Original Article
Rab13 silencing causes inhibition of growth and induction of apoptosis in human glioma cells

Bo Diao, Xiaoyong Huang, Chenying Yang, Sheng Guo, Lei Fei, Yongwen Chen, Yuzhang Wu

Department of Immunology, The Third Military Medical University, Chongqing, China

Abstract: Glioma is the most common malignant tumor in the central nervous system worldwide with a low five-year survival rate. Several members of Rab GTPases have been found associated with glioma. Here, Rab13 (Rab GTPase 13), a member of Rab GTPase, was significantly increased in glioma tissues compared to non-tumorous brain tissues. Furthermore, Rab13 level was notably higher in high-grade glioma (WHO III-IV) than in low-grade glioma (WHO II). The down-regulation of RAB13 by specific-siRNA transfection in two glioma cell lines (U87 and U251) significantly decreased cell proliferation, while remarkably induced G0/G1 cell arrest and cell apoptosis. Rab13 siRNA is significantly downregulating the protein expression of p53, Caspase7, Bim and Bcl-2 in glioma cells. Our present study indicated that Rab13 may work as an oncogene and serve as a new target for glioma treatment.

Keywords: Rab13, glioma, proliferation, cell cycle arrest, apoptosis, p53

Introduction

Gliomas are the most frequent primary tumors in the central nervous system (CNS) [1]. According to the World Health Organization (WHO) classification, gliomas can be grouped into four grades (I, II, III and IV) [2]. Despite significant advances in surgical resection, chemotherapy and radiotherapy, no significant improvement was obtained in the overall survival of patients with glioma [3-5]. The five-year overall survival rate for glioma is less than 10% [5]. The median survival of the most malignant form of glioma, glioblastoma multiforme (GBM, WHO grade IV), is merely 12-15 months [6]. Therefore, new therapeutic strategies represent as urgent medical need. Identification of the molecular regulators related to the oncogenesis and development of glioma may guide future therapeutic strategies of this disease.

Rab13 (Rab GTPase 13) is a member of the Rab subfamily of small GTPases. Rab small GTPases have been demonstrated to be associated with various key cellular functions, including membrane trafficking, cell growth, cell differentiation, cell-matrix and cell-cell adhesion [7]. Increasing evidence has revealed the altered expression of the Rab small GTPases in several human malignancies including glioma. Rab3a is reported as a novel oncogene involved

in glioma initiation and progression. Overexpression of Rab3a in glioma cell lines could promote cell proliferation by increasing cyclin D1 expression [8]. Rab38 expression level was closely related with grade progression and prognosis in gliomas [9]. Ectopic expression of Rab27a in glioma cell U251 could promote cell proliferation and inhibit cell apoptosis [10]. Here, we aimed to evaluate Rab13 expression and functions in human glioma.

In the current study, we compared Rab13 expression between glioma and normal brain tissues. The effects of Rab13 knockdown in the proliferation, cell cycle and cell apoptosis of glioma cells were then assessed. The involved possible mechanisms were also explored.

Materials and methods

Sample collection

Sixty-two glioma samples and 11 non-neoplastic normal brain tissues of patients with epilepsy were obtained from Department of Immunology, The Third Military Medical University between July 2013 and June 2014. None of the patients had received chemotherapy or radiation therapy before surgery. All glioma samples, including 15 cases of low-grade glioma samples (WHO II, n = 15) and 47 cases of high-grade

Table 1. Primers sequences for quantitative PCR

Primer	Primer sequence	Size (bp)
Rab13 (NM_001272038.1)	F: 5'-GGAGGGTCACATAGGTAG-3'	196
	R: 5'-CCCAGGTAAGGTCTGAAG-3'	
GADPH (NM_001256799.1)	F: 5'-CACCCACTCCTCCACCTTTG-3'	110
	R: 5'-CCACCACCCTGTTGCTGTAG-3'	
P53 (NM_000546.5)	F: 5'-CCACCATCCACTACAACTAC-3'	135
	R: 5'-AAACACGCACCTCAAAGC-3'	
Caspase7 (NM_001227.4)	F: 5'-ACCTATCCTGCCCTCACATC-3'	108
	R: 5'-TCTTCTCCTGCCTCACTGTC-3'	
Bax (NM_004324.3)	F: 5'-AGCTGAGCGAGTGTCTCAAG-3'	248
	R: 5'-TGTCCAGCCCATGATGGTTC-3'	
Bim (NM_001204106.1)	F: 5'-CCACCAGCACCATAGAAG-3'	131
	R: 5'-GAGCAGGCACAGAGAAAG-3'	

cols. The qRT-PCR assay was performed using an ABI 7300 real-time thermal cycler (Applied Biosystems, Foster City, USA) with SYBR Green PCR kit (Thermo, Rockford, IL, USA). Primer sequences were provided in **Table 1**. The PCR reaction conditions were as follows: at 95°C for 5 min, and 40 cycles at 95°C for 15 sec and at 60°C for 45 sec. The relative expression level of mRNA was calculated with ΔCt method. GAPDH was used as the internal control.

glioma samples (WHO III, n = 14; WHO IV, n = 33) were obtained through surgical resection. The tissue samples were immediately collected after resection and stored at -80°C until used. All patients were provided written informed consent. This study was conducted in accordance with the ethical standards of the Helsinki Declaration and authorized by the ethical committed of the Third Military Medical University.

