LRIG2 promotes the proliferation and cell cycle progression of glioblastoma cells in vitro and in vivo through enhancing PDGFRβ signaling

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Abstract. The leucine-rich repeats and immunoglobulin-like domains (LRIG) gene family, comprising LRIG1, 2 and 3, encodes integral membrane proteins. It has been well established that LRIG1 negatively regulates multiple growth factor signaling pathways and is considered to be a tumor suppressor; however, the biological functions of LRIG2 remain largely unexplored. It was previously demonstrated that LRIG2 positively regulates epidermal growth factor receptor (EGFR) signaling, the most common aberrant receptor tyrosine kinase (RTK) signaling in glioblastoma multiforme (GBM), which promotes GBM growth. In the present study, the effect of LRIG2 on the proliferation of GBM cells was further addressed, as well as the possible mechanisms underlying the regulatory effect of LRIG2 on platelet-derived growth factor receptor β (PDGFRβ) signaling, another common oncogenic RTK signaling pathway in GBM. First, the expression levels of endogenous LRIG2 and PDGFRβ were found to vary notably in human GBM, and the LRIG2 expression level was positively correlated with the expression level of PDGFRβ. Furthermore, to the best of our knowledge, this is the first study to demonstrate that LRIG2 promoted the PDGF-BB-induced proliferation of GBM cells in vitro and in vivo through regulating the PDGFRβ signaling-mediated cell cycle progression. Mechanistically, LRIG2 has the ability to physically interact with PDGFRβ, promoting the total expression and the activation of PDGFRβ, and enhancing its downstream signaling pathways of Akt and signal transducer and activator of transcription 3 and the effectors of key regulators of cell cycle progression, resulting in increased GBM cell proliferation. Collectively, these data indicated that LRIG2 may serve as a tumor promoter gene in gliomagenesis by positively regulating PDGFRβ signaling, another important oncogenic RTK signaling pathway, in addition to the previously reported EGFR signaling in GBM modulated by LRIG2, and validated LRIG2 as a promising therapeutic target for the treatment of GBM characterized by multiple aberrant RTK signaling.

Introduction

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor in adults. Despite treatment optimization and improved standard of care, the median survival of patients with GBM is only 14.5-16.6 months (1). Thus, the development of novel therapeutic targets is crucial for the future treatment of GBM.

Over the last decade, substantial efforts have been focused on elucidating the molecular characteristics of GBM, in order to identify molecular subsets as targets for specific targeted therapies, potentially improving the outcome (2). The most common genetic characteristics of GBM are activated oncogenic receptor tyrosine kinase (RTK) signaling pathways via epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR), and inactivated tumor suppressor pathways regulated by retinoblastoma protein and tumor protein p53 (3). Among these, PDGFR signaling plays a key role in the pathogenesis of GBM and represents a target for tyrosine kinase inhibitors (4), whereas PDGFR inhibitors to date have failed to elicit significant responses in patients with GBM. Due to the key role of PDGFR activation in GBM progression and the yet obscure mechanisms underlying the poor clinical outcomes with PDGFR inhibitors, the understanding of the endogenous regulators of PDGFR signaling has been an area of intensive research.
The leucine-rich repeats and immunoglobulin-like domains (LRIG) gene family is composed of three paralogs, namely LRIG1, LRIG2 and LRIG3, which encode a family of integral membrane proteins, with a signal peptide, an extracellular part consisting of 15 leucine-rich repeats (LRR) and three immunoglobulin-like domains, followed by a transmembrane domain and a cytoplasmic tail (5). LRIG proteins have attracted attention as key regulators of growth factor receptors, including receptor tyrosine and serine/threonine kinases (6). LRIG1, the most extensively investigated LRIG family member, is considered to be a tumor suppressor by negatively regulating the signaling pathways mediated by ErbB (7), MET (8) and RET (9) receptor tyrosine kinases. LRIG1 is downregulated and associated with a favorable prognosis in various types of cancer (6,10-15), including glioma (16,17); however, our knowledge on the biological and molecular functions of mammalian LRIG2 in tumors is limited. It has been reported that LRIG2 expression is associated with poor survival in oligodendroglioma (18) and uterine cervical carcinoma (19), and wild-type mice developed PDGFB-induced gliomas at a higher frequency and of higher malignancy compared with LRIG2-deficient mice (20). Over the past decade, our research team has focused on the functions of LRIGs in gliomagenesis, and demonstrated that LRIG2 promotes the growth of GBM by positively modulating EGFR-mediated signaling (21,22), which adds to the evidence supporting the hypothesis that LRIG2 may play an oncogenic role in the progression of glioma, possibly contrary to the role of LRIG1 (23,24); however, more compelling evidence is required to support the concept of LRIG2 as a tumor promoter in GBM and to elucidate the mechanistic insights.

In the present study, the role of LRIG2 in the progression of GBM and the possible underlying mechanisms by which LRIG2 modulates PDGFRβ signaling were investigated. First, the association between the expression levels of LRIG2 and PDGFRβ in human GBM was evaluated. Furthermore, stable GBM cells with LRIG2 overexpression or knockdown were established and the effects of LRIG2 on PDGF-BB-induced proliferation and cell cycle progression were investigated in vitro and in vivo. Finally, the mechanism underlying these effects was explored. To the best of our knowledge, the present study is the first to investigate the effect of LRIG2 on PDGFRβ signaling pathways, and thereby on the proliferation and cell cycle progression of human GBM.

