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Original Article

Knockdown of NUPR1 inhibits the proliferation of U87 cells in vivo and vitro

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Abstract: Nuclear protein-1 (NUPR1), also called p8 or Com1, plays an important role in the growth and migration of human malignant tumour cells as a stress response factor. However, the role of NUPR1 in glioma is poorly understood. In this study, quantitative real-time PCR and western blot were used to confirm knockdown efficiency of U87 cells infected with lentiviral vector, Wound-healing, MTT, BrdU assay and tumorigenesis in nude mice were used to analysis migration and proliferation of U87 cells. Fluorescence-activated cells sorting (FACS) and western blot were applied to detect mechanism in cell apoptosis function of NUPR1 in U87 cells. We confirmed NUPR1 was up-expressed in glioma tissues compared with normal brain (NB) tissues. Down-regulation of NUPR1 suppressed cell migration and proliferation, and promoted cell apoptosis in U87 cells in vitro and in vivo. Furthermore, the expression levels of phosphorylated Erk1/2 (P-Erk1/2) and phosphorylated p38 (P-p38) MAPK were decreased by lowering NUPR1 expression in U87 cells. In conclusion, NUPR1 play an important role in the growth and migration of U87 cells. Knockdown of NUPR1 could suppress U87 cells growth by inducing cell apoptosis via decreasing expression of P-Erk1/2 and P-p38.

Keywords: NUPR1, glioma, proliferation, apoptosis

Introduction

NUPR1, located on chromosome 16P11.2, was first described and induced in the acute phase of pancreatitis of rats as a stress-related molecule [1]. NUPR1 is a widely expressed gene conserved in mammals, Xaenopus, Caenorhabditis elegans and Drosophila. Its open reading frame is 249 bp with a molecular weight of 8872.7 Da [2]. Studies have showed that the expression level of NUPR1 existed differences in digestive system carcinomas, urologic neoplasms and multiple myeloma tumor tissue, which affects tumor cells proliferation, migration and apoptosis [3].

Glioma, the most common primary brain tumor, has high mortality, high relapse rate and low cure rate. But survival time of high grade gliomas (WHO III/IV) is still poor, even when we take an individual and systematic treatment [4]. With the development of molecular biology and genetics, more and more biomarkers were identified during the progression of glioma, such as mentioned molecular markers IDH (Isocitrate dehydrogenase), 1P19q (codeletion) loss and TP53 mutations in glioma treatment guidelines, which help to predict prognosis and therapy of patients with glioma [5].

In this study, we find that NUPR1 was up-expressed in human glioma tissues. The capacity of affecting cell proliferation, migration and associated mechanisms of NUPR1 were explored by knockdown expression of NUPR1 in U87 cell lines in vitro and vivo. With the further understanding, NUPR1 will improve the diagnosis and prognosis of patients with gliomas as a new biological marker.

Materials and methods

Tissue samples

Glioma tissues and normal brain samples (acquired during the gliomas operations) were obtained from the Department of Neurosurgery of the First Affiliated Hospital of Dalian Medical University, Liaoning, China. The frozen specimens after resection were stored at -80°C. This study was approved by the Human Ethics

Committee of the First Affiliated Hospital of Dalian Medical University (approval number: LCKY2014-20) conforming to institutional medical requirements, and written informed consents were obtained from all of the enrolled patients.

Animals

The procedures and animal care were in accord with NIH Guidelines approved by the Ethics Committee of The First Affiliated Hospital of Dalian Medical University in China. About five week old female nude mice (BALB/c-nu/nu) were bought and raised at the Center of Experimental Animals, Dalian Medical University (China).

Cell culture

Human glioma U87, U251, U373, and A172 cell lines were provided by Institute of Neuroscience, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences and cultured in the Dulbecco's Modified Eagle Medium (DMEM, Corning, US) with 10% fetal bovine serum (FBS, Ausbian, Australia) at 37°C in 5% CO_2.

