RESEARCH COMMUNICATION

# Integrin-linked Kinase Functions as a Tumor Promoter in Bladder Transitional Cell Carcinoma

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## Abstract

**The aim of this study was to elucidate the role of the integrin-linked kinase (ILK) gene in development of human bladder transitional cell carcinoma (BTCC). Expression of ILK protein and ILK mRNA in 56 cases of human BTCC tissue and in 30 cases of adjacent normal bladder tissue was detected by immunohistochemistry S-P and reverse transcription polymerase chain reaction (RT-PCR), respectively. Four specific miRNA RNAi vectors targeting human ILK were synthesized and transfected into BIU-87 cells by liposome to obtain stable expression cell strains. The influence of ILK on proliferation of BTCC was detected by MTT, FCM on athymic mouse tumorigenesis. The positive rate of ILK protein in BTCC tissue (53.6%) was much higher than adjacent normal bladder tissue (10.0%) ($p<0.05$). Similarly, expression of ILK mRNA in BTCC tissue (0.540±0.083) was significantly higher than in adjacent normal bladder tissue (0.492±0.070) ($p<0.05$). MTT showed that the proliferation ability of miRNA–ILK transfected group was clearly decreased ($p<0.05$), the cell cycle being arrested in G0/G1-S, an tumorigenesis in vivo was also significantly reduced ($p<0.05$). ILK gene transcription and protein expression may be involved in the development of BTCC, so that ILK might be the new marker for early diagnosis and the new target for gene treatment.**

**Keywords:** Bladder neoplasms - integrin-linked kinase - miRNA - RNAi - stable transfection

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## Introduction

Bladder carcinoma is one of the common malignant tumors. An estimated 386, 300 new cases and 150, 200 deaths from bladder cancer occurred in 2008 world wide. According to 2008 estimates for the 20 world regions including more or less developed regions, bladder carcinoma was the seventh-ranked after lung & bronchus cancer, prostate cancer, colorectal cancer, stomach cancer, liver cancer and esophagus cancer for male (Jemal et al., 2011). Bladder carcinoma is the most common malignant tumors of urinary tract in China, more than 90% of bladder transitional cell carcinoma (BTCC), accounted for 1% to 2% of malignant tumors. Bladder carcinoma is a complex multi-step process involving the activation and/or abrogation of signal transduction pathways resulting in a variety of cellular activities.

Recent studies found that many oncogenes and tumor suppressor genes participated in cell growth, proliferation, differentiation, and ultimately apoptosis signal transduction pathway members. Integrin signaling pathways is an important one in the cell proliferation and differentiation and apoptosis process (Giancotti and Ruoslahti, 1999). Integrin-linked kinase (ILK) is a Ser/Thr protein kinase activity of cellular protein, can be activated by integrin or growth factor depending on the way of PI-3K and make other extracellular signal to be passed to the downstream by phosphorylation of the downstream substrate PKB/Akt and GSK-3β, to regulate cell growth, proliferation, differentiation and apoptosis (Naska et al., 2006). A series of studies had found that ILK abnormally over-expressed and was activated in prostate cancer (Kieffer et al., 2005), ovarian cancer (Ahmed et al., 2003), colon cancer (Marotta et al., 2003), gastric cancer (Ito et al., 2003) and other multi-malignant tumors, ulitizing many mechanism: such as inhibiting cell apoptosis, and promoting cell proliferation, these changes move forward a single step to affect biological function (Gu et al., 2012), integrin and other element enhance tumor tissue invasion force through the endothelial cells of the adhesion strength changes (Kaiser et al., 2012). But the expression of ILK in the BTCC and its role in occurrence and development of BTCC are still unclear.

RNA interference (RNAi) is a new technology that can block the functional gene expression from the mRNA levels and lead to post-transcriptional gene silencing. Recent new studies proved that the growth of prostate cancer could be inhibited in vitro and in vivo when the

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activity of ILK was silenced and inhibited via RNAi in human prostate cancer cells (Li et al., 2010), more recently, there was a report of silencing of the ILK gene induces the apoptosis in ovarian carcinoma (Liu et al., 2012). This indicated that ILK over-expression could promote cell proliferation. When RNAi technology is used to knockout the ILK gene, tumor cell proliferation might be inhibited significantly.

In this study, the expression of ILK protein and mRNA were detected in BTCC and adjacent normal tissue collected in our institute, then the relationship of ILK protein and mRNA expression with the pathological grade and clinical stage of BTCC was analyzed. In addition, the role of the ILK gene in human bladder cancer BIU-87 cell growth and proliferation and tumor formation ability was studied by RNAi technology.

