

# LRIG1 Modulated Radioresistance of Glioma via Regulating CTLA-4/AKT Signaling Pathway

Shiqi Cheng

The second affiliated hospital of Nanchang University <https://orcid.org/0000-0003-1984-4169>

Xiangqun Huang

Nanchang University Second Affiliated Hospital

Raorao Yuan

The Second Affiliated Hospital of Nanchang University

Yan Zhang (✉ [doctorzhangyan@outlook.com](mailto:doctorzhangyan@outlook.com))

The second affiliated hospital of Nanchang University

---

## Research

**Keywords:** LRIG1, glioma, radioresistance, CTLA4, AKT

**Posted Date:** December 21st, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-131093/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

**Background:** Radioresistance has a great impact on prognosis of glioma patients. However, the potential mechanism underlying the radioresistance of glioma cells remains largely unknown.

**Methods:** LRIG1 overexpression model was firstly established by using Flag-LRIG1 plasmid. The expression of LRIG1, CTLA-4 proteins were detected by western blot and IHC in cells and human tissue. Real-time PCR was used for deterring mRNA expression. Cell viability and apoptosis were detected using CCK-8 and Annexin-V/propidium iodide (PI), respectively. Co-Immunoprecipitation was used for detecting the combination of LRIG1 and CTLA-4 proteins.

**Results:** LRIG1 was significantly down-regulated in radioresistant glioma cells. Overexpressed LRIG1 could promote the radiosensitivity of glioma cells, meanwhile, inhibit the expression of p-AKT and CTLA-4 protein in radioresistant glioma cells. Furthermore, LRIG1 combined with CTLA-4 and promoted CTLA-4 degradation. In human glioma tissue, LRIG1 was down-regulated, while CTLA-4 was highly expressed in glioma tissue. Finally, correlation analysis showed that the expression of LRIG1 was negatively correlated with expression of CTLA-4 and radioresistance of glioma patients.

**Conclusion:** Our findings demonstrated that LRIG1 facilitates radioresistance glioma cells by regulating CTLA4 /AKT signaling pathway.

## 1 Introduction

Glioma is considered to be one of the most commonly-diagnosed intracranial tumor of the central nervous system, representing 81% of malignant brain tumors in adult <sup>[1, 2]</sup>. Some subtypes of glioma, e.g. glioblastoma, cause significant mortality <sup>[3]</sup>. In recent years, the therapeutic interventions for treating glioma are surgery combined with chemotherapy and/or radiotherapy, which provide benefit to patients due to their different merits <sup>[4]</sup>. However, even following proper surgical resection, many tumors show a high resistance to irradiation therapy, further cause highly recurrence rate and poor prognosis for glioma patients <sup>[5]</sup>. Thus, the potential mechanism underlying resistance to irradiation therapy is urgently needed.

Radioresistance of glioma cells has an important effect on prognosis of glioma patients. In numerous clinical studies, improvement of radiosensitivity of tumor cells, e.g., combining PARP inhibitors, using hyperbaric oxygenation, could significantly improve survival outcomes of glioma patients <sup>[6, 7]</sup>. However, due to the heterogeneity of tumor cells, i.e the different self-renewal capacities of each cell population, the radioresistance remains a big challenge in clinical practice <sup>[8]</sup>. So far, multiple factors have been identified responsible for radioresistance of tumor cells, e.g. Wnt.<sup>[9]</sup>,  $\beta$ -Catenin<sup>[10]</sup>, AKT <sup>[11]</sup>, STAT3 <sup>[12]</sup>, however, a detailed, integrative picture of the molecular network has not yet clarified.

Leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1), a type 1 transmembrane protein whose extracellular domain contains 15 leucine-rich repeats (LRRs) and three immunoglobulin (Ig)-like

domains, was firstly cloned by Hedman *et al*/in 2002 [13]. The gene of the LRIG1 localized in chromosome band 3p14.3, and the protein is well-known as a cell transmembrane protein that widely expressed in human cells [14]. Over decades, accumulating evidence has suggested that LRIG1 might act as a tumour suppressor, for its expression usually correlates with better patient survival in several human cancers including prostate, bladder, breast, cervical, colon, cervical, non-small-cell lung cancers and gliomas [15–19]. For molecular mechanism, two studies from independent groups [20, 21] demonstrated that LRIG1 negatively regulates all four members of the ERBB receptor family (ERBB1/EGFR, ERBB2/HER2/Neu, ERBB3/HER3, and ERBB4/HER4) by heterologous expression. LRIG1 dramatically reduce receptor levels by promoting receptor ubiquitylation and lysosomal degradation [22]. In addition to ERBBs, LRIG1 also affected other oncogenic signaling molecules, e.g. mutant EGFR (EGFRvIII) [23], Stat3 [24] and TNF $\alpha$  [25].

Despite the large amount of knowledge on LRIG1 in many tumor tissues, little is known about its role in glioma radioresistance. In fact, few papers have been reported that LRIG1 could improve the radiosensitivity of glioma cells by attenuating EGFR/Akt signaling pathway [26], or its impression could affect by certain microRNAs, e.g. microRNA-590-3p [27], miR-183 [28]. In order to have a better understanding of LRIG1 and its down-stream factors on glioma radioresistance, in the present study, we tried to further investigate the potential role of LRIG1 in glioma radioresistance and its underlying mechanisms, moreover, the interaction between LRIG1 and cytotoxic T lymphocyte antigen 4 (CTLA-4) / AKT pathway in glioma radioresistance will also be clarified. Our study might set a novel sight into pathologic mechanism of glioma and may provide therapeutic strategies in treating this disease.

## 2 Materials And Methods

### 2.1 Cell culture and treatment

Human glioma cell line U251 was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). U251 cells were cultured in a standard humidified incubator (5% CO<sub>2</sub>; 37 °C) with RPMI-1640 (Invitrogen, Carlsbad, CA, USA) culture medium which supplemented with 10% fetal bovine serum (FBS, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA).

For treatment, as previously described [28], briefly, U251 cells were irradiated by a linear accelerator 6-MV X-rays with the dose rate of 4 Gy/min. The procedure was performed daily for 5 months, starting with 1 Gy/fraction and ending with 10 Gy/fraction. The X-ray treated U251 cells were labeled as U251R cells.

### 2.2 Quantitative real time-PCR (qRT-PCR)

Total RNA was isolated from glioma cells with an RNA extraction kit (Takara Biotechnology, Japan). Then, Reverse Transcription Kit (Takara Biotechnology, Japan) was used to obtain cDNA and reverse transcribe RNA. The primers used in this study were synthesized by Yingjun Technology (Shanghai, China) which showed in Table 1. qRT-PCR was conducted using the SYBR Green Realtime PCR Master

Mix (Toyobo, Japan) and performed on ABI 7900 fast Real-time PCR Systems (Applied Biosystems, USA). GAPDH were used as internal controls.

