Ribosomal Protein S27-Like is overexpressed in glioma cells and inhibition of RPS27L expression suspends the tumorigenesis

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Abstract

Background: Ribosomal Protein S27-Like is an evolutionarily conserved ribosomal protein and the role of RPS27L influencing the malignance of several cancers has been reported. However, its effects on glioma were still unknown. This investigation aims to characterize the clinical significance and the biological functions of RPS27L in gliomas.

Methods: TCGA databases were explored to analyze the correlation between RPS27L expression and the clinical characteristics of glioma patients. Immunohistochemical staining was performed on glioma cases and normal brain tissues. The function of RPS27L in glioma was further explored using U87MG and A172 cell lines and a orthotopic xenograft model of nude mice.

Results: Data obtained from TCGA database showed higher expression of RPS27L in glioma than normal, and the overall survival was lower in the high expression group. Immunohistochemistry showed the expression levels of RPS27L were increased with the tumor grade rising in gliomas. Functional assays showed knockdown of RPS27L inhibited proliferation, cell cycle transition, migration and invasion, while promoted apoptosis. Data of western blot indicated that knockdown of RPS27L increased the level of p21^{Bax} and Cleaved Caspase-3 while decreased the level of CDK4, cyclinD1, cyclinE1, Bcl-2 and MMP2, MMP9 in glioma cells. In vivo, the growth of orthotopic glioma xenografts was suppressed by expression of RPS27L shRNA, and the tumors with RPS27L shRNA showed less aggressiveness and reduced expression of Ki67, Bcl-2 and MMP2.

Conclusions: RPS27L is overexpressed in glioma cells. Knockdown of RPS27L could inhibit the proliferation, migration and invasion while promote apoptosis of glioma cells in vitro and in vivo. RPS27L might be a potential prognostic biomarker and possible target for future therapy in glioma.

Background

Gliomas which account for 80% of primary central nervous system tumors are prevailing and mortal in adults [1]. It is characterized by a high mortality rate, recurrent malignancy, difficulty in complete surgical resection, and insensitivity to radiotherapy and chemotherapy [2, 3]. Gliomas can be divided into various subtypes according to their histological characteristics [4]. Although great progress has been made in the diagnosis and treatment of glioma in the past decades, the prognosis is still poor [5, 6]. The 5-year survival rate of low-grade glioma patients is about 30~70%, while the median survival time of GBM patients is 9~12 months [7, 8]. Therefore, the identification of new biomarkers involved in the development of glioma is very necessary for developing new treatment strategies and improving the clinical efficacy of glioma patients.

Ribosomal protein (RPs) are not only the essential components of the ribosome but also play important roles in assembly of ribosome particles, a process termed as ribosome biogenesis [9]. RPS27L is an evolutionarily conserved ribosomal protein with 84-amino acid, which differs from its family member RPS27 only by three amino acids (R5K, L12P, K17R) [10]. It has been reported that RPS27L is a direct p53

transcriptional target and participates in DNA damage response and plays an important role in regulating cell cycle checkpoints, DNA repair, and chromosome stability [11]. Aberrant RPS27L expression has been reported to influence the malignancy of various cancers and diseases, such as colorectal cancer, Li-Fraumeni syndrome and so on [9-12]. How RPS27L influences glioma is still unclear, so this is the focus of our investigation.

We investigated that RPS27L was overexpressed in glioma cells and associated with the tumor grade and prognosis of glioma patients. RPS27L knockdown inhibited glioma cell proliferation, migration and invasion while promoted apoptosis in vitro and in vivo. These findings indicate that RPS27L has the potential to be a novel biomarker and a new therapeutic target in the treatment of human gliomas.

Methods

TCGA databases and clinical specimens

The mRNA expression chip data and accompanying clinical information were downloaded from the TCGA Research Network. The data was analyzed using GraphPad Prism. Paraffin-embedded glioma tissues (WHO II-IV) was obtained from patients undergoing neurosurgery in Qilu Hospital of Shandong University (n=30). Normal brain tissue samples were collected from 5 patients with severe traumatic brain injury who had partially resected normal brain.