Cell cultured and RNA interference

The human glioma cell lines T98G, U251, SHG44, U373 and U87 were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone, UT, USA) at 37°C in a humidified atmosphere of 5% CO_2.

The siRNA targeting human Rab13 (siRab13, 5'-GGCCACCAUUUGCACAAAU-3') and nonspecific siRNA (siNC) were purchased from Gene-Pharma Company (Shanghai, China). U87 and U251 cells were transfected with siRNAs using Lipofactamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacture's instruction. Assays were performed 48 h after transfection.

RNA extraction and quantitative RT-PCR (qRT-PCR) analysis

Total RNA was extracted from tissue samples and cells using TRizol reagent (Invitrogen) and reverse transcribed to cDNA using Promega (Madison, WI, USA) reverse transcriptase enzyme according to the manufacturer's proto-

Western blotting

Cells were lysed with RIPA buffer (JRDUN, China) and the protein concentration was determined by using a BCA Kit (Beyotime, Shanghai, China) accordance with the manufacturer's protocols. Equal amounts of protein were resolved on an SDS-PAGE gel and then transferred to nitrocellulose membranes (Millipore, Bedford, USA). After blocking in 5% skim milk for 30 min, the membranes were incubated with primary antibodies at 4°C overnight. The specific primary antibodies were Rabbit anti-Rab13 (Abcam, Cambridge, USA; 1:1000 dilution), Mouse anti-p53 (Cell Signaling Technology, Danvers, MA, USA; 1:1000 dilution), Rabbit anti-Caspase7 (Abcam; 1:500 dilution), Rabbit anti-Bim (Abcam; 1:500 dilution), Rabbit anti-Bax (Santa Cruz Biotechnology, Dallas, TX, USA, 1:200 dilution) and Mouse anti-GAPDH (Cell Signaling Technology; 1:1500 dilution). After washing, the membrane was incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (Beyotime; 1:1000 dilution) at room temperature for 1 h. The signal was detected using Enhanced Chemiluminescence system (Millipore). The band intensity was analyzed with Image J software (NIH, USA) and the protein levels were normalized by GAPDH.

Cell proliferation assay

The cells were seeded in 96-well plates at a density of 1×10^4/well and incubated at 37°C in a humidified atmosphere overnight. The cells were transfected with siRab13 or siNC by using lipofectamine 2000 (Invitrogen) based on the
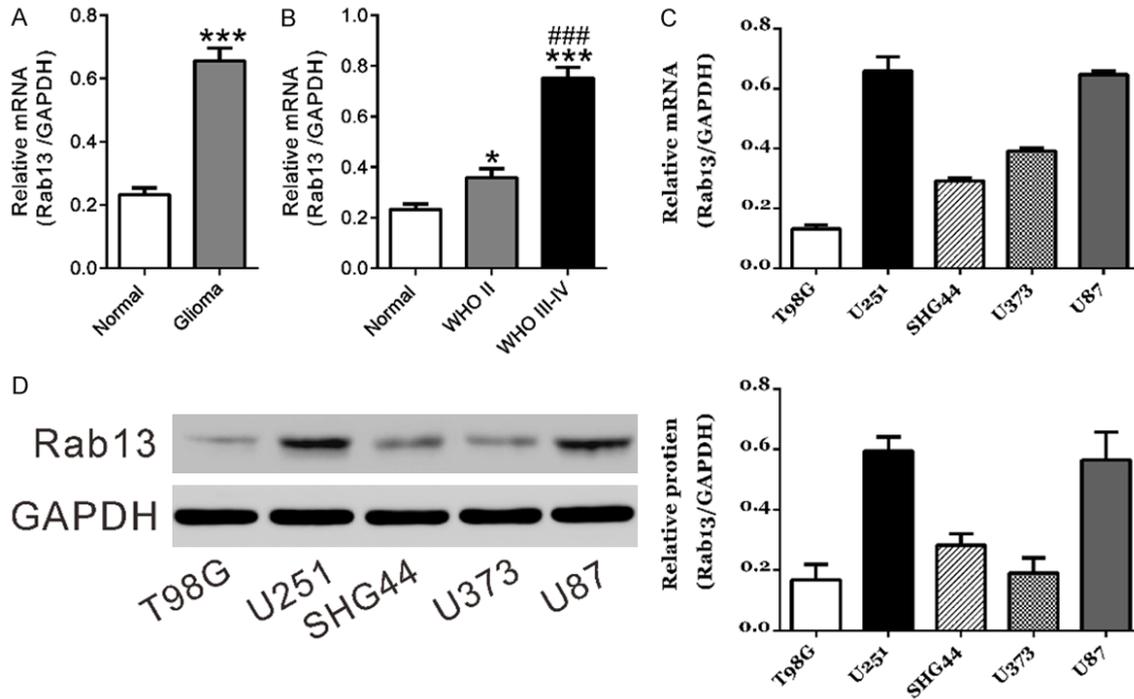
Figure 1. Rab13 expression in human glioma tissues and cells. A. qRT-PCR was performed to evaluate Rab13 mRNA in glioma tissues (n=62) and normal brain tissues (n=11). B. Rab13 mRNA expression levels in normal brain tissues (n=11), WHO Grade II and WHO Grade III-IV glioma (*$P<0.05$, ***$P<0.001$ as compared with normal tissues; ###$P<0.001$ as compared with WHO Grade II glioma). C, D. mRNA and protein levels of Rab13 in five glioma cell lines were determined by qRT-PCR and Western blot, respectively.

