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Nuclear factor I A promotes temozolomide resistance in glioblastoma via activation of nuclear factor κB pathway

Xiao Yu[a,1], Maode Wang[a,b,1], Jie Zuo[c], Alafate Wahafu[a], Ping Mao[a], Ruichun Li[a], Wei Wu[a], Wanfu Xie[a], Jia Wang[a,b,*]

[a] Department of Neurosurgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, 710061, PR China
[b] Center of Brain Science, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, 710061, PR China
[c] The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, 710004, PR China

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ABSTRACT

Aims: To investigate the underlying mechanism by which glioblastoma (GBM) cells gain temozolomide (TMZ) resistance and to clarify novel therapeutic targets and new prognostic biomarkers for GBM.

Main methods: A genome-wide hierarchical bi-clustering based on previously published microarray databases identified Nuclear Factor I A (NFIA) as one of the most significantly upregulated genes correlated to TMZ resistance in GBM. Then, the potential biological functions of NFIA in oncogenesis and chemoresistance were clarified by qRT-PCR, Western blotting and *in vivo* xenograft models with artificially induced TMZ-resistant U87 cells. Additionally, immunohistochemistry (IHC) assays were performed to explore the clinical significance of NFIA in glioma patients. Last, luciferase reporter assay was performed to study the transcriptional regulation of NFIA on the nuclear factor κb (NF-kB) pathway.

Key findings: NFIA was correlated with TMZ resistance in GBM. Clinically, elevated NFIA expression was significantly correlated with adverse outcomes of glioma patients, especially in GBM patients. Moreover, NFIA contributed to the acquired TMZ resistance of GBM cells, while suppression of NFIA via lentivirus reduced cell proliferation, tumorigenesis and resistance to TMZ of GBM. Additionally, NFIA promoted transcription activity that regulated the expression of NF-kB. Last, NFIA induced phosphorylation of NF-kB p65 at serine 536, thus inducing TMZ resistance in GBM cells. Altogether, our study suggests that NFIA-dependent transcriptional regulation of NF-kB contributes to acquired TMZ resistance in GBM.

Significance: Abnormally activated NFIA-NF-kB signaling was strongly correlated with acquired TMZ resistance and poor prognosis in GBM, and it could be a new therapeutic target for TMZ-resistant GBM.

1. Introduction

Glioma is one of the most common primary brain tumors in humans, representing 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 have 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 growing glioma that has acquired resistance to either chemotherapeutics or radiation, which leads to more frequent recurrence compared to low-grade gliomas [3]. A wide range of molecular pathways and regulation factors have been proved to be required for GBM growth and therapy resistance, and these might be potential therapeutic targets for GBM treatment [4]. Therefore, it is essential to deeply investigate the mechanism included in the biological behaviors of GBM.

GBM was often characterized 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 the nervous system [6]. NFIA has been proven to be functionally required for glial lineage specification, glial progenitors and astrocyte terminal differentiation, as well as for the tumorigenesis

* Corresponding author. Department of Neurosurgery, Center of Brain Science, The First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Road, Xi'an, Shaanxi, 710061, PR China.
E-mail address: jiawang_xjtu@163.com (J. Wang).
[1] These authors contributed equally to this study and should be considered as co-first authors.

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of GBM [6]. Moreover, an increased NFIA expression could be found in astrocytoma and GBM; moreover, it functions as a critical component of the oncogenic network in glioma [7]. Lee et al [7] also reported that NFIA promotes cell growth and inhibits cell apoptosis of glioma through a negative regulation of multiple tumor suppressors such as p65 and p21.

The Nuclear Factor κB (NF-κB) pathway is well known for a variety of functions on cellular responses and disease development. Abnormal activation of the NF-κB pathway mediates multiple cellular processes related to tumorigenesis, including the reduction of cell apoptosis, oncogene mutations and immune stimulation in human cancers [8–10]. The activation of NF-κB in cancers most likely occurs though either inflammatory stimulation such as tumor necrosis factor (TNF) or through an upstream regulator such as NF-kB-inducing kinase (NIK), respectively [11,12]. As an important regulator in GBM, elevated NF-κB activity is related to poor prognosis and enhanced resistance to chemotherapy and radiation [13]. Kim et al [14] has reported that MLK4-dependent activation of IkB kinase-α (IKKα) enhances NF-kB activity in GBM and induces the subtype transition from proneural to mesenchymal, thus promoting the cell proliferation and radioresistance of GBM. Moreover, NFIA was identified as an upstream transcriptional regulator of NF-kB via the transcriptional regulation of NF-kB p65 promoter activity [6]. However, the mechanism of NFIA on phosphorylation and om the activation of NF-kB signaling in GBM are still not fully understood. Herein, this study was performed to investigate the mechanism by which NFIA-dependent regulation of NF-kB signaling activity contributes to the acquired temozolomide (TMZ) resistance in GBM.

2. Materials and methods

2.1. Ethical statement

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.

2.2. Reagents

Dulbecco's modified Eagle'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 Tasly Group Co, Ltd (Jiangsu province, China).

2.3. Antibodies

Anti-NFIA primary antibodies was purchased from Thermo Fisher Scientific (Cat log no. PA5-52252, Waltham, MA, USA). Anti-NF-κB p65 primary antibody was purchased from Thermo Fisher Scientific (Cat log no. 701079, Waltham, MA, USA). Anti-NF-kB-p65(Phospho-Ser536) primary antibody was purchased from Signalway Antibody (Cat log no. 11014, College Park, MD, USA). Anti-IKKβ antibody was purchased from Origene (Cat log no. AM06154SU-N, Rockville, MD, USA). Anti-β-actin antibody was purchased from Abcam (Cat log no. ab115777, Cambridge, 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.

