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ORIGINAL ARTICLE

# Extracellular and cytoplasmic regions of LRIG1 play a negative role in EGFR activity: Findings of a radioligand-binding assay*

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## Abstract

**Objective** Leucine-rich repeats and immunoglobulin-like domains 1 (*LRIG1*) is a newly identified human gene that inhibits the epidermal growth factor receptor (EGFR), which on combining with a ligand, can drive tumor growth. This study investigated the interaction between human LRIG1 and EGFR and attempted to delineate the functions of as well as the mechanisms used by the extracellular (ECD) and cytoplasmic (CPD) domains of the human LRIG1 protein to downregulate human EGFR signaling activity.

**Methods** Two constructed chimeric eukaryotic expression vectors, pIRES2-EGFP-3XFLAG-LRIG1-ET and p3FLAG-LRIG1-TC, encoding the extracellular and transmembrane regions (LRIG1-ET) and the transmembrane and cytoplasmic regions (LRIG1-TC), respectively, and the plasmid p3XFLAG-CMV-9-LRIG1 encoding full-length LRIG1 (LRIG1-FL) were transfected into the human glioma cell line U251 or primary astrocytoma cells by using liposomes. The number and affinity of cell surface EGFR on transfected cells was determined by $^{125}$I-EGF binding assay.

**Results** The dissociation constant (KD) values for EGFR were higher, and the maximum increase was observed in the cells transfected into LRIG1-ET (1.36 folds). The number of maximal binding sites (Bmax) of the receptors was decreased in all transfected cells; the maximum decrease was noted in the cells transfected into LRIG1-FL (40.05%).

**Conclusion** Both the ECD and CPD of LRIG1 are important to negate EGFR signaling. The ECD may interfere with the binding between EGFR and its ligand and facilitate the functions of CPD. The CPD may, when brought in proximity to EGFR, enhance receptor degradation. These two mechanisms can contribute to the downregulation of EGFR-mediated signaling by LRIG1.

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Leucine-rich repeats (LRRs) and immunoglobulin-like (Ig-like) domains 1 (*LRIG1*) is a newly identified human gene, which was formally named by the Human Genome Organization (HUGO) Gene Nomenclature Committee in 2001 [1]. With a sequence of 4763 bp, LRIG1 codes for a transmembrane glycoprotein (1093 amino acids) that contains an extracellular region (796 amino acids) with one potential signal peptide, 15 leucine-rich repeats, 3 immunoglobulin-like domains, and 6 potential N-glycosylation sites, a transmembrane region (23 amino acids), and a cytoplasmic region (274 amino acids). The latter contains 1 consensus phosphorylation site for protein kinase C, one consensus phosphorylation site for cAMP- and cGMP-dependent kinases, and 6 consensus phosphorylation sites for casein kinase II. It displays structural relativeness to *Drosophila Kek-1* gene and mouse *LIG-1* gene. *Drosophila* Kekkon-1 (Kek-1) and murine Lrig1 (LIG-1) are both transmembrane proteins. In the extracellular region, Kekkon-1 contains 3 LRRs and 1 Ig-like domain, Lrig1 contains 15 LRRs and 3 Ig-like domains and shares 83% homology with LRIG1 [1–2]. Interestingly, the Kekkon-1 transmembrane protein acts in a feedback loop to negatively regulate the epidermal growth factor receptor (EGFR) during *Drosophila*

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oogenesis and involves a physical and direct association between the extracellular or transmembrane domains of both proteins [3–4]. Further study revealed that the 6 LRRs of Kekkon-1 are necessary for the recognition of EGFR and for consequent inhibition of activation by growth factors [4–5]. Mouse Lrig1 also presents some possibility of negating EGFR. Disruption of *Lrig1* in mice resulted in psoriasiform hyperplasia [6], which may cause excessive activity of tyrosine kinases; treatment with relevant inhibitors might be a therapeutic alternative [7–8].

