Targeting Integrin-linked Kinase Suppresses Invasion and Metastasis through Downregulation of Epithelial to Mesenchymal Transition in Renal Cell Carcinoma

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Abstract

Renal cell carcinoma (RCC) is the most common malignancy in the kidney. Anti-angiogenic targeted therapies inhibit the progression of RCC, but have limited impacts on invasion or metastasis of tumor cells. Integrin-linked kinase (ILK) is a serine/threonine kinase implicated in the regulation of cell growth/survival, cell cycle progression, epithelial-mesenchymal transition (EMT), invasion/migration, and angiogenesis. However, the role of ILK in RCC has not been evaluated. We investigated the role of ILK on cancer progression and metastasis and the therapeutic potential of ILK inhibition in RCC. Our investigation reveals that ILK is expressed at a low level in normal cells and low-stage RCC cells and is highly expressed in advanced and metastatic cells. Caki-1, a metastatic RCC cell line showed higher expression of molecular EMT markers including Snail and Zeb1, but decreased activity of GSK3β. Knockdown of ILK using small interference (si)-ILK minimally inhibited tumor proliferation and cell cycle progression was not significantly affected. However, ILK knockdown suppressed the formation of stress fibers and focal adhesions and impeded phenotypic EMT markers, including cell migration and invasion, in Caki-1 and UMRC-3 cells. Finally, in vivo knockdown of ILK suppressed the progression, invasion, and metastasis of primary RCC in nude mice by downregulation of EMT markers (Snail, Zeb1, vimentin, and E-cadherin). Our results show that ILK may be essential for invasion and metastasis in RCC and regulates vimentin and E-cadherin expression by regulating the EMT-related transcription factors Snail and Zeb1. These results suggest that ILK may be a potential target in RCC.
Introduction

Renal cell carcinoma (RCC) is the most common renal malignancy, and the incidence and mortality rates have been increasing steadily worldwide at a rate of 2-3% per decade (1,2). Despite an overall shift in earlier diagnosis of RCC, approximately 20-30% of all patients are diagnosed with metastatic disease; even with surgical extirpation, 20% of patients will relapse and develop metastasis during follow-up (3). Anti-angiogenic therapies targeting vascular-endothelial growth factor (VEGF) have shown clinical benefits in patients with advanced RCC, but the results are generally thought to be cytostatic and do not cure patients. These treatments effectively inhibit tumor progression through deprivation of oxygen and nutrition from the tumor microenvironment, but cannot block metastasis of RCC cells. Moreover, there is growing evidence that anti-angiogenic therapies can accelerate invasion and metastasis by making the tumor microenvironment more fertile (4,5).

Epithelial-mesenchymal transition (EMT), defined as the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype, is now known to also occur in a variety of disease states, including during cancer progression (6,7). EMT is characterized by a combined loss of epithelial cell junction proteins, such as E-cadherin, and the gain of mesenchymal markers, such as vimentin, and is believed to play an essential role in tumor invasion and metastasis. A number of distinct molecular processes are engaged to initiate an EMT and allow it to reach completion (8). These include activation of transcription factors, expression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins, and the production of extracellular matrix (ECM)-degrading enzymes [8]. Upon activation, EMT-inducing transcription factors, including Snail, Slug, zinc finger E-box binding homeobox 1 (Zeb1), and Twist, act pleiotropically to choreograph the mesenchymal transition (9). Implementation of EMT by these cells depends on cell surface proteins such as β4 integrins, α5β1 integrin and αVβ6 integrin (9,10). Activation of EMT programs is also facilitated by the disruption of cell-cell adherence junctions and integrin-mediated cell-ECM adhesions (11-13).
Integrins are cell surface proteins representing the main class of receptors for ECM proteins, such as collagen and laminin, and are located in the basement membrane. Attachment to, and movement along, the ECM is often integrin-dependent. Integrin-linked kinase (ILK), originally identified as a β1-integrin subunit cytoplasmic domain interactor, is a widely expressed serine/threonine protein kinase located in focal adhesions (14). ILK is an essential protein that connects integrins to the actin cytoskeleton and regulates actin polymerization. Localization of ILK induces focal adhesion plaques and coordinates cell spreading and actin organization. These adhesion sites are major intracellular signaling centers where ILK plays a central role in transducing many of the biochemical signals initiated by cell-matrix interactions that regulate fundamental processes such as growth, proliferation, survival, differentiation, migration, invasion, and angiogenesis (15-19).

