Int J Clin Exp Med 2016;9(2):3441-3448
www.ijcem.com /ISSN:1940-5901/IJCEM0014369

Original Article

LRIG1-modulated invasion and metastasis of human gliomas through regulation of SNAI2 and E-cadherin

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Received August 12, 2015; Accepted November 13, 2016; Epub February 15, 2016; Published February 29, 2016

Abstract: The molecular mechanism that activates invasion and metastasis of human gliomas remains obscure. Although several studies have implicated the function of LRIG1 in the inhibition of the invasion, the downstream genes coordinating this process are poorly known. Therefore, we prepared a full-length expression vector to overexpress LRIG1 in the U251 malignant glioma cell line and simultaneously we used small interfering RNA (siRNA) to block LRIG1 gene in the U251 malignant glioma cell line. Introduction of exogenous LRIG1 into glioma cells inhibited cell invasion and metastasis. Silencing of LRIG1 promoted cell invasion and metastasis as manifested by transwell assay, and transwell migration assay *in vitro*. On the other hand, with qRT-PCR and western blot, LRIG1 overexpression inhibited cell invasion and metastasis, leading to, leading to reduced SNAI2 expression and elevated Elevated E-cadherin expression. By contrast, knockdown of endogenous LRIG1 promoted cell invasion and metastasis, leading to, leading to reduced E-cadherin expression and elevated Elevated SNAI2 expression. Our data suggested that LRIG1 as a tumor suppressor restricted glioma invasion and metastasis by regulating SNAI2 and SNAI2 levels. Restoration of LRIG1 in glioma cells could be a novel therapeutic strategy.

Keywords: LRIG1, gliomas, SNAI2, E-cadherin, invasion, metastasis

Introduction

Generally speaking, malignant gliomas invade the surrounding part of the central nervous system or migrate to other tissue and organs, implying extremely poor prognosis, in spite of being treated with surgery, radiotherapy, chemotherapy and immunotherapy. Glioma cell invasion and metastasis depend on multiple factors such as growth factors, extra-cellular matrix (ECM) molecules, and the activity of intracellular pathways regulating cell motility [1]. Leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) is a Type 1 transmembrane protein whose extracellular domain contains 15 leucine-rich repeats (LRRs) and three immunoglobulin (Ig)-like domains. Recently, LRIG1 was cloned and characterized as a putative tumor suppressor gene often downregulated in many human tumors like gliomas [2]. Its precise role and potential molecular mechanisms remain unknown although several studies have implicated the function of LRIG1 as inhibiting the glomas invasion and metastasis.

Recent studies have revealed the existence of several transcription factors and intracellular molecules that promote glioma metastasis and invasion in human cancers such as head and neck cancers [3]. The transcription factors we studied are SNAI2 and E-cadherin.

The SNAI2 gene encoding Slug-which is an oncogenic transcriptional repressor acting as a master regulator of cell metastasis in various tissues-has been found overexpressed in numerous cancers including lung cancer, leukemia, colorectal cancer, esophageal cancer, prostate cancer, breast cancer and ovarian cancer [4]. It acts as an inducer of the epithelial to mesenchymal transition (EMT), which mediates metastasis and invasion of tumor cells [5]. A latest research has identified SNAI2/Slug as a regulator of celluar growth and invasion

in human gliomas [4], but its precise role and potential molecular mechanisms remain obscure.

Cadherin family are a group of calcium-dependent cell-surface adhesion molecules involved in intercellular adhesion that reorganizes the actin cytoskeleton via interaction with the catenins [6]. Dysregulation of the cadherin/catenin assembly has been implicated in various cancers such as gliomas [6], pancreatic cancer [7], gastric cancer [8], colorectal cancer [9], breast cancer [10] and oropharyngeal squamous cell cancer [11]. Modulation of the cadherin/catenin system plays a role in cell motility, a key step of malignant tumor cell invasion and metastasis. E-cadherin is the major subtype of cadherin family expressed in epithelial tissues. Shortage of E-cadherin directly leads to the loss of epithelial phenotypes, including the change of cell polarity and the loss of specialized cell-cell contacts. The epithelial cell acquires a migratory function which enables it to move away from the cell community and to integrate into the surrounding tissue through this transition [12].

