mice were moribund or cachectic, the brain tumor tissue were harvested, fixed in 10% formalin, embedded in paraffin, cut into sections for HE staining.

Statistical analysis

Data analysis was performed and visualized using GraphPad Prism or R software. Each experiment was performed at least in three times, and all results are presented as the means±SD. Comparisons between groups were made with Student t test to assess statistical significance. The data were considered significant with the following P values: * P value < 0.05, ** P value < 0.01, *** P value < 0.001, and **** P value < 0.0001, P values > 0.05 were considered not significant and are denoted by “ns”.

Results

TNFAIP8 overexpression could serve as an indicator of mesenchymal subtype

Significant higher expression of TNFAIP8 was discovered in GBM compared with lower grade gliomas (LGG) according to the Cancer Genome Atlas (TCGA) and Chinese Glioma Genome Atlas (CGGA) database (LGG VS GBM, all P<0.0001, **Fig. 1A, left two panels**). Immunohistochemical (IHC) staining (**Fig. 1C-D**) for clinical samples showed that TNFAIP8 was predominantly expressed in nucleus in glioma cells. Immune reactive score (IRS) analysis for IHC verified obvious higher expression of TNFAIP8 in GBM (mean IRS = 9.200, n=30) than in grade 2 (mean IRS = 3.917, n=24, P= 0.0001) and 3 (mean IRS = 6.472, n=36, P=0.0004) gliomas, which was in line with bioinformatic findings. In addition, western blot assay also illustrated that TNFAIP8 expression was markedly upregulated in GBM (**Fig. 1E**).

In the meantime, an elevation of TNFAIP8 level was observed in IDH1 wild type GBM compared with their mutant type counterparts, though the differences in TCGA failed to reach the statistical significance (TCGA P=0.0535, CGGA P<0.0001, **Fig. 1A, middle panels**). Besides, TNFAIP8 mRNA level among proneural, neural, classical and mesenchymal subtype was compared and significantly increased TNFAIP8 was found in mesenchymal glioma (proneural, neural and classical VS mesenchymal, all

P<0.001, **Fig. 1A, right panels**). These results established an association between high expression of TNFAIP8 and mesenchymal subtype. The specificity and sensitivity of TNFAIP8 in indicating mesenchymal subtype were evaluated. As expected, the area under curve (AUC) was 0.955 and 0.888 in CGGA and TCGA, respectively, suggesting that TNFAIP8 could serve as an indicator for mesenchymal glioma (**Fig. 1B**). In general, TNFAIP8 played a vital role in tumorigenesis and development of glioma. TNFAIP8 was highly expressed in GBM and could be used as an indicator of mesenchymal subtype.

TNFAIP8 could predict adverse prognosis for glioma patients

Glioma patients in TCGA or CGGA cohorts were divided into two subgroups according to TNFAIP8 mRNA abundance and higher expression of TNFAIP8 was correlated with shortened survival in both GBM and LGG glioma patients (TCGA LGG P<0.0001, GBM P<0.05, CGGA LGG P<0.001, GBM P<0.05, **Fig. 2A**). In addition, similar association was found when astrocytoma and oligodendroglioma was analyzed separately (TCGA astrocytoma P<0.001, oligodendroglioma P<0.001, CGGA astrocytoma P<0.05, oligodendroglioma P<0.05, **Fig. 2A**). Multivariate (HR = 1.81, P = 0.007) and univariate (HR = 4.41, P < 0.0001) Cox regression analysis was performed using the patient clinical characteristics including age, gender, WHO grade, IDH status and therapeutic regimen (chemotherapy and radiotherapy, **Table1**). The prognostic nomogram with a risk classification system for 1-, 3- and 5-year survival rates of glioma was established based on TCGA database (n = 545, **Fig. 2B**). We found that TNFAIP8 was a robust and independent prognostic biomarker for evaluating patient outcomes (**Fig. 2B**). The calibration curves showed optimal conformity between the prediction by nomogram and actual observation in TCGA database (**Fig. 2C**). To sum up, TNFAIP8 was confirmed to serve as an unfavorable predictor for survival in glioma patients.

