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Original Article

LRIG1 regulates proliferation and invasion of tongue squamous cell carcinoma via EGFR pathway

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Abstract: Tongue squamous cell carcinoma (TSCC) is an aggressive neoplasm which typically spreads through invasive growth with serious morbidity and mortality. Previous studies suggested that the expression of LRIG1 functions as the vital suppressor gene in various human cancer types by inhibiting invasion and proliferation. However, little is known about the downstream genes coordinating this process in tongue squamous cell carcinoma. In this study, we overexpressed LRIG1 with a full-length expression vector and used small interfering RNA (siRNA) to block LRIG1 gene in the Tca8113 TSCC cell line. The expression of LRIG1 and EGFR at mRNA and protein level were tested by qRT-PCR and Western blot, respectively. The transwell assay and CCK8 assay were performed to detect the invasion and proliferation of TSCC cells. LRIG1 overexpression reduced EGFR expression, and significantly inhibited the invasion and proliferation of TSCC cells. In addition, silence of endogenous LRIG1 expression resulted in elevated EGFR expression and increased cell invasion and proliferation. Our result suggested that LRIG1 restricted TSCC invasion and proliferation via regulating the EGFR pathway. Restoration of LRIG1 in TSCC cells could be used as a novel therapeutic strategy.

Keywords: LRIG1, TSCC, EGFR, invasion, proliferation

Introduction

Oral cancer is the sixth most common human malignant tumor with an overall 50% of the five-year survival rate [1, 2]. Tongue squamous cell carcinoma (TSCC) is the most common type of oral cancer, typically characterized by its wide spreads resulting from malignant proliferation, progressive local invasion and lymph nodal metastases [4]. Therefore, the primary effort has been made to reveal the molecular mechanisms underlying its invasive behavior and malignant proliferation of TSCC [5].

Leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) is a transmembrane leucine-rich repeat and immunoglobulin (Ig)-like domain-containing protein. LRIG1 gene is located at chromosome 3p14.3, a region frequently deleted in various types of human malignant tumor [6]. Recently, LRIG1 was cloned and characterized as a putative tumor suppressor gene which is downregulated in human malignant tumors such as gliomas, bladder cancer, breast cancer, and prostatic cancer [7, 8]. Although many studies have implicated LRIG1 as an inhibitor of cancer cell invasion and metastasis, there were studies showing that the upregulation of LRIG1 inhibits the proliferation and attenuates cell invasion [9]. LRIG1 was downregulated in several squamous cell carcinomas, correlated with poor differentiation status and increased proliferation in primary tumors [10]. However, the effects of LRIG1 on biological behaviors of aggressive TSCC cells and the possible mechanisms have not been reported.

Receptor tyrosine kinases (RTKs), which convey signals from polypeptide growth factors, are important for tumor cell regulation. Cellular effects mediated by RTKs typically include stimulated proliferation, enhanced viability, and increased migration [11]. Notable RTKs that stimulate migration include the epidermal growth factor receptor (EGFR), the receptor for

the EGF family [12]. Previous studies suggested that LRIG1 is a candidate suppressor of EGFR activity by interacting with EGFR and enhancing its ubiquitination and degradation [13, 14]. In our study, we found that LRIG1 inhibits invasion and proliferation of TSCC cells and attenuates EGFR.

Materials and methods

Cell culture and transfection

The human tongue squamous cell carcinoma cell line Tca8113 cell was purchased from Shanghai YuBo Biological Technology. Cells were cultured in Dulbecco's modified Eagle media (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. The medium was replaced every 2 to 3 days and cells were passaged at confluence.

By using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions, Tca8113 were transfected with expression vector pLRIG1-GFP that encodes human LRIG1 protein or pEGFP-N1 (Clontech) as vector control. Cell clones resistant to G418 (GIBCO) were cloned and amplified for further experiments. LRIG1 upregulation was identified and confirmed at the mRNA and protein levels.

