

# RIP2 promotes glioma cell growth by regulating TRAF3 and activating the NF- $\kappa$ B and p38 signaling pathways

XIN CAI<sup>1\*</sup>, YONGCHANG YANG<sup>2\*</sup>, WENGRONG XIA<sup>1</sup>, HAIBO KONG<sup>3</sup>, MIN WANG<sup>1</sup>,  
WENLIANG FU<sup>1</sup>, MINHUI LONG<sup>1</sup>, YUHUA HU<sup>3</sup> and DONGGANG XU<sup>1</sup>

<sup>1</sup>Laboratory of Genome Engineering, Beijing Institute of Basic Medical Sciences, Beijing 100850;  
<sup>2</sup>Department of Clinical Laboratory, PLA Army General Hospital, Beijing 100700; <sup>3</sup>Department of Neurosurgery,  
The Second Hospital Affiliated to Hebei Medical University, Shijiazhuang, Hebei 050000, P.R. China

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**Abstract.** Receptor-interacting protein 2 (RIP2) has recently been reported to be involved in tumor infiltration and cancer metastasis. However, the function of RIP2 in human astrocytoma remains unclear. In the present study, we showed that the expressions of RIP2 and Bcl-xL were positively correlated with the malignant grade in 28 cases of astrocytoma of various grades and 6 cases of normal human tissues. In addition, increased activity of the NF- $\kappa$ B and p38 signaling pathways in astrocytoma tissue was observed. Cytological experiments indicated that RIP2 promoted human glioblastoma cell proliferation by inducing expression of Bcl-xL, and knockdown of endogenous RIP2 promoted cell apoptosis. Mechanistically, knockdown of RIP2 suppressed downstream events including the canonical and alternative NF- $\kappa$ B pathway as well as the mitogen-activated protein kinase (p38) pathway. In addition, the present study also demonstrated that tumor necrosis factor receptor-associated factor 3 (TRAF3), as a novel RIP2 binding partner, was downregulated in glioma tissues and functionally was a negative regulator involved in RIP2-induced glioma cell growth. Taken together, the present study established a negative link between RIP2 and TRAF3 proteins and identifies a new pathway for regulating astrocytoma progression.

## Introduction

Glioma is a tumor that originates in the glial cells of the brain or the spine. Gliomas comprise ~30% of all brain and central nervous system tumors, and 80% of all malignant brain tumors (1). According to the histopathological features and clinical presentation, primary brain tumors can be graded from I to IV, of which glioblastoma multiforme (GBM) is the most malignant brain glioma subtype (2). Despite several advances achieved currently in multimodal treatments, the average lifespan expectancy of patients with GBM is still <14 months (3). In the process of genesis, development and malignant transformation, the expression of different signaling molecules all can accelerate or delay the progress of the condition of the patient. It has been reported that NF- $\kappa$ B and p38 signaling pathways play crucial roles in the invasion and metastasis of glioma cells (4-8), blocking of which are considered treatment targets in the activated state in GBM (4,9).

Receptor-interacting protein 2 (RIP2), also called RICK/CARDIAK, consists of an N-terminal serine/threonine kinase domain and a CARD domain for protein-protein interaction (10). RIP2 is an inducible transcriptional product of NF- $\kappa$ B activation, and serves as a positive regulator of the NF- $\kappa$ B pathway by binding to the IKK complex (11). In addition, RIP2 has been associated with activation of the c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p-38 pathways (12-14). RIP2 expression is regulated at the transcriptional level. Its upregulated expression has been associated with inflammatory disease states and models such as peritoneal dialysis (PD)-associated peritonitis, Crohn's disease, multiple sclerosis (MS) and allergic asthma (15-18). Recent research demonstrated that RIP2 is involved in the invasion and metastasis of triple-negative breast cancer and bladder cancer (19,20), which highlights a novel role of RIP2 in cancer. However, whether RIP2 plays a role in human glioblastoma remains unclear.

Here, we showed the upregulation of RIP2 and NF- $\kappa$ B and p38 pathway activation in human glioma tissues and cell lines. In addition, the role of RIP2 in glioma cells was investigated. Our results suggest that RIP2 may induce proliferation of glioma cells by NF- $\kappa$ B pathway activation. Most recently, we demonstrated that RIP2 physically and functionally interacts with tumor

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*Correspondence to:* Dr Donggang Xu, Laboratory of Genome Engineering, Beijing Institute of Basic Medical Sciences, 27 Taiping Road, Beijing 100850, P.R. China  
E-mail: xudg@bmi.ac.cn

Dr Yuhua Hu, Department of Neurosurgery, The Second Hospital Affiliated to Hebei Medical University, 215 Heping West Road, Shijiazhuang, Hebei 050000, P.R. China  
E-mail: chenhuihua@126.com

\*Contributed equally

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necrosis factor receptor-associated factor 3 (TRAF3) (21), which is an important regulator in carcinogenesis by negatively regulating mitogen-activated protein kinase activation and alternative nuclear factor- $\kappa$ B signaling (22,23). To further elucidate the effects of RIP2 on the regulation of glioma cell proliferation, we started from the interaction protein of RIP2 to explore the possible mechanism of RIP2 in glioma. The present study identified that RIP2 and TRAF3 exist as a negative regulation link and TRAF3 functionally is a negative regulator involved in RIP2-induced glioma cell growth.