manufacture's instruction. At 0 h, 24 h, 48 h and 72 h post-transfection, 10% CCK-8 solution (Beyotime) was added to each well and incubated at 37°C for 1 h. Optical density values (OD_{450nm}) was measured at using a microplate reader (Bio-Rad). Each assay was performed in triplicate. Results of cell proliferation were normalized to the initial cell numbers (100%).

Cell cycle assay

At 48 h after transfection, the cells were trypsinized, washed, collected, fixed with 70% cold ethanol at 4°C overnight, and stained by propidium iodide (PI) at room temperature for 30 min in the dark. The percentages of cells in G0/G1 phase, S phase and G2/M phase were analyzed using flow cytometry (BD FACScaliber, Franklin Lakes, NJ, USA).

Cell apoptosis assay

Double staining with Annexin V-FITC/PI was carried out to evaluate cell apoptosis. At 48 h post-transfection, the cells were collected and incubated with 195 µl binding buffer containing 5 µl Annexin V-FITC (eBioscience, San Diego, CA,

USA) at 4°C for 15 min in the dark. Then, 5 µl propidium iodide was added to each sample and incubated at 4°C for another 5 min. The cells were evaluated by flow cytometry (BD Biosciences). Early (Annexin V positive, PI negative) and late (Annexin V positive, PI positive) apoptotic cells were quantitated.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). Each experiment was repeated at least three times and the data were expressed as the mean ± SD. Differences between two groups were evaluated for significance using Student's t-test. For all tests, P values less than 0.05 were considered statistical significance.