Silencing of LRIG1 was achieved by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Short interfering RNAs (siRNAs) (5'-ACTCTCTGAGATTGACC CT-3') targeting against LRIG1 were constructed and transfected into Tca8113 cells, siRNAs (5'-ACTACCGTTG TTATAGGTG-3') were used as negative controls [15]. LRIG1 silence was identified and confirmed at the mRNA and protein levels.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. qRT-PCR was employed to detect the expression levels of mRNA. The amount of total RNA was determined by ultraviolet (UV) spectrophotometry, and 1 μg of total RNA was used as a template for reverse transcription. Oligonucleotide primer sequences of LRIG1 used were as follows: sense 5'-ACACCGAAGTGGACTGTTACTCC-3' and antisense 5'-CCGGGTGATACAACCTTGCT-3'. The reference gene we used here was β-actin, primer sequences were as follows: the sense 5'-GGGACCTGACTGACTACCTC-3' and antisense 5'-TCATACTCCTGCTTGCTGAT-3'. Delta Delta CT method was used for the calculation.

Western blot

RIPA buffer supplemented with proteinase inhibitors was used to extract total protein. Tca8113 cells were treated in pre-cold RIPA buffer (Beyotime Biotech, Nantong, China) for 10 min. Protein separation were performed by Gel electrophoresis with 8% SDS-PAGE-Tris-HCl gels. The proteins were transferred to nitrocellulose membranes and detected by using a specific antibody of LRIG1. The primary and secondary antibodies were used as follows: monoclonal rabbit anti-GAPDH (1:10000; Abcam, Cambridge, USA); polyclonal rabbit anti-LRIG1 (1:1000; Abcam, Cambridge, USA). The membranes were incubated in the specific secondary antibody, and the immunoreactive bands were visualized using the enhanced chemiluminescence system.

Cell invasion assay

The invasive capability of Tca8113 cells *in vitro* was measured by transwell chamber assay. The transwell chamber was purchased from Millipore Corporation (Billerica, MA). The transwell assay with a matrigel coating was performed as described previously [15].

Cell proliferation assay

The proliferation of Tca8113 cells was detected by CCK8 assay. Cells of each group were planted into 96-well plates and CCK8 reagent was added into each well at 24 h, 48 h and 72 h after transfection. The plates were incubated for 1 h at 37°C and then the absorbance at 450 nm was measured using a microplate reader. CCK-8 Kit was purchased from Dojindo Company (Japan).

Statistical analysis

Statistical analyses of mRNA levels, protein expression and the invasion or proliferation ability of Tca8113 cells were performed by *t*-test employing GraphPad Prism version 5.00