Materials and methods

Cell culture. The U87 human GBM cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). U87 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO₂ at 37°C. According to Allen et al (25), the fact that U87 from ATCC originated from an unknown patient and is not the original U87 established at the University of Uppsala does not affect the authenticity of U87 as a human GBM cell line. Thus, the use of U87 from ATCC in the present study is considered appropriate and the results from the use of U87 as a GBM cell line are not affected.

shRNA-mediated gene knockdown. To knock down LRIG2 expression, a vector-based short hairpin RNA (shRNA) expression system was used. A total of two nucleotide sequences, targeting LRIG2 (NM_014813) nucleotides 451-471 (shRNA1) and 1379-1399 (shRNA2), and one non-silencing scrambled shRNA (scr) were designed and synthesized (Table 1). The shRNA inserts were digested with EcoRI and AgeI prior to ligation into the pLKO.1-TRC cloning vector (Addgene Inc., Cambridge, MA, USA) according to the manufacturer's protocol. The constructed plasmid contained the ampicillin resistance gene for selection of ampicillin-resistant colonies in bacteria. All the inserted sequences were verified by DNA sequencing.

Stable cell transduction. The stable LRIG2-overexpressing U87 cells (U87-LRIG2) and the corresponding control cells (U87-Con) had been established and used previously (22). U87 cells were stably infected with lentivirus expressing pLKO.1-TRC-scr or pLKO.1-TRC-LRIG2shRNA using Lenti-X lentiviral Expression Systems (Clontech Laboratories, Inc., Mountainview, CA, USA) according to the manufacturer's instructions as described previously (22). In brief, 1 day before the transfection, 4x10⁵ 293T cells (ATCC) were seeded in a 100-mm plate and cultured in DMEM containing 10% Tc-free FBS (Clontech Laboratories, Inc.). A total of 3 µg of each plasmid DNA, including the constructed pLKO.1-TRC-scr and pLKO.1-TRC-LRIG2sh, was mixed with Lenti-X HT Packaging Mix (Clontech Laboratories, Inc.) and added to the 293T cell culture medium. After 48 h of incubation, the lentivirus-containing supernatants were harvested and filtered through a 0.45-µm filter (EMD Millipore, Billerica, MA, USA). The Lenti-X qRT-PCR Titration Kit (Clontech Laboratories, Inc.) was used to determine the viral titer. U87 cells were seeded into 6-well plates at a density of 3x10⁵ cells/well and incubated overnight prior to transduction. The viral supernatant was mixed with culture medium containing 4 µg/ml polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), added to the cells and transduced for 24 h. Then, the transduced cells were selected with culture medium containing 1 µg/ml puromycin (Sigma-Aldrich; Merck KGaA) for 2 weeks and the puro-resistant clones that represented possible stably transduced cells were expanded for further experiments.

Patients and tissue samples. A total of 40 GBM samples were obtained by surgical resection from Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China), between 2012 and 2013. The subset of patients with GBM included 24 men and 16 women aged 38-76 years (median age, 58 years). All the patients provided written informed consent for the use of their tissues, and the study was approved by the Institutional Review Board of Tongji Hospital (ID: 20121202). Following surgical resection, fresh tumors were immediately fixed with formalin and embedded in paraffin for routine histopathological evaluation and immunohistochemical staining.

Immunohistochemical staining. The immunohistochemical staining procedures were performed as previously described (22). Briefly, GBM samples from humans and nude mice were fixed in
a phosphate-buffered 4% formaldehyde solution and embedded into paraffin blocks using standard methods. Tissue sections (4-µm) were labeled with the following primary antibodies at the indicated concentrations: Anti-LRIG2 rabbit polyclonal antibody 1:100 (cat. no. ab157452; Abcam, Cambridge, UK), anti-PDGFRβ rabbit monoclonal antibody 1:100 (cat. no. 3169), anti-pDGFRβ rabbit monoclonal antibody 1:50 (cat. no. 4549), anti-pAkt rabbit monoclonal antibody 1:100 (cat. no. 4058), anti-pStat3 rabbit monoclonal antibody 1:100 (cat. no. 9145) (all from Cell Signaling Technology, Inc., Danvers, MA, USA), anti-cyclin B1 rabbit polyclonal antibody 1:100 (cat. no. BA0766), anti-Cyclin D1 rabbit polyclonal antibody 1:100 (cat. no. BA0770) (both from Boster Biological Technology), anti-Ki67 rabbit monoclonal antibody 1:100 (cat. no. ab16667), anti-PCNA rabbit monoclonal antibody 1:100 (cat. no. ab92552) (both from Abcam). The evaluation of the immunostaining was performed by two independent observers in a blinded manner. In brief, positive immunohistochemical staining in tumor cells was evaluated with respect to the intensity of positive staining and the percentage of positive tumor cells. From these scoring data, a four-grade semiquantitative score was defined as follows: 0, no or very faint immunoreactivity (IR) or immunopositive cells <5%; 1, weak IR or immunopositive cells 5-20%; 2, moderate IR or immunopositive cells 26-50%; 3, strong IR or immunopositive cells >50%. A total score based on low intensity and fraction (scored as 0 or 1) vs. high intensity and fraction (scored as 2 or 3) was created for each patient and statistically analyzed.

**Immunofluorescence.** Cells were fixed with 4% paraformaldehyde for 1 h at 4°C, permeabilized with 0.25% Triton X-100 for 5 min, blocked with 10% bovine serum albumin (Boster Biological Technology) for 1 h and labeled with primary anti-LRIG2 1:50 (cat. no. ab157452; Abcam), anti-PDGFRβ 1:100 (cat. no. 3169; Cell Signaling Technology, Inc.), and anti-Flag 1:100 (cat. no. F1804; Sigma-Aldrich; Merck KGaA) antibodies overnight at 4°C, as previously described (22). Following washing with phosphate-buffered saline (PBS), cells were incubated with fluorescein isothiocyanate or cyanine-labeled secondary antibodies (1:100; ProteinTech Group, Inc., Chicago, IL, USA) and subjected to immunofluorescence microscopy using appropriate filters.