Quantitative real-time PCR

Cell lines were collected after washing with PBS twice. And then their RNA was extracted using Trizol agents (KeyGEN, Cat: KGA1203) according to the product description. cDNA was taken by reverse-transcribing the total RNA using PrimeScript® RT Reagent Kit (Takara, Cat: RR037A) and amplified using SYBR® Premix Ex Taq™ Kit (Takara, Cat: RR820A) at the condition of 40 cycles in a total volume of 20 μl with 7500 Real-Time PCR System. We used $2^{-\Delta\Delta CT}$ method to analyze the relative expression. The primer sequences of NUPR1 were: 5'-AGCCTGGATGAATCTGACCTCTAT-3' (forward) and 5'-GGTGTT GGCAGCAGCTTCTC-3' (reverse). The primer sequences of GAPDH were: 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGACGCCAGTGGA-3' (reverse).

Construction of shRNA lentiviral vector and cell infection

ShRNA (synthesized by GeneChem Co, Ltd, Shanghai, China) targeting NUPR1 sequence (CCGGAACTGGTGACCAAGCTGCAGATTCAAGAGATCTGCAGCTTGGTCACCAGTTTTTTT) was selected according to the best kinetic parameters, and Control nonsilencing RNA was TTCTCCGAACGTGTCACGT. ShRNA-containing lentiviruses (shCtrl: No.LVPSC3741, shNUPR1: No.LVpGCSIL-004PSC1501-1, GeneChem, China) were synthesized by transferring pGCSIL-GFP vector with pHelper1.0 and pHelper2.0 into 293T cells. U87 cell suspensions including 5×10^4 cells were cultured in six-well plates for 24 h, and then infected with lentiviruses according to multiplicity of infection (MOI). Medium was changed after 8 h and cells were continued to screen using puromycin (2 μg/ml) if GFP fluorescence cells were more than 80% observed under the fluorescent microscope (OLYMPUS IX71) after infection for 72 h. Levels of NUPR1 mRNA were detected using Quantitative real-time PCR analysis.

Western blot

Protein quantification was measured by BCA method (Cat: PC0020, Solarbio, China). Equal amounts of protein were separated by SDS-PAGE (15%), transferred to PVDF (0.2 μm, Millipore, USA), and then blocked with 5% non-fat milk. Membranes were incubated with rabbit anti-human NUPR1 antibody (1:500, SANTA CRUZ, SC-30184, US), anti-GAPDH (Proteintech, Cat60004-1-Ig, US) overnight at 4°C and incubated in second antibodies for 2 h after washing by TBS next day. Protein bands were detected by enhanced chemiluminescence (ECL) with Bio-Spectrum Gel Imaging System (UVP, USA). The Erk (Cat: 7695S), P-Erk (Cat: 4370S, Cat: 13148), P-p38 (Cat: 4511s) and anti-rabbit IgG (Cat: 7074P2) secondary antibodies were all purchased from Cell Signaling Technology, Inc. (MA, USA). Anti-GAPDH (Cat: 60004-1-Ig, US) was purchased from Proteintech.

Immunohistochemistry (IHC)

Paraffinized sections were dipped in xylene for 15 min and then rehydrated in a graded ethanol series for 5 min per solution. Antigen retrieval was performed by heating the sections in a citrate-buffered solution (0.01 M, pH 6.0) in a microwave oven at thawing temperature for 20 min. Endogenous peroxidase activity was eliminated via incubation in 3% hydrogen peroxide diluted in deionized water for 15 min. After blocking with goat serum (ZSGB-BIO), the sections were incubated with a rabbit anti-human NUPR1 antibody (1:100, SANTA CRUZ, SC-30184, US) overnight at 4°C. The primary antibody was detected with a biotin-labeled goat anti-rabbit antibody (ZSGB-BIO) for 20 min at room temperature after washing the slides

with phosphate-buffered saline (PBS, pH 7.4). Subsequently, the sections were incubated in horseradish peroxidase (HRP) for 15 min. Finally, chromogenic reactions were performed using 3, 3'-diaminobenzidine (DAB, ZSGB-BIO) and hematoxylin, and then, the samples were analyzed using a biomicroscope (LEICA, DM6000-B, GERMANY).

Wound-healing assay

Wound-healing assay was used to observe Cell migration in vitro. U87 cells transfected steadily with shRNA were cultured in 6-well plates. A cell-free scratched gap was made with a pipette tip at the bottom of every plate when cells became confluent, then cells were incubated for another 6 h and 12 h. The lengths between two initial walls of every wound were measured with Image J software.