Immense efforts have been made to investigate whether human LRIG1 negatively regulates EGFR, as reported by Goldoni [9], and whether the extracellular domain (ECD) of LRIG1 inhibits cancer cell growth by attenuating basal and ligand-dependent EGFR activity. A recent study [10] showed that the direct binding of LRIG1 ECD to EGFR cannot be observed by gel filtration or biosensor analysis. However, we found that an immunoligand assay can help identify the direct binding of LRIG1 ECD and cytoplasmic domain (CPD) to EGFR, which negatively regulates EGFR activity.

## Materials and methods

### Plasmid construction

The plasmid pFLAG-LRIG1, encoding a protein with a vector-encoded pre-protrypsin signal peptide (PS) and a 3X FLAG epitope and followed by full-length LRIG1 lacking its signal peptide (GenBank accession no. AF381545), was generated in the Department of Radiation Sciences, Oncology, Umea University, Sweden, by assembling PCR fragments of LRIG1 and a synthetic linker into the p3XFLAG CMV-9 expression vector (Sigma-Aldrich, Sweden) [11]. The plasmid pFLAG-LRIG1-ET, encoding a protein containing the PS and 3X FLAG epitope, followed by the extracellular and transmembrane region of LRIG1 (LRIG1-ET) lacking its signal peptide (GenBank accession no. AF381545), and another protein, namely, enhanced green fluorescent protein (EGFP), were generated in the Department of Neurosurgery, Tongji Hospital of Huazhong University of Science and Technology, Wuhan, China, by cloning the PCR fragments of LRIG1-ET from pFLAG-LRIG1 into the pIRES2-EGFP expression vector (Clontech, USA). The p3XFLAG-LRIG1-TC, encoding a protein with the PS and 3X FLAG epitope, followed by the transmembrane and cytoplasmic regions of LRIG1 (LRIG1-TC) lacking its signal peptide (GenBank accession no. AF381545), was produced by the same laboratory and method as the plasmid pFLAG-LRIG1-ET, but using the p3XFLAG CMV-9 expression vector (Sigma-Aldrich, Sweden) instead of the pIRES2-EGFP vector. The nucleotide sequences of the respective constructs were confirmed by sequencing (can be obtained from the authors upon request).

### Cell culture and gene transfection

U251 cells (China Center for Type Culture Collection; CCTCC, Wuhan, China) and human primary astrocytoma cells (50-year female, grade III according to WHO classification) were cultured in RPMI 1640 medium (Hyclone, USA) supplemented with 10% (v/v) Newborn Calf Serum (Hyclone, USA), 50 U/mL penicillin (Jiehui Company, Wuhan, China), and 50 μg/mL streptomycin (Juehui Company, Wuhan, China) in 95% O2, 5% CO2 at 37℃. The plasmids of pFLAG-LRIG1, pFLAG-LRIG1-ET, and pFLAG-LRIG1-TC were transfected into these cells by using Lipofectamine 2000 reagent (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. All the cells were divided into 5 groups for transfection with pFLAG-LRIG1 (LRIG1-FL Group), pFLAG-LRIG1-ET (LRIG1-ET Group), pFLAG-LRIG1-TC (LRIG1-TC Group), empty vectors (the p3XFLAG CMV-9 or the pIRES2-EGFP expression vector) (Empty Plasmid Group), and empty Lipofectamine 2000 reagent (Non-transfected Group). After 24 h of transfection, the medium was renewed, and the cells were treated or continuously incubated according to the different experiments detailed below.

