

Upregulation of LGMNP1 confers radiotherapy resistance in glioblastoma

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Abstract. Glioblastoma is a lethal brain tumor type, which is frequently resistant to radiotherapy. The aim of the present study was to explore the function of legumain pseudogene 1 (LGMNP1) on radioresistance in glioblastoma. Reverse transcription-quantitative PCR was used to detect the relative expression of LGMNP1 in glioma cell lines after radiotherapy. Ectopic expression of LGMNP1 was achieved by transfection of a lentiviral vector. A clonogenic assay was used to determine the colony formation ability following radiotherapy. A comet assay, flow cytometry and western blot analysis were applied to detect DNA damage, the apoptotic rate, and levels of apoptotic proteins, respectively. The results revealed that LGMNP1 was significantly upregulated in glioma cells after radiation. Glioma cells stably overexpressing LGMNP1 were successfully established. Overexpression of LGMNP1 in glioma cells reduced DNA damage processes and the percentage of apoptotic cells after radiotherapy. In addition, overexpression of LGMNP1 in glioblastoma multiforme cells decreased apoptotic protein expression after radiotherapy. The present results indicated that upregulation of LGMNP1 conferred radiotherapy resistance by increasing the ability of DNA damage protection and reducing the apoptotic population in glioma cells.

Introduction

Glioblastoma multiforme (GBM) is recognized as the most aggressive type of diffuse glioma of astrocytic lineage and is

equivalent to grade IV based on the World Health Organization (WHO) Classification from 2007; it is divided into isocitrate dehydrogenase (IDH) wild-type and IDH mutant-type according to molecular typing in the most recent WHO Classification (1). GBM is the most common malignancy of the central nervous system with an average annual age-adjusted incidence rate of 19/100,000 individuals, accounting for 54% of all glioma cases (2). GBM is a refractory malignant tumor type with a median survival time of only 15 months (3). Treatment is complex, and initially consists of maximal safe surgical resection and subsequent radiation therapy with concurrent temozolomide chemotherapy, followed by 6-10 or even more cycles of maintenance temozolomide chemotherapy (4-6). Radiation therapy has a crucial role in GBM treatment, particularly for the tumor that is not totally surgically removed (7,8). However, for a long time, the problem of radiotherapy resistance of GBM, which is one of the reasons why the survival of affected patients cannot be prolonged remains an obstacle for clinicians. Therefore, it is important to elucidate the mechanisms underlying radiotherapy resistance of GBM.

Legumain (LGMN), also known as asparagine endopeptidase, is a lysosomal cysteine protease originally identified in the seeds of legumes, which is also present in the human body. The gene is located on chromosome 14 and it is associated with a variety of tumor types at the stages of development, metastasis and invasion (9). A previous study by our group indicated that once tumor-associated macrophages, which highly expressed LGMN on their surface, were selectively ablated by using a doxorubicin-based prodrug activated by LGMN, tumor growth and metastasis were markedly inhibited in a murine tumor model, this implying an important role of LGMN in cancers (10). LGMN pseudogene 1 (LGMNP1) is a pseudogene of LGMN located on chromosome 13 and its expression in GBM is much higher than that in normal tissues (11,12), implying that LGMNP1 has a certain association with GBM. To study the function of LGMNP1 on the radioresistance of GBM, the present study assessed whether LGMNP1 was altered after radiotherapy and whether overexpression of this gene promoted the radiotherapy resistance of GBM using *in vitro* experiments. In addition, the underlying mechanisms were explored.

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Materials and methods

Cell culture. The human GBM cell lines U87-MG (glioblastoma of unknown origin; cell line was authenticated by STR profiling) and T98G (purchased in 2014 from the Cell Bank of the Chinese Academy of Sciences) were cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare Life Sciences, Logan UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained in a humidified atmosphere at 37°C with 5% CO₂.

Radiation treatment. Cells in culture were treated with an irradiator (GE 3000; GE Healthcare Life Sciences) using a ¹³⁷Cs source at an exact dose of 0 or 6.0 Gy. During irradiation, the cultures were stored in the cell culture incubator (5% CO₂ at 37°C). The cells were harvested exactly at the end of the irradiation.

Reverse transcription-quantitative PCR. Total RNA was extracted from cellular samples using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. UV spectrophotometry was used to determine the RNA concentration and quality. Reverse transcription of total RNA was performed using an iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following the manufacturer's instructions. A 7500 Fast PCR instrument (Applied Biosystems, Thermo Fisher Scientific, Inc.) was used for quantitative PCR amplification. The primer and probe sequences were as follows: LGMNP1 forward primer, 5'-GGA CGTGGAAGATCTGACTAACC-3', reverse primer, 5'-ATG ATGTGGCTGGTATTGGTGTAT-3' and probe, 5'-VIC-CAA GCAGTGCCGCC-MGB-3'. The probe was modified with MGB at the 3'-end. GAPDH forward primer, 5'-GAAGGA CTCATGACCACAGTCCA-3', reverse primer, 5'-GCAGGG ATGATGTTCTGGAGAG-3' and probe, 5'-ROX-CGGCCA TCACGCCACAGTTTCC-3'-BHQ2. Gene alignments and primer specificity analysis were used to choose specific primers and probes. The composition of the reaction mixture contained 10 µl of 2X TaqMan Universal Master Mix II with UNG (Applied Biosystems, Thermo Fisher Scientific, Inc.), 1 µl (100 ng) of cDNA, 1 µl of probe-primer mix and 8 µl of nuclease-free water, constituting to a final volume of 20 µl. The thermocycling conditions for qPCR were as follows: 50°C for 2 min, 95°C for 10 min and 45 cycles of 95°C for 15 sec and 60°C for 1 min. Data were acquired at the end of the annealing/extension phase. Melting curve analysis was performed at the end of each run from 50 to 95°C.

