

Nuclear Factor I A Promotes Temozolomide Resistance in Glioblastoma via Transcriptional Regulation of Nuclear Factor κ B Pathway

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Research article

Keywords: Glioblastoma, Temozolomide Resistance, NFIA, NF- κ B, Transcriptional Regulation

Posted Date: July 19th, 2019

DOI: <https://doi.org/10.21203/rs.2.11554/v1>

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Abstract

Background: Glioma is one of the most common primary brain tumors in human with severe mortality based on its therapy resistance and recurrence. Many molecular pathways and regulation factors have been proved to be required for GBM growth and therapy resistance, however, the underlying molecular mechanisms still remains unclear. **Methods:** Nuclear factor I-A (NFIA) was identified as a key candidate kinase encoding gene in chemoresistance regulation by using kinome-wide bioinformatic analysis. Afterwards, the potential biological functions of NFIA in oncogenesis and chemoresistance were clarified by qRT-PCR, western blotting and in vivo xenograft models followed by temozolomide (TMZ) resistant U87 cell induction. Additionally, immunohistochemistry (IHC) assays were performed to explore the clinical significance of NFIA in glioma patients. At last, lentiviral silencing of NFIA was used to explore the potential downstream targets for NFIA in acquired TMZ resistance in GBM.. **Results:** We identified NFIA was the most correlated gene for TMZ resistance in GBM. Clinically, elevated NFIA expression was significantly correlated to adverse outcomes of glioma patients especially in GBM patients. Moreover, NFIA was functionally required for TMZ resistance of U87 cells while suppression of NFIA via lentivirus infection reduced cell proliferation, tumorigenesis as well as resistance to TMZ in GBM cells. Lastly, NFIA promoted acquired TMZ resistance in GBM via transcription activity thus regulated the expression of nuclear factor κ B (NF- κ B). **Conclusions:** Altogether, our study suggests that NFIA-dependent transcriptional regulation of NF- κ B contributes to the acquired TMZ resistance in GBM, indicating that NFIA-NF- κ B axis could be a new therapeutic target for TMZ resistant GBM.

Background

Glioma is one of the most common primary brain tumors in human, representing as more than 80% of all primary intracranial malignant neoplasms[1]. Glioblastoma (GBM), also defined as WHO grade IV glioma, is the most severe and lethal subtype of glioma[1]. Efforts has been made during the past decades to improve the outcomes of GBM, however, the median survival for GBM patients still remains less than 15 months despite the maximum therapies including surgery, radiation and chemotherapy[2]. GBM represents a more invasive and faster growth as well as acquired resistance to either chemotherapeutics or radiation, which leads to more frequent recurrence compared to low grade glioma[3]. A wide range of molecular pathways and regulation factors have been proved to be required for GBM growth and therapy resistance, which might be potential therapeutic targets for GBM treatment[4]. Therefore, it is meaningful for researchers to deeply investigate the mechanism included in the biological behaviors of GBM.

GBM was often chartered by aberrant proliferation and differentiation, indicating a deregulation of the neurodevelopmental process[5]. Accumulating evidence shows that glial fate regulator, nuclear factor I-A (NFIA), is essential for both embryonic development and tumorigenesis for nervous system[6]. NFIA has been proved to be functionally required for glial lineage specification, glial progenitors and astrocyte terminal differentiation, as well as the tumorigenesis of GBM[6]. Moreover, an increased NFIA expression could be found in astrocytoma and GBM and functions as a critical component of the oncogenic network in glioma[7]. Lee et al[7] also reports that NFIA promotes cell growth and inhibits cell apoptosis of glioma

through a negative regulation of multiple tumor suppressors such as p65 and p21. However, the molecular mechanisms of NFIA-induced proliferation and drug resistance in GBM are still not clearly understood.

Nuclear Factor κ B (NF- κ B) pathway is well known for its variety of functions on cellular responses and disease development. Abnormal activation of NF- κ B pathway mediates a wide range of cellular processes related to tumorigenesis, including reduction of cell apoptosis, oncogene mutations and immune stimulation in multiple types of human cancers[8-10]. Activation of NF- κ B in cancers is most likely through either inflammatory stimulation such as tumor necrosis factor (TNF) or upstream regulator such as NF- κ B-inducing kinase (NIK), respectively[11, 12]. As an important regulator in GBM, elevated NF- κ B is related to poor prognosis and enhanced resistance to chemo-therapy or radiation[13]. Kim et al[14] has reported that MLK4-dependent activation of I κ B kinase- α (IKK α) enhances NF- κ B activity in GBM and induces the subtype transition from proneural to mesenchymal thus promotes the cell proliferation and radio-resistance of GBM. Moreover, NFIA was identified as an upstream transcription factor for NF- κ B via transcriptional regulation of NF- κ B p65 promoter activity[6]. Herein, this study was performed to investigate the mechanism of NFIA-dependent transcriptional regulation of NF- κ B contributes to the acquired temozolomide (TMZ) resistance in GBM.

Methods

Reagents

Dulbecco's modified Eagle's medium-nutrient mixture F12 (DMEM-F12), fetal bovine serum (FBS), AlamarBlue reagent, PageRuler plus prestained protein ladder and Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Accutase solution, radio immunoprecipitation assay (RIPA) lysis buffer, phosphatase inhibitor and protease inhibitor were purchased from Merck KGaA (Darmstadt, Germany). Bradford reagent and iScript Reverse Transcription SuperMix were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Bovine serum albumin (BSA) was purchased from New England BioLabs (Ipswich, MA, USA). TMZ was provided by Tasyly Group Co, Ltd (Jiangsu province, China).

Antibodies

Anti-NFIA primary antibodies were purchased from Thermo Fisher Scientific (Cat log no. PA5-52252, Waltham, MA, USA). An Anti-NF- κ B p65 primary antibody was purchased from Thermo Fisher Scientific (Cat log no. 701079, Waltham, MA, USA). Anti-rabbit IgG was purchased from Abcam (Cat log no. ab171870, Cambridge, MA, USA) and used as a negative control. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Cat log no. ab97051) and goat anti-mouse IgG were purchased from Abcam (Cat log no. ab205719, Cambridge, MA, USA) and used as secondary antibodies.