2.4. 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.

2.5. Bioinformatics analysis

Gene expression profiles were derived from the Cancer Genome Atlas (TCGA) database. All data were preprocessed by background correction, gene ID transformation and normalization by R. Furthermore, these data were ordered by the expression of NFIA to divide all samples into two groups, $NFIA^{high}$ and $NFIA^{low}$ (by median cutoff). Subsequently, limma package [17] was used to calculate the differentially expressed genes in these two groups and Pearson analysis was used to examine the correlation.

2.6. In vitro cell culture

GBM cell lines U87 an U251 were provided by BeNa Culture Collection (Kunshan, China). Cells were cultured in DMEM-F12 with 10% vol FBS at 37 °C with 5% CO_2. 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 10^6 cells/10 mL.

2.7. Inducing TMZ resistance in GBM cells

U87 and U251 cells were cultured in 6-well plates with DMEM-F12 containing 10% FBS at 37 °C with 5% CO_2 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 500 μM.

2.8. 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 100 μL fresh medium. Cell number was calculated by alamarBlue according to the manufacturer's protocol at day 0, 2, 4, 6 and 8 after seeding.

2.9. In vitro cell viability assay

Single cell suspension was seed into 96 wells plate at a density of 2000 cells/100 μL per well and cultured for 12 h at 37 °C with 5% CO_2 then added 100 μL 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.

2.10. 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-κB p65-forward: CCGCACC TCCACTCCATCC; NF-κB p65-reverse: ACATCAGCACCCAAGGACACC; FLIP-forward: TCAAGGAGCAGGGACAAGTTA; FLIP-reverse: GACAAT GGGCATAGGGTGTTATC; CIAP2-forward: AAGCTACCTCTCAGCCTAC TTT; CIAP2-reverse: CCACTGTTTTCTGTACCCGGA; BFL1-forward: TACAGGCTGGCTCAGGACTAT; BFL1-reverse: CGCAACATTTTGTAGC ACTCTG; BCL2-forward: GGTGGGGTCATGTGTGTGG; BCL2-reverse: CGGTTCAGGTACTCAGTCATCC; GAPDH-forward: GAAGGTGAAGGTC GGAGTCA; GAPDH-reverse: TTGAGGTCAATGAAGGGGTC. Relative quantitation of cDNAs to GAPDH was determined via $2^{-\Delta\Delta Ct}$ method.

2.11. 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 concentrations of protein were measured by using the Bradford method. 10 μg/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 1 h then incubated with the primary antibody overnight at 4 °C then incubated with the secondary antibody at room temperature for 1 h. Protein expression was visualized by using ECL methods according to the manufacturer's instructions (GE Healthcare Life Sciences). β-actin served as a control.

2.12. 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.I 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.

2.13. Lentivirus production and transduction

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

2.14. 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-κB p65 activity was determined by using the NF-κB Reporter kit (BPS Bioscience, Cat log no. 60614, San Diego, CA, USA). The attached cells were transfected with NF-κB reporter and negative control reporter for 24 h following the manufacturer's protocol. Normalized luciferase activity for NF-κB 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 ± SD.

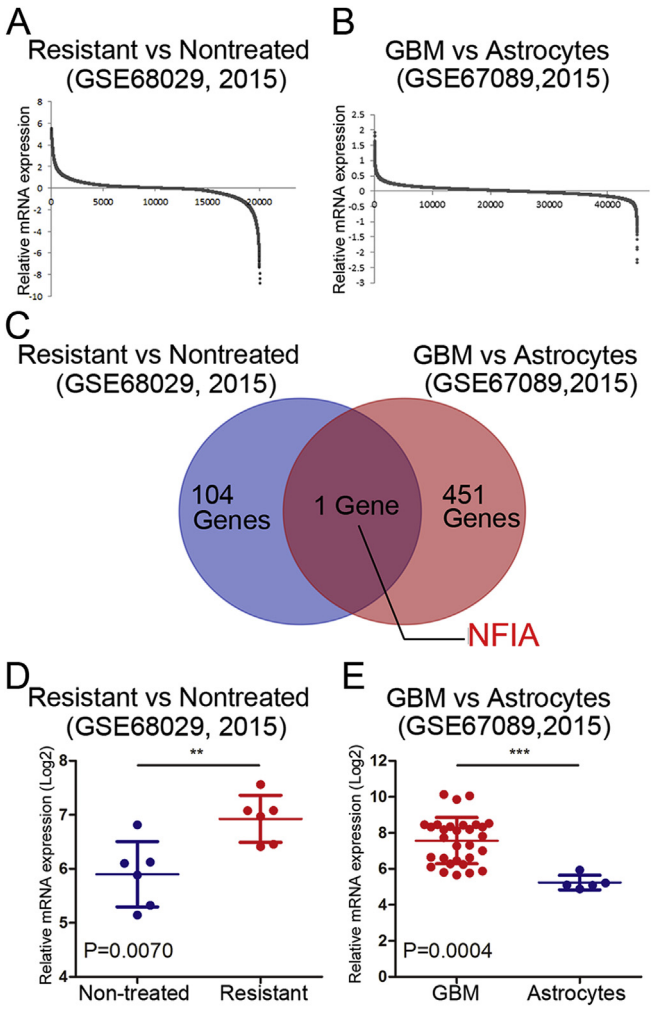


Fig. 1. Enriched NFIA expression could be related to TMZ resistance in GBM. (A) Genome-wide transcriptome microarray analysis for 45043 genes in TMZ-resistant GBM compared to the naïve GBM (GSE 68029). (B) Genome-wide transcriptome microarray analysis for 19988 genes in GBM compared to astrocytes (GSE 67089). (C) Venn diagram indicated NFIA was the only gene candidate that was upregulated in GBM and TMZ resistance when the 2 databases were merged 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).