Immunohistochemistry (IHC)

Sections were obtained from paraffin-embedded tissues of normal brains and of different grades of human gliomas. The sections were dewaxed and deparaffinized in xylene and rehydrated in graded alcohol solutions. Sections were heated in tris-EDTA buffer for 30 mins to extract antigens. The slides were blocked with 10% normal goat serum (#5425; Cell Signaling Technology; USA) and incubated with primary antibody (rabbit anti-RPS27L monoclonal antibody, 1:100 dilution, #AP33216PU-N; Acris Antibodies GmbH; GERMANY) (mouse anti-Ki67 antibody, 1:800 dilution, #9449; Cell Signaling Technology; USA) (rabbit anti-Bcl-2 antibody, 1:150 dilution, #40994; Cell Signaling Technology; USA) (rabbit anti-MMP2 antibody, 1:150 dilution, #40994; Cell Signaling Technology; USA) at 4 °C overnight. The images were visualized by following standard protocols using horseradish-peroxidase-conjugated secondary antibody and 3, 3'-diaminobenzidine (DAB) as the substrate. Sections were avoided light with hematoxylin, then dehydrated and secured. Images were caught with Leica DM 2500 microscope.

Cell culture

GBM cell lines U87MG and A172 human were purchased from the Chinese Academy of Sciences (Shanghai) culture bank. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific; Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA). Cells were maintained at 37 °C in a humidified chamber containing 5% CO₂.

RPS27L silencing

siRNA targeting RPS27L was synthesized (GenePharma; Shanghai, China). Lipofectamine™ 3000 reagent (Thermo Fisher Scientific; USA) was used to transfect siRNAs according to manufacturer's protocol. Stable knockdown of RPS27L in cells were produced by sh-RPS27L lentivirus (GenePharma; Shanghai, China). Knockdown efficiency was evaluated by qRT-PCR and Western blotting. siRNA sequences (n = 2) are the following: si-RPS27L#1: 5'-GCCUUUGGCUAGAGAUUUATT-3'; and si-RPS27L#2: 5'-GAAGGGUGUUCAUUUAGAATT-3'. The first sequence of siRNA was used for stable knockdown and for the functional assays in vitro.

Quantitative real-time PCR (qRT-PCR)

RNA isolation, reverse transcription and quantitative expression were carried out according to the instructions. Total RNA was isolated from tissue or cultured cells using Trizol reagent (Invitrogen, USA) and DNA was generated using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA was subjected to real-time PCR using the quantitative PCR System Mx-3000P (Stratagene). The RPS27L primers were 5-GTCTGGTAGGGCTGAGCTTG-3 and 5-GGTGATCTTGTAGCAACCTGGA-3. The primers for GAPDH were 5-GCACCGTCAAGGCTGAGAAC-3 and 5-TGGTGAAGACGCCAGTGGA-3.

Western blotting

Harvested cells are thermally denatured in the RIPA cell lysis buffer. The protein lysate was run on SDS-PAGE and the protein was transferred to the PVDF membrane. Blots are cultured with primary antibodies against RPS27L (1:500 dilution; #AP33216PU-N; Acris Antibodies GmbH; GERMANY); p21, CDK4, Cyclin D1, Cyclin E1, MMP2, MMP9, Bcl-2, Bax, cleaved Caspase-3, GAPDH (Cell Signaling Technology; Danvers, MA, USA). Specific proteins were detected with enhanced chemiluminescence (ECL, Millipore, Bedford, MA, USA). GAPDH was used as the loading control.

5-ethynyl-2'-deoxyuridine (EdU) cell proliferation assay

An EdU cell proliferation assay kit (RiboBio, #C10310-1; Guangzhou, China) was used to measure the glioma cell proliferation according to the manufacturer's protocol. The glioma cells were incubated in 250 µl EdU solution for 2 h. Cells were fixed in 4% paraformaldehyde for 15min, 0.4% Triton X-100 penetrability for 10min, and incubated with 250 µl Apollo® reagent for 30min. Then the cells were stained with Hoechst 33342 for 30min. The ratio of EDU positive cells (red) to the total number of Hoechst 33342 positive cells (blue) was used as cell proliferation rate.