Results

Expression of Rab13 in human glioma tissues and cell lines

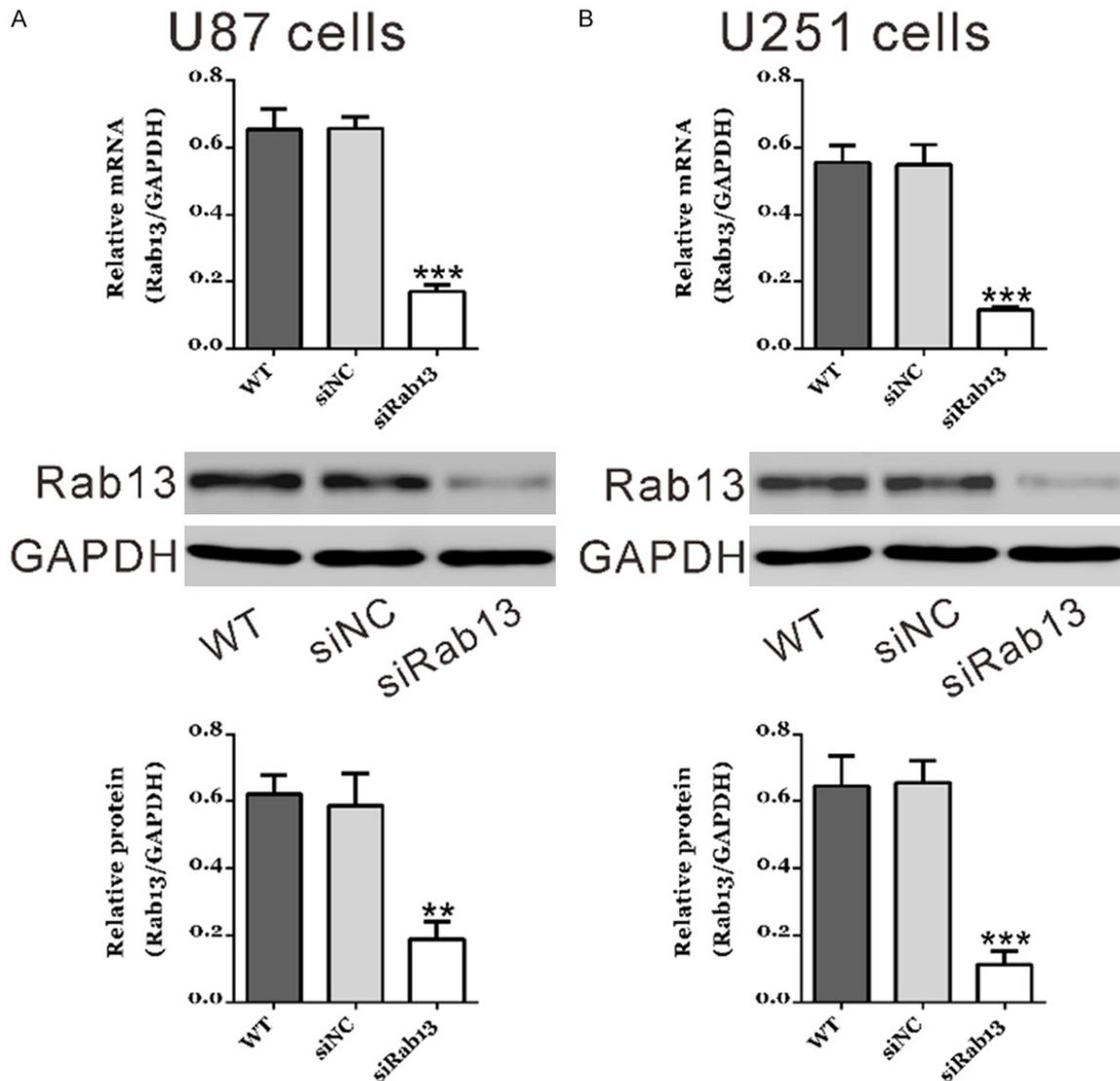To explore the potential roles of Rab13 in human glioma, we first assessed the expres-

Figure 2. Knockdown of Rab13 in glioma cells. Downregulation of Rab13 mRNA and protein expression in U87 (A) and U251 (B) cells by Rab13 siRNA was determined by RT-PCR and Western blot, respectively. **P<0.01, ***P<0.001 compared with the siNC group.

sion of Rab13 in human glioma tissues. As demonstrated by qRT-PCR, Rab13 was highly expressed in human glioma tissues (n=62) compared with normal brain tissues (n=11) (P<0.001) (**Figure 1A**). We then compared Rab13 mRNA expression between low-grade (WHO II, n=15) and high-grade (WHO III-IV, n=47) glioma. As shown in **Figure 1B**, Rab13 mRNA was gradually increased with increasing histologic tumor grade in glioma.

Additionally, Rab13 expression was detected in all tested five human glioma cell lines, among

which U251 and U87 cells exhibited higher levels of Rab13 expression (**Figure 1C** and **1D**).

