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Knockdown of ribosomal protein S15A induces human glioblastoma cell apoptosis

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Abstract

Background: RPS15A is a ribosome protein that is highly conserved in many organisms from yeast to human. A number of studies implied its role in promoting cancer cell growth.

Methods: Here, we firstly conducted RPS15A gene expression analysis in brain cancer using Oncomine database and found RPS15A was remarkably overexpressed in glioblastoma (GBM) compared with that in normal tissues. Then, the expression of RPS15A was specifically silenced in GBM cell line U251 using lentiviral-mediated RNA interference technique. We further investigated the effect of RPS15A knockdown in U251 cells using MTT assay, colony formation test, and flow cytometry analysis. We detected the protein level of Bcl-2 and poly (ADP-ribose) polymerase (PARP) as well as activation of caspase-3.

Results: Our results showed that the knockdown of RPS15A could inhibit cancer cell growth and colony formation in vitro, as well as induced cell cycle arrest at G0/G1 phase and cell apoptosis. In addition, Western blot analysis indicated that the knockdown of RPS15A could significantly inhibit bcl-2 and activate caspase-3 and PARP.

Conclusions: Our findings suggest RPS15A may play an important role in the progression of GBM and lentiviral-mediated silencing of RPS15A could be an effective tool in GBM treatment.

Keywords: Glioblastoma, RPS15A, Cell proliferation, Apoptosis

Background

Glioma is the most common and most aggressive malignant brain tumor in the human central nervous system. According to histological progression, gliomas are classified into four grades, including low-grade astrocytomas (grade I–II), anaplastic astrocytomas (grade III), and glioblastoma (GBM, grade IV) [1, 2]. As a frequent form of malignant glioma [3], GBM is invariably lethal for patients at most 2 years following diagnosis [4]. Current standard treatment for patients with GBM is concurrent TMZ and radiotherapy followed by six maintenance cycles of adjuvant chemotherapy to improve progression-free survival and overall survival [5]. Despite the standard of care therapy, recurrence rates remain higher in patients with GBM. Therefore, it is urgent to

identify new molecular targets to improve the prognosis against human GBM.

Ribosome is the fundamental translational machinery for protein synthesis from messenger RNAs. In eukaryotes, a mature ribosome consists of 60S and 40S subunits [6]. Ribosome biogenesis and protein translation are fine-tuned to match the cell growth rate, proliferation, and differentiation during development. However, this type of regulation is abnormal in cancer cells [7]. Several tumor suppressors could inhibit tumor cell growth by affecting ribosome biogenesis [8]. On the other hand, oncogenes often stimulate cell growth and proliferation by enhancing ribosome biogenesis in cancer cells [9]. RPS15A, ribosomal protein s15a, is one of the subunits of the 40S ribosomal protein [10, 11]. To date, there are several preliminary studies which show that down-regulation of RPS15A suppresses proliferation of lung cancer cells, osteosarcoma cells, and hepatic cancer cells in vitro [12–14]. Despite the ubiquitous presence and important functions of ribosomal complex, the role

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of ribosomal protein RPS15A in GBM cells remains not to be fully understood.

Herein, we aimed to investigate the effect of RPS15A knockdown in GBM progression. We firstly found the messenger RNA (mRNA) levels of RPS15A were significantly up-regulated in glioma compared that in normal tissues using the datasets from Oncomine database. Functional analysis showed knockdown of RPS15A obviously suppressed cell proliferation in U251 by inducing cell cycle arrest and apoptosis. More interestingly, we found RPS15A knockdown induced apoptosis through cell intrinsic mitochondria pathway. Our results suggested a prominent role of RPS15A in GBM cell proliferation and provide a new insight to a potential therapeutic target for GBM.

Methods

Cell lines and culture

The human GBM cell line U251 and the human embryonic kidney cell line 293T (HEK293T) were purchased from Cell Bank of Chinese Academy of Science (Shanghai). Cells were cultured in DMEM (Hyclone) supplemented with 10 % FBS (Biological Industries) at 37 °C in an incubator containing 5 % CO₂.