Lentiviral vector-mediated gene overexpression. The LGMNP1 overexpression sequence was constructed by Shanghai Hanyin Co., Ltd. (Shanghai, China). The recombinant lentivirus and negative control (NC) lentivirus were prepared and titrated to 10⁹ transfection U/ml. After 48 h, the efficiency of overexpression was confirmed via RT-qPCR. To obtain stably transfected cells (LGMNP1-OE), GBM cells were seeded in 6-well dishes at a density of 1x10⁵ cells/well. The cells were then infected with the same virus titer on the following day with 8 µg/ml Polybrene. At 72 h post-viral infection, the culture medium was replaced with selection medium containing 4 µg/ml

puromycin. The puromycin-resistant cells were amplified in medium containing 2 µg/ml puromycin for 7 days and then transferred to medium without puromycin.

Colony formation assay. The isolated cells were seeded at 300 cells/well in a 6-well tissue culture plate and grown for 14 days until macroscopic cell clones were visible. The cells were then fixed with 95% cold methanol for 15 min at 4°C and stained with 0.5% methylene blue for 2 min in order to determine the number of colonies by microscopy. The colony forming efficiency was calculated as the percentage of single cells that generated colonies on the 14th day. The colony formation rate was calculated as follows: Colony formation rate = (number of clones/300) x 100%.

Comet assay. Cells were lysed by placing the slides in a Coplin jar (Thomas Scientific, Swedesboro, NJ, USA) containing 2.5 M NaCl, 0.1 M Na₂EDTA, 0.1 M Tris and 1% Triton X-100 (pH 10) at 4°C for at least 1 h. Subsequently, slides were immersed in electrophoresis solution (0.3 M NaOH and 1 mM Na₂EDTA, pH >13) for 30 min. Electrophoresis was then performed at 1.3 V/cm for 20 min in the same solution. Slides were washed twice in cold phosphate-buffered saline (PBS) for 10 min and in water for another 10 min. Comets were fixed by immersing the slides in 70% ethanol for 15 min and in absolute ethanol for a further 15 min prior to placing them on the bench to dry overnight. Comets were stained with SYBRGold at the dilution recommended by the manufacturer in a bath at 4°C with agitation. After 40 min, SYBRGold solution was removed and the slides were rinsed twice with water and left to dry at room temperature. On the day of analysis, gels were hydrated by adding a drop of water on top of each minigel, and a glass coverslip (24x60 mm) was used to cover all the minigels on the slide. The semi-automated image analysis system Comet Assay IV (Perceptive Instruments Ltd., Bury St. Edmunds, UK) was used to evaluate 100 comets/gel in the case of the H₂O₂ experiments. Percentage DNA in the tail was the parameter selected to describe each comet. The number of comets per gel (including so-called hedgehogs) was counted by direct observation.

Cell apoptosis analysis. Apoptosis was analyzed by translocation of phosphatidylserine to the cell surface using an Annexin and DAPI apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Cells were treated with 6 Gy of radiation, then collected and washed in cold PBS. Cells were resuspended in Annexin V-FITC and DAPI for 30 min in the dark. Cell apoptosis was analyzed on a FACS Aria flow cytometer (BD Biosciences) and quantified using CellQuest software 5.1 (BD Biosciences). Fluorescence was captured with an excitation wavelength of 480 nm.

Western blot analysis. The total proteins of cells were extracted with cell lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40 and 1 mM PMSF) and determined by BCA methods. Protein samples (30 µg) were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were incubated with blocking buffer [5% skimmed milk in Tris-buffered saline containing Tween-20 (TBS-T)] at