## Materials and methods

**Patients and tissue samples.** All the tissue samples were collected during surgery from inpatients at the Department of Neurosurgery, The Second Hospital of Hebei Medical University, and the study protocol was approved by the Local and Medical Ethics Committee (The Second Hospital of Hebei Medical Research Ethics Committee, approval no. 2012003). Written informed consent was obtained from all patients or the next of kin, and the data for samples were analyzed anonymously. Our clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki. Among the 34 samples, 28 cases were diagnosed as astrocytoma and graded according to WHO's Histopathological Grading (2000): 8 cases of grade II (mean age at diagnosis, 45.4 $\pm$ 16.9 years; 4 males and 4 females), 11 cases of grade III (mean age at diagnosis, 46.6 $\pm$ 17.8 years; 6 males and 5 females), 9 cases of grade IV (mean age at diagnosis, 54.0 $\pm$ 13.9 years; 3 males and 6 females). In addition, 6 normal control samples were obtained from resected tissues from patients with traumatic brain injury treated by brain decompression (mean age at diagnosis, 47.8 $\pm$ 14.8 years; 2 males and 4 females). Meanwhile, all the pathological results were diagnosed by two senior physicians from the Department of Pathology. The fresh tissue samples were stored in liquid nitrogen, and used for analysis of the expression levels of RIP2, TRAF3 and Bcl-xL at the mRNA or protein levels. The phosphorylation level of I $\kappa$ B $\alpha$  and p38 MAPK in the tissues was detected by western blot analysis.

**Total RNA preparation and real-time quantitative PCR (qPCR) assay.** RNA pure reagent kit for rapid extraction of ultrapure RNA (Biomed Technology Development Co., Ltd., Beijing, China) was used to extract the total RNA from the clinical tissue samples according to the manufacturer's instructions. Primer Script Reverse Transcriptase (Takara Bio, Inc., Otsu, Japan) was used for the reverse transcription of 1  $\mu$ g total RNA of tissue samples strictly following the operation manual enclosed with the kit. qRT-PCR assay of RIP2, TRAF3 and Bcl-xL mRNA transcript was performed with the SYBR-Green Master Mix (Fermentas, Burlington, ON, Canada) within the 20  $\mu$ l reaction system. The primer sequences for RT-qPCR were: RIP2-*qf*, 5'-CCCTGCCAGCTCCTCAAGACA-3'; RIP2-*qr*, 5'-GGCTATACGAGCTGCAGACGTT-3'; TRAF3-*qf*, 5'-AGGCGTGTAATACCGGGAAGC-3'; TRAF3-*qr*, 5'-GGGCCTTGATCTGCTGGTTTGT-3'; Bcl-xL-*qf*, 5'-GAGGCAGGCGACGAGTTTGAAC-3'; Bcl-xL-*qr*, 5'-GCTGCGATCCGACTCACCAATAC-3'; GAPDH-*qf*, 5'-GAGTCAA CGGATTTGGTCGT-3'; GAPDH-*qr*, 5'-TTGATTTGGAG

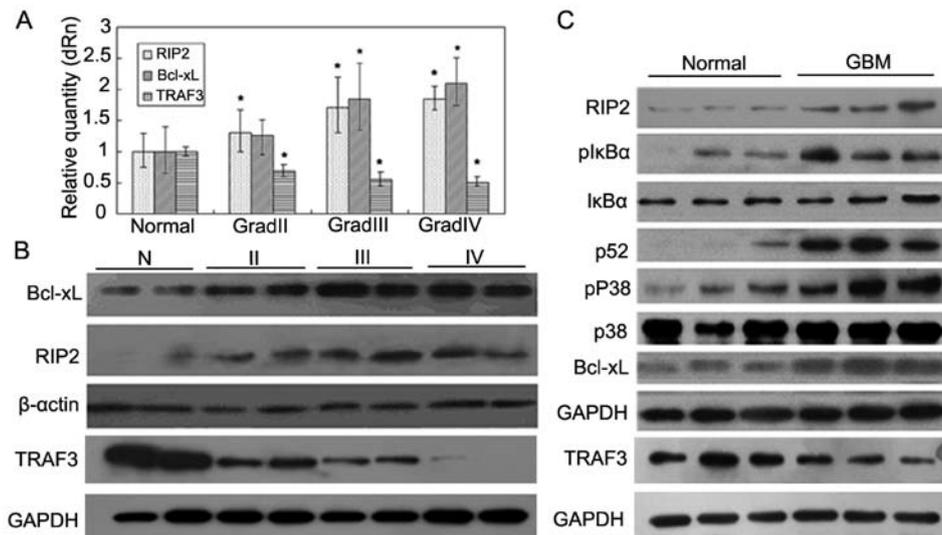
GGATCTCG-3'. The endogenous control GAPDH was used to normalize the sample with the 2<sup>- $\Delta$ ACq</sup> method for relative quantification analysis (24).

**Cell lines.** Human glioblastoma U87MG (HTB-14) and U251MG cells (the Cell Center of Union Medical College, Beijing, China) were used in the study. It has been shown that the DNA profile of U87MG (HTB-14) cell line is different from that of the original cells (25), but the gene expression profile indicates that the U87MG (HTB-14) cell line is of CNS origin and probably is a GBM cell line from an unknown patient. Thus, the U87MG (HTB-14) cell line was used as a cell model of GBM. To ensure the integrity of our research results, the U87 cell line was authenticated by STR profiling (Shanghai Biowing Applied Biotechnology Co., Ltd., Shanghai, China). Cells were cultured respectively in RPMI-1640 medium and MEM/EBSS media (both from HyClone; GE Healthcare, Logan, UT, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), at 37°C with 5% CO<sub>2</sub>.