## Materials and Methods

*Patients and tissue specimens*

In the present for this preparation of this research, the bladder cancer specimens were obtained from the First Affiliated Hospital of Chongqing Medical from January 2006 to November 2008, which consisted of 56 cases of bladder cancer (pathology confirmed BTCC), 30 cases of adjacent normal bladder tissue (3 cm from the tumor or more than 3 cm was confirmed by pathology of normal bladder tissue), This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. Written informed consent was obtained from all participants and each specimen was divided into two pieces equal parts. One was drawn into a Haoe frozen pipe after treated by DEPC water, liquid nitrogen frozen condensate, cryopreserved -80 °C for RT-PCR experiments; another was placed in 10% formalin solution for immunohistochemistry experiments. Ages of the 56 patients with BTCC ranged from 34 to 85 years (mean, 50 years), the gender of 56 patients were 42 males and 14 females. The extent of pathology was graded as follow grade I 16 cases, grade II 25 cases, grade III 15 cases; TNM stage included Tis-T1 9 cases, T2-4 47 cases.

*Immunohistochemistry*

For immunohistochemical staining, after deparaffinization of specimens, antigen retrieval was performed by microwave vacuum histoprocessor (RHS-1, Milestone, Bergamo, Italy) at 121 °C for 15 min. After peroxidase blocking, sections were incubated with mouse anti-human monoclonal antibody anti-ILK (Santa Cruz, SanDiego, CA) diluted 1:2000 in Antibody Diluent (Glolen Bridge, Mukilteo, WA) at 4°C overnight. The sections were treated with rabbit secondary antibody (Santa Cruz, U.S.) and DAB. Slides were counterstained with hematoxylin (bluegene, Shanghai, China) after immunostaining. Primary antibody was utilized instead of PBS solution as negative control; it was known a positive specimen as positive control. A semiquantitative scoring criterion for tissue specimens of ILK was used, in which both staining intensity and positive areas were recorded. The intensity of ILK positive staining was calculated scale 0-3 (i.e., negative = 0, weak = 1, moderate = 2 or strong =3), and the proportion of immuno-positive cells was calculated scale 1-4 (i.e., <25% = 1, 25–50% = 2, >50% to <75% = 3 or ≥75% = 4). Intensity of reactivity was scored using a four-tier system: 0-1 is divided into negative (-), 2-3 divided into weakly positive (+), 4-5 is divided into medium positive (++), 6-7 is divided into strongly positive (+++). A certified pathologist reviewed all specimens.

*RT-PCR*

Total cellular RNA of each group was extracted by One-step extraction of Trizol (GIBCOL / BRL Inc. U.S.). The RNA quality was detected by UV spectrophotometer and agarose gel (SABC companies, China) electrophoresis, and 1μg total RNA was transcribed reversely for the cDNA, PCR reaction amplification of ILK gene, and β-actin as internal reference. Primers of ILK mRNA: upstream 5' GCACTCAATAGCCGTAGTG 3', downstream 5' CCTACTTGTCCTGCATCTTC 3', target fragment was 406 bp. internal reference β actin upstream: 5' GAGC GGGAAATCGTGCGTGAC 3', downstream: 5' ATGGTGGTGCCGCCAGACAG 3', target fragment was 303 bp. PCR products was subjected to agarose gel electrophoresis, UV light observation and photography. Bio-Rad gel formatter was used to analyze the original band.

*Cell culture*

BTCC BIU-87 cells were obtained from the Southwest Hospital, Third Military Medical University, Chongqing China. It was grown in RPMI-1640 (Gibco Company) medium containing 10% fetal bovine serum (Hangzhou Sijiqing Company, china) at 37 °C, 5% $CO_2$ in the culture flask. When the cells reached density up to 90% -95%, 0.1% trypsin was used to digest adherent growing cells for subculture.

*Design and Synthesis of ILK miRNA*

According to the human ILK gene sequence Genbank (NM_004517), Invitrogen website rnaidesigner. invitrogen.com/rnaiexpress / online designing sequences were as follows:

| | |
|---|---|
| miR-1-F | TGCTGTGGAGAGGTCAGCAAAGGGTAGTTT |
| | TGGCCACTGACTGACTACCCTTTTGACCTCTCCA |
| miR-1-R | CCTGTGGAGAGGTCAAAAGGGTAGTCAGTCA |
| | GTGGCCAAAACTACCCTTTGCTGACCTCTCCAC |
| miR-2-F | TGCTGTTCCAATGCCACCTTCATTCCGTTTT |
| | GGCCACTGACTGACGGAATGAATGGCATTGGAA |
| miR-2-R | CCTGTTCCAATGCCATTCATTCCGTCAGTCA |
| | GTGGCCAAAACGGAATGAAGGTGGCATTGGAAC |
| miR-3-F | TGCTGTACAATATCACGGTGTCCATGGTTTT |
| | GGCCACTGACTGACCATGGACAGTGATATTGTA |
| miR-3-R | CCTGTACAATATCACTGTCCATGGTCAGTCA |
| | GTGGCCAAAACCATGGACACCGTGATATTGTAC |
| miR-4-F | TGCTGTCATGTAGTACATTGTAGAGGGTTTT |
| | GGCCACTGACTGACCCTCTACAGTACTACATGA |
| miR-4-R | CCTGTCATGTAGTACTGTAGAGGGTCAGTC |
| | AGTGGCCAAAACCCTCTACAATGTACTACATGAC |
| Neg-F | tgctgAAATGTACTGCGCGTGGAGACGTTTTGGC |
| | CACTGACTGACGTCTCCACGCAGTACATTT |
| Neg-R | cctgAAATGTACTGCGTGGAGACGTCAGTCA |
| | GTGGCCAAAACGTCTCCACGCGCAGTACATTTc |

Anterior sequences were compared to Blast (www.ncbi.nlm.nih.gov/BLAST) from the published EST sequence database; four pairs of specific sequences were confirmed in addition to human ILK genes, not found any genetic homology with the other genes. Four pairs miRNA duplex oligonucleotides targeting on human ILK mRNA were synthesized by Invitrogen.