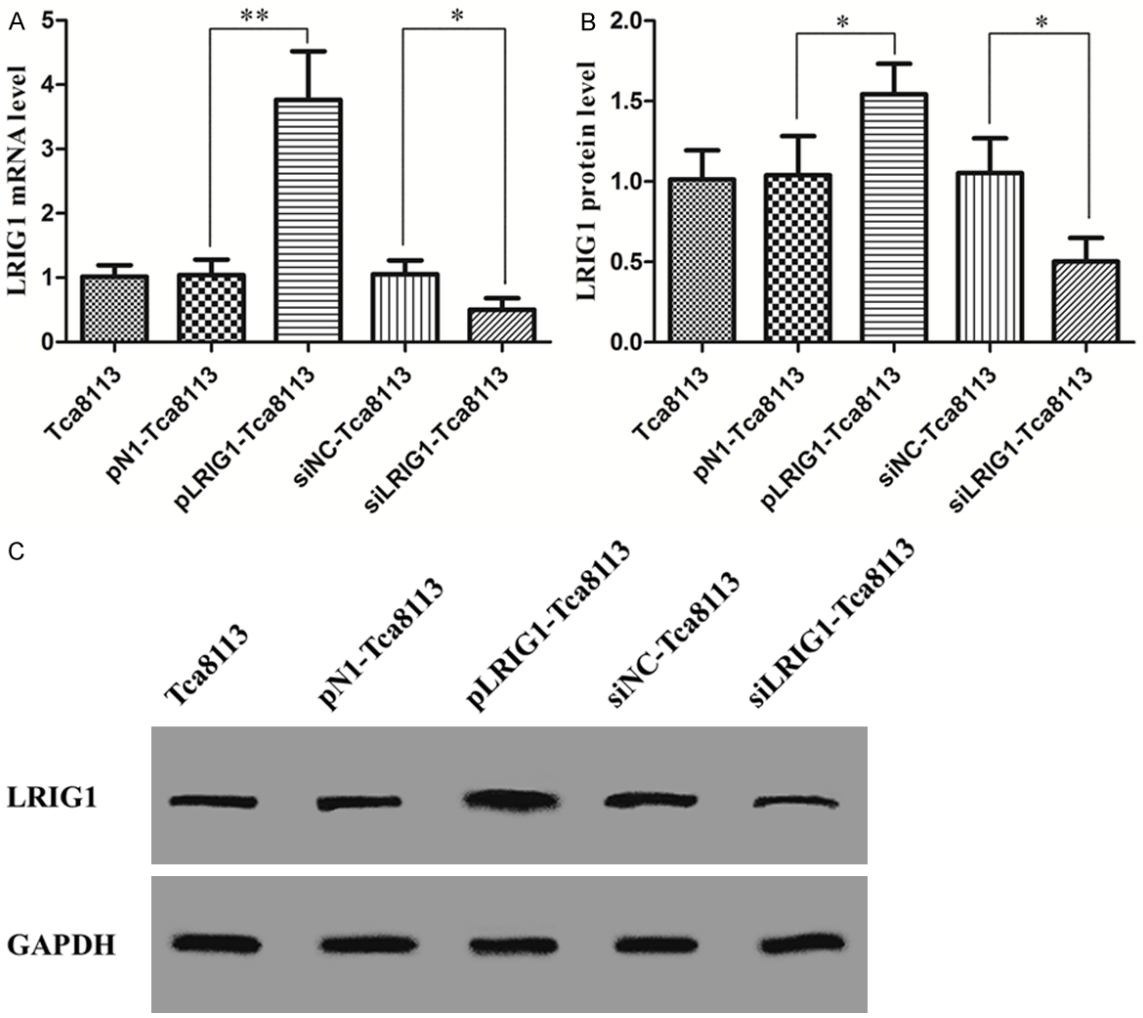

Figure 1. Up or down regulation of LRIG1 in Tca8113 cells after vector pLRIG1 and siLRIG1 transfected respectively. A. Tca8113 cells were transfected with pLRIG or silenced by siLRIG1, and negative control groups were constructed. LRIG1 mRNA was quantified by qRT-PCR. LRIG1 mRNA level was increased by 3.8 times in pLRIG1-Tca8113 cells and reduced by 60.5% in siLRIG1-Tca8113 cells. B. LRIG1 protein level was quantified by Western blot. LRIG1 protein level was increased by 1.5 times in the pLRIG1-Tca8113 and reduced by 58.3% in siLRIG1-Tca8113 cells. C. Western blot of LRIG1 protein expression in the indicated cell lines. Data are presented as the means ± SD (*P<0.05, **P<0.01).

software for Windows (GraphPad, La Jolla, CA, USA). The data were expressed as the mean ± standard deviation (SD), and statistical significance defined as a *P* value <0.05.

Results

Vector expressing LRIG1 caused significant upregulation of LRIG1

Vector pLRIG1 and the vector control pN1 were transfected into Tca8113 cells. Cell clones resistant to G418 were ring-cloned and amplified for the following experiments. Quantitative real-time PCR and Western blot were employed to evaluate the LRIG1 mRNA and protein levels of different groups of Tca8113 cells LRIG1 mRNA level in pLRIG1-Tca8113 cells was significantly increased by 3.8 times comparing with the control pN1-Tca8113 cells (**Figure 1A**). In line with the promotion of mRNA level, the LRIG1 protein level was increased by 1.5 times comparing with the control (**Figure 1B** and **1C**).

The specific anti-LRIG1 siRNA caused significantly LRIG1 silence

Accordingly, we constructed a specific anti-LRIG1 siRNA and a negative control siRNA, and