Gene expression analysis

Gene expression data was extracted from GEO datasets (Tso et al[15], GSE 68029, 2015 and Mao et al[16], GSE67089, 2015). Hierarchical bi-clustering was performed to analyze the expression of the targeting genes via Cluster 3.0. Euclidean distance and average linkage were used as similarity metric and clustering methods, respectively. The comparison of the relative gene expression between the naïve and resistant GBM cells was presented as fold-changes.

In vitro cell culture

GBM cell lines U87 was purchased from BeNa Culture Collection (Kunshan, China). Cells were cultured in DMEM-F12 with 10% vol FBS at 37°C with 5% CO₂. The medium was replaced every 2-3 days and cells were dissociated with accutase before seeded. The number of cells was measured by using cell counter with trypan blue and was seeded with a density of 10⁶ cells/10 mL.

Inducing TMZ resistance in GBM cells

U87 cells were cultured in 6-well plates with DMEM-F12 containing 10% FBS at 37°C with 5% CO₂ overnight. Cells were treated with TMZ at a starting dose of 100 µM. Medium containing TMZ was replaced every 24 h for the first 5 days continuously. TMZ concentration was added every 2 weeks for 3 months and the maintenance dose was 500µM.

In vitro cell proliferation assay

Adhered GBM cells were dissociated into single cell suspension with accutase before using. Cell number was measured by using cell counter with trypan blue. Cells were seeded into 96 wells plate at a density of 1000 cells per well with 100µL fresh medium. Cell number was calculated by alamarBlue according to the manufacturer's protocol at day 0, 2, 4, 6 and 8 after seeding.

In vitro cell viability assay

Single cell suspension was seed into 96 wells plate at a density of 2000 cells/100µL per well and cultured for 12 h at 37°C with 5% CO₂ then added 100µL of fresh medium containing TMZ at different amount. The cell number was measured by using alamarBlue according to the manufacturer's instructions. IC50 was calculated with SPSS 19.0.

Quantitative RT-PCR (qRT-PCR)

Total RNA was prepared by using the RNeasy mini kit according to the manufacturer's instructions. Concentration of RNA was determined by Nanodrop 2000. cDNA was synthesized by using iScript reverse transcription5 supermix for RT-qPCR according to the manufacturer's protocol. qRT-PCR was performed by using StepOnePlus real-time PCR system with SYBR Select Master Mix (Applied Biosystems). GAPDH was used as an internal control. Running cycles for DNA amplification used in this study is described as below: 94°C for 2 min, 50 cycles of 94°C (30 s), 60°C (30 s), and 72°C (40 s). The sequences of the primers were shown as below: NFIA-forward: TAATCCAGGGCTCTGTGTCC; NFIA-reverse:

CCTGCAGCTATTGGTGTCTG; NF- κ B p65-forward: CCGCACCTCCACTCCATCC; NF- κ B p65-reverse: ACATCAGCACCCAAGGACACC; GAPDH-forward: GAAGGTGAAGGTCCGAGTCA; GAPDH-reverse: TTGAGGTCAATGAAGGGGTC. Relative quantitation of cDNAs to GAPDH was determined via $2^{-\Delta\Delta C_t}$ method.

Western blotting

Western blotting analysis was performed as described previously[4]. Cell lysates were prepared with RIPA buffer containing protease and phosphatase inhibitor cocktail on ice then then concentrations of protein were measured by using the Bradford method. 10ug/lane of protein were fractionated on NuPAGE Novex 4-12% Bis-Tris Protein gel (Invitrogen) and then transferred to PVDF membrane (Invitrogen). Membranes were blocked with 5% skimmed milk for 1h then incubated with the primary antibody overnight at 4°C then incubated with the secondary antibody at room temperature for 1h. Protein expression was visualized by using ECL methods according to the manufacturer's instructions (GE Healthcare Life Sciences). β -actin served as a control.

Immunohistochemistry (IHC)

IHC was performed as previously described[4]. All glioma samples used in this study had been pathologically diagnosed and the recurrence was confirmed by computed tomography (CT) or magnetic resonance imaging (MRI). Nuclei were counterstained with hematoxylin. German immunohistochemical scoring (GIS) was used to measure the expression of NFIA. Immunoreactivity score = positive cell score x staining intensity score. The positive cell score was calculated as below: 0, negative; 1, <10% positive; 2, 11-50%; 3, 51-80%; 4, >80%. Staining intensity score was graded as below: 0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive. Immunoreactivity score >3 was considered positive staining.

Lentivirus production and transduction

Lentivirus production and transduction was performed as mentioned in the previous study[4]. The lentivirus for shNFIA and NFIA overexpression were purchased from Genechem (Shanghai, China). The lentivirus infection was performed according to the manufacturer's protocol.

Luciferase reporter assay

After lentivirus infection, pre-treated 293T or U87 cells were seeded at a concentration of 10^6 cells per well in six-well plates. NF- κ B p65 activity was determined by using the NF- κ B Reporter kit (BPS Bioscience, Cat log no. 60614, San Diego, CA, USA). The attached cells were transfected with NF- κ B reporter and negative control reporter for 24 h following the manufacturer's protocol. Normalized luciferase activity for NF- κ B p65 reporter was measured as a ratio of firefly luminescence to Renilla luminescence. 5 replicates were used for each sample and the results were represented as mean \pm SD.

Flow cytometry

Flow cytometry was performed as previously described[4]. The Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis kit was used to measure U87 cell apoptosis according to the manufacturer's protocol.

In vivo intracranial xenograft tumor model

All the usage of experimental animals in this study was adheres to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines. 6-week-old female nude mice cultured under specific pathogen free condition (provided by the Experimental Animal Centre of Xi'an Jiaotong University) were used for *in vivo* xenograft of GBM cells. Prepared GBM cell suspension (pre-transduced with shNT or shNFIA lentivirus) was diluted to the density of 1×10^5 cells in 2 μ L PBS then implanted into the mice brains as previously described[4, 17]. 5 mice were used for each group. TMZ (50mg/kg/d) or DMSO was taken by tail vein injection after 7 days injection of glioma cells. Mice were monitored once a day until the following symptoms appeared: arched back, leg paralysis, unsteady gait or bodyweight loss for more than 10%. When neuropathological symptoms developed, the mice were anaesthetized and sacrificed with overdose Ketamine (80mg/kg) and Xylazine (20mg/kg).