Overexpression of ILK is often a prominent feature of human malignancies and its increased abundance in tumor tissues correlates with poor outcome (20-24). Recent reports suggest that overexpression of ILK in epithelial cells induces the EMT by repressing E-cadherin expression, activating nuclear β-catenin and inducing a transformed, tumorigenic phenotype (25). However, the role of ILK in RCC cell survival, invasion and metastasis is unclear. Here, we show that, in metastatic RCC cells, both ILK and its downstream effectors related to the EMT are highly expressed. Overexpression of ILK increased tumor cell activity for migration and invasion. Finally, we show that ILK is essential for invasion and metastasis of RCC both in vitro and in vivo, and demonstrate its potential as a therapeutic target as anti-metastasis therapy in RCC.

Materials and Methods

Cell culture and reagents

The human RCC cell line Caki-1 was obtained from the American Type Culture Collection (ATCC) and maintained in McCoy’s 5A Medium (Hyclone, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) in 2011. Two additional human RCC cell lines, UMRC-3 and UMRC-6, were gifts
from Dr. P. Black (Vancouver Prostate Centre, University of British Columbia, Canada) in 2011 and maintained in Minimal Essential Medium (MEM; Invitrogen) supplemented with 10% FBS and 2 mmol/l L-glutamine. Cell line authentication for UMRC-3 and UMRC-6 from Dr. P Black was performed using the fingerprinting by AmplFLSTR identifier amplification kit (Applied Biosystems, Foster, CA) in Korean Cell Line Bank, Korea. The human RCC cell line 786-O was purchased from the ATCC and maintained in RPMI-1640 Medium (Hyclone, Thermo Fisher Scientific) supplemented with 10% FBS in 2011. The human renal epithelial cell line HK-2 was purchased from the ATCC and cultured in DMEM/Ham’s F12 (Invitrogen) supplemented with 10% FBS and 2 mmol/L L-glutamine in 2012. All cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. For all experiments, cell lines were maintained for no more than 2 months.

**Small-interfering RNAs**

Caki-1 and UMRC-3 cells were transiently transfected with 21-bp siRNA molecules targeting the integrin-binding domain of ILK (5’-GACGCTCAGCAGACATGTGGA-3’) or a non-silencing sequence siRNA (si-Scr). Caki-1 and UMRC-3 cells were plated onto 6-well plates at a density of 1.2 × 10⁵/well and then transfected at 30-50% confluence for 16 h with siRNAs by Lipofectamine 2000 (Invitrogen Life Technologies) diluted with OPTIMEM (Invitrogen Life Technologies) according to the manufacturer’s instructions. After transfection, media was replaced and cells were then incubated for 48-72 h based on the purpose of the experiment.

**Stable transfections**

To generate ILK-overexpressing RCC cells, UMRC-6 cells were transfected with either pcDNA3.1 His-V5-tagged kinase constitutively active ILK (ILK S343D) expressing constitutively kinase-active mutant ILK or pcDNA 3.1 His-V5 as a control (mock) (provided by Dr. Shouket Dedhar of the BC Cancer Agency,
Canada) (26). Cells grown in 6-cm dishes were transfected for 4 h with 3-μg DNA using a 1:3 ratio of Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s recommendations (26). Clones were selected with G418 (Invitrogen). For stable knockdown of ILK in Caki-1 cells, oligonucleotides for ILK small hairpin RNA (shRNA) (5'-CCGGGCAGTACAAGGCAGACATCAACTCGAGTTGATGTCTGCC-TTGTACTGCTTTTTG-3') were purchased from Sigma-Aldrich. To generate lentiviral particles, the pLKO.1-puro ILK1 shRNA plasmid was co-transfected with the gal/pol plasmid pMDLg/pRRE, envelop plasmid pRSV-REV and pMD2.G by Lipofectamine 2000 reagent (Invitrogen) in the human embryonic kidney cell line 293FT transformed with SV40 large T antigen. After 48 h, the resulting supernatant was collected, filtered through a 0.45-μm syringe-driven filter unit (Sartorius Stedim Biotech), and infected with 1 μg/ml polybrane (hexadimethrine bromide) (Sigma-Aldrich) into Caki-1 cells. After incubation with virus supernatant for 72 h, cells were selected with 1 μg/ml puromycin (Sigma-Aldrich). pLKO.1-puro non-silencing shRNA was used as a control. Similarly, to develop the orthotopic renal tumor model (Ckai-1-luc), Caki-1 cells were stably transduced with lentiviral particles containing the firefly luciferase gene. The infectious virus was generated by co-transfection of 293FT cells with the FULLBW vector [derived from FUGW (a generous gift from David Baltimore), but GFP was replaced with the firefly luciferase gene using the Invitrogen Gateway System] and the packaging plasmids pCMVDR8.2 and pMD.G. After lentivirus transduction, Caki-1 cells were further selected with Blasticidin (Invitrogen). Stable cell clones were then confirmed by Western blotting analysis. After selection and confirmation, stable cell lines were frozen at early (less than 10) passages.