TNFAIP8 could enhance viability and motility in GBM cells

To explore the function of TNFAIP8 on glioma *in vitro*, loss/gain of function experiments were employed in GBM cell lines. We searched Cancer Cell Line Encyclopedia derived from Betastasis (https://betastasis.com/) for TNFAIP8 mRNA expression in different GBM cell lines. Discrepant TNFAIP8 mRNA level was found in various GBM cell lines, among which U87, U138, U118 and T98 cells highly expressed TNFAIP8, whereas U251 GBM cells exhibited lowest abundance of TNFAIP8 (data not shown). We thereafter utilized U87 and U251 cell lines for TNFAIP8 knockdown and overexpression, respectively (**Fig. 3A**). Results for CCK8, ATP production and colony formation assays uniformly demonstrated that overexpression of TNFAIP8 enhanced cell proliferation in GBM cells and *vice versa*, suggesting a proliferation-promoting role for TNFAIP8 (all P<0.01, **Fig. 3B-D**). The elevated migration and invasion ability is one of the most significant biological features of GBM(24). Herein, TNFAIP8-knockdown in GBM cells significantly hampered migration and invasion while TNFAIP8-overexpression boosted cell motility (migration assay, all P<0.001, invasion assay, all P<0.01, **Fig. 3E**). Moreover, TNFAIP8-overexpression elicited upregulation of N-cadherin, vimentin and decreased the expression of E-cadherin, implying an occurrence of epithelial-to-mesenchymal transition process (**Fig. 3F**). In summary, we demonstrated that TNFAIP8 promoted proliferation, migration and invasion in GBM cells.

Knockdown of TNFAIP8 could affect nucleotide metabolic process and TCA cycle

RNA sequencing was performed for U87 GBM cells transfected by sh-TNFAIP8.
KEGG pathway enrichment analysis for differential expressed genes (DEGs; 3098 upregulated DEGs and 4493 downregulated DEGs) in U87-shTNFAIP8 cells illustrated that TNFAIP8 was involved in multiple biological pathways related to metabolism, cell cycle, transcriptional regulation, cell polarity and cell junction, most of which were potentially associated with tumor progression (**Fig. 4A-B**). Since metabolic process accounted for a considerable proportion among top twenty altered pathways, mass spectrum was performed to examine metabolite changes after TNFAIP8 knockdown. In line with the results for RNA sequencing, metabolites relevant to purine (IMP, xanthine, allantoin) and pyrimidine (orotate, UMP, cytidine) metabolism and TCA cycle (citrate/isocitrate, orotate, malate) were drastically affected by TNFAIP8 (all $P<0.05$, **Fig. 4C-D**), implying that aberrant nucleotide metabolism and dysfunction of TCA cycle potentially contributed to TNFAIP8-associated poor outcomes in glioma patients.

TNFAIP8-knockdown could impair GBM growth *in vivo*

Xenografted models were established orthotopically in nude mice to further validate tumor promoting effect of TNFAIP8. U87 cells knocking down TNFAIP8 were injected intracranially. Consistent with the above findings, tumor burden of mice in shTNFAIP8 group was much smaller than that in control group ($P<0.01$, **Fig. 5A-B**). In the meantime, TNFAIP8 knockdown significantly prolonged the life span of nude mice (median survival time, vector group=33 days, sh-TNFAIP8-1 group=52 days, $P<0.0005$, **Fig. 5C**). Additionally, HE staining for xenografted GBM samples displayed smaller tumor volume in TNFAIP8-knockdown groups (**Fig. 5D**). Altogether, these findings suggested that TNFAIP8-knockdown impaired GBM growth *in vivo*.

Discussion

Glioma remained an incurable disease despite progression in therapeutic regimen. The prognosis of glioma varies by molecular subtype, tumor grade, performance status and age(1). However, there is a large variability in overall survival that cannot be explained by these prognostic factors(25, 26). In the current study, we found that higher expression of TNFAIP8, an oncogene defined in other types of tumors, indicated unfavorable outcomes in both GBM and LGG patients. TNFAIP8 could therefore serve as a novel and independent prognostic biomarker for glioma.

TNFAIP8 plays a pivotal role in regulation of a variety of tumors and inflammatory diseases(27). Kumar et al. first identified a partial cDNA clone for TNFAIP8 which amplified in a metastatic and radioresistant head and neck squamous cell carcinoma (HNSCC)-derived cell line(8), endorsing its importance in cell survival, tumor growth and progression. TNFAIP8 transcript was detectable in most human normal tissues, yet its expression level was extremely low in normal adult brain(8). Likewise, our current findings illustrated that TNFAIP8 positive cells were seldom found in normal brain tissue but widely distributed in gliomas. Moreover, the proportion of TNFAIP8 positive cells increased drastically along with the elevation of WHO grade. Previous researches have proved that expression of TNFAIP8 was induced by TNF-α

through nuclear translocation of a NF-κB(8, 9). Of note, TNF-α/NF-κB pathway is notorious for its pro-glioma effects(8, 9), leading to aberrant cell viability and motility(28), immunocompromise(28) and mesenchymal transition(29). These facts, along with our findings, determined TNFAIP8 as an oncogene in glioma.