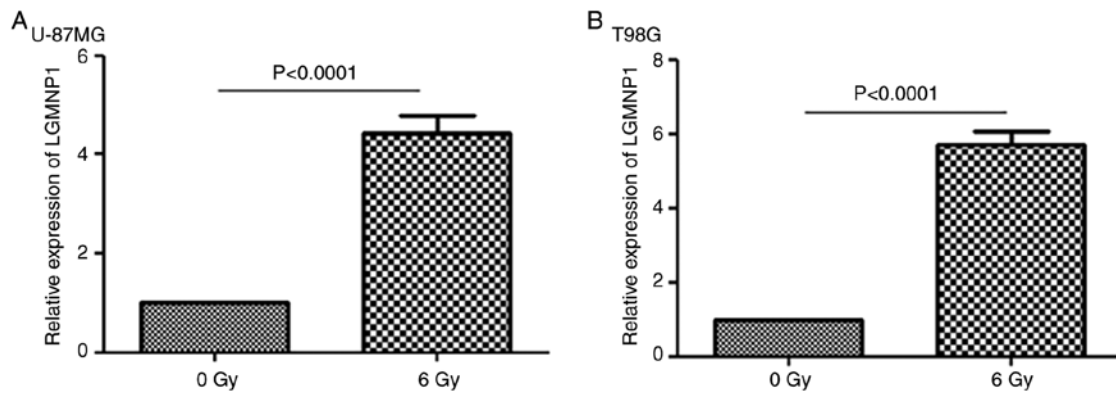


Figure 1. LGMNP1 is significantly upregulated in glioma cells after radiation. (A) Relative LGMNP1 mRNA levels in U87-MG cells treated with 6 or 0 Gy. (B) Relative LGMNP1 mRNA levels in T98G cells treated with 6 or 0 Gy. LGMNP1, legumain pseudogene 1.

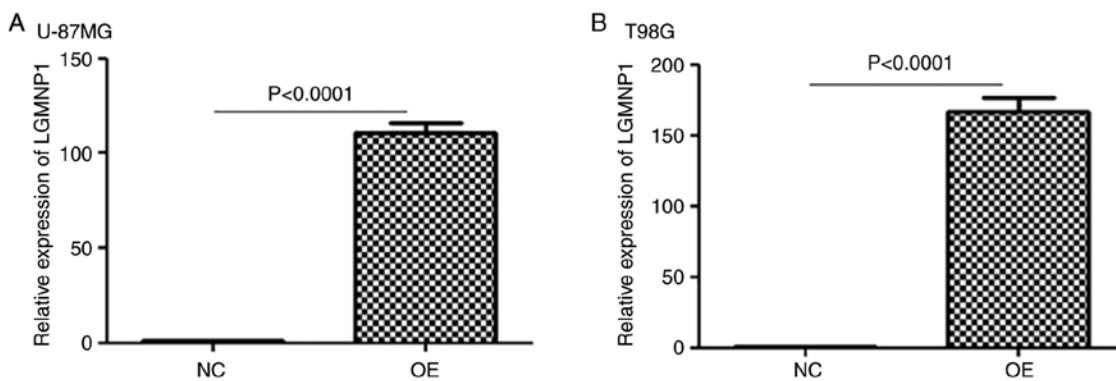


Figure 2. Overexpression of LGMNP1 in glioma cells. (A) Relative LGMNP1 mRNA levels in U87-MG cells. (B) Relative LGMNP1 mRNA levels in T98G cells. LGMNP1, legumain pseudogene 1.

room temperature for 1 h. The membranes were then incubated with the following antibodies at a 1:500 dilution at 4°C overnight: caspase-3 antibody (cat. no. 9662), caspase-7 antibody (cat. no. 12827), Bax antibody (cat. no. 14796; all from Cell Signaling Technology, Inc., Danvers, MA, USA), active caspase-3 antibody (cat. no. ab2302; Abcam, Cambridge, UK) and β -actin antibody (cat. no. 4970; Cell Signaling Technology, Inc.). The membranes were washed with TBS-T, then incubated with horseradish peroxidase-conjugated anti-rabbit (cat. no. R2655) or anti-mouse antibody (cat. no. M8270; both from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a 1:10,000 dilution at room temperature for 2 h. Detection was performed using western blot detection reagents (Odyssey; LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis. Values are expressed as the mean \pm standard error of the mean. One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed for comparison of multiple groups. All statistical analyses were performed using SPSS for Windows v. 17.0 (SPSS, Inc., Chicago, IL, USA). A two-tailed $P < 0.05$ was considered to indicate a statistically significant difference.

Results

LGMNP1 is significantly upregulated in response to radiotherapy. To determine the potential role of LGMNP1

in GBM radiotherapy resistance, RT-qPCR was employed to detect the relative expression of LGMNP1 in two glioma cell lines after exposure to a radiation dose of 6 Gy compared with that in the control group (0 Gy) (Fig. 1A and B). The result indicated that in each of the two cell lines, the relative expression of LGMNP1 in the experimental group was significantly higher than that in the control group ($P < 0.0001$). It was, therefore, indicated that the expression of LGMNP1 is associated with radiotherapy.

Upregulation of LGMNP1 enhances the colony formation ability after radiotherapy. To verify the function of LGMNP1 in radiotherapy resistance of GBM, U87-MG and T98G cells with stable overexpression of LGMNP1 were established using the lentiviral vector-mediated method. As is presented in Fig. 2A and B, LGMNP1 was effectively upregulated in LGMNP1-OE cells compared with that in the NC cells. The clonogenic assay indicated that the colony formation ability of LGMNP1-OE cells after radiotherapy was greater than that of the NC cells (Fig. 3). These results suggested that overexpression of LGMNP1 contributes to radiation resistance.