Cell counting kit (CCK)-8 assay

Cell viability was assessed using CCK-8 assay according to the manufacturer's instructions (Dojindo, Kumamoto, Japan). Cells (2×10^3 /well) were incubated in 96 well plates for 24, 48, and 72 h. CCK-8

solutions (10 μ L) were added to each well, the plates were incubated for 1 h at 37°C, and the absorbance of OD450 was measured with a Microplate Reader (Bio-RAD).

Flow cytometry

In the cell cycle analysis, U87MG and A172 glioma cells were collected, re-suspended and stained with propidium iodide (PI) (BD Biosciences; USA) for 20 min in the presence of RNase A. In the apoptosis assay, U87MG and A172 cells were stained with Annexin V-FITC (BD Biosciences; USA) and PI for 20 min. Cells were analyzed using flow cytometry (BD Biosciences; USA) according to manufacturer's instructions.

3D tumor spheroid invasion assay

Glioma cells were seeded into a 3D culture qualified 96-well spheroid-formation plate (5×10^3 cells/well) and incubated in the spheroid-formation matrix (Trevigen, Gaithersburg, MD, USA) and DMEM containing 10% FBS for 72 h. When the spheroids grew to a diameter of >200 μ m, the invasion matrix (Trevigen, Gaithersburg, MD, USA) was infused. The spheroids at 24 h were regarded as a reference point for measuring the area invaded by the sprouting cells. Spheroids were imaged every 24 h using a Leica microscope.

Transwell invasion and migration assays

To further assess the invasiveness of glioma cells, we coated the filter with Matrigel. The glioma cells were added to the superior lumen in serum-free medium. The bottom chambers were filled with DMEM (10% FBS). After incubation for 24 h, the superior lumen cells were taken out with a cotton swab, fixed with 4% paraformaldehyde for 15 min, and stained with crystal violet for 15 min. The adherent cells in each well were randomly imaged in 5 fields. To measure migration, the filter was not precoated with a substrate.

Orthotopic glioma xenografts

To evaluate the tumorigenesis in vivo, U87MG luciferase cells (1×10^6) were infected with Lentivirus sh-RPS27L (the sequence was same as si-RPS27L#1) or Lentivirus control. Then we implanted them stereotactically into the brain of nude mice (SLAC laboratory animal Center; Shanghai, China). We detected the intracranial tumor growth by Bioluminescence imaging on days 7, 14, 21 and 28. Kaplan-Meier survival curves were plotted to show survival time. The brain tissues with tumor were collected, formalin-fixed and paraffin-embedded, sectioned (4 μ m) and incubated with antibodies against RPS27L, Ki-67, MMP2 and Bcl2.

Statistical analysis

We used oneway ANOVA test or Student's *t* test to data comparisons with the help of GraphPad Prism 6 software. The data was presented as the mean $\pm$ standard error of three independent experiments.

Kaplan-Meier survival curves were also analyzed by log-rank tests using GraphPad Prism software. All tests were two-sided, and P-values <0.05 were considered to be statistically significant.

Results

Expression level of RPS27L is associated with glioma patient survival

The prognostic value of RPS27L in glioma patients in TCGA was inspected by Kaplan-Meier survival curve. It showed patients with higher RPS27L expression (>median value) had significantly poorer prognosis than those with lower RPS27L expression in all glioma patients ($P < 0.001$) and LGGs ($P < 0.05$). In the separate GBM patients, there was no statistical significance ($P=0.083$) in TCGA (Fig. 1a).

Increased expression of RPS27L is associated with increasing tumor grade in glioma

To determine the role of RPS27L in glioma development, we analyzed the gene expression levels of RPS27L in GBMs and LGGs as well as normal brain tissues in the TCGA. RPS27L mRNA levels in GBMs and LGGs were significantly increased in TCGA compared with normal brain tissues ($P < 0.001$; Fig. 1b).

Immunohistochemical was used to detect RPS27L protein levels in gliomas (30 cases) and normal brain tissues (5 cases) of Qilu Hospital of Shandong University. Consistent with the mRNA microarray results, the protein level of RPS27L was significantly increased with tumor grade rising in glioma (Fig. 1c). Therefore, the expression level of RPS27L was positively correlated with tumor grade both in the public database and in our primary clinical specimens.