**Cell viability assay**

Cells were seeded onto six-well plates at a density of 1.2 × 10^5 per well and incubated overnight. After transfection, cells were incubated for 72 h and then fixed with 1% glutaraldehyde and stained with 0.5% crystal violet solution. Cells were washed with water and the remaining crystal violet was resolved with
Sorensen’s solution. Absorbance was measured at 562 nm by spectrophotometry. All experiments were performed in triplicate.

Cell migration assay

A wound-healing assay was used to assess directional cell migration. Cells were plated onto six-well plates and allowed to form a confluent cell monolayer. Wounds were made in each well using a 200-µl pipette tip. After the scratch, floating cells and debris were washed out with phosphate-buffered saline (PBS) twice and cells were then incubated in culture media for an additional 36 h. Wound-healing was recorded every 6 h by microscopy. Each experiment was performed in triplicate and, in each experiment, the area of cells that migrated across the wound mark was determined in five microscopic fields. The percentage of wound healing was calculated using the equation: (percent wound healing) = average of ([gap length: 0 h] - [gap length: 18–24 h]) / [gap length: 0 h]).

Cell invasion assay

Matrigel-coated inserts in 24-well plates (BD Matrigel™ Invasion Chamber, 8.0 µm PET membrane) were used to assess cell invasion. A cell suspension containing 1 × 10^6 cells/ml in serum free media was added to the inside of the insert. Lower wells were filled with 500-µl media containing 10% FBS. Plates were incubated for 24 or 48 h in a cell culture incubator. Non-migratory cells on the top of the membrane were carefully removed using the ends of cotton-tipped swabs three times. The remaining migratory cells were stained with 0.5% crystal violet solution, gently washed several times with water, and allowed to air dry. Each experiment was performed in triplicate. Quantification was performed by counting migratory cells using light microscopy in three individual fields per insert.

Cell cycle analysis
Cells were harvested and fixed with 70% alcohol as a single-cell suspension. The fixed cells were collected by centrifugation and washed with phosphate-buffered saline (PBS). The cells were incubated with RNase A (1 mg/mL) for 30 min at 37°C and then incubated with propidium iodide (20 μg/mL; Sigma Chemical) for 30 min. Relative DNA contents were analyzed on a FACSCanto II flow cytometer and FACSDiva software (BD Bioscience).

**TUNEL assay**

Cells were fixed with 4% paraformaldehyde after 48 h of treatment and stained using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit (Promega, Madison, WI). Apoptotic (fluorescent green) and total cells were counted under a fluorescence microscope and data were recorded. Images were obtained using a confocal microscope (LSM Meta 700, Carl Zeiss, Oberkochen, Germany).

**Antibodies and reagents**

Anti-ILK (C-19), anti-vinculin and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pGSK3b, anti-Snail, anti-Zeb1, and anti-vimentin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-E-cadherin antibody was purchased from BD Biosciences (Franklin Lakes, NJ). Anti-goat, anti-rabbit and anti-mouse IgG secondary antibodies were used (1:5000) for Western blotting analysis or immunocytochemical staining.

**Western blotting analysis**

Proteins were harvested in RIPA lysis buffer containing vanadate, phosphatase inhibitor and phenylmethanesulfonylfluoride (PMSF). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-
PAGE) was performed, and gels were then transferred to a nitrocellulose membrane using the trans-blot system (Bio-Rad, Hercules, CA) as described previously (26). Membranes were blocked in 5% skim milk diluted with Tris-buffered saline containing Tween-20 (TBST) for 1 h at room temperature and then immunoblotted with primary antibodies diluted in 3% skim milk diluted with TBST for 1 h at room temperature or overnight at 4°C. Amersham™ (GE Healthcare, UK) or Supersignal (Thermo Scientific, US) was used as Western blotting detection reagents.

**Immunocytochemistry and confocal microscopy**

To assess the expression of ILK in cells, cells were plated onto glass coverslips in six-well plates overnight to allow cell attachment, washed with ice-cold PBS, fixed with ice-cold methanol mixed with acetone (3:1) for 10 min at room temperature, and treated with 0.25% Triton X-100 in PBS solution for 10 min. After blocking with 3% bovine serum albumin for 30 min, cells were incubated with anti-ILK at room temperature for 1 h. Cells were washed three times with TBS and incubated with Alexa Fluor 488 anti-goat antibody for 1 h in a dark room. Nuclei were stained with DAPI. To evaluate the expression of ILK, Snail, Zeb1, vimentin, E-cadherin, actin, vinculin, and Ki-67 in cells after ILK knockdown, cells were processed in a similar manner with anti-actin and -vinculin antibodies. Immunofluorescence was visualized using a Zeiss confocal laser-scanning microscope.