### Radioligand-binding assay

After 24 h of transfection, the cells were suspended in 0.02% EDTA and harvested by centrifugation. After washing twice with no-serum RPMI 1640 medium at 37 °C and once with the medium at 4 °C, the cells were re-suspended into 4 °C RPMI 1640 medium containing 0.01% bovine serum albumin (BSA; Sigma, USA) to reach a concentration of $2.0 \times 10^5$ /mL. The cell suspension was added into 36 tubes, each containing 100 μL. These tubes were divided into 2 series at random: one group was for saturation binding assay and another for non-specific binding assay. Each series included 6 groups, and each group included 3 parallel tubes. With a final volume of 250 μL, each tube contained 10–100 μL (with a radioactivity of about 1200–12 000 cpm) $^{125}$I-EGF (Beijing Atom Hightech Company, Beijing, China); in addition, the tube for the non-specific binding assay contained 50 μL of EGF (an excess of 500-fold $^{125}$I-EGF, compared to the saturation binding assay group) (Sigma, USA). After incubation for 3 h at 37 °C, the tubes were washed with 0.01 M PBS at 4 °C, followed by immediate measurement of radioactivity by using the gamma counter (Shanghai Hefu, China). Using Microsoft Excel 2003 software, the KD and Bmax values of EGFR were determined using the Scatchard Plot Analysis and Double Reciprocal Plot Analysis [12–13]. Statistical differences among the groups were calculated using the Student's test and multifactorial ANOVA by using the SPSS 11.5 software.

## Results

The affinity of the ligand-binding reaction can usually be determined by measuring the concentration of ligands bound to a constant amount of binder at varying ligand concentrations. In the present study, the kinetics of EGFRs was analyzed using radioisotope-labeled ligands ($^{125}$I-EGF) and by measuring their binding to the receptors. The radioligands were added to the cultured U251 cells and incubated until the reaction reached equilibrium. Then, the unbound radioligands were washed and the radioactivity was measured. This was marked as saturation binding (total binding) of the ligands and receptors on the cell surface. When excess free unlabeled ligands were added into another parallel cell system and measured as above, the non-special binding value was obtained. The difference between non-special and saturation binding yields the special binding value. We got 3 sigmoidal curves (Fig. 1a) with $^{125}$I-EGF concentration as the X-axis and radioactivity as the Y-axis, which is called the ligand-receptor (L-R) binding curve (also called the saturation curve, competition curve, or adsorption isotherm). The curves indicate that specific binding was over 75% of the total binding. Using the concentration of bound ligand-receptor [LR] as the X-axis and [LR] / [L] ([L] indicating the concentration of free ligand) as the Y-axis (Scatchard plot, Fig. 1b), the KD and Bmax of EGFRs were determined from the obtained tendency curve. KD and Bmax can be derived from the Lineweaver-Burk Plots (or Double Reciprocal Plots) also, which are, in turn, derived from the Michaelis-Menten equation. These can also provide the linear regression by regarding 1 / [L] as the X-axis and 1 / [LR] as the Y-axis (Fig. 1c). Our results showed that the KD value of the non-transfected U251 cells was (87.11 ± 0.79) pmol/L, and the Bmax was (2.64 ± 0.10) × $10^4$ /cell. They were not significantly different between the Empty Plasmid Group cells and the Non-transfected Group cells (both $P > 0.05$). After being transfected into full-length LRIG1 or LRIG1 mutants (LRIG1-ET or LRIG1-TC), however, the cells' KD value increased ($P < 0.01$) and Bmax decreased ($P < 0.01$) (Table 1). The most obvious increase in KD value was noted in the LRIG1-ET group (1.40 folds). The most obvious decrease in Bmax was noted in the LRIG1-FL group (43.56%) (Fig. 2).

For the saturation binding assay, varied concentrations of $^{125}$I-EGF with radioactivity ranging from 1100 to 12 000 cpm were added into 6 groups (each containing 3 parallel tubes) of U251 cells (density: 2.0 × $10^4$). For the specific binding assay, conditions were identical, except that each tube was additionally contained 1 mM EGF. After incubating for 3 h at 37 °C and washing with 4 °C 0.01 M PBS, the radioactivity of each tube was measured using the gamma counter.