2.15. 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.

2.16. In vivo intracranial xenograft tumor model

6-week-old female nude mice 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 μL PBS then implanted into the mice brains as previously described [4]. 5 mice were used for each group. Mice were monitored once a day until the following symptoms appeared: arched back, leg paralysis, unsteady gait or bodyweight loss for more than10%.TMZ (50 mg/kg/d) or DMSO was taken by oral after 7 days injection of glioma cells.

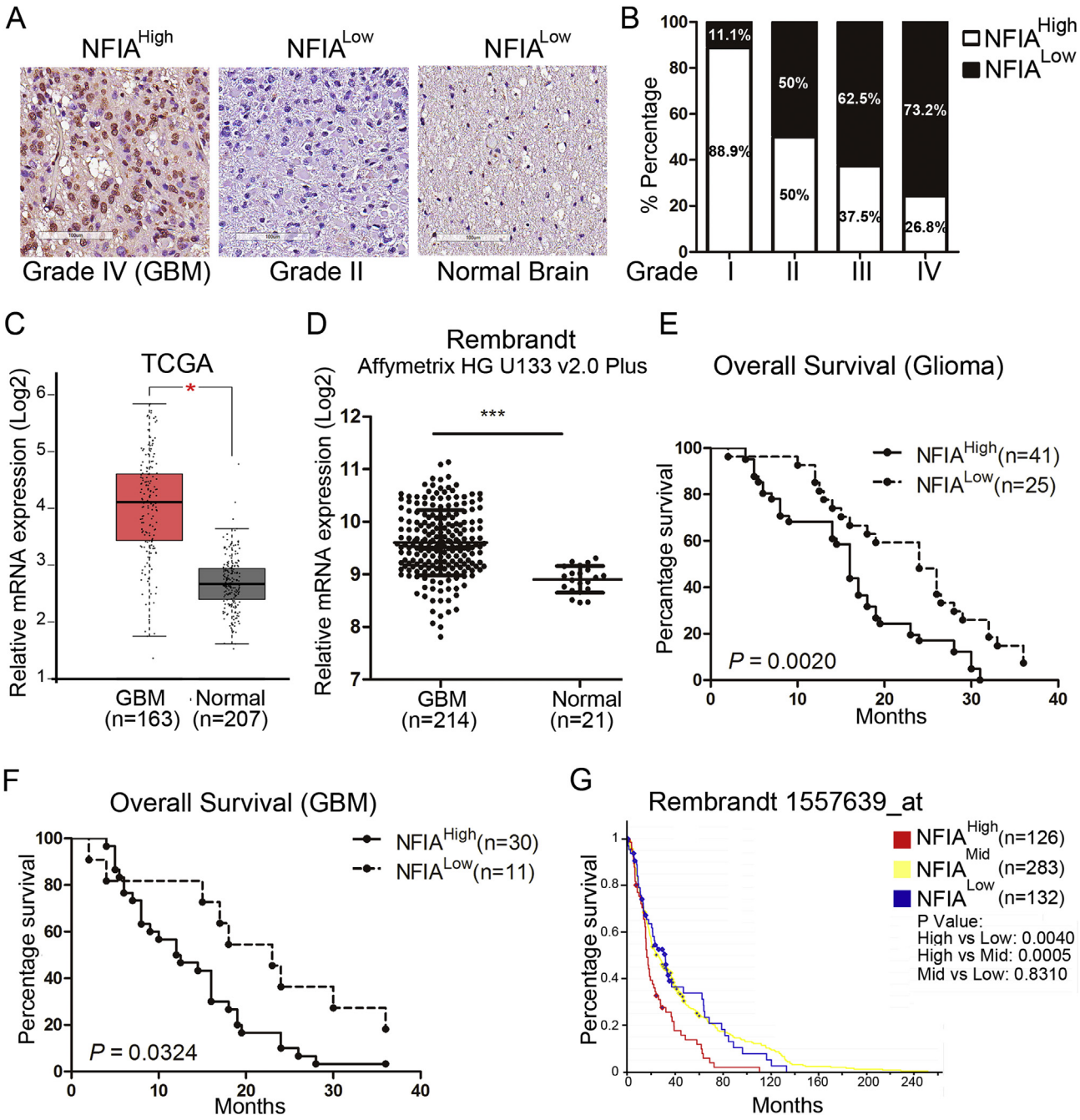


Fig. 2. Elevated NFIA was clinically related to poor prognosis in glioma patients. (A) Representative IHC images of NFIA in glioma samples. Brain tissue from epilepsy surgery was used as a negative control. (B) NFIA was enriched in high-grade glioma samples (WHO III-IV), compared with low-grade glioma samples (WHO I-II). (C) Gene expression analysis of the 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 of the 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).

2.17. Statistical analysis

All the results in this study are presented as mean ± 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.