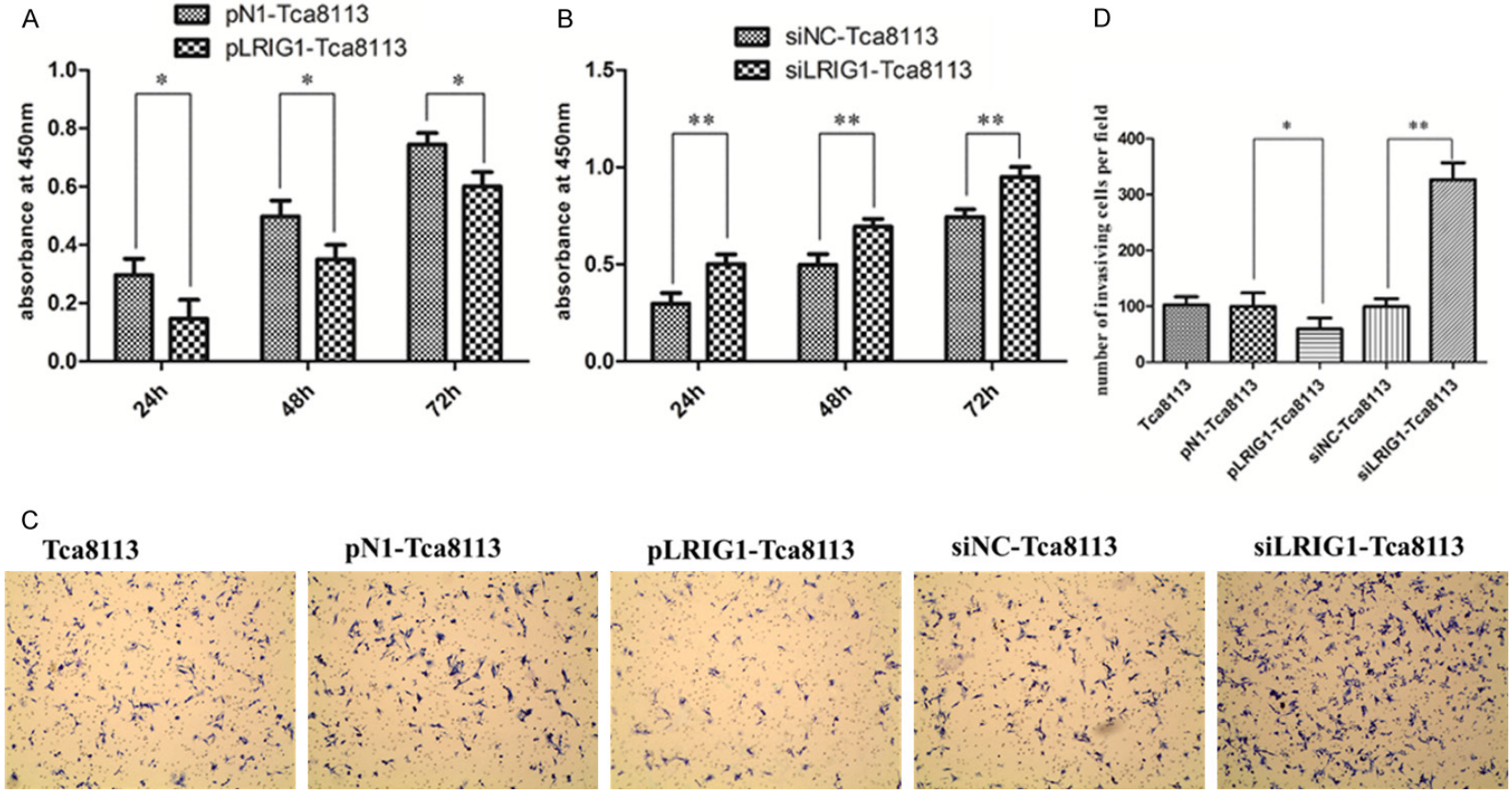

Figure 2. Up or down regulation of LRIG1 modulates cell proliferation and invasion of Tca8113 cells. A. The proliferation of Tca8113 cells was detected by CCK8 assay. The proliferation of pLRIG1-Tca8113 cells was significantly reduced by 53.3% at 24 h, 30.2% at 48 h and 20.0% at 72 h in comparison with pN1-Tca8113 cells. B. The proliferation ability of siLRIG1-Tca8113 cells was significantly promoted by 1.67 times at 24 h, 1.4 times at 48 h and 1.27 times at 72 h in comparison with control. C. The invasion of Tca8113 cells were measured by transwell invasion assay. pLRIG1-Tca8113 cells has reduced invasive ability and on the contrary siLRIG1-Tca8113 cells has raised invasive ability. D. The number of invading cells per field was 62.5±10.2 in the pLRIG1-Tca8113 cells and 100.3±16.7 in the pN1-Tca8113 cells. Accordingly, the number of invading cells per field was 331.7±19.0 in siLRIG1-Tca8113 cells and 109.3±9.4 in siNC-Tca8113 cells. Data are shown as the means ± SD (*P<0.05, **P<0.01).