Knockdown of Rab13 in human glioma cells by siRNA transfection

To further evaluate the functional role of Rab13 in the proliferation of glioma cells, synthetic human Rab13 siRNA (siRab13) and nonpecific siRNA (siNC) were transfected into U87 and U251 cells. As demonstrated in **Figure 2**, them RNA and protein expression of Rab13 was decreased by Rab13 siRNA transfection in
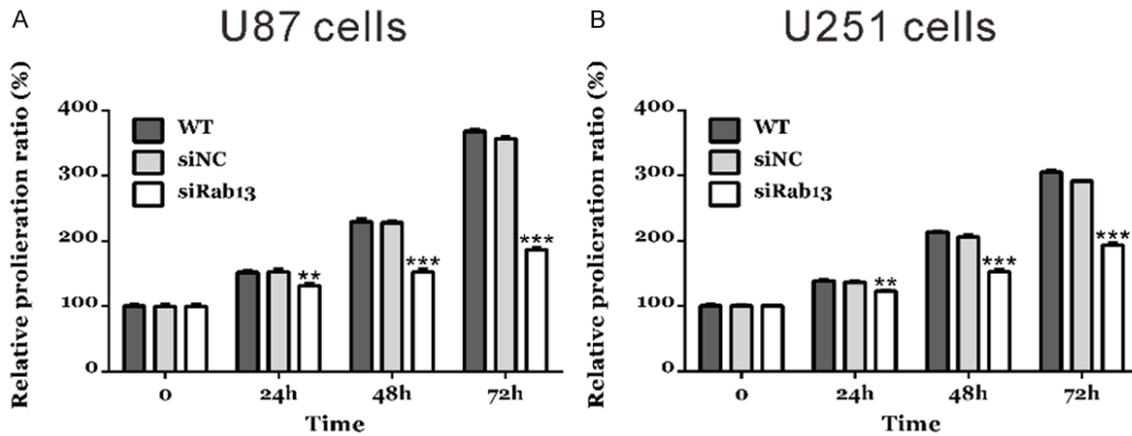
Figure 3. Rab13 knockdown inhibits glioma cell proliferation. Human Rab13 siRNA were transfected into U87 and U251 cells, followed by cell proliferation determination by CCK-8 assay. Results of cell proliferation were normalized to the initial cell numbers (100%). **$P<0.01$, ***$P<0.001$ compared with the siNC group.
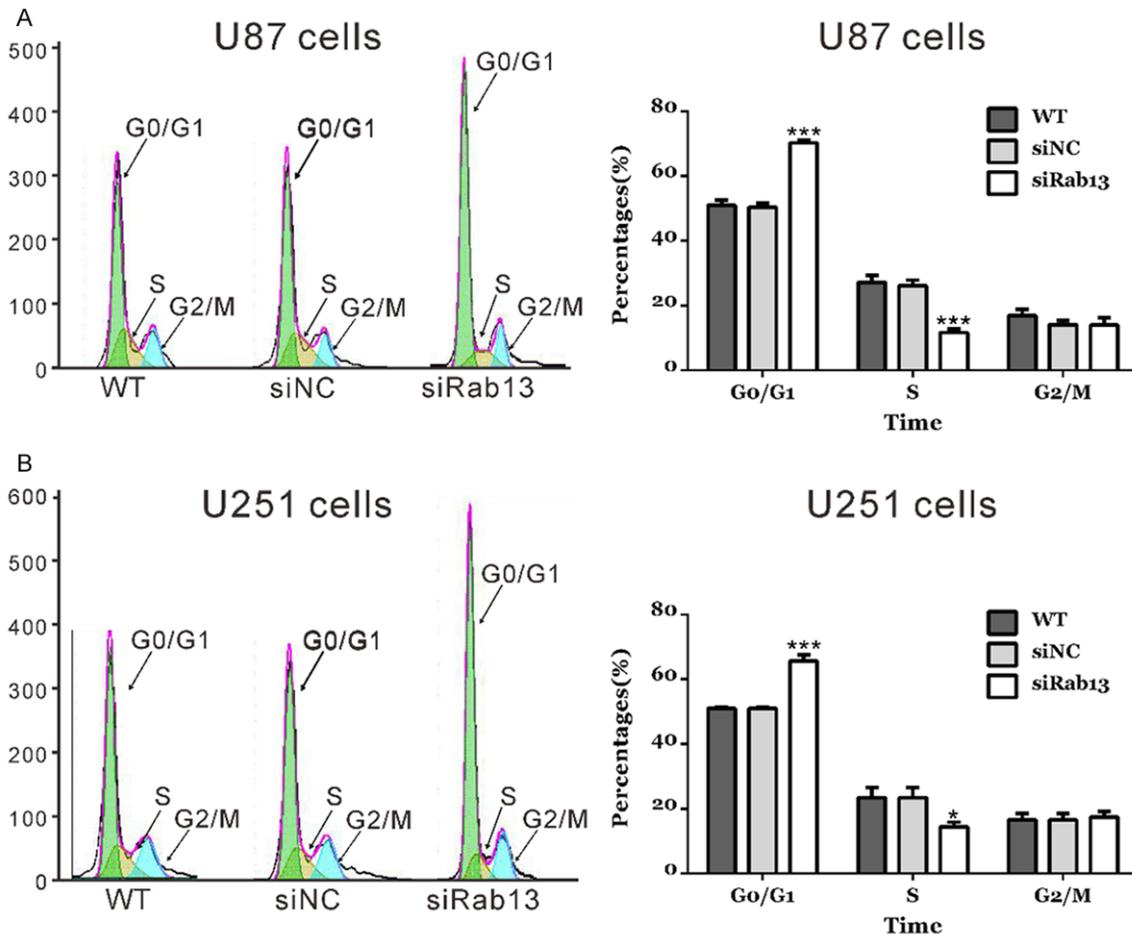