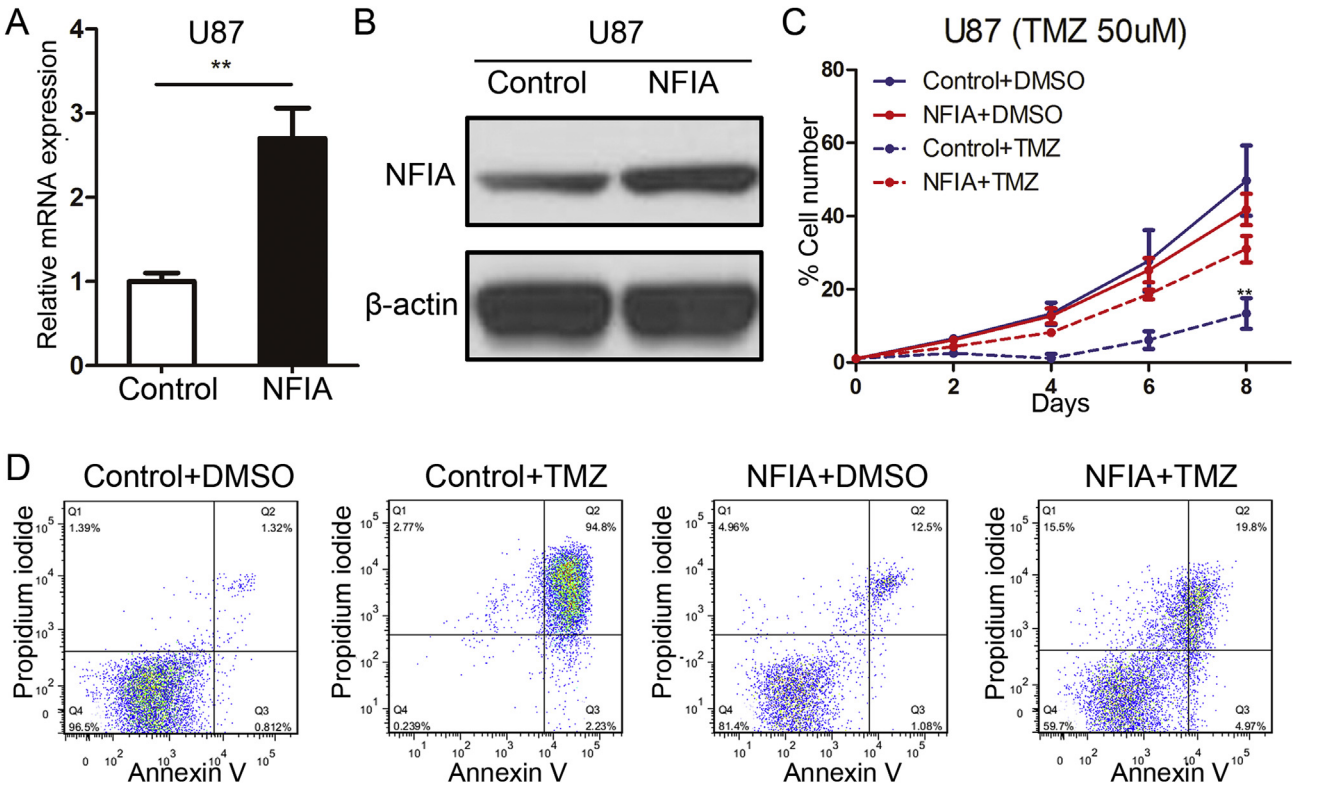


Fig. 3. Increased NFIA could be observed in TMZ-resistant GBM. (A) qRT-PCR analysis of NFIA in U87 cells transduced with NFIA overexpression lentivirus (NFIA) or control lentivirus (Control) (**$P < 0.01$, with *t*-test). (B) Western blotting analysis of NFIA in U87 cells transduced with NFIA overexpression 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 (Control + TMZ versus NFIA + 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.