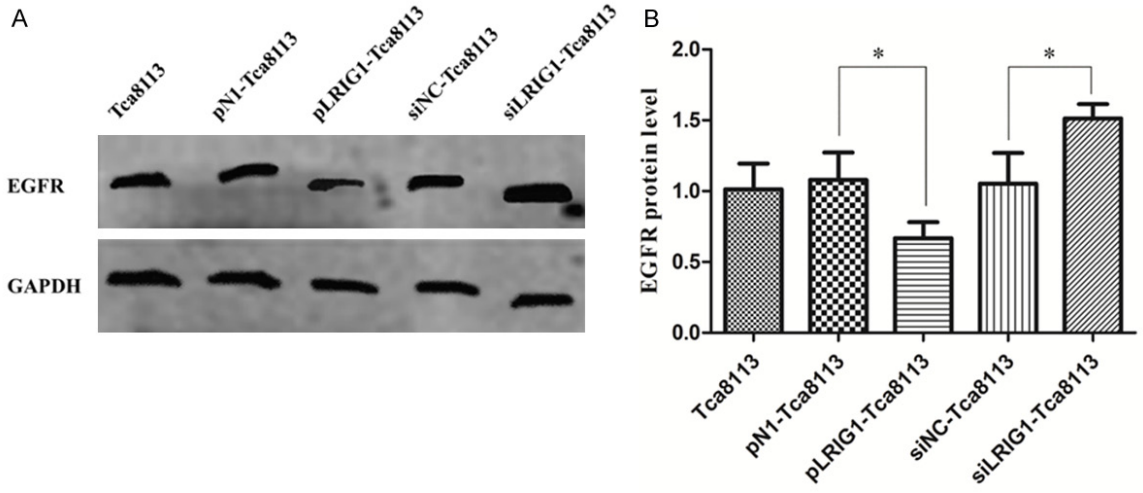

Figure 3. Effect of LRIG1 silent or overexpression on EGFR level. A. The protein level of EGFR was examined by western blot. LRIG1 overexpression significantly decreased endogenous EGFR protein and silent of endogenous LRIG1 would lead to significant promotion of EGFR signaling. B. The protein level of EGFR in pLRIG1-Tca8113 cells was reduced by 31.7% compared with pN1-Tca8113 cells and in siLRIG1-Tca8113 cells was promoted 1.56 times in comparison with siNC-Tca8113 group. Data are shown as the means ± SD (*P<0.05, **P<0.01).

transfected them into Tca8113 cells. LRIG1 and GAPDH mRNA and protein levels were then measured by qRT-PCR and Western blot. LRIG1 transcription was significantly downregulated by 60.5% in the siLRIG1-Tca8113 cells, compared with the negative control siNC-Tca8113 cells (**Figure 1A**). In proportion to the downregulation of LRIG1 mRNA level, the LRIG1 protein expression was reduced by 58.3% in siLRIG1-Tca8113 cells in comparison with that of siNC-Tca8113 cells (**Figure 1B** and **1C**).

Modulation of LRIG1 expression affects the invasion and proliferation of Tca8113 cells

To investigate the role of LRIG1 in invasion and proliferation of Tca8113 cells *in vitro*, we utilized the LRIG1 expression vector to overexpress LRIG1 and specific LRIG1 siRNA to silence LRIG1 in TSCC Tca8113 cells. Whereafter, transwell invasion assay and CCK8 assay were employed to detect the invasion and proliferation of Tca8113 cells. A significant attenuation of invasion and proliferation ability was revealed in LRIG1 overexpressed Tca8113 cells compared with control group (**Figure 2**). According to the transwell invasion assay, the number of invading cells per field was 62.5±10.2 in the pLRIG1-Tca8113 cells and 100.3±16.7 in the pN1-Tca8113 cells (**Figure 2C** and **2D**). In addition, the proliferation ability of pLRIG1-Tca8113 cells was significantly reduced by 53.3% at 24 h, 30.2% at 48 h and 20.0% at 72 h in comparison with that of pN1-Tca8113 cells (**Figure 2A**). In addition, we revealed that the invasion and proliferation ability of siLRIG1-Tca8113 cells was greatly increased (**Figure 2**). The number of invading cells per field was 331.7±19.0 in siLRIG1-Tca8113 cells and 109.3±9.4 in the siNC-Tca8113 cells (**Figure 2C** and **2D**). The proliferation of siLRIG1-Tca8113 cells was significantly increased by 1.67 fold at 24 h, 1.4 fold at 48 h and 1.27 fold at 72 h in comparison with that of the control groups (**Figure 2B**).

