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High expression of immunity-related GTPase family M protein in glioma promotes cell proliferation and autophagy protein expression

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ABSTRACT

Glioma is the commonest malignant tumor in the central nervous system (CNS), characterized by rapid growth. However, the molecular mechanism underlying the growth remains unclear. Immunity-related GTPase family M protein (IRGM) participates in immune response to pathogen and tumorigenesis. Proliferation and autophagy are two crucial functions contributing to aggressive growth. Therefore, our aims were to probe whether IRGM regulates glioma proliferation and autophagy. In this study, we found that 47 glioma specimens had more IRGM expression than 11 non-cancerous brain tissues with immunohistochemistry. IRGM was also up-regulated in human glioma cell lines U87, U251 and A172 and so on compared with immortalized astrocytes. Importantly, overexpression of IRGM significantly increased the cell colonies formation, cell proliferation and Akt activation (Thr308 and Ser473 sites) than matched control. On another hand, all of IRGM, autophagy marker LC3II and autophagy adaptor p62 gradually increased after starvation 2 and 4 h. Furthermore, western blot and immunofluorescence results showed that knockdown of IRGM inhibited the formation of LC3-II and the expression of p62. Our data uncovered that IRGM acted in glioma proliferation and autophagy, providing a new target with dual roles for the future translation research.

1. Introduction

Glioma is the commonest malignant tumor in the CNS and characterized by aggressive growth, strong invasion and high recurrence rate, which lead to its poor prognosis and short survival time [1]. Glioblastoma multiforme (GBM) is the most malignant type among glioma four grades as WHO grade IV. Although the standard treatment strategies including surgery, chemotherapy, and radiotherapy are widely used, median survival time of patients with malignant glioma is typically 2–6 years and only 12–16 months for patients with GBM [2]. Therefore, it is essential to identify novel effective therapy targets for glioma.

IRGM belongs to a member of a large family of GTPases, and is involved in resistance to the infections of intracellular bacterium by regulating autophagy [3]. Recent studies focus on the association between IRGM and chronic infectious diseases or inflammatory bowel diseases [4,5]. Interestingly, there have been studies shown that IRGM can promote the tumorigenesis of melanoma and hepatocellular carcinoma [6,7]. However, whether IRGM is responsible for the rapid growth of glioma and the related mechanisms are unclear.

Phosphatidylinositol 3-kinase (PI3K) activates protein kinase B (Akt), a downstream protein of PI3K through phosphorylating amino

Abbreviations: Akt, protein kinase B; CCK-8, cell counting kit-8; CNS, central nervous system; DAB, diaminobezidin; DAPI, 4'6-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle medium; GBM, glioblastoma multiforme; IRGM, immunity-related GTPase family M protein; LC3, microtubule-associated protein 1 light chain 3; OD, optical density; PI3K, phosphatidylinositol 3-kinase