MTT assay

U87 cells in logarithmic phase were trypsinized and resuspended after lentivirus infection for 3 days, and then incubated in 96-well plate at 2000 cells per well at 37°C in 5% CO_2. From the second day, MTT (10 μL, 5 mg/mL, Genview, Cat: JT343) was added into each well with no need of replacing medium and then 100 μL DMSO (Corning Cat: 10-013-CVR) was added after 4 h once a day. Absorbance of each well was detected at 490 nm in a microplate reader (Tecan infinite, Cat: M2009PR) at the end of incubation.

BrdU analysis

The transfected U87 cells were incubated in 96-well plate for 24 h and then 10 μl BrdU (1:100) was added to each well. Cell proliferation was observed using BrdU kit (Roche, Cat: 11647229001, USA) according to the manufacturer instructions. Absorbance of each well was observed at 450 nm at the first and fourth day of incubation.

Analysis of the cells apoptosis assay by fluorescence-activated cells sorting (FACS)

More than 5×10^5 infected U87 cells were centrifuged at 1300 rpm for 5 min and then washed with D-Hanks. Next, cells were washed by 1× binding buffer, centrifuged at 1300 rpm for 3 min. Finally, after adding 5 μl annexin V-APC (eBioscience, Cat: 88-8007), cells resuspended by 400 μl binding buffer were transferred into tube of flow cytometer (Millipore, Cat: Guava easy Cyte HT) for analysis.

Statistical analysis

The qualifying data were expressed as the mean ± SD of experiments which were performed in triplicate. SPSS 17.0 software, Graph Pad Prism 5.0 and excel software were used for statistical analysis and drawing graphs. Two-tailed Student's t-test or One-way ANOVA were used evaluate the differences between groups. $P<0.05$ was considered as statistical significance.

Results

NUPR1 is overexpressed in U87 cells and in gliomas tissues compared with NB tissues

Quantitative real-time PCR was performed to detect the expression level of NUPR1 mRNA in 25 glioma samples and 15 NB tissues. Glioma tissues presented a much higher expression levels of NUPR1 mRNA than NB tissues ($P<0.05$) (**Figure 1A**). Similarly, the data of western blot also showed that the NUPR1 protein levels in NB tissues were lower than glioma tissue samples ($P<0.05$) (**Figure 1B**). Furthermore, NUPR1 expression levels of different representative human glioma cell lines were detected by quantitative real-time PCR. The result showed NUPR1 was expressed highly in U87, U251 and U373 cells (**Figure 1C**).

Establishment of U87 cell line with steadily down-regulated NUPR1 expression

In this study, U87 cell was chosen to investigate biological function of NUPR1 because it was obtained from the malignant gliomas. Transfection efficiency of cells was observed by fluorescence microscope, real-time PCR and Western blotting were used to measure NUPR1 mRNA and protein expression levels respectively after using lentiviral vector (PLV-Ctrl vs. sh-NUPR1) to infect U87 cells. GFP fluorescence cells were observed more than 80% (**Figure 1D**). Compared with the shCtrl-transfect cells, the mRNA level of shNUPR1-transfect cells was significantly reduced ($P<0.001$) (**Figure 1E**), and Western blotting indicated the same result ($P<0.05$) (**Figure 1F**).

Down-regulation of NUPR1 suppressed U87 cells migration and proliferation

Wound healing, MTT and BrdU assay were used to analysis migration and proliferation of U87