Despite the evidence that LRIG1 is involved in proliferation [13], apoptosis [14] and drug-resistance [15] of gliomas cells, LRIG1's role in human gliomas cells and its molecular mechanisms have not yet been identified. We found in our study that the upregulation of LRIG1 inhibited invasion and metastasis of gliomas cells, leading to, leading to reduced SNAI2 expression and elevated E-cadherin expression. We also found that downregulation of LRIG1 promoted the invasion and metastasis, leading to elevated SNAI2 expression and reduced E-cadherin expression.

Materials and methods

Cell culture and transfection

The human glioma cell line U-251 MG was kindly donated by Dr Baohui Liu (Dept. of Neurosurgery, Renmin Hospital of Wuhan University) and 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% CO_2 at 37°C. Normally, the medium was replaced every 2 days or 3. Cells were passaged every 5 days or 6 and routinely examined.

Lipofectamine 2000 (Invitrogen, USA) was used according to the manufacturer's instructions. U251 cells were transfected with expression vector pLRIG1-GFP that encodes an LRIG1-GFP fusion protein or pEGFP-N1 (Clontech) as vector control encoding GFP. Cell clones resistant to G418 were ring-cloned and amplified for further experiments.

For LRIG1 knockdown, siRNA (5'-ACTCTCTGAGATTGACC CT-3') were constructed and transfected into U251 cell using LipofectamineTM 2000 (Invitrogen) according to the manufacturer's instructions. On the other hand, siRNAs (5'-ACTACCGTTG TTATAGGTG-3') were used as negative controls [16]. To enable the selection of kanamycin-resistant colonies in bacteria and G418-resistant clones in mammalian cells, each constructed plasmid contained the neomycin/kanamycin-resistance gene and all the inserted sequences were verified by DNA sequencing. LRIG1 knockdown was identified and confirmed at the mRNA and protein levels.

RNA isolation and qRT-PCR

Total RNAs from cultured cells were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. As described earlier, qRT-PCR was used to evaluate the expression levels of mRNAs. 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'-ACACCGAAGTGGACTGTTACTCC-3' and antisense 5'-CCGGGTG ATACAACCTTGCT-3'; SNAI2 sense 5'-ACACCGAAGTGGACTGTTA CTCC-3' and antisense 5'-CCCAGGCTCACATATTCCTTGT-3'; E-cadherin sense 5'-ACTGGTGCCATTTCCACTCG-3' and antisense 5'-ATAGTTCGAGGTTCTGGTATGGG-3'; actin sense 5'-GTCCACCG CAAATGCTTCTA-3' and antisense 5'-TGCTGTCACCTTCACCGTTC-3'.

Western blot

Total protein was extracted using RIPA buffer supplemented with proteinase inhibitors. Glioma cells were scraped off into pre-cold RIPA buffer (Beyotime Biotech, Nantong, China) for 10 min. All subsequent manipulations were

performed in ice. Protein extracts were then separated by gel electrophoresis using 8% SDS-PAGE-Tris-HCl gels. The proteins were transferred to nitrocellulose membranes and detected using a specific antibody. Immunoreactive bands were visualized using the enhanced chemiluminescence system after being rinsed and incubated in the appropriate secondary antibody. The primary and secondary antibodies used in this study were as follows: monoclonal rabbit anti-GAPDH (1:10000; Abcam, Cambridge, USA); polyclonal rabbit anti-LRIG1 (1:1000; 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); HRP labelled goat anti-rabbit IgG (1:10000; Santa Cruz Biotechnology, USA).

Cell metastasis and invasion assay

The transwell migration assay with or without a matrigel coating was performed as described previously [17].

Statistical analysis

Relationships between mRNA levels, protein expression and the invasion or metastasis ability of glimoma cells were compared using Student's t-test. GraphPad Prism version 5.00 software for Windows (GraphPad, La Jolla, CA, USA) was used to analyze the experiment, and the data were expressed as the means ± standard deviation (SD) and statistical significance defined as a P value <0.05.

Results

Vector expressing LRIG1 caused effective up-regulation of LRIG1 expression

U251 cells were transfected with vector pLRIG1-GFP and pEGFP-N1 were used as vector control. Cell clones resistant to resistant G418 possible were ring-cloned and amplified for further experiments. The mRNA and protein levels of LRIG1 and its housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were measured by quantitative real-time PCR and western blotting. LRIG1 mRNA level was increased by 4.6 times in the PEGFP-LRIG1-U251 cells, compared with the control PEGFP-N1-U251 cells (**Figure 1A**). In line with the real-time PCR results, the level of the LRIG1 protein was increased by 3.6 times in the in the PEGFP-LRIG1-U251 cells (**Figure 1D** and **1G**).