Construction of RPS15A short hairpin RNA containing lentivirus

For the RNA interfering experiment, two short hairpin RNA (shRNA) sequences were designed against the RPS15A gene (NM_001019): shRPS15A-1, 5'-GTGC AACTCAAAGACCTGGAACCTCGAGTTCCAGGTCTT TGAGTTGCACTTTTT-3' and shRPS15A-2, 5'-GCA TGGTTACATT-GGCGAATTCTCGAGAATTCCGCA ATGTAACCATGCTTTTT-3'). The control shRNA (shCon) sequence was 5'-GCGGAGGGTTTGAAAGAA TATCTCGAGATATTCTTCAAACCCTCCGCTTTTT T-3'. Each nucleotide sequence was synthesized and inserted into pFH-L shRNA expressing vector. For lentiviral production, recombinant plasmid described above and pHelper plasmids (pVSVG-1 and pCMVΔ8.92) were co-transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. After 72 h, virus supernatant was harvested and concentrated by ultracentrifugation (4000g at 4 °C) for 10 min.

Cell infection

For cell infection, U251 cells were seeded at a density of 50,000 cells per well in six-well plates and transduced with constructed lentiviruses (shCon, shRPS15A-1, and shRPS15A-2) at a multiplicity of infection of 40. After 72-h infection, the infection efficiency was determined by observing the green fluorescence protein (GFP) expression under a fluorescence microscope.

Quantitative PCR analysis

After 6-day infection, total RNA was isolated from cells by using the Trizol reagent (Invitrogen), according to the manufacturer's protocol. Reverse transcribed was performed Bio-Rad Connect Real-Time PCR (polymerase chain reaction) platform (Bio-Rad Laboratories Inc, Hercules, CA, USA) using an SYBR Green Master Mix Kit. Data were analyzed using the 2^{-ΔΔCt} method, and the levels of mRNA were normalized to β-actin. The PCR primers used were as follows: RPS15A (forward-CCTCCTTTTTTCGGTTTCCTC; reverse-AGAGATGG AA-TGGTGGTTGG); β-actin (forward-GTGGACATCC GCAAAGAC; reverse-AAAGGGTGT-AACGCAACTA).

Western blot analysis

Western blot analysis was done 5 days post infection. Proteins were extracted from cells using 2× SDS sample buffer (100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4 % SDS, 10 Glycine). A total of 30 ug proteins were separated in 12 % SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with primary antibodies (rabbit anti-RPS15A, 1:1000, Ab175054, Abcam; rabbit anti-caspase-3, 1:500, #9661, Cell signaling; rabbit anti-poly (ADP-ribose) polymerase (PARP), 1:1000, #9542, Cell signaling; rabbit anti-bcl-2, 1:1000, #2876, Cell signaling; rabbit anti-GAPDH, 1:500000, #2876, Cell signaling) for 1 h at room temperature followed by incubation with secondary antibody goat anti-rabbit HRP (Santa Cruz) at room temperature for 1 h. The bands were visualized using an ECL kit (Beyotime). Protein bands were quantified using Gel-pro analyzer software (MediaCybernetics). GAPDH was used as the reference control.

MTT assay

Cells were seeded in a 96-well plate with 2500 cells per well. Growth curve and MTT assay was carried out 96 h post transduction of virus. Briefly, MTT solution was added to each well, followed by 4 h of incubation at 37 °C. Then, cells were washed and dissolved in acidic isopropanol (10 % SDS, 5 % isopropanol, and 0.01 mol/L HCl) for 10 min. Cell density was measured at 595 nm using the microplate reader using enzyme-linked immunosorbent assay. The cell growth curves were drawn according to the OD values.

Colony formation analysis

Cells were seeded at the density of 500 cells per well in a six-well plate. After 96 h infection with shRNA virus, followed by additional eight days of incubation, cells were washed with PBS twice, fixed with absolute methanol for 15 min. Then, fixed cells were stained with 1 % crystal violet for 20 min. After cleaning with PBS, colonies were counted under light microscope.

Cell cycle and apoptosis analysis

Cells were seeded in a 6-cm dish at 100,000 cells per well. Four days after infection with lentivirus, the cells were fixed with pre-cooled 70 % ethanol at 4 °C overnight and incubated with 1 mg/ml RNase A (QIAGEN) for 30 min at 37 °C. Then, cells were added propidium iodide (50 ug/mL, ebioscience) at 4 °C for 30 min to stain DNA. The DNA content of cells was determined by a FACS Calibur II sorter and Cell Quest FACS system (BD Biosciences).