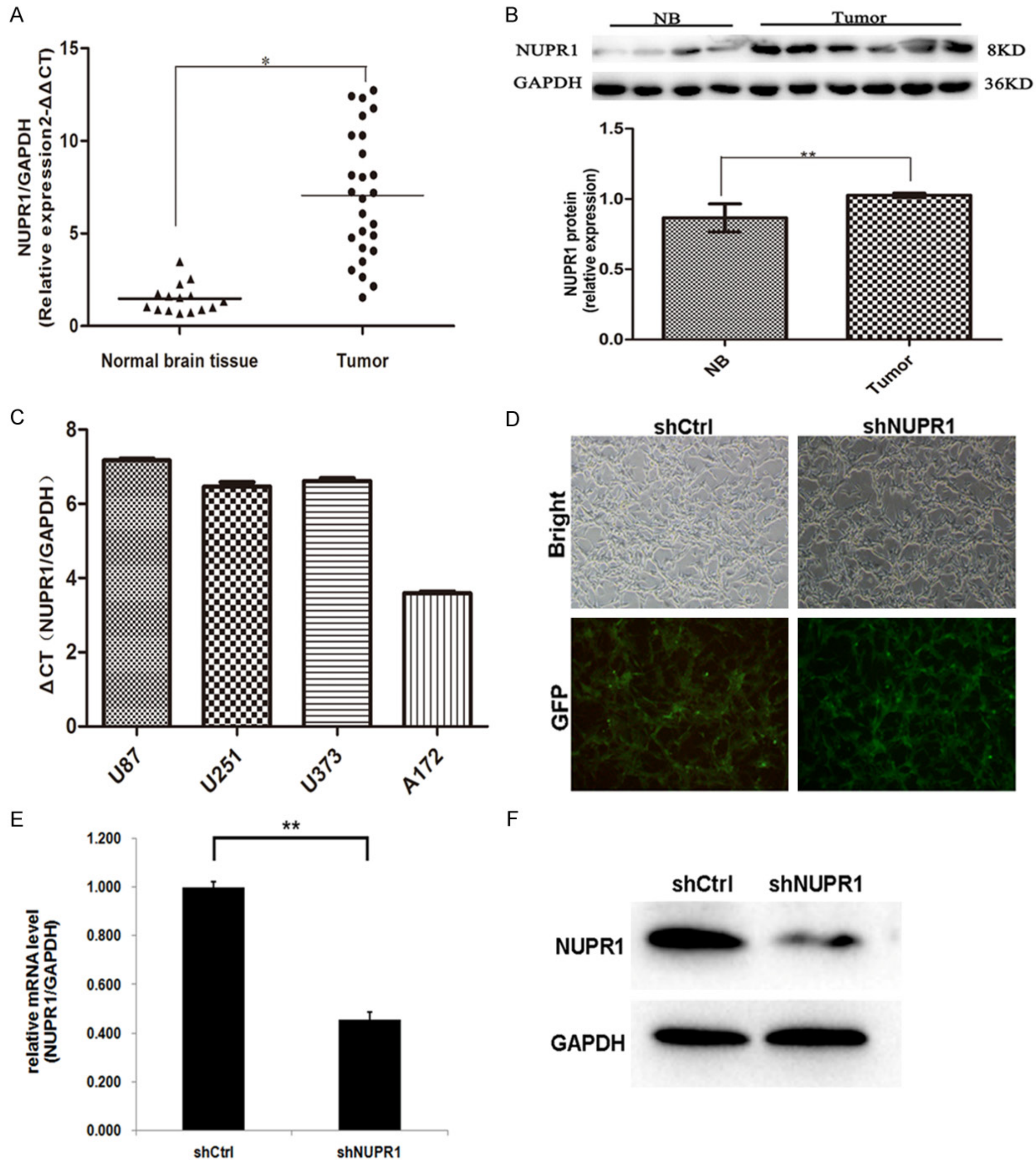

Figure 1. The expression of NUPR1 in human glioma tissues was increased compared with normal brain tissues. A. 25 cases glioma tissues presented higher mRNA expression than NB (15 cases) by Q-RT-PCR assay. GAPDH was a loading control. B. Western blot assay was performed the NUPR1 protein expression between NB and glioma tissues, and GAPDH was a loading control. ShRNA stably down-regulates NUPR1 expression in the human glioma cell line U87. C. Q-RT-PCR assay shows the NUPR1 mRNA levels in U87, U251, U373 and A172 cell lines. D. GFP fluorescence images of U87 cells infected with lentivirus for 3 days were shown (100×). E. Q-RT-PCR assay was used to detect the transcriptional levels of the NUPR1 between ShCtrl and shNUPR1 U87 cells. F. Western blot assay shows that the NUPR1 expression levels in ShCtrl and shNUPR1 treatments. GAPDH was used as the loading control. Data are presented as the mean ± SD for three independent experiments. *P<0.05, **P<0.01.

cells in vitro. The result of the wound healing assay revealed that LV-shCtrl-infected cells performed a higher speed into the gap than LV-shNUPR1-infected cells (P<0.05) (**Figure 2A**), revealing that the reduced expression of NUPR1 could suppress cell migration.

