LRIG1 regulates invasion and migration of human gliomas through SNAI2 and E-cadherin

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Abstract

The particular molecular mechanisms that activates invasion and migration of human gliomas remains obscure. Our previous study has indicated the function of leucine-rich repeats and immunoglobulin-like domains 1(LRIG1) in the inhibition of the cell invasion and downstream genes snail homolog 2(SNAI2) and E-cadherin were involved in this process. In this study we give an insight into the role SNAI2 and E-cadherin played in this process and the relationship between them in glioma tissue specimen. We employed a full length expression plasmid to overexpress LRIG1 in the malignant glioma cell line U251. Introduction of exogenous LRIG1 into U251 significantly inhibited cell invasion and metastasis detected by transwell assay and scratch test, respectively. On the other hand, LRIG1 overexpression leads to reduced SNAI2 expression and elevated E-cadherin expression, manifested by qRT-PCR and western blot, which was consistent with the results in glioma tissue specimens. Further research revealed that 4-chloro-DL-phenylalanine (PCPA), a small molecule inhibitor of SNAI2, can significantly promote the inhibitory effect of cell invasion and migration caused by overexpressed LRIG1. Our data suggested that LRIG1 as a tumor suppressor restricted glioma invasion and migration by regulating SNAI2 and E-cadherin axis and low expression of LRIG1 is associated with poor prognosis in glioma. In conclusion, restoration of LRIG1 in glioma cells could be a novel therapeutic strategy.

Introduction

Gliomas are the most common primary intracranial tumors which cause significant mortality and morbidity in spite of the advances in diagnostic imaging, microneurosurgical techniques, radiation therapy, and chemotherapy. Tumor invasion and migration are the major contributors to the death in glioma patients. Our previous study has indicated the function of LRIG1 in the inhibition of the cell invasion, but the particular molecular mechanisms remains obscure. LRIG1 is a type 1 transmembrane protein whose extracellular domain contains 15 leucine-rich repeats (LRRs) and three immunoglobulin (Ig)-like domains, mainly function as regulators of growth factor signaling[1]. LRIG1 has been identified as a tumor suppressor in human cancers, such as prostate cancer[2], non-small cell lung cancer[3], thyroid cancer[4] as well as gliomas[5]. The SNAI2 gene encoding Slug, an oncogenic transcriptional repressor acting as a key regulator of cell migration, has been found overexpressed in many cancers such as leukemia, lung cancer, esophageal cancer, colorectal cancer, prostate cancer, breast cancer and ovarian cancer[6]. Cadherin family are a group of cell-surface adhesion molecules which played important roles in intercellular adhesion and E-cadherin is the major subtype of cadherin family expressed in epithelial tissues. E-cadherin is a key component of the adherens junctions which are indispensable in cell adhesion and maintaining cell epithelial phenotype[7]. E-cadherin is often inactivated or functionally inhibited, which resulting in tumor development or progression[8].The mechanism of glioma invasion and migration is an intricate program called epithelial-to-mesenchymal transition (EMT), in which process SNAI2 and E-cadherin play an important role[9]. Glioma cells undergo a series of molecular and conformational changes shifting towards mesenchymal traits, which include cytoskeletal re-patterning, extracellular matrix (ECM) remodeling, and stem-like trait acquisition [10]. It has been reported that LRIGI1

regulates EMT in melanoma cells[11] and basal-like breast cancer cells[12] to inhibit cell invasion, whether this is present in gliomas is still unknown. We found in our study that low expression of LRIG1 is associated with poor prognosis in glioma and LRIG1 inhibits glioma invasion and migration by regulating downstream SANI2-E-cadherin axis.

Materials And Methods

Clinical specimen and data collection. Human glioma specimens were collected from the inpatients in Department of Neurology, Renmin Hospital of Wuhan University from 2015 to 2017. This study was reviewed and approved by Medical Ethics Committee of Wuhan University. Written informed consent was obtained from each patient included. Normal contral brain tissue that corresponds to glioma tissues were taken from patients undergoing surgical treatment for craniocerebral trauma.

Immunohistochemistry (IHC) and evaluation. The IHC was performed as described previously[13]. LRIG1-positive cells displayed brownish blue granules on the cytoplasm. According to the percentage of immunoreactive cells and intensity of the staining, cell were divided into four parts from + to ++++ through immunohistochemical scores (IHC scores). The percentage was rated on a scale of 0–4 as follows: 0, < 5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%. The evaluation of the staining intensity was rated and scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. By multiplying the percentage and intensity score we obtained the IHC score. The final grouping criteria was as follows: IHC scores 0–3 were considered as group +, scores 4–6 were considered as group ++, scores 7–9 were considered as group +++ and scores 10–12 were considered as group ++++. These scores were independently determined by two independent senior pathologists. Group + and group + + were considered as low expression, group +++ and group ++++ were considered as high expression.