Statistical analysis

All the results in this study are presented as mean \pm SD. Number of replicates is mentioned in the related figure legends. Statistical differences between 2 groups were evaluated by using 2-tailed *t* tests. Multiple groups were compared with one-way ANOVA followed by Dunnett's posttest. Kaplan-Meier survival analysis was compared by log-rank analysis. All statistical analysis was performed with SPSS 19.0 or GraphPad Prism 6 software. Statistically significance was considered when P value was less than 0.05.

Results

Enriched NFIA expression could be related to TMZ resistance in GBM

To deeply explore the key regulator and related molecular mechanism of acquired TMZ resistance in glioma, hierarchical bi-clustering based on the previously published microarray databases (Tso et al[15], GSE 68029, 2015 and Mao et al[16], GSE67089, 2015) then the genes were ranked according to their fold changes (Figure 1A and Figure 1B). Genes with more than 5 fold up-regulation were picked up from these 2 databases. Finally 105 genes were identified for TMZ resistance characters as well as 452 genes for GBM compared to astrocytes. Then we merged the 2 gene lists together and found that NFIA was the only gene which was significantly enriched in both 2 phenotypes (Figure 1C). Additionally, we confirmed that NFIA was significantly increased in TMZ resistant glioma cells compared to their naïve control lines (Figure 1D), demonstrating that NFIA could be essential for glioma cells to gain TMZ resistance. Moreover, an elevated NFIA expression could be observed in GBM, which was considered as the most lethal type of glioma (Figure 1E). Taken together, these results indicated that NFIA was elevated in GBM and might be essential for therapy resistance and tumor recurrence.

Increased NFIA expression was associated with poor outcomes in GBM patients

Given the results from the previous data, it raised up the question whether NFIA could be a prognostic marker for glioma. To this end, IHC was performed to examine NFIA expression in 68 glioma tumor tissues which were collected from patients underwent surgical therapy from 2008 to 2017 in the First Affiliated Hospital of Xi'an Jiaotong University. As a result, NFIA was found to be expressed in the nucleus of glioma cells (Figure 2A). GIS was used to determine the expression level and NFIA was found to be markedly enriched in GBM, contrarily to low grade glioma samples (Figure 2A and Figure 2B). We next assessed the expression of NFIA in GBM by analyze TCGA database and Rembrandt database. The results demonstrated that NFIA was elevated in GBM compared to normal brain tissue, which were similar with our study (Figure 2C and Figure 2D). When look into the survival for those patients, patients with lower NFIA expressed glioma represented longer overall survival compared to those with higher NFIA expression (Figure 2E). Similar results were achieved when we specifically focused on GBM patients (Figure 2F). Moreover, an analysis of overall survival was performed among 541 glioma patients from Rembrandt database and the results showed that the post-surgical survival for the patients with low NFIA expression was significantly prolonged than that in the patients with increased NFIA expression (Figure 2G). Altogether, the results showed the possibility that NFIA is supposed to be a specific clinical relevant oncogene for glioma and GBM.

NFIA was functionally required for TMZ resistance in GBM

To investigate the function of NFIA in the acquirement of TMZ resistance in GBM, an exogenous overexpression of NFIA was performed in U87 cells by using lentivirus infection. qRT-PCR and western blotting analysis confirmed that NFIA expression was markedly increased in NFIA overexpressed U87 cells (Figure 3A and 3B). *In vitro* cell growth assay indicated that the proliferation and TMZ resistance was obviously enhanced after NFIA overexpression (Figure 3C). Moreover, U87 cells with or without NFIA overexpression were treated with TMZ thus the apoptosis analysis was performed. The results showed increased TMZ resistance after NFIA overexpression (Figure 3D), demonstrating that NFIA was functional required for acquired TMZ resistance in GBM.

Suppression of NFIA enhanced the TMZ sensitivity of TMZ-resistant GBM

To thoroughly study the functional role of NFIA in acquired resistance to TMZ in GBM, we established *in vitro* TMZ resistant U87 cell line according to the previous publications[18, 19]. After 3 months culturing with TMZ-contained medium, U87 cells gained stable resistance to TMZ (Figure 4A). qRT-PCR analysis was performed among the TMZ resistant GBM cell lines and their naïve control lines. The results indicated dramatically increased NFIA expression in TMZ resistant U87 cells (Figure 4B). Moreover, western blotting results also showed up-regulated NFIA in TMZ resistant population of U87 cells (Figure 4C).

For further assessment of the functional role of NFIA, TMZ resistant U87 was transduced with a lentiviral shRNA clone for NFIA (shNFIA) or a non-targeting lentivirus (shNT) as a negative control. Both qRT-PCR and western blotting analysis showed dramatic down-regulation of NFIA at mRNA level (Figure 4D and 4E). Additionally, to test the function of NFIA on TMZ resistance, U87 TMZ resistant cells treated with or

without NFIA suppression then exposed to TMZ treatment at 300 μ M. *In vitro* cell growth assay exhibited decreased cell proliferation and increased TMZ sensitivity in U87 TMZ resistant cells transduced with shNFIA lentivirus (Figure 4F). Similarly, flow cytometry assays for apoptosis were performed with shNFIA or shNT pre-transduced U87 TMZ resistant cells followed with or without TMZ treatment (300 μ M). The proportions of cells that undergoing early and late apoptosis were both dramatically increased when cells received combined treatment of TMZ and NFIA silencing compared with TMZ alone (Figure 4G). Next, we investigated the function of NFIA knock-down on *in vivo* tumorigenesis by using mouse intracranial tumor models. The results indicated that the control mice with xenografts of shNT-transduced U87 TMZ resistant cells rapidly represented tumor-related symptoms compared with those transplanted with shNFIA-transduced U87 TMZ resistant cells combined with TMZ treatment (Figure 4G), highlighting a potent anti-TMZ resistance effects of NFIA knock-down in TMZ resistant GBMs.

NFIA-dependent transcriptional regulation of NF- κ B contributes to the acquired TMZ resistance in GBM

Previous study showed NFIA contributes to tumor progression through regulating NF- κ B expression. In our study, we found that NF- κ B expression was significantly increased in TMZ resistant U87 cells compared to the original non-treated U87 (Figure 5A and Figure 5B). Furthermore, inhibition of NFIA induced reduction of NF- κ B in TMZ resistant U87 (Figure 5C and Figure 5D). Altogether, these data suggested suggests the presence of a tumor-promoting regulation between NFIA and NF- κ B in GBM.