*miRNA vector construction and miR-ILK expression*

Four miRNA interference plasmid vectors-pcDNATM6.2-GW/EmGFP- ILK-miR-1, 2, 3, 4, and a negative control vector were constructed by Invitrogen. pcDNATM6.2-GW/EmGFP-ILK-miR was transfected into bladder cancer BIU-87 cells by application of Lipofectamine 2000 (Invitrogen Corporation, U.S.). The day before transfection, BIU-87 cells at various growth stages, were inoculated into 6 well plates, consistent 2x105/holes into apatures, each holes was added 2 ml RPMI-1640 medium containing 10% fetal bovine serum. Transfecting step was executed strictly in accordance with Lipofectamine 2000 product instructions. Green fluorescence was observed with an inverted fluorescent microscope in 24 h after transfection. The next day adding into blasticidin (Invitrogen Corporation, U.S.) which the concentration was 4 μg/ml, pressure screening was done, and the non-transfected BIU-87 cells was acted as blank control. The medium was changed every 2-3 days. About 2 weeks later, clusters could be seen in the inverted fluorescence microscope. The fluorescent cells in clusters were selected out, fostered by a limited dilution to 96 well plates. And 3 μg/ml blasticidin was added into medium, until the stable monoclonal cell lines was formed.

*RT-PCR*

The cells of 4 stable transfection experimental groups, a negative control group, and a non-transfected group were collected. The details of RT-PCR analysis was referred to the above Two-step RT-PCR. ILK/β-actin (The ratio of absorbance) indicated the relative expression levels of ILK mRNA, ILK mRNA expression inhibition ratio (%) = (1-transfected group/negative control group) × 100%.

*Western blot*

The cells of 4 stable transfection experimental groups, a negative control group, and a non-transfected group were collected. The total protein was extracted by lysate (Beyotime, Shanghai, China), and protein concentration was determined by protein quantitated kit (Beyotime, Shanghai, China), and the total protein was separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane (Beyotime, Shanghai, China). Blots were then probed with monoclonal anti-β-actin (Sigma), polyclonal anti-ILK (Santa Cruz, U.S.) antibody overnight at 4 °C. Membranes were washed with TBS containing 0.05% Tween-20 (TBS-T), and then incubated with horseradish peroxidase-conjugated anti-mouse and rabbit secondary antibody (Santa Cruz, U.S.). Band absorbance was analyzed by Bio-Rad software, and β-actin as internal reference. ILK/β-actin (The ratio of absorbance) indicated the relative expression levels of ILK protein, ILK protein expression inhibition ratio (%) = (1-transfected group/negative control group) × 100%.

*Cell proliferation assays*

pcDNATM6.2-GW/EmGFP-ILK-miR-3 with the most efficient inhibition effect was selected as research object. The cells of ILK- miR-3 transfected group and non-transfected group were cultured respectively in 96 well plates, and each plate was cultured 0-7 days. Cell morphology, refraction, growth and adherence were observed. 4 h before the end of culturing, each aperture was added into 5 g/L MTT (Sigma Corporation, U.S.) 20 μl, and discarded the supernatant. DMSO 200 μl was added into each hole. Absorbances were measured at 570 nm using a SpectraMax 250 Microplate Reader (Molecular Devices, Sunnyvale, CA).

*The detection of cell cycle*

The cells of pcDNATM6.2-GW/EmGFP-ILK-miR-3 transfected group and non-transfected group were cultured abundantly. For cell cycle analysis, BIU-87 cells stably expressing pcDNATM6.2-GW/EmGFP-ILK-miR-3 were seeded in a 100-mm culture dish the day before experiment. The cells were incubated in RPMI-1640 without serum for 30 h and then the cell cycle was initiated with RPMI-1640 containing 10% FBS for 12 h. The cells were harvested and fixed in 70% ethanol for 30 min at -20 °C. Fixed cells were stained with 50 μg/μl propidium propidium idodie (PI, BD Biosceinces) and 100 μg/μl RNase (Sigma, U.S.). Cell cycle profile of BIU-87 cells transfected pcDNATM6.2-GW/EmGFP-ILK-miR-3 was measured by FACS, and resulting data were analyzed.