Knockdown of RPS27L inhibits proliferation and induces cell cycle arrest in glioma cells

To explore the effect of RPS27L on glioma cell survival and proliferation, we transfected cells with siRNA to downregulate RPS27L. qRT-PCR and western blot were used to verify the efficiency (Fig. 2a and b). Then EdU and CCK-8 assays were performed. The downregulation of RPS27L resulted in significant decreases in the percentage of EdU positive cells as well as the OD450 values in U87MG and A172 cells 48 h after transfection (Fig. 3a-c). Cell cycle analysis also showed that RPS27L silencing increased the number of U87MG and A172 cells in G0/G1 phase (Fig. 3d and e). Cyclin D1, Cyclin E1 and CDK4 are cell cycle regulators that prevent cell cycle arrest in the G0/G1 phase and accelerate S phase progression [13-15]. Western blot showed that the expression levels of them in si-RPS27L cells were downregulated compared with the si-NC group (Fig. 3f). The levels of the cyclin-dependent kinase inhibitors P21 increased in si-RPS27L group (Fig. 3f).

Knockdown of RPS27L induces apoptosis in human glioma cells

It was verified that RPS27L silencing promoted cell apoptosis compared with controls in U87MG ($P < 0.001$) and A172 ($P < 0.01$) cell lines (Fig. 4a and b). Apoptosis regulators were further examined. Bax and cleaved Caspase-3 expression increased, while Bcl-2 expression levels were decreased in RPS27L knockdown cell lines (Fig. 4c).

Knockdown of RPS27L inhibits invasion and migration of glioma cells

In order to investigate the influence of RPS27L on the migration and invasion of glioma cells, we first conducted a 3D collagen spheroid invasion assay. Silencing RPS27L reduced the area invaded by U87MG and A172 spheroids relative to controls (Fig. 5a and b). In Transwell migration assay, the number of migratory glioma cells in the si-RPS27L group was significantly lower than that in the si-NC group (Fig. 5c and d). The invasion assay showed that si-RPS27L significantly reduced the number of glioma cells that invaded through the Matrigel-coated membrane (Fig. 5c and d)..

MMP2 and MMP9, members of the MMP family, play important roles in the ECM degradation, migration and invasion of tumor cells [16]. It was proved that RPS27L knockdown induced the down-regulation of MMP2 and MMP9 proteins in U87MG and A172 cells (Fig. 5e). These results suggested that RPS27L silencing significantly inhibits glioma cell migration and invasion in vitro.

RPS27L downregulation inhibits tumorigenesis in vivo

To enhance our viewpoints, orthotopic xenografts were assessed in vivo. Nude mice bearing sh-RPS27L U87MG cells showed significantly smaller tumor volume via bioluminescence imaging (Fig. 6a). The sh-RPS27L group exhibited a longer survival rate relative to controls (Fig. 6b). HE staining of tumors that were collected at the same time (14 days after implantation) in the two groups also showed a smaller size in the sh-RPS27L group, and tumors of the sh-RPS27L cells displayed significantly more circumscribed borders (Fig. 6c). IHC validated again that RPS27L protein levels were downregulated in xenografts generated with sh-RPS27L cells (Fig. 6d). The proliferation index marker Ki-67, the apoptosis marker Bcl-2 and the invasion marker MMP2 were also downregulated in sh-RPS27L xenografts model (Fig. 6d). These results verified that RPS27L silencing led to slower growth and less aggressiveness of glioma cells in vivo.

Discussion

Even with the use of multimodal treatment strategies, the prognosis of glioma patients remains poor [17]. In this context, revealing the feasible molecular mechanisms contributing to the formation and progression of this tumor might be a pivotal step in the generation of new and more effective precision therapies [18-21]. In this study, we explored RPS27L overexpression in gliomas and demonstrated that RPS27L expression increased as an increase in tumor grade and that RPS27L was associated with the overall survival of patients, which suggested that RPS27L played an important role in glioma malignancy. In support of this finding, we demonstrated for the first time that RPS27L downregulation inhibited glioma cell proliferation, migration and invasion while promoted apoptosis in vitro and in vivo.