We then performed a luciferase reporter assay with constructs driven by a human NF- κ B promoter. As expected, overexpression of NFIA led to a significant increase of NF- κ B promoter activity in both 293T and U87 cells (Figure 5E). Contrarily, shRNA-mediated-knockdown of NFIA resulted in a marked decrease in transcription activity of NF- κ B promoter region in U87 cells, especially in TMZ resistant U87 cells which exhibited a higher expression of NF- κ B (Figure 5F). These data suggest that the NFIA-dependent transcriptional regulation of NF- κ B contributes to the acquired TMZ resistance in GBM.

Discussion

Accumulating data demonstrates that acquired resistance to radio therapy and chemotherapy is essential for the recurrence and lethal mortality of GBM[20]. Multiple mechanisms have been identified to be functionally for GBM cells to gain therapy resistance[4, 21]. Our findings here indicates that NFIA promotes TMZ resistant in GBM via a transcriptional regulation of NF- κ B thus contributes to poor prognosis and recurrence for GBM patients, suggesting that NFIA-NF- κ B axis could be a new therapeutic target for TMZ resistant GBM.

The NFI family of site specific DNA-binding proteins, which includes NFIA, NFIB, NFIC, and NFIX, was first identified to be required for viral replication and regulation of gene expression[22]. NFI family members comprise a set of vertebrate nuclear proteins which recognize and bind to the particular DNA sequence thus activate or repress transcription and DNA replication[6]. Among the NFI family, NFIA has been proved to be essential for the development of glial lineage specification and regulation of astrocyte terminal differentiation[23]. At the meantime, NFIA has been implicated in a wide range of human tumor including

esophageal squamous cell carcinoma, esophagogastric junction adenocarcinoma, astrocytoma, as well as glioma[6, 24, 25]. Additionally, NFIA was reported to be responsible for proliferation and recurrence via negatively regulating of tumor suppressors including p65 and p21, indicating NFIA is a critical oncogene for tumorigenesis in glioma, especially in GBM[7]. Our study identified that NFIA was highly expressed in GBM compared to normal brain, moreover, was significantly enriched in TMZ resistant GBM. Additionally, high expression of NFIA implied poor prognosis of glioma patients. Similar results could be observed when we look into databases such as TCGA and Rembrandt in which more patients are included, providing a potential therapeutic target for GBM treatment. Due to the limitation of the *in vitro* research and small amount of data, it still remains to be seen whether these findings could be extended to the more complex *in vivo* situations. Also, an analysis of a larger cohort is planned to strengthen the conclusion.

NF- κ B p65 is a well-recognized anti-apoptotic transcription factor and abnormal expression or activation of NF- κ B p65 has been found in multiple types of malignant cancers such as cervical cancer, esophageal squamous cancer, ovarian cancer, breast cancer and glioma et al[13, 26-29]. NF- κ B promotes tumorigenesis and therapy resistance mainly through induction of master transcription factors including signal transducer and activator of transcription 3 (STAT3) and TAZ[30]. As well known, NF- κ B activity is primarily enhanced by interaction with inhibitor of kappa B (I κ B) with contributions from positive and negative upstream regulators including TNF, NIK, et al[31]. Lee et al[6] reported that NFIA increased NF- κ B transcription activity thus increased NF- κ B expression at both mRNA level and protein level. Interestingly, this study also demonstrates a feed-forward loop between NFIA and NF- κ B, which may increase GBM cell survival and protect GBM cells from chemotherapy-induced apoptosis[30]. Herein, we found abnormal up-regulated expression of NFIA and NF- κ B in TMZ resistant U87 cell line and artificial lentivirus-dependent suppression of NFIA induced a reduction of NF- κ B expression thus decreased the cell growth and resensitized TMZ resistant U87 cells to TMZ. Moreover, shRNA-mediated inhibition of NFIA reduced the activity of NF- κ B promoter, contrarily, exogenous overexpression of NFIA led to a significant increase of NF- κ B promoter activity in both 293T and U87 cells, suggesting that NFIA-dependent transcriptional regulation of NF- κ B contributes to the acquired TMZ resistance in GBM. A further study including ChIP-sequencing and ChIP-sequencing needs to be performed to investigate either NFIA binds to NF- κ B promoter directly or indirectly, in purpose to clarify the mechanism for NFIA-dependent regulation of NF- κ B in GBM.

Conclusions

Our study suggests that NFIA-dependent transcriptional regulation of NF- κ B contributes to the acquired TMZ resistance in GBM, indicating that NFIA-NF- κ B axis could be a new therapeutic target for TMZ resistant GBM.

Abbreviations

GBM: Glioblastoma; NFIA: nuclear factor I-A; NF- κ B: Nuclear Factor κ B; TNF: tumor necrosis factor; NIK: NF- κ B-inducing kinase; IKK: α kinase; TMZ: temozolomide; qRT-PCR: Quantitative RT-PCR; IHC: Immunohistochemistry; ARRIVE guidelines: Animal Research: Reporting In Vivo Experiments guidelines.

Declarations

Ethics approval and consent to participate

The usage of the experimental animals in this study was approved by the Ethics Committee of the School of Medicine, Xi'an Jiaotong University (approval no. 2016-085). The usage of the tumor samples and patient information was approved by the patients and the Scientific Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (approval no. 2016-18). All necessary consent forms or documents were signed before the surgery.

Consent for publication

Not Applicable

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the GEO repository. (GSE 68029: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68029>; GSE67089: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67089>).

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the National Natural Science Foundation of China (no. 81802502) and the Fundamental Research Funds of Xi'an Jiaotong University (no. 1191329177).

Authors' contributions

XY and JW designed the study concept and performed the molecular biology experiments. JW, MW, AW and WX participated in data acquisition and wrote the manuscript. RL, WW, LS, HL and CL performed the animal experiments and the survival analysis. JW, JZ and RL revised the manuscript and contributed to data analysis. All authors have read and approved the manuscript.

Acknowledgements

The authors would thank all the member of the Department of Neurosurgery and the Center of Brain Science of the First Affiliated Hospital of Xi'an Jiaotong University.