For cell apoptosis analysis, cells were harvested after 3-day infection with lentivirus and resuspended in 100 ul 1× binding buffer (ebioscience). Then, cells were stained with 2 uL Annexin V-APC (20 ug/ml; ebioscience) for 15 min on ice. Samples were diluted to 400 uL and added 1 uL 7-AAD (50 ug/ml; ebioscience) before detection on FACS Calibur II sorter and Cell Quest FACS system (BD Biosciences).

Statistical analysis

All experiments were at least repeated in triplicate. All data were analyzed using GraphPad Prism software and expressed as the mean ± standard deviation (SD) of three independent experiments. Comparisons between groups were conducted by Student's *t* test. The *p* value <0.05 was considered as statistically significant.

Results

RPS15A is overexpressed in GBM

In order to evaluate the differential expression of RPS15A in GBM, we used the Oncomine database (www.oncomine.org), a cancer microarray database of genome-wide expression analysis [15]. A quick search of RPS15A gene in GBM revealed that this gene was up-regulated in several types of GBM, including anaplastic oligoastrocytoma [16, 17], glioblastoma [16, 18], oligodendroglioma [19], and diffuse astrocytoma [20] vs. normal tissues (Table 1). The comparison showed an average increase of one- to sixfold in RPS15A expression

in GBM compared to normal tissues. Therefore, we concluded that RPS15A might play an important role in GBM progression. Thus, we focused on this particular gene in following studies by using U251, a cell line derived from a malignant glioblastoma.

RPS15A gene knockdown in U251 cells

To investigate the biological function of RPS15A in glioblastoma cells, we constructed two individual shRNA vectors for RPS15A knockdown using lentiviral vectors carrying green fluorescent protein (GFP). Both control vectors (shCon) and two target vectors (shRPS15A-1 and shRPS15A-2) showed good infection rates 72 h post infection in U251 cells, determined by a fluorescence microscopy of the GFP expression (Fig. 1a, b). We performed RT-PCR and western blot to evaluate the efficiency of shRNA knockdown of RPS15A. As shown in Fig. 1c, d, both of two target shRNAs showed significant reduction of RPS15A mRNA compared to the shCon ($p < 0.001$). Western blot confirmed that, at protein level, target shRNAs efficiently knocked down RPS15A expression in U251 cells (Fig. 1e). Together, these results suggested that we successfully constructed RPS15A knockdown cell model, which could be applied to the following studies.

RPS15A knockdown affects cell proliferation and colony formation ability

We next examined the effect of RPS15A on cell proliferation and viability. We measured cell proliferation by MTT assay, a colorimetric assay assessing cell metabolic activity depending on NADPH oxidoreductase enzymes, over the course of 5 days. As shown in Fig. 2a, shRPS15A-treated cells showed significant inhibition of cell proliferation compared to cells treated with shCon ($p < 0.001$). We also did colony formation assay with cells treated by shCon vs. shRPS15A. As shown in Fig. 2b, c, shRPS15A-treated cells failed to form any colony, whereas shCon-treated cells can form colonies

Table 1 RPS15A differential transcript expression in human glioblastoma extracted from multiple studies in the Oncomine database

Study	Comparison (specimen number in each group)	Fold change	<i>p</i> value
Bredel M et al.	Anaplastic oligoastrocytoma (6) vs. normal(4)	2.125	0.005
Bredel M et al.	Anaplastic oligodendroglioma (3) vs. normal (4)	2.337	0.004
Bredel M et al.	Glioblastoma (5) vs. normal (4)	1.916	6.64E-04
Bredel M et al.	Oligodendroglioma (27) vs. normal (4)	2.014	0.006
French PJ et al.	Anaplastic oligodendroglioma (23) vs. normal(6)	2.373	4.09E-05
Murat A et al.	Glioblastoma (80) vs. normal(4)	1.985	1.51E-08
Shai R et al.	Oligodendroglioma (3) vs. normal (7)	5.803	5.49E-06
Sun L et al.	Diffuse astrocytoma (7) vs. normal (23)	2.163	8.40E-07
TCGA	Brain glioblastoma (542) vs. normal (10)	1.408	7.04E-04

TCGA The Cancer Genome Atlas-Glioblastoma Multiforme Gene Expression Data (<http://tcga-data.nci.nih.gov/tcga/>)