**Orthotopic xenograft model of human RCC**

All animal experiments were conducted in compliance with accepted standards of the University of British Columbia Committee on Animal Care. Six- to eight-week-old female nude mice (nu/nu) were anesthetized with isoflurane. To develop the orthotopic renal tumor model (Caki-1 cells) the right kidney was exposed via a small incision in the flank area and a suspension of $1 \times 10^6$ Caki-1-luc cells in 50-µl PBS was injected into the subscapular area of the exposed kidney through a 30-gauge needle. The punctured site
was pressed by a cotton-tipped swab until bleeding ceased. The kidney was returned to the normal position and the flank incision was closed with 6-0 vicryl sutures. Orthotopic growth of RCC was confirmed via bioluminescence. Invasion and metastasis were also traced using bioluminescence. Animals were imaged every week using an IVIS2000 Imaging system (Caliper Life Sciences). Following treatment, mice were euthanized by a CO$_2$ chamber and cervical dislocation. All tumors were fixed in 10% formalin for immunohistochemical staining or frozen at -80°C for protein and RNA analysis.

**Immunohistochemistry**

All tissues were fixed in 10% neutral buffered formalin and embedded in paraffin using standard surgical pathology protocols. Tissue sections (5 μm) were dewaxed and antigen retrieval was performed in citrate buffer (pH 6) using an electric pressure cooker set at 120°C for 5 min. Sections were incubated for 5 min in 3% hydrogen peroxide to quench endogenous tissue peroxidase. Staining was performed using the Ventana Discover XT Autostainer (Ventana Medical Systems, Tucson, AZ) with a biotin streptavidin system and a solvent-resistant DAB Map kit. Primary monoclonal antibodies were directed against ILK (Santa Cruz, CA, USA), snail (Santa Cruz, CA, USA), Zeb 1 (Abcam, Cambridge, UK), and vimentin (Abcam, Cambridge, UK), which were diluted with phosphate-buffered saline at a ratio of 1:100. All tissue sections were counterstained with hematoxylin, dehydrated, and mounted. The MetaMorph 4.6 software was used for computerized quantification of immunostained target proteins.

**Statistical analysis**

All observations were confirmed by at least three independent experiments. The results are presented as the means ± SE. Statistical analysis was performed using SigmaPlot 12 (Systat Software Inc., Chicago, IL, USA). Student’s t-test was used for two-group comparisons. Differences were considered significant when P values were 0.05 or less.
Results

ILK is highly expressed in advanced RCC

To investigate the expression of ILK in RCC, we first examined baseline expression of ILK by immunofluorescence using a panel of human RCC cell lines and normal human renal epithelial cells. Non-cancerous human renal epithelial cells (HK-2) poorly expressed ILK and RCC cell lines generally showed higher expression of ILK compared to normal renal cells (Fig. 1A). Additionally, ILK was more highly expressed in cell lines derived from high-stage tumors (UMRC-3 from a stage IV primary tumor and Caki-1 from a metastatic tumor) compared to a cell line from a low-stage tumor (UMRC-6 from a stage I primary tumor), suggesting that ILK may be related to the aggressive features of high-stage RCC (Fig. 1B).

ILK expression is correlated with the expression of EMT markers in RCC

Markers related to the EMT were assessed using a panel of human RCC cell lines and normal human renal epithelial cells. Phosphorylation of GSK3β was low in Caki-1 cells. ILK expression was correlated with the expression of transcription factors Snail and Zeb1, as well as vimentin. E-cadherin was detected in HK-2 cells, but not in all RCC cell lines (Fig. 1B). We confirmed this correlation, shown with the Western blots, using immunofluorescence confocal imaging (Fig. S1 A-E). These results show that ILK expression is related to EMT markers in various RCC cells.

Overexpression of ILK induces phenotypic changes by upregulating Snail and Zeb-1 in RCC
We next developed ILK-overexpressing UMRC-6 cells to assess the role of ILK expression in RCC. UMRC-6 cells have low ILK expression compared to other RCC cell lines, but ILK-overexpressing UMRC-6 cells demonstrated high expression of ILK compared with mock cells (Fig. 2B). *In vitro*, ILK-overexpressing cells showed a more fibroblast-like morphology and loss of polarization and contact inhibition, suggesting a morphologic transition to a mesenchymal phenotype by ILK overexpression. To determine whether these morphologic changes are related to the EMT, we assayed EMT markers in ILK-overexpressing UMRC-6 cells (Fig 2A). Western blotting analysis confirmed that ILK overexpression was associated with the expression of Zeb-1 and Snail, critical transcription factors in the EMT, and also vimentin, a major cytoskeletal component of mesenchymal cells (Fig. 2B). EMT characteristics, migration and invasion, as well as a morphological change, were evaluated using a wound-healing assay and Matrigel invasion assay. These assays confirmed that ILK overexpression increased the migration and invasion potential in RCC cells (Fig 2C-F). In summary, these results suggest that ILK induces the EMT through upregulation of Zeb-1 and Snail in RCC cells.