Table 1  
Primers used in the present study

Gene	Primer sequence
LRIG1	forward: 5'-GAAAAGGGACTCTGGTTGGGAT-3'
	reverse: 5'-AGGAAGTCATCGCACACGAA-3'
CTLA-4	forward: 5'-AGGTGACTGAAGTCTGTGCG-3'
	reverse: 5'-CATGAGCTCCACCTTGCAGA-3'
GAPDH	forward: 5'-CCGGGAAACTGTGGCGTGATGG-3'
	reverse: 5'-AGGTGGAGGAGTGGGTGTCGCTGTT-3'

### 2.3 Western blot

Total proteins from human tissue or glioma cells were extracted by using a radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology Co., Ltd., Shanghai, China). The protein samples were separated using 8% SDS-PAGE. After transferred onto PVDF membrane, the primary antibodies were added: anti-LRIG1 (PA5-52860, 1:500, Invitrogen, Carlsbad, CA, USA), anti-Akt (ab235958, 1:1000, Abcam, UK), anti-Akt (phospho S473) (ab81283, 1:1000, Abcam, UK), anti-CTLA-4 (PA5-47547, 1:250, Invitrogen, Carlsbad, CA, USA), anti-Tubulin (ab210797, 1:1000, Abcam, UK). Tubulin served as internal control. After 24 hours of incubation, membrane was incubated along with secondary antibody immunoglobulin G (IgG) at room temperature for 2 hours.

### 2.4 Cell transfection

The LRIG1 was overexpressed or knockdown by using LRIG1 overexpression plasmid (Flag- LRIG1) or Short hairpin RNAs (shRNA) targeting LRIG1 (sh-LRIG1), respectively. The plasmid or empty vectors were purchased from GeneChem Corporation (Shanghai, China). Lipofectamine 3000 (Invitrogen, USA) was used for cell transfection following manufacturer's instructions.

### 2.5 CCK-8 assay

Transfected U251 cells or U251R cells ( $2 \times 10^5$  per well) were seeded into a 96-well plate. CCK-8 Kit (Abcam, UK) and RPMI-1640 were added into each well. After incubation with CCK-8 solution for 2 hours, optical density of cells was measured at 450 nm at different time points by using micro-plate reader (Thermo Fisher, USA).

### 2.6 Flow cytometry assay

Cell apoptosis was detected using Annexin-V-propidium iodide (PI) apoptosis assay. Annexin V-FITC Apoptosis Detection Kit (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instruction. Briefly, different groups of transfected U251R cells were with propidium iodide (PI) at 37°C for 30min, followed by flow cytometry (Beckman Coulter FC500, CA, USA).

## **2.7 Co-Immunoprecipitation**

Co-Immunoprecipitation was performed to detect the combination of LRIG1 and CTLA-4. Briefly, cells were lysed in 500 µl co-IP buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri, USA). Then, 20 µl immobilized protein A/G beads was incubated with cell lysates for 1h at 4°C. Primary antibody or control Ig G were incubated with lysates for 24 hours. Finally, immobilized protein A/G beads were added, then, the proteins were prepared for western blot.

## **2.8 Human glioma tissue collection**

Tissue samples of paired glioma tissues and paracancerous tissues were collected from patients who underwent glioma resection at the second affiliated hospital of Nanchang university. All patients enrolled in this study have given their informed written consents prior to conduct the clinical research related procedure and this study was approved by the second affiliated hospital of Nanchang university Ethical Committee (No. 2019003). All patients have received 5 weeks' radiation therapy. Tissue samples were collected and immediately snap-frozen in liquid nitrogen after surgery (-80°C) for further use.

## **2.9 Immunohistochemistry (IHC)**

The expression of LRIG1 and CTLA-4 proteins was determined in human glioma tissues by IHC. Briefly, paraffin-embedded sections were dewaxed and dehydrated by xylene and ethanol. Then, after incubation with 50 µl of 10% goat serum for 60min, the sections were probed with primary antibodies: anti-LRIG1 (ab197985, 1:1000, Abcam, UK), anti-CTLA-4 (ab227709, 1:1000, Abcam, UK) for 24 hours. Then, peroxidase-labeled secondary antibody (Invitrogen, Carlsbad, CA, USA) were added and incubated for 30min. The sections were developed using diaminobenzidine (DAB).

## **2.10 Statistical analysis**

All cell experiments were repeated triplicate. The data were exhibited as Mean ± SD. SPSS21.0 (IBM Corp. Armonk, NY) software was used for data analysis. Two-tailed Student's t-test, one-way ANOVA, Pearson correlation coefficient were used in this study. P < 0.05 was considered as statistically significant difference.

# **3 Results**

## **3.1 LRIG1 was down-regulated in radioresistant glioma cells**

Firstly, the expression of LRIG1 mRNA was determined in U251 cell or radioresistance U251 cells (U251R) by using qRT-PCR. As shown in Fig. 1A, the expression of LRIG1 mRNA in U251R cells was significantly decreased compared with that in U251 cells ( $P<0.05$ ). Subsequently, LRIG1 protein expression in both groups was detected by western blot. Result demonstrated that in U251R cells, the level of LRIG1 protein was much lower compared with that in U251 cells (Fig. 1B). These data suggested that in both mRNA and protein levels, LRIG1 was down-regulated in radioresistant glioma cells.

### **3.2 Overexpressed LRIG1 increased radiosensitivity of glioma cells**

To explore the potential role of LRIG1 in radioresistant glioma cells, we firstly used the plasmid to overexpress LRIG1 in U251R cells. Results revealed that LRIG1 protein was highly expressed in Flag-LRIG1 group compared with NC group (Fig. 2A), suggesting that the LRIG1 overexpressed system can be used in further studies. Next, the effect of LRIG1 on cell phenotypes were detected by CCK-8 assay and Flow cytometry assay. We found that the expression of LRIG1 significantly inhibited the cell proliferation (Fig. 2B;  $P<0.01$ ), meanwhile, in U251R cells, the apoptosis of cells was promoted by overexpression of LRIG1 proteins (Fig. 2C). To sum up, the above results provide the evidence that overexpressed LRIG1 increased radiosensitivity of glioma cells.