Previous studies have found that RPS27L is a direct p53 target that regulates apoptosis [22]. RPS27L directly binds to MDM2 and is subjected to MDM2-mediated ubiquitylation and degradation [23]. Thus, RPS27L interplays with the MDM2-p53 axis to regulate p53 activity [22-24]. It has been reported that elevated RPS27L may improve the prognoses of certain colorectal cancer patients by enhancing the DNA

repair capacity of their colonic cells [12]. In our studies, we just found the phenomenon that RPS27L was overexpressed in glioma and downregulation of RPS27L inhibited the proliferation, migration and invasion while promoted apoptosis of glioma cells. However, the mechanism was still unclear. We plan to investigate it in the following studies.

Abnormal cell proliferation is the main characteristics of human gliomas [25]. We performed EdU and CCK-8 assays and the results both demonstrated that RPS27L downregulation could obviously inhibit glioma cell proliferation. Flow cytometry revealed that RPS27L knockdown induced cell cycle arrest at G0-G1. Western blot analysis indicated that RPS27L knockdown led to significantly reduced levels of oncogenic factors including cyclinD1, cyclinE1 and CDK4, which play an important role in cell cycle G0-G1 arrest [14, 15]. In addition, the tumor suppressor p21 was induced after RPS27L depletion [26]. In view of the perspective that RPS27L is a direct P53 inducible target and is related to the apoptosis [22-24], we performed the flow cytometry. As expected, Knockdown of RPS27L induces apoptosis of glioma cells, and Western blot verified apoptosis regulators, such as Bax and cleaved Caspase-3 expression increased, while Bcl-2 expression levels were decreased in RPS27L knockdown cell lines [27, 28].

Previous studies barely reported RPS27L influenced cell migration and invasion or EMT of tumor cells. However, certain ribosomal proteins have been previously reported that they could influence the migration and invasiveness of glioma cells [29]. We first performed a 3D tumor spheroid invasion assay, which is considered an objective assay [30]. The results showed that RPS27L downregulation distinctly inhibited the aggressiveness of glioma cells. Next, Transwell assay was performed to confirm this phenomenon. Western blot analysis showed that RPS27L knockdown decreased the expression of MMP2 and MMP9, which are considered markers of cell invasion [31]. Thus, RPS27L might influence migration and invasion of glioma cells. Orthotopic xenografts of nude mice also supported the in vitro results. Notably, we found that the sh-RPS27L tumors were obviously smaller and less aggressive than the control group. Moreover, IHC of Ki67, Bcl2 and MMP2 showed convincing results.

Conclusions

In summary, RPS27L is overexpressed in glioma cells and knockdown of RPS27L inhibits glioma cell proliferation, migration and invasion while promotes apoptosis. Although RPS27L is implicated in p53 pathway of several cancers, more studies are needed to explain the particular mechanisms by which RPS27L influencing the tumorigenesis of gliomas.

List Of Abbreviations

ANOVA, Analysis of Variance; CCK-8, cell counting kit-8; CDK4, cyclin-dependent kinase 4; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EdU, 5-ethynyl-2'-deoxyuridine; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; GBM, glioblastoma multiforme; HE, Hematoxylin-eosin staining; HR, Hazard Ratio; IHC, immunohistochemistry; LGG, low-grade glioma; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9; OD450, Optical Density 450; PI,

propidium iodide; qRT-PCR, quantitative real-time PCR; RIPA, Radio Immunoprecipitation Assay; RPS27L, Ribosomal Protein S27-Like; siRNA, small interfering RNA; TCGA, The Cancer Genome Atlas.

Declarations

Acknowledgement

Not applicable.