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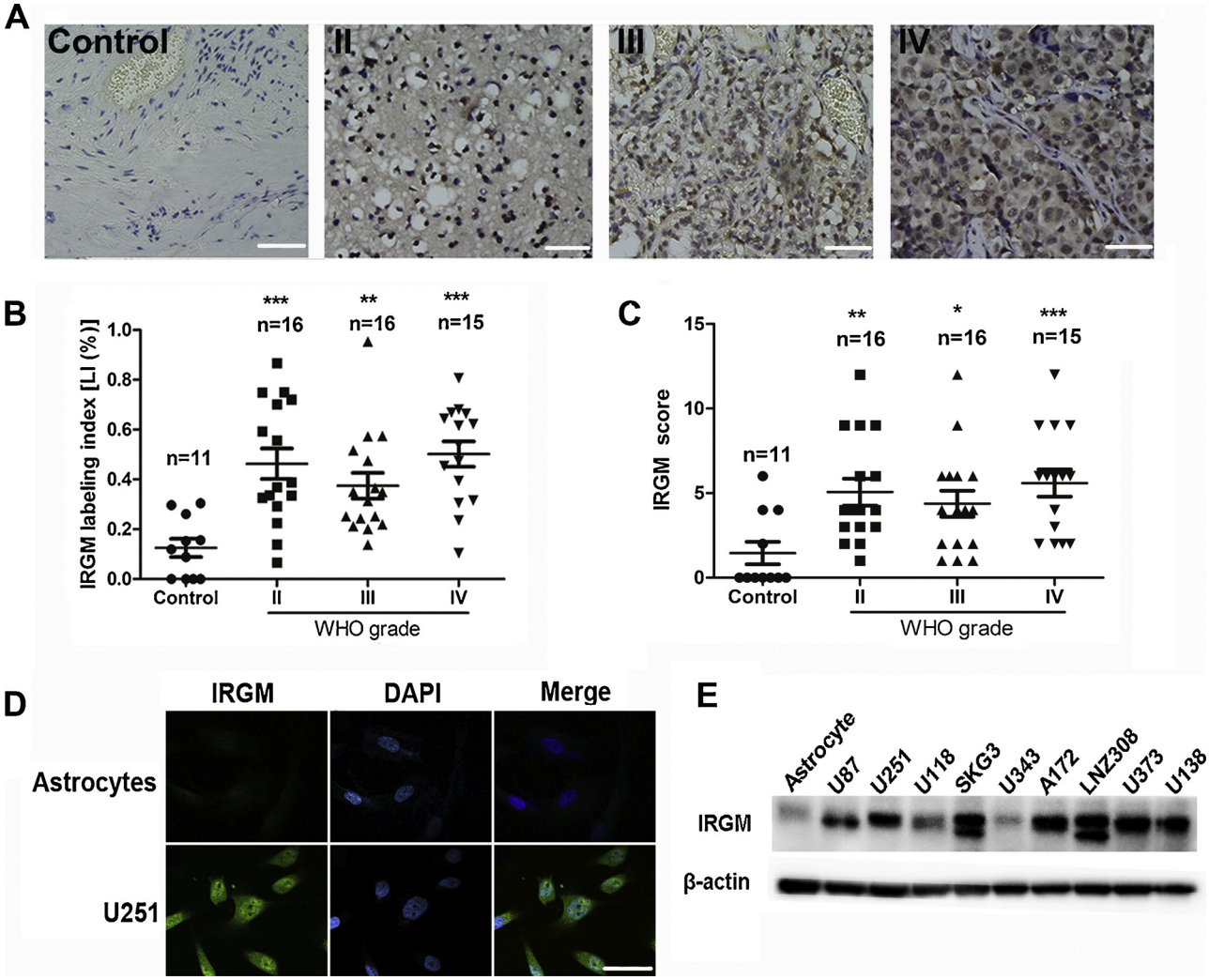

Fig. 1. IRGM was highly expressed in human glioma tissue and cell line. (A) Nontumorous brain tissues (Control), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma (grade IV) specimens were stained with immunohistochemistry to detect IRGM expression. The positive cell rate (B) and total staining score of IRGM (C) in glioma tissues were counted and analyzed. (D) Immunofluorescence analysis was performed in U251 to test IRGM protein level. Astrocytes were used as control. DAPI (blue) was used to stain nuclei. IRGM (green) was stained with FITC-anti-human-IRGM. (E) The expression of IRGM and β-actin in glioma and astrocyte cell lines was detected with western blot. β-actin was referred as control. Experiments were performed in triplicate and representative results were shown. Scale bars represent 50 μm; Data were calculated as Mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

acid residues such as Thr308 and Ser473. The PI3K/Akt pathway is one of the most significant pathways during tumorigenesis and cell proliferation [8,9]. In addition, human glioma displays activation or overexpression of PI3K and Akt [10], but it is unknown that whether IRGM regulates glioma cell proliferation through PI3K/Akt signaling.

Besides high speed of proliferation, constant autophagy is absolutely indispensable for the crazy growth of tumor [11,12]. Autophagy refers to a cellular degradation process that involves identifying intracellular components and transferring them to the lysosome for degrading and recycling [13]. Autophagy occurs at a low basal level, but it can be induced by starvation and other stress conditions [13,14]. Microtubule-associated protein 1 light chain 3 (LC3) has two forms: LC3I and LC3II, and LC3II contributes to the elongation of autophagosomes. LC3II is regarded as the most reliable marker for quantification of the autophagy level due to the conversion of LC3I to LC3II during autophagy. The autophagy adaptor p62 – also termed sequestasome 1 (SQSTM1) targets ubiquitinated substrats to autophagosomes via its interaction with LC3B [15]. On one hand, autophagy supplies renewable nutrition to cope with energy need through degradation; on another hand, autophagy cleans up protein misfolding to reduce stress occurrence. Therefore, it is crucial to find out the key protein regulating autophgy in glioma for potential targets [16].