### **3.3 Overexpressed LRIG1 inhibited the expression of p-AKT in U251R cells**

To further validate the mechanisms of LRIG1 in radioresistant glioma cells, the expression of many proteins was determined in different groups by western blot. First, the expression of LRIG1, p-AKT, AKT proteins was detected in U251 cells or U251R cells. As shown in Fig. 3A, in radioresistant glioma cells, the expression of p-AKT was highly expressed, suggesting that p-AKT was activated. Then, the expression of LRIG1, p-AKT, AKT proteins was also measured in either overexpressed LRIG1 group or NC group. Result demonstrated that the activated p-AKT can be inhibited by overexpression of LRIG1 in U251R cells (Fig 3B).

### **3.4 Overexpressed LRIG1 inhibited the expression of CTLA-4 protein in U251R cells**

Subsequently, the effect of overexpression of LRIG1 on CTLA-4 expression was analyzed in U251R cells. We found that overexpression of LRIG1 dramatically suppressed the protein level of CTLA-4 in U251R cells (Fig. 4A). However, in mRNA level, overexpression of LRIG1 was not able to affect the expression of CTLA-4 mRNA (Fig. 4B). These data indicated that LRIG1 only have effect on CTLA-4 post-translational modification, while not change the mRNA level of CTLA-4.

### **3.5 LRIG1 combined with CTLA-4, and promoted CTLA-4 degradation**

Furthermore, the relationship between LRIG1 and CTLA-4 was identified by conducting Co-immunoprecipitation and western blot. As shown in Fig 5A, coimmunoprecipitated proteins were analyzed by western blot with indicated antibodies. Results showed that endogenous LRIG1 protein was immunoprecipitated with anti-CTLA-4, meanwhile, CTLA-4 also combined with LRIG1. Moreover, MG132 was used to inhibit the proteasome in each group. In Fig. 5B, results demonstrated that by blocking

proteasome, overexpression or knockdown of LRIG1 did not have any effect on expression of CTLA-4 protein in U251R cells. These data indicated that LRIG1 combined with CTLA-4, and the same time, promoted CTLA-4 degradation.

### **3.6 LRIG1 was down-regulated, while CTLA-4 was highly expressed in glioma tissue**

Additionally, we had performed experiments on human glioma tissue. The expression of LRIG1 and CTLA-4 protein was detected in human glioma tissue by IHC and western blot. As shown in Fig. 6A, compared with paracancerous tissue, the expression level of LRIG1 was much lower. On the contrary, CTLA-4 was highly expressed in glioma tissue. To better quantify the protein expression, western blot was performed in each group. In tumor tissue, LRIG1 was down-regulated, while CTLA-4 was highly expressed compared with paracancerous tissue (Fig. 6B).

### **3.7 The expression of LRIG1 was negatively correlated with expression of CTLA-4 and radioresistance of glioma patients**

Finally, the correlation analysis was performed to better clarify the relationship between LRIG1 and CTLA-4. In Fig 7A, the correlation analysis between expression of LRIG1 and radioresistance of glioma was performed. It was shown that the expression of LRIG1 was negatively correlated with radioresistance of glioma. However, the expression of CTLA-4 was positively correlated with radioresistance of glioma (Fig. 7B). Furthermore, result in Fig 7C showed that the expression of LRIG1 was negatively correlated with expression of CTLA-4 in glioma patients. These data indicated that LRIG1 may have positive effect on glioma in clinic.

## **4 Discussion**

Gliomas make up the largest proportion of malignant brain tumors with very limited treatment strategies. Radiotherapy seems to be the most effective nonsurgical treatment for gliomas, however, its efficacy is severely suppressed due to the high intrinsic radioresistance of glioma cells [29]. Thus, to explore the potential mechanism underlying gliomas radioresistance is critical to design novel strategies and improve the prognosis of glioma patients. In our study, we firstly found that LRIG1 was down-regulated in radioresistant glioma cells in both mRNA and proteins levels. Due to the fact that expression of LRIG1 can be affected by irradiation, we further established LRIG1 overexpression system in radioresistant glioma cells (U251R) for further studies.

The biological effects of irradiation on cells mainly due to DNA lesions, e.g., disruption of the phosphate DNA backbone [30]. The DNA lesions can be direct caused by interactions between particles or indirect following interactions with reactive oxygen species (ROS) generated by cell water ionization [31]. These breaks can be either repaired or can lead to cell cycle arrest. As such, we can observe the effect of irradiation which is manifested as viability or apoptosis of tumor cells. Results from our study showed that in radioresistant glioma cells, overexpressed LRIG1 dramatically inhibited the cell proliferation and promote cell apoptosis, indicating that LRIG1 act as a tumor suppressor in radioresistant glioma cells. As

an important cell transmembrane protein, LRIG1 was widely studied in gliomas. For instance, a study includes 404 patients with gliomas showed that expression of LRIG1 positively correlated with the prognosis of glioma patients and negatively correlated with WHO histological grade<sup>[32]</sup>. Moreover, in Ye *et al's* study, after clinical surgical resections of astrocytoma tumor tissue, the results showed that LRIG1 expression was significantly decreased in tumor tissue compared to the corresponding surrounding non-tumor tissues<sup>[18]</sup>.

In molecular level, the underlying mechanism of LRIG1 on glioma radioresistance was further clarified in our studies. We found that in radioresistant glioma cells, overexpression of LRIG1 could inhibit the expression of phosphorylated AKT (p-AKT), but not AKT, suggesting that LRIG1 might play a role in AKT activation. AKT is a serine/threonine kinase which can be activated by translocation to the plasma membrane or phosphorylation<sup>[33]</sup>. Activation of AKT, i.e, high level of p-AKT has been reported to correlate with a poor prognosis for patients with glioma<sup>[34]</sup>. AKT-related pathways have been also widely studied in molecular level in the oncogenic and development of cancers cells<sup>[35-37]</sup>. Previously in our group, we found that the glioblastoma radioresistance can be promoted by overexpressed miR-183, further, expression of LRIG1 and activation of EGFR/Akt pathway can be regulated by miR-183<sup>[28]</sup>. In the present study, we further clarified that overexpression of LRIG1 inhibited the expression of CTLA-4 protein, but not CTLA-4 mRNA. In addition, results also confirmed that LRIG1 could combined with CTLA-4, and promoted CTLA-4 degradation. CTLA-4, known as a member of a family of Immunoglobulin-related receptors, has been confirmed play a key role in tumorigenesis<sup>[38]</sup>. For its machoism, CTLA-4 can bind to phosphatidylinositol 3-kinase (PI3K) as well as phosphatases PP2A and SHP-2, which considered to be the key factors in AKT-related pathway<sup>[39,40]</sup>. However, whether CTLA-4 involved in the resistant to irradiation was still un-documented.