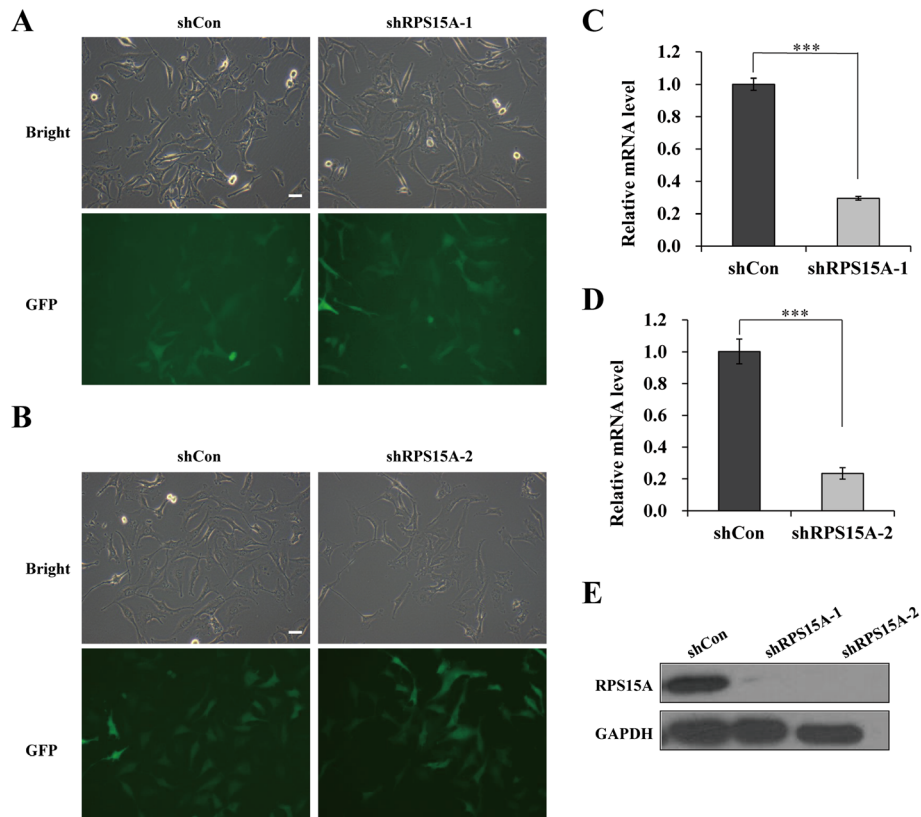


Fig. 1 Verification of RPS15A silencing in U251 cells after shRPS15A infection. **a, b** Infection efficiency was assessed by counting GFP-positive cells with fluorescence microscopy. **c, d** The mRNA levels of RPS15A in U251 cells with three treatments (shCon, shRPS15A-1, and shRPS15A-2) measured by qPCR analysis. The experiment was performed in triplicate and repeated three times. **e** The protein levels of RPS15A in U251 cells with three treatments (shCon, shRPS15A-1, and shRPS15A-2) determined by Western blot analysis. *** $p < 0.001$

naturally ($p < 0.01$). Therefore, RPS15A could promote cell viability and proliferation ability.