In this study, we found that IRGM was up-regulated in human glioma tissues and cell lines. Overexpression of IRGM significantly promoted the cell proliferation and Akt activation. Under starvation condition, more IRGM expression was induced, and promoted the formation of LC3-II and the expression of p62. Our study confirmed the role of IRGM in proliferation and autophagy of glioma, suggesting that IRGM inhibitors might be novel anti-tumor agents.

2. Material and methods

2.1. Patients and samples

47 glioma specimens were collected from 2014 to 2016. 11 non-cancerous brain tissues were collected from patients with trauma, epilepsy and vascular malformation after surgery. Surgeries were performed before carrying out chemotherapy or radiation. After tumor resection, all samples were immediately snapped frozen in liquid nitrogen and stored in -80 ℃. For histological examination, the specimens

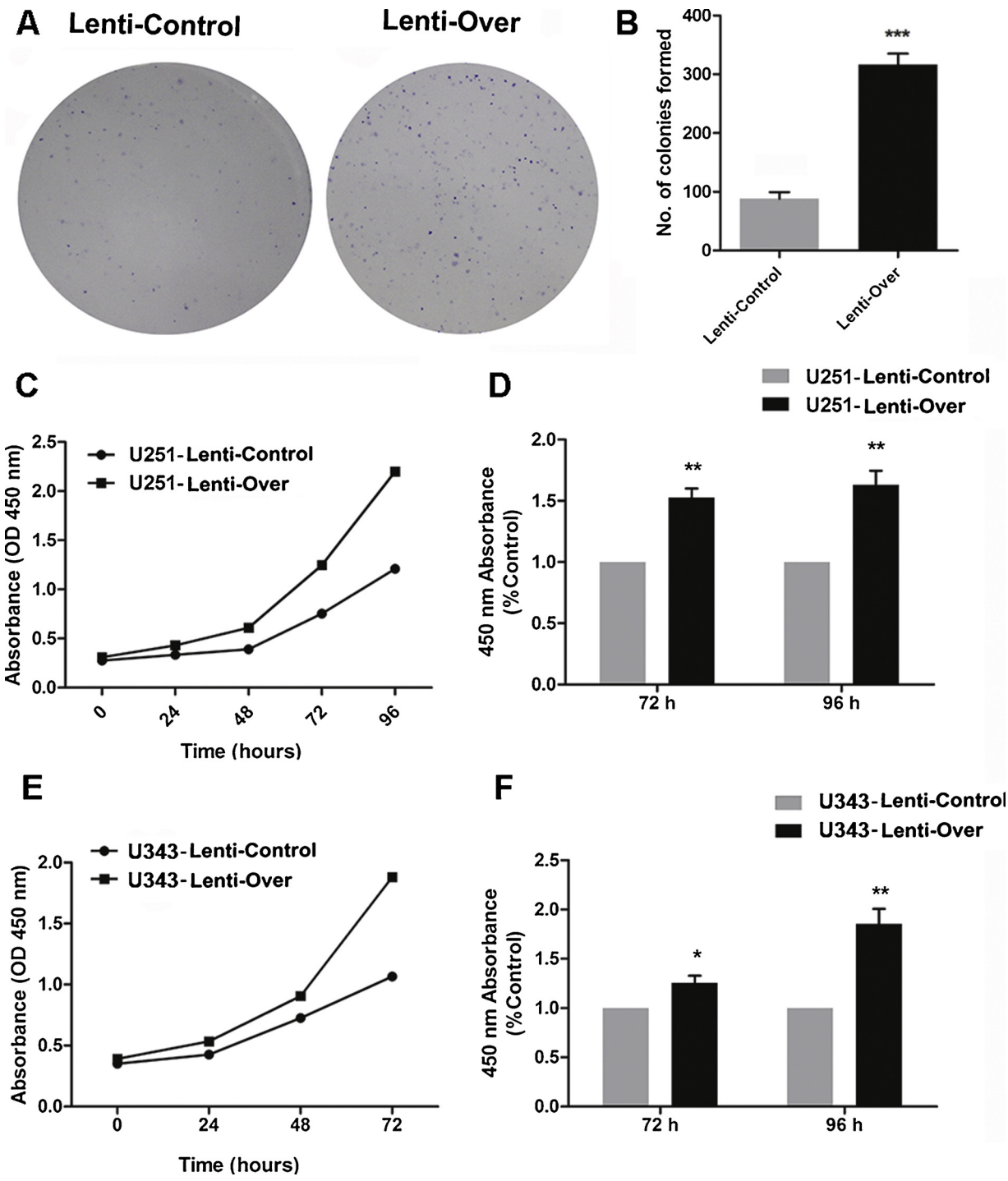

Fig. 2. Overexpression of IRGM enhanced proliferation ability of glioma cells. (A and B) U343 cell line stably overexpressing IRGM was established with lentivirus vector transfection. Colony formation assays were performed using Lenti-Control and Lenti-Over cells (IRGM overexpression shRNA Lentivirus tranfection). Representative images of colony formation assays and statistical result were shown. (C and E) Cell viability of U251 and U343 cell lines overexpressing IRGM were monitored by CCK-8 assays. Representative results were shown. (D and F) OD values were calcultated at 72 h and 96 h, and data are presented as Mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

were fixed in 10% buffered formalin and embedded in paraffin for sectioning. Clinical information of these specimens was described in Supplementary Table. The tumor specimens were re-evaluated according to the 2016 WHO classification system by two experienced pathologists. The tumor types and grades were distributed as follows: WHO grade II diffuse astrocytoma (n = 16), WHO grade III anaplastic astrocytoma (n = 16), and WHO grade IV GBM (n = 15). The study was performed after obtaining informed consent from all patients and was approved by the Ethics Committee of the Shenzhen Second People's Hospital.

2.2. Cell lines and culture

Astrocyte and human glioma cell lines including U87, U251, U118, SKG3, U343, A172, LNZ308, U373 and U138 were obtained from the Institute of Biochemistry and Cell Biology (Shanghai Institutes for