**MTT assay.** Cells were respectively seeded in 96-well plates at 5,000 cells/well in 10% FBS-supplemented RPMI-1640 and MEM media. On the following day, the cell monolayers were incubated in serum-free medium for 24 h, and then treated with various concentrations of rhRIP2, as previously described (22). In another experiment, cells were transfected with pCMV-Myc/TRAF3 or pCMV-Myc for 24 h, and then treated with various concentrations of rhRIP2. After incubation for 48 h, cell growth was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay. Absorbance at 490 nm was measured using a Bio-Rad model 550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The data represent the means of three independent experiments.

**Plasmids, siRNAs, and transfection.** The pCMV-Myc/TRAF3 and pCMV-HA/RIP2 expression constructs have been described previously (21), as well as the cDNA target sequence of siRNAs for RIP2 (26). All of the plasmids were purified using the Plasmid Mini kit (Omega Bio-Tek, Inc., Norcross, GA, USA). siRNAs were synthesized by the Shanghai GenePharma Co., Ltd. (Shanghai, China) and transfected into glioma cell lines using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA).

**Detection of apoptosis and flow cytometric analysis.** Apoptotic cells were quantified by measuring externalized phosphatidylserine (PS) assessed by uptake of Annexin V-EGFP and propidium iodide (PI). After various experimental treatments, cells were stained using an Annexin V-EGFP apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Briefly, the harvested cells were rinsed once with phosphate-buffered saline (PBS), and then resuspended in 500  $\mu$ l of 1X binding buffer and 5  $\mu$ l Annexin V-FITC and 5  $\mu$ l of PI, and then incubated at room temperature for 15 min in the dark. For flow cytometric analysis, the cells were stained with Annexin/PI, and the population of apoptotic cells was analyzed immediately by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). Flow cyto-



**Figure 1.** RIP2 is upregulated in human astrocytoma malignancies compared with normal brain tissues. (A) Real-time PCR analysis of RIP2, Bcl-xL and TRAF3 in normal brain tissues and glioma specimens. Columns represent means and bars represent SD. (B) Representative western blots showing RIP2 protein levels in normal brain tissues and glioma specimens.  $\beta$ -actin and GAPDH were used as a loading control. Normal, n=3; grade II, low-grade astrocytoma (n=9); grade III, anaplastic astrocytoma (n=8); grade IV, GBM (n=11). \* $P < 0.05$ . (C) The correlation of expression difference of RIP2 between normal and glioma tissues with activation status of canonical and alternative NF- $\kappa$ B signaling pathway and p38 MAPK pathways. RIP2, receptor-interacting protein 2; TRAF3, tumor necrosis factor receptor-associated factor 3; GBM, glioblastoma multiforme.

metric analysis was conducted at the flow cytometry facility (FACSCalibur; BD Biosciences). Briefly, after 48-72 h of transfection, the cells were fixed and then incubated in PBS containing RNase A (100  $\mu$ g/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and PI (50  $\mu$ g/ml; Sigma-Aldrich; Merck KGaA) at room temperature for 30 min prior to analysis.

**Western blot analysis.** The ground clinical tissue samples or 48 h-transfected U87MG and U251MG cells were harvested and washed twice with ice-cold PBS and lysed in RIPA lysis (Applygen Technologies, Inc., Beijing, China) for 15 min on ice. Cell lysates were then clarified by microcentrifugation at 12,000  $\times$  g for 10 min at 4°C. Equal amounts of protein (40  $\mu$ g) were quantified using the Lowry Protein Assay kit (Nanjing KeyGen Biotech Co., Ltd.), and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Bedford, MA, USA). Membranes were then blocked with PBST (PBS with 0.05% Tween-20) containing 5% non-fat dry milk for 1 h at room temperature. The specific antibodies (primary antibodies) against Bcl-xL (cat. no. 2764) and TRAF3 (cat. no. 4729; both from Cell Signaling Technology, Inc., Danvers, MA, USA), and RIP2 (cat. no. ab75257) (Abcam, Cambridge, MA, USA) were prepared with 5% defatted milk powder and diluted 1,000 times (1:1,000); polyclonal p100/p52 antibodies (cat. no. ab31409; Abcam) and the phosphorylated anti-pP38 antibody (cat. no. ab4822) and p38 MAPK monoclonal antibodies (cat. no. ab32142; Abcam) were prepared with 5% BSA and diluted 1,000 times (1:1,000). GAPDH monoclonal antibodies (cat. no. TA-08; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) were prepared with 5% defatted milk powder and diluted 2,000 times (1:2,000) overnight at 4°C, respectively. The membranes were then washed with TBST for three times followed by incubation

with HRP-conjugated anti-mouse (cat. no. ZB-2305) or HRP-conjugated anti-rabbit immunoglobulin G (IgG) (cat. no. ZB-2301; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) at room temperature for 1 h. Finally, the membranes were analyzed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech; GE Healthcare, Oakville, Canada).

**Statistical analysis.** All data are expressed as mean  $\pm$  SD. The difference between two groups was analyzed using the Student's t-test, while differences among more than two groups were analyzed using one-way ANOVA method. Multiple comparisons between the groups was performed using the Student-Newman-Keuls (SNK) method. Statistical analysis was performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) and MxPro 3000-qPCR statistical software, respectively.  $P < 0.05$  was used to indicate a statistically significant difference.

## Results

**Analysis of RIP2 expression in normal brain tissues and astrocytoma tissues of various grades.** The expression of RIP2, TRAF3 and Bcl-xL was detected in 34 tissue samples from control samples and patients with glioma by qPCR. The results demonstrated that the expression of RIP2 and Bcl-xL was markedly increased in the lower (II) and higher (III, IV) grades of glioma in comparison with that in the normal brain tissues ( $P < 0.05$ ;  $P < 0.05$ ), whereas the expression of TRAF3 was decreased in the higher grades (III, IV) of glioma ( $P < 0.05$ ) (Fig. 1A).