Table 1 Maximal binding sites (Bmax) of EGFR on U251 cell surface with different types of transfected material

| Groups | Transfected material | *n* | Bmax [($\bar{\chi}$ ± *s*) × $10^4$ sites /cell] | *P* value |
|---|---|---|---|---|
| 1 | Empty lipofectamine | 3 | 2.64 ± 0.10 | > 0.05 (2); < 0.001 (3, 4, 5) |
| 2 | Empty plasmid | 3 | 2.69 ± 0.19 | > 0.05 (1); < 0.001 (3, 4, 5) |
| 3 | LRIG1-ET | 3 | 2.05 ± 0.07 | < 0.001 (1, 2, 5); < 0.05 (3) |
| 4 | LRIG1-TC | 3 | 1.83 ± 0.10 | < 0.001 (1, 2); < 0.05 (3, 5) |
| 5 | LRIG1-FL | 3 | 1.53 ± 0.16 | < 0.001 (1, 2); < 0.01 (3); < 0.05 (4) |

Note: the digits in the signs of aggregation in *P* value railing point the group that being compared. 1: empty plasmid; 2: pIRES2-EGFP-3XFLAG-LRIG1-ET; 3: p3XFLAG-LRIG1-TC; 4: p3XFLAG-CMV-9-LRIG1

## Discussion

LRIG1, a transmembrane protein, is known to inhibit growth factor signal transduction from oncogenic receptor tyrosine kinases (RTKs), including EGFR, and MET and RET proto-oncogenes [14]. Downregulation of LRIG1 expression results in tumor formation, which suggests that LRIG1 may serve as a tumor suppressor in some types of cancer. Furthermore, high LRIG1 expression shows good prognosis and correlates with a longer disease-free survival and/or overall survival in squamous cell skin carcinoma [15], breast cancer [16], cervical cancer [17], hepatocellular carcinoma [18], and oropharyngeal cancer [19]. Although the expression and functional mechanisms of LRIG1 have been long documented, this is, to the best of our knowledge, the first study to confirm a direct negative action of LRIG1 on EGFR in primary human gliomas. We transfected LRIG1 and LRIG1 mutants into primary human astrocytomas and found they can restrict EGFR activity and inhibit cellular proliferation. As a potential negative regulator for human EGFR, LRIG1 has been proved to negate EGFR signaling in some cell lines [9]. Our results, along with those of our previous study on LRIG1 expression in astrocytomas [20], further confirm that LRIG1 acts as an EGFR antagonist and tumor suppressor factor in the development and progression of human brain gliomas. These are fully consistent with the behavior of LRIG1 in renal cell carcinomas [21], but only partially with that in breast cancer [22].

To determine the aspects involved in the functions of LRIG1, we designed and constructed two vectors: pIRES2-EGFP-3XFLAG-LRIG1-ET, containing the extracellular and transmembrane domains of LRIG1 (LRIG1-ET), and p3FLAG-LRIG1-TC, containing the transmembrane and cytoplasmic domains of LRIG1 (LRIG1-TC). When