Cell culture and PCPA treatment. The human glioma cell line U-251 MG was purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cell was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) under a humidified atmosphere with 5% CO2 at 37˚C. Normally, the medium was replaced every 2 or 3 days. Cells were passaged every 5 days or 6 and routinely examined. PCPA (Aladdin Biochemical Technology, Shanghai, China) is prepared as 50 mmol/L PCPA solution dissolved in ultrapure water, and stored at 4 ℃ for further use. The final concentration of PCPA was 100 μmol/L when the cells were treated for further qRT-PCR, Western blot or cell invasion and migration assay.

RNA isolation and qRT-PCR. Total RNA were extracted from fresh frozen glioma tissues or glioma cell line using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One μg of total RNA was used as a template for reverse transcription using ReverTra Ace-A (Toyobo, Osaka, Japan), after the determination of the amount of total RNA by ultraviolet (UV) spectrophotometry. Oligonucleotide primer sequences used were as follows: LRIG1 sense 5'CCGGGTGATACAACCTTGCT3' and antisense 5'ACACCGAAGTGGACTGTTACTCC3'; SNAI2 sense 5'CCCAGGCTCACATATTCCTTGT3' and antisense

5’ACACCGAAGTGGACTGTTA CTCC3’; E-cadherin sense 5’ATAGTTCGAGGTTCTGGTATGGG3’ and antisense 5’ACTGGTGCCATTTCCACTCG3’; GAPDH sense 5’GTCCACCGCAAATGCTTCTA3’ and antisense 5’TGCTGTCACCTTCACCGTTC3’. RT-PCR was performed to quantify the mRNA expression using the SYBR-Green PCR Master Mix (Takara). Glyceraldehyde-3-phosphate dehydrogenase(GAPDH) was used as reference for mRNA and each sample was analyzed in triplicate.

Transfection of plasmid. The pLRIG1-GFP, which was kindly donated by Dr Jianming Liao (Department of Neurosurgery, Renmin Hospital of Wuhan University), is a mammalian expression vector carrying a GFP-coding sequence upstream from its cloning sites. U251 cells were transfected with pLRIG1-GFP using LipofectamineTM 2000 (Invitrogen) according to the manufacturer’s instructions. The empty vector p-EGFP-N1 was transfected into U251 cells for control. Cell clones resistant to G418 for 3 weeks were ring-cloned and amplified for further experiments.

Western blot analysis. Total protein of glioma tumor tissues or glioma cell line was extracted using RIPA buffer supplemented with proteinase inhibitors. The primary and secondary antibodies used in this work were as follows: polyclonal rabbit anti-LRIG1 (1:1000; Abcam, Cambridge, USA); monoclonal rabbit anti-GAPDH (1:10000; Abcam, Cambridge, USA); polyclonal rabbit anti-SNAI2 (1:1000; Cell Signaling Technology, Boston USA); polyclonal rabbit anti-E-cadherin (1:1000; Cell Signaling Technology, Boston USA) and HRP labelled goat anti-rabbit IgG (1:10000; Santa Cruz Biotechnology, USA). The intensities of bands were semiquantified by using densitometry.

Cell invasion and migration assay. The cell invasion was performed with 8-μm pore size Transwell chambers (Corning, Corning, NY, USA) precoated with matrigel (R&D Systems, Minneapolis, MN, USA). Cells were suspended in serum-free DMEM. Then, 5×10^4 cells in 100 μl of serum-free DMEM were plated into the upper chamber and 600 μl of DMEM containing 10% FBS in the lower chamber. These chambers were cultured at 37°C with 5% CO_2 for 36 h. The cells in the upper chamber were collected with a cotton swab and cells that had adhered to the lower surface were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and counted under a microscope (Olympus BX51). The migration assay had the same steps except that the absence of matrigel and the cells were cultured for 24 h.

Scratch test. 5×10^5 cells in 1 ml of DMEM containing 10% FBS were plated into 6-well plate. These plates were cultured at 37°C with 5% CO_2 and observed under inverted microscope (Olympus). Remove the culture solution when the cells are spread over the bottom of the 6-well plate. Scratch from top to bottom in the center of the 6-well plate perpendicularly with a sterile 200 μl gun head and wash the cells with 2 ml PBS solution for 5 times. Then observe and record the scratch width at 0 h and 24 h under the inverted microscope and take photos. Cell migration ability is expressed as (0 h scratch width − 24 h scratch width) / time.

Statistical analysis. T-test or one-way analysis of variance (ANOVA) was employed to analyze the significant differences between groups. Significant difference between clinicopathological characteristics of glioma tissues were determined with Chi-square Test. Survival was analyzed by the Kaplan-Meier

survival curve. The aforementioned statistical tests all performed with GraphPad Prism 6.0 version (GraphPad Software, Inc., La Jolla, CA, USA) and comparisons were two-tailed and p 0.05 was considered significant.