Furthermore, western blot analysis revealed that the protein expression levels of both RIP2 and Bcl-xL in the glioma patient tissues were also higher than these levels in the normal tissues, and the expression of TRAF3 was decreased in the glioma patient tissues, which was consistent with the qPCR

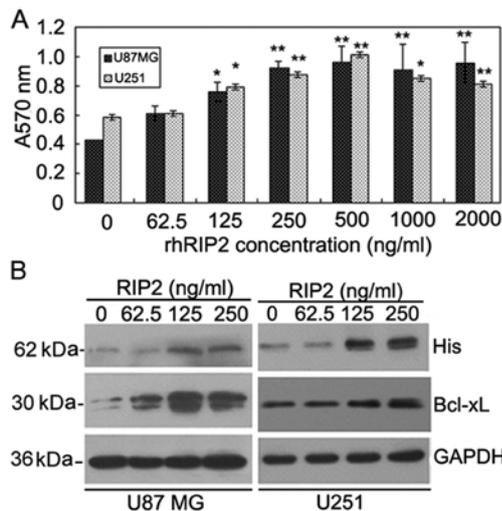


Figure 2. Effect of RIP2 on the growth of U87MG and U251 cells. (A) Indicated amounts of RIP2 were added to 96-well plates containing 5,000 cells/well. After incubation for 48 h, cell growth was measured by MTT assay. The data represent the means of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.001$  (Student's *t*-test). (B) U87MG and U251 cells were treated with indicated amounts of RIP2 for 48 h. Western blot analysis was used to analyze the protein expression levels of His-RIP2 and Bcl-xL from rhRIP2-treated cell lysates. GAPDH was used as loading control. RIP2, receptor-interacting protein 2.

results (Fig. 1B). The results showed that the mRNA and protein expression of RIP2 had a positive correlation with the degree of tumor malignancy, whereas the expression of TRAF3 had a negative correlation with the degree of tumor malignancy.

As RIP2 is associated with activation of the NF- $\kappa$ B and p38 pathways (12-14,21), we evaluated the NF- $\kappa$ B signaling pathway including both canonical NF- $\kappa$ B and alternative NF- $\kappa$ B as well as p38 MAPK activation in glioma samples by western blot analysis. As shown in Fig. 1C, the phosphorylation levels of I $\kappa$ B $\alpha$  and p38 MAPK in glioma tissues were markedly increased compared with the levels in the normal brain tissues. In addition, we also observed that the expression of p52 and Bcl-xL in glioma tissues was also markedly increased (Fig. 1C) and positively correlated with the malignant degree of glioma, which was concurrent with inhibition of the expression of TRAF3 mRNA in glioma tissues. Taken together, the above results indicate that RIP2 may take part in the regulation of NF- $\kappa$ B and p38 MAPK pathway-mediated anti-apoptosis by negative regulation of the expression of TRAF3.

**Exogenous RIP2 promotes glioma cell proliferation.** The effect of RIP2 on the U87MG and U251 cells was examined by MTT assay. As shown in Fig. 2A, RIP2 stimulated the proliferation of U87MG and U251 cells at a concentration of 62.5 ng/ml. The effect of rhRIP2 on the proliferation of U87MG and U251 cells displayed a dose-dependent manner from 62.5 to 500 ng/ml, which was decreased at concentrations >1,000 ng/ml (Fig. 2A). The results demonstrated that RIP2 facilitated U87MG and U251 cell proliferation.

We next investigated whether the entry of the RIP2 fusion protein into U87MG and U251 cells caused a specific biological effect by regulation of an anti-apoptosis-related protein. As shown in Fig. 2B, the entry of RIP2 fusion protein into glioma

cells was markedly upregulated when U87MG and U251 cells were pretreated with rhRIP2 from 62.5 to 500 ng/ml. Moreover, we found that RIP2 induced upregulation of the anti-apoptotic protein Bcl-xL in glioma cells in a dose-dependent manner (Fig. 2B). These data indicated that the entry of RIP2 fusion protein into glioma cells may facilitate cell proliferation by upregulating the expression of Bcl-xL.

**Knockdown of endogenous RIP2 promotes glioma cell apoptosis.** To assess whether the observed increase in proliferation in response to exogenous RIP2 was due to the anti-apoptotic role of RIP2 in glioma cells, apoptosis assay was evaluated by Annexin V-FITC/PI double staining method after knockdown of endogenous RIP2 (Fig. 3A) in the U87MG and U251 cells.

Apoptosis assay confirmed that the apoptotic rate was increased by 18 and 21.83%, respectively, in the U87MG and U251 cells in comparison with the control group (Fig. 3B and D). ANOVA test indicated that the percentage of apoptotic U87MG and U251 cells increased after RIP2 knockdown as compared with Nc siRNA transfection ( $P < 0.05$ , Fig. 3C and E). The results demonstrated that knockdown of endogenous RIP2 increased the apoptosis in U87MG and U251 cells, which was consistent with the effect of RIP2-promoting U87MG and U251 cell proliferation.

**Knockdown of endogenous RIP2 inhibits NF- $\kappa$ B and p38 MAPK activation in glioma cells.** Next, we aimed at identifying the underlying molecular mechanisms responsible for the RIP2-stimulated cell proliferation. The effects of the knockdown of endogenous RIP2 were examined in U87MG and U251 cells, since U87MG and U251 cells express endogenous RIP2 at high levels. The loss of RIP2 expression was confirmed by western blot analysis (Fig. 4A and B). Following, we firstly focused on the effect of RIP2 on the activation of the canonical NF- $\kappa$ B signaling pathway in glioma cells. Western blot analysis indicated that knockdown of RIP2 markedly decreased the level of I $\kappa$ B $\alpha$  phosphorylation at Ser32, while the protein level of total I $\kappa$ B $\alpha$  was unchanged (Fig. 4A).