**Cell proliferation assay.** In order to examine cellular proliferation, cell counting kit-8 (CCK8) assays and cell count analysis were performed. For CCK8 assays, 5x10^3 cells in 200 µl suspension per well were seeded in triplicate in 96-well flat-bottomed plates and allowed to attach overnight at 37°C. Subsequently, the cells were treated with indicated concentrations of PDGF-BB for indicated times. At the indicated time points, CCK8 (CK04; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to the wells prior to incubation for 2 h at 37°C. The optical density was measured with a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using a wavelength of 450 nm. For cell count determination, U87 GBM cells with LRIG2 overexpression or downregulation and the corresponding control cells were seeded in triplicate at a density of 1x10^5 cells/well in 6-well plates. Similar to the CCK8 assay, cells were allowed to attach, followed by serum starvation with DMEM for 24 h.

**Table I. Oligonucleotide sequences of LRIG2-specific siRNA.**

| Name       | siRNA sequences (5’-3’)          | Target sequence on LRIG2 cDNA |
|------------|----------------------------------|------------------------------|
| shRNA1     | AAATCGGTTGTCTAACTGGGAAC          | 451-471                      |
| shRNA2     | AAGGAAACCAGATTAAGTCAA            | 1379-1399                    |
| Scramble   | CAACAGATGAGAGACCAAC             |                              |

LRIG2, leucine-rich repeats and immunoglobulin-like domain 2.

**Flow cytometric analysis of the cell cycle.** The cells were synchronized to the G0/G1 stage by 24 h of serum deprivation and then cultured in DMEM with 0.5% FBS with or without PDGF-BB for an additional 24 h. Cell cycle analysis was performed by propidium iodide (PI) staining and flow cytometry. In brief, cells were collected and washed twice with cold PBS and resuspended in pre-cooled 70% ethanol at 4°C overnight. Subsequently, fixed cells were collected by centrifugation and incubated with RNase (25 µg/ml) and PI (50 µg/ml) at 37°C for 30 min in the dark. The number of cells in the G0/G1, S and G2/M phases was analyzed by flow cytometry using a FACSCalibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

**Soft agar colony formation assay.** Anchorage-independent cell proliferation was determined by soft agar colony formation assay as previously described (22). Briefly, 6-well plates were first covered with a bottom layer of 0.6% agar made in 10% FBS DMEM and 4x10^4 cells per well were embedded in triplicate into 0.3% top agar gel containing DMEM supplemented with 10% FBS. Cells were fed with 0.5% FBS DMEM with or without PDGF-BB (50 ng/ml) every third day and allowed to grow for 2 weeks. For each well, 30 randomly selected fields were photographed under a microscope (ECLIPSE TS100; Nikon, Tokyo, Japan) with a x10 microscopic lens and a x60 optical lens (magnification, x60). The colonies were then...
counted and the mean number of colonies per field was calculated. Each experiment was performed in triplicate.

In vivo tumor formation in nude mice. All animal procedures were conducted in accordance with the guidelines for animal experimentation, and the animal protocol used in this study was approved by the Institutional Review Board of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (IRB ID: 2011A01). A total of 10 immune-deficient female nude mice (BALB/c), aged 6 weeks and weighing 20-22 g, were purchased from the Wuhan Laboratory Animal Center, bred at the facility of laboratory animals at Tongji Medical College, and randomly assigned to two groups (n=5 per group): U87-scr and U87-LRIG2sh. For subcutaneous inoculation, a total of 2x10^6 cells in 100 µl PBS were injected subcutaneously into the right flank of each mouse as previously described (22). Tumor volume was measured with calipers every 5 days and calculated using the formula: Volume = length x width^2 x (π/6) (length, larger diameter in mm; width, smaller diameter in mm). At the end of the experiment, the mice were euthanized and the tumors were surgically harvested, measured, weighed, fixed in 4% paraformaldehyde overnight and analyzed by immunohistochemistry.

Western blotting and co-immunoprecipitation. Western blotting and co-immunoprecipitation were performed as previously described (22,24). The primary antibodies used in western blotting at the indicated concentrations were as follows: Anti-pPDGFRβ rabbit monoclonal antibody 1:500 (cat. no. 4549), anti-PDGFβ rabbit monoclonal antibody 1:500 (cat. no. 3169), anti-pAkt rabbit monoclonal antibody 1:1,000 (cat. no. 4058), anti-Akt rabbit polyclonal antibody (cat. no. 9273), anti-Stat3 rabbit monoclonal antibody 1:1,000 (cat. no. 9145), anti-Stat3 rabbit monoclonal antibody 1:1,000 (cat. no. 4904) (all from Cell Signaling Technology, Inc.), anti-cyclin D1 rabbit polyclonal antibody 1:500 (cat. no. BA0766), anti-cyclin D1 rabbit polyclonal antibody 1:500 (cat. no. BA0770), β-actin mouse monoclonal antibody 1:1,000 (cat. no. BM0267) (all from Boster Biological Technology). Co-immunoprecipitation of LRIG2 and PDGFRβ were performed using flag-tagged LRIG2-overexpressing U87 cells. Briefly, cells were washed twice with ice-cold PBS and lysed with IP lysis buffer (cat. no. 87877; Thermo Fisher Scientific, Inc.). Lysates were pre-cleared by adding 20 µl of Protein A/G Agarose (cat. no. A1000; Abmart, Berkeley Heights, NJ, USA), followed by overnight incubation with anti-Flag (cat. no. F1804; Sigma-Aldrich; Merck KGaA) or anti-PDGFRβ (cat. no. 3169; Cell Signaling Technology, Inc.) on a rocker at 4°C. Immunocomplexes were captured by incubating with Protein A/G Agarose for 2 h at 4°C, followed by washing with ice-cold lysis buffer to eliminate non-specific interactions. The agarose-bound immunocomplexes were then eluted by boiling the beads in 2X SDS loading buffer (cat. no. P0015B; Beyotime Institute of Biotechnology, Shanghai, China) for 10 min at 100°C. The whole-cell lysates were used as positive controls. In negative controls, primary antibodies used for co-IP were replaced with corresponding isotype IgG controls. Eluted proteins and the input (whole lysates) were subjected to western blotting.