Results

Low expression of LRIG1 is associated with poor prognosis in glioma. We employed IHC to detect the expression of LRIG1 in 78 glioma specimens, 40 normal brain tissue specimens were used as a control group. IHC results showed that LRIG1 was significantly down-regulated in glioma tissues compared with the control group (Figure 1A and 1B). To avoid possible false positive results, we used qRT-PCR to further verify the expression of LRIG1 mRNA in another independent group of fresh frozen glioma tissues and the control group, a total of 30 glioma tissues and 20 normal controls. Consistently, Results of qRT-PCR corroborated what was achieved by IHC, exhibiting that LRIG1 was markedly reduced in glioma tissues in comparison with normal controls (Figure 1C). Subsequently, clinicopathological characteristics and LRIG1 expression in 78 cases of glioma tissues were performed with Chi-square Test. The results displayed that expression of LRIG1 was remarkably associated with tumor WHO grade and relapse, but no significant correlation can be identified between LRIG1 expression and other clinicopathological characteristics comprising age, sex and histological subtype (Table 1). Kaplan-Meier survival analysis revealed that reduced LRIG1 dramatically led to inferior overall survival (Figure 1D) and disease-free survival (Figure 1E) in glioma. Furthermore, multivariate COX regression analysis was performed suggesting that LRIG1 expression was an independent prognostic factor in glioma (Table 2), in addition to tumor WHO grade and relapse. In conclusion, the data demonstrated down-regulated LRIG1 was significantly linked with poor prognosis in glioma.

The LRIG1 expression plasmid pLRIG1-GFP and empty control pEGFP-N1 were transfected into U251 cells. Fluorescence microscopy (488nm, Nikon, Japan) showed transfected cells pLRIG1-GFP-U251 and pEGFP-N1-U251 emitted green fluorescence, and U251 cells emitted none 24h after transfection (Figure 2A). Cells were selected by culture medium containing G418 48h after transfection and neomycin-resistant clones were generated after 3 weeks. LRIG1 mRNA level was significantly up regulated in pLRIG1-GFP-U251 cells compared with controls, as shown by qRT-PCR (Figure 2B). Further analysis by western blot showed that LRIG1 protein level in pLRIG1-GFP-U251 cells increased accordingly (Figure 2C).

LRIG1 regulates downstream genes SNAI2 and E-cadherin in glioma. LRIG1, SNAI2 and E-cadherin protein level in 30 cases of fresh frozen glioma tissues and 20 cases normal controls were evaluated by western blot analysis. LRIG1 and E-cadherin were down regulated while SNAI2 was up regulated in glioma tissue ($p<0.05$) (Figure 3A). SNAI2 and E-cadherin mRNA level were evaluated by qRT-PCR in four groups of U251 cells, which were p-EGFP-N1, p-EGFP-N1+PCPA, pLRIG1-GFP, pLRIG1-GFP+PCPA U251 cells, respectively. The results indicated that LRIG1 can down regulated SNAI2 mRNA level in glioma ($p<0.05$) and PCPA treatment has no effect on the mRNA level of SNAI2 (Figure 3B). LRIG1 can also up regulated E-cadherin mRNA level, and PCPA has a positive effect on this process ($p<0.05$) (Figure 3C). SNAI2 and E-cadherin protein level in four groups of U251 cells abovementioned were evaluated by western blot

analysis (Figure 3D). The results indicated that LRIG1 can down regulated SANI2 protein level in glioma ($p<0.05$) (Figure 3E). LRIG1 can also up regulated E-cadherin protein level, and PCPA has a positive effect on this process ($p<0.05$) (Figure 3F). There result indicated that LRIG1 modulate SANI2-E-cadherin axis in glioma.

LRIG1 inhibits glioma invasion and migration by regulating SANI2-E-cadherin axis. Invasion **(Figure 4A)**, migration **(Figure 4B)** and speed of scratch healing **(Figure 4C)** of four groups of cells were detected by transwell invasion, migration assay and scratch test, respectively. The representative images are shown (magnification, x100), and the invaded, migrated cells and scratch healing speed of each image are presented as the mean ± standard deviation (SD). One way ANOVA was employed to analyze the significant difference ($P<0.001$). The number of invaded cells were 420±40, 310±32, 198±43 and 99±29 from front to back and the migrated cells were 386±35, 295±29, 220±33, 86±25.The results show that LRIG1 inhibits invasion, migration and speed of scratch healing, and PCPA, the small molecule inhibitor of SNAI, has obvious promotive effect on such a process. In summary, we believe that LRIG1 inhibits the invasion and migration of glioma by regulating the SANI2-E-cadherin axis.