Given the identification of RIP2 as a novel binding partner for TRAF3 and its involvement in the regulation of the alternative NF- $\kappa$ B pathway in Ramos cells in our previous study, we sought to determine whether RIP2 is involved in the TRAF3-regulated alternative NF- $\kappa$ B pathway in glioma cells. The expression level of TRAF3 and proteolytic processing of p100 to p52 were assessed. The results showed that knockdown of RIP2 markedly inhibited the processing of p100 to p52 and increased the expression level of TRAF3. Meanwhile, RIP2 knockdown induced downregulation of anti-apoptotic protein Bcl-xL in glioma cells (Fig. 4A). Moreover, the activation of the p38 MAPK signaling pathway after RIP2 knockdown was also investigated. As shown in Fig. 4B, knockdown of RIP2 resulted in suppressed phosphorylation of p38 MAPK protein. Taken together, these findings suggest that RIP2 may be involved in the development of glioma by activating the canonical and alternative NF- $\kappa$ B pathways as well as the p38 MAPK signaling pathway.

**Endogenous TRAF3 overexpression inhibits the RIP2-induced glioma cell proliferation.** Given that RIP2 interacts with TRAF3, we investigated whether TRAF3 affects RIP2-induced

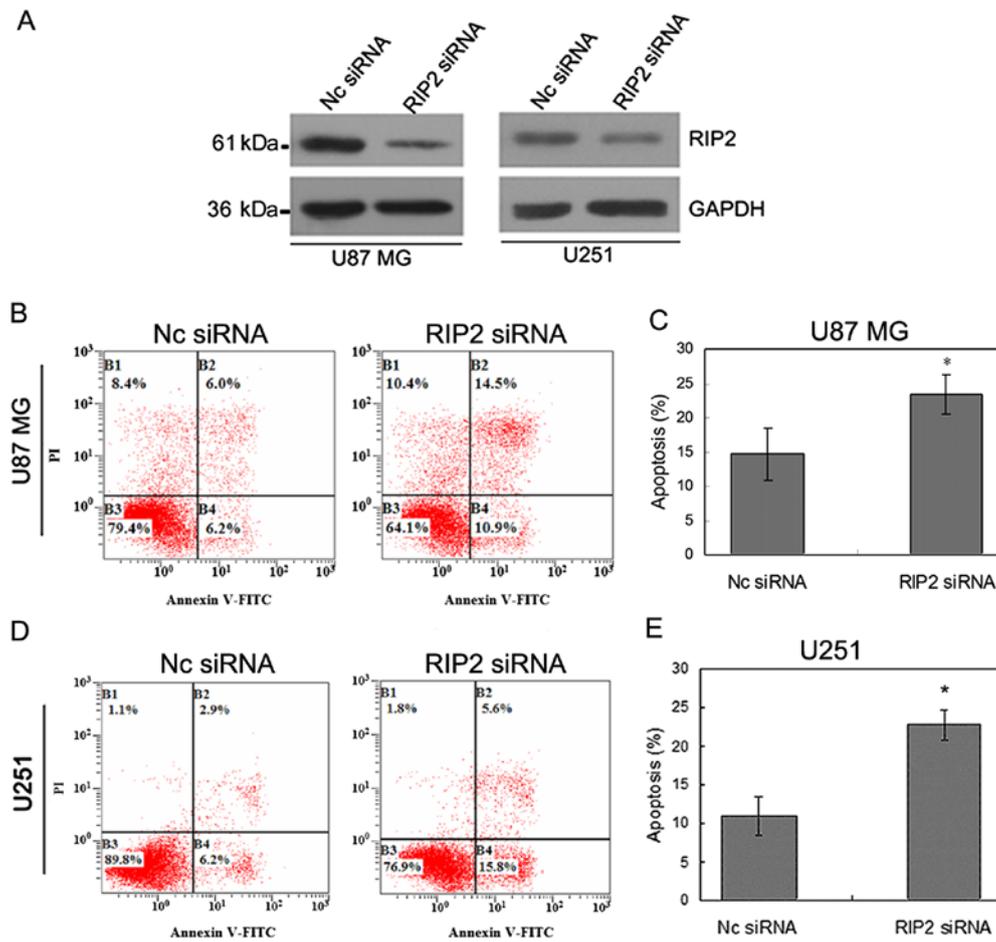


Figure 3. Effect of knockdown of RIP2 on U87MG and U251 apoptosis was analyzed by double staining with Annexin V-FITC/PI and flow cytometry. (A) U87MG and U251 cells were transfected with RIP2 siRNA or NC siRNA. (B and D) Forty-eight hours post-transfection the cells were lysed and probed with anti-RIP2 or anti-GAPDH antibodies and analyzed by double staining with Annexin V-FITC/PI and flow cytometry. Representative graphs illustrate the effect of knockdown of RIP2 on the apoptosis status of U87MG and U251 cells by flow cytometry analysis. (C and E) Following flow cytometric analysis, the percentage of cell undergoing apoptosis was calculated. Triplicate assays were performed. \* $P < 0.01$ . RIP2, receptor-interacting protein 2; PI, propidium iodide.

