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Over-expression of LRIG3 Suppresses Growth and Invasion of Bladder Cancer Cells

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Summary: The purpose of this study was to investigate the impact of leucine-rich repeats and immunoglobulin-like domains 3 (LRIG3) on the biological features of bladder cancer cell lines. The plasmids of over-expressed LRIG3 and the blank plasmid serving as control were transfected into the bladder cancer cell lines, T24, EJ and BIU-87, and the expression levels of LRIG3 mRNA and protein were detected by using real-time PCR and Western blotting. The changes in the cell cycle and apoptosis were examined by using flow cytometry. The invasive ability was measured by Transwell assay, and CCK-8 assays were used to measure the proliferation of cells. As compared with the control group, the LRIG3 mRNA and protein expression levels in LRIG3 cDNA-transfected group were raised significantly (P<0.05). The average number of cells with up-regulated LRIG3 passing through the inserted filter was decreased significantly as compared with the control group (P<0.05). Up-regulation of LRIG3 also could inhibit proliferation and induce apoptosis of T24, EJ and BIU-87 cells. Except BIU-87, the T24 and EJ cells transfected with LIRG3 cDNA were arrested in G_0/G_1 phase compared to the control group (P<0.05). In conclusion, the over-expression of LRIG3 could influence the cell cycle and invasion, inhibit proliferation and induce apoptosis in the three bladder cancer cell lines.
Key words: LRIG3; bladder cancer cell lines; cell cycle; apoptosis; invasion

Bladder cancer is the fourth most common cancer in men after prostate, lung, and colorectal cancers, accounting for 7% of all cancer cases[1]. Bladder cancer is a highly malignant urinary tumor. With the development of the molecular biology, genes involved in tumorigenesis have been targeted for the treatment of tumor. The human leucine-rich repeats and immunoglobulin-like domains (LRIG) family comprises three paralogous genes, namely LRIG1 (formerly LIG1)[2], LRIG2[3] and LRIG3[4]. Among the three human LRIG family members, LRIG1 has been shown to function as an epidermal growth factor receptor (EGFR) signaling inhibitor by enhancing EGFR ubiquitination[5, 6], and has also been linked to hepatocyte growth factor (HGF) signaling as a negative regulator[7]. The LRIG3 gene was blast mapped to chromosome 12q13.2, which lies within a region that is amplified in a subset of glioblastomas[8]. The LRIG3 is closely related to the onset, development and prognosis of the glioblastomas. However, the relationship of LRIG3 and bladder cancer has not been demonstrated. Therefore, we designed this study to clarify the effect of LRIG3 on bladder cancer cells.

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1 MATERIALS AND METHODS

1.1 Cell Lines and Cell Culture

Three human bladder cancer cell lines (T24, EJ, and BIU-87) used in this study were obtained from the American Type Cell Collection (ATCC, USA), and grown in complete growth medium supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified 5% CO_2 atmosphere at 37°C.

1.2 Construction and Transfection of Plasmids

Plasmid pEGFP-LRIG3, containing a full-length of human LRIG3 structural cDNA gene, was subjected to the double digestion by *Xma* I and *Eco*R I. And then the target sequence was connected to the plasmid vector pLVX-DsRed-Monomer-N1, which was transfected into cells as experiment groups. And the plasmid pLVX-DsRed-Monomer-N1 was transfected as control vector group. Control cells were incubated with growth medium alone without plasmid and lipofectamine. The bladder cancer cells were transfected through lipofection, and grown in complete growth medium containing 5 μg/mL G418. After 2 weeks, G418-resistant clones for both LRIG3 targeted cells and control cells that represented possible stably transfected cells were individually selected and expanded for further experiments.

1.3 Quantitative Real-time RT-PCR

Total RNA was extracted from the transfected bladder cancer cells by using Trizol (Invitrogen, USA), and reverse transcription was carried out according to the manufacturer's instructions. The expression of β-actin mRNA was used as an internal standard. DNA primer sequences were designed as follows: for human LRIG3, sense 5'-CAC ATC AAT GGA ACC TGG GTA TTT TGAC-3' and antisense 5'-GTT TCG GTT CAA TTC GAG ATG TTG CAG TT-3'. The amplicon size was 139 bp. RNA samples were run in triplicate using 20 ng of RNA perreaction. The resulting cDNA samples were amplified by real-time PCR using gene-specific primer sets in conjunction with the SYBR Premix Ex Taq (TaKaRa, Japan) in a Mx3000p instrument. The qPCR was performed with the following conditions: activation at 95°C for 5 min followed by 40 cycles of denaturation at 94°C for 15 s, amplification at 60°C for 30 s, elongation at 72°C for 30 s. In the last, a cycle of solubility curve was added to examine the amplification quality.