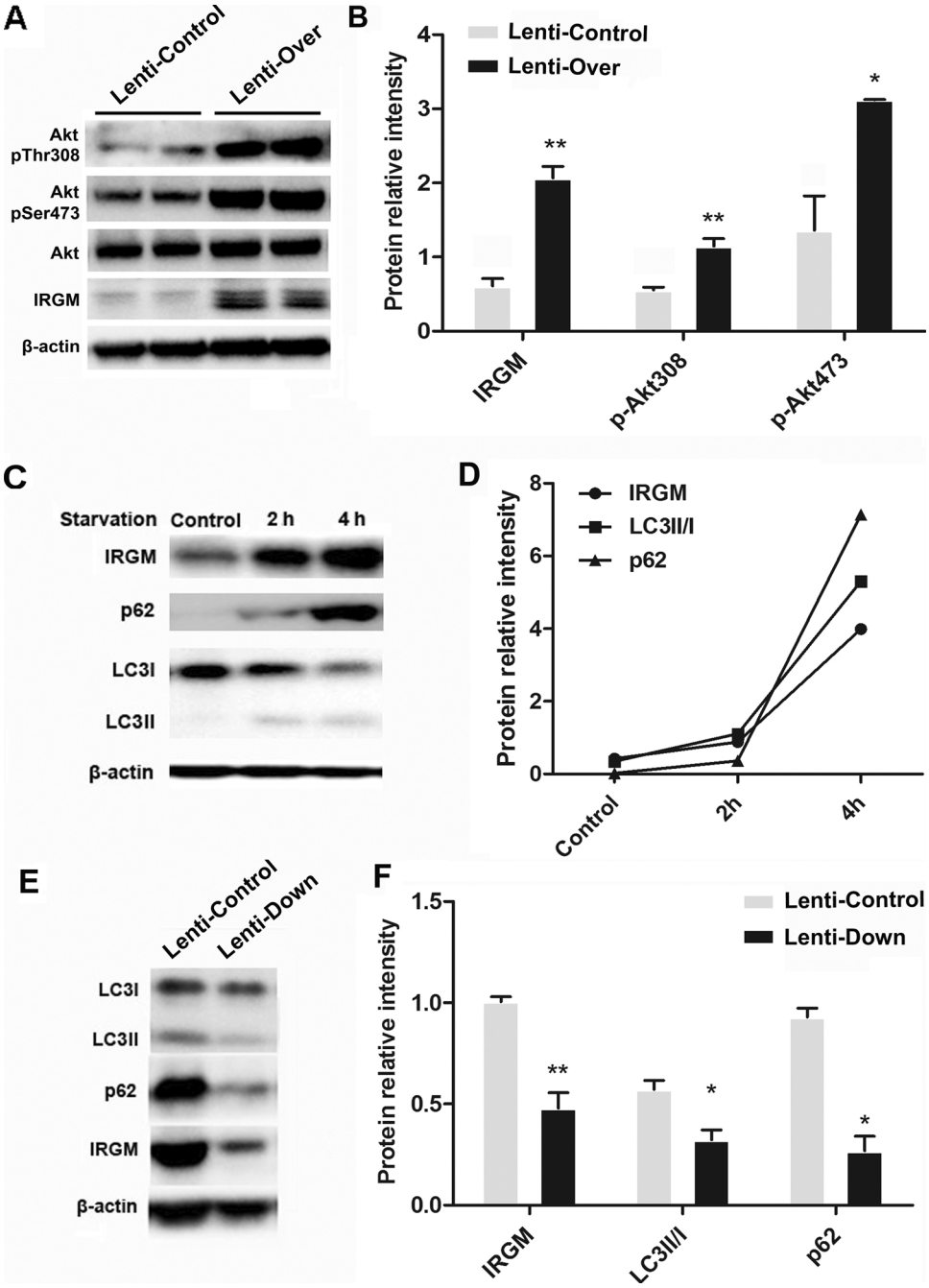

Fig. 3. IRGM activated Akt by promoting phosphorylation of Thr308 and Ser473 amino acid residues. (A) Representative western blotting results for IRGM, p-Akt (Thr308), p-Akt (Ser473), Akt protein expression. (B) Statistical results of protein expression ratios of IRGM to β-actin, p-Akt (Thr308) and p-Akt (Ser473) to Akt in U343 cell lines overexpressing IRGM were calculated. (C and D) U343 cells were cultured under starvation condition for 2 and 4 h, then IRGM, LC3I/II and p62 were analyzed with western blot. Change trend of expression also was shown as 3D. (E and F) A172 cell line transfected IRGM knockdown lentivirus vector (shown as Lenti-Down) was used to explore LC3II and p62 expression. Protein relative expression of IRGM and p62 related to β-actin, LC3II to LC3I was calculated and data were presented as Mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$.

Biological Sciences, Chinese Academy of Sciences, Shanghai, China). Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Life Technologies, Carlsbad, CA, USA), and were stored in a humidified incubator at 37 ℃ containing 5% CO_2. Cell starvation was created by using serum-deprived low-glucose conditioned medium.

2.3. CCK-8 cell proliferation assay

Cells were seeded in 96-well plates at a density of 1000 cells per well. CCK-8 (Beyotime Technology, Shanghai, China) was added to each well and incubated for 2 h at 37 ℃. The optical density (OD) was measured at the wavelength of 450 nm using a V Max kinetic microplate reader at different time points.

2.4. Colony formation assay

Cells were seeded in 60 mm culture dishes at a density of 1000 cells per dish. The colonies were stained with crystal violet after 1 week, then the colonies size and amount were observed.

2.5. Western blotting

Whole cell lysates were extracted with proteinase inhibitor cocktail and phosphatase inhibitor cocktail (Roche, Alameda, CA, USA). The protein concentration was determined by the bicinchoninic acid (BCA, CWBIO) assay. Protein was electrophoretically separated in 10% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Danvers, MA, USA). The membranes were blocked with 5% skimmed milk in Tris Buffered Saline Tween, then incubated with primary antibodies at 4℃ overnight. The primary antibodies used here included the