Statistical analysis. Statistical analyses were performed using SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL, USA). The significance of the correlation between LRIG2 and PDGFRβ in the immunohistochemical staining analysis was determined using Pearson’s χ² test. Multiple comparisons were performed using one-way analysis of variance followed by Bonferroni as a post-hoc test. Statistical differences between the two groups were analyzed with the Student’s t-test. Values are expressed as the mean ± standard deviation of at least three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Protein expression level of LRIG2 is positively correlated with the level of PDGFRβ in human GBM. To investigate the correlation between the expression levels of LRIG2 and PDGFRβ in human GBM, the protein expression levels of LRIG2 and PDGFRβ were determined in 40 patient-derived biopsies of GBM, obtained from surgical resections using immunohistochemical staining. According to the total score based on the relative intensity of LRIG2 expression, the patients were categorized into two groups: LRIG2 high expression (scored as 2 or 3) and LRIG2 low expression (scored as 0 or 1). Of the 20 cases in the LRIG2 high expression group, the PDGFRβ IR of 7 cases was defined as low expression and of 13 cases as high expression. Of the 20 cases in the LRIG2 low expression group, the PDGFRβ IR of 14 cases was scored as low expression and of 6 cases as high expression. The difference in PDGFRβ IR was significant between the LRIG2 high expression and low expression groups (P<0.05; Fig. 1 and Table II), which indicated that the protein expression level of PDGFRβ was higher in GBMs with high LRIG2 expression, compared with GBMs with low LRIG2 expression.

Establishment of the stable LRIG2 knockdown GBM cell line. In order to evaluate the role of LRIG2 in GBM, the expression of LRIG2 was downregulated via shRNA mediated-knockdown in U87 cells. A total of three plasmids expressing two nucleotide sequences targeting LRIG2 (shRNA1 and shRNA2) and one non-silencing scramble shRNA (scr) (Table I) were constructed and stably transduced into U87 GBM cells. RT-qPCR and western blotting were used to verify the mRNA and protein expression levels of LRIG2, respectively, in stably transduced cells. Compared with the scramble control cells, LRIG2 transcripts were markedly reduced in the shRNA1- and shRNA2-transduced cells (Fig. 2A). In line with the RT-qPCR results, LRIG2 protein levels were also significantly decreased in the shRNA1- and shRNA2-transduced cells (Fig. 2B). These results indicated that the expression levels of LRIG2 were notably downregulated by LRIG2 shRNAs, and the transduced cells with shRNA1, which exhibited higher knockdown efficiency, were selected for the subsequent experiments. Furthermore, fluorescence microscopy was used to investigate the expression of LRIG2 and PDGFRβ in U87 cells with LRIG2 knockdown by shRNA1. The results demonstrated that the fluorescent intensity of LRIG2 was markedly attenuated in the U87-LRIG2sh cells (Fig. 2C). Notably, the fluorescent intensity of PDGFRβ was also significantly decreased (Fig. 2C), similar to the result of LRIG2 expression being positively correlated with the expression of PDGFRβ in human GBM samples.
LRIG2 promotes PDGF-BB-stimulated proliferation of human GBM cells in vitro. To determine the potential effects of the LRIG2 protein on PDGF-BB-induced cell proliferation of GBM cells, U87 cells were first treated with different concentrations of PDGF-BB for various durations. The CCK8 assay was used to evaluate cell proliferation. Overexpression of LRIG2 was found to markedly promote the PDGF-BB-induced proliferation of U87 GBM cells in a concentration- and time-dependent manner (Fig. 3A and B). Congruently, downregulation of LRIG2 markedly inhibited the PDGF-BB-induced proliferation of U87 cells in a concentration- and time-dependent manner (Fig. 3C and D). Subsequently, we investigated the effects of LRIG2 on the PDGF-BB-induced anchorage-independent growth of U87 cells, a property that mimics tumorigenesis in vivo. Using soft agar colony formation assays, it was observed that, when stimulated with PDGF-BB, LRIG2-overexpressing GBM cells formed more colonies compared with the control cells (Fig. 4A); however, when LRIG2 was downregulated in U87 cells, the effect of LRIG2 on enhancement of PDGF-BB-induced anchorage-independent cell growth was abrogated (Fig. 4B), indicating that the LRIG2 protein enhances the ability of PDGF-BB-stimulated anchorage-independent proliferation in GBM cells.

Furthermore, STI571, the most promising PDGFRβ inhibitor, was used to investigate whether the impact of LRIG2 on the proliferation of GBM cells relies on PDGFRβ RTK regulation. U87 GBM cells with LRIG2 overexpression or downregulation and the corresponding control cells were pretreated with or without STI571 to block PDGFRβ signaling, and then stimulated with or without PDGF-BB for 24 h. As shown in Fig. 3E and F, pretreatment of LRIG2-overexpressing U87 cells with STI571 resulted in marked attenuation of the increased proliferation induced by PDGF-BB; however, when LRIG2 was downregulated, the PDGF-BB-induced proliferation of GBM cells was markedly decreased, and pretreatment with STI571 exerted no significant effect on PDGF-BB-induced proliferation. Taken together, these findings confirmed that the LRIG2 protein promotes PDGF-BB-induced proliferation of human GBM cells in vitro by regulating the activation of PDGFRβ.