1.4 Western Blotting

The transfected bladder cancer cells were collected and washed with 0.01 mol/L phosphate-buffered saline (PBS) three times. Then the cells were added into 200 μL pre-cold RIPA-PICT cell disruption liquor and centrifuged. All subsequent manipulations were performed on ice. After centrifugation, the supernatant was collected. The protein concentration of each sample was measured with micro-BCA protein assay reagent. The protein samples were added into 5×loading buffer. The mixture was heated to 100°C for 5 min to denature the proteins. The protein from each sample was subjected to electrophoresis on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. After TBST buffer containing 5% non-fat dry milk was used to block non-specific binding for 1 h at room temperature, 1:1000 diluted LRIG3 primary antibody and 1:1000 diluted GAPDH primary antibody were added overnight at 4°C. Then secondary antibody labeled with alkaline phosphatase was added at room temperature. One h later, the samples were washed three times with TBST, and then visualized using DAB detection system.

1.5 Cell Invasion Assay

Motility and invasion capability *in vitro* were measured by using Transwell chamber assay. 100 μL matrigel was put into upper chambers of the Transwell inserts. The inserts were incubated at 37°C for 4 h for gelling and then pretreated with serum-free medium at 37°C for 1 h before seeding cells at a density of 2×10^4/mL with 1% FCS. The lower chambers of the Transwells were filled with 600 μL medium containing 10% FCS. Then the Transwell chambers were incubated at 37°C in 5% CO_2 for 24 h to allow cells to migrate. Thereafter, the cells on the upper side were removed by wiping with cotton swab. Cells that had invaded through matrigel were fixed in paraformaldehyde and crystal violet stained according to the manufacture's instruction. Cells that had invaded the matrigel and reached the lower surface of the filter were counted under a light microscope at a magnification of 200×. We chose five fields of vision and counted the number of the invading cells and the results from three separate chambers were then averaged. The experiment was performed in triplicate.

1.6 Apoptosis Analysis

Annexin V-FITC/7-AAD double staining assay was used to detect cell apoptosis. After transfected and incubated for 3 days, cells were collected, centrifuged and washed with PBS two times. Binding buffer was then added to each tube and cells were re-suspended. The cells were incubated with 5 μL of annexin V-FITC and 5 μL of 7-AAD for 15 min at room temperature in the dark. Then, the apoptotic analyses were done by flow cytometry within 1 h.

1.7 Cell Cycle by Flow Cytometry Analysis

The treated cells were then collected and washed with PBS, fixed with 70% ethanol at –20°C overnight. On the next day, after cells were washed with PBS, they were incubated with PI (20 μg/mL) and RNase (100 μg/mL) for 15 min at room temperature in the dark. Data were collected from the flow cytometry and analyzed with the accompanying software.

1.8 Survival Assay by CCK-8

The growth of T24, EJ and BIU-87 cells after LRIG3 gene transfection was evaluated by Cell Counting Kit-8 (CCK-8) assays. Untreated cells, cells treated with liposome alone and cells treated with the vector control were used for comparison. Cell suspensions (at 1×10^3/mL) were transferred to 96-well plates in triplicate and incubated for 24, 48 and 72 h. Subsequently, CCK-8 (10 μL) was added to each well, and cells were incubated for an additional 4 h. Then, The value of each well was measured by microplate reader at 450 nm.

1.9 Statistical Analysis

Statistical analysis was performed using the software of Statistical Package for the Social Sciences Version 17 for Windows. Student's *t* tests and variance analysis were used to determine the statistical significance of the differences between the experimental groups. A *P*-value of less than 0.05 was considered significant. Graphs were created with Excel software (Microsoft Office for Windows 2003).