Overexpression of LGMNP1 confers radioresistance by enhancing the ability of DNA damage protection and reduction of apoptosis. One of the mechanisms by which radiotherapy kills tumor cells is the induction of DNA double-strand breaks. In order to determine whether overexpression of

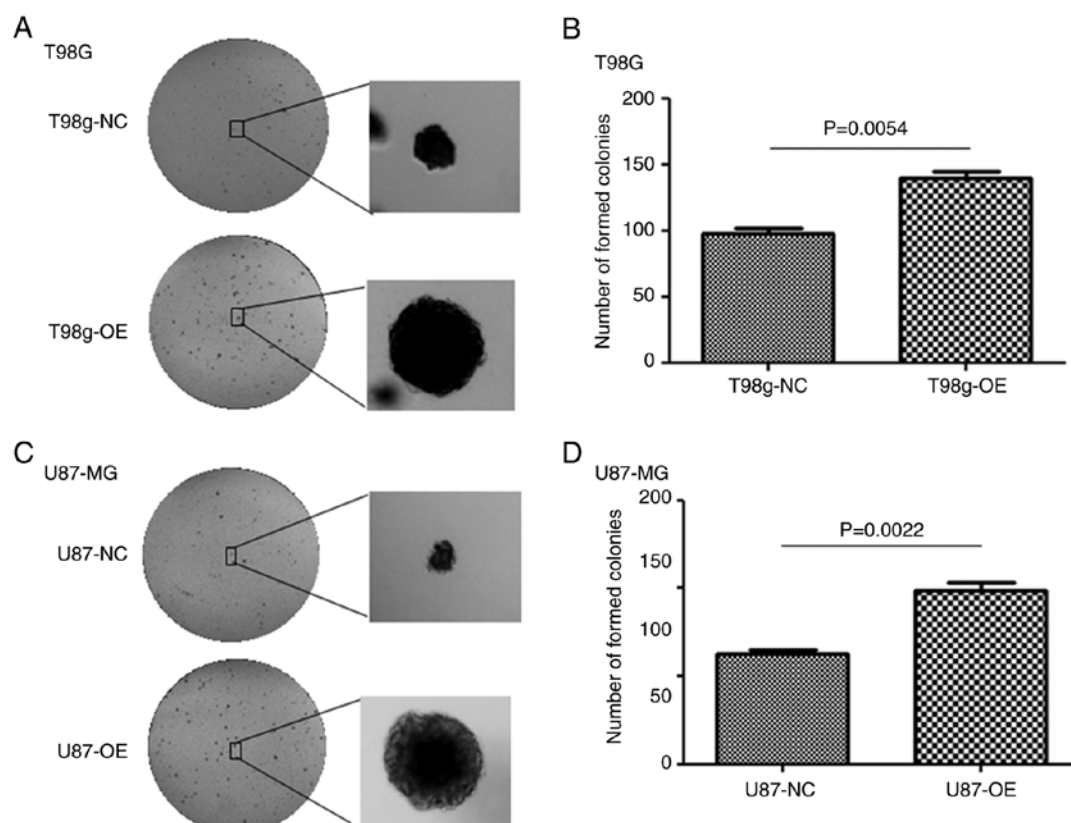


Figure 3. Overexpression of LGMNP1 in glioma cells increases the colony formation ability after radiotherapy. (A and B) Size and number of colonies formed by LGMNP1 overexpressing or native control T98G cells treated with 6 Gy. (C and D) Size and number of colonies formed by LGMNP1 overexpressing or native control U87-MG cells treated with 6 Gy. LGMNP1, legumain pseudogene 1.

LGMNP1 improves the capacity for DNA damage protection after exposure to radiation damage, the cell lines were subjected to the comet assay. The results indicated that the percentage of tail DNA was higher in the cells exposed to 6 Gy of radiation compared with that of cells that received 0 Gy (Fig. 4). Furthermore, the percentage of tail DNA in the LGMNP1-OE group was less than that in the NC group, in the T98G cell line (Fig. 4A and B) and also in the U87-MG cell line (Fig. 4C and D). These results suggested that overexpression of LGMNP1 improves the ability of glioma cells to perform DNA damage protection. To detect apoptosis in glioma cell lines, flow cytometric analysis was employed, demonstrating a lower ratio of apoptosis in LGMNP1-OE vs. NC glioma cells after radiotherapy (Fig. 5). For further verification, the levels of apoptotic proteins were assessed by western blot analysis. The results revealed that the levels of apoptotic proteins, including caspase-3, active caspase-3, caspase-7 and Bax in LGMNP1-OE glioma cells treated with radiotherapy were decreased compared with those in the NC group, and almost the same results were obtained with the two cell lines (Fig. 6). Overall, these results indicated that overexpression of LGMNP1 in GBM cell lines enhances the capacity for DNA damage protection and reduces apoptosis following radiation-induced damage.