*Bladder cancer xenografts in nude mice*

Female BALB/c-nu/nu athymic mice (4-5 weeks old), from Beijing Slac Laboratory Animal Co. Ltd. (Beijing, China), were kept under specific pathogen-free conditions and cared for in accordance with the guidelines of the Laboratory Animal Ethics Committee of Chongqing Medical University. For the xenograft tumor growth assay, ILK-miR-3 transfected BIU-87 cells or the control cells were injected subcutaneously into the right flank of the mice (5 mice/group). Two weeks after inoculation, tumor size was measured every 7 days until the tumors grew to a diameter of 10 mm or when the tumor burden exceeded 10% of their bodyweight, at which time the mice were sacrificed by cervical dislocation. Tumor volume was calculated by the formula $V=ab^2/2$(a=longest axis, b=shortest axis).

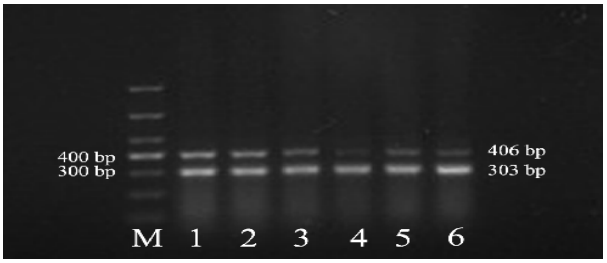


**Figure 1. Expression of ILK mRNA and β-actin mRNA by RT-PCR** (1, 2, 3: BTCC tissues; 4, 5, 6: normal bladder tissues). The expression of ILK mRNA in normal bladder tissue was obviously weaker than BTCC ($P<0.05$)

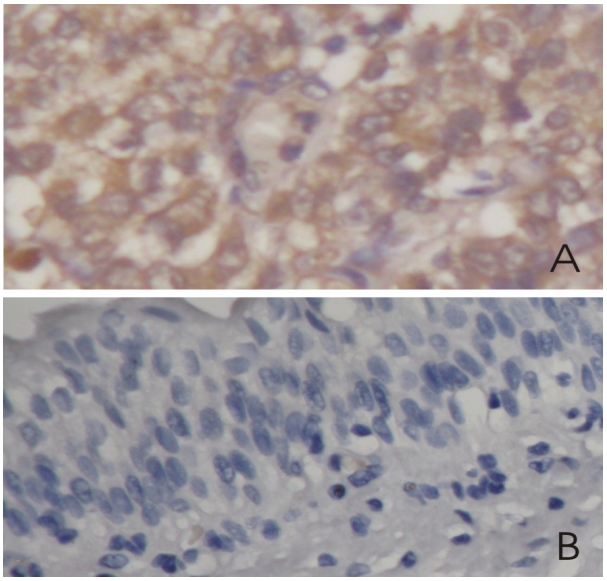


**Figure 2. Expression of ILK Protein by Immunohistochemistry (×400).** A: Positive expression of ILK protein in BTCC; B: Negative expression of ILK protein in adjacent normal bladder tissue. BTCC tissue staining was deeper than the normal bladder tissue

**Table 1. Expression of ILK Protein and mRNA in the BTCC Tissue and the Adjacent Normal Bladder Tissue**

| Groups | n | ILK expression + | - | Postive (%) | P | ILK mRNA / β-actin | P |
|---|---|---|---|---|---|---|---|
| BTCC tissue | 56 | 30 | 26 | 53.60% | 0 | 0.540±0.083 | 0.009 |
| Adjacent bladder tissue | 30 | 3 | 27 | 10% | | 0.492±0.070 | |

**Table 2. Correlation of ILK Expression with Clinicopathologic Features of BTCC**

| Groups | n | ILK expression +++ | ++ | + | - | Positive (%) | P |
|---|---|---|---|---|---|---|---|
| WHO Grade | | | | | | | 0.045 |
| G1 | 16 | 2 | 2 | 2 | 10 | 37.50% | |
| G2 | 25 | 4 | 5 | 3 | 13 | 48.00% | |
| G3 | 15 | 8 | 3 | 1 | 3 | 80.00% | |
| TNM Stage | | | | | | | |
| Tis-T1 | 9 | 1 | 1 | 1 | 6 | 33.30% | 0.184 |
| T2-T4 | 47 | 13 | 9 | 5 | 20 | 57.40% | |

*Statistical analysis*

Statistical analysis was performed with SPSS software (SPSS Standard version 13.0, SPSS). Chi-square trend test was used to assess the differential expression of ILK in different bladder tissues. Data derived from cell line experiments were presented as mean ± SE (X± SE) and assessed by a 2-tailed Student's t test and analysis of variance. p values <0.05 were considered significantly.

## Results

*Expression of ILK mRNA and protein*

The expression of ILK mRNA in the BTCC tissue and the normal bladder tissue aside cancer were 0.540±0.083 and0.492±0.070. The expression of ILK mRNA in BTCC tissue was significantly higher than that in adjacent normal bladder tissue (p<0.05); The positive rates of ILK protein expression in the BTCC tissue was 53.6% (30/56), most of which was strongly positive; but the positive rates of expression in the normal tissue was 10% (3/30), and most of which was weakly positive. The positive rate of ILK protein in BTCC tissue was much higher than adjacent normal bladder tissue (p<0.05) (Figure 1, 2, Table 1).