To the best of our knowledge, our study was the first one to clarify the relationship between LRIG1 and CTLA4/PI3K/AKT pathway in glioma radioresistance. We found that in human glioma tissue, LRIG1 was down-regulated, while CTLA-4 was highly expressed. By conducting correlation analysis, results shown that the expression of LRIG1 was negatively correlated with radioresistance of glioma and expression of CTLA-4. However, the expression of CTLA-4 was positively correlated with radioresistance of glioma.

To sum up, our data provided information that LRIG1 could increase the radiosensitivity of glioma cells, overexpressed LRIG1 had a significantly impact on activation of AKT and CTLA-4 expression. These findings provided a novel insight into the mechanism of radiotherapy in glioma and could be the potential targets for treating gliomas in the future.

## Declarations

### Competing interests

The authors declare that they have no financial or other conflicts of interest.

## Acknowledgments

This study has received funding from the Educational department foundation of Jiangxi Province, China (Grant No. GJJ190145)

## Funding

No applicable.

## Availability of data and materials

The data used and analyzed in this paper are available from the corresponding author under reasonable request.

## Authors' contributions

SQ and XQ wrote the manuscript and performed most of the experiments. RR participated in the study data acquisition and analysis. YZ edited and revised final manuscript.

## Ethics approval and consent to participate

Ethical approval was obtained from the Biomedical Ethics Committee of The Second Affiliated Hospital of Nanchang University.

## Consent for publication

Patients provided their consent for publication.

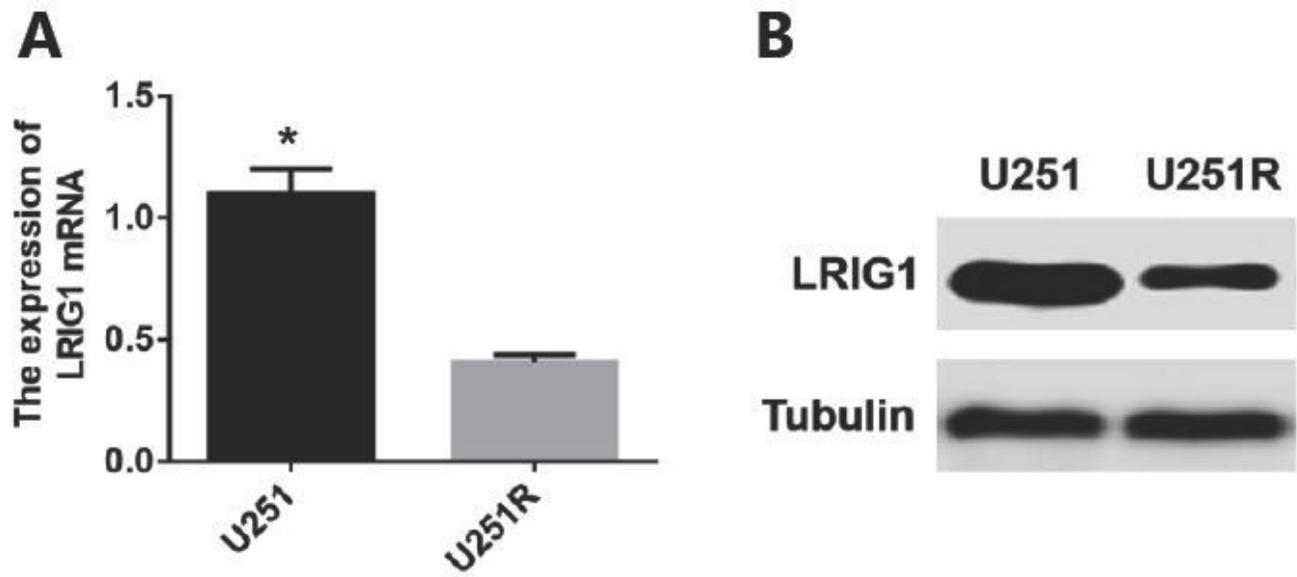
## References

1. Ostrom, Q.T., et al., *The epidemiology of glioma in adults: a "state of the science" review*. Neuro Oncol, 2014. 16(7): p. 896-913.
2. Ostrom, Q.T., et al., *Epidemiology of gliomas*. Cancer Treat Res, 2015. 163: p. 1-14.
3. Omuro, A. and L.M. DeAngelis, *Glioblastoma and other malignant gliomas: a clinical review*. Jama, 2013. 310(17): p. 1842-50.
4. Ghotme, K.A., et al., *Gliomas: New Perspectives in Diagnosis, Treatment and Prognosis*. Curr Top Med Chem, 2017. 17(12): p. 1438-1447.
5. Nahed, B.V., et al., *Management of patients with recurrence of diffuse low grade glioma: A systematic review and evidence-based clinical practice guideline*. J Neurooncol, 2015. 125(3): p. 609-30.
6. Lesueur, P., et al., *Phase I/IIa study of concomitant radiotherapy with olaparib and temozolomide in unresectable or partially resectable glioblastoma: OLA-TMZ-RTE-01 trial protocol*. BMC Cancer, 2019. 19(1): p. 198.