**Cell survival is not significantly regulated by ILK in RCC**

To evaluate the effects of ILK on tumor growth and cell cycle progression in RCC, we transiently knocked-down ILK in RCC cells using RNA interference targeting ILK. siILK successfully inhibited ILK expression at a concentration of 40 nmol (Fig 3A). However, a cell growth assay using HK-2, UMRC-6, UMRC-3, and Caki-1 cells showed that *in vitro* proliferation of RCC cells was not suppressed by ILK knockdown compared to the normal renal epithelial cell line HK-2 (< 20%) (Fig 3B). Also, the insufficient inhibition of proliferation was unaffected by increasing siRNA concentration in Caki-1 cells (Fig 3C). Cell cycle analysis showed that ILK knockdown did not induce apoptosis or significant cell cycle arrest in Caki-1 cells. In summary, ILK plays a minimal role in cell survival and cell cycle pathways in RCC cells. We confirmed the minimal effect of ILK knockdown on cell proliferation and apoptosis using a TUNEL assay and Ki-67 staining (Fig. 3E and F).
Knockdown of ILK suppresses migration and invasion in RCC

To study the effect of ILK inhibition on tumor migration in RCC cells, we used a wound-healing assay after transient knockdown of ILK in RCC cells using siILK. The wound-healing assay confirmed that cell migration into the wound was delayed significantly by ILK knockdown in Caki-1 cells (Fig. 4A and B). Cell growth assay and Ki-67 staining suggested that ILK has minimal effects on the proliferation of RCC cells (Fig. 3), but to completely rule out the effect of cell proliferation on migration, the wound-healing assay was repeated after mytomycin C treatment. The results confirmed that ILK knockdown suppressed the migration of RCC cells (Fig. 4C and D). We also assessed cell invasion using a Matrigel invasion assay, which showed that invasion through the Matrigel pore was reduced by ILK knockdown in Caki-1 and UMRC-3 cells (Fig. 4E-G). Immunocytochemical staining showed that E-cadherin expression was induced by knockdown of ILK (Fig. 4H). These results suggest that the invasive potential of RCC can be suppressed by ILK inhibition.

ILK knockdown impedes cell attachment by regulating stress fiber formation and focal adhesion in RCC

After transient knockdown of ILK, cells exhibited less attachment to cell culture plates (Fig. 5A). From this finding, we hypothesized that ILK knockdown affects both cell attachment and movement by changing cytoskeleton organization. Then, we tested if knockdown of ILK reduces cell attachment to fibronectin-coated plates. ILK knockdown by siILK impeded the attachment of renal cell carcinoma cells (Fig. 5B). Rhodamine-phalloidin staining showed that ILK knockdown impeded stress fiber formation and organization compared with control cells (Fig. 5C and D). To evaluate actin filament organization and focal adhesions, immunofluorescence staining using anti-actin and vinculin antibodies in UMRC-3 cells replated after transient knockdown of ILK was used. ILK knockdown significantly reduced actin filaments and organization and reduced focal adhesions (Fig. 5E). These results suggest that ILK plays an
important role in stress fiber formation and focal adhesion in RCC cells, and inhibition of ILK impedes cell attachment by suppressing the organization of stress fibers.

**ILK is necessary for invasion and metastasis in an orthotopic model of human RCC**

To investigate the role of ILK in *in vivo* tumor invasion and metastasis, we created an orthotopic RCC mouse model by inoculating stably transfected ILK knockdown luciferase-expressing Caki-1 cells into the subscapular renal parenchyma of nude mice (Fig 6A). The growth rate of the primary tumors inoculated into the kidney was much lower in shILK transfectants than in vector control transfectants (Fig 6B and C). In addition, it was clear that invasion and metastasis were reduced in animals inoculated with shILK transfectants compared to in vector control transfectants. Direct invasion of renal tumors into adjacent tissues or organs, including perirenal tissues, intestines, liver, and abdominal muscles, and disseminated seeding nodules in the intestine, spleen, and peritoneum were found in xenografts expressing mock shRNA, whereas no invasion or metastasis was observed and only intrarenal tumors were found in xenografts expressing shILK (Fig. 6D). Immunohistochemical staining showed that ILK was successfully knocked down *in vivo*, and also that Snail, Zeb-1, and vimentin were downregulated and E-cadherin was upregulated by ILK knockdown (Fig. 6E and F). These results suggest that ILK is essential for invasion and metastasis in RCC, and also show that ILK may serve as a therapeutic target for anti-metastasis therapy of RCC. In vitro knockdown of ILK had minimal effect on cell proliferation in RCC cells, whereas in vivo knockdown of ILK showed marked inhibition of primary orthotopic tumor growth, as well as of invasion and metastasis. To confirm this, we evaluated the anti-angiogenic effect of ILK using immunohistochemical staining and confirmed significant suppression of tumor angiogenesis by ILK knockdown in RCC xenografts (Fig. S2).
Discussion