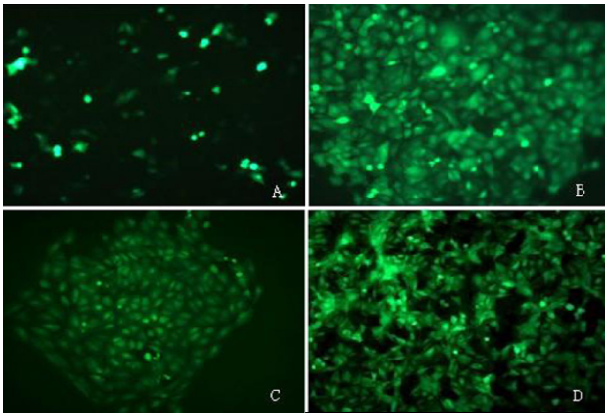


**Figure 3. The Results Observed in Fluorescence Microscope** (A: The fluorescence after transfected 24 h; B: The fluorescent cells after screened 2 weeks; C: The formation of monoclonal cells; D: The stable expressing cell strain). The green fluorescence was seen from the successfully transfected cells

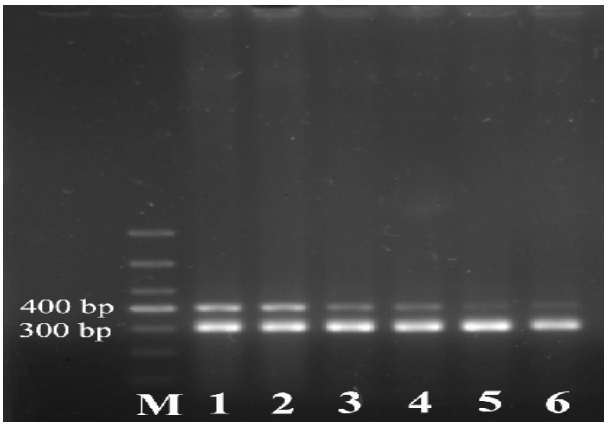


**Figure 4. The Suppression Effect of ILK mRNA After ILK- miRNA Transfected** (M: DNA Marker, 1: non-transfected group, 2: negative control group, 3: miR-1, 4: miR-2, 5: miR-3, 6: miR-4). The negative control group, compared with the non-transfected, ILK mRNA expression was not significantly different (P>0.05). pcDNATM6.2- GW/EmGFP- ILK-miR could effectively reduce the expression level of ILK mRNA compared with the negative control group

*Correlation of ILK expression with clinicopathologic features of BTCC*

The positive rates of ILK expression in grade I, II, III were 37.5 % (6/16), 48.0 % (12/25), 80.0 % (12/15). The positive rate of ILK expression showed an increasing trend from grade I to grade III (P<0.05). In non muscle-invasived bladder cancer (Tis, Ta, T1) and muscle-invasive bladder cancer (T2-T4), the positive rate of ILK expression was 33.3% (3/9) and 57.4% (27/47). There was no obvious relationship between the expression of ILK with BTCC clinical stage (P> 0.05) (Table 2).

*BIU-87 cells after transfected with interference plasmid*

After pcDNATM6.2-GW/EmGFP-ILK-miR plasmid

**Table 3. The Relative Expression Level of ILK mRNA in Each Group**

| Group | mRNA ILK/β-actin | P |
|---|---|---|
| non-transfection group | 0.572±0.007 | |
| negative control group | 0.566±0.003 | 0.219 |
| miR-1 | 0.189±0.006 | 0.00 |
| miR-2 | 0.176±0.002 | 0.00 |
| miR-3 | 0.087±0.004 | 0.00 |
| miR-4 | 0.094±0.005 | 0.00 |

**Table 4. The Relative Expression Level of ILK Protein in Each Group**

| Group | ILK protein/β-actin | P |
|---|---|---|
| non-transfection group | 0.929±0.006 | |
| negative control group | 0.856±0.008 | 0.07 |
| miR-1 | 0.494±0.006 | 0.00 |
| miR-2 | 0.333±0.007 | 0.00 |
| miR-3 | 0.185±0.004 | 0.00 |
| miR-4 | 0.211±0.004 | 0.00 |

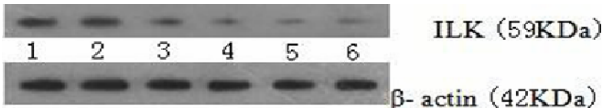


**Figure 5. The Suppression Effect of ILK Protein After ILK-miRNA Transfected** (1: non-transfected group, 2: negative control group, 3: miR-1, 4: miR-2, 5: miR-3, 6: miR-4) The transfected group ILK-miRNA inhibited the ILK protein expression effectively (P<0.05), while ILK protein was not significantly different in the negative control group, compared with the non-transfected group (P>0.05)