7. Beppu, T., et al., *A phase II study of radiotherapy after hyperbaric oxygenation combined with interferon-beta and nimustine hydrochloride to treat supratentorial malignant gliomas*. J Neurooncol, 2003. 61(2): p. 161-70.
8. Barker, F.G., 2nd, et al., *EGFR overexpression and radiation response in glioblastoma multiforme*. Int J Radiat Oncol Biol Phys, 2001. 51(2): p. 410-8.
9. Kim, Y., et al., *Wnt activation is implicated in glioblastoma radioresistance*. Lab Invest, 2012. 92(3): p. 466-73.
10. Liu, X., et al.,  *$\beta$ -Catenin overexpression in malignant glioma and its role in proliferation and apoptosis in glioblastoma cells*. Med Oncol, 2011. 28(2): p. 608-14.
11. Squatrito, M., et al., *Loss of ATM/Chk2/p53 pathway components accelerates tumor development and contributes to radiation resistance in gliomas*. Cancer Cell, 2010. 18(6): p. 619-29.
12. de la Iglesia, N., S.V. Puram, and A. Bonni, *STAT3 regulation of glioblastoma pathogenesis*. Curr Mol Med, 2009. 9(5): p. 580-90.
13. Hedman, H., et al., *Is LRIG1 a tumour suppressor gene at chromosome 3p14.3?* Acta Oncol, 2002. 41(4): p. 352-4.
14. Wang, Y., E.J. Poulin, and R.J. Coffey, *LRIG1 is a triple threat: ERBB negative regulator, intestinal stem cell marker and tumour suppressor*. Br J Cancer, 2013. 108(9): p. 1765-70.
15. Willis, S., et al., *Single Gene Prognostic Biomarkers in Ovarian Cancer: A Meta-Analysis*. PLoS One, 2016. 11(2): p. e0149183.
16. Li, Q., et al., *LRIG1 is a pleiotropic androgen receptor-regulated feedback tumor suppressor in prostate cancer*. Nature Communications, 2019. 10(1): p. 5494.
17. Ljuslinder, I., et al., *LRIG1 expression in colorectal cancer*. Acta Oncol, 2007. 46(8): p. 1118-22.
18. Ye, F., et al., *Upregulation of LRIG1 suppresses malignant glioma cell growth by attenuating EGFR activity*. J Neurooncol, 2009. 94(2): p. 183-94.
19. Li, F., et al., *Suppression of bladder cancer cell tumorigenicity in an athymic mouse model by adenoviral vector-mediated transfer of LRIG1*. Oncol Rep, 2011. 26(2): p. 439-46.
20. Gur, G., et al., *LRIG1 restricts growth factor signaling by enhancing receptor ubiquitylation and degradation*. Embo j, 2004. 23(16): p. 3270-81.
21. Laederich, M.B., et al., *The leucine-rich repeat protein LRIG1 is a negative regulator of ErbB family receptor tyrosine kinases*. J Biol Chem, 2004. 279(45): p. 47050-6.
22. Hedman, H. and R. Henriksson, *LRIG inhibitors of growth factor signalling - double-edged swords in human cancer?* Eur J Cancer, 2007. 43(4): p. 676-82.
23. Stutz, M.A., et al., *LRIG1 negatively regulates the oncogenic EGF receptor mutant EGFRvIII*. Oncogene, 2008. 27(43): p. 5741-52.
24. Nakamura, T., et al., *LRIG1 inhibits STAT3-dependent inflammation to maintain corneal homeostasis*. J Clin Invest, 2014. 124(1): p. 385-97.

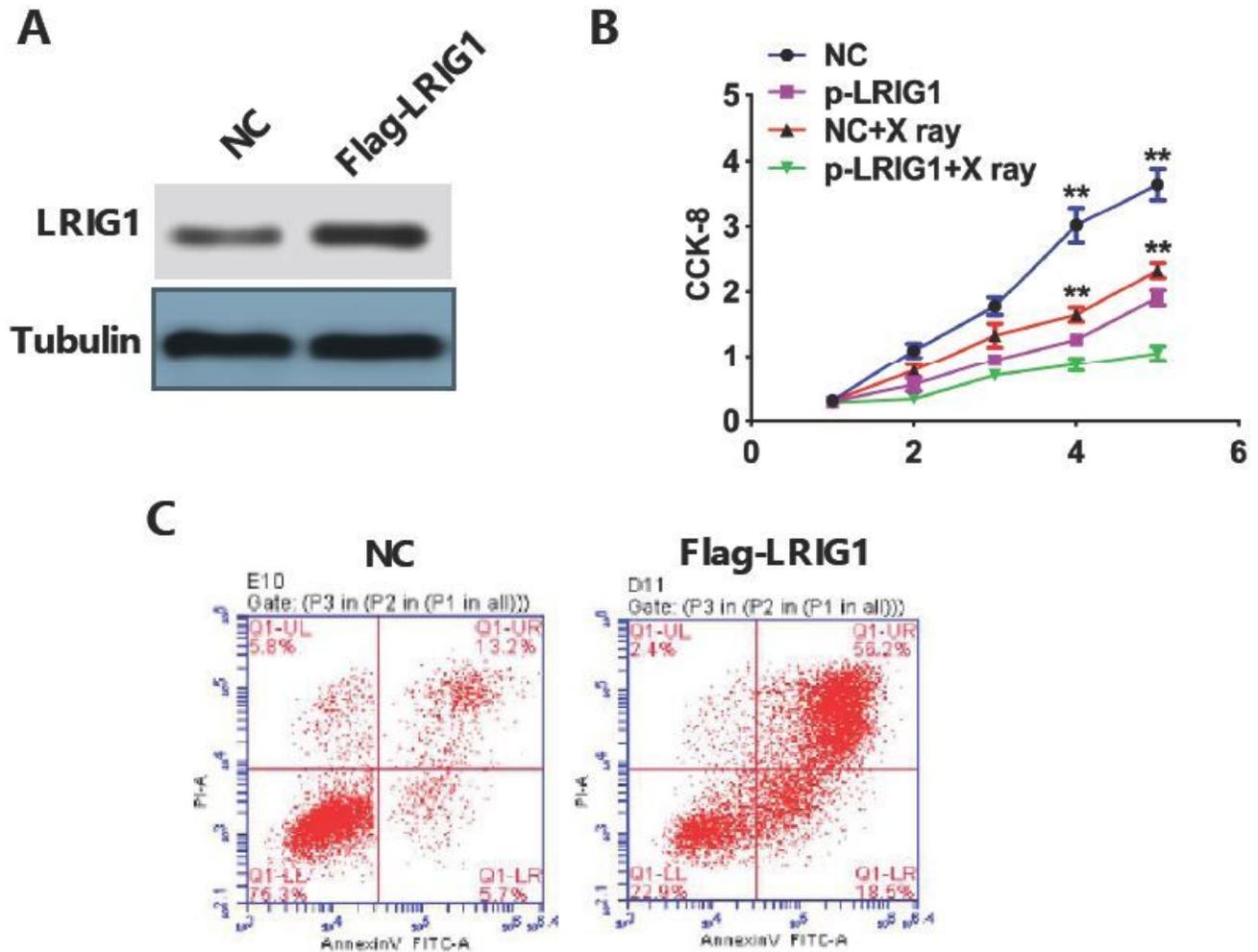
25. Bai, L., et al., *LRIG1 modulates cancer cell sensitivity to Smac mimetics by regulating TNF $\alpha$  expression and receptor tyrosine kinase signaling*. *Cancer Res*, 2012. 72(5): p. 1229-38.
26. Yang, J.A., et al., *LRIG1 enhances the radiosensitivity of radioresistant human glioblastoma U251 cells via attenuation of the EGFR/Akt signaling pathway*. *Int J Clin Exp Pathol*, 2015. 8(4): p. 3580-90.
27. Chen, L., et al., *MicroRNA-590-3p enhances the radioresistance in glioblastoma cells by targeting LRIG1*. *Exp Ther Med*, 2017. 14(2): p. 1818-1824.
28. Fan, H., et al., *Overexpressed miR-183 promoted glioblastoma radioresistance via down-regulating LRIG1*. *Biomed Pharmacother*, 2018. 97: p. 1554-1563.
29. Maucort-Boulch, D., et al., *Rationale for carbon ion therapy in high-grade glioma based on a review and a meta-analysis of neutron beam trials*. *Cancer Radiother*, 2010. 14(1): p. 34-41.
30. Lomax, M.E., L.K. Folkes, and P. O'Neill, *Biological consequences of radiation-induced DNA damage: relevance to radiotherapy*. *Clin Oncol (R Coll Radiol)*, 2013. 25(10): p. 578-85.
31. Biedermann, K.A., et al., *scid mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair*. *Proc Natl Acad Sci U S A*, 1991. 88(4): p. 1394-7.
32. Guo, D., et al., *Perinuclear leucine-rich repeats and immunoglobulin-like domain proteins (LRIG1-3) as prognostic indicators in astrocytic tumors*. *Acta Neuropathol*, 2006. 111(3): p. 238-46.
33. Testa, J.R. and A. Bellacosa, *AKT plays a central role in tumorigenesis*. *Proc Natl Acad Sci U S A*, 2001. 98(20): p. 10983-5.
34. Suzuki, Y., et al., *Higher pAkt expression predicts a significant worse prognosis in glioblastomas*. *J Radiat Res*, 2010. 51(3): p. 343-8.
35. Han, F., et al., *The critical role of AMPK in driving Akt activation under stress, tumorigenesis and drug resistance*. *Nat Commun*, 2018. 9(1): p. 4728.
36. Yang, L., X. Hu, and Y.Y. Mo, *Acidosis promotes tumorigenesis by activating AKT/NF- $\kappa$ B signaling*. *Cancer Metastasis Rev*, 2019. 38(1-2): p. 179-188.
37. Guo, J., et al., *AKT methylation by SETDB1 promotes AKT kinase activity and oncogenic functions*. *Nat Cell Biol*, 2019. 21(2): p. 226-237.
38. Mao, H., et al., *New insights of CTLA-4 into its biological function in breast cancer*. *Curr Cancer Drug Targets*, 2010. 10(7): p. 728-36.
39. Schneider, H., et al., *CTLA-4 binding to the lipid kinase phosphatidylinositol 3-kinase in T cells*. *J Exp Med*, 1995. 181(1): p. 351-5.
40. Lee, K.M., et al., *Molecular basis of T cell inactivation by CTLA-4*. *Science*, 1998. 282(5397): p. 2263-6.