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Figures

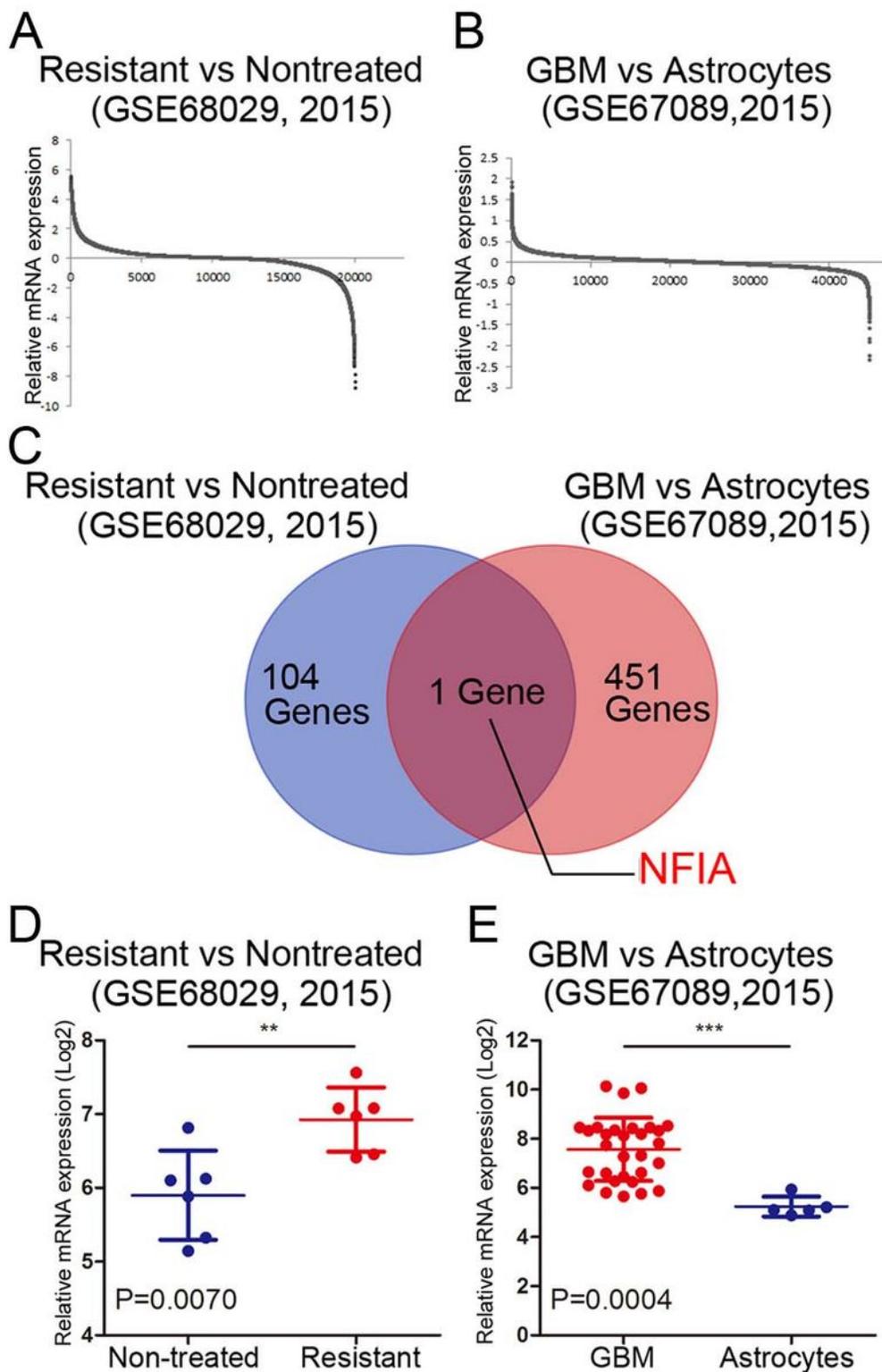


Figure 1

Enriched NFIA expression could be related to TMZ resistance in GBM (A) Genome-wide transcriptome microarray analysis for 45043 genes in TMZ resistant GBM compared to the naïve GBM (GSE 68029). (B) Genome-wide transcriptome microarray analysis for 19988 genes in GBM compared to astrocytes (GSE 67089). (C) Venn diagram indicated NFIA was the only gene candidate which was up-regulated in GBM and TMZ resistance when merged the 2 databases together. (D) Gene expression analysis indicated that

NFIA expression was elevated in TMZ resistant GBM cells (GSE 68029, $**P < 0.01$, with t test). (E) Gene expression analysis indicated that NFIA expression was elevated in GBM (GSE 67089, $***P < 0.001$, with t test).