Discussion

LRIG1 is a cell surface transmembrane protein, according to the existing research, it mainly has the following functions: an endogenous feedback regulator of receptor tyrosine kinases (RTKs), a tumor suppressor, an intestinal stem cell marker and a modulator of different cellular phenotypes[14, 15]. It has been reported to be a tumor suppressor gene in malignant glioma, involved in the proliferation[16], radioresistance[17], chemosensitivity[18] of glioma cells. LRIG1 inhibits hypoxia-induced vasculogenic mimicry formation via suppression of the EGFR/PI3K/AKT pathway[19]. Xie R, *et al* reported that down regulation of LRIG1 RNA interference promotes the aggressive properties of glioma cells via EGFR/Akt/c-Myc activation[20], other than that, no more detailed reports. Our previous study have focused on the role LRIG1 played in glioma invasion inhibition, however the molecular mechanism involved is still unknown. In this study we revealed low expression of LRIG1 is associated with poor prognosis in glioma and indicated higher WHO grade, relapse rate and shorter survival. To our knowledge, the invasive growth and migration of gliomas to surrounding normal brain tissues is an important reason why gliomas are extremely prone to relapse. Tumor relapse was the chief reason for poor prognosis of glioma, is mainly attributed to glioma stem cells (GSCs) and epithelial-mesenchymal transition (EMT). The latest report states another transmembrane protein containing leucine-rich repeat, leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), promotes EMT by activating the Wnt/β-catenin pathway and predicts poor prognosis of glioma[21]. Many proteins or microRNAs regulate glioma cell invasion, and the detailed mechanism is by modulating EMT[22–24]. EMT is regulated by key transcription factors, including SNAI2. We showed in our data that SNAI2 and E-cadherin were involved in the inhibition of cell invasion and migration by LRIG1, and this provides guidance and direction for us to further study its detailed mechanism. EMT mainly involving an E-cadherin to N-cadherin shift, led to tumor invasion or migration and therapeutic resistance. E-cadherin is a transmembrane glycoprotein which connects epithelial cells

together at adherens junctions. E-cadherin functional loss has frequently been associated with poor prognosis and survival in patients of various cancers[25]. In our study, E-cadherin loss was detected in both tissue specimens and cell lines and the ability of cell invasion and migration rises sharply with the loss of E-cadherin,.

In conclusion, we demonstrated for the first time that LRIG1 is down-regulated in glioma tissues, resulting in the activation of SANI2 and loss of E-cadherin, lead to malignant prognosis of gliomas. LRIG1regulated invasion and migration of gliomas by modulating SNAI2-E-cadherin axis, EMT may play a decisive role in this process but more details still need further study.

Declarations

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Disclosure

The authors report no conflicts of interest in this work.

Author contributions and consent to publication

Xu Haitao and Chen Zhibiao co-designed the study and Chen Zhibiao provided all the reagents involved in the study. Tao Xiang and Zhang Wenfei collected all the data.

, Liu Junhui and Zhu Xiaonan collected all the clinical samples. Tao Xiang and Ji Baowei drafted the manuscript and analyzed the data. Xu Haitao and Ji Baowei made critical discussion. All authors agree to the publication.

Abbreviations

LRIG1: leucine-rich repeats and immunoglobulin-like domains 1; SNAI2: snail homolog 2; PCPA: 4-chloro-DL-phenylalanine; LRRs: leucine-rich repeats;

Ig: immunoglobulin; EMT: epithelial-to-mesenchymal transition; ECM: extracellular matrix; IHC: Immunohistochemistry; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; ANOVA: analysis of variance; WHO: World Health Organization; RTKs: receptor tyrosine kinases; GSCs: glioma stem cells; LGR5: leucine-rich repeat-containing G protein-coupled receptor 5.

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Tables

Table 1. clinicopathological characteristics of LRIG1 expression in 78 cases of glioma tissues.

Variables	Group	No.	LRIG1 expression		χ^2	*P* value
			High	Low		
Glioma		78	28	50	28.29	< 0.0001
Normal control		40	35	5		
Age	≥50	38	13	25	0.092	0.762
	<50	40	15	25		
Sex	Male	36	14	22	0.260	0.610
	Female	42	14	28		
Histological subtype	Astrocytic	25	11	14	1.097	0.578
	Oligoastrocytic	30	10	20		
	Oligodendroglial	23	7	16		
WHO grade	I+II	28	15	13	5.929	0.015
	III+IV	50	13	37		
Relapse	Yes	60	18	42	3.930	0.047
	No	18	10	8		

Notes: High +++, ++++; Low +, ++.

Abbreviation: LRIG1, Leucine-rich repeats and immunoglobulin-like domains protein 1; WHO, World Health Organization.