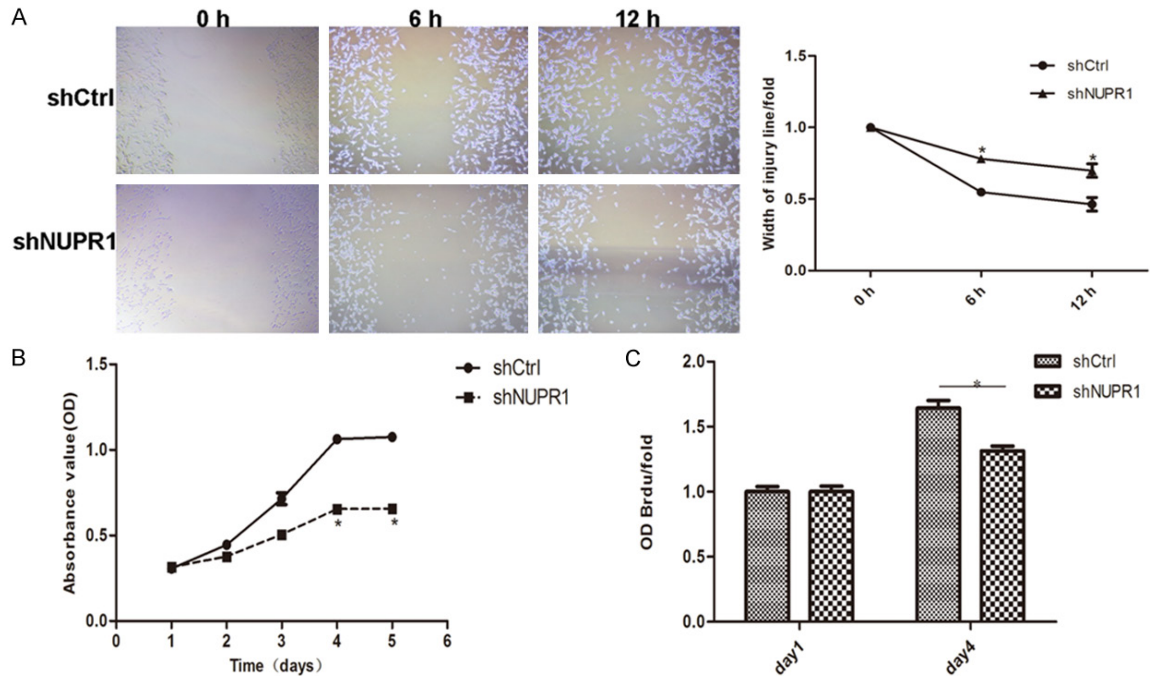

Figure 2. Migration and proliferation of U87 cells were suppressed by knockdown of NUPR1. A. Wound-healing assay was used to test the migration ability between shCtrl and shNUPR1-infected cells. B. MTT assay showed cell proliferation in the shNUPR1 group was significantly inhibited. C. BrdU assay showed the 490 nm absorbance fold change in the first day and fourth day, indicating the number of shNUPR1-infected cells was significantly decreased. Data are presented as the mean ± SD for three independent experiments. *P<0.05.

Compared with LV-shCtrl-infected cells, the growth curve obtained from MTT assay showed shNUPR1 cells grew quite slowly (P<0.001) (**Figure 2B**), and BrdU assay indicated that the number of LV-shNUPR1-infected cells was significantly decreased (P<0.05) (**Figure 2C**), suggesting that down-regulated NUPR1 expression could significantly inhibit cell proliferation.

Knockdown of NUPR1 could promote cell apoptosis in U87 cells

The data of U87 cells apoptosis was obtained from FACS (**Figure 3A**). As shown in **Figure 3B**, the average apoptosis percentage of shNUPR1-infected U87 cells was 6.05%, and shCtrl group was 2.34% (P<0.001). The result revealed that knockdown of NUPR1 expression could promote apoptosis significantly in U87 cells.