The development of novel therapies that suppress cancer metastasis is an urgent therapeutic need in RCC, as most existing approved therapies for metastatic disease have been documented to prevent disease progression rather than clearly improve survival (27,28). We have shown that ILK may be important in invasion and metastasis in advanced RCC. Additionally, we showed that ILK might promote EMT by regulating the transcription factors Snail and ZEB1, as well as cell movement by inducing focal adhesions and actin organizations in RCC. Inhibition of ILK suppresses both migration and invasion in RCC by impeding the formation of stress fibers and focal adhesion plaques. Inhibition of the primary tumor was supported by significant suppression of tumor angiogenesis after ILK knockdown in RCC xenografts. Suppression of metastasis, as well as primary tumor size, in orthotopic renal xenografts is consistent with the hypothesis that ILK may serve as a therapeutic target for inhibiting invasion and metastasis in advanced RCC.

ILK plays a central role in cell signaling and is related to tumor proliferation and cell cycle progression in many types of cancer, but the role of ILK in RCC is less clear. A recent study suggested a possible role for ILK in the progression of RCC by showing that ILK expression is correlated with tumor severity in clear-cell RCC (29). In this study, the RCC cell line from a metastatic lesion showed the highest expression of ILK and displayed higher expression of EMT-related transcriptional factors Snail and Zeb1, which suggests that ILK might be related to the metastatic potential of RCC cells. Although our study showed that ILK is not a main driver of tumor proliferation according to in vitro experiments, marked suppression of primary tumor size was observed in orthotopic xenografts containing shILK transfectants, suggesting that inhibition of ILK may suppress tumor progression by blocking cell movement and angiogenesis rather than direct suppression of tumor proliferation in RCC.

Metastasis requires the interaction of tumor cells with the ECM, which is mediated by the formation of elongated, integrin β1-containing adhesion plaques (30). The development of such plaques requires the prior assembly of integrin β1-containing filopodium-like protrusions. Actin-rich protrusions morphologically resembling filopodia formed by cells growing in monolayer culture and knockdown of ILK
reduced the number of filopodium-like protrusions in mouse mammary carcinoma cells with metastatic potential (30). We showed that ILK is essential for focal adhesions and actin organizations required for metastatic movement of RCC cells, and that inhibition of ILK suppresses the formation of focal adhesion plaques. In our study, ILK knockdown caused a reduction in focal adhesion plaques and stress fiber formation, and also changed the morphology of RCC cells from a spindle shape to a rounded shape, which is consistent with data showing reduced filopodium-like protrusions in mammary carcinoma cells. In summary, these results suggest that ILK plays a central role in the formation of focal adhesion plaques through reorganization of cytoskeletons for metastasis in RCC.

The key mechanism by which ILK regulates the EMT is through inhibition of transcription of E-cadherin and vimentin. Multiple signaling pathways can regulate the EMT through modulating Snail, Twist and Zeb family transcription factors (31). Our study showed that, through the overexpression of ILK in cells derived from low-stage RCC, ILK expression induces vimentin through upregulating the expression of Snail and Zeb1 in RCC. Snail, a zinc-finger transcription factor, triggers the EMT process by repressing E-cadherin expression and is regulated by GSK-3β (32). Zeb-1 is the Zeb family zinc finger transcription factor. The expression of Zeb proteins in epithelial cells strongly inhibits CDH1 gene expression, and this mechanism has been implicated in the EMT, tumorigenesis and metastasis. Zeb factors can also regulate the expression of various EMT- and tumor-related genes, such as genes encoding proteins critical to maintain the epithelial phenotype, such as E-cadherin, plakophilin 2 and ZO3 (31). Although the mechanism underlying ILK-mediated regulation of Zeb-1 and Snail is not defined, it was noted that overexpression of ILK in epithelial cells increases the mRNA levels of both Zeb-1 and Snail (33). Also, the major ILK responsive element, SIRE (Snail ILK responsive element) is located in the -134 to-69 region of the Snail-1 promoter and ILK knockdown was shown to downregulate Snail-1 promoter activity and upregulate E-cadherin promoter activity (34). Since it is known that the transcription of Zeb-1 is regulated by Snail-1 (33), we propose that ILK indirectly regulates the transcription of Zeb-1 via Snail, though we cannot exclude the possibility that ILK also directly regulates Zeb-1. Likewise, PARP-1 was identified as a SIRE-binding protein. ILK silencing inhibited PARP-1 binding to SIRE, and PARP-1 silencing decreased both Zeb1 and Snail, which resulted in the upregulation of E-cadherin (35).
Vimentin, the intermediate filament protein, is an important marker of the EMT and a requisite regulator of mesenchymal cell migration (36). Recent studies showed that vimentin also contributes functionally to EMT phenotypes including cell migration and invasion. This suggests that ILK plays a critical role in directly regulating EMT signals, as well as mediating EMT signals to reorganization of the cytoskeleton in RCC cells (36,37). Our data show that vimentin expression is regulated by ILK expression, which suggests that ILK regulates the EMT in RCC in various ways. In conclusion, our study shows that ILK regulates the EMT in RCC cells, and targeting ILK suppresses invasion and metastasis through inhibition of the EMT in RCC. These results suggest that ILK may serve as a target for anti-metastasis therapy for RCC.
References