Table 2. Univariate and multivariate analysis of LRIG1 expression and clinicopathological characteristics in glioma.

	Univariate analysis		Multivariate analysis	
	95% CI	*P* value	95% CI	*P* value
LRIG1 expression	0.418 to 0.886	0.015	0.514 to 0.914	0.012
Age(≥50 vs <50)	1.123 to 2.862	0.321	1.109 to 1.856	0.486
Sex	0.605 to 1.756	0.856	0.556 to 1.243	0.587
WHO grade	1.089 to 1.686	0.028	1.059 to 1.985	0.043
Relapse	1.259 to 4.561	0.012	0.985 to 1.854	0.036

Abbreviation: LRIG1, Leucine-rich repeats and immunoglobulin-like domains protein 1; WHO, World Health Organization.

Figures

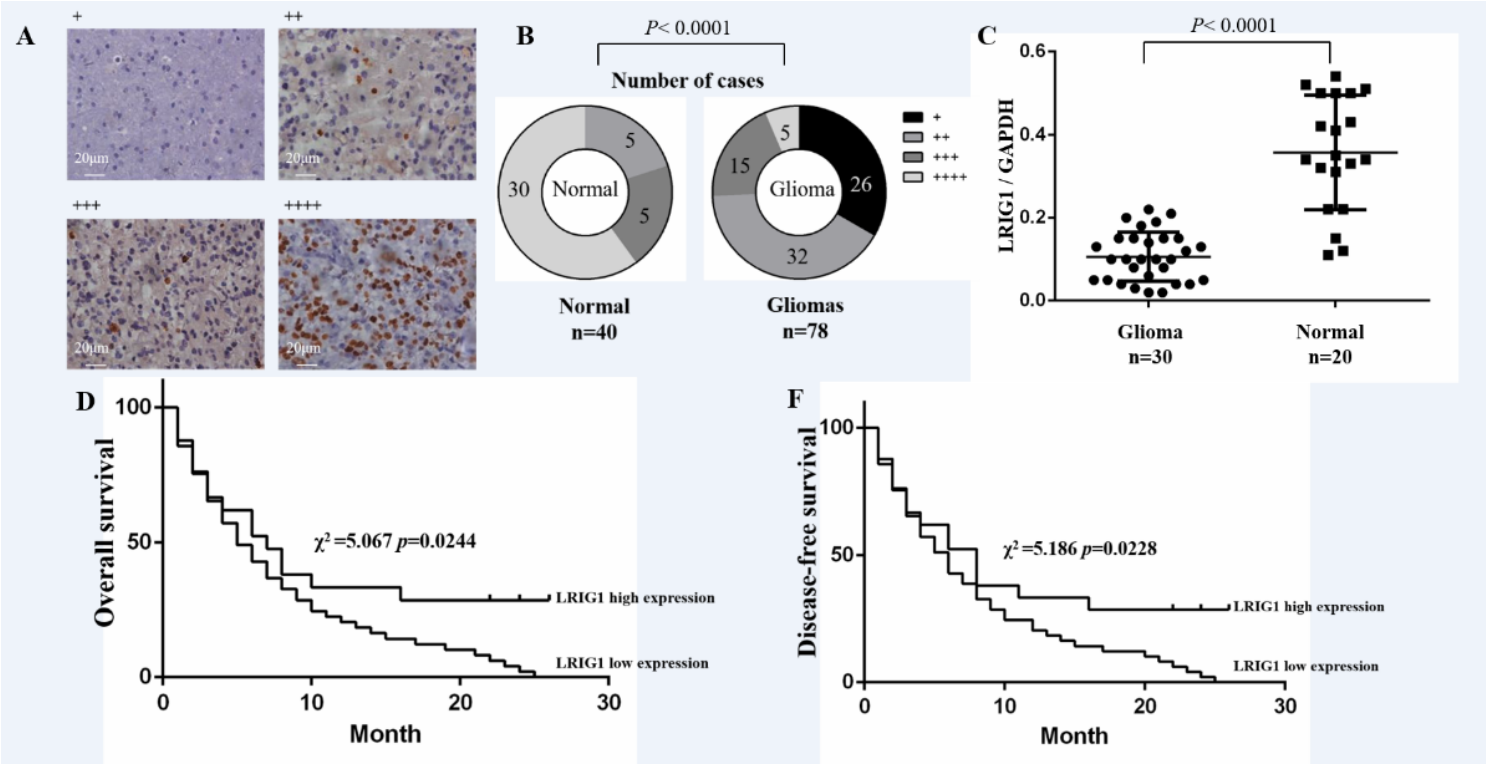

Figure 1

Low expression of LRIG1 is associated with poor prognosis in glioma (A) Heterogeneous expression of LRIG1 in glioma specimens as well as normal control brain tissue were detected by IHC and observed with a microscope (x 400). Scale bar, 20μm. LRIG1 expression being varied from + to ++++ by IHC scores. (B) Distribution of LRIG1 expression evaluated by IHC in 78 glioma specimens and 40 normal controls. (C) LRIG1 mRNA in another