Figure 4. Effect of Rab13 siRNA on cell cycle distribution in glioma cells. After transfection with Rab13 siRNA or Rab13pecific siRNA for 48 hours, the cell cycle distribution of U87 (A) and U251 (B) cells was determined by flow cytometry. Representative images of cell cycle distribution alterations, and cell percentages in different cell cycle phases after Rab13 knockdown were shown. *$P<0.05$, ***$P<0.001$ compared with siNC group.
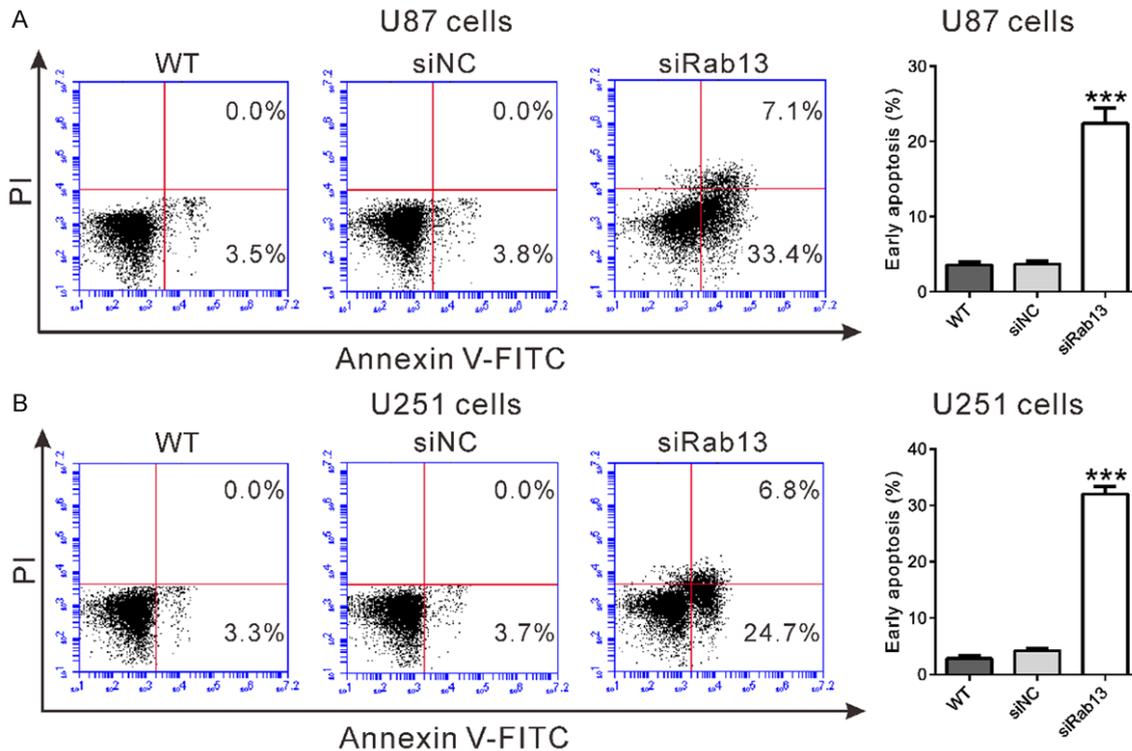
Figure 5. Rab13 knockdown induces cell apoptosis in glioma cells. After transfection with Rab13 siRNA or Rab-13pecific siRNA for 48 hours, the cell apoptosis of U87 (A) and U251 (B) cells was assessed by flow cytometry analysis. Representative images of cell apoptosis alterations, and apoptosis rate after Rab13 knockdown were shown. ***$P < 0.001$ compared with siNC group.

both cell lines with a suppression ratio of greater than 80%.

Rab13 knockdown inhibits glioma cell proliferation

Following knockdown of Rab13 gene expression with siRNA, the proliferation of U87 and U251 cells was measured using the CCK-8 assay at 24 h, 48 h and 72 h post-transfection (**Figure 3**). The cell proliferation in the Rab13 siRNA transfected group (siRab13) was significantly reduced compared with both the WT and siNC groups ($P < 0.01$). No significant difference was observed in the proliferation between the WT and siNC group ($P > 0.05$).

Rab13 knockdown in glioma cells induces cell cycle arrest in G0/G1 phase

To investigate the underlying mechanisms that inhibit glioma cell proliferation by Rab13 knockdown, PI staining and flow cytometry analysis was used to determine the cell cycle phase distributions in glioma cells at 48 h after

Rab13 siRNA transfection. As shown in **Figure 4**, a significant G0/G1 cell cycle arrest accompanied by a reduction in S-phase cell percentage was observed in both U87 and U251 cells after Rab13 knockdown, suggesting that Rab13 knockdown could induce human glioma cell cycle arrest in G0/G1 phase.

Rab13 knockdown induces cell apoptosis in human glioma cells

We then examined cell apoptosis using PI/Annexin V staining followed by flow cytometry analysis. As demonstrated in **Figure 5**, early stage (Annexin V+/PI-) and late stage apoptotic population (Annexin V+/PI+) was higher in in both glioma cell lines transfected with Rab13 siRNA, suggesting the promotion effects of Rab13 siRNA on human glioma cells.