Discussion

With the development of surgical techniques and the routine use of radiotherapy and chemotherapy, the two-year survival of

GBM patients has increased from 7% among cases diagnosed between 1993 and 1995 to 17% in cases diagnosed between 2005 and 2007 (3). In recent years, further treatments have been implemented for the treatment of GBM, among which immunotherapy, chimeric antigen receptor (CAR) T-cell therapy, tumor vaccines, viral therapy and tumor-treating fields (TTF) therapy have provided certain benefits. A patient with recurrent multifocal glioblastoma receiving CAR-engineered T cells targeting the tumor-associated antigen interleukin-13 receptor $\alpha 2$ exhibited regression of all intracranial and spinal tumors (13). Tetanus toxoid pre-conditioning may improve the migration of the dendritic cell vaccine and suppress tumor growth in mice and glioblastoma patients (14). Oncolytic viruses are the most extensively studied type of virus in glioma treatment and have been developed for brain cancer treatment. They were demonstrated to be safe and such therapies may also direct long-lasting immune responses toward the tumor while reducing early antiviral reactions (15). TTF therapy has been evaluated in randomized phase 3 trials in GBM and was demonstrated to prolong progression-free survival and overall survival when administered together with standard maintenance temozolomide chemotherapy in patients with newly diagnosed GBM (16,17). However, this is far from enough, all of the aforementioned methods are only effective in a limited number of patients. Therefore, traditional radiotherapy still remains crucial for the treatment of GBM. For newly diagnosed GBM, accurate, timely and efficient radiotherapy is required and for recurrent GBM, radiation is also recommended if the cancer is still susceptible (18-20). The resistance of GBM to

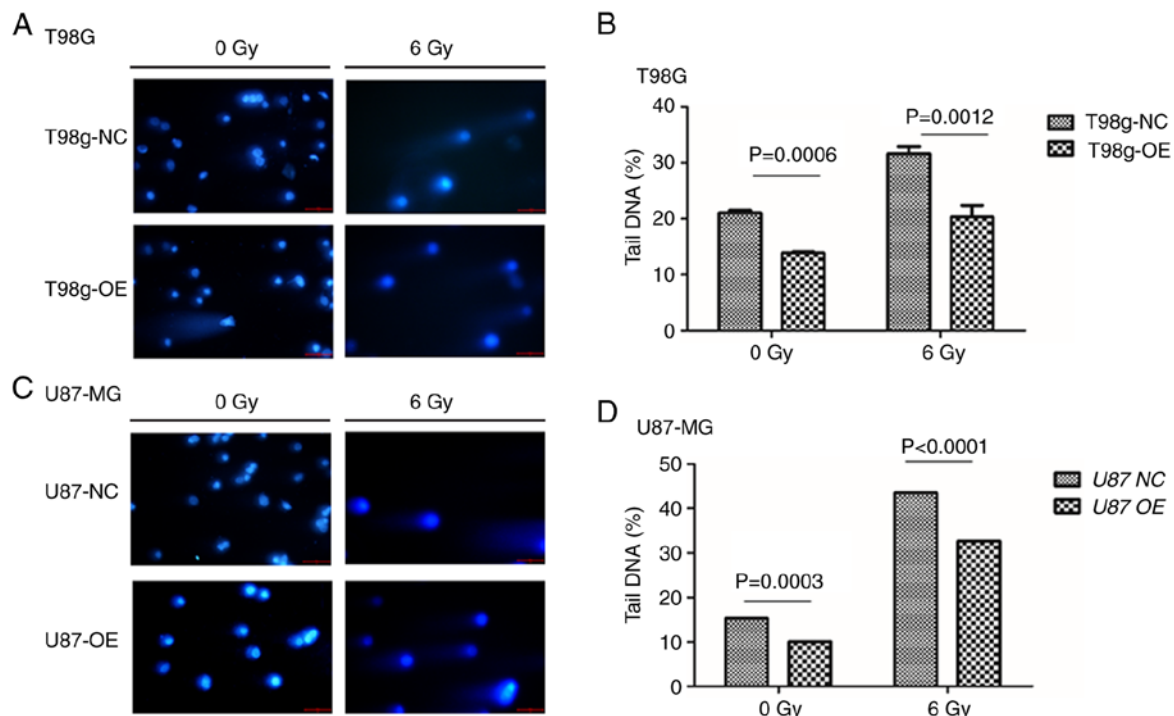


Figure 4. Overexpression of LGMNP1 in glioma cells enhances DNA damage protection processes after radiotherapy. (A) Fluorescent comet tails of LGMNP1-OE or NC T98G cells treated with 6 or 0 Gy. (B) Tail DNA (%) of LGMNP1-OE or NC T98G cells treated with 6 or 0 Gy. (C) Fluorescent comet tails of LGMNP1-OE or NC U87-MG cells treated with 6 or 0 Gy. (D) Tail DNA (%) of LGMNP1-OE or NC U87-MG cells treated with 6 or 0 Gy. LGMNP1-OE, legumain pseudogene 1 overexpression; NC, negative control.