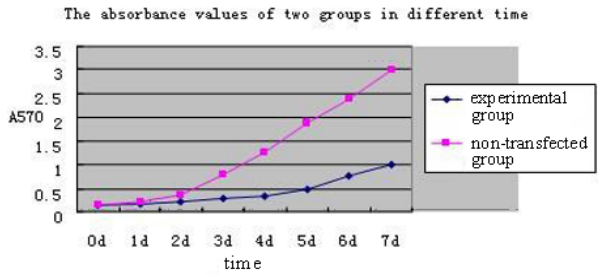


**Figure 6. Cell Growth Curve.** The growth rate of the transfected group cells was slower than that of the non-transfected group cells significantly (P <0.05). It showed that cells growth and proliferation were inhibited effectively, when the expression of ILK in human bladder cancer BIU-87 cells was inhibited by ILK- miRNA

was transfected into BIU-87 cells for 48 h, the expression of green fluorescent protein was observed by inverted fluorescent microscope in each group. It suggested that every group was transfected successfully. After 2 weeks, green fluorescent cell clusters were seen by blasticidin screening. Monoclonal cell clusters were observed after 2 weeks by limited dilution method subculture, and then were expanded, all cells with green fluorescence were stably transfected cell lines (Figure 3).

*ILK mRNA expression*

RT-PCR results showed the 406bp purpose bands and 303bp internal reference band (Figure 4). The gel image analysis showed relative expression levels of ILK mRNA (Table 3). The expression of ILK mRNA in each group was significant difference by the single factor variance

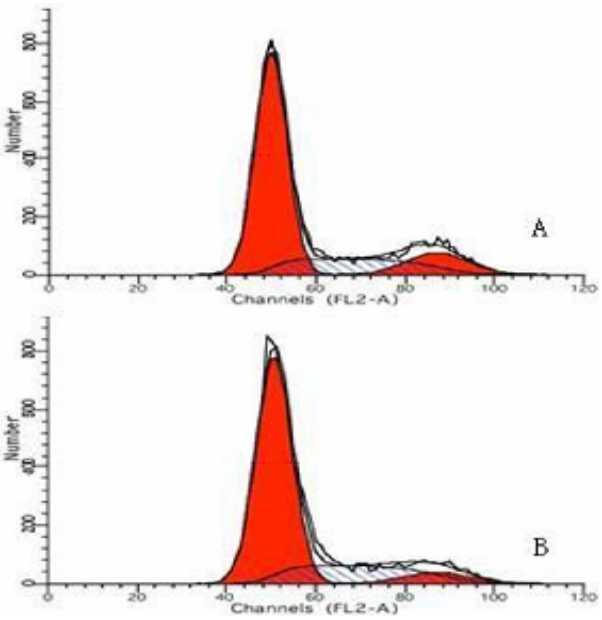


**Figure 7. Cell Cycle Distribution of Each Group** (A: non-transfected group, B: transfected group) Cell cycle of the transfected group was compared with the non-transfected group, G0/G1 phase was significantly increased, while the S phase was significantly reduced, It suggested that ILK miRNA can inhibit cell growth effectively via inducing G0/G1 arrest (P <0.05)

(Oneway ANOVA) analysis (P<0.05). LSD-t test multiple comparison showed that ILK mRNA in ILK-miRNA plasmid transfected group was significantly lower than that in non-transfected and the negative control group (P>0.05). The inhibition efficiency showed that miR-1 was 66.7%, miR-2 was 68.8%, miR-3 was 84.6%, miR-4 was 83.4%, and pcDNATM6.2-GW/EmGFP- ILK-miR-3 was the most efficient vector of interference plasmid.

*ILK protein expression*

Western-blot quantitative detection results showed ILK protein bands 59 KDa and β-actin protein bands 42KDa (Figure 5). ILK protein relative expression level results showed that ILK protein expression significantly decreased in each transfected group compared with non-transfected group and negative control group by the single factor variance (Oneway ANOVA) analysis and LSD-t test multiple comparison (P<0.05, Table 4). The inhibition efficiency of protein expression showed that miR-1 was 34.6%, miR-2 was 56.0%, miR-3 was 75.5%, and miR-4 was 72.1%, while there was no significant difference of ILK protein expression between the negative control group and non-transfected group (P>0.05). Thus ILK-miRNA in the transfected group inhibited the ILK protein expression effectively.

*MTT*

The absorbance value of the pcDNATM6.2-GW/EmGFP- ILK-miR-3 transfected group was significantly weaker than non-transfected group with increasing time of cells culture (Figure 6). The two-factor analysis of variance showed that the value of A from the third day difference between the two groups was statistically significant (P <0.05). It showed that cells growth and proliferation were inhibited effectively when the expression of ILK

**Table 5. The Volume of Tumor at Different Times in Different Groups**

| Group | 1 w | 2 w | 3 w | 4 w |
|---|---|---|---|---|
| transfected group | 20.36±2.15 | 36.27±3.12 | 44.36±3.78 | 56.67±4.32 |
| non-transfected group | 38.27±3.16 | 86.16±10.45 | 269±21.38 | 389.56±36.49 |

