

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

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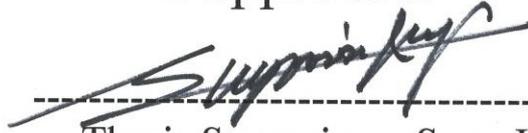
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Kyung Seok Han

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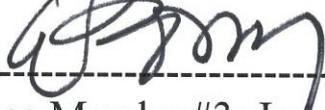
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ABSTRACT

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

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(Directed by Professor Sung Joon Hong)

Renal cell carcinoma (RCC) is the most common malignancy in the kidney and advanced RCC is related to a poor prognosis. Anti-angiogenic targeted therapies inhibit the progression of RCC, but have no or negative effects on invasion and metastasis of tumor cells. Integrin-linked kinase (ILK) is a serine/threonine kinase implicated in the regulation of cell growth and survival, cell cycle progression, epithelial-mesenchymal transition (EMT), invasion and migration, and angiogenesis. However, the role of ILK in RCC has not been evaluated. Here, we investigated the role of ILK on cancer progression and metastasis and the therapeutic potential of ILK inhibition in RCC. Our investigation revealed that ILK is expressed at a low level in normal cells (HK-2) and low-stage RCC cells (UMRC-6), but highly expressed in advanced and metastatic cells (UMRC-3 and Caki-1). Caki-1, a metastatic RCC cell line showed higher expression of molecular EMT markers including Snail and Zeb1, but decreased activity of glycogen synthase kinase 3 beta (GSK3 β). Knockdown of ILK using small interference (si)-ILK inhibited tumor proliferation, but the inhibition rate was minimal and cell cycle progression was not significantly affected.

However, ILK knockdown suppressed the formation of stress fibers and focal adhesions in UMRC-3 and Caki-1 cells, 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. Immunohistochemical studies showed that Snail, Zeb1, vimentin, and E-cadherin were downregulated upon ILK knockdown. Critically, in advanced RCC, ILK is highly expressed and its expression is related to EMT-related proteins. Thus, ILK is essential for invasion and metastasis in RCC and regulates vimentin expression by regulating EMT-related transcription factors Snail and Zeb1. These results suggest the potential of ILK inhibition as an anti-metastasis therapy for advanced RCC.

Key words : renal cell carcinoma, integrin-linked kinase, metastasis, invasion, epithelial-mesenchymal transition

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I. 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 and an additional 20% of patients undergoing nephrectomy 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 β 4 integrins, α 5 β 1 integrin and α V β 6 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 β 1-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.

II. MATERIALS AND METHODS

Cell culture and reagents

The human RCC cell line Caki-1 was obtained from the American Type Culture Collection and maintained in McCoy's 5A Medium (Hyclone, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS). 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) and maintained in Minimal Essential Medium (MEM; Invitrogen) supplemented with 10% FBS and 2 mmol/l L-glutamine. The human renal epithelial cell line HK-2 was provided by Dr. C. Du (Vancouver Prostate Centre, University of British Columbia, Canada) and cultured in DMEM/Ham's F12 (Invitrogen) supplemented with 10% FBS and 2 mmol/L L-glutamine. 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'-GACGCTCAGCAG ACATGTGGA-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, human ILK cDNA was subcloned into the pcDNA3.1 vector and transfected into UMRC-6 cells using Lipofectamine 2000 (Invitrogen Life Technologies) diluted with

OPTIMEM (Invitrogen Life Technologies). ILK-overexpressing UMRC-6 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- μ m syringe-driven filter unit (Sartorius Stedim Biotech), and infected with 1 μ g/ml polybrene (hexadimethrine bromide) (Sigma-Aldrich) into Caki-1 cells. After incubation with virus supernatant for 72 h, cells were selected with 1 μ g/ml puromycin (Sigma-Aldrich). pLKO.1-puro non-silencing shRNA was used as a control. 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- μ l 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 the number of cells that migrated across the wound mark was determined in five microscopic fields.

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.

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 phenylmethanesulfonyl fluoride (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 actin and vinculin in cells after ILK knockdown, cells were processed in a similar manner with anti-actin and anti-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. The right kidney was exposed via a small incision in the flank area and a cell suspension containing 1×10^6 tumor cells in 50 μ l 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 CO₂ 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 (5µm) 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. 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 haematoxylin, dehydrated, and mounted. MetaMorph 4.6 software was used for computerized quantification of immunostained target protein.

Statistical analysis

All observations were confirmed by at least three independent experiments. The results were presented as the mean ± SE. Statistical analysis was performed with SigmaPlot 12 (Systat Software Inc., Chicago, IL, USA). The Student t test was applied for two-group comparison. Difference was considered significant when P values were 0.05 or less.