Regarding the intrinsic gene signature, GBM is classified into three subtypes namely proneural, mesenchymal and classical subtypes, among which the mesenchymal tumor (MES) constitutes 34% of GBM samples in TCGA dataset and tends to have the worst survival rates compared to other subtypes(30). In this study, TNFAIP8 was found to enable the stratification for glioma regarding to their molecular subtypes. MES glioma exhibited higher expression of TNFAIP8 compared with their non-MES counterparts. We thereby determined TNFAIP8 as a critical indicator for mesenchymal glioma. TNFAIP8 promoted GBM migration, invasion and proliferation, implying a transition towards MES subtype. Moreover, other researches have illustrated that TNFAIP8 exerts pro-angiogenic, anti-radiotherapy and immune suppressive effects in tumors(11, 27). Considering that malignant behavior such as resistance to therapy, angiogenesis, increased motility and viability or recruitment of immune suppressive cells were hallmarks for MES GBM(31, 32), it is reasonable to propose that TNFAIP8 exerted oncogenic effects through induction of MES phenotype in GBM. Indeed, an elevation of mesenchymal markers (N-cadherin and vimentin) was observed after TNFAIP8 overexpression in this study, although we preferred to consider these alterations as evidences of epithelial-mesenchymal transition (EMT). It is reported that phenotypic transition from proneural/classical to mesenchymal subtype were molecular events similar to EMT(33). Therefore, induction of N-cadherin and vimentin was another evidence for MES transition triggered by TNFAIP8.

Cancer cells exhibit altered metabolic pathways to meet the needs of biosynthesis and reduction-oxidation during tumor progression(34). Nucleotide metabolism, including purine metabolism and pyrimidine metabolism, is critical for DNA replication and RNA production in rapid growing tumors such as GBM(35). With regard to energy metabolism, Warburg et al. noted that glycolysis was often enhanced in cancers despite the presence of abundant oxygen, leading to a switch in means of energy generation from oxidative phosphorylation (OXPHOS) to glycolysis(36-38). However, emerging evidences suggest that energy metabolism in some cancers extends beyond the Warburg effect and exists an intact mitochondrial TCA cycle(39-41). For example, lactate, the by-product of the Warburg effect, was reported to enhance the self-renewal and mitochondrial respiration in glioma stem cells via the acidification of tumor microenvironment(42). Thus, the significance of TCA cycle in tumor energy metabolism as well as in tumor progression has been reconsidered these years(43). In this context, the overall decline in TCA cycle-related metabolites represented drastic detriment in energy metabolism after TNFAIP8 knockdown. Recent studies have revealed that in some circumstances, activated TCA cycle and nucleotide metabolic process could concordantly exert an oncogenic effect in GBM(35). Consistently, we also found a simultaneous impairment of TCA cycle and nucleotide metabolism in TNFAIP8-abrogated GBM cells. The decrease of intermediate products in nucleotide metabolism suggested that TNFAIP8 possibly stimulated GBM progression *via de novo* purine and pyrimidine metabolic process. It is worth noting that IDH wildtype GBM cells were enriched for pathways involved in *de novo* nucleotide synthesis(44); our findings

might explain this phenomenon: IDH wildtype GBM are rich for TNFAIP8, which activates the purine and pyrimidine metabolic process and aggravates GBM malignancy eventually.

Taken together, this study identified a novel oncogene, TNFAIP8, in glioma with tremendous potential for prognostic prediction and subtype identification. Alterations in metabolic process and transition towards mesenchymal subtype were supposed to be the underlying mechanisms for the oncogenic function of TNFAIP8. However, there were still some points remained elusive. Our previous studies have paid great attention on D-2-hydroxyglutarate (D-2-HG), the metabolite closely associated with IDH mutation status in glioma(23). Considering that D-2-HG is a downstream molecule for α-ketoglutarate (α-KG), it is necessary to determine whether TNFAIP8 affects the expression of D-2-HG via TCA cycle. Therefore, further studies focused on a detailed molecular mechanism would help to explain the interactions among TNFAIP8, cell metabolites, mesenchymal markers and IDH mutant status in glioma.

Declarations

Data availability statement

The original data in this study are available from the corresponding author.

Ethics statement

The studies involving human participants were approved by the Human Ethics Committee of Huashan Hospital. The written informed consents of participant were obtained before study.

Author contributions

JZ designed and conducted the study, ZZ drafted the original manuscript. HZ, XX, MF, and YF helped collect the databases. QW helped conduct the animal study. WH supervised the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Tables

Table 1. Univariate and multivariate analysis of overall survival in the TCGA database

Variables	Univariate Analysis		Multivariate Analysis	
	HR (95%CI)	P	HR (95%CI)	P
Age (<45 vs >45)	1.02 (1.01-1.03)	<0.0001	1.02 (1.01-1.03)	0.003
Gender (female vs male)	1.07 (0.79-1.46)	0.66	-	-
WHO Grade	4.86 (3.78-6.26)	<0.0001	3.52 (2.64-4.72)	<0.0001
IDH1 (mutation vs wildtype)	2.78 (1.92-4.02)	<0.0001	1.46 (0.98-2.18)	0.06
Chemotherapy (yes vs no)	0.85 (0.61-1.19)	0.35	-	-
Radiotherapy (yes vs no)	0.81 (0.56-1.16)	0.26	-	-
TNFAIP8 (low vs high)	4.41 (3.08-6.31)	<0.0001	1.81 (1.17-2.78)	0.007