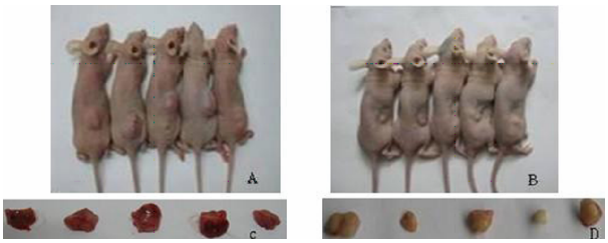


**Figure 8. Two Groups of Mice were Sacrificed After 1 Month Growth and Tumor Tissue** (A, C: non-transfected group, B, D: transfected group) Tumor of the non-transfected group infiltrated surrounding tissue and skin, and was difficultly separated, with ill-defined margin and larger size. Tumor of the transfected group was smaller size and easily separated in nude mice, without infiltration of surrounding tissue

was inhibited by ILK- miRNA in human bladder cancer BIU-87 cells.

*The influence of the cell cycle*

The cell cycle of BIU-87 cells in non-transfected group showed that G0/G1 phase, S phase and G2/M phase were 49.67%, 32.68% and 17.64%, respectively, while the distribution of pcDNATM6.2-GW/EmGFP- ILK-miR-3 transfected cells in those three phase was 67.22%, 9.98% and 22.81%, respectively. The cell cycle of transfected group compared with non-transfected group, G0/G1 phase was significantly increased, while the S phase was significantly reduced analysised by t test. It suggested that ILK miRNA can inhibit cell growth effectively via cell cycle arrest ($P < 0.05$) (Figure 7).

*The influence of tumorigenesis ability*

To evaluate the effect of ILK down-expression on bladder carcinoma growth in vivo, athymic nude mice were subcutaneously injected with pcDNATM6.2-GW/EmGFP- ILK-miR-3 transfected cells or control group BIU-87 cells. Tumor volumes were measured every 7 days after the appearance of the xenografts tumors. The neoplasms in mice which were injected with BIU-87 cells grew faster than those were injected with the pcDNATM6.2-GW/EmGFP- ILK-miR-3 transfected cells. Athymic nude mice were sacrificed when cells were inoculated subcutaneously 1 month. The mean volume of the neoplasms which were injected with BIU-87 cells was significantly larger than that of mice were injected with pcDNATM6.2- GW/EmGFP- ILK-miR-3 transfected cells (Table 5, $P<0.05$). The weight of neoplasms in non-transfected group was 0.965 ± 0.02g, while that in the transfected group was 0.518 ± 0.03g, the independent sample t test showed that the difference is significant ($P<0.05$) (Figure 8). Tumor tissue sections were observed under microscope, non-transfected group tumor cells were dense, having abundant cytoplasm, large and deeply stained nucleus, marked atypia, and more common mitotic figures. The number of tumor cells in the transfected group was relatively rare, small nucleus,

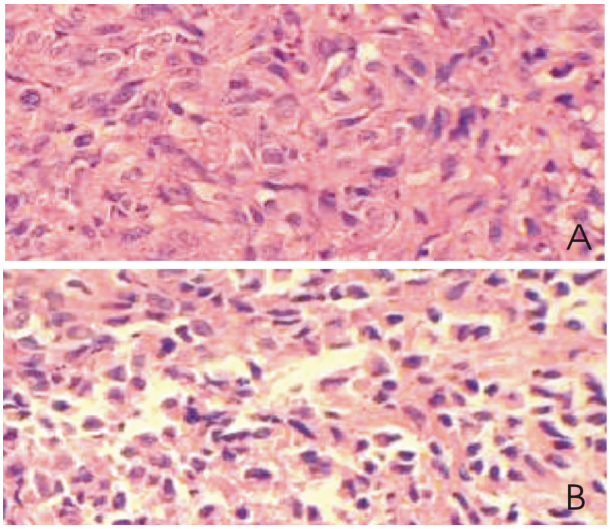


**Figure 9. Tumor Pathological Slices (× 200)** (A: non-transfected group, B: transfected group). The number of tumor cells in the transfected group was relatively rare, small nucleus, little cytoplasm, rare mitotic figures. Condensation and necrosis-like changes in the nucleus and cytoplasm could be seen scattered. In the interstitial, blood vessels were less, neutrophil and lymphocytic infiltration were seen

little cytoplasm, rare mitotic figures, and condensation and necrosis-like changes in the nucleus and cytoplasm could be seen. In the interstitial, blood vessels were fewer, neutrophil and lymphocytic infiltration could be seen. Further immunohistochemistry staining of ILK in the bladder carcinoma xenografts revealed that the staining intensity of ILK in BIU-87 cells was stronger than the pcDNATM6.2-GW/EmGFP-ILK-miR-3 trnasfected cells (Figure 9).