III. 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 cell lines from a low-stage tumor (UMRC-6 from a stage I primary tumor), suggesting that ILK might be related to aggressive features of high-stage RCC (Fig 1B).

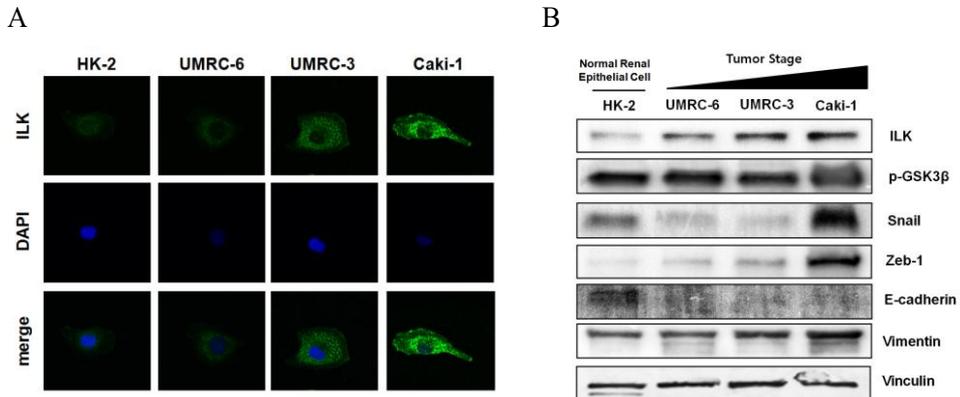


Figure 1. Expression of ILK protein and its downstream effectors in normal renal epithelial cells and RCC cells. (A) ILK expression is shown in fluorescent green. ILK is poorly expressed in normal renal epithelial cells (HK-2), but is highly expressed in RCC cells (UMRC-6, UMRC-3, and Caki-1), 40 X magnification. Expression of ILK correlates with the original tumor stage of the cell lines, with Caki-1 having the highest expression and UMRC-6 with the lowest. ILK is primarily expressed in the cytoplasm. (B) Western blotting analysis shows that Zeb-1 and vimentin expression is correlated with ILK expression, and Snail is highly expressed in Caki-1 cells, a metastatic cell line, and E-cadherin is not expressed in RCC cells. Expression of ILK is similar to expression found in (A).

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 GSK3 β 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). These results show that ILK expression is related to markers of EMT 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 induces Zeb-1 and Snail, critical transcription factors in the EMT, and also vimentin, a major cytoskeletal component of mesenchymal cells. 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.