Figures

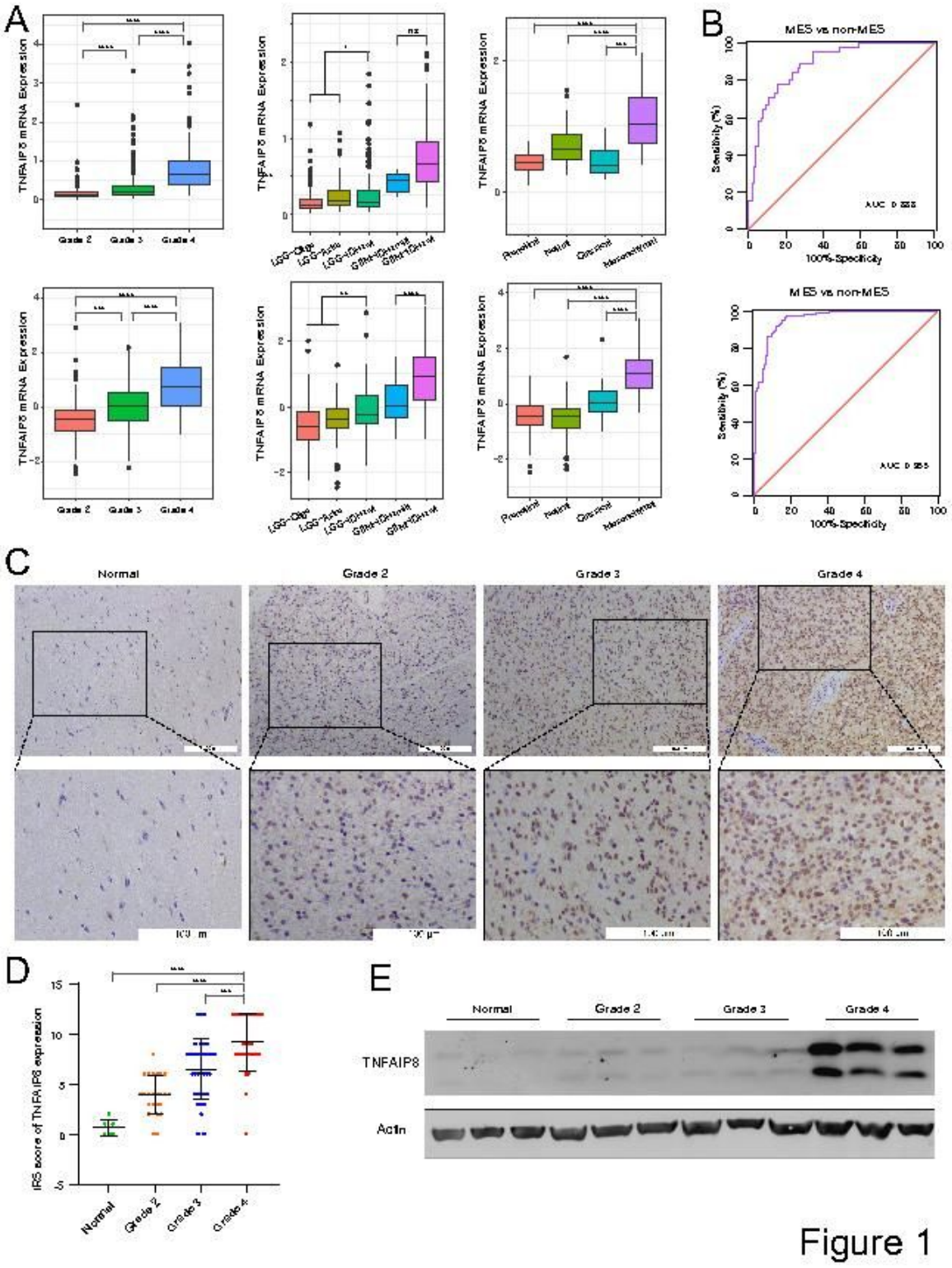

Figure 1

Figure 1

TNFAIP8 is enriched in GBM and associated with IDH-wild type status and mesenchymal subtype

(A) TNFAIP8 is highly expressed in GBM, IDH-wild type tumors or mesenchymal gliomas. The upper three box blots were results for TCGA dataset and the lower three for CGGA. Data are shown as the mean±SD, n(TCGA)=667, n(CGGA)=301, one-way ANOVA (*, P < 0.05). (B) Receiver operating characteristic (ROC)

curve for mesenchymal subtype prediction in TCGA (upper, AUC=0.888) and CGGA (lower, AUC=0.955) dataset. (C) and (D) IHC staining for TNFAIP8 in normal brain tissues or different grades of glioma samples (C). The quantification (D) for TNFAIP8 positive cells is shown in histogram. Data are shown as the mean±SD, n=96, one-way ANOVA (*, P < 0.05). (E) Western blot assays for TNFAIP8 expression in normal brain tissues or different grades of glioma samples. n=48.