Figure Legends

Figure 1. Expression of ILK protein and its downstream effectors in normal renal epithelial cells and RCC cells. A, expression and localization of ILK protein in normal kidney tubular cells (HK-2) and RCC cells (UMRC-6, UMRC-3, and Caki-1). Cells on coverslips were fixed and stained for ILK expression and Hoescht and representative confocal micrographs were taken at 40× magnification. B, association of ILK expression with EMT markers in normal kidney tubular cells and RCC cells. Cells were harvested and whole-cell lysates were prepared and subjected to Western blotting to examine the expression of ILK and indicated molecules in the EMT pathway. Vinculin was used as a loading control.

Figure 2. Morphologic and molecular changes after stable transfection with ILK in UMRC-6 cells (low-stage RCC cells). A, morphologic changes in UMRC-6 cells after stable transfection with ILK. When cells reached confluence, photographs were obtained using a light microscope at 20× magnification. B, changes in the expression of EMT markers after ILK overexpression. Whole-cell lysates from UMRC-6 cells transfected with pcDNA3.1 His-V5-tagged kinase constitutively active ILK (ILK S343D) expressing constitutively kinase-active mutant ILK or vector control were subjected to Western blotting to examine the expression of ILK and EMT markers. β-actin was used as a loading control. C and D, effect of ILK overexpression on migration of UMRC-6 cells. When cells reached confluence, a wound was made by scratching and the percentage of wound healing was calculated. The photos were taken at 20× magnification. E and F, effect of ILK overexpression on the invasion of UMRC-6 cells. A cell suspension was plated on Matrigel-coated insert plates and migratory cells were stained with 0.5% crystal violet solution and counted under a light microscope. The photos were taken at 20× magnification. ** P <0.05 vs. mock.

Figure 3. Effects of ILK inhibition on tumor growth and the cell cycle in RCC in vitro. A, Western blot analysis following transfection with si-ILK or control siRNA in Caki-1 cells. Vinculin was used as a loading
control. B, growth of Caki-1 cells after transient knockdown with si-ILK at the indicated concentrations. Caki-1 cells were transiently knocked down with si-ILK or control siRNA (20-160 μM), and cell viability was measured by crystal violet assay. C, growth of a panel of RCC cell lines after transient knockdown of ILK. Five RCC cell lines were used for the screen. Cell growth was measured by crystal violet assay after transient transfection with si-ILK, control siRNA, or reagent control. D, cell cycle analysis of Caki-1 cells treated with si-ILK. The cell cycle was analyzed in Caki-1 cells treated with si-ILK or control siRNA using FACS flow cytometry with PI staining. E, cells were fixed and stained using a TUNEL assay kit, and apoptotic (fluorescent green) and total cells were visualized and counted. F, immunofluorescence images from the Ki-67 staining. Cells were fixed and incubated with anti-Ki-67 antibody and Alexa Fluor 488 anti-goat antibody. The representative photographs were obtained using a confocal microscope (E and F). * P <0.05 vs. control, ** P <0.01 vs. control.

Figure 4. Changes in cell migration and invasion after knockdown of ILK in advanced RCC in vitro. A and B, scratches were made when Caki-1 cells reached confluence after transient transfection with si-ILK or control si-RNA. The photographs were obtained 36 h after scratching at 20× magnification. C and D, when Caki-1 cells reached confluence after transient transfection with si-ILK or control si-RNA, cells were treated with mitomycin C (5μg/ml) and scratches were made. E, Caki-1 cells and UMRC-3 cells were used for invasion assay. A cell suspension was placed onto Matrigel insets and after 24 h, the non-migratory cells were washed out and the migratory cells were counted after crystal violet staining. The photographs were obtained using a light microscope at 20× magnification. F and G, quantification of migratory cells in E. H, immunocytochemical staining of Caki-1 cells stably expressing shILK or mock using an anti-E-cadherin antibody and Hoescht. Representative confocal micrographs were obtained at 40× magnification. * P <0.05 vs. control, ** P <0.01 vs. control.