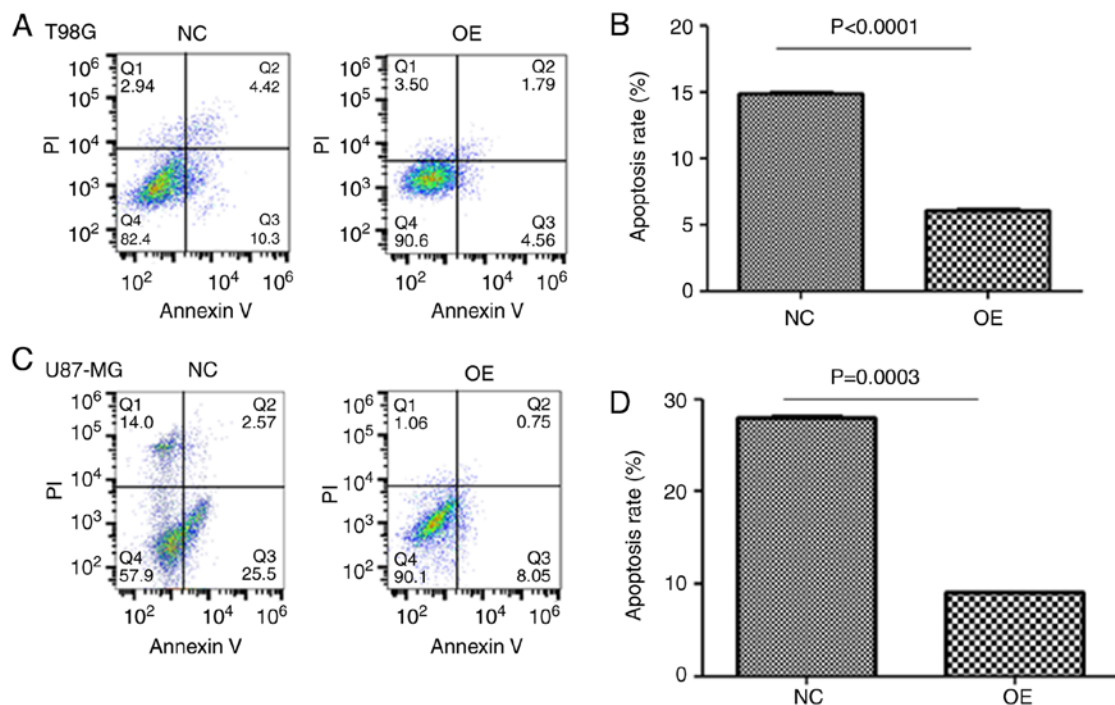


Figure 5. Overexpression of LGMNP1 in glioblastoma multiforme cells reduces the apoptotic cell population after radiotherapy. (A and B) Apoptosis rate of LGMNP1-OE or NC T98G cells after radiotherapy. (C and D) Apoptosis rate of LGMNP1-OE or NC U87-MG cells after radiotherapy. LGMNP1, legumain pseudogene 1.

radiation therapy is a common problem, and the underlying mechanisms remain to be fully elucidated. For tumor cells, abnormal activation of the DNA damage repair pathway is not only the root cause of tumorigenesis, but also an important

mechanism for its resistance to radiotherapy. p53, as a widely known tumor suppressor gene interacts with Rad51 promoter and Rad51 protein to downregulate Rad51, thereby inhibiting homologous recombination and DNA repair (21). After