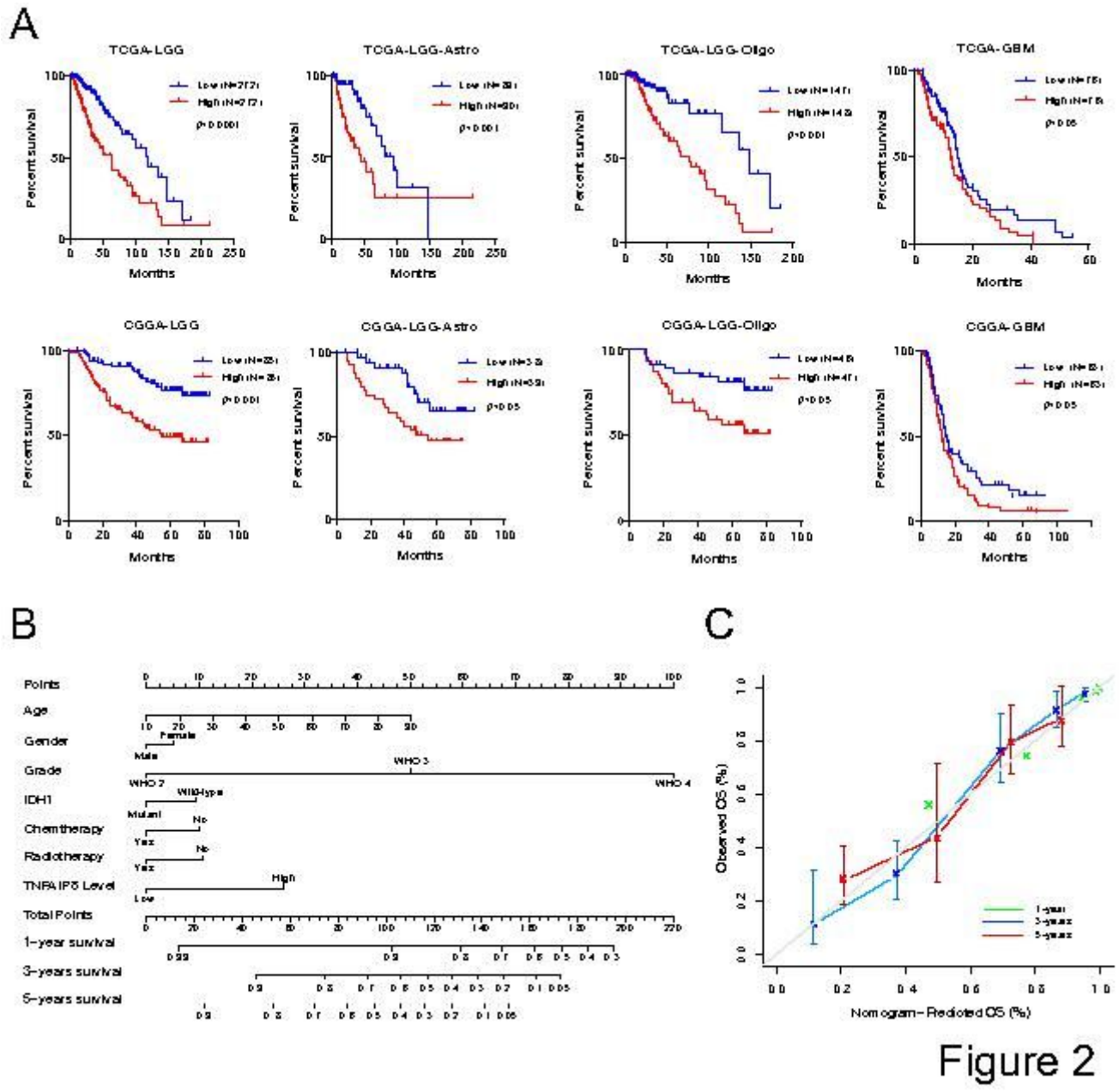

Figure 2

Figure 2

TNFAIP8 could predict adverse prognosis for glioma patients

(A) Glioma patient survival are significantly shortened in TNFAIP8-high groups. Gliomas from the TCGA and CGGA databases was separated into TNFAIP8-high and -low groups. The cut-off level was set at the median value of TNFAIP8 mRNA. n(TCGA-LGG) = 544; n(TCGA-GBM) = 152; n(CGGA-LGG) = 170; n(CGGA-

GBM) = 126. Log-rank test (*, P < 0.05). (B) Nomogram for predicting 1-, 3- or 5-year survival in glioma patients. The top row represents the point value for each variable. Rows 2–8 display the variables included in the nomogram. Each variable fits to a point value based on glioma characteristics. The Total Points axis equals to the sum of the point values, and the lines downward to the total points is used to establish the liability of 1-, 3- or 5-year survival. (C) Calibration curves for predicting patient survival in TCGA dataset at 1, 3 and 5 years.