Effects of LRIG2 on the PDGF-BB-stimulated cell cycle distribution of GBM cells. To investigate the mechanism underlying LRIG2 promoting the proliferation of PDGF-BB-induced GBM cells, an experiment was performed to assess the effects of LRIG2 on U87 cell cycle progression stimulated by PDGF-BB. The synchronized cells were harvested, cultured in DMEM with 0.5% FBS with or without PDGF-BB for 24 h, and the cell cycle distribution was analyzed by flow cytometry. The results revealed that the percentage of cells in the Go/G1 phase was markedly decreased and the percentage of cells in the S or G2/M phase was markedly increased in the PDGF-BB-induced LRIG2-overexpressing U87 cells compared with the control cells (Fig. 5A). Concordantly, downregulation of LRIG2 caused increased accumulation of cells in the G0/G1 phase and a significantly decreased percentage of cells in the S or G2/M phase (Fig. 5B), which was in line with the results reported previously (21). More importantly, when stimulated with PDGF-BB, LRIG2-knockdown GBM cells exhibited markedly increased accumulation in the G0/G1 phase and a strikingly decreased percentage of cells in the S or G2/M phase compared with the scramble control cells (Fig. 5B). Taken together, these results demonstrated that the LRIG2 protein promoted PDGF-BB-induced DNA synthesis and the G0/G1 to S phase cell cycle transition in GBM cells, resulting in a higher number of cells entering the G2/M phase.

LRIG2 promotes the growth of U87 tumor xenograft through regulating the PDGFRβ signaling pathway in vivo. The aforementioned data confirmed the role of LRIG2 in promoting the proliferation of human GBM cells in vitro; therefore, the present study was further extended in vivo to investigate the effects of LRIG2 knockdown on U87 GBM xenografts, and to explore the possible underlying mechanisms. As mentioned above, stably transduced U87 GBM cells expressing LRIG2 shRNA or scramble RNA were inoculated via subcutaneous injection to the right flank of mice with severe combined immunodeficiency (SCID). Tumor volume was recorded every 5 days from day 15 post-implantation, and the tumor growth curve results demonstrated that tumor growth was markedly

Table II. Protein expression levels of LRIG2 and PDGFRβ in human glioblastoma tissues.

| LRIG2 expression | PDGFRβ expression |
|------------------|-------------------|
| High             | High              |
| Low              | Low               |
|                  |                   |
| LRIG2            | 13                |
| PDGFRββ          | 7                 |
|                  |                   |
| LRIG2            | 6                 |
| PDGFRββ          | 14                |

‘High’ or ‘Low’ indicates the protein expression level based on the total score of immunohistochemical staining. LRIG2, leucine-rich repeats and immunoglobulin-like domain 2; PDGFRβ, platelet-derived growth factor receptor β.

Figure 1. LRIG2 expression levels positively correlates with the expression levels of PDGFRβ in primary glioblastoma tissues. Representative images of immunohistochemical staining of glioblastoma specimens for LRIG2 and PDGFRβ in high-expressing cases and low-expressing cases, respectively (scale bar, 20 μm). LRIG2, leucine-rich repeats and immunoglobulin-like domain 2; PDGFRβ, platelet-derived growth factor receptor β.
inhibited in mice injected with LRIG2-knockdown U87 cells compared with the scramble group (Fig. 6A and B). At the end of the experimental period (day 40), the xenografts were surgically removed, weighed and subjected to immunohistochemical staining for Ki-67 and proliferating cell nuclear antigen (PCNA), two classical markers of cell proliferation. Consistently, the results revealed that tumor weight was also notably lower in tumors with LRIG2 downregulation (Fig. 6B), and the expression levels of Ki-67 and PCNA were both significantly decreased in LRIG2-shRNA-expressing U87 xenograft tumor tissues (Fig. 6C). Combining the effects of LRIG2 knockdown on tumor growth of in vivo, which were in line with the in vitro data, with the previously reported results that overexpression of LRIG2 promoted the growth of GBM xenografts in vivo (22), the evidence supporting a role of LRIG2 in promoting the proliferation of human GBM cells in vivo is compelling.

Based on the aforementioned in vitro results that LRIG2 promoted the PDGF-BB-induced proliferation of GBM cells via regulating the cell cycle distribution, the possible PDGFRβ signaling pathways mediated by LRIG2 was further explored in vivo. Fresh LRIG2-overexpressing and LRIG2-silenced tumor xenografts were subjected to immunohistochemical staining for LRIG2, PDGFRβ and its downstream pathways and effectors. Notably, the expression levels of phosphorylated and total PDGFRβ, as well as its downstream targets, phosphorylated Akt (pAkt) and phosphorylated Stat3 (pStat3), were significantly upregulated in LRIG2-overexpressing GBM xenografts compared with the control xenografts (Fig. 7), whereas the levels of those proteins were markedly decreased in LRIG2-downregulated tumor xenografts (Fig. 7). Furthermore, the expression levels of PDGFRβ downstream effectors cyclin D1 and cyclin B1, a key regulator of the G1/S phase transition and a G2/M regulator, respectively, were positively correlated with the expression levels of the LRIG2 protein in GBM xenografts (Fig. 7), providing a convincing explanation for the effects of LRIG2 on the PDGF-BB-stimulated cell cycle distribution of GBM cells in vitro. Collectively, these results