The specific anti-LRIG1 siRNA caused effective LRIG1 knockdown

A specific anti-LRIG1 siRNA and a negative control siNRA were constructed and transfected into U251 cells. The mRNA and protein levels of LRIG1 and GAPDH were measured by quantitative real-time PCR and western blotting. LRIG1 transcripts were reduced by 50.2% in the si-LRIG1-U251 cells, compared with the negative control si-NC-U251 cells (**Figure 1A**). The level of the LRIG1 protein expression was reduced by 64.7% in the si-LRIG1-U251 cells, in comparison with si-NC-U251 cells (**Figure 1D** and **1G**).

Modulation of LRIG1 expression was associated with changes in SNAI2-E-cadherin signaling

In this study, we further investigated the effect of LRIG1 knockdown or overexpression in the downstream signaling pathways of SNAI2-E-cadherin signaling. We found that LRIG1 overexpression would lead to reduced SNAI2 expression and elevated E-cadherin expression, and that knockdown of endogenous LRIG1 would lead to reduced E-cadherin expression and elevated SNAI2 expression at the mRNA level, as shown in quantitative real-time PCR and by western blotting. Compared with PEGFP-N1-U251 cells, SNAI2 in PEGFP-LRIG1-U251 cells was reduced by 57.1% on mRNA level (**Figure 1B**) and 66.2% at protein level (**Figure 1E** and **1G**); E-cadherin was increased by 2.9 times at mRNA level (**Figure 1C**) and 3.1 times at protein level (**Figure 1F** and **1G**). Compared with si-NC-U251 cells, SNAI2 in si-LRIG1-U251 cells was increased by 4.3 times at mRNA level (**Figure 1B**) and 3.4 times at protein level (**Figure 1E** and **1G**); E-cadherin was reduced by 66.9% on mRNA level (**Figure 1C**) and 65.6% on protein level (**Figure 1F** and **1G**).

Modulation of LRIG1 expression affects the invasion and metastasis of U251 cells

To validate the effects of LRIG1 on the invasion and metastasis of U251 cells in vitro, we employed transwell assay and transwell migration assay respectively. A reduction of invasion and metastasis was noticed in the LRIG1 overexpressed groups compared with control cells (**Figure 2A**). The number of migrating cells per