3. Results

3.1. Enriched NFIA expression could be related to TMZ resistance in GBM

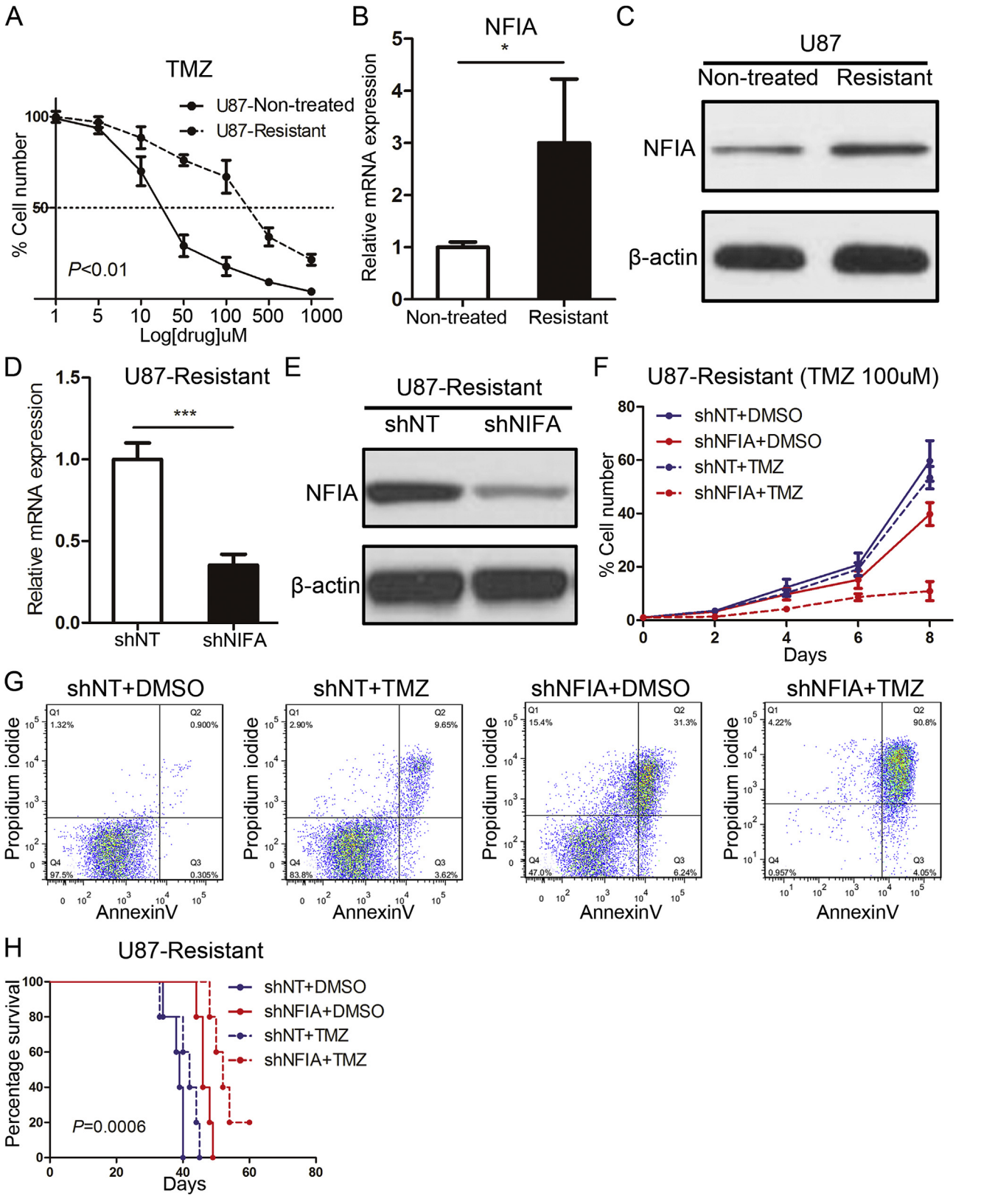
To deeply explore the key regulators and related molecular mechanisms of acquired TMZ resistance in glioma, hierarchical bi-clustering based on the previously published microarray databases (Tso et al [15], GSE 68029, 2015 and Mao et al [16], GSE67089, 2015) was performed, and the genes were ranked according to their fold changes (Fig. 1A and B). Genes with more than 5-fold upregulation were picked up from these two databases. Finally, 105 genes were identified for TMZ resistance, and 452 genes were identified as upregulated for GBM compared to astrocytes. Then, we merged the 2 gene lists together and found that NFIA was the only gene that was significantly enriched in both phenotypes (Fig. 1C). Additionally, we confirmed that NFIA was significantly increased in TMZ-resistant glioma cells compared to their naïve control lines (Fig. 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 (Fig. 1E). Taken together, these results indicated that NFIA was elevated in GBM and might be essential for therapy resistance and tumor recurrence.

3.2. Increased NFIA expression was associated with poor outcomes in GBM patients

The results from the previous data raised the question as to whether NFIA could be a prognostic marker for glioma. To this end, IHC was performed to examine NFIA expression in 66 glioma tumor tissues that were collected from patients who underwent surgical therapy from 2008 to 2017 in the First Affiliated Hospital of Xi'an Jiaotong University. The baseline information of those 66 patients can be found in Table S1. As a result, NFIA was found to be expressed in the nucleus of glioma cells (Fig. 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 (Fig. 2A 2B). We next assessed the expression of NFIA in GBM by analyzing the TCGA and Rembrandt databases. The results demonstrated that NFIA was elevated in GBM compared to normal brain tissue, which were similar to the results of our study (Fig. 2C and D). When we look into the survival for the 66 glioma patients mentioned above, glioma patients with lower NFIA expression represented longer overall survival compared to those with higher NFIA expression (Fig. 2E). Similar results were achieved when we specifically focused on GBM patients (Fig. 2F). Moreover, an analysis of overall survival was performed among 541 glioma patients from the Rembrandt database, and the results showed that the post-surgical survival for the patients with low NFIA expression was significantly prolonged compared to that in the patients with increased NFIA expression (Fig. 2G). Altogether, the results showed the possibility that NFIA is supposed to be a specific clinically relevant oncogene for glioma and GBM.

3.3. 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 or U251 cells using lentivirus infection. qRT-PCR and Western blotting analysis confirmed that NFIA expression was markedly increased in NFIA overexpressed U87 and U251 cells (Figs. 3A, 3B, S1A and S1B). *In vitro* cell growth assay indicated that TMZ resistance was obviously enhanced after NFIA overexpression in U87 and U251 cells (Figs. 3C and S1C). Moreover, U87 cells with or without NFIA



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overexpression were treated with TMZ, thus an apoptosis analysis was performed. The results showed that TMZ promoted an apoptotic cellular population and that NFIA overexpression significantly decreased the TMZ-induced apoptosis in U87 cells (Fig. 3D). Similar results could be observed when using U251 cells (Fig. S1D). These results showed increased TMZ resistance after NFIA overexpression, demonstrating that NFIA was functionally required for acquired TMZ resistance in GBM.

Fig. 4. NFIA was functionally required for acquired TMZ resistance in GBM. (A) *In vitro* cell viability assay for TMZ in U87 naïve cells (Nontreated) and U87 TMZ-resistant cells (Resistant) ($P < 0.01$, with one-way ANOVA). (B) qRT-PCR analysis showed that the NFIA mRNA expression level was substantially increased in TMZ-resistant U87 cells (*$P < 0.05$, with *t*-test). (C) Western blotting analysis showed that 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 nontargeting 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 nontargeting 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 (shNT + TMZ versus shNFIA + TMZ, *$P < 0.05$, 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 treated with or without TMZ. (H) Kaplan-Meier analysis for mice after the intracranial transplantation of U87 TMZ-resistant cells pretreated with shNFIA or shNT lentivirus and then followed continuously with 10-day TMZ (50 mg/kg/d) treatment or placebo (DMSO) by tail vein injection (shNT + TMZ versus shNFIA + TMZ, $P = 0.0006$, with log rank test).