## Figures



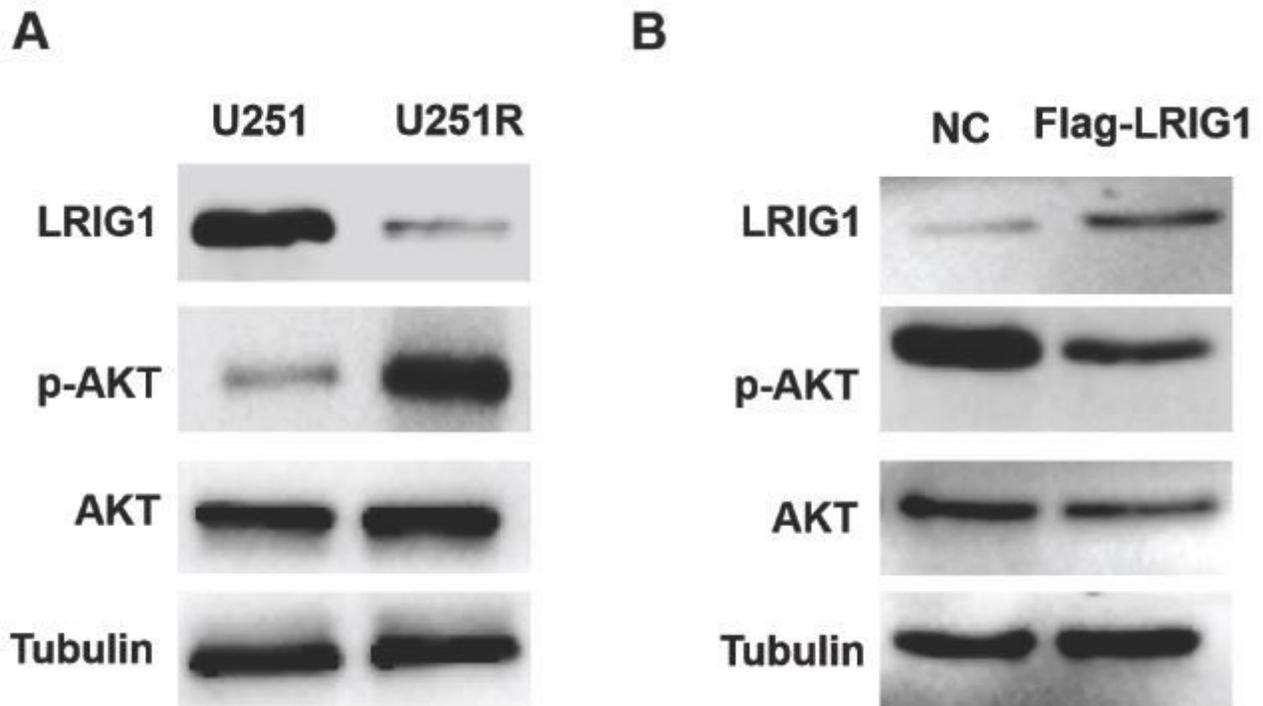
**Figure 1**

The expression of LRIG1 in radioresistant glioma cells. (A) The relative expression level of LRIG1 mRNA in U251 and U251R cells were determined by qRT-PCR. (B) The expression of LRIG1 protein in U251 and U251R cells were checked by western blot. Tubulin served as internal control. Data were expressed as mean  $\pm$  SD. (\* $P < 0.05$ ).