RPS15A plays a role in cell cycle progression in U251 cells

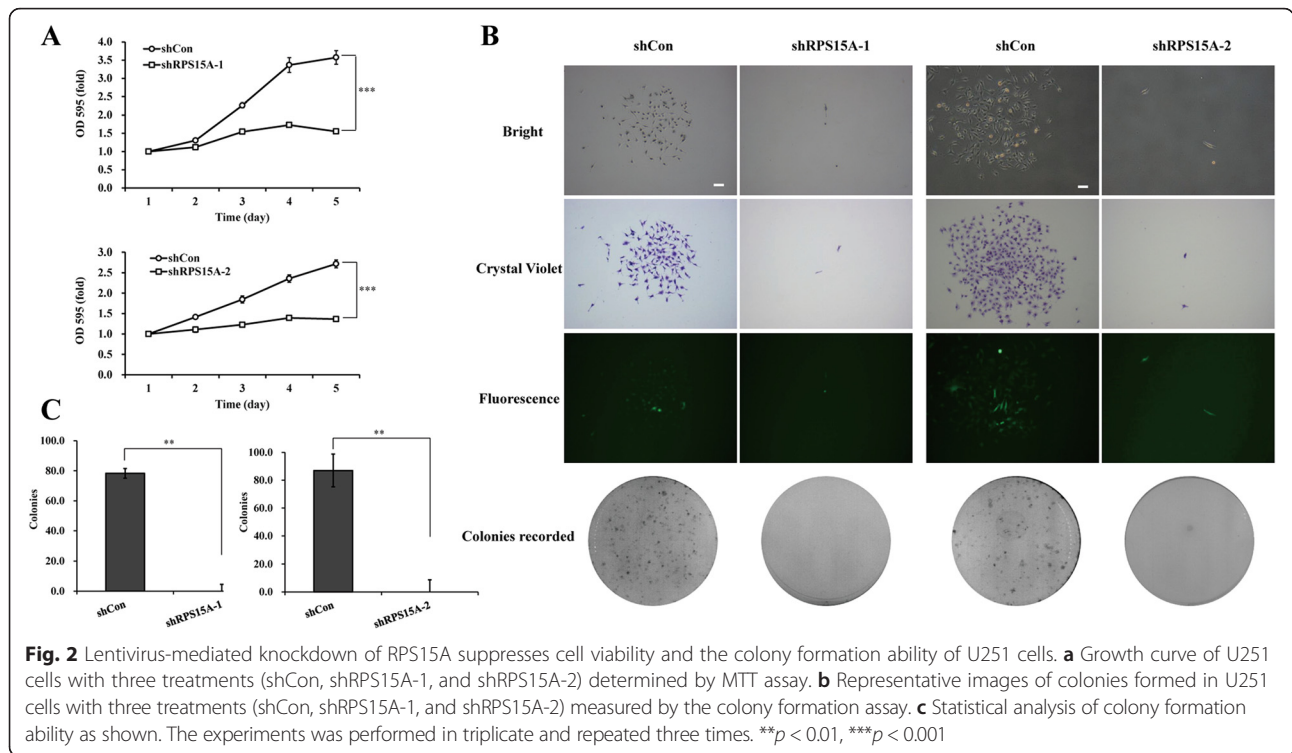
Inhibition of cell proliferation could occur at any stage of the cell cycle. To investigate the mechanisms of the U251 cell proliferation inhibition induced by shRPS15A, we used flow cytometry to examine the cell cycle progression under the treatment of shCon or shRPS15A (Fig. 3a). We found that 72 h after shRNA treatment, shRPS15A-treated cells showed significantly reduced cells in S phase compared to shCon-treated cells ($p < 0.001$); instead, there were more cells accumulated in G0/G1 phase ($p < 0.001$) after shRPS15A infection, but no significant difference was found in G2/M phase (Fig. 3b). This cell cycle results strongly indicated that knockdown of RPS15A arrested cell cycle at G0/G1 phase, which means RPS15A plays an important role in cell cycle progress from G1 to S phase.

RPS15A knockdown induces apoptosis in U251 cells

To further investigate the effect of RPS15A on cell apoptosis, Annexin V combined with 7-Aminoactinomycin D (7-AAD) staining was used to distinguish apoptosis

(Fig. 4a) between early and late stages of apoptosis based on the exposure of phosphatidylserine to the surface of cell apoptosis [21]. We found that knockdown of RPS15A significantly induced cell early (Annexin V+/7-AAD-) and late apoptosis (Annexin V+/7-AAD+) in U251 cells (Fig. 4b, $p < 0.001$).

We further extended our study to investigate the pathway through which RPS15A knockdown induced apoptosis. Apoptosis is typically induced through two distinct signaling pathways: the death receptor pathway and mitochondrial pathway [22]. Because RPS15A is a cytosolic protein, we hypothesized it affected mitochondria pathway, in which bcl-2, caspase 3, and PARP are critical components. Thus, we carried out Western blot analysis to determine the expression of these three proteins in shRPS15A-treated cells. As shown in Fig. 4c, the anti-apoptotic and mitochondria localized protein Bcl-2 was down-regulated in U251 cells after RPS15A knockdown. The expression levels of cleaved caspase-3 and PARP were up-regulated in U251 cells following shRPS15A infection. This result indicated that RPS15A knockdown activated the mitochondrial pathway of apoptosis. What is more, shRPS15A-1 showed more strong effect on