3.4. 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* a TMZ-resistant U87 cell line according to the previous publications [18,19]. After 3 months of culturing the cells with TMZ-contained medium, the U87 cells gained stable resistance to TMZ (Fig. 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 (Fig. 4B). Moreover, Western blotting results also showed upregulated NFIA in the TMZ-resistant population of U87 cells (Fig. 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 nontargeting lentivirus (shNT) as a negative control. Both qRT-PCR and Western blotting analysis showed dramatic downregulation of NFIA at the mRNA level (Fig. 4D and E). Additionally, to test the function of NFIA on TMZ resistance, U87 TMZ-resistant cells either exposed or not exposed to NFIA suppression were then treated with 300 μM TMZ. An *in vitro* cell growth assay exhibited decreased cell proliferation and increased TMZ sensitivity in U87 TMZ-resistant cells transduced with shNFIA lentivirus (Fig. 4F). Similarly, flow cytometry assays for apoptosis were performed with shNFIA or shNT pre-transduced U87 TMZ-resistant cells treated with or without TMZ for 24 h (300 μM). The proportions of U87 cells that were undergoing early and late apoptosis were both dramatically increased when they received the combined treatment of TMZ and NFIA silencing compared with TMZ alone (Fig. 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 (Fig. 4G), highlighting a potent anti-TMZ resistance effect of NFIA knock-down in TMZ-resistant GBMs.

3.5. NFIA-dependent transcriptional regulation of NF-kB contributes to the acquired TMZ resistance in GBM

A previous study showed that NFIA contributes to tumor progression through the regulation of NF-κB expression. In our study, we found that NF-κB expression as well as phosphorylated NF-kB p65 were significantly increased in TMZ-resistant U87 cells compared to the original nontreated U87 (Fig. 5A and B). As a downstream target of NF-κB, MGMT expression was significantly elevated in U87 TMZ-resistant cells (Fig. 5B). Furthermore, inhibition of NFIA reduced NF-κB expression and phosphorylated NF-kB p65 protein in TMZ-resistant U87 (Fig. 5C and D). Interestingly, we also found that MGMT expression was significantly decreased after knock-down of NFIA (Fig. 5D). We then performed a luciferase reporter assay with constructs driven by a human NF-κB promoter. As expected, overexpression of NFIA led to a significant increase of NF-κB promoter activity in both 293T and U87 cells (Fig. 5E). Contrarily, shRNA-mediated-knockdown of NFIA resulted in a marked decrease in the transcription activity of the NF-κB promoter region in U87 cells, especially in TMZ-resistant U87 cells, which exhibited a higher expression of NF-κB (Fig. 5F). Altogether, these data suggest the presence of a tumor-promoting regulation between NFIA and NF-κB in GBM.

NF-kB signaling has been reported to be an essential pathway which was abnormally activated thus enhanced tumor proliferation and therapy resistance in cancers [20]. To further evaluate the underlying signaling pathways by which NFIA functions as an apoptosis regulator, the TCGA database was grouped into two groups according to the mRNA expression of NFIA. Hierarchical bi-clustering analysis indicated significant gene signatures in $NFIA^{High}$ GBM compared with $NFIA^{Low}$ GBM. Through grouping the TCGA GBM samples with a median cutoff, the results showed that a wide range of apoptosis correlated genes were significantly enriched in $NFIA^{High}$ GBM samples (Fig. S2). Moreover, 4 genes including FLIP, CIAP2, BFL1 and BCL2 were picked as the apoptosis genes which are correlated with NF-kB signaling. Pearson correlation analysis was performed by using TCGA database. The results indicated that BCL2 was strongly correlated with NFIA while no significant correlation was observed in the other 3 genes (Figs. S3A, S3B, S3C and S3D). Interestingly, when we look into the Chinese Glioma Genome Atlas (CGGA) database, expression of NFIA and BCL2 exhibited remarkable correlation in recurrent GBM but not in primary GBM (Figs. S3E and 3F). Additionally, qRT-PCR analysis was performed to examine the potential NF-kB-targeted apoptosis genes in U87 cells pre-transduced with lentivirus against NFIA. As a result, silencing of NFIA decreased the expression of BCL2 in U87 cells but no significant reduction of FLIP, CIAP2 or BFL1 was detected (Figs. S4A, 4B, 4C and 4D).

As we know that NF-kB is biologically activated after it is phosphorylated, it is important to investigate whether NFIA-dependent TMZ resistance occurs through the phosphorylation of NF-kB signaling. To this end, IKKβ, IKKα or NEMO was overexpressed in U87 cells to directly active NF-kB signaling, and the cells were either exposed or not exposed to lentiviral suppression of NFIA. Western blotting analysis indicated that IKKβ overexpression dramatically increased the expression of phosphorylated NF-kB in U87 cells, while this effect could be reversed, at least partially, by NFIA knock-down (Fig. 5G). Additionally, *in vitro* cell growth assay showed that increased IKKβ enhanced TMZ resistance in U87 cells (Fig. 5H). By contrast, the phosphorylation level of NF-kB in U87 TMZ-resistant cells was significantly decreased by IKKβ knock-down; however, it could be markedly increased when combined with exogenous NFIA overexpression (Fig. 5I). Cell proliferation and TMZ resistance of U87-resistant cells was suppressed by lentivirus-induced IKKβ inhibition (Fig. 5J). Moreover, western blotting and cell proliferation assay showed that both expression of NF-kB and *in vitro* cell growth were decreased in shNFIA U87 cells, however, could be partially rescued by IKKα and NEMO overexpression, although not as significant as IKKβ overexpression (Figs. S5A and S5B). Altogether, these data suggest that the NFIA-dependent regulation of NF-kB signaling contributes to the acquired TMZ resistance in GBM.