2 RESULTS

2.1 Transfection Efficiency with LRIG3 cDNA

The mRNA and protein levels of LRGI3 were detected by using real time-PCR and Western blotting. The expression of LRIG3 mRNA and protein in the three bladder cancer cells was increased significantly after transfection as compared with control groups ($P<0.05$, fig. 1). There was no significant difference in the transfection efficiency among the three bladder cancer cells.

2.2 Effect of LRIG3 cDNA on Cell Invasion

The effect of upregulation of LRIG3 on cell invasion was measured by using the Matrigel *in vitro* invasion assay. As shown in fig. 2, as compared with the negative control cells, T24, EJ and BIU87 cells, which were stably transfected with LRIG3 cDNA, showed low invasion potential, suggesting that the enhanced expression of LRIG3 was associated with reversed invasive ability.

2.3 Effect of Up-regulated LRIG3 on Cell Growth and Apoptosis of Bladder Cancer Cells

Changes in cell number caused by upregulated LRIG3 expression were detected by CCK-8 cell prolif-

eration assay after cells had been treated for 24, 48 and 72 h, respectively. As shown in fig. 3, the proliferation rate of transfected cells was significantly lower than that of untransfected cells. There was no significant difference in proliferation rate in T24, EJ and BIU87 cells transfected with LRIG3 cDNA.

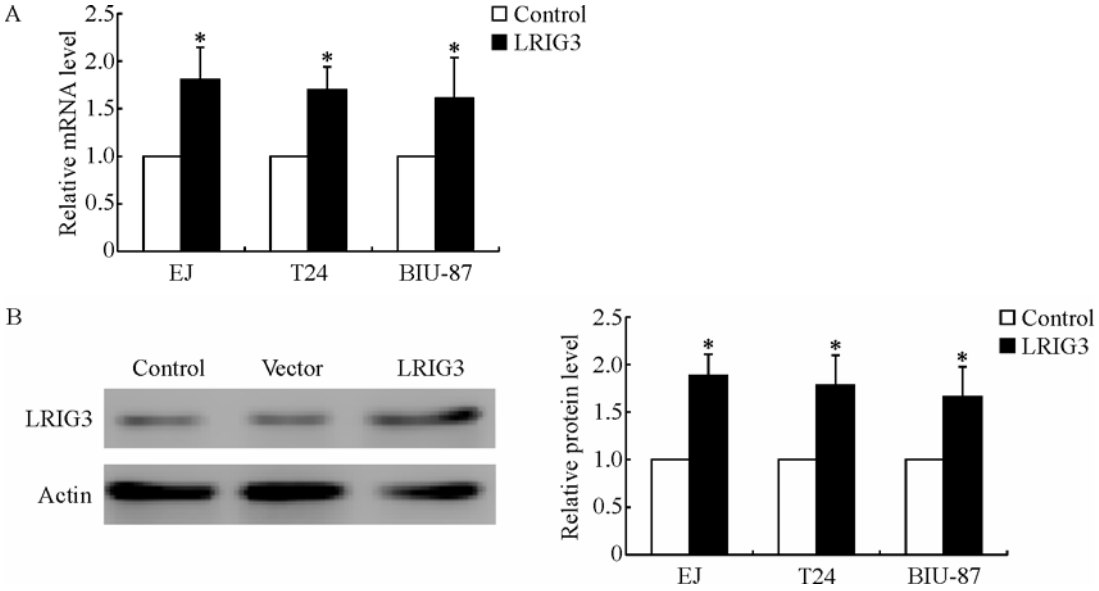

Fig. 1 Transfection efficiency of T24, EJ and BIU87 cells after transfection

A: The up-regulated expression of LRIG3 mRNA detected by using real time-PCR; B: The LRIG3 cDNA up-regulated the expression of LRIG3 protein detected by using Western blotting. $^{*}P<0.05$ *vs.* control