NUPR1 knockdown suppressed cell growth via phosphorylation of ERK1/2 and p38

As previous reports, NUPR1 played a role in apoptosis function of cancer via MAPK signal pathway and p53 [2, 6, 7]. To explore the underlying mechanism of NUPR1 in glioma cells apoptosis, P44/42 (ERK1/2, Thr^{202}/Try^{204}), p38 (Thr^{180}/Try^{182}) and P53 were detected in U87 cells with LV-shNUPR1 against LV-shCtrl. The analysis of western blot revealed that the expression levels of phosphorylated P44/42 (ERK1/2, Thr^{202}/Try^{204}) and p38 (Thr^{180}/Try^{182}) were significantly decreased in shNUPR1 U87 cells compared with the shCtrl group (P<0.05), whereas p53 were not affected in the knockdown of NUPR1 group (P>0.05) (**Figure 3C**). Above data showed that down-regulation of NUPR1 inhibited U87 cell growth and induced apoptosis via phosphorylation of ERK1/2 and p38. Thus, it's inferred that knockdown of NUPR1 could suppress glioma cells growth by inducing cell apoptosis.

Knockdown of NUPR1 suppresses cell tumourigenicity in vivo

The nude mouse xenograft model was performed to confirm the growth effects of NUPR1 after implanting U87 cells. The average weights of the tumors removed from mice injected with shPLV-Ctrl U87 cells was 2.008 g, and shNU-