independent 30 cases of fresh frozen glioma tissues and 20 cases normal controls were evaluated by qRT-PCR. Two tailed, paired T-test was employed to analyze the significant difference (P<0.0001). (D) Overall survival analysis of LRIG1 by Kaplan-Meier survival curve in 50 low expression and 28 high expression cases. Log-Rank Chi-square was used to analyze the difference of survival (χ2=5.067, P=0.0244). (E) Likewise and incidentally, Disease-free survival was also analyzed (χ2=5.186, P=0.0228).

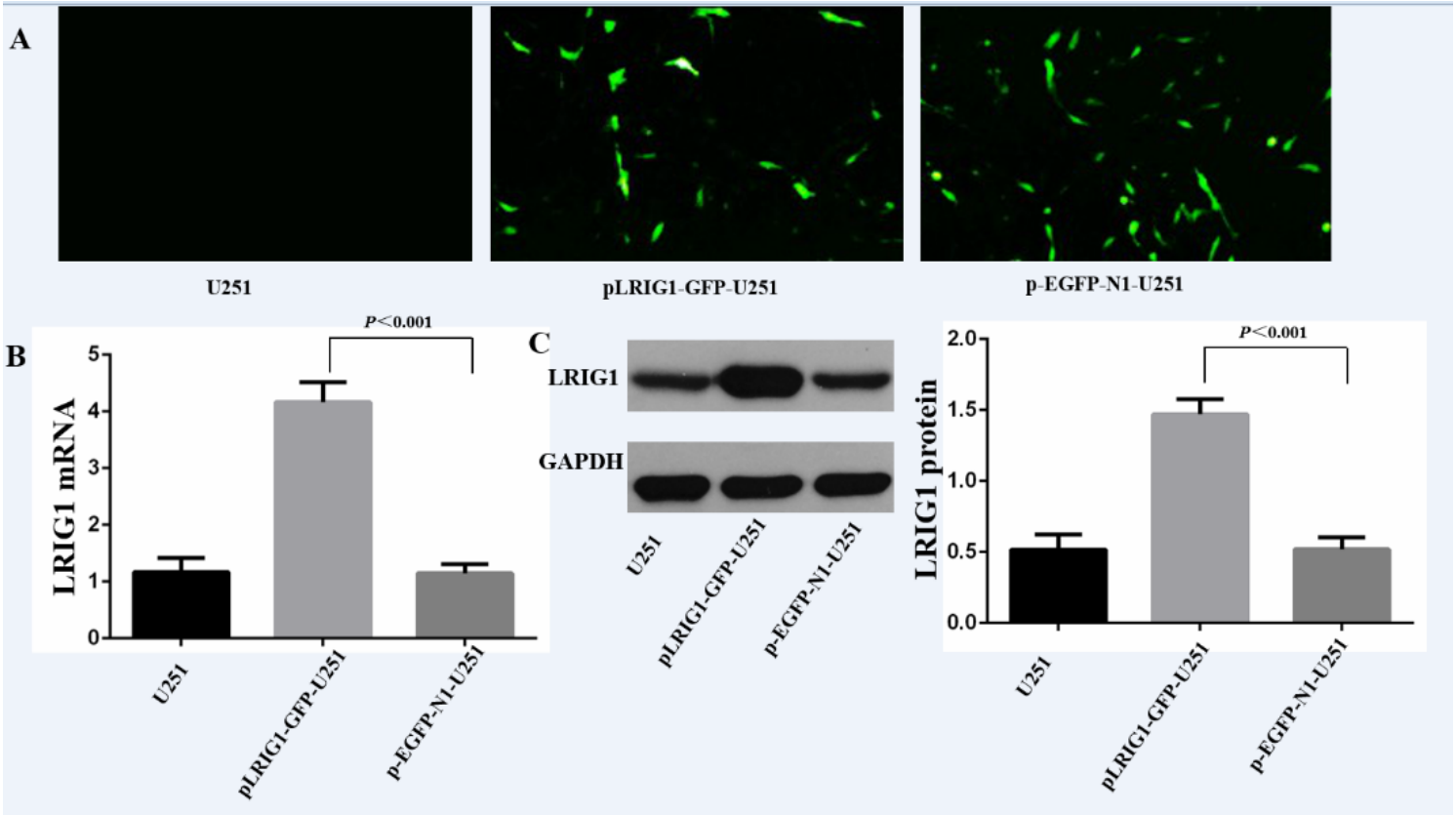

Figure 2

The LRIG1 expression plasmid pLRIG1-GFP and pEGFP-N1 were transfected into U251 cells. (A) 24h after transfection, fluorescent microscopy (488 nm) showed pLRIG1-GFP-U251 cells and p-EGFP-N1 emitted green fluorescence. (B) LRIG1 mRNA and (C) protein level in three groups of cells were evaluated by qRT-PCR and western blot analysis, respectively (T Test, P<0.001). LRIG1 mRNA and protein level of pLRIG1-GFP-U251 group was significantly up regulated and there was no significant difference between p-EGFP-N1-U251 and U251 cells.