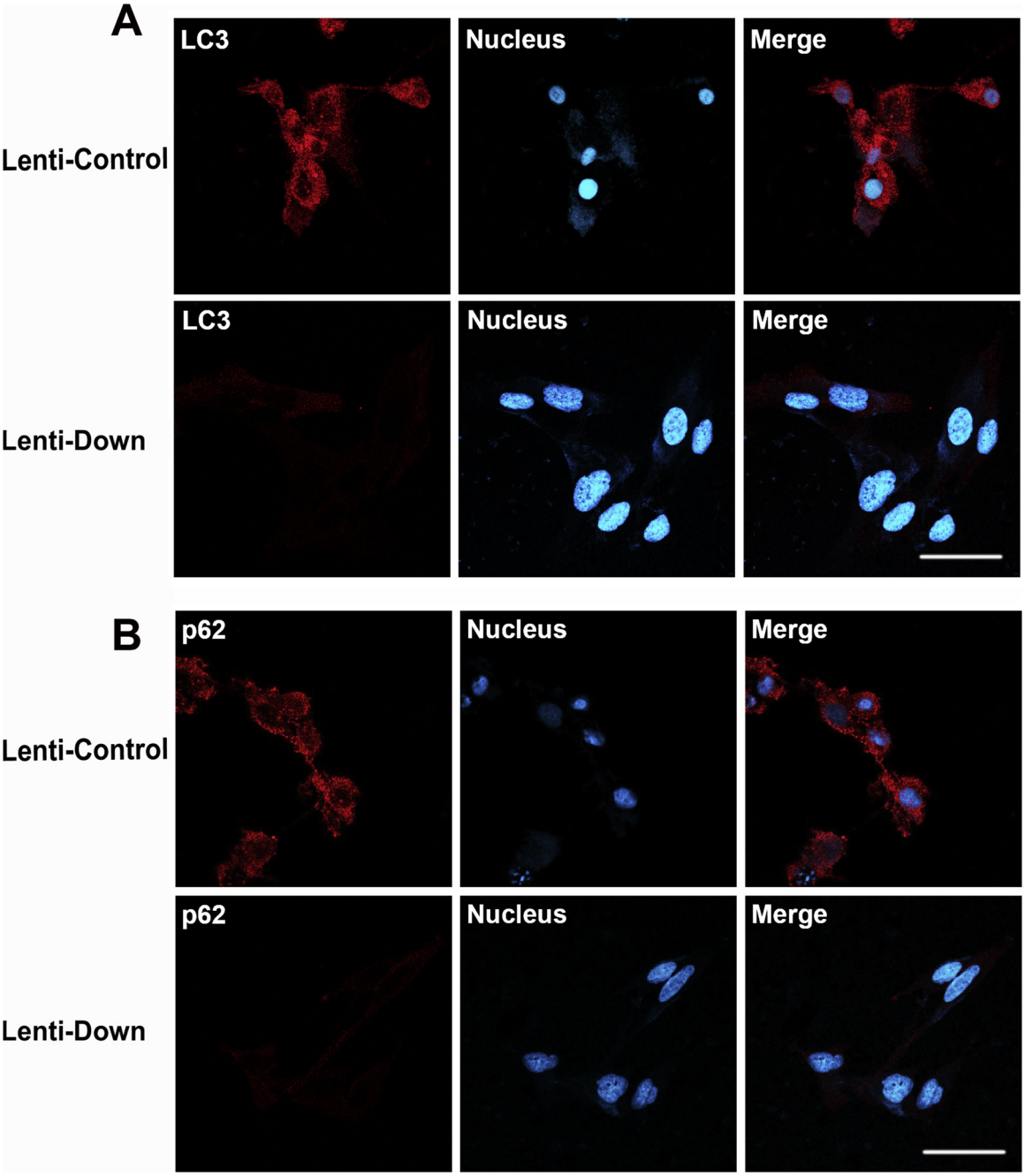

Fig. 4. Immunofluorescence results confirmed the effect of IRGM on LC3 and p62 expression. (A and B) LC3 and p62 were stained and shown as red, the nucleus displayed blue. Both autophagy protein reduced in the Lenti-Down group than control. Experiments were performed in triplicate and representative results were shown. Scale bars represent 50 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

following: anti-human β-actin, IRGM, LC3 and p62 antibodies (1:1000, Abcam), anti-human p-Akt (Thr308), p-Akt (Ser473), Akt (1:1000, Cell Signaling Technology, USA). Anti-rabbit HRP-linked antibody and anti-mouse HRP-linked antibody (1:5000, Cell Signaling Technology, USA) were used the next day. Results were observed by chemiluminescence (Thermo Fisher Scientific, Grand Island, NY, USA) and quantified using Quantity One (Bio-Rad, Hercules, CA, USA).

2.6. Immunohistochemistry

Formalin-fixed, paraffin-embedded, sectioned tissues (4 μm thick) were performed with clinical samples. IRGM expression was detected with an anti-human IRGM rabbit polyclonal antibody (1:200, Abcam). After steps of deparaffinization and rehydration, tissue sections were incubated with 3% hydrogen peroxide in methanol for 30 min to block endogeneous peroxidase activity. The primary antibody reaction was carried out at 4 ℃ overnight. Subsequent steps were performed according to the manufacturer's instructions. DAB was used as the substrate to detect antigen-antibody binding. Then the sections were stained by hematoxylin. The degree of immunohistochemical staining was evaluated by two independent pathologists based on the positive cells proportion and intensity of staining.