Roles of EGFR signaling in the regulation of invasion and proliferation by LRIG1

By Western blotting, we revealed that LRIG1 overexpression attenuated EGFR signaling and silence of endogenous LRIG1 led to activation of EGFR signaling (**Figure 3**). EGFR level in pLRIG1-Tca8113 cells was significantly reduced by 31.7% compared with pN1-Tca8113 cells (**Figure 3A** and **3B**) and in siLRIG1-Tca8113 cells it was increased by 1.56 fold compared with that of siNC-Tca8113 group (**Figure 3A** and **3B**).

Discussion

Our findings revealed that LRIG1 strongly suppressed the invasion and proliferation of TSCC. Furthermore, genetic modulation of LRIG1 al-

tered the expression of EGFR. Thus LRIG1 regulated the invasion and proliferation of TSCC, at least in part, via the regulation of EGFR signaling.

TSCC is the most common subtype of oral cancer and is typically characterized by malignant proliferation, progressive local invasion and lymph nodal metastases. Both invasion and proliferation of cancers cells require coordination of numerous proteins and intracellular pathways.

LRIG1, a transmembrane leucine-rich repeat and immunoglobulin (Ig)-like domain-containing protein and endogenous suppressor of EGFR, was considered as a candidate of tumor suppressor gene which downregulates a large amount of human malignant tumors. LRIG1 gene is located at chromosome 3p14.3, a region which has been identified in as the frequent loss of heterozygosity (LOH) region in various types of human cancers [6]. Numerous studies have demonstrated the association of deregulation of LRIG1 with cancers such as gliomas, bladder cancer, oropharyngeal cancer, breast cancer, and prostatic cancer [7, 8, 16-18]. That makes us curious about the role of LRIG1 in the occurrence and progress of TSCC. A previous study reported that LRIG1 was downregulated in squamous cell carcinoma (SCC) cell lines and squamous cell carcinomas compared with primary human keratinocytes and adult oral mucosa. Decrease of LRIG1 is associated with poor differentiation status and increased proliferation in primary tumours [10]. However, the effects of LRIG1 on biological behaviors of aggressive TSCC cells *in vitro* and the possible mechanisms have not been reported yet.

EGFR, a 170 kDa tyrosine kinase receptor functioning as a versatile signal transducer, has been reported to be overexpressed in numerous types of tumors including lung cancer, bladder cancer, gliomas, squamous cell carcinoma of skin, colorectal cancer, colon carcinoma, renal cancer and prostate cancer [9, 19-25]. EGFR is composed of a transmembrane lipophilic domain, an extracellular ligand-binding domain, an intracellular tyrosine kinase domain and the C-terminus region with multiple tyrosine residues. Activation of EGFR leads to signal transduction cascades stimulating cell proliferation, migration, and metastasis of several types of tumour [26, 27].

LRIG1 was first discovered as an endogenous negative regulator of EGFR, and numerous subsequent studies has confirmed its function as tumor suppressor via interaction with EGFR signaling. LRIG1 antagonizes the activity of EGFR and acts within a framework of a negative feedback loop by organized leucine-rich repeats (LRRs) interacting with extracellular region of EGFR [16]. In general, LRIG1 suppresses EGFR activity by interacting with EGFR and enhancing its basal and ligand-stimulated ubiquitination and degradation in a Cbl-dependent manner [13, 14]. On the other hand, Stutz MA et al has reported that LRIG1 negatively regulated the oncogenic EGF receptor mutant EGFRvIII in a Cbl-independent manner, inhibiting proliferation, motility and invasion of glioblastoma cells [14].

In conclusion, we demonstrated for the first time that the invasion and proliferation of TSCC cells are regulated, at least in part, by the level of LRIG1 via the regulation of EGFR signaling. Further investigation regarding the underlying molecular mechanisms and more biological effects of LRIG1 on TSCCs is justified. In addition, additional experiments are required to confirm our conclusions by examining animal models *in vivo*.

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Disclosure of conflict of interest

None.

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