Meanwhile, expression of apoptosis-associated protein was measured. As shown by qRT-PCR and Western blot (**Figure 6**), depletion of Rab13 by siRNA resulted in a significant increase in the expression of apoptotic pro-
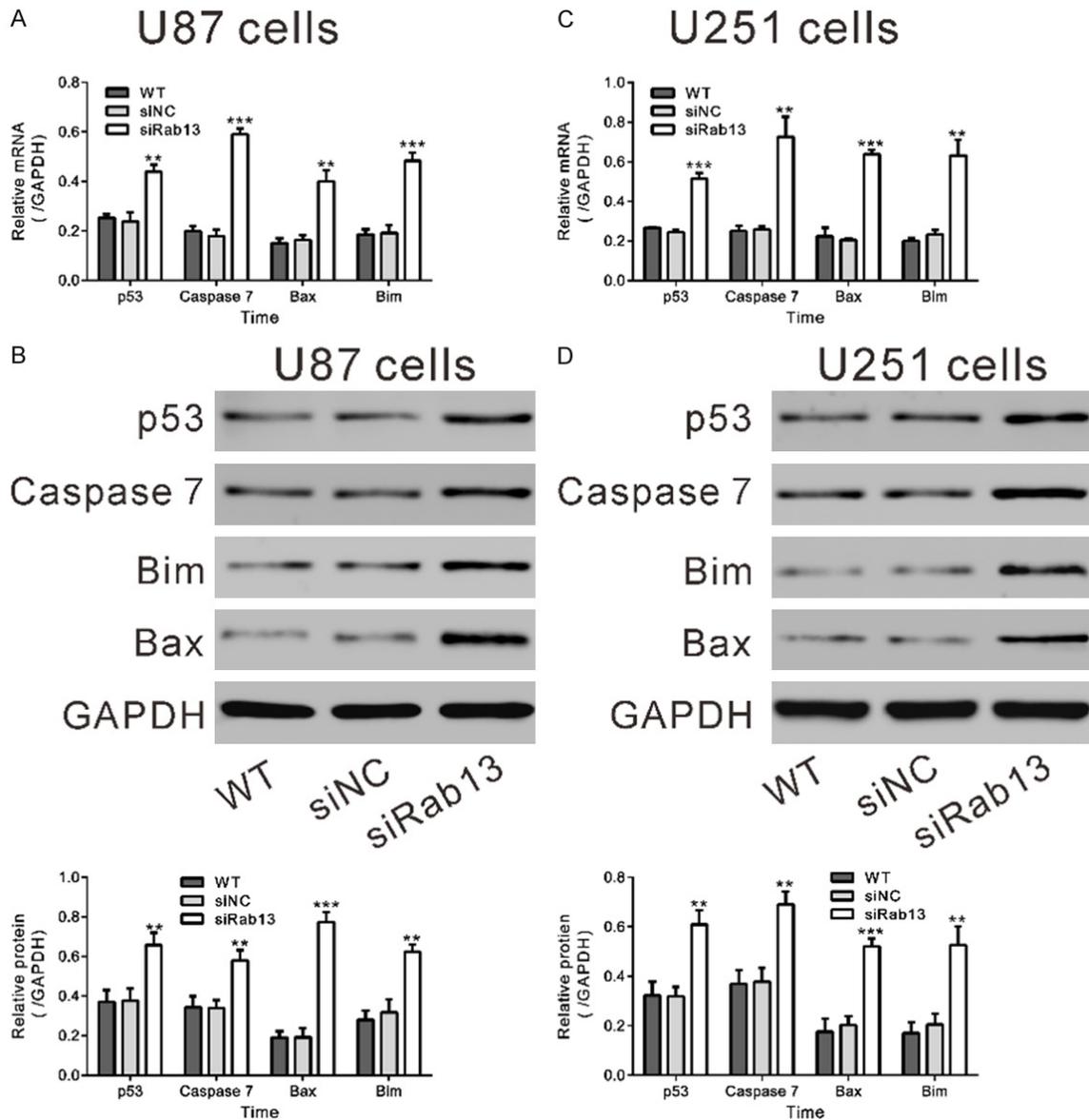
Figure 6. Effects of Rab13 siRNA on the expression of apoptosis-related proteins. After transfection with Rab13 siRNA or Rab13 specific siRNA for 48 hours, mRNA and protein levels of apoptosis-related proteins were determined by qRT-PCR (A, C) and Western blot (B, D), respectively. **$P<0.01$, ***$P<0.001$ compared with siNC group.

teins, including p53 [11], Caspase7 [12], Bax and Bim [13] in both U87 and U251 cells.