Figure 2. Establishment of stable U87 glioblastoma cells with LRIG2 knockdown, and the effects of LRIG2 downregulation on the expression level of PDGFRβ. U87 cells were transduced with two LRIG2 shRNAs and one control scr, as indicated. (A) The LRIG2 mRNA expression levels were assessed by reverse transcription-quantitative polymerase chain reaction and (B) the LRIG2 protein levels were examined by western blotting (*P<0.05 vs. scr). (C) Stably transduced cells were subjected to immunohistochemical staining for LRIG2 (green) or PDGFRβ (red), and counterstained with DAPI to show the nuclei (blue). Representative images of three independent experiments are depicted (scale bar, 50 µm). LRIG2, leucine-rich repeats and immunoglobulin-like domain 2; PDGFRβ, platelet-derived growth factor receptor β; shRNA, short hairpin RNA; scr, scrambled shRNA.
demonstrated that LRIG2 played a pivotal role in promoting the growth of GBM xenografts through regulating PDGFRβ signaling and its downstream effectors in vivo.

**LRIG2 physically interacts with PDGFRβ RTK in GBM cells.** Having demonstrated that LRIG2 promotes the growth of GBM and the possible role of LRIG2 in the enhancement of PDGFRβ signaling activation, in order to gain insight into the molecular mechanisms underlying the activation of PDGFRβ by LRIG2, confocal microscopy was performed to evaluate the expression pattern of the two fluorescent proteins in U87 cells. In line with previous studies regarding fluorescent LRIG2 subcellular distribution (17,26), the present study demonstrated via immunofluorescence staining that Flag-tagged LRIG2 was primarily localized in the cytoplasm, and it is noteworthy that the distribution of PDGFRβ fluorescent signals mirrored the LRIG2 distribution (Fig. 8A), indicating a colocalization of LRIG2 and PDGFRβ expression in GBM cells. Based on the co-expression pattern and the similar protein structure of LRIG2 and PDGFRβ, both containing extracellular immunoglobulin loops and an intracellular tail (27), it is reasonable to hypothesize that LRIG2 may physically interact with PDGFRβ in GBM cells. To confirm this hypothesis, the Flag-tagged LRIG2 was precipitated from the cell lysates of LRIG2-overexpressing cells using an anti-Flag antibody. Coprecipitation of PDGFRβ was observed by probing the resulting blot with anti-PDGFRβ, and when PDGFRβ was immunoprecipitated from the cell lysates, coprecipitation...
of the Flag-LRIG2 was also detected by western blotting with the anti-Flag antibody (Fig. 8B), indicating that LRIG2 physically associates with PDGFRβ. In conclusion, the results presented in Fig. 8 indicate that LRIG2 physically interacts with the PDGFRβ RTK in GBM cells, which may result in the enhancement of PDGFRβ signaling activation.

**LRIG2 promotes PDGF-BB-induced activation of PDGFRβ RTK and the downstream Akt and Stat3 signaling pathways and effectors.** Subsequently, the physical association of LRIG2 and PDGFRβ prompted an investigation into whether LRIG2 can modulate the ligand-dependent activation of the PDGFRβ receptor and its downstream signaling. Stably transduced U87 cells with LRIG2 overexpression or LRIG2 knockdown were synchronized for 24 h and then treated with or without various concentrations of PDGF-BB for a further 24 h. As depicted in Fig. 9A, the western blotting results demonstrated that the total expression level and the phosphorylation of PDGFRβ following stimulation with PDGF-BB (the primary activator of PDGFRβ) were significantly increased in LRIG2-overexpressing cells and drastically reduced in LRIG2-knockdown cells, as compared with the corresponding control cells. Consistently, the levels of PDGF-BB-induced phosphorylation of Akt and Stat3, two key downstream signaling pathways of PDGFRβ, were markedly increased in LRIG2-overexpressing cells, resulting in substantial accumulation of PDGF-BB-induced downstream effectors of cyclin B1 and cyclin D1, which are key regulators of cell cycle progression (Fig. 9A). Notably, when LRIG2 was downregulated in GBM cells, the PDGF-BB-induced activation of Akt, Stat3 and the downstream effectors of cyclin B1 and cyclin D1 was markedly attenuated (Fig. 9A). To further substantiate the role of LRIG2 in the regulation of PDGFRβ signaling pathways, STI571, the most promising PDGFRβ inhibitor, was used to inhibit the phosphorylation of PDGFRβ prior to detecting the aforementioned downstream pathways. Cells were serum-starved for 24 h and then treated with STI571 for 2 h prior to a 10-min exposure to PDGF-BB, and the total cell lysates were subjected to western blotting. As expected, the expression level of total PDGFRβ was increased and the activation of PDGFRβ, as well as its downstream targets Akt and Stat3, was significantly enhanced following a 10-min stimulation with PDGF-BB in LRIG2-overexpressing cells, whereas the PDGFRβ expression level was decreased and the activation of PDGFRβ, Akt and Stat3 was attenuated in cells with LRIG2 knockdown (Fig. 9B). Notably, the effects of LRIG2 overexpression or knockdown on PDGF-BB-induced enhanced or attenuated activation of Akt and Stat3 were significantly abrogated following the inhibition of PDGFRβ phosphorylation by STI571 (Fig. 9B), which demonstrated that LRIG2 played a pivotal role in the regulation of PDGF-BB-induced PDGFRβ activation and its downstream pathways. Taken together, the aforementioned results provide compelling evidence supporting that LRIG2
physically interacts with PDGFRβ, leading to stabilization and activation of PDGFRβ and enhancing the downstream Akt and Stat3 pathways, ultimately resulting in accumulation of the downstream pro-proliferative effectors of cell cycle proteins to promote the proliferation of GBM cells.