Authors' contributions

GL and CWW led study design and prepared the manuscript. ZZS, HX and YW performed the research and wrote the manuscript. ZZS, RRZ and JYX collected clinical samples and corresponding clinical data. ZZS, MYQ and SBW performed cell culture and the assessment of cell functions in vitro. ZZS and CCW conducted the in vivo work. GL and CWW revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

We confirm that the research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All experimental protocols were approved by the Ethics Committee of the Qilu Hospital (Jinan, China) and performed in accordance with the relevant guidelines and regulations. Written informed consent was obtained from all patients. All animal experiments took place in the animal laboratory of the Qilu Hospital (Jinan, China), and were approved by the Institutional Animal Care and Use Committee (IACUC) of Shandong University (Jinan, China).

Consent for publication

Not applicable.

Competing interests

The authors have no competing interest to disclose.

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Figures

Figure 1

RPS27L expression is associated with patient survival and tumor grade in gliomas. (a) The prognostic features of RPS27L expression in LGG and GBM patients were showed in the TCGA database. The cut-off level was set at the median value of the RPS27L levels. (b) Quantification of RPS27L mRNA expression levels in LGGs, GBM and normal brain tissue in TCGA. (c) Representative images of IHC staining for

RPS27L in normal brain specimens and different grade gliomas. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. ns: no statistical significance.

Figure 2

RPS27L downregulation was confirmed by qRT-PCR and western blot. (a) qRT-PCR was used to verify RNA expression variation in U87MG and A172 cells transfected with RPS27L siRNA or control; (b) Western blot analysis was performed to verify protein expression; GAPDH was used as a loading control; * $P<0.05$; ** $P<0.01$; *** $P<0.001$, relative to control. Control: nonsilencing siRNA; si-RPS27L: siRNAs targeting RPS27L.

Figure 3

RPS27L downregulation inhibits cell proliferation, induces cell cycle arrest. (a, b) The result of EdU assay 48 h after transfection was showed (scale bar: 200 μ m); (c) In the CCK-8 assay growth curve based on OD450 values was detected; (d, e) Cell cycle distribution was detected via flow cytometry analysis using PI staining; (f) Western blot was used to detect the expression levels of proteins which regulates the cell cycle. GAPDH was used as a loading control. The data are shown as the mean $\pm$ SEM from three

independent experiments. *P<0.05; **P<0.01; ***P<0.001, relative to control. Control: nonsilencing siRNA; si-RPS27L: siRNAs targeting RPS27L.

Figure 4

RPS27L downregulation induces apoptosis of glioma cells. (a, b) Apoptosis of the indicated cells was analyzed using flow cytometry; (c) Western blot was used to detect the expression levels of apoptosis regulatory factors. GAPDH was used as a loading control. The data are shown as the mean±SEM from three independent experiments. *P<0.05; **P<0.01; ***P<0.001, relative to control. Control: nonsilencing siRNA; si-RPS27L: siRNAs targeting RPS27L.

Figure 5

RPS27L knockdown decreases the invasiveness of glioma cells. (a, b) Representative images showing invading spheroids in the 3D invasion assay for U87MG and A172 cells transfected with si-RPS27L and control evaluated at 24 h, 48 h and 96 h (scale bar: 200 μ m). (c, d) Effect of si-RPS27L on cell movement ability was assessed using Transwell migration and Matrigel invasion assays (scale bar: 50 μ m). (e) Expression levels of the MMP2 and MMP9 proteins were detected via western blot analysis. GAPDH was used as the loading control. The data are shown as the mean $\pm$ SEM from three independent experiments.

*P<0.05; **P<0.01; ***P<0.001, relative to control. Control: nonsilencing siRNA; sh-RPS27L: siRNAs targeting RPS27L.

Figure 6

RPS27L downregulation inhibits tumorigenesis and aggressiveness in vivo. (a) Bioluminescence imaging showed the change of tumor size as days elapsed. (b) Survival analysis was performed of mice implanted with sh-RPS27L or control cells (P<0.05 by log-rank test). (c) HE staining of sections was performed from mouse brains with control and sh-RPS27L xenografts at 2 weeks after implantation, where the tumor size and sections at tumor margins were shown. (d) IHC for RPS27L, Ki-67, Bcl-2 and

MMP2 in sections from the indicated xenografts. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, relative to the control.
Control: nonsilencing shRNA; sh-RPS27L: shRNAs targeting RPS27L.