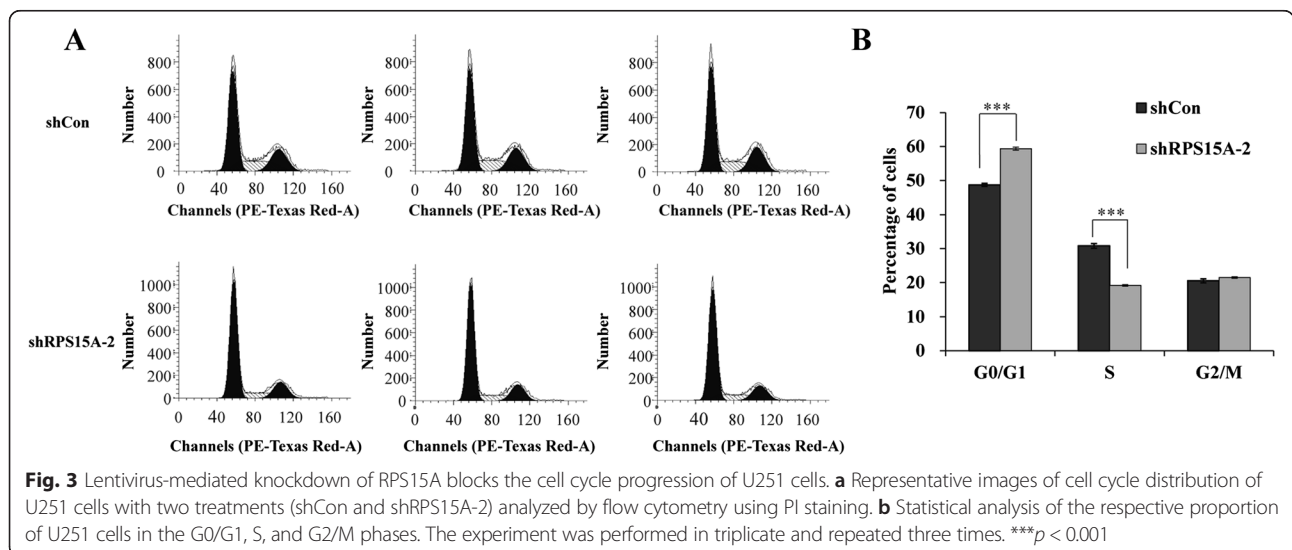


apoptosis induction than shRPS15A-2. Taken together, we found that RPS15A knockdown induced U251 cells apoptosis through mitochondrial pathway.

Discussion

RPS15A encodes a 40S ribosomal protein S15a, one of the components of the 40S subunit in human [23]. Ribosomes are required for basic cellular functions, especially for cancer cells, whose growth and proliferation are uncontrolled, thus demand a large amount of protein

synthesis [7]. GBM is invariably lethal glioma characterized by extremely high aggressiveness. However, little is known about the biological function of ribosome formation in GBM progression. In this study, we firstly evaluated the relative expression of RPS15A in glioma tissues and normal tissues and found it was significantly up-regulated in GBM tissues compared with that in normal tissues. To further investigate its role in GBM, the functional role of RPS15A in human GBM cell growth was studied by RNAi-mediated knockdown. Our results