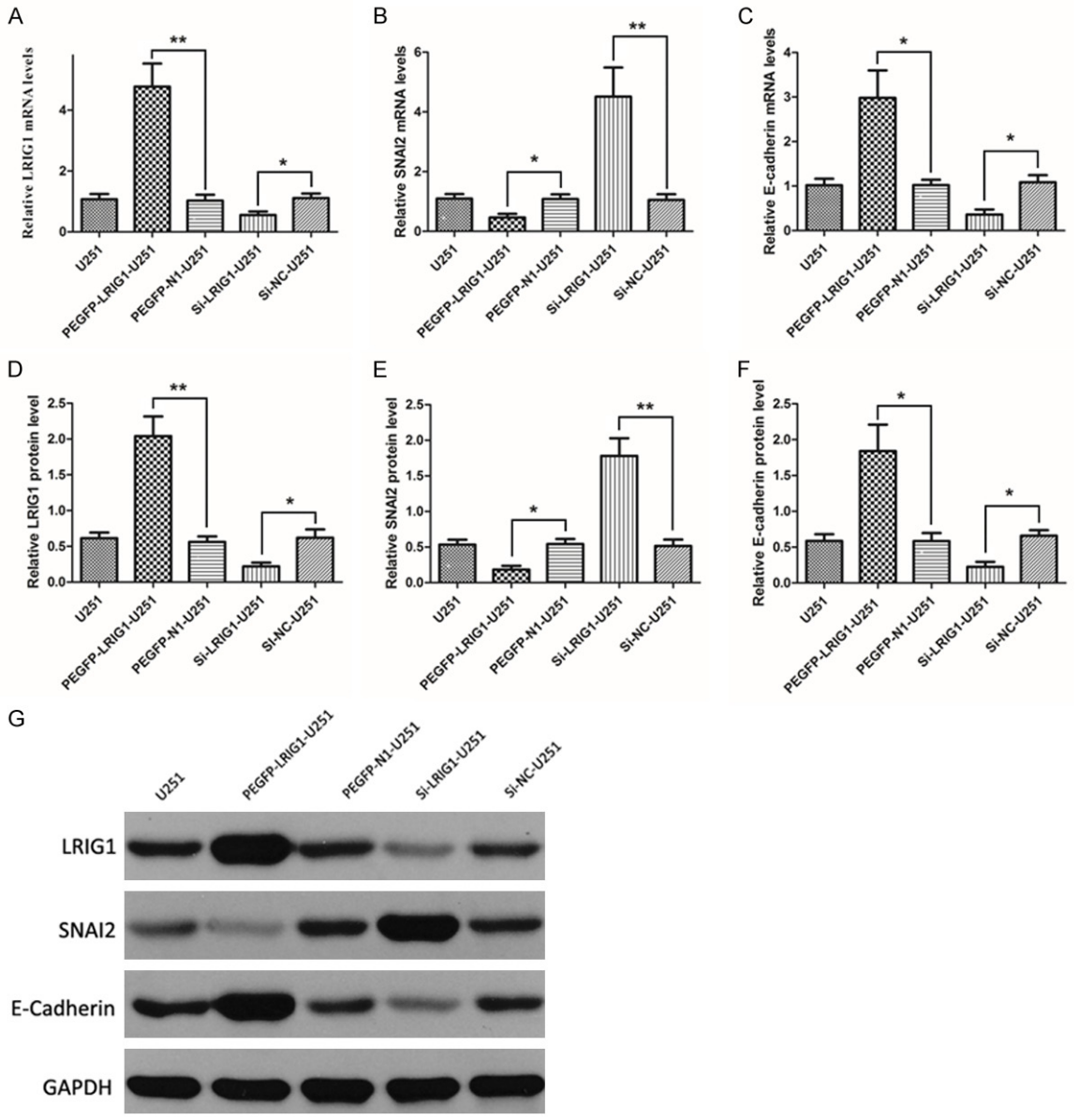

Figure 1. Effect of LRIG1 overexpression or silence on SNAI2 and E-cadherin. A. U251 cells were transfected with PEGFP-LRIG1 and negative control construct or knocked down by Si-LRIG1 and negative control construct. LRIG1 mRNA was quantified by real-time RCR. LRIG1 mRNA level was increased 4.6 times in the PEGFP-LRIG1-U251 cells and reduced by 50.2% in the si-LRIG1-U251 cells. B. SNAI2 was reduced by 57.1% in PEGFP-LRIG1-U251 cells and raised 4.3 times in Si-LRIG1-U251 cells on mRNA level. C. E-cadherin was raised 2.9 times in PEGFP-LRIG1-U251 cells and reduced by 66.9% in Si-LRIG1-U251 cells on mRNA level. D. LRIG1 protein was quantified by Western blot. LRIG1 protein level was increased 3.6 times in the PEGFP-LRIG1-U251 cells and reduced by 64.7% in the si-LRIG1-U251 cells. E. SNAI2 was reduced by 66.2% in PEGFP-LRIG1-U251 cells and raised 3.4 times in Si-LRIG1-U251 cells on protein level. F. E-cadherin was raised 3.1 times in PEGFP-LRIG1-U251 cells and reduced by 65.6% in Si-LRIG1-U251 cells on protein level. G. The expression of LRIG1, SNAI2 and E-cadherin was quantified by Western blot. Data are presented as the means ± SD (*P<0.05, **P<0.01).

field was 183.0±11.9 in the PEGFP-LRIG1-U251 group and 376.7±14.5 in the PEGFP-N1-U251 group (**Figure 2B**). In proportion the number of invading cells was 210.3±17.3 in PEGFP-LRIG1-U251 group and 376.0±15.8 in control group (**Figure 2C**). In further study we knocked down the LRIG1 of U251 cells and the invasion and metastasis of Si-LRIG1-U251 cells have risen significantly (**Figure 2A**). The number of migrating cells per field was 443.3±15.3 in the