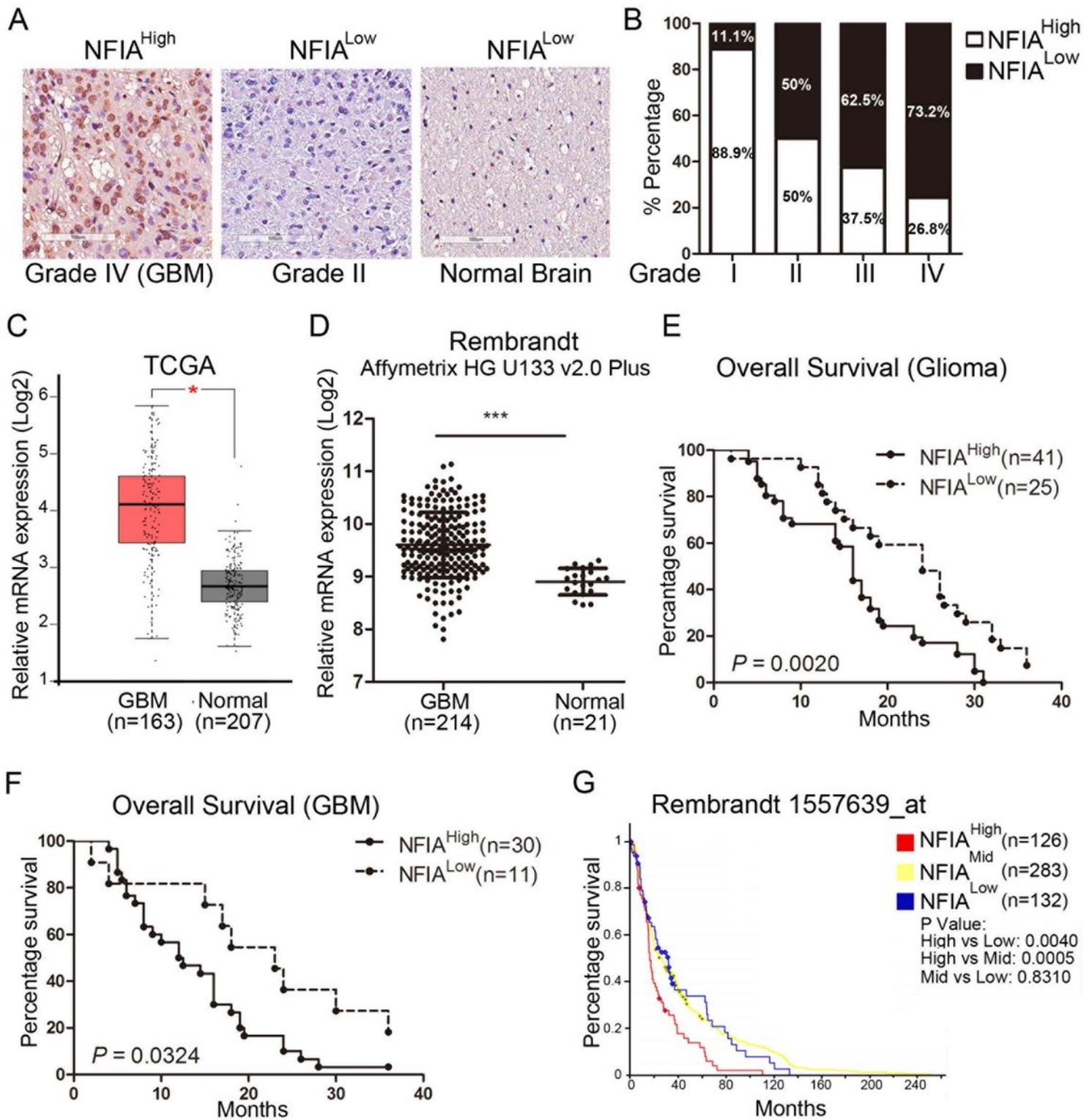


Figure 2

Elevated NFIA was clinically related to poor prognosis in glioma patients (A) Representative IHC images of NFIA in glioma samples. Brain tissue from epilepsy surgery was used as a negative control. (B) NFIA

was enriched in high grade glioma samples (WHO III-IV), compared with low grade glioma samples (WHO I-II). (C) Gene expression analysis by using TCGA database indicated that NFIA expression was elevated in GBM compared to normal brain tissue (n=163 for GBM, n=207 for normal brain tissue, *P<0.05, with t test). (D) Gene expression analysis by using Rembrandt database indicated that NFIA expression was elevated in GBM compared to normal brain tissue (n=214 for GBM, n=21 for normal brain tissue, ***P<0.001, with t test). (E) Kaplan-Meier analysis exhibited a longer overall survival in samples with a lower NFIA expression, compared with samples with a higher NFIA expression among 66 glioma patients (P=0.0020, with log-rank test). (F) Kaplan-Meier analysis exhibited a longer overall survival in samples with a lower NFIA expression, compared with samples with a higher NFIA expression among 41 GBM patients (P=0.0324, with log-rank test). (G) Analysis of the Rembrandt data indicated that NFIA expression was elevated in GBM samples (P=0.0040 for NFIA^{High} GBM patients compared to NFIA^{Low} GBM patients, P=0.0005 for NFIA^{High} GBM patients compared to NFIA^{Mid} GBM patients, P=0.8310 for NFIA^{Mid} GBM patients compared to NFIA^{Mid} GBM patients, with one-way ANOVA followed by Dunnett's posttest).