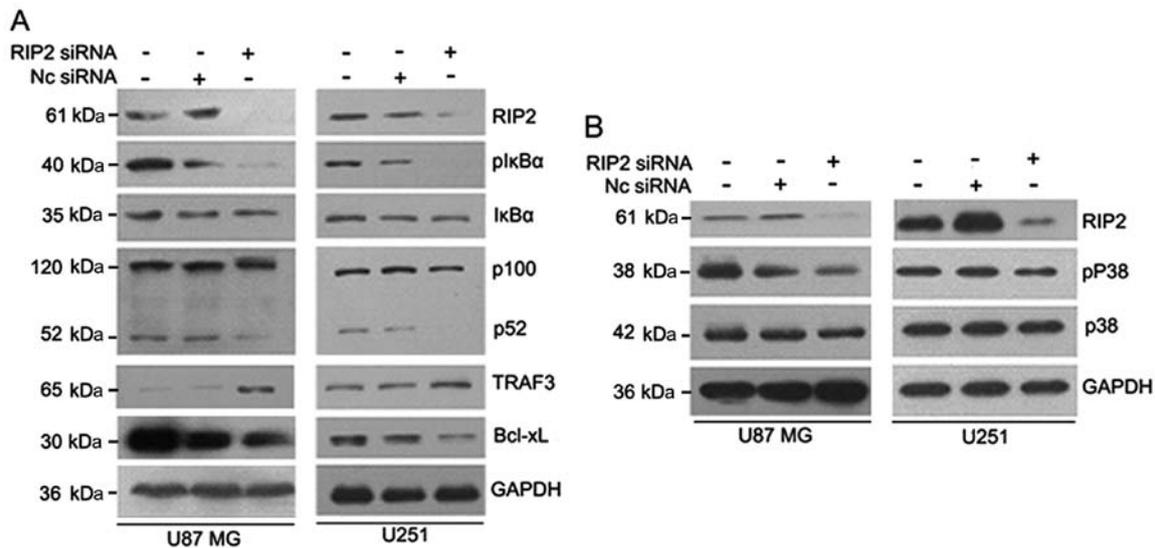


Figure 4. Knockdown of endogenous RIP2 inhibits the activation of canonical NF- $\kappa$ B and alternative NF- $\kappa$ B pathways as well as p38 MAPK signaling pathways in U87MG and U251 cells. (A) Detection of knockdown of endogenous RIP2 downregulation of the activation of canonical NF- $\kappa$ B and alternative NF- $\kappa$ B pathways in glioma cells. U87MG and U251 cells were respectively transfected with RIP2 siRNA or NC siRNA. RIP2, TRAF3, phosphorylated I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$ , p100, p52 and Bcl-xL were analyzed by western blot analysis. Expression of GAPDH served as loading control. (B) Knockdown of endogenous RIP2 inhibits the activation of p38 MAPK pathway in glioma cells. Western blots showing phosphorylated p38 and p38 protein levels in glioma cell lines transiently transfected with the control siRNA or RIP2 siRNA. GAPDH was used as a loading control. RIP2, receptor-interacting protein 2; TRAF3, tumor necrosis factor receptor-associated factor 3.