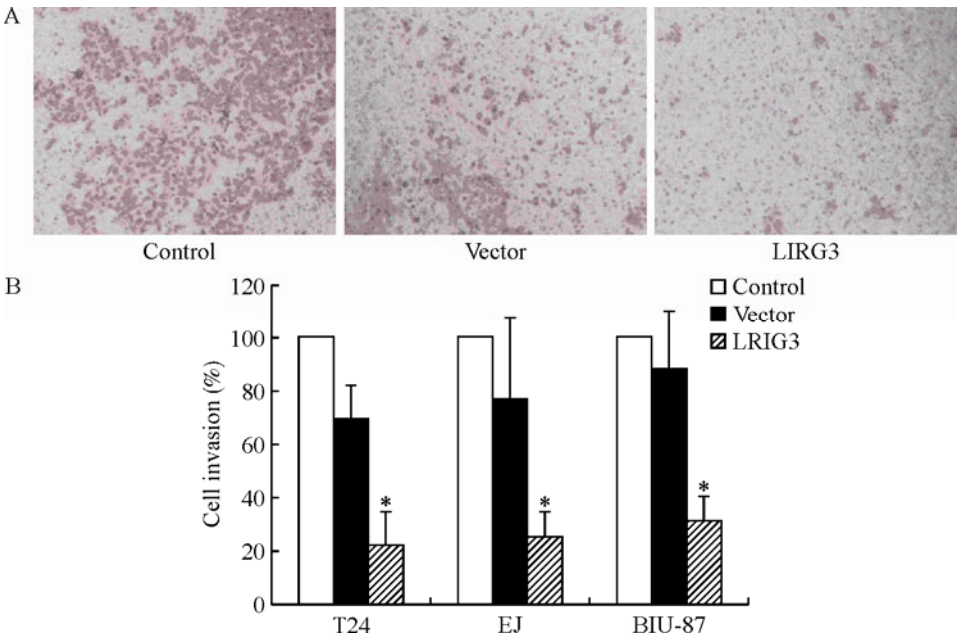

Fig. 2 Effects of up-regulated LRIG3 expression on invasion of bladder cancer cells

A: Effect of LRIG3 gene transfection on the invasion of human bladder cancer cells (×200); B: Data showed transfection of LRIG3 cDNA significantly inhibited the cell invasion as compared with vector and control cells ($^{*}P<0.05$).

The effect of LRIG3 on cell cycle was detected by using flow cytomtry with PI straining. The proportion of cells in G_0/G_1 phase was increased significantly after transfection as compared with control group in T24 and EJ cells, suggesting that upregualtion of LRIG3 induced an arrest in G_0/G_1 phase (fig. 3). But in BIU-87 cells, there was no evident regularity of changes in cell cycle.

The apoptotic effect of LIRG3 on bladder cancer cell lines was detected through Annexin V-FITC/7-AAD double staining assay. Results demonstrated that LRIG3 over-expression had an effect on increasing apoptosis in all of the three bladder cancer cells. With Annexin V-FITC staining, early apoptosis was clearly detectable in cells transfected with LRIG3 cDNA, and the apoptosis rate in control group was very low (fig. 4).