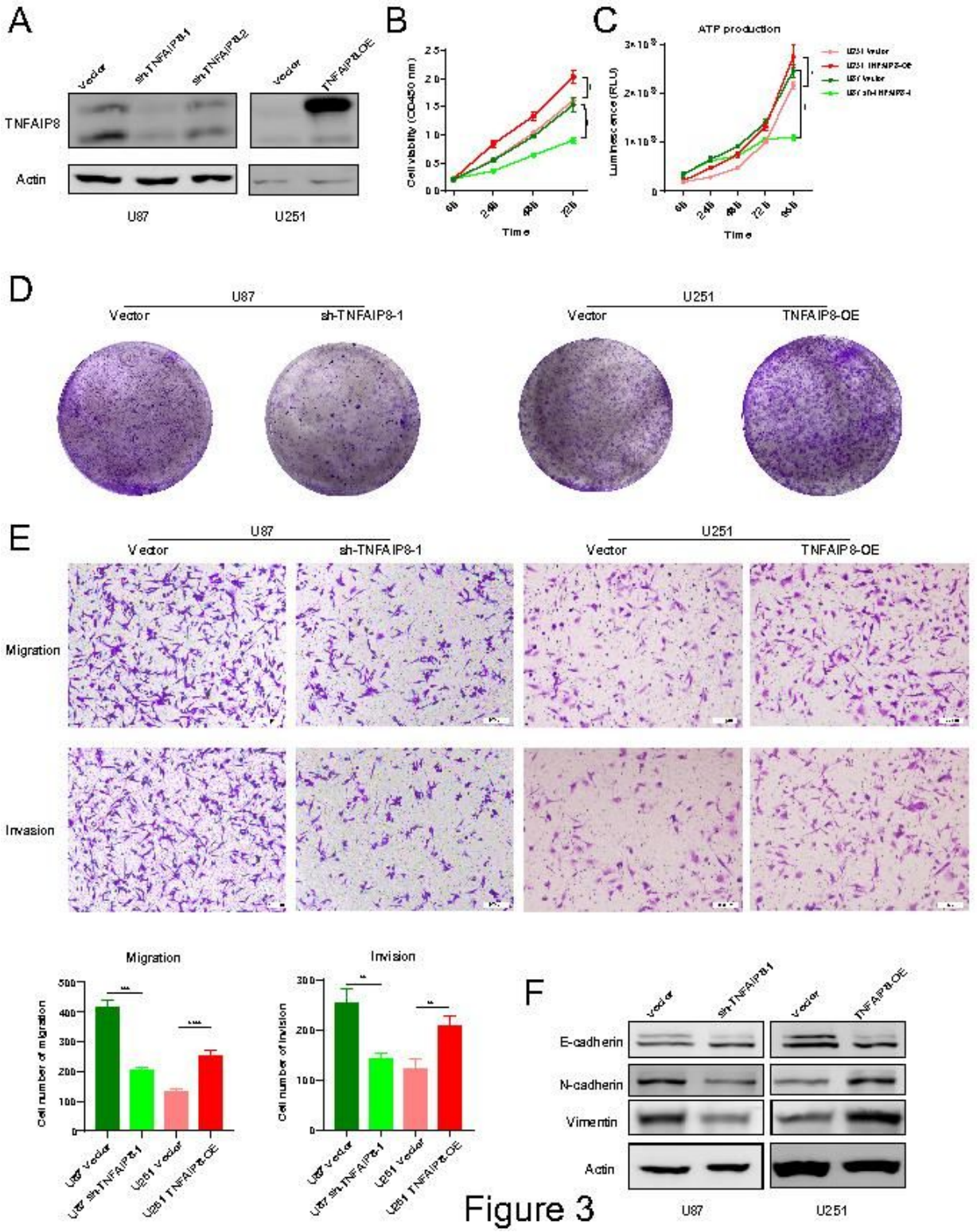

Figure 3

Figure 3

TNFAIP8 could promote glioma proliferation, migration and invasion

(A) TNFAIP8 protein level in U87 and U251 cell lines after TNFAIP8 knockdown or overexpression. n=3. (B) CCK8 cell proliferation assay for cells after TNFAIP8 knockdown or overexpression. Data are shown as the mean±SD, n=3, one-way ANOVA (*, P < 0.05). (C) ATP production assay for cells after TNFAIP8 knockdown or overexpression. Data are shown as the mean±SD, n=3, one-way ANOVA (*, P < 0.05). (D) Representative images for colonal formation after TNFAIP8 knockdown or overexpression. n=3. (E) Transwell migration and invasion assays for cells after TNFAIP8 