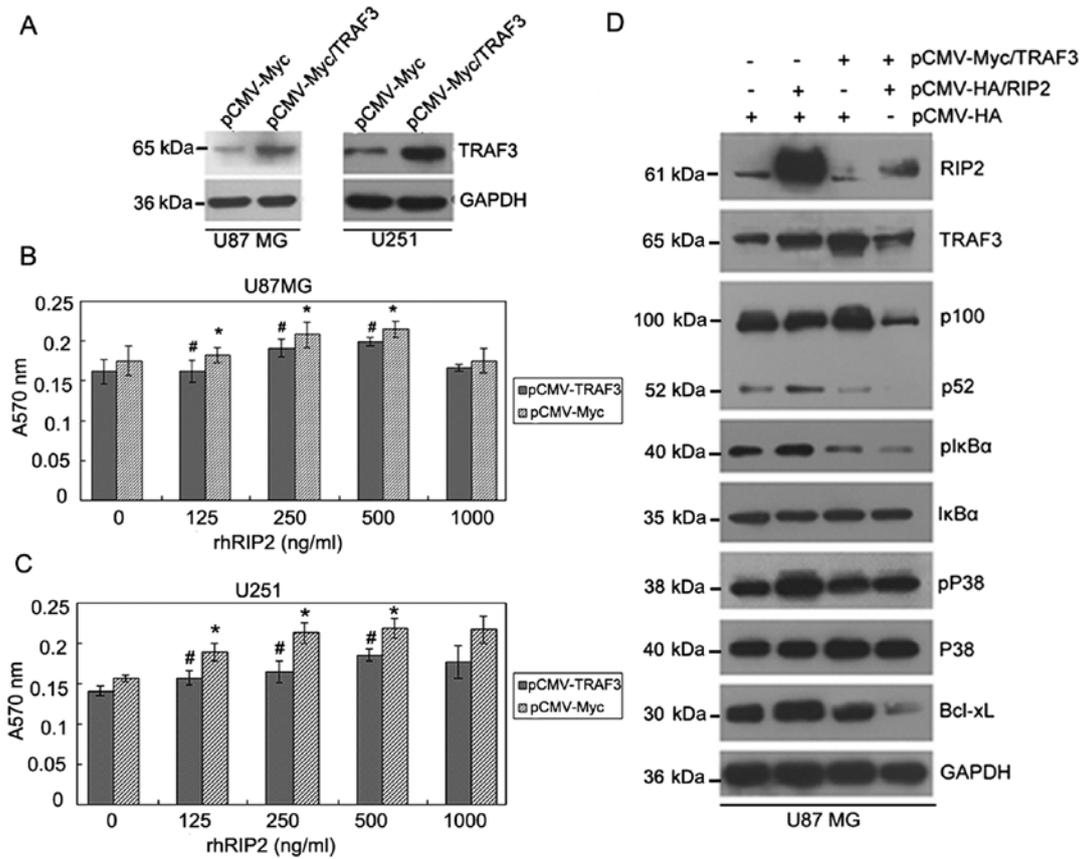


Figure 5. A negative regulation link exists between RIP2 and TRAF3 proteins in U87MG cells. (A) Overexpression of TRAF3 decreased the RIP2-induced proliferation effect on U87MG and U251 cells. The expression of TRAF3 protein of U87MG and U251 cells transfected with pCMV-Myc/TRAF3 or control pCMV-Myc plasmid was determined by western blot analysis. GAPDH was used as loading control. (B and C) Indicated amounts of rhRIP2 were added to 96-well plates containing 5,000 cells/well in transfected with pCMV-Myc/TRAF3 or control pCMV-Myc cells. After incubation for 48 h, cell growth was measured by MTT assay. The data represent the means of three independent experiments. \*P<0.05; p-values were calculated by comparing absorbance at 570 nm between rhRIP2 stimulation and control groups. #P<0.05; p-values were calculated by comparing absorbance at 570 nm between transfected with pCMV-Myc/TRAF3 and control pCMV-Myc. (D) U87MG cells were respectively transfected with the control pCMV-HA, pCMV-HA/RIP2, pCMV-Myc/TRAF3 plasmids or cotransfected with pCMV-HA/RIP2 and pCMV-Myc/TRAF3 plasmids. The expression of RIP2 and TRAF3, phosphorylated IκBα, IκBα, p100, p52 and Bcl-xL were analyzed by western blot analysis. Expression of GAPDH served as loading control. RIP2, receptor-interacting protein 2; TRAF3, tumor necrosis factor receptor-associated factor 3.

proliferation in glioma cells. As U87MG and U251 cells express endogenous TRAF3 at low levels, cells were transfected with pCMV-Myc/TRAF3 or pCMV-Myc plasmid before treatment with various concentrations of rhRIP2. The results showed that overexpression of TRAF3 effectively increased its expression as compared with the negative control (pCMV-Myc) (Fig. 5A). After confirmation of the efficient overexpression of TRAF3, transfected cells were stimulated with RIP2 for 48 h in order to investigate the role of TRAF3 in the RIP2-induced proliferation effects on glioma cells. MTT analysis indicated that TRAF3 overexpression decreased the RIP2-induced proliferation effect on U87MG and U251 cells (Fig. 5B and C). These data showed that TRAF3 negatively regulated the RIP2-induced proliferation effects on glioma cells.

Furthermore, we investigated how TRAF3 overexpression mediates the inhibition of RIP2-induced glioma cell proliferation by western blot analysis. We found that the expression of endogenous TRAF3 in U251 cells was higher than that in U87MG cells. Upregulation of TRAF3 expression in U251 cells was not as obvious as that in U87MG cells after transfection of the TRAF3 expression plasmid. Therefore, representative U87MG cells were selected to overexpress TRAF3, in order to

better understand its effect in RIP2-induced glioma cell proliferation. As shown in Fig. 5D, markedly higher expression of TRAF3 and RIP2 was observed in the U87MG cells which were respectively transfected with TRAF3 and RIP2 expression plasmids. Western blot analysis indicated that overexpression of endogenous TRAF3 downregulated the expression of RIP2 and Bcl-xL. Meanwhile, TRAF3 overexpression suppressed the activation of p100 and phosphorylation of IκBα and p38 in the transfected U87 cells. Conversely, overexpression of endogenous RIP2 downregulated the expression of TRAF3 and increased the expression of Bcl-xL. Accordingly, RIP2 overexpression induced the activation of p100 and phosphorylation of IκBα and p38 in transfected U87MG cells. Moreover, we examined the effect of TRAF3 and RIP2 cotransfection in U87MG cells on the expression of each other. The result showed that overexpression of RIP2 and TRAF3 could inhibit each other's expression. We also observed that overexpression of endogenous TRAF3 could inhibit RIP2-induced activation of p100 and phosphorylation of IκBα and p38 by cotransfection with RIP2 and TRAF3 in U87MG cells. Taken together, these results suggested that RIP2 and TRAF3 proteins may exist in a negative regulation link.

## Discussion

RIP2 is a kinase with known central roles in inflammation and immunity (27-29). Recent study highlights RIP2 as a pro-metastasis kinase in patients with advanced breast cancer and shows that targeted knockdown of RIP2 may improve outcomes in advanced breast cancer patients (19). Additionally, RIP2 may mediate bladder cancer surveillance by involvement in the development and recruitment of granulocytic myeloid-derived suppressor cells (MDSCs) and highlight the contribution of MDSCs to the development of metastases in bladder cancer (20). These results show a novel function of RIP2 in cancer in addition to its known role in inflammation. Moreover, research has found that RIP1, a member of the RIP kinase family, is overexpressed in GBM, but not in low grade gliomas, and that increased expression of RIP1 confers a worse prognosis (30). However, whether or not the expression level of RIP2 is associated with glioma progression remains unknown. Glioma is one of the common tumors seriously threatening human health. Despite several advances achieved currently in multimodal treatments, the 5-year survival of glioma patients ranks third, just after that of pancreatic and lung cancers (3). Therefore, a greater understanding of the biological mechanisms underlying glioma pathogenesis may contribute to the development of targeted therapies that can improve patient outcome.