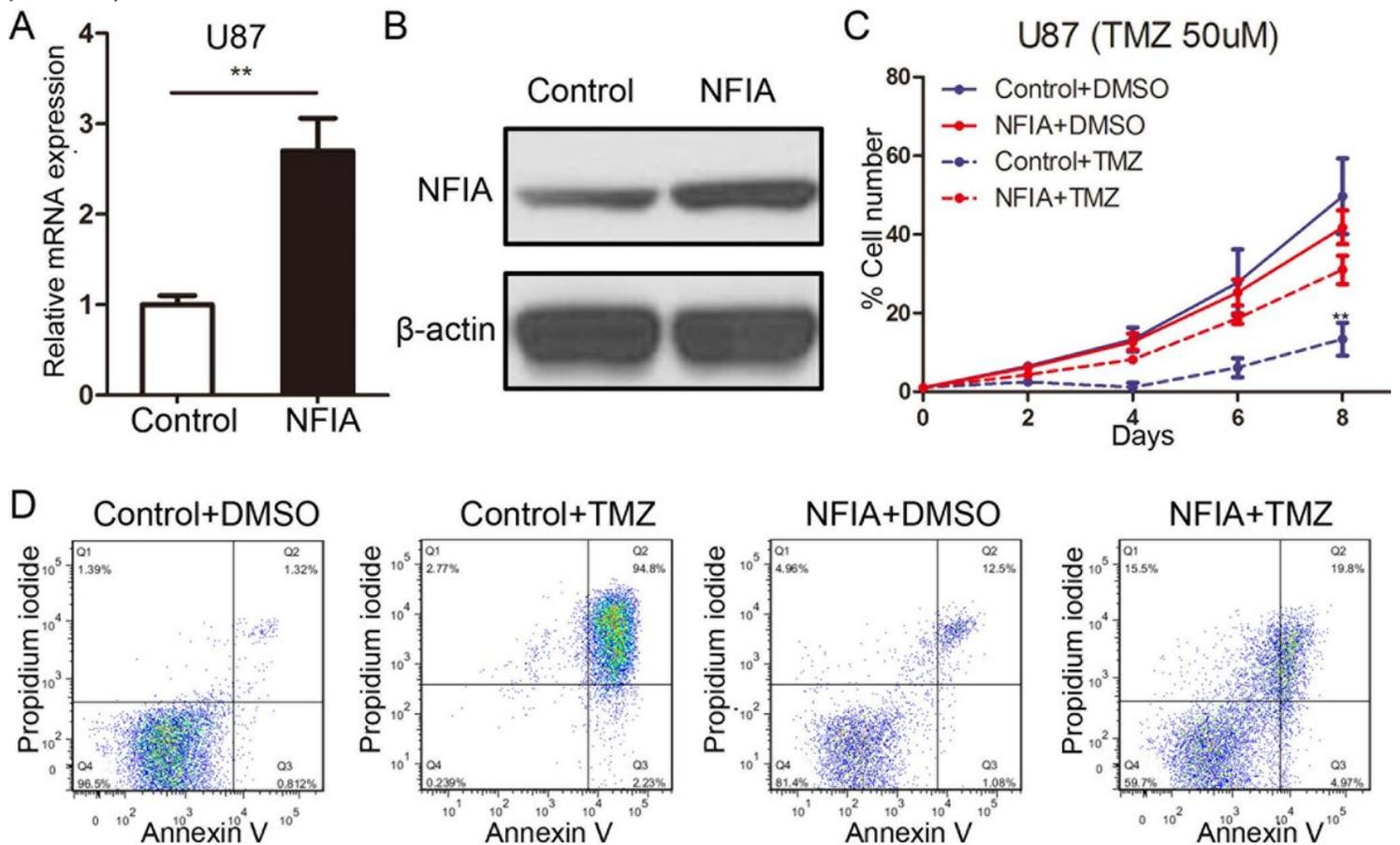


Figure 3

Increased NFIA could be observed in TMZ resistant GBM (A) qRT-PCR analysis of NFIA in U87 cells transduced with NFIA overexpression lentivirus (NFIA) or control lentivirus (Control) (**P<0.01, with t test). (B) Western blotting analysis of NFIA in U87 or U251 cells transduced with NFIA overexpression lentivirus (NFIA) or control lentivirus (Control). β -actin served as a loading control. (C) In vitro cell growth assay for

NFIA overexpressed U87 cells treated with TMZ (** $P < 0.01$, with one-way ANOVA). (D) Flow cytometry analysis for apoptosis with Annexin V antibody and Propidium Iodide using NFIA overexpressed U87 cells treated with TMZ.

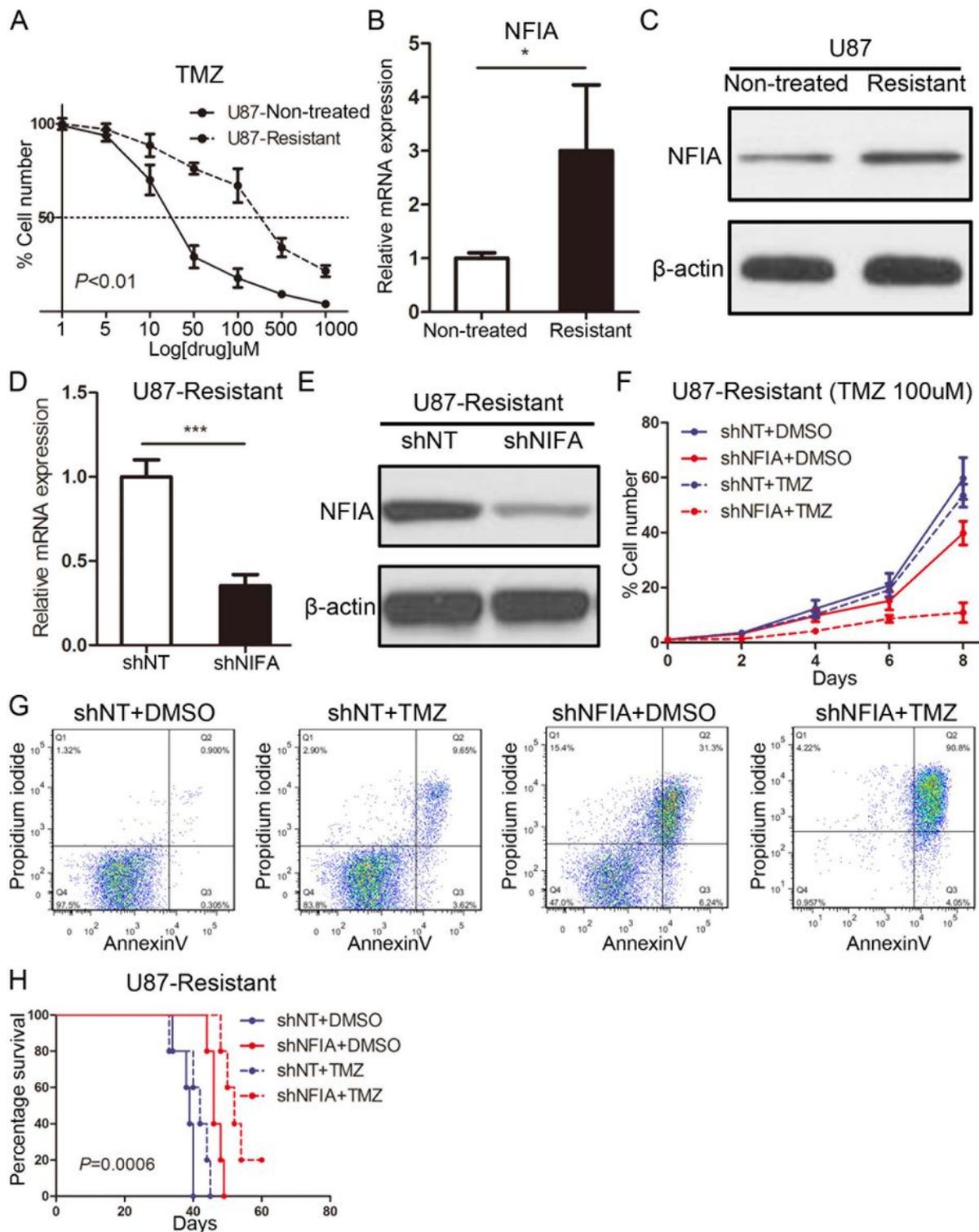


Figure 4

NFIA was functional required for acquired TMZ resistance in GBM (A) In vitro cell viability assay for TMZ in U87 naïve cells (Non-treated) and U87 TMZ resistant cells (Resistant) ($P < 0.01$, with one-way ANOVA).