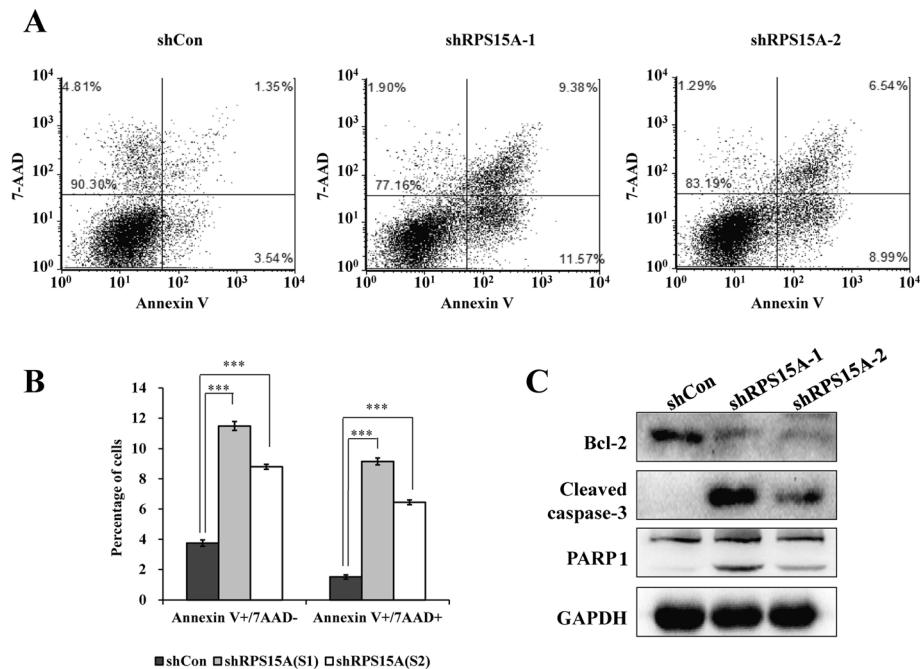


Fig. 4 Knockdown of RPS15A induces cell apoptosis via activation of mitochondrial pathway. **a** Representative images of cell apoptosis of U251 cells with three treatments (shCon, shRPS15A-1, and shRPS15A-2) analyzed by flow cytometry using Annexin V/7-AAD double staining. **b** Statistical analysis of the proportions of U251 cells corresponding to early (Annexin V⁺/7-AAD⁻) and late (Annexin V⁺/7-AAD⁺) apoptotic cells. **c** Activation of bcl-2, caspase-3, and PARP signaling pathways measured by Western blot analysis. The experiment was performed in triplicate and repeated three times. *** $p < 0.001$

showed knockdown of RPS15A remarkably suppressed U251 cell growth, possibly due to induction of cell cycle arrest and apoptosis.

Similar effects of RPS15A were demonstrated in previous studies in other cancer types [13, 24]. Our study demonstrated that RPS15A was important for survival and proliferation of glioblastoma cancer cells. In the absence of this gene, the cells underwent apoptosis through intrinsic mitochondria pathway.

The mitochondrial pathway of apoptosis typically involves the release of mitochondrial cytochrome c into cytosol. The released cytochrome c combines with apoptotic protease activating factor 1 (Apaf-1) and procaspase-9, resulting in the activation of caspase-9 and caspase-3 [25]. Bcl-2 plays an important role in releasing cytochrome c from mitochondria. Our western blot result of Bcl-2 showed a significant decrease upon RPS15A knockdown. Thus, wastage of RPS15A led to the increase of anti-apoptotic signals (Bcl-2), which was in favor of the occurrence of apoptosis. Caspase-3 cleavage and PARP cleavage are the downstream of Bcl-2 [26, 27]. Our western blot confirmed these two cleavage events were both enhanced upon RPS15A knockdown. These results suggested that RPS15A knockdown induced apoptosis mainly through the activation of mitochondrial pathway. We hypothesize that RPS15A might be directly

involved in the translation of Bcl-2 mRNA; thus, knockdown of RPS15A resulted in the down-regulation of Bcl-2 protein.

Many types of cancers, including glioblastoma, are resistant against many anti-cancer treatments. One of the main reasons is that they do not activate their intracellular apoptosis pathway [28]. Current standard chemotherapy treatment for glioblastoma is the use of alkylating agents, temozolomide, in combination with radiation therapy. Temozolomide has been proven somewhat effective in prolonging survival; however, tumor often returns after treatment, which begs the exploration of new drugs and therapies. Our finding of induction of apoptosis in GBM cell line by down-regulating RPS15A provides a new avenue, which targets ribosome proteins.

Conclusions

From above analysis, we can see that RPS15A was significantly up-regulated in GBM tissues compared with that in normal tissues. Knockdown of RPS15A remarkably suppressed U251 cell growth; then, the results demonstrated that RPS15A was important for survival and proliferation of glioblastoma cancer cells. In the absence of this gene, the cells underwent apoptosis through activation of intrinsic mitochondria pathway.

In summary, our findings suggest RPS15A may play an important role in the progression of GBM and lentiviral-mediated silencing of RPS15A could be an effective tool in GBM treatment.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CZ carried out the construction of RPS15A short hairpin RNA containing lentivirus, participated in the cell cycle and apoptosis analysis, and drafted the manuscript. JF carried out the cell infection and quantitative PCR analysis. FX participated in the cell cycle and apoptosis analysis. TZ participated in the cell culture. SZ participated in the Western blot analysis. JS participated in the MTT assay. ZS participated in the colony formation analysis. LZ participated in the design of the study and performed the statistical analysis. XC conceived of the study and participated in its design and coordination and helped to draft the manuscript. BR and XX participated in the grammar mistakes correcting and statistical analysis. All authors read and approved the final manuscript.

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