Discussion

In the present study, it was demonstrated that LRIG2, the least investigated and documented member of the LRIG gene family, is positively correlated with the expression of PDGFRβ RTK in human GBM, and markedly promoted the PDGF-BB-induced growth of GBM cells *in vitro* and *in vivo* through modulating PDGFRβ activation and its downstream signaling pathways and effectors of cell cycle progression. These data indicated that LRIG2 exerts its pro-tumor effects on GBM cells by positively regulating PDGFRβ signaling, another important aberrant oncopgenic RTK signaling modulated by LRIG2 in GBM, similar to our previously reported EGFR signaling (22).

GBM, the most common and lethal type of malignant primary brain tumor (1), is a devastating and intractable disease with a poor outcome. A hallmark of malignant glioma is activation of aberrant RTK signaling pathways, most commonly caused by EGFR amplification/mutation or PDGFR amplification/overexpression (28,29), the genomic alterations of which ultimately lead to enhanced tumor cell proliferation, increased transition through the cell cycle and resistance to treatment. PDGFRs are well-characterized RTKs in GBM that have been found to be overexpressed in human gliomas of all grades, are most highly expressed in GBM, and are closely associated with GBM initiation and progression (20,28). Similar to previous studies (30), we observed that overexpression of PDGFRβ was a frequent event.

Figure 5. Effects of LRIG2 on PDGF-BB-induced cell cycle distribution. Synchronized U87 glioblastoma cells with (A) LRIG2 overexpression or (B) LRIG2 knockdown were treated with or without PDGF-BB (50 ng/ml) for 24 h, then stained with propidium iodide and analyzed for cell cycle distribution by using flow cytometry. Three independent experiments were performed and a representative plot is displayed. The percentage of cells in the G0/G1, S and G2/M phases was quantified and plotted. Data are expressed as the mean ± standard deviation of three independent experiments (*P<0.05, **P<0.01). LRIG2, leucine-rich repeats and immunoglobulin-like domain 2; PDGF, platelet-derived growth factor.
in human GBM. To the best of our knowledge, this is the first study to demonstrate that the expression levels of PDGFRβ vary and are positively correlated with the expression levels of LRIG2 in human GBM, indicating that the inter-individual variation in PDGFRβ expression may be determined by the expression pattern of LRIG2, and PDGFRβ expression may be positively regulated by LRIG2 in human GBM, which was demonstrated in vitro and in vivo in the present study. Congruently, Rondahl et al. (20) used animal models of PDGFB-induced glioma to demonstrate that Lrig2E12−/− mice, generated by the ablation of Lrig2 exon 12, developed lower-grade tumors (77%) or had no detectable tumors (23%), and Lrig2E12+/− mice developed lower-grade tumors (82%) or high-grade GBM-like tumors (18%). The Lrig2E12−/− mice developed PDGFB-driven gliomas at a higher frequency and of higher malignancy compared with Lrig2E12+/− mice (20), suggesting a key promoting role of Lrig2 in the regulation of PDGF signaling and in the development and/or progression of glioma, particularly in mice, consistently with the findings of the present study, which demonstrated that LRIG2 positively regulates PDGFRβ signaling in human GBM. Furthermore, the present results, which indicated that LRIG2 is positively correlated with PDGFRβ in human GBM, are also in line with those of a previous study reporting that LRIG2 expression is associated with poor survival of patients with oligodendrogliaoma, in which dysregulated PDGFR signaling is a common feature (18). To the best of our knowledge, this is the first study demonstrating a positive association between LRIG2 and PDGFR signaling in human GBM, providing compelling evidence in support of the hypothesis that LRIG2 serves as a tumor promoter in human glioma, which is distinct from the functions of LRIG1 (23,24) and LRIG3 in glioma (6). Furthermore, in order to provide further evidence to support the concept that LRIG2 acts as a tumor promoter by positively regulating PDGFRβ signaling in GBM, PDGF-BB-induced biological effects and signaling events were analyzed in GBM...
cells with LRIG2 overexpression or downregulation. The pathogenesis of GBM is complex, due to a highly deregulated tumor genome with a network of interconnected signaling pathways of three steps: i) An input step in which membrane receptors are triggered from the signals outside the cell; ii) a core system in which protein kinases transmit the signal to the nucleus; and iii) an output step in which transcription factors regulate the genes that affect various cellular functions (29). Congruently, LRIG2 initially plays a critical role in the first input step by promoting the PDGF-BB-induced activation of PDGFRβ, followed by enhancing the activation of Akt kinases and the Stat3 transcription factor, the core system of the second step, ultimately resulting in the output step, in which the pro-proliferative cell cycle proteins are accumulated and the uncontrolled proliferation of GBM cells is promoted. Due to the modulation of PDGFRβ signaling by LRIG2 upstream and the importance of abnormal PDGFRβ signaling in gliomagenesis, it is reasonable to propose that LRIG2 fits the role of a single critical oncogene, the antagonism of which should abrogate the important downstream deregulated signaling cascades and interrupt cell proliferation. It is well documented that deregulation of the phosphoinositide 3-kinase/Akt pathway is an obligate event frequently observed in gliomagenesis (2), with Akt activation reported in ~80% of human GBMs (31). Stat3 is also aberrantly activated in human GBM tissues and serves as a ‘molecular hub’ connecting extracellular signals to the transcriptional control of critical cellular events in gliomagenesis, including cell cycle progression, proliferation, angiogenesis and immune evasion (32). Of note, our findings demonstrated that LRIG2 positively regulated