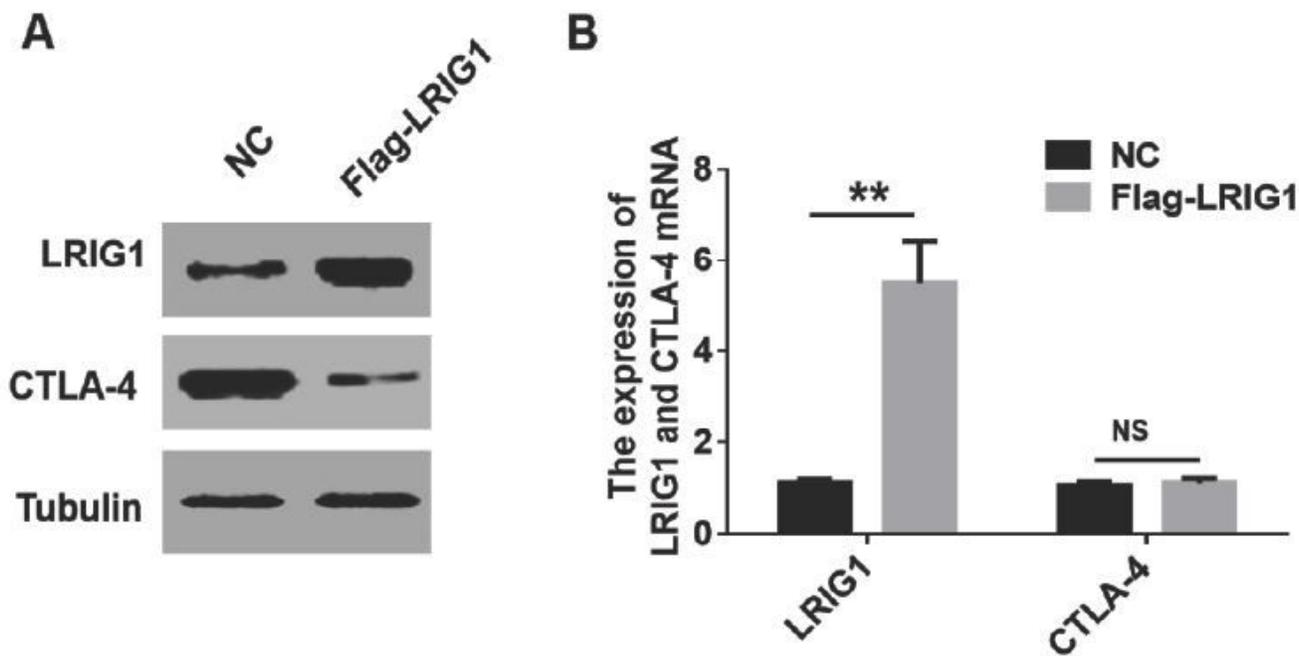
**Figure 2**

Effects of LRIG1 on radioresistance of glioma cells. (A) The expression of LRIG1 protein in LRIG1 overexpressed or control group were determined by western blot. (B) The effect of overexpressed LRIG1 on cell viability were detected by CCK-8 assay. (C) The cell apoptosis of glioma cells was determined in LRIG1 overexpressed or control group by flow cytometry assay. Data were expressed as mean  $\pm$  SD. (\*\*P < 0.01, NC vs p-LRIG1; NC + X ray vs p-LRIG1 + X ray)



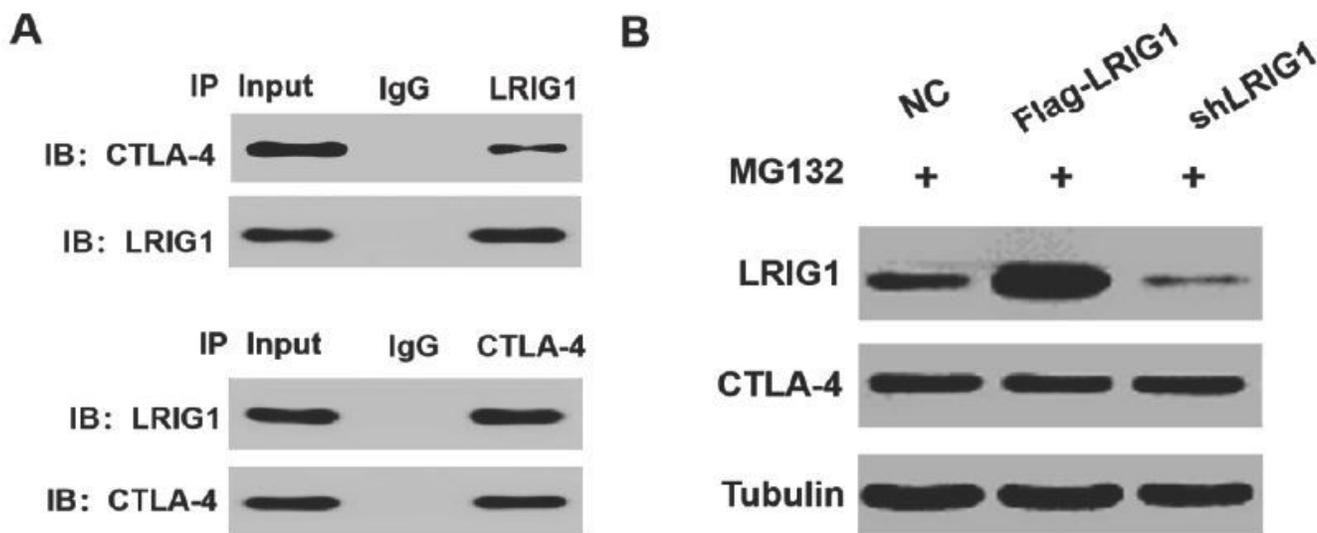
**Figure 3**

Effects of LRIG1 on expression of p-AKT and AKT in glioma cells. (A) The expression of protein levels of LRIG1, p-AKT, AKT in U251 and U251R cells were determined by western blot. (B) The expression of protein levels of LRIG1, p-AKT, AKT in LRIG1 overexpressed or control group were determined by western blot.