4. Discussion

Accumulating data demonstrates that acquired resistance to radio therapy and chemotherapy is essential for the recurrence and lethal

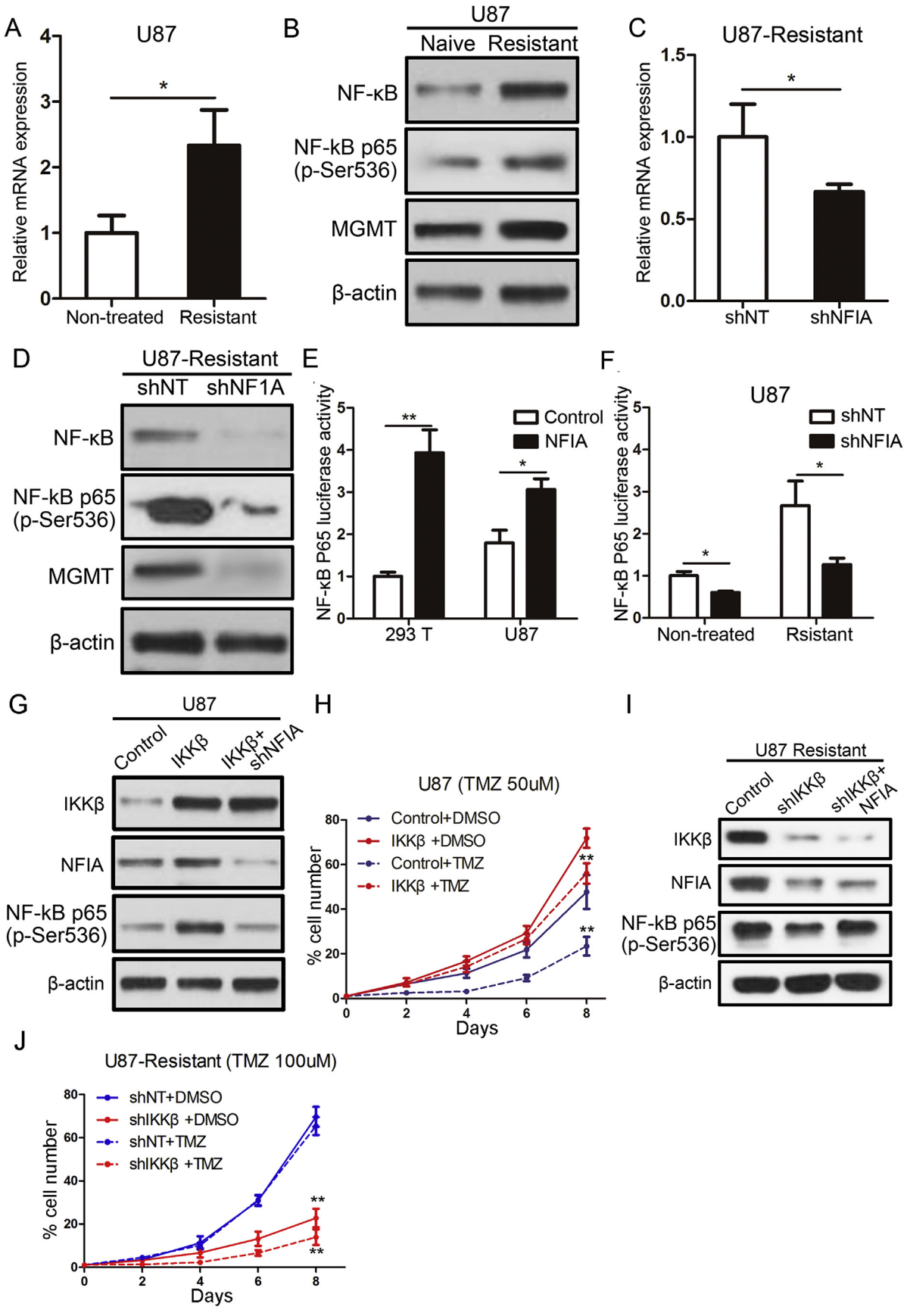

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Fig. 5. NFIA transcriptional regulation of NF-kB-induced TMZ resistance in GBM. (A) qRT-PCR analysis showed that the NF-kB p65 mRNA expression level was substantially increased in TMZ-resistant U87 cells (*$P < 0.05$, with *t*-test). (B) Western blotting analysis showed that NF-kB p65 protein, phosphorylated NF-kB p65 protein and MGMT were increased in TMZ-resistant U87 cells. β-actin served as a loading control. (C) qRT-PCR analysis of NF-kB p65 in U87 TMZ-resistant cells transduced with shRNA against NFIA (shNFIA) or nontargeting control (shNT) (*$P < 0.05$, with *t*-test). (D) Western blotting analysis of NF-kB p65, phosphorylated NF-kB p65 and MGMT in U87 TMZ-resistant cells transduced with shRNA against NFIA (shNFIA) or nontargeting control (shNT). β-actin served as a loading control. (E) Relative luciferase activity of NF-κB p65 promoter transfected into 293T cells or U87 GBM cells pretreated with NFIA overexpression lentivirus (NFIA) or control lentivirus (Control) (*$P < 0.05$, **$P < 0.01$, with *t*-test). (F) Relative luciferase activity of NF-κB p65 promoter transfected into TMZ-resistant U87 cells pretreated with shRNA against NFIA (shNFIA) or nontargeting control (shNT). (*$P < 0.05$, with *t*-test). (G) Western blotting analysis of IKKβ, NFIA and phosphorylated NF-kB p65 in U87 cells transduced with control lentivirus (Control) and IKKβ overexpression lentivirus (IKKβ) combined with or without shRNA against NFIA (shNFIA). β-actin served as a loading control. (H) *In vitro* cell growth assay for IKKβ overexpressed U87 cells treated with TMZ (**$P < 0.01$, with one-way ANOVA). (I) Western blotting analysis of IKKβ, NFIA and phosphorylated NF-kB p65 in U87 TMZ-resistant cells transduced with control lentivirus (Control) and IKKβ knock-down lentivirus (shIKKβ) in the presence or absence of NFIA overexpression lentivirus (NFIA). β-actin served as a loading control. (J) *In vitro* cell growth assay for IKKβ knock-down U87 TMZ-resistant cells treated with TMZ (**$P < 0.01$, with one-way ANOVA).

mortality of GBM [21]. Multiple mechanisms have been identified for how GBM cells acquire therapy resistance [22,23]. Our findings here indicate that NFIA promotes TMZ resistance in GBM via the transcriptional regulation of NF-κB, thus contributing to poor prognosis and recurrence for GBM patients and suggesting that the NFIA-NF-κB 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 [24]. NFI family members comprise a set of vertebrate nuclear proteins that recognize and bind to a particular DNA sequence, thus activating or repressing transcription and DNA replication [6]. Among the NFI family, NFIA has been proved to be essential for the development of glial lineage specification and for the regulation of astrocyte terminal differentiation [25]. Meanwhile, NFIA has been implicated in a wide range of human tumors, including esophageal squamous cell carcinoma, esophagogastric junction adenocarcinoma, astrocytoma, and glioma [6,26,27]. Additionally, NFIA was reported to be responsible for the proliferation and recurrence of glioma via the negative regulation of tumor suppressors, including p65 and p21, indicating that 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, it 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. These findings suggest that NFIA is a potential therapeutic target for GBM treatment. Due to the limitation of the *in vitro* research and to the small amount of data, it still remains to be seen whether these findings could be extended to the more complex *in vivo* situations. Additionally, an analysis of a larger cohort is planned to strengthen the conclusion.