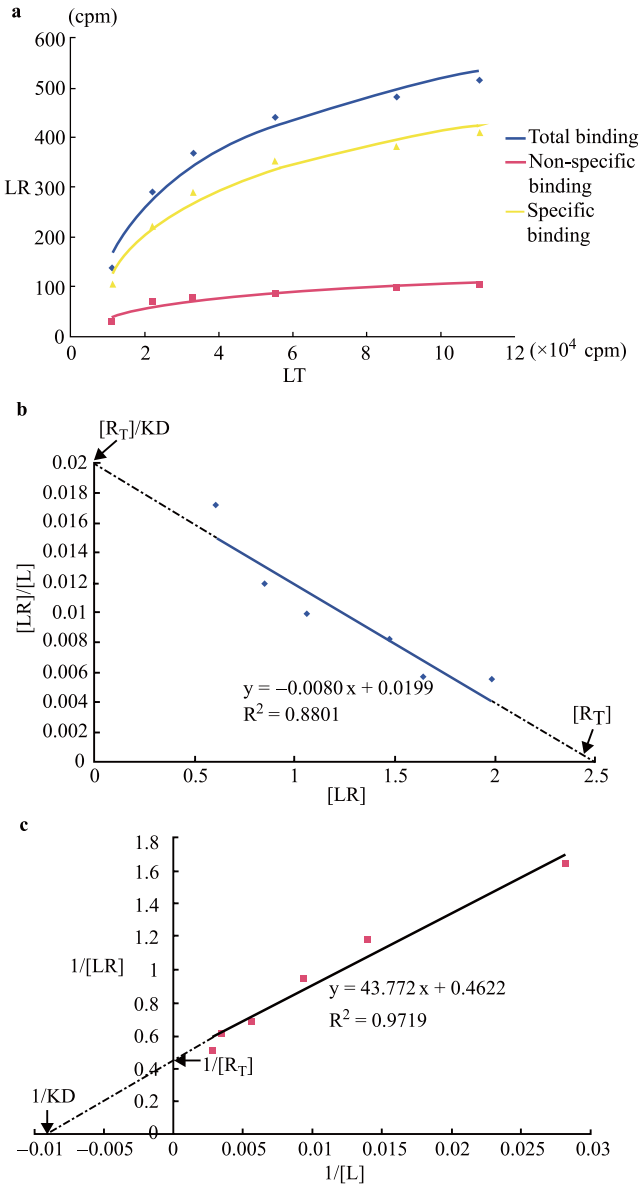


Fig. 1 Binding studies of $^{125}$I-EGF and EGFR in U251 cells. (a) Binding curves of $^{125}$I-EGF and EGFR. The abscissa (X-axis) shows the original radioligand activity of each group. The ordinate (Y-axis) marks the total binding-associated radioactivity (blue line), specific binding-associated radioactivity (yellow line), and non-specific binding-associated radioactivity (red line) in the cells. (b) Scatchard plot for $^{125}$I-EGF and EGFR binding. X-axis indicates specific binding [LR], while Y-axis indicates specific binding divided by the concentration of free radioligands [LR] / [L]. Bmax is the X intercept [RT] and KD is the negative reciprocal of the slope of linear regression (slope = –1 / KD) in this plot. (c) Lineweaver-Burk plot for $^{125}$I-EGF and EGFR binding. X-axis is the reciprocal of the concentration of free radioligands (1 / [L]); Y-axis is the reciprocal of specific binding (1 / [LR]). In this plot, Bmax is the reciprocal of the Y intercept (1 / [RT]) and KD is the reciprocal of the X intercept (1 / KD)

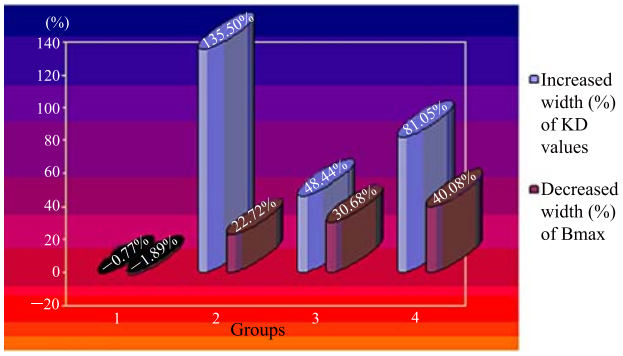


Fig. 2 Variation rate (%) of the dissociation constant (KD) and maximal binding sites (Bmax) of EGFR on the U251 cell surface with different transfected material, on comparison with non-transfected U251 cells