2.7. Immunohistochemical evaluation

Classification of staining intensity was graded according to the

following criteria: an intensity score from 0 to 3 (I0, I1 - 3), the proportion score of positive cells from 0 to 4 (P0, P1 - 4). Intensity score: negative = 0; weakly positive = 1; positive = 2; strongly positive = 3. Proportion score of positive cells: 0 (no staining); 1 (1–25%, including 25%); 2 (25–50%, including 50%); 3 (50–75%, including 75%); 4 (> 75%). The final score (range 0–12) was calculated as final score = I × P. We defined the score ≥ 6 as high expression while the score < 6 was considered as low expression.

2.8. Immunofluorescence

For immunofluorescence analysis, cell lines growing on glass coverslips were washed with PBS and then fixed with 4% paraformaldehyde for 15 min, and blocked with BSA (Amresco) for 30 min. The cells were incubated with IRGM, LC3 or p62 antibody (1:200, Abcam) at 4 ℃ overnight, then incubated with fluorescence dye-conjugated secondary antibodies (1:1000, Abcam) at 37 ℃ for 1 h. The cells were stained with DAPI, and imaged with a confocal microscopy (OLYMPUS BX50/BXFLA/DP70; Olympus Co., Japan).

2.9. Lentiviral vector transfection

IRGM overexpression or knockdown assays were performed according to the manufacturer's protocols. pHBLV-CMV-MCS-3flag-EF1-ZsGreen-T2 A-PURO vectors of Lenti-Over Lenti-Down (shRNA sequence: GAAAGCAGTGCTGTGTAACAGAAAT) group and Lenti-HK control were purchased from Hanbio Biotechnology (Shanghai, China). Stably infected cells were selected using puromycin (Gibco Life Technologies, Carlsbad, CA, USA). Lentiviruses were harvested 72 h after transfection, centrifuged to remove cell debris and filtered through 0.45 mm membrane (Millipore, Bedford, MA). To avoid multiple genomic integrations of the target gene, a total of 8×10^4 cells seeded in a 12-well plate were infected by 2.4×10^5 infectious units of lentiviruses (multiplicity of infection [MOI] = 3).

2.10. Statistical analysis

Results are expressed as means ± SEM. Difference between 2 groups was analyzed by 2-tailed Student' s t-test, and statistical significance of the correlations between IRGM expression and the clinicopathologic features was analyzed by Pearson χ^2-test using GraphPad Prism 5 software. The level of significance was set at $P < 0.05$.

3. Results

3.1. IRGM was highly expressed in glioma tissues and cell lines

In the present study, we first examined the expression level of IRGM in human glioma and non-cancerous tissues. The results indicated that IRGM was highly expressed in glioma of grade II, III and IV than non-cancerous control brain tissues (Fig.1A). Analysis of immunohistochemical results showed that 47 cases of glioma tissues had higher positive rate and total score of IRGM compared with non-cancerous brain tissues (Fig.1B and C). The correlation between IRGM expression and the pathological features was analyzed and summarized among 47 glioma tissues in Supplementary Table. As shown in Supplementary Table, IRGM was not associated with other clinical pathological features except age. The expression of IRGM in glioma cell lines was detected with immunofluorescence and western blot. Immunofluorescence showed that IRGM was more expressed in glioma cells U251 than immortalized astrocytes (Fig. 1D). The level of IRGM was up-regulated in 8 glioma cell lines (e.g., U87, U251, U118, SKG3, A172, LNZ308, U373 and U138) in contrast with astrocytes (Fig. 1E).

3.2. Overexpression of IRGM enhanced proliferation ability of glioma cells and Akt activation

Next, to explore the function of IRGM in glioma proliferation, we established stable glioma cell lines U343 and U251 overexpressing IRGM with lentivirus vector transfection, then we confirmed that IRGM was more expressed in Lenti-Over group than Lenti-Control with western blot (Fig. 3A and B). Subsequently, U343 cell lines overexpressing IRGM were used to determine colony formation, which reflected the effect of IRGM on glioma cells growth. Both U251 and U343 cell lines overexpressing IRGM were used to analyze cell proliferation and viability. Finally, Lenti-Over group displayed stronger ability of colony formation compared with Lenti-Control (Fig. 2A and B). The OD values of CCK-8 also indicated that Lenti-Over group also remarkably enhanced cell viability in contrast with Lenti-Control cells (Fig. 2C–F). Results above confirmed that high expression of IRGM promotes glioma cells proliferation.