Figure 7. LRIG2 enhances the activation of PDGFRβ and regulates its downstream pathways and effectors in vivo. A total of four groups of indicated stable U87 glioblastoma cells (U87-LRIG2, LRIG2-overexpressing; U87-Con, control U87 cells; U87-LRIG2sh, LRIG2-knockdown cells; and U87-scr, scramble U87 cells) were injected subcutaneously into the flanks of mice with severe combined immunodeficiency. At the end of the animal experiments, tumor xenografts were isolated and immunohistochemistry was performed to evaluate the expression of LRIG2, PDGFRβ and its downstream pathways, as indicated. Representative images are depicted (scale bars, 20 µm). LRIG2, leucine-rich repeats and immunoglobulin-like domain 2; PDGFRβ, platelet-derived growth factor receptor β.
Akt and Stat3 pathways to promote cell cycle progression and proliferation of GBM cells by modulating PDGFRβ activation. However, Rondahl et al. (20) reported that Lrig2 exerted no effects on PDGFR protein levels or the phosphorylation events.
of PDGFR and Akt in mouse embryonic fibroblasts (MEFs), which was distinct from the results of the present study. Due to the fact that the experiments by Rondahl et al were carried out in MEFs, rather than in tumor cells or GBM cells, we hypothesized that the positive regulation of LRIG2 on PDGFRβ and its downstream pathways may be prominent in GBM cells or other tumor cells, but subtle in non-tumor cells, highlighting that the role of LRIG2 in the modulation of PDGFR signaling is oncogenic and tumor-specific.

In addition to the pivotal role of LRIG2 in the positive regulation of PDGFR signaling in GBM, we previously demonstrated that LRIG2 also positively regulated EGFR signaling, another common dysregulated RTK signaling pathway in GBM, to exert its pro-tumor effects on GBM (21,22), indicating that LRIG2 may play a key role in the genesis and progression of GBM by positively regulating EGFR and PDGFR signaling, the most commonly dysregulated RTKs in GBM. Over the past decade, GBM research has primarily focused on determining and investigating specific inhibitors targeting key RTK signaling pathways, in order to develop novel and effective therapeutic strategies to improve the prognosis of GBM. However, accumulating clinical trial data to date, evaluating RTK inhibitors, including EGFR tyrosine kinase inhibitors (TKIs) and PDGFR TKIs, support the conclusion that RTK inhibitors are not beneficial for patients with GBM and the underlying mechanisms have not yet been fully elucidated (33). It has been demonstrated that multiple RTKs may be activated simultaneously in GBM cells (34,35), which is a plausible explanation for the disappointing effects of RTK inhibitors, in that targeting specific RTK signaling may result in compensatory activation of alternative signaling mediators. Our determination of the positive regulation of LRIG2 on EGFR and PDGFR, the most common dysregulated RTKs in GBM, not only improved our understanding of the endogenous regulatory mechanisms of EGFR and PDGFR, but also provided one potential explanation for the limited clinical responses to anti-EGFR or anti-PDGFR therapies using corresponding inhibitors, which is that targeting specific RTK signaling may confer activation of alternative RTK signaling through LRIG2, the key mediator of multiple RTK signals. Our data provide the rationale for targeting LRIG2 as a strategy to achieve the dual inhibition of EGFR and PDGFR signaling in GBM, highlighting the possibility of targeting LRIG2 as a promising therapeutic strategy for the future treatment of GBM.

To the best of our knowledge, this is the first study to demonstrate that LRIG2 positively regulates multiple RTKs, including PDGFRβ, EGFR (22) and other RTKs in GBM (data not published). The role of LRIG2 in the positive regulation of RTKs in glioma is contrary to the role of LRIG1, which has been well documented to negatively regulate multiple RTKs in glioma (6,23,24). LRIG1 has been demonstrated to restrict RTK signaling through different mechanisms, such as enhancing the degradation of EGFR in a ligand-induced cbl-mediated ubiquitylation process (7,36), destabilizing the MET receptor in a lysosome-dependent and cbl-independent manner (8), and inhibiting glial cell-derived neurotrophic factor binding with RET (9). Little is known regarding the mechanisms through which LRIG2 positively regulates RTKs. In the present study, it was demonstrated that LRIG2 could physically interact with PDGFRβ in the cytoplasm and increase the PDGF-BB-induced total expression level and phosphorylation of PDGFRβ, which was in line with the effects of LRIG2 on EGFR (22). We hypothesized that the physical interaction of LRIG2 with RTKs may stabilize the expression level of RTKs in GBM cells; however, the critical questions of how LRIG2 stabilizes RTKs, and why the function of LRIG2 as a tumor promoter is largely distinct from that of LRIG1 in glioma, remain unanswered and require further investigation.

Collectively, it was demonstrated that LRIG2 promoted cell cycle progression and proliferation of GBM cells in vitro and in vivo by positively regulating the PDGFRβ signaling pathways, a critical aberrant oncogenic RTK signaling modulated by LRIG2 in GBM, similar to the previously reported effects on EGFR signaling. Targeting LRIG2, the key oncogenic regulator of multiple RTKs signals, appears to be promising for developing novel therapeutic strategies for the future treatment of GBM.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

QX performed the cell biology experiments, is responsible for the animal experiments, and drafted the manuscript. MD and WZ contributed to western blotting experiments. FC and KW collected the fresh human GBM samples. FM performed the immunohistochemistry experiments. HW and RX carried out the statistical analyses. BW, TL and DG designed the experiments and critically revised the manuscript. All the authors provided final approval for the submission.

Ethics approval and consent to participate

Collection and use of patient tumor tissue samples was approved by the local ethics committees (Institutional Review Board, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, IRB ID: 20121202) and the consents of all the patients were written and approved. All animal experiments were conducted in accordance with the institutional animal research guidelines approved by local Ethics Committee (Institutional Review Board of Experimental Animals, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, IRB ID: 2011A01).

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.
Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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