Discussion

Gliomas are the most common tumors in the CNS with an extremely low five-year overall survival rate [1, 5]. It is urgent needed to identify novel molecular regulators associated with the oncogenesis and development of glioma. Several members of Rab small GTPases have been linked to oncogenesis or prognosis of glioma [8-10]. Here, we report a tumorigenic role

of Rab13 in glioma. We demonstrated that Rab13 expression was progressively increased with grade progression of glioma (**Figure 1**), which was similar to previous findings of Rab38 [9]. The major finding of study was that Rab13 siRNA markedly suppressed cell proliferation (**Figure 3**), and induced G0/G1 cell cycle arrest (**Figure 4**) as well as cell apoptosis (**Figure 5**) in U87 and U251 cells, which indicates an anti-proliferation effect of Rab13 siRNA. The above findings provided a new idea to glioma treatment.

Understanding apoptosis in tumors will not only give an insight into the pathogenesis of tumors, but also leave clues on the tumor treatment. Similar to other types of cancer, essential pathways regulating apoptosis, including p53, are disrupted in gliomas [11, 14]. Here, the possible mechanism of how Rab13 siRNA promoted the apoptosis of glioma cells was explored. It has been extensively explored that Caspase7 was one of key downstream effectors in p53-dependent apoptosis [15]. Bim and Bcl-2 are two pro-apoptosis proteins [13]. Our study found that the expression of p53, Caspase7, Bim and Bcl-2 was down-regulated in U87 and U251 cells transfected with Rab13 siRNA (**Figure 6**). The findings described above indicated that Rab13 siRNA exerted anti-tumor effects by downregulating p53/ Caspase7 in glioma cells.

In summary, our present study strongly indicates that the specific siRNA-mediated silencing of Rab13 in glioma cells resulted in the reduction of cell proliferation via the cell cycle arrest followed by apoptotic cell death. Therefore, Rab13 may serve as a potential target in the treatment of human glioma.

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Yuzhang Wu and Yongwen Chen, Department of Immunology, The Third Military Medical University, 30 Yanzheng Rd, Chongqing 400038, China. E-mail: yzwucq@sina.com

References

[1]   Adamson C, Kanu OO, Mehta AI, Di C, Lin N, Mattox AK and Bigner DD. Glioblastoma multiforme: a review of where we have been and where we are going. Expert Opin Investig Drugs 2009; 18: 1061-1083.

[2]   Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW and Kleihues P. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 2007; 114: 97-109.

[3]   Wang Y and Jiang T. Understanding high grade glioma: molecular mechanism, therapy and comprehensive management. Cancer Lett 2013; 331: 139-146.

[4]   Pollack IF. Neuro-oncology: Therapeutic benefits of reirradiation for recurrent brain tumors. Nat Rev Neurol 2010; 6: 533-535.

[5]   Khasraw M and Lassman AB. Neuro-oncology: late neurocognitive decline after radiotherapy for low-grade glioma. Nat Rev Neurol 2009; 5: 646-647.

[6]   Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO; European Organisation for R, Treatment of Cancer Brain T, Radiotherapy G; National Cancer Institute of Canada Clinical Trials G. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005; 352: 987-996.

[7]   Schwartz SL, Cao C, Pylypenko O, Rak A and Wandinger-Ness A. Rab GTPases at a glance. J Cell Sci 2007; 120: 3905-3910.

[8]   Kim JK, Lee SY, Park CW, Park SH, Yin J, Kim J, Park JB, Lee JY, Kim H and Kim SC. Rab3a promotes brain tumor initiation and progression. Mol Biol Rep 2014; 41: 5903-5911.

[9]   Wang H and Jiang C. RAB38 confers a poor prognosis, associated with malignant progression and subtype preference in glioma. Oncol Rep 2013; 30: 2350-2356.

[10]   Wu X, Hu A, Zhang M and Chen Z. Effects of Rab27a on proliferation, invasion, and anti-apoptosis in human glioma cell. Tumour Biol 2013; 34: 2195-2203.

[11]   Haupt S, Berger M, Goldberg Z and Haupt Y. Apoptosis-the p53 network. J Cell Sci 2003; 116: 4077-4085.

[12]   Zhivotovsky B, Samali A, Gahm A and Orrenius S. Caspases: their intracellular localization and translocation during apoptosis. Cell Death Differ 1999; 6: 644-651.

[13]   Strasser A, O'Connor L and Dixit VM. Apoptosis signaling. Ann Rev Biochem 2000; 69: 217-245.

[14]   Steinbach JP and Weller M. Apoptosis in gliomas: molecular mechanisms and therapeutic implications. J Neurooncol 2004; 70: 245-254.

[15]   Shen Y and White E. p53-dependent apoptosis pathways. Adv Cancer Res 2001; 82: 55-84.