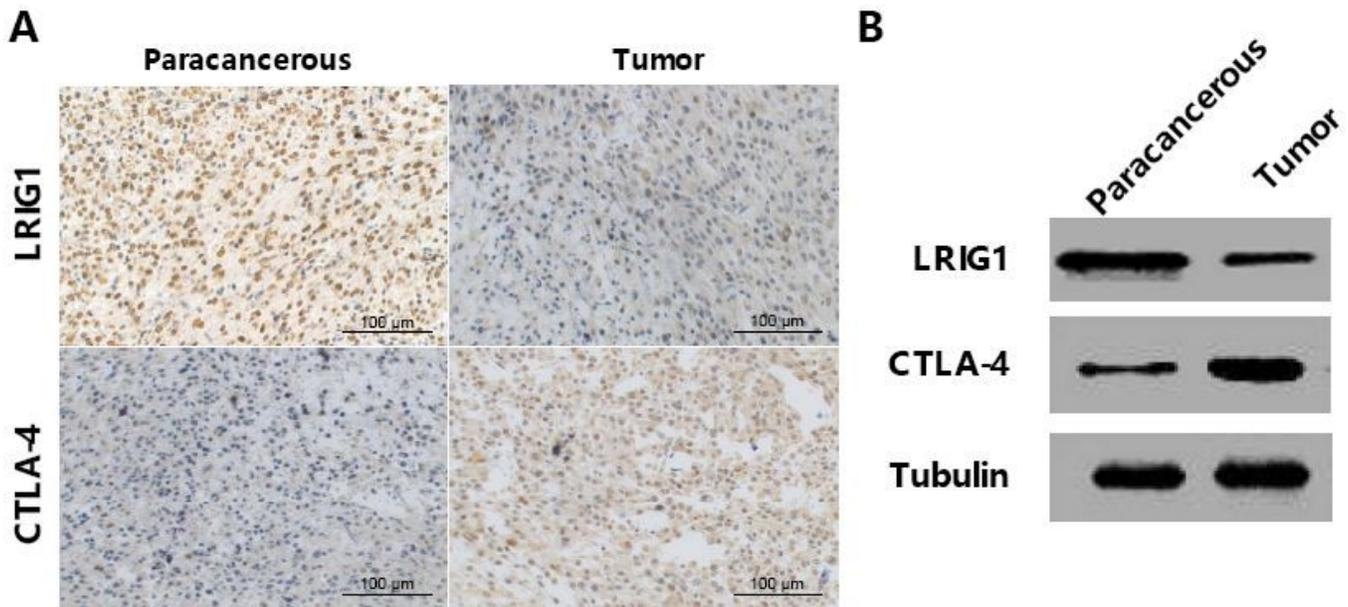
**Figure 4**

Effects of LRIG1 on expression of CTLA-4 in U251R cells. The effect of overexpressed LRIG1 on expression of CTLA-4 protein (A) and CTLA-4 mRNA (B) were determined by western blot and qRT-PCR, respectively. Data were expressed as mean  $\pm$  SD. (\*\*  $P < 0.01$  vs NC; NS,  $P > 0.05$  vs NC)



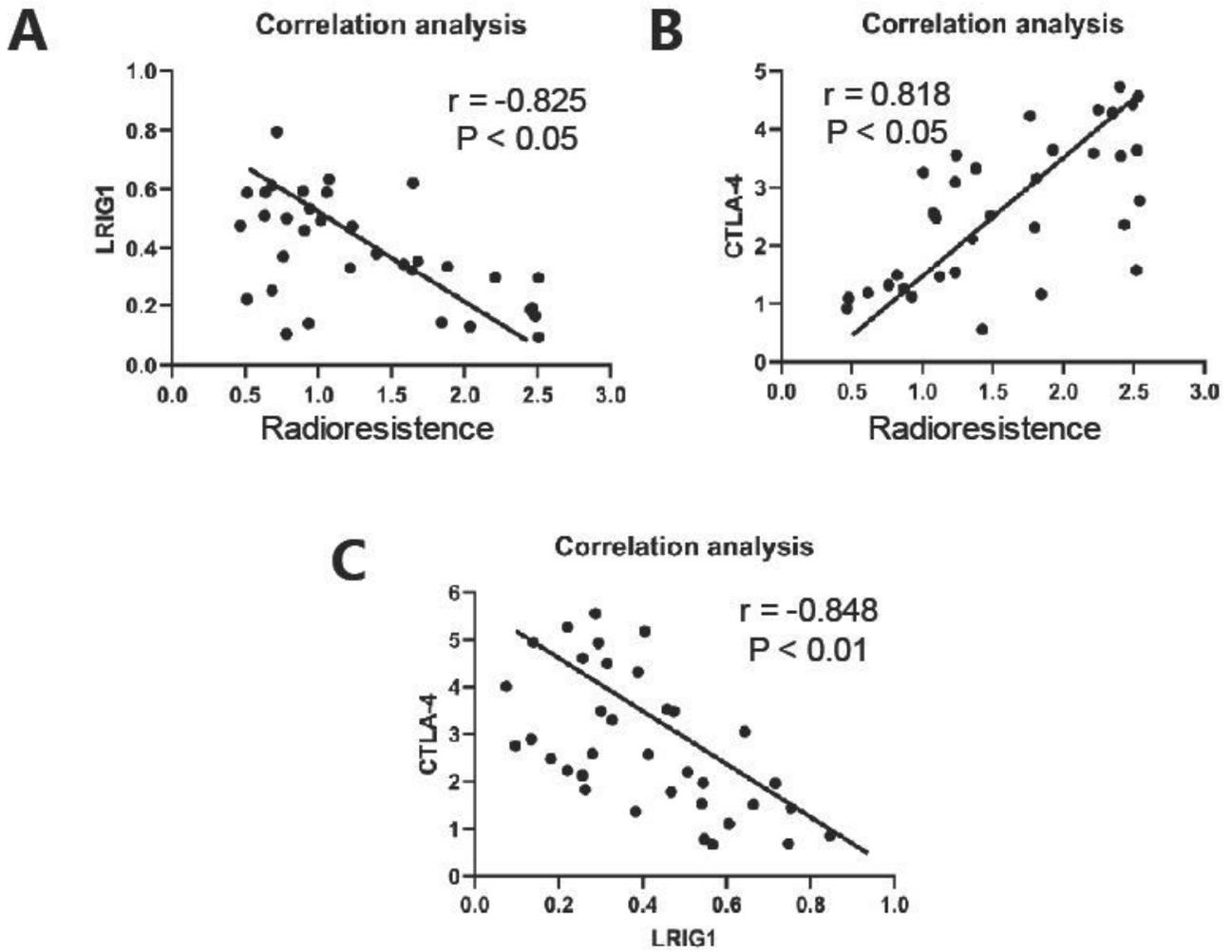
**Figure 5**

LRIG1 affects the degradation of CTLA-4 protein. (A) The binding of LRIG1 to CTLA-4 protein was determined by immunoprecipitation assay. (B) The effect of LRIG1 on the expression of CTLA-4 protein was determined by western blot after blocking the ubiquitin-proteasome.



**Figure 6**

The expression of LRIG1 and CTLA-4 in glioma tissue. The expression of LRIG1 and CTLA-4 were detected in tumor tissue or paracancerous tissue by immunochemistry (A) and western blot (B), respectively.



**Figure 7**

Correlation analysis of LRIG1, CTLA-4 and radioresistance of glioma. Correlation analysis between LRIG1 and radioresistance of glioma (A), CTLA-4 and radioresistance of glioma (B), LRIG1 and CTLA-4 were conducted by Pearson correlation analysis.