PI3K/Akt pathway is regarded as an important indication of cell proliferation [8]. Akt is activated by PI3K through phosphorylating amino acid residues of Akt. Using western blot assay, data further indicated that Akt activation was strongly induced by enhancing phosphorylation of amimo acid sites Thr308 and Ser473 without influence on the expression of Akt (Fig. 3A and B), suggesting that IRGM may regulate glioma cell proliferation via activating PI3K/AKT signal pathway.

3.3. IRGM knockdown inhibited autophagy protein LC3II and p62 expression

Studies show that IRGM is also involved in regulating cell autophagy [6,7]. Autophagy usually occurs under nutrition deficiency and stress and plays a key role in tumorigenesis [13,17]. In this article, we discussed whether IRGM regulates glioma another important biological function - autophagy. U343 cell line has less IRGM expression under normal nutrition condition. However, IRGM expression was gradually up-regulated after starvation 2 and 4 h, which is accompanied by LC3II and p62 increasing (Fig. 3C and D). We established A172 cell line with stable IRGM knockdown using lentivirus transfection (Fig. 3E and F). Western blot results showed that IRGM Lenti-Knockdown significantly reduced the ratio of LC3II /LC3I than Lenti-Control group (Fig. 3E and F), and IRGM knockdown also reduced the expression level of p62, a protein receptor of autophagy (Fig. 3E and F). In addition, the effect of IRGM on LC3 and p62 expression was determined with immunofluorescence analysis, indicating that IRGM knockdown strongly inhibited LC3 and p62 level (Fig. 4A and B). These results demonstrate that IRGM obviously promotes the expression of autophagy protein, suggesting a new mechanism by which IRGM regulates autophagy.

4. Discussion

In the present study, we probed the expression level of IRGM and the effect on proliferation and autophagy of glioma cells using samples from glioma patients, glioma cell lines and molecular analysis. Previously, IRGM was identified due to elimiating intracellular mycobacteria by inducing autophagy [18]. After that, the role of IRGM in Crohn's disease and other inflammation-related diseases during cell autophagy was studied [19,20]. However, whether IRGM plays a role in glioma proliferation and autophagy has not been studied.

Based on research above, we investigated the role of IRGM in glioma growth and related mechanisms. In our report, we observed that IRGM could promote glioma cells proliferation (Fig. 2). Further, more activation of Akt was detected in cells with IRGM overexpression, and we confirmed that IRGM significantly promoted phosphorylation of amimo acid sites Thr308 and Ser473 (Fig. 3A and B). Combined with previous study that PI3K/Akt regulates tumor growth [8,9], we speculated IRGM should promote glioma cells growth though PI3K/Akt signal pathway.

The interaction between IRGM and PI3K will be explored in our following study.

Next, we wanted to check whether IRGM could also regulate autophagy of glioma cell. Autophagy is a highly conserved cellular lysosomal degradation pathway in which proteins and organelles are redigested to maintain nutrition supply [21]. Autophagy occurs at a low basal level, but it can be induced by starvation and other stress conditions [13]. Due to rapid growth, it is rational for glioma to induce autophagy. This study displayed that an increase of antophagy protein in glioma cells under starvation condition (Fig. 3C and D). In line with previous report, we found that IRGM promoted the formation of autophagy marker LC3II and the expression of autophagy adaptor p62 (Figs. 3E and F; Fig. 4), indirectly demonstrating that IRGM was a key molecule for glioma growth under nutrition deficiency. In our next study, we will research the direct effect of IRGM on other molecules in autophagy flow under nutrition stress.

Taken together, we propose that IRGM play a crucial role in glioma growth, which is not reported before. Our findings suggest that IRGM was more highly expressed in glioma than control brain tissues or cell lines. Overexpression of IRGM obviously enhanced the capacity of proliferation and the activation of Akt. Furthermore, starvation induced autophagy protein and IRGM up-regulation, in return IRGM promoted autophagy protein LC3II and p62 expression, suggesting IRGM could be a potential target to treatment glioma.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prp.2018.10.004.

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