knockdown or overexpression. Representative images and quantification are shown here. Data are shown as the mean±SD, n=3, one-way ANOVA (*, P < 0.05). (F) Western blot analysis for EMT-related protein after TNFAIP8 knockdown or overexpression. n=3.

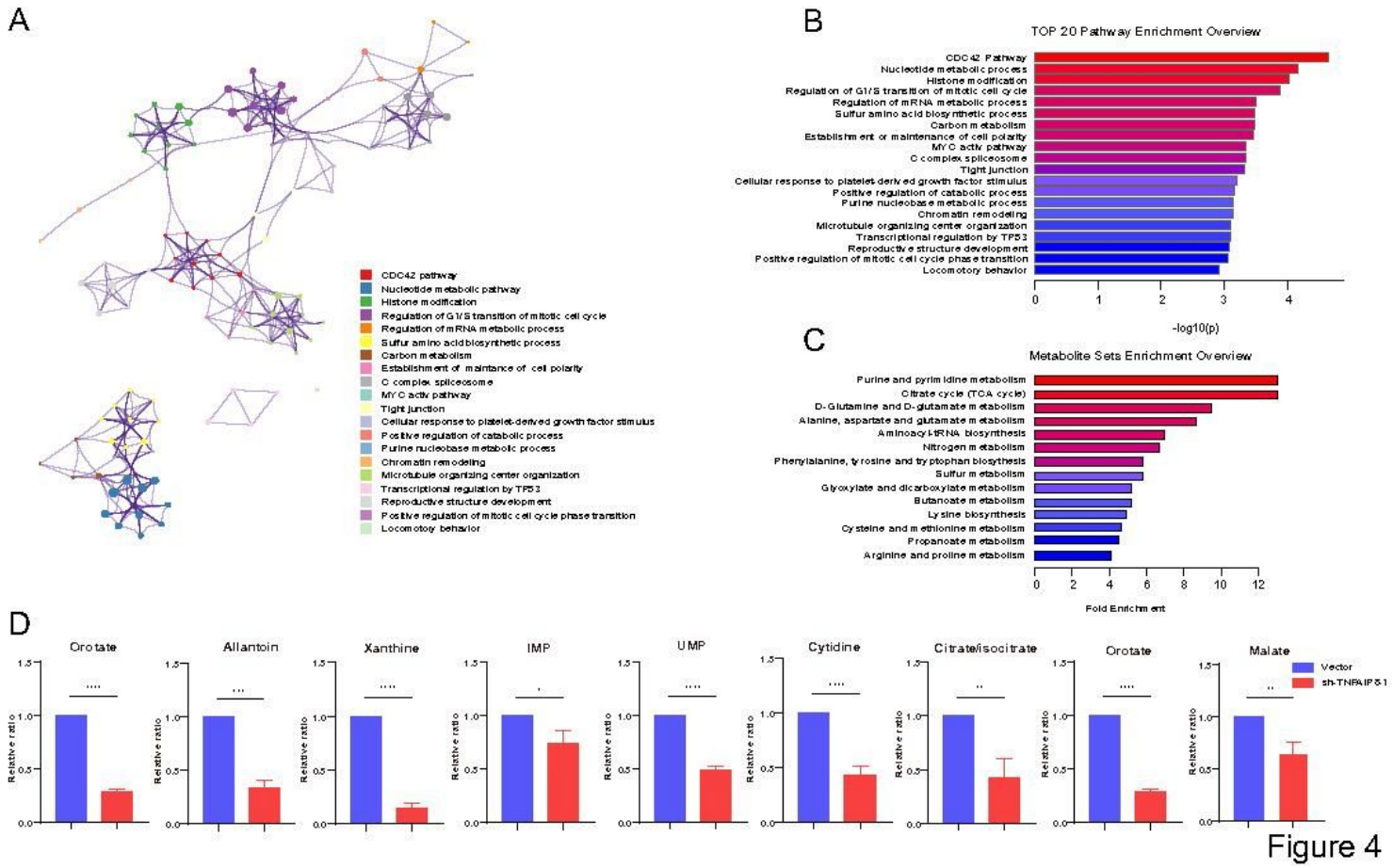

Figure 4

Figure 4

Knockdown of TNFAIP8 could affect nucleotide metabolic process and TCA cycle

(A) and (B) Top twenty affected pathways after TNFAIP8 knockdown. The cytoscape of enrichment map results are shown in (A). Nodes represent gene-sets, which were automatically arranged so that highly similar gene-sets are placed close together, and node size represents the number of genes in the gene-set.