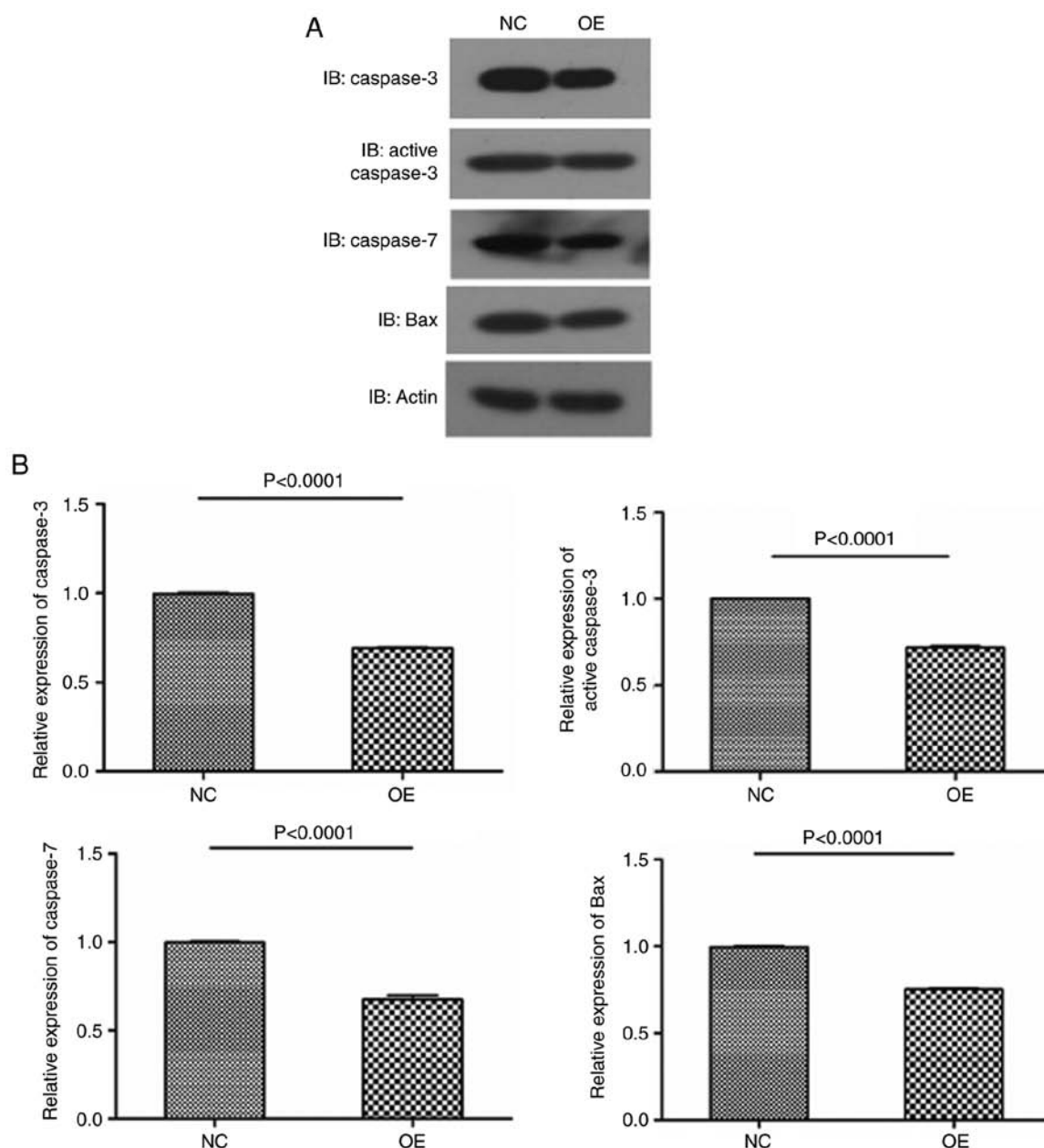


Figure 6. Overexpression of LGMNP1 in glioblastoma multiforme cells decreases apoptotic proteins after radiotherapy. (A) Protein bands of caspase-3, active caspase-3, caspase-7, Bax and β -actin from LGMNP1-OE or NC T98G cells after radiotherapy. (B) Relative expression of caspase-3, active caspase-3, caspase-7 and Bax in LGMNP1-OE or NC T98G cells after radiotherapy.

radiation, an accelerated senescence response was observed in p53 wild-type GBM cells (22). In addition, it is known that glioma stem cells expressing CD133 as a biomarker contribute to radioresistance of glioma through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity (23). Beyond that, vascular endothelial growth factor (VEGF) has been thoroughly studied, since it is one of the angiogenic growth factors that is highly expressed in GBM. A tumor blood flow study indicated that the blood volume increased following radiotherapy, which may be associated with the expression of VEGF (24). Furthermore, VEGF binds to the vascular endothelial growth factor receptor 1 and activates a signal transduction cascade, neutralizing antibodies to the receptor inhibited angiogenesis and enhanced the radiation-induced response, and a greater additive effect

was achieved in GBM when VEGF blocking antibody was combined with radiation (25,26). Advanced research on the epidermal growth factor receptor (EGFR) using *in vitro* and *in vivo* experiments indicated that high levels of EGFR or EGFRvIII were able to facilitate DNA double-strand break repair, which was mediated by the AKT pathway (26-28). The occurrence of tumor radiotherapy resistance is thought not to be a single signaling process. Studies have revealed that blockade of transforming growth factor β signaling enhanced the response of glioblastoma patients to radiation therapy and prolonged their survival (29). Furthermore, combined inhibition of poly(ADP-ribose) polymerase and heat shock protein 90 enhanced the radiosensitivity of human glioma cells (30). The mechanisms require to be elucidated in further studies.