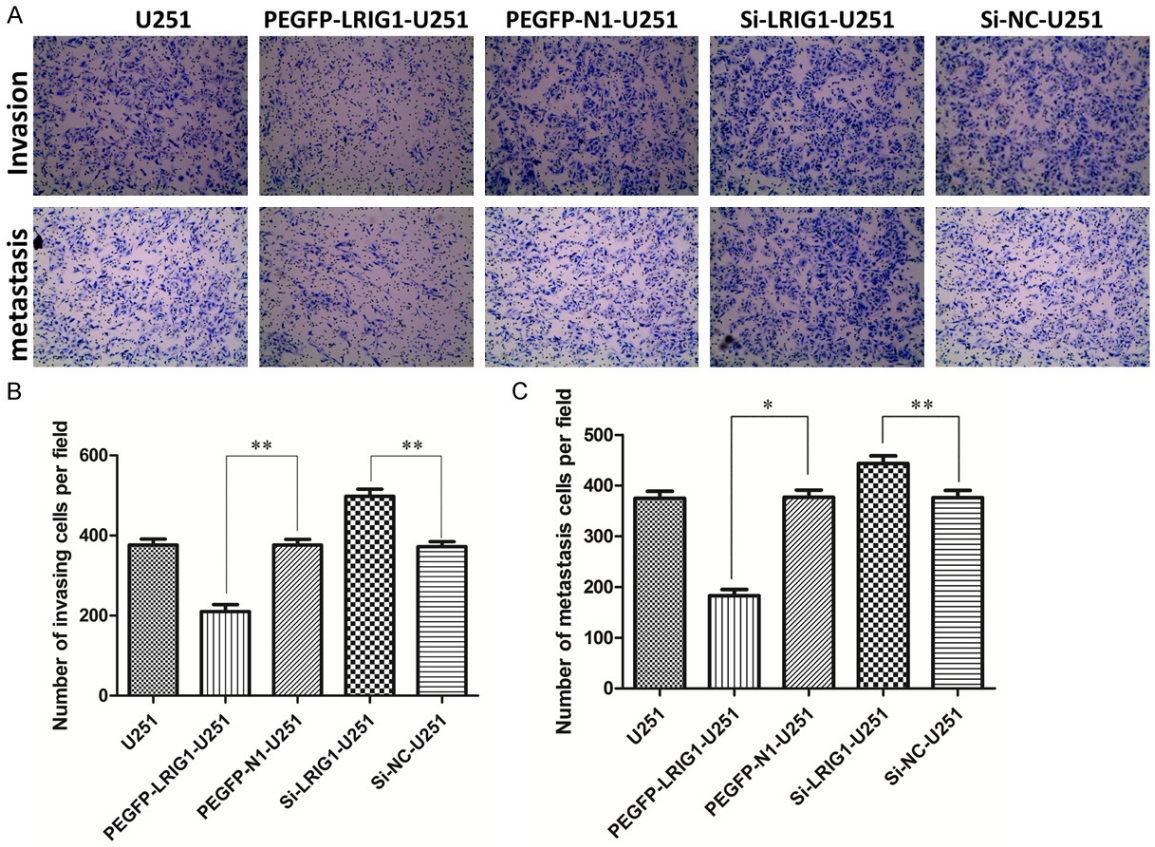

Figure 2. Effect of LRIG1 overexpression or silence on invasive or metastasis capability of U251 cells. A. Invasive and migrate ability was measured by the transwell assay and transwell migration assay respectively. PEGFP-LRIG1-U251 cells has reduced invasive and migrate ability and on the contrary Si-LRIG1-U251 cells has raised invasive and migrate ability. B. The number of migrating cells per field was 183.0±11.9 in the PEGFP-LRIG1-U251 group and 376.7±14.5 in the PEGFP-N1-U251 group and in proportion 443.3±15.3 in Si-LRIG1-U251 group and 376.0±14.5 in control group. C. The number of invading cells was 210.3±17.3 in PEGFP-LRIG1-U251 group and 376.0±15.8 in control group and in proportion 497.7±17.9 in Si-LRIG1-U251 group and 372.3±12.2 in control group. Data are shown as the means ± SD (*P<0.05, **P<0.01).

Si-LRIG1-U251 group and 376.0±14.5 in the Si-NC-U251 group (**Figure 2B**). In proportion the number of invading cells was 497.7±17.9 in Si-LRIG1-U251 group and 372.3±12.2 in control group (**Figure 2C**).