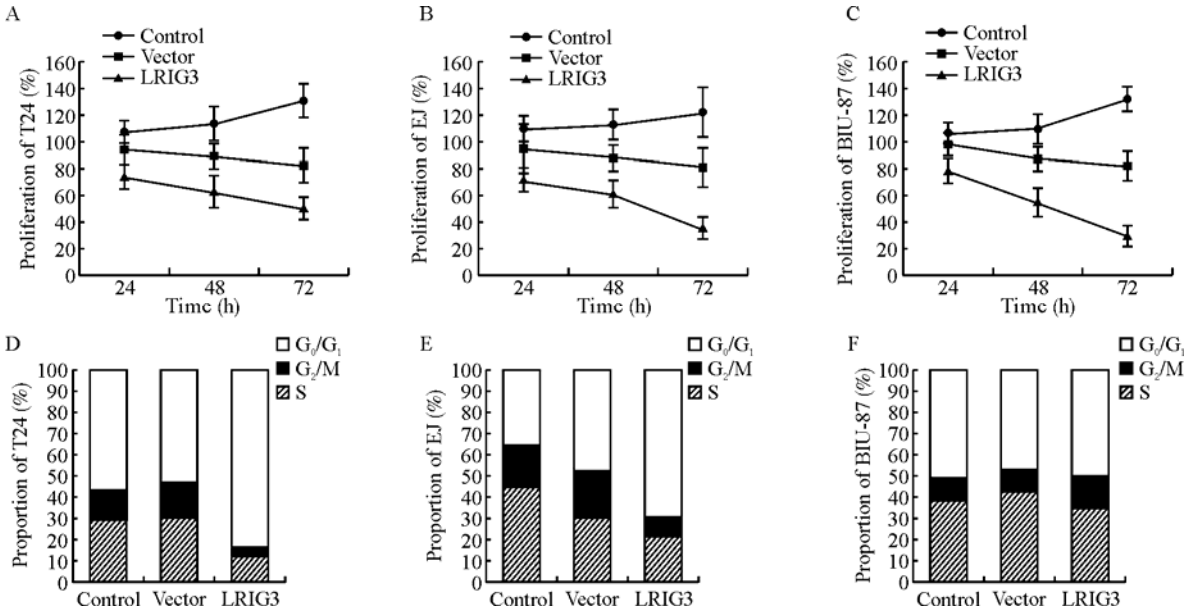

Fig. 3 Effects of up-regulated LRIG3 expression on proliferation and cell cycle of bladder cancer cells
Upregulation of LRIG3 inhibited proliferation of T24 (A), EJ (B) and BIU-87 (C) cells. Upregulation of LRIG3 induced an arrest in G_0/G_1 phase in T24 (D) and EJ (E) cells, but not in BIU-87 (F) cells.