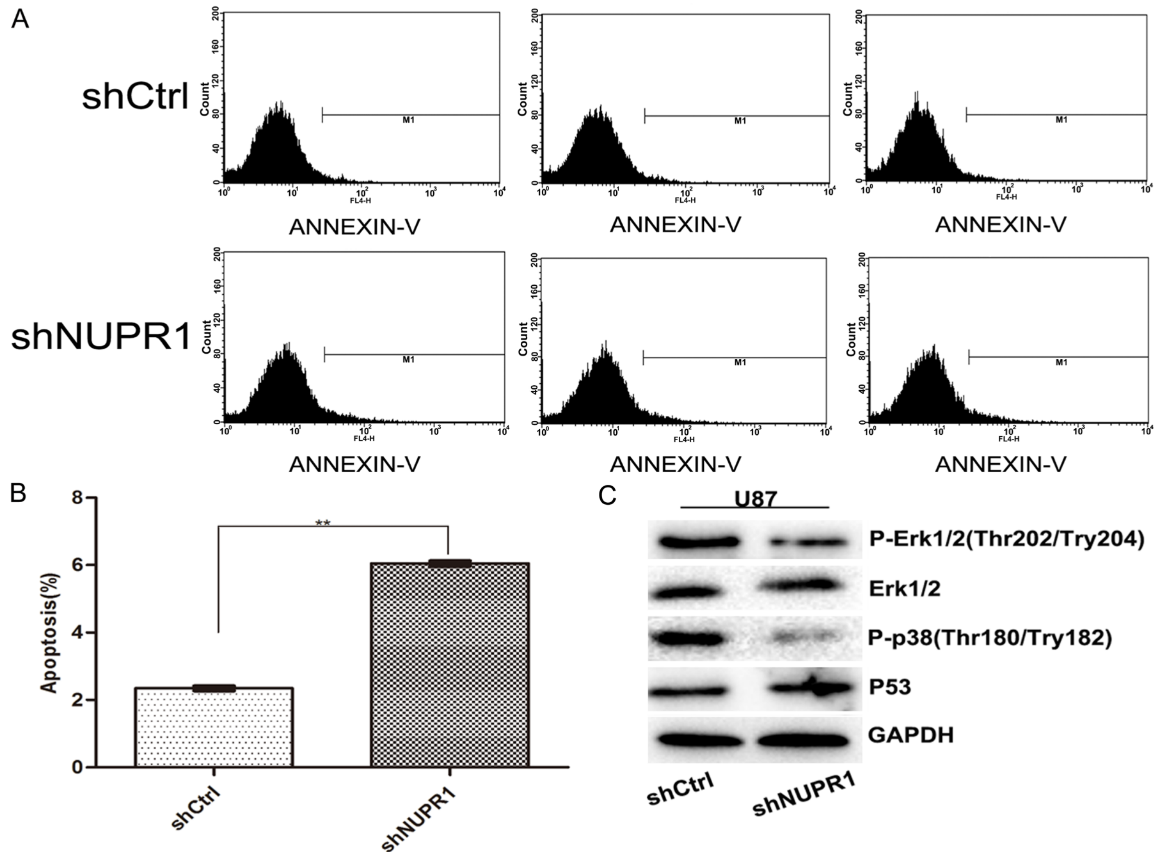

Figure 3. Knockdown of NUPR1 promotes apoptosis in U87 cells. A. Apoptosis was detected by annexin V-APC staining. B. Percentage of apoptotic cells showed a significant increase in the Lv-shNUPR1 group. C. The gray value of western blot revealed that the expression levels of phosphorylated P44/42 (ERK1/2, Thr^{202}/Try^{204}), ERK1/2, phosphorylated p38 (Thr^{180}/Try^{182}) and P53 were significantly decreased in shNUPR1 group compared with shCtrl group. Data are presented as the mean ± SD for three independent experiments. **P<0.01.

PR1 was 1.161 g (P<0.05) (**Figure 4A** and **4B**). Furthermore, the results of immunohistochemistry staining showed that the NUPR1 protein expression level in the shNUPR1-xenografted tumors was reduced compared with shPLV-Ctr-xenografted tumors (**Figure 4C**).

Discussion

Glioma, originated from glial cell deterioration, which has a poorer overall prognosis than other primary intracranial tumor, even though great progress have been made in surgery, chemotherapy and radiotherapy in recent years [8]. At the same time, more and more studies about molecular biology are performed to predicate the prognosis accurately and select appropriate treatment plan for patients with glioma, especially high WHO grade [9]. The secondary structure of NUPR1 was similar to high mobility protein (High Mobility Group proteins, HMG), particularly HMG-I/Y protein, which were increased rapidly when some organs in the body including pancreas, liver and kidney were damaged [10]. Previous studies revealed that expression levels of NUPR1 were related to tumor proliferation, migration and prognosis in variety of tumors, such as breast cancer [11], thyroid cancer [12], lung cancer [13], liver cancer [14], pancreatic cancer [15], bladder cancer [16] and uterine cancer [17]. In the present study, the role of NUPR1 was discussed in glioma potentially. NUPR1 mRNA was detected by PCR in 25 glioma samples and 15 NB tissues, which showed that the expression of NUPR1 was higher in glioma than in NB tissues. Similarly, western blot showed the same result. So, NUPR1 may be a specific molecular marker of glioma and potential therapeutic targets.

It was firstly discovered that NUPR1 was high expression in Cos-7 and AR4-2J cell lines which