Discussion

The findings shown here indicate that LRIG1 has a strong suppressive effect on gliomas invasion and metastasis. Furthermore, genetic modulation of LRIG1 altered the expression of EMT-related factors including SNAI2 and E-cadherin.

Glioblastoma multiforme (GBM) is the most common and devastating malignant tumor with strong ability of rapid proliferation and diffusive invasion. GBM can fast invade into its surrounding normal brain tissues or metastasize to other organs. Traditional therapeutic modalities are ultimately ineffective in curing this cancer, due to its diffusive infiltrative growth pattern. Metastasis and invasion in cancers is a complex process requiring the coordinated action of numerous proteins and intracellular pathways. Both our overexpression and knockdown studies indicate that LRIG1 could suppress gliomas invasion and metastasis. Moreover LRIG1 overexpression would lead to reduced SNAI2 expression and elevated E-cadherin expression and knockdown of endogenous LRIG1, would lead to reduced E-cadherin expression and elevated SNAI2 expression.

LRIG1 was cloned and characterized as a putative tumor suppressor gene downregulated in

various human tumors and in the search for endogenous negative regulators of EGFR. Stutz MA et al. reported that LRIG1 negatively regulated the oncogenic EGF receptor mutant EGFRvIII in a Cbl-independent manner, inhibiting proliferation, motility and invasion of glioblastoma cells [18]. A few studies have reported that LRIG1 inhibited the invasion or metastasis of gliomas *in vitro* or *in vivo* experiments but the effect of LRIG1 on metastasis of gliomas remains unknown. Feng Mao et al. indicated that LRIG1 inhibited U-251 MG cell invasion and metastasis by attenuating MMP2 and MMP9 production and exerting an inhibitive effect on the PI3K/AKT pathway [2]. LRIG1 also functions as a tumor suppressor in the invasion into the GL15 human glioma cell line via EGFR/Akt/c-Myc activation [16]. Upregulation of LRIG1 induced cell growth inhibition and cell apoptosis and further reversed invasion of glioma cell lines and primary glioma cells, This would lead to of the decreased EGFR on their cytomembranes and posing an inhibitive effect on phosphorylation of downstream MAPK and AKT signaling pathways [19]. The signaling pathways mentioned above may explain about the activation of SNAI2-E-cadherin signaling activated by the upregulation or downregulation of LRIG1, but the specific molecular mechanism needs further investigation.

What SNAI2 and E-cadherin have in common is that they both are key molecules in the process of epithelial-to mesenchymal transition (EMT). SNAI2 as an EMT regulator regulates downstream EMT effectors like E-cadherin. Numerous studies have indicated that EMT related factors or its biochemical process (glial-mesenchymal transition) were central to glioma invasion and progression. More and more studies show that EMT induces and promotes the invasion and metastasis of gliomas [20, 21] although the role of EMT in malignant gliomas remains unclear. SNAI2 acts as an oncogene inherent in hematopoietic and other peripheral tissues [22] and it functions as a regulator of the EMT, which is closely correlated with tumor recurrence and invasion in GBM [23, 24]. A recent study shows that the level of E-cadherin m reflect different biological features of gliomas and it might strengthen cell metastasis, representing a hallmark of glioma invasion and growth [25]. Fenouille et al. indicates that SPARC (also known as osteonectin), a secreted extracellular matrix-associated factor that promotes EMT-like changes, may promote EMT-associated tumor invasion and E-cadherin loss by supporting PI3 kinase/AKT-dependent upregulation of SNAI2 in melanoma cells [26]. The strong correlation between SNAI2-E-cadherin signaling and EMT factors may therefore give explanations about the enhanced invasiveness and metastasis of glioma cells.

In conclusion, we demonstrated for the first time that the invasion and metastasis of gliomas cells are regulated by the level of LRIG1 via the activation of SNAI2-E-cadherin signaling. Further investigation regarding the underlying mechanisms and biological effects of LRIG1 knockdown or overexpression is justified and the applicability of amplifying LRIG1 in gliomas therapy needs further investigation. The limitation of this study is that this research was performed *in vitro*, which only focused on one gliomas cell line. Further experiments are required to confirm our conclusions by studying other cell lines *in vitro* and animal models *in vivo*.

Acknowledgements

This study was supported by National Natural and Science Foundation of China, No. 8137-2683.

Disclosure of conflict of interest

None.

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