NF-κB p65 is a well-recognized anti-apoptotic transcription factor, and abnormal expression or activation of NF-κB p65 has been found in multiple types of malignant cancers such as cervical cancer, esophageal squamous cancer, ovarian cancer, breast cancer and glioma [13,28–31]. NF-κB promotes tumorigenesis and therapy resistance mainly through the induction of master transcription factors, including signal transducer and activator of transcription 3 (STAT3) and TAZ [32]. It is well known that NF-κB activity is primarily enhanced by interaction with inhibitor of kappa B (IkB) with contributions from positive and negative upstream regulators, including TNF and NIK, among others [33]. Lee et al [6] reported that NFIA increased NF-κB transcription activity, thus increasing NF-κB expression at both the mRNA and protein levels. Interestingly, this study also demonstrates a feed-forward loop between NFIA and NF-κB that may increase GBM cell survival and protect GBM cells from chemotherapy-induced apoptosis [32]. Herein, we found abnormal upregulated expression of NFIA and NF-κB in a TMZ-resistant U87 cell line, and artificial lentivirus-dependent suppression of NFIA induced a reduction of NF-κB expression, thus decreasing the cell growth and re-sensitizing TMZ-resistant U87 cells to TMZ. Moreover, shRNA-mediated inhibition of NFIA reduced the activity of the NF-κB promoter. On the contrary, exogenous overexpression of NFIA led to a significant increase of NF-κB promoter activity in both 293T and U87 cells, suggesting that NFIA-dependent transcriptional regulation of NF-kB contributes to the acquired TMZ resistance in GBM. However, as NF-kB is biologically active only after it is phosphorylated, the regulation of NFIA on the phosphorylation of NF-kB signaling should be investigated. In this study, our results indicated that NFIA promotes GBM cells to acquire TMZ resistance via phosphorylation of NF-kB p65 at serine 536, which was identified as an important functional site of NF-kB signaling. Moreover, IKKβ could partially reverse the reduction of phosphorylated NF-kB after NFIA knock-down, indicating that IKKβ was functionally required for NFIA-induced activation of NF-kB signaling in GBM. Further research that includes ChIP-sequencing needs to be performed to investigate the binding site for NFIA on NF-kB and to clarify the mechanism for NFIA-dependent regulation of NF-κB in GBM.

5. Conclusion

NFIA promotes TMZ resistance in GBM via the activation of NF-κB, thus contributing to poor prognosis and recurrence for GBM patients. These findings suggest that the NFIA-NF-κB axis could be a new therapeutic target for TMZ-resistant GBM.

Author contributions

XY and JW designed the study concept and performed the molecular biology experiments. JW, MW, AW, PM and WX participated in data acquisition and wrote the manuscript. RL, WW, and JZ performed the animal experiments and the survival analysis. JW, JZ and RL revised the manuscript and contributed to data analysis.

Declaration of competing interest

No conflicts of interest exists in the submission of the manuscript and the manuscript is approved by all authors for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116917.

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