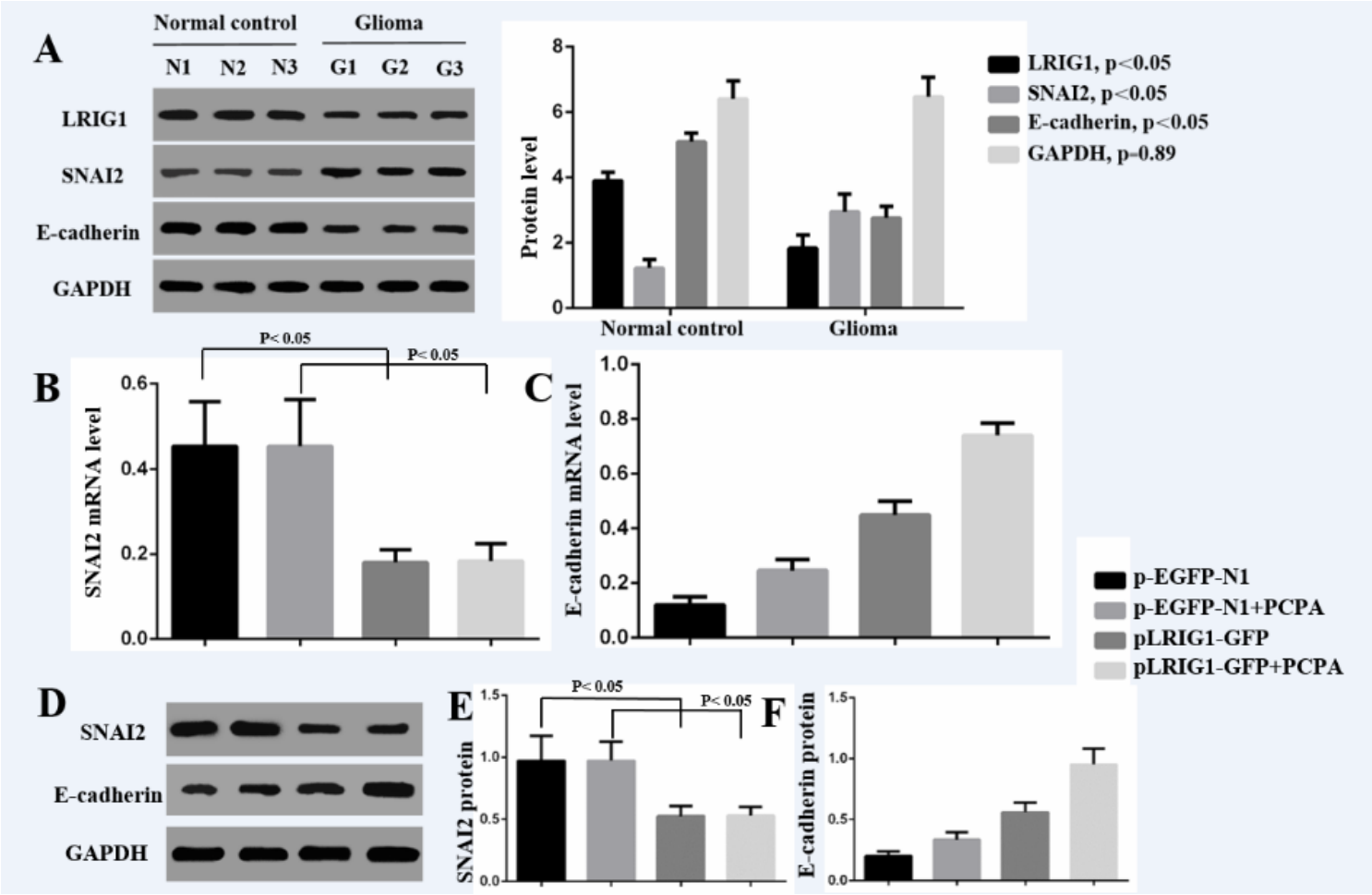

Figure 3

LRIG1 regulates downstream genes SNAI2 and E-cadherin in glioma. (A) LRIG1, SNAI2 and E-cadherin protein level in 30 cases of fresh frozen glioma tissues and 20 cases normal controls were evaluated by western blot analysis. (B) SNAI and E-cadherin mRNA level were evaluated by qRT-PCR in four groups of U251 cells, which were p-EGFP-N1, p-EGFP-N1+PCPA, pLRIG1-GFP, pLRIG1-GFP+PCPA U251 cells, respectively (T Test, $p<0.05$). (C) LRIG1 up regulated E-cadherin mRNA level in glioma, and PCPA has a positive effect on this process (one way ANOVA, $p<0.05$). (D) SNAI2 and E-cadherin protein level in four groups of U251 cells abovementioned. (E) SNAI protein level