Figure 5. ILK regulates stress fiber formation and focal adhesions in RCC cells. A, representative images of morphologic changes in cells replated after transient transfection with siScr or siILK. Photographs were
obtained at 20× magnification. B, representative images of stress fiber formation in UMRC-3 cells transfected with siScr or siILK. C, representative images of stress fiber formation and focal adhesion. Cells were stained with 1 unit of phalloidin-rhodamine (Invitrogen) and anti-vinculin antibody for 30 min and Hoechst 33258 (20 mmol/L) for 5 min. Fluorescence staining was visualized using a fluorescence confocal microscope. (B and C, 40× magnification). D, quantification of stress fiber staining intensity in C. Data were obtained from triplicate experiments. * P <0.05 vs. control, ** P <0.01 vs. control. E, cells were seeded on non-tissue culture plates coated with fibronectin for 1 h at 37°C. Attached cells were fixed and stained with crystal violet and the absorbance at 590 nm was measured.

**Figure 6.** ILK knockdown inhibits tumor progression and distant metastasis in an orthotopic RCC animal model. A, cell lysates were harvested from Caki-1 cells stably expressing shILK or control vector and subjected to Western blotting to assess knockdown efficiency. Vinculin was used as a loading control. B, growth, invasion, and metastasis of orthotopic tumors were traced using bioluminescence imaging for 6 weeks. Photon images were obtained using an IVIS instrument. C, quantification of photons in Caki-1-shILK or –mock mice was performed using the IVIS software. D, all mice were sacrificed at the end of the study and the extent of the tumor was grossly evaluated. Representative photographs were obtained. A yellow arrow indicates the original tumor implanted into the kidney and white arrows indicate metastatic nodules. E and F, immunohistochemical staining with antibodies for the indicated EMT markers, including Snail, Zeb1, vimentin, and E-cadherin, in orthotopic xenografts. Magnification, 20×. Quantification of positive staining was performed using the Metamorph 6.0 software (Universal Imaging Corp., Downingtown, PA, USA). ** P <0.01 vs. control.
**Figure 1**

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Figure 2

A

UMRC-6-Mock

UMRC-6-ILK

B

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C

RC-6-Mock

RC-6-ILK

D

Area of migrating cells

Mock

ILK

E

RC-6-Mock

RC-6-ILK

F

Number of Invading Cells

Mock

ILK

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Figure 4

(A) Control, si-Scr, si-ILK at 0h and 36h.

(B) Bar graph showing cell migration (% of control) for control, si-Scr, and si-ILK.

(C) si-Scr, si-ILK at 0h and 18h.

(D) Bar graph showing cell migration (% of control) for si-Scr and si-ILK.

(E) Caki-1, UMRC-3 with control, si-Scr, si-ILK at 0h.

(F) Bar graph showing the number of invading cells for control, si-Scr, and si-ILK in Caki-1.

(G) Bar graph showing the number of invading cells for control, si-Scr, and si-ILK in UMRC-3.

(H) DAPI, E-cadherin, and merge images for Mock and sh-ILK.
**Figure 5**

A. Control, si-Scr, si-ILK

B. Relative attached cell (%)

C. si-Scr, si-ILK

D. Relative ratio of cells with stress fiber (%)

E. Actin, Vinculin, DAPI, merge

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Figure 6

A) Western blot analysis showing the expression levels of ILK and Actin in Mock and sh-ILK groups.

B) Luciferase images of tumor bioluminescence in Mock and sh-ILK groups over 8 weeks.

C) Graph showing the tumor bioluminescence over time (week) for Caki-1-mock and Caki-1-shILK groups.

D) Photographs of tumor samples from Mock and sh-ILK groups, with arrows indicating differences.

E) Immunohistochemical staining for ILK, Snail, Zeb1, Vimentin, and E-cadherin in Mock and sh-ILK groups.

F) Bar charts showing the intensity of ILK, Snail, Zeb1, Vimentin, and E-cadherin in Mock and sh-ILK groups.
Targeting Integrin-linked Kinase Suppresses Invasion and Metastasis through Downregulation of Epithelial to Mesenchymal Transition in Renal Cell Carcinoma

Kyung Seok Han, Na Li, Peter Raven, et al.

Mol Cancer Ther Published OnlineFirst February 5, 2015.

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