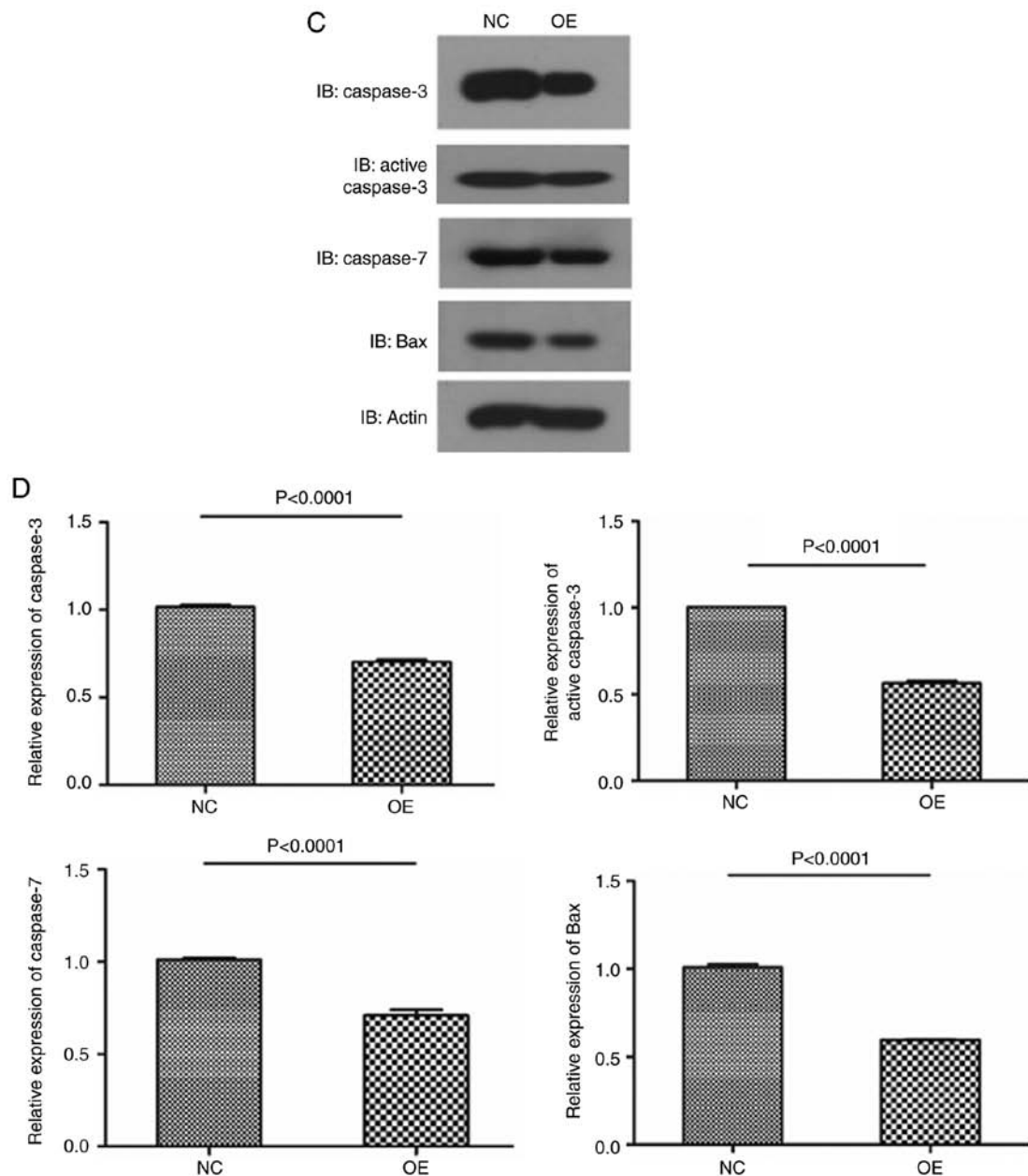


Figure 6. Continued. (C) Protein bands of caspase-3, active caspase-3, caspase-7, Bax and β -actin from LGMNP1-OE or NC U87-MG cells after radiotherapy. (D) Relative expression of caspase-3, active caspase-3, caspase-7 and Bax in LGMNP1-OE or NC U87-MG cells after radiotherapy. LGMNP1-OE, legumain pseudogene 1 overexpression; NC, negative control.

LGMN is known for its cysteine endopeptidase activity in lysosomes, where it contributes to antigen processing for class II major histocompatibility complex presentation. However, it also occurs extracellularly and even translocates to the cytosol and the nucleus. LGMN has also been reported to be associated with the development of a variety of tumor types, including breast cancer, gastric carcinoma, ovarian and colorectal cancer, and it may even serve as a biomarker for such tumors (31-34). Furthermore, LGMN was indicated to promote the proliferation and invasiveness of prostate cancer cells via the PI3K/AKT signaling pathway (35). In addition, knockdown of LGMN was reported to suppress cervical cancer cell migration and invasion (36). Pseudogenes are not truly non-functional genes, although they do not encode proteins, but non-coding RNAs formed by transcription have

certain functions. It has been indicated that abnormal expression of pseudogenes may have vital roles in tumors (37). For instance, phosphatase and tensin homolog (PTEN) is under the regulatory control of PTEN pseudogene-expressed non-coding RNA-PTENpg1, which encodes antisense RNA (asRNA) that regulates PTEN transcription by balancing the two PTENpg1 asRNA isoforms, α and β (38). It is possible that LGMNP1 regulates LGMN expression in a similar manner to enhance the DNA repair capacity and produce radiotherapy resistance. According to the results of the present study, LGMNP1 was upregulated once the glioma cells were exposed to radiotherapy, and when LGMNP1 was ectopically overexpressed, the glioma cells were more resistant to radiotherapy. The comet assay and the apoptosis detection assays indicated less amount of DNA double-strand

breaks and the amounts of apoptotic cells and proteins were decreased. In summary, upregulation of LGMNP1 was indicated to enhance the radioresistance of glioma and targeting LGMNP1 may be a novel strategy to increase sensitivity to radiotherapy, however the mechanism still requires further exploration.

In conclusion, LGMNP1 was upregulated in GBM cell lines after administration of ionizing radiation. LGMNP1 conferred radiotherapy resistance by increasing the capacity for DNA damage protection and reducing apoptosis in glioma cells *in vitro*. The therapy targeting LGMNP1 may be a promising method to reverse radioresistance of GBM.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

LR and YL designed and supervised the research, YQ was also involved in the conception of the study. HX and BC took part in the fund raising, experimental design, data acquisition, manuscript writing, performed the majority of the experiments and drafted the manuscript. ZW and JX helped with the design and performance of the experiments of western blotting and lentiviral vector-mediated gene overexpression. CL helped with the colony formation and comet assays. YL and YQ contributed to the revising of the manuscript. All authors were involved in the conception of the study, 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

Not applicable

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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