in four groups of U251 cells abovementioned (T Test, $p<0.05$). (F) LRIG1 up regulated E-cadherin protein level in glioma, and PCPA has a positive effect in this process (one way ANOVA, $p<0.05$).

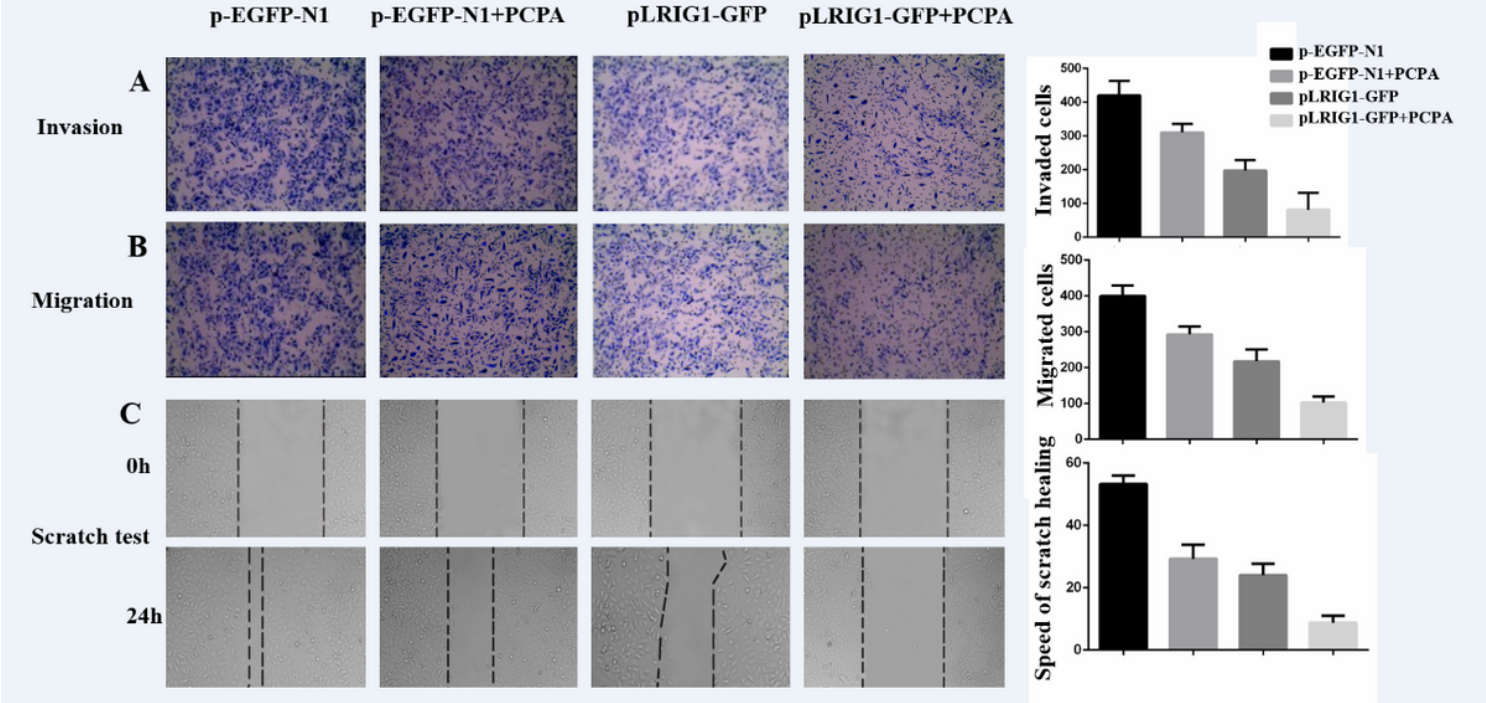

Figure 4

LRIG1 inhibits glioma invasion and migration by regulating SANI2-E-cadherin axis. Invasion (A), migration (B) and speed of scratch healing (C) of four groups of cells were detected by transwell invasion, migration assay and scratch test, respectively. The representative images are shown (magnification, x100), and the invaded, migrated cells and scratch healing speed of each image are presented as the mean ± standard deviation (SD) (one way ANOVA, $P<0.001$).