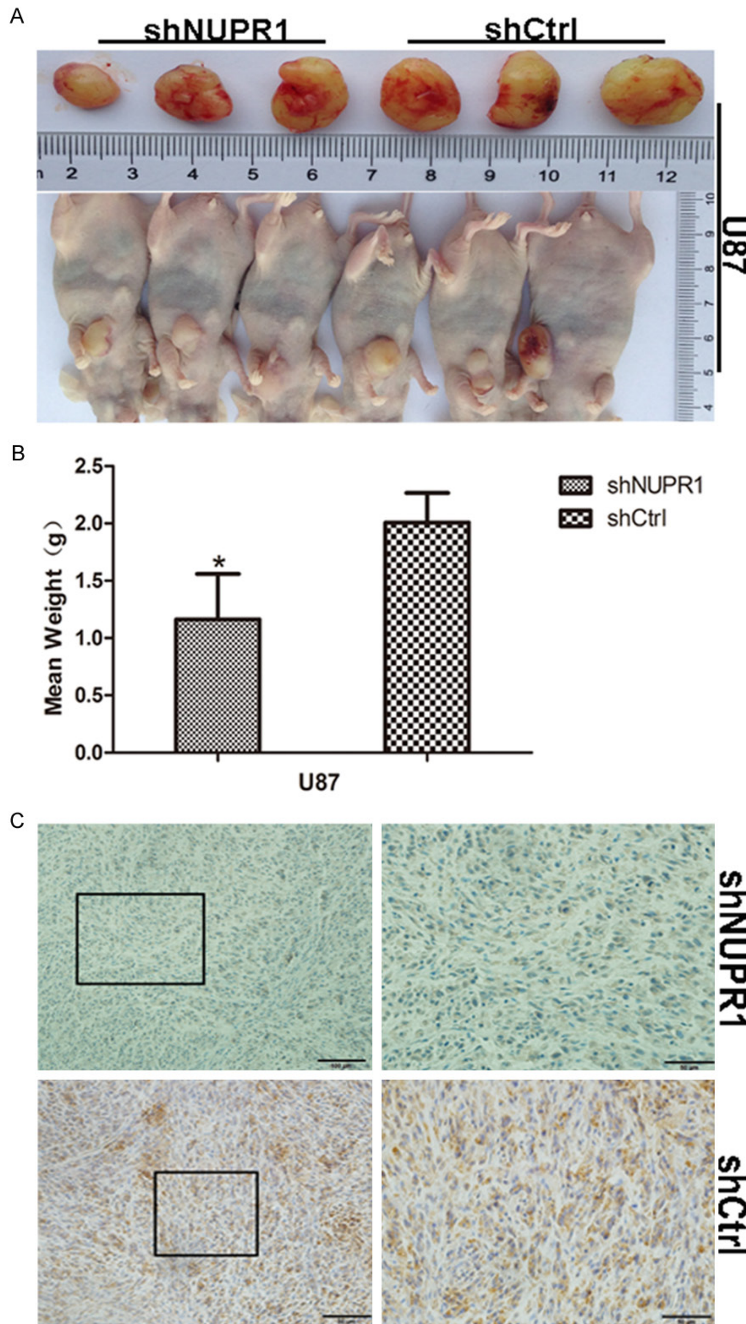

Figure 4. Down-regulation of NUPR1 suppresses cell tumourigenicity. A. The tumors were removed from mice injected with shPLV-Ctrl and shNUPR1 U87 cells for one month. B. Tumorigenicity of shNUPR1-U87 cells was reduced in vivo compared with shCtrl group. Five mice were used for each treatment. *P<0.05. C. IHC staining showed the NUPR1 expression in shCtrl and shNUPR1 subcutaneous tumors. Original magnification ×400, ×100.

enable cells to proliferate 1.5-2 times [1], and in HepG2 cells, knockdown of NUPR1 significantly decreased the migration of cells, and MTT and colony formation assay revealed that decreased NUPR1 expression could significantly suppress cell proliferation [18]. Above date indicated that NUPR1 affected migration and proliferation of tumor cells. In addition, Knockdown of NUPR1 expression also inhabited lung cancer cell line H1299 growth in vivo [13]. In this experiment, Wound-healing, MTT and BrdU assays revealed that knockdown of NUPR1 expression could suppress the migration and proliferation of U87 cell lines vitro and decrease tumorigenesis in vivo, which was in line with previous studies.

Previous studies showed that NUPR1 affected the biological functions of tumor cells via regulating the apoptosis function via ERK signaling pathway [19, 20]. As an anti-apoptotic molecule, NUPR1 exerted anti-apoptotic effect in breast cancer specimens and pancreatic cancer [11, 21, 22]; However, it was found knockdown NUPR1 expression promoted apoptosis in MEF cells, suggesting that NUPR1 was a promoting apoptosis molecule [23]; Some results support that serum deprivation sensitizes astrocytes to oxidative stress via a p38 MAPK-dependent NUPR1 upregulation that leads in turn to decreased HO-1 expression and NUPR1 could be combined with p53 and then affected initiation factor of p21 [6, 7]. Previously, researchers have reported that the Erk and p38 pathways are involved in the regulation of cell proliferation, migration and apoptosis [19, 24, 25]. In this experiment, the result of FACS demonstrated that knockdown of NUPR1 in U87 cells could significantly promote apoptosis, which was consistent with results of previous studies about NUPR1 regulating apoptotic function of tumor cell. According to previous research, to initially

explore the related mechanisms of NUPR1 affecting apoptosis function of glioma cells in this study, P44/42 MAPK (ERK1/2), p38 MAPK phosphorylated proteins expression level, P53 and the total protein levels of Erk were detected by western blot between shCtrl and shNUPR1 U87 cells, which indicated that knockdown of NUPR1 may suppress glioma cells growth by inducing cell apoptosis via decreasing expression of P-ERK1/2 and P-p38.

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Disclosure of conflict of interest

None.

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