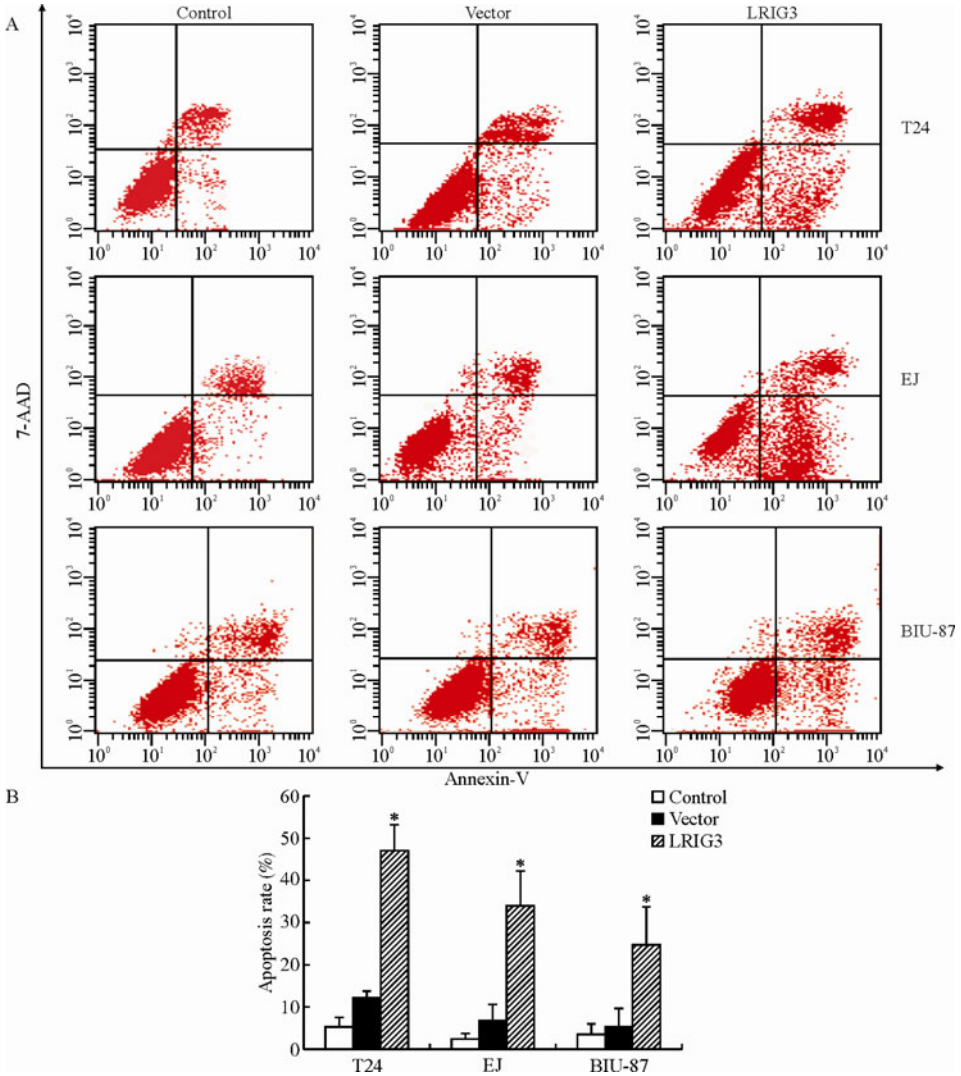

Fig. 4 LRIG3 gene transfection-induced apoptosis in human T24, EJ and BIU-87 cell lines by flow cytometry
A: LRIG3 gene transfection induced apoptosis in three bladder cancer cell lines by flow cytometry; B: Comparison of apoptosis rates ($\bar{x}\pm s$). $^*P<0.05$ *vs*. vector and control cells

3 DISCUSSION

The LRIG gene family contains LRIG1, 2 and 3. LRIG1 is a negative regulator of EGFR, but little is known about the function of LRIG3. The LRIG proteins share the same domain structure with signal peptide, an extracellular part containing 15 leucine-rich repeats and three immunoglobulin-like domains, a transmembrane domain, and a cytoplasmic tail[3]. The extracellular/lumenal part, the transmembrane region and the cytoplasmic tail all contain amino acid stretches which are highly conserved among the three LRIG proteins[4], implying that the LRIG proteins share some, if not all, of their molecular functions. LRIG1 is proposed to be a human tumor suppressor gene[9], but the function and the role of LRIG3 in bladder cancer are not well known. As a member of LRIG gene family, LRIG3 has been previously reported to be down-regulated in multiple tumors with unknown actions. It was reported that LRIG3 signaling is able to regulate cell growth, proliferation and apoptosis in glioblastoma cell line (GL15)[10].

In this study, three bladder cancer cell lines (T24, EJ, BIU-87), which were stably transfected with plasmid vector carrying LRIG3 cDNA, were successfully screened out. RT-PCR and Western blot results showed that the expression of LRIG3 mRNA and protein was significantly increased in experiment groups as compared with that in control groups.

Invasion is necessary for tumor metastasis formation[11-13]. As we expected, the over-expression of LRIG3 in T24, EJ and BIU87 cells inhibited the cell invasion activity *in vitro*. Certainly, in order to prove the effect of LRIG3 on the invasion of bladder cancer more powerful, several important factors which are involved in the process of invasion will need to be detected in future. The over-expression LRIG3 in the bladder cancer cells (T24, EJ) also showed an arrest in G_0/G_1 phase by flow cytometry. And T24, EJ and BIU87 cells transfected by LRIG3 cDNA have exhibited increased apoptosis by flow cytometry. Moreover, over-expression of LRIG3 also inhibited proliferation of T24, EJ, BIU-87 cells. So, we deduced that LRIG3 is able to regulate cell growth and apoptosis at least in T24, EJ, and BIU-87 cells.

EGFR is a protein tyrosine kinase that is overexpressed in many types of tumor cells, including lung, colon and prostatic carcinoma, and up-regulation of EGFR is associated with poor clinical prognosis[14, 15]. EGFR mediates signals that stimulate proliferation, migration, and metastasis in many tumor types[15, 16], and its signal transduction is regulated by stimulatory and inhibitory inputs. It was reported that up-regulation of LRIG1, followed by a decrease of EGFR on the cytomembrane of the cells, induced cell apoptosis and cell growth inhibition, and further reversed invasion in glioma cell lines[17]. Meanwhile, suppression of bladder cancer cells by LRIG1 is also related to the activity of EGFR[18]. In our study, we found that up-regulation of LRIG3 could induce apoptosis and inhibit invasion of bladder cancer cells. Whether EGFR, a member of LRIG gene family, is involved in this process need to be verified.

In conclusion, our study demonstrated that over-expression of LRIG3 in bladder cancer cell lines could result in apoptosis and invasion reversal. Summarizing the observations by us and others, we favor the hypothesis that LRIG3 may play a critical role in the inhibition of a wide spectrum of cancer cells. In next stage, we will design more experiments to detect the mechanism of how LRIG3 influences the biological characteristics of bladder cancer cell lines and verify whether EGFR plays an important role in this phenomenon. Furthermore, the expression of LRIG3 in different staging and grading of bladder cancer tumor also needs to be demonstrated.

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