Many previous studies have confirmed that the tumor malignant degree is closely related with the activation of various intracellular signaling pathways, such as NF- $\kappa$ B, PI3K/AKT/mTOR, MAPK and JAK/STAT, among which much more attention has been paid to the role of the NF- $\kappa$ B and MAPK pathways in the genesis and progression of glioma (20). Research has demonstrated that the NF- $\kappa$ B pathway is constitutively activated and upregulated in glioma patient samples or glioma cells in response to different stimuli (30,31). Moreover, the invasion and metastasis of glioma cells require specific intracellular signaling cascade activations, among which the p38 signaling pathway is considered crucial (5-8), and the levels of numerous NF- $\kappa$ B and p38 target genes are elevated in glioma cells (8,32). However, whether or not activation of the alternative NF- $\kappa$ B and p38 pathways is associated with the genesis of glioma is unknown. Therefore, we explored the activation status of alternative NF- $\kappa$ B and p38 pathways in addition to detection of activation of the canonical NF- $\kappa$ B pathway in glioma patient samples. Compared to the control samples, our analyses of 28 cases of glioma demonstrated that RIP2 expression was positively correlated with the malignant degree of the glioma tissues. In addition, we also found that the phosphorylation levels of I $\kappa$ B $\alpha$  and p38 and the expression levels of p52 and Bcl-xL in glioma were markedly higher than these levels in the normal brain tissues. These findings indicated that the activation of the alternative NF- $\kappa$ B and p38 pathways is correlated with the genesis of glioma. These results imply that RIP2 expression may positively correlate with canonical NF- $\kappa$ B and alternative NF- $\kappa$ B as well as p38 status. Our previous study demonstrated that RIP2 interacts with TRAF3 (21), which acts as a negative regulator of mitogen-activated protein kinase activation and alternative NF- $\kappa$ B signaling (22,23). Therefore, we also examined whether TRAF3 is associated with glioma progression.

In the present study, we showed that the expression of TRAF3 is negatively correlated with the malignant grades of glioma, which was concurrent with activation of the alternative NF- $\kappa$ B pathway and p38 pathway status in glioma tissues. These results suggest that RIP2 and TRAF3 may be involved in the genesis of glioma.

To understand the role of RIP2 in glioma, we further analyzed the functional significance of RIP2 upregulation *in vitro* and *in vivo*. Our *in vitro* studies provided several insights into the mechanism of RIP2 action. First, RIP2 promoted U87MG and U251 cell proliferation, and downregulation of RIP2 induced cell apoptosis. In addition, RIP2 upregulated the expression level of anti-apoptotic protein Bcl-xL in U87MG and U251 cells. Previous studies have demonstrated that RIP2 is involved in multiple signaling pathways such as the NF- $\kappa$ B pathway and mitogen-activated protein kinase (p38) pathways (12-14). Collectively, together with the findings of activation of the NF- $\kappa$ B and p38 pathways in glioma patient samples in this study, we proposed that the anti-apoptotic activity of RIP2 in glioma may be accomplished by regulating the NF- $\kappa$ B and p38 pathways. Using a small interfering RNA strategy, we found that RIP2 was involved in the positive regulation of canonical NF- $\kappa$ B and p38 pathways by regulation of the phosphorylation of I $\kappa$ B $\alpha$  and p38. Given the downregulation of TRAF3 expression and activation of alternative NF- $\kappa$ B pathway in glioma tissues, we then further investigated the effects of RIP2 knockdown on TRAF3 expression and alternative NF- $\kappa$ B activation. Knockdown of RIP2 inhibited proteolytic processing of p100 to p52 and promoted TRAF3 expression, suggesting that RIP2 may have positive regulation on the activation of alternative NF- $\kappa$ B by controlling the expression level of TRAF3.

TRAF3, a member of the TRAF family of cytoplasmic adaptor proteins, is employed in signaling by the tumor necrosis factor receptor (TNFR) superfamily and Toll-like receptors (TLRs) (33,34). Recently, it was identified that TRAF3 function mutations or complete gene deletions promoted multiple myeloma cell survival by preventing the interaction of TRAF3 with NIK and constitutive activation of NF- $\kappa$ B signaling in malignant cells from patients (35,36). Moreover, specific ablation of TRAF3 in B lymphocytes resulted in severe peripheral B-cell hyperplasia (37). The above-mentioned results further suggested that TRAF3 may play an important role in carcinogenesis. In our previous study, the specific interaction between TRAF3 and RIP2 was confirmed by co-immunoprecipitation and GST pull-down assays, and RIP2 was found to be involved in the positive regulation of the alternative NF- $\kappa$ B pathway by controlling the expression level of TRAF3 in Ramos cells (21). To better clarify the relationship between RIP2 and TRAF3 in the genesis of glioma, we explored the effects of TRAF3 overexpression on RIP2-induced glioma cell proliferation. Our experiments showed that TRAF3 overexpression suppressed RIP2-induced glioma cell proliferation. Based on previous and present experimental results, TRAF3 appears to play a tumor suppressor-like role in cancers. Furthermore, we explored how TRAF3 affects RIP2-induced glioma cell proliferation. Our study showed that endogenous TRAF3 could inhibit RIP2-induced activation of p100 and phosphorylation of I $\kappa$ B $\alpha$  and p38. We also discovered that TRAF3 overexpression

inhibited the expression of RIP2. These findings led us to conclude that RIP2 and TRAF3 proteins exist in a negative regulation link. In conclusion, the present study established a negative regulation link between RIP2 and TRAF3 and identified a new pathway for regulating glioma progression.

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### Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

XC, DX, and YH conceived and designed the study. XC, YY, WX, HK, MW and ML performed the experiments. HK, YH and WF contributed to collection and analyze the samples from patients. XC and YY wrote the paper. XC, YY, DX and YH reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

The study protocol was approved by the Local and Medical Ethics Committee (The Second Hospital of Hebei Medical Research Ethics Committee, approval no. 2012003). Written informed consent was obtained from all patients or the next of kin.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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