(B) qRT-PCR analysis showed NFIA mRNA expression level was substantially increased in TMZ resistant U87 cells (* $P < 0.05$, with t test). (C) Western blotting analysis showed NFIA protein was increased in TMZ resistant U87 cells. β -actin served as a loading control. (D) qRT-PCR analysis of NFIA in U87 TMZ resistant cells transduced with shRNA against NFIA (shNFIA) or non-targeting control (shNT) (** $P < 0.0001$, with one-way ANOVA followed by Dunnett's posttest). (E) Western blotting analysis of NFIA in U87 resistant cells transduced with shRNA against NFIA (shNFIA) or non-targeting control (shNT). β -actin served as a loading control. (F) In vitro cell growth assay for NFIA knock-down combined with TMZ treatment in U87 TMZ resistant cells (* $P < 0.05$, with one-way ANOVA). (G) Flow cytometry analysis for apoptosis with Annexin V antibody and Propidium Iodide using TMZ resistant U87 cells pretreated with shNFIA then followed with or without TMZ treatment. (H) Kaplan-Meier analysis for mice after the intracranial transplantation of U87 TMZ resistant cells pre-treated with shNFIA or shNT lentivirus then followed continuously 10-day TMZ (50mg/kg/d) treatment or placebo (DMSO) by tail vein injection ($P = 0.0006$, with log rank test).

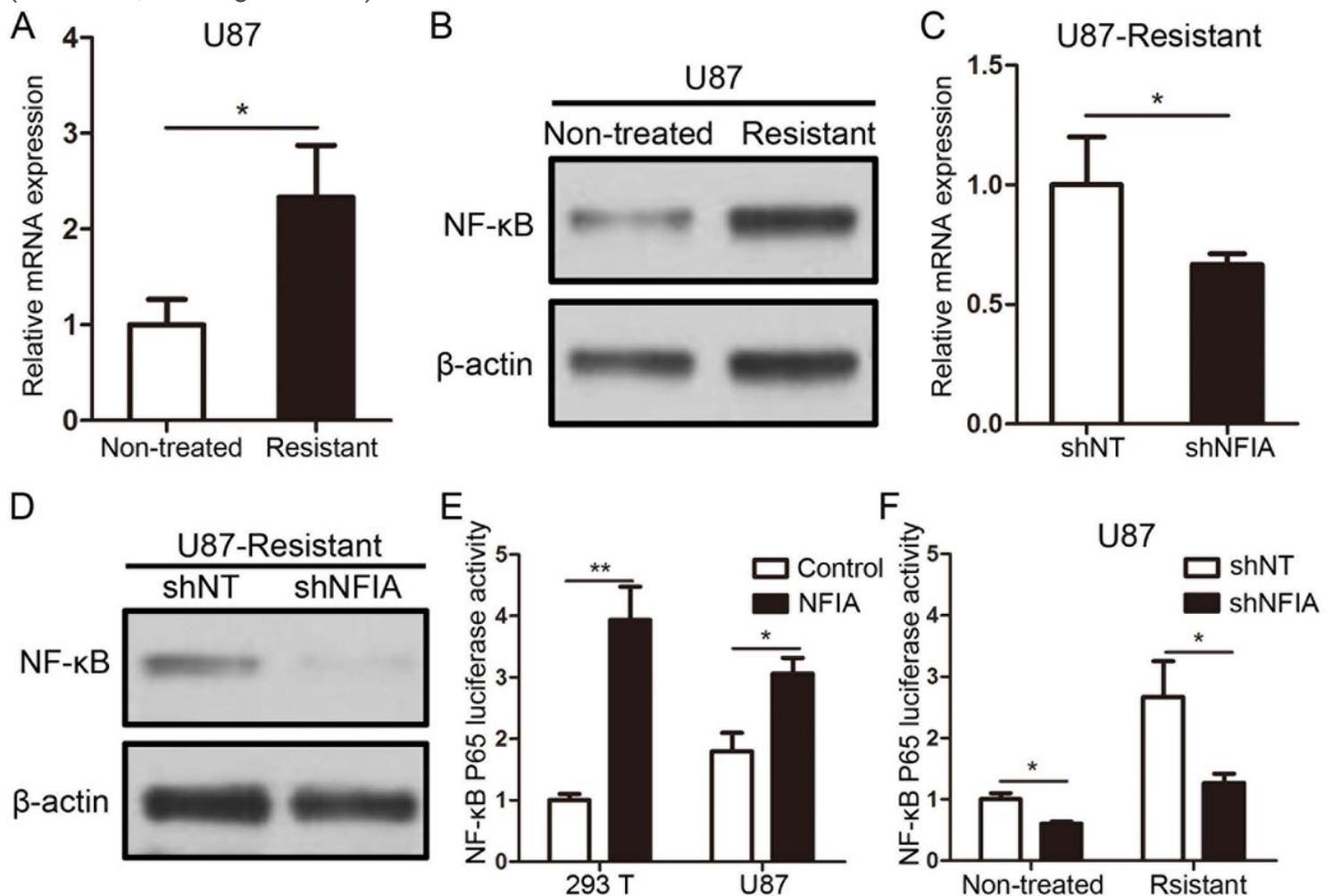


Figure 5

NFIA transcriptional regulation of NF- κ B induced TMZ resistance in GBM (A) qRT-PCR analysis showed NF- κ B p65 mRNA expression level was substantially increased in TMZ resistant U87 cells (* $P < 0.05$, with t test). (B) Western blotting analysis showed NF- κ B p65 protein was increased in TMZ resistant U87 cells.

β -actin served as a loading control. (C) qRT-PCR analysis of NF- κ B p65 in U87 TMZ resistant cells transduced with shRNA against NFIA (shNFIA) or non-targeting control (shNT) (*P<0.05, with t test). (D) Western blotting analysis of NF- κ B p65 in U87 TMZ resistant cells transduced with shRNA against NFIA (shNFIA) or non-targeting control (shNT). β -actin served as a loading control. (E) Relative luciferase activity of NF- κ B p65 promoter transfected into 293T cells or U87 GBM cells pre-treated with NFIA overexpression lentivirus (NFIA) or control lentivirus (Control) (*P<0.05, **P<0.01, with t test). (F) Relative luciferase activity of NF- κ B p65 promoter transfected into TMZ resistant U87 cells pre-treated with shRNA against NFIA (shNFIA) or non-targeting control (shNT). (*P<0.05, with t test).

Supplementary Files

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