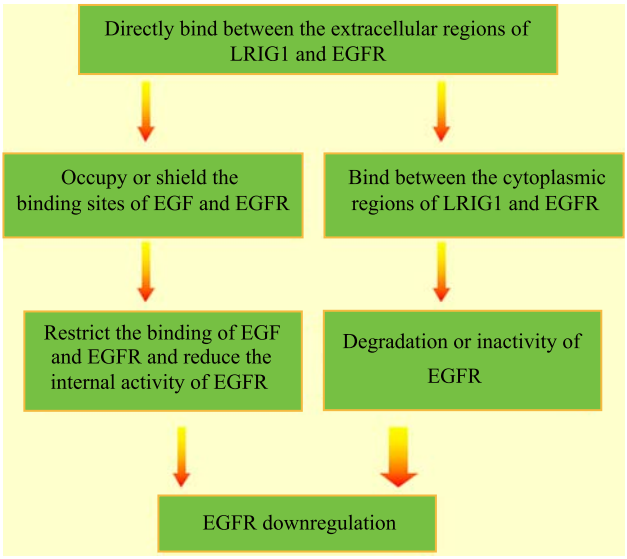


Fig. 3 A model of LRIG1 restricting EGFR

these two plasmids and the full-length LRIG1 (LRIG1-FL) plasmid were transformed into U251 cells, the KD of EGFRs on the cell surface increases and the Bmax decreases. The radioligand-binding assay showed that overexpression of LRIG1-ET can cause maximum increase in the KD value, but exerts minimum influence on the Bmax value. This shows that the extracellular part of LRIG1 can efficiently reduce the affinity of EGF-EGFR and hamper this binding, by using a competitive antagonism-like mechanism. However, its Bmax is still smaller than that of LRIG1-FL. It is implicated that, on the other hand, LRIG1-ET overexpression can decrease the internal activity (binding ability) or structural integrity (number) of EGFRs. It is suspected that the extracellular region of LRIG1 can directly bind to EGFR, thereby restricting the binding of EGF and EGFR (something like Kekkon1 and EGFR in *Drosophila*) and inhibiting EGFR activity or accelerating the degeneration of receptors.

LRIG1-TC overexpression can improve the KD of cell

surface EGFR, but only to a small extent, less than that observed in case of LRIG1-ET. However, it can reduce EGFR Bmax, downstream signaling, and proliferation of the transfected cells to a large extent (higher than that observed for LRIG1-ET). It is suggested that the chief mechanism underlying LRIG1-induced inhibition of EGFR does not depend on its interaction with the receptor, but on the reduction in the internal activity or number of the receptors. With respect to EGFR inhibition, the cytoplasmic region of LRIG1 contributes more than the extracellular region. Furthermore, because LRIG1-TC causes an increase in the KD of EGFR, it is suggested that the cytoplasmic region of LRIG1 can influence the binding between EGF and EGFR too.

Regarding the efficacy of EGFR inhibition, full-length LRIG1 seems to be the best alternative; when LRIG1-FL was overexpressed in the cells, the functions of EGFR were optimally reduced irrespective of the Bmax value, phosphorylation of downstream signals, or cellular proliferation. It seems that although both extracellular and cytoplasmic regions of LRIG1 are able to restrict EGFR alone, full-length LRIG1 is an excellent EGFR inhibitor. When LRIG1-FL is overexpressed, the increase in the KD of EGFR is smaller than that observed in the case of LRIG1-ET; this proves that the extracellular region of LRIG1 is an excellent binding factor for EGFR.

Considering the constitution of LRIG1 and EGFR, we mapped a model of the restriction mechanisms exerted by LRIG1 on EGFR (Fig. 3). LRIG1 binds to EGFR at the extracellular sites, on one hand, occupying the binding sites of EGF and EGFR or shielding them by causing conformational changes to EGFR, and more importantly, on the other hand, allowing interaction between the cytoplasmic regions of LRIG1 and EGFR, which results in the degeneration or reduction in the activity of EGFR.

The extracellular region of human LRIG1 is indeed similar to that of *Drosophila* Kekkon1, capable of binding to EGFR and restricting its binding to EGF. However, what is different from Kekkon1 is that the extracellular part of human LRIG1 is not the predominant functional part. c-Cbl, an E3 ubiquitin ligase, can bind and sort EGFR for degradation and ubiquitylation [23-26]. The binding of LRIG1 and EGFR can activate c-Cbl [27]; this aspect might play a role in the mechanisms underlying the degeneration and activity reduction of EGFRs after binding to LRIG1. One or some functional areas in the cytoplasmic LRIG1 can probably reduce the number or activity of EGFRs and cause receptor degradation and inactivation of c-Cbl binding. The efficacy, by this approach, is higher than that of direct inhibition by the extracellular region.

Other mechanisms might be involved in the determination of the structure of LRIG1, in particular, the cytoplasmic region. The detailed mechanisms underlying these interactions need to be studied further. These findings help understand how human tumors overcome negative regulation to abnormally activate EGFR signals, which, in turn, might be useful to determine the therapeutic relevance of some growth factors in tumor formation.

### Conflicts of interest

The authors indicated no potential conflicts of interest.

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