## Discussion

Cancer, in effect, is the loss of normal cell communication, which produces unregulated cell proliferation, migration, and an inhibition of apoptosis. Integrin-linked kinase (ILK) is a molecular target which can lead to various oncogenic-related events including cell migration, invasion and an inhibition of apoptosis. Increased ILK activity is correlated to several varieties malignant tumors including prostate (Kieffer et al., 2005), colon (Marotta et al., 2003), gastric (Ito et al., 2003), and so on. ILK expression was significantly associated with tumor grade, T status, lymph node metastasis and stage (Graff et al., 2001; Takanami, 2005), and tumor angiogenesis (Tan et al., 2004). There is sufficient evidence that ILK is a valid molecular target, playing a central role not only for the formation, progression and maintenance of cancer, but is also a key component in the cross-talk that can exist between pathways (i.e. the PI3K/PKB/AKT and Ras/Raf/MAPK pathway) (Attwell et al., 2000). When the prostate epithelial cancer cells with high expression of ILK were inoculated into nude

mice, tumor could be rapidly formed, and tumor cells displayed a highly invasive phenotype (Zhang et al., 2003; McDonald et al., 2008). When the expression of ILK and protein kinase B (PKB / AKT) activity was inhibited, the cell cycle was arrested and apoptosis was inducted, tumor growth was inhibited (Persad et al., 2000). It suggested that ILK might be an oncogene. ILK over-expression or activation will lead to persistent non-adherence-dependent cell survival, tumorigenic transformation, increased tumorigenicity and invasive potentiality of tumor (Oloumi et al., 2004).

In this study, ILK mRNA and protein expression in BTCC tissue and adjacent normal tissue was studied the first time. Immunohistochemistry and RT-PCR were used to detect ILK protein and mRNA in 56 cases of BTCC and 30 cases of adjacent normal bladder tissue samples. In BTCC tissues, ILK protein and mRNA expression was significantly higher than in adjacent normal bladder tissue ($P < 0.05$). This result was consistent with the findings of ILK in most other tumors, suggesting that ILK might be closely related to the occurrence of BTCC. Correlation analysis showed that, ILK protein expression was significantly correlated to mRNA expression, indicating that the ILK gene transcription and protein expression were parallel. Further integrating with clinical and pathological data comprehensive analysis showed that gene transcription and protein expression of ILK increased with the rise of tumor grade. We found that the positive rate of ILK gene transcription and protein expression exhibited the increasing trend with the increased clinical stage (from the non-invasive to invasive carcinoma). In the early stage of BTCC, there was high expression of ILK, and the higher expression of ILK was accompanied with the higher degree of malignant tumor. ILK might be involved in the occurrence and development process of BTCC and it might act as a tumor marker for early detection of BTCC.

Recent studies have found that, miRNAs (microRNAs) is a class of small molecule non-coding RNA, regulation of intracellular gene expression (Tang, 2005). miRNAs is a stem-loop structure with approximately 70-90 bp single-stranded RNA precursors, generate of about 21-23 bp size of mature miRNAs after processing by Dicer enzyme. Then it forms a miRNA-containing RNA-induced silencing complex (miRISC) when it combines with ribozyme complex. It can inhibit mRNA translation or degraded mRNA to achieve the purpose of preventing protein expression (Mello and Conte, 2004). In this study, we designed ILK miRNA sequences and constructed the specific miRNA interference plasmid pcDNATM6.2- GW/EmGFP-ILK- miR as a vector by Invitrogen, targeting on ILK gene. With applying Gateway expression vector technology, when the precursor miRNA was cloned into pcDNATM6.2-GW/EmGFP-miR, a precursor miRNA expression complex was formed. When expressed in eukaryotic cells, intra-molecular stem-loop structure was formed, then it formed a mature miRNA molecules on endogenous Dicer enzyme. Specific miRNA RNAi vectors targeting on human ILK was transfected into the BIU-87 cells through liposome, obtained cell strain stably expressing pcDNATM6.2-GW/EmGFP-ILK-miR. We observed the expression of EmGFP in BIU-87 cells, indicating that the constructed interference plasmid was transfected into the purpose cells effectively and the cell strains stably expressing interference plasmid were established successfully. It was further verified that ILK mRNA and protein were inhibited effectively by RT-PCR, Western-blot. Through flow cytometry, MTT colorimetric assay and tumorigenesis in nude mice, we found that the BIU-87 cells proliferation ability in vitro and tumor formation ability in vivo were significantly decreased, when the ILK gene was suppressed. This result was consistent with the findings of ILK in most other tumors (Zhao et al., 2011). The activity of PKB was inhibited by inhibiting ILK which could lead to human glioblastoma procedure death and G2~M phase cell cycle arrest (Koul et al., 2005). The ILK antisense oligonucleotide was transfected into malignant glioma cell to inhibit the activity of PKB and induced apoptosis of cancer cells and then slowed tumor growth (Fuchs et al., 2008). ILK-miRNA might inhibit BTCC cell proliferation due to inhibition of PKB activity and prohibition of Akt phosphorylation in this signaling pathway. The BIU-87 cells tumor formation ability in vivo significantly decreased maybe relate to the decrease expression of VEGF when the ILK gene was suppressed (Tan et al., 2004). ILK gene may also influences on the bladder cancer cell growth and proliferation through other signaling pathways; we will further investigate it in the future. Our research provides a new target and idea for gene therapy of bladder cancer.

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