Gene ontology (GO) analysis results are shown in (B). (C) Metabolites alterations after TNFAIP8 knockdown. LC-MS was performed after TNFAIP8 knockdown to detect altered metabolites. (D) Represented metabolites in nucleotide metabolic process and TCA cycle after TNFAIP8 knockdown. Expression of TNFAIP8 in control group is set to 1. Data are shown as the mean±SD, n=3, Student's t test (*, P < 0.05).

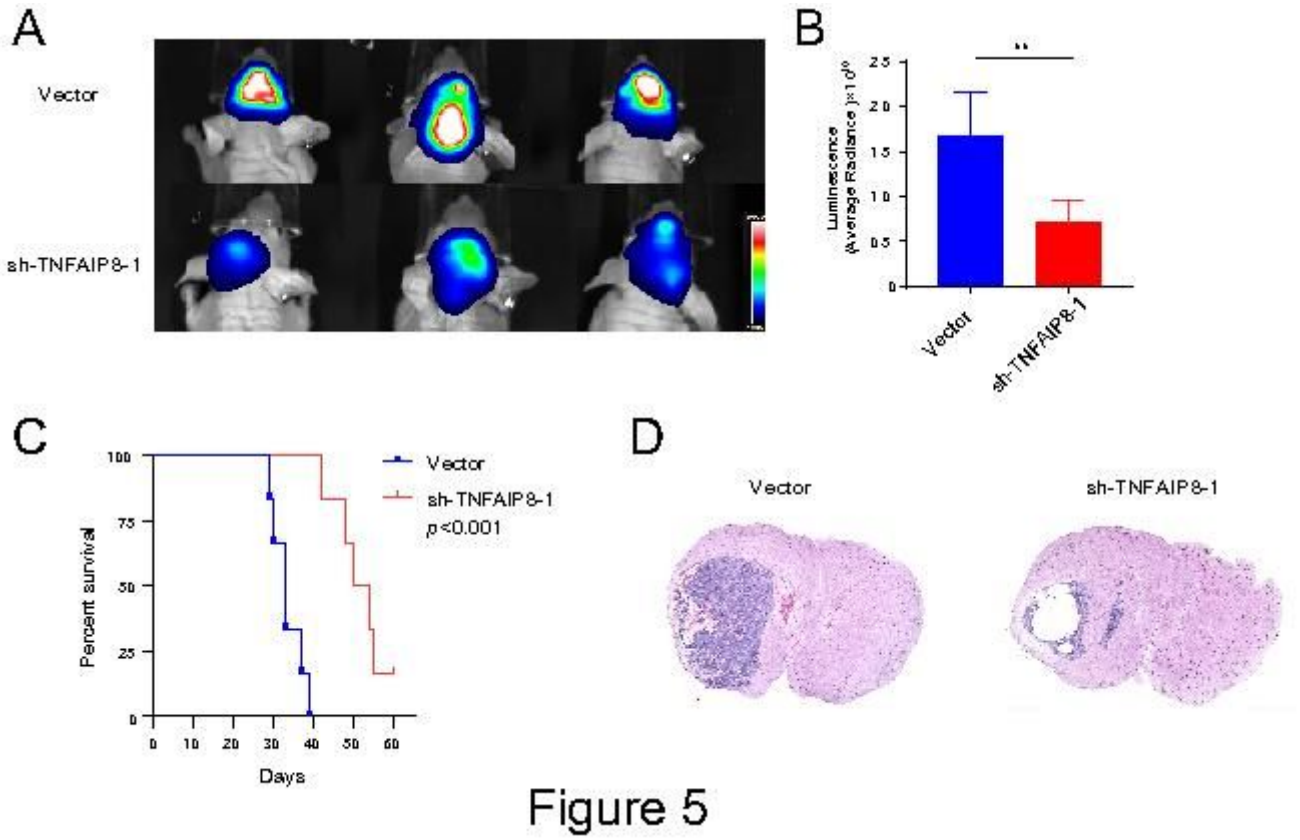

Figure 5

Figure 5

TNFAIP8-knockdown could impair glioma growth *in vivo*

(A) *Ex vivo* bioluminescent imaging of tumor growth in xenograft nude mice bearing tumors. n=3. (B) Quantification of luminescence (indicating the tumor volume) in mice from (A). Data are shown as the mean±SD, n=3, Student's t test (*, P < 0.05). (C) Kaplan-Meier survival curves for animals in different groups. n=3. Log-rank test (*, P < 0.05). (D) Representative images for HE staining for brain sections in (A).