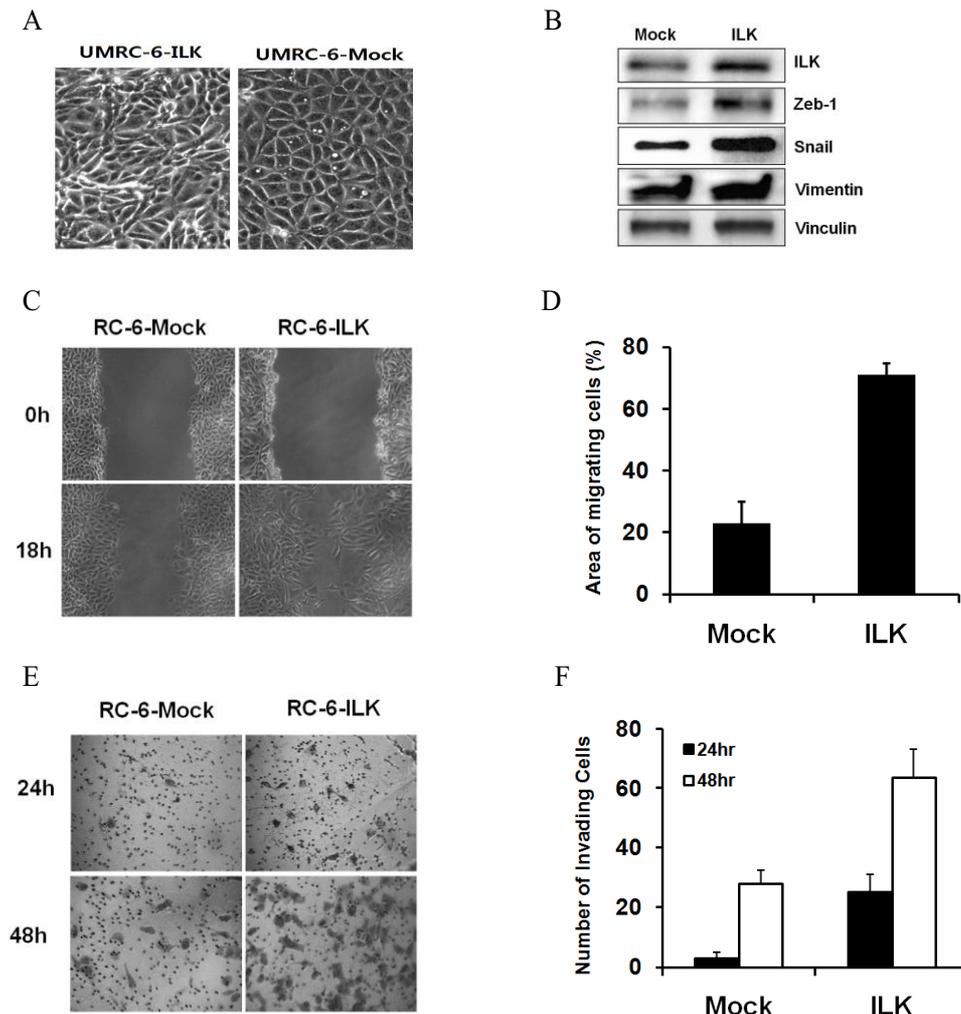


Figure 2. Morphologic and molecular changes after stable transfection of ILK in UMRC-6 cells (low-stage RCC cells). UMRC-6 cell morphology after stable transfection of ILK changed to a more irregular and spindle shape in vitro, 20X magnification (A). Western blot of UMRC-6-Mock and UMRC-6-ILK cells, confirming that UMRC-6-ILK overexpressed ILK. UMRC-6-ILK also is associated with increased Zeb-1, Snail and vimentin, molecular EMT-related transcription factors and proteins in UMRC-6 cells (B). ILK-overexpressing RC-6 cells show increased migration and invasion ability compared to -mock-transfected cells (C-F). Scratch test showing increased migration in UMRC-6-ILK compared to UMRC_6-Mock cells (C and D, 20X magnification). Invasion assay showing increased invasion of UMRB-6-ILK compared to UMRC-6-Mock (E and F, 20X magnification).

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 confirmed 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.

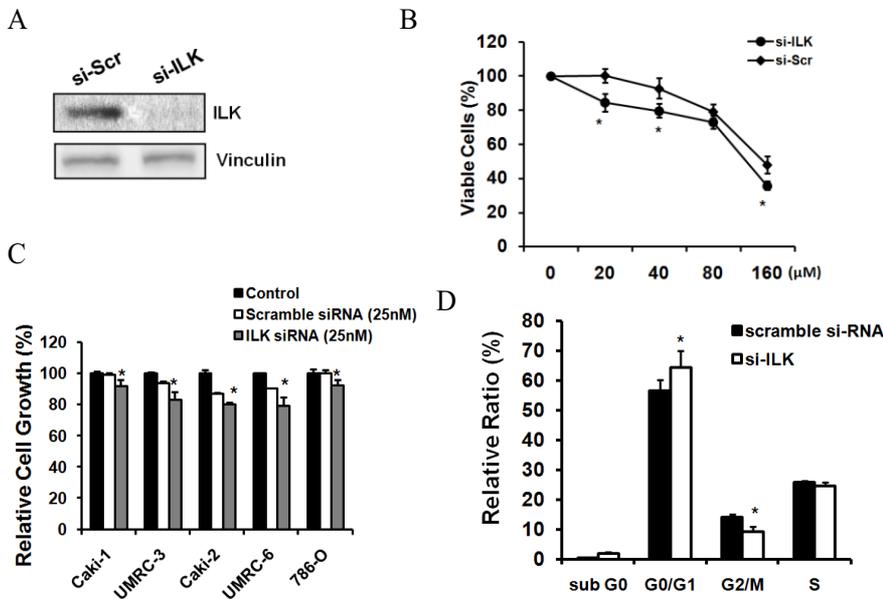


Figure 3. Effects of ILK inhibition on tumor growth and the cell cycle in RCC in vitro. Western blot analysis showing that transfection of Caki-1 with si-ILK effectively knocks down of ILK expression (A). ILK knockdown with siRNA inhibits tumor growth only minimally as shown in (B and C). Crystal violet assay showing that si-ILK has only minimal effects on cell viability in a variety of cell lines compared to scr-ILK and lipofectamine only control (B in Caki-2 and C in other cell lines). FACS analysis of Caki-1 cells treated with si-ILK showed minimal alteration of cells cycle (D). * P < 0.01 vs. control, ** P < 0.001 vs. control

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). 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. 4C and D). These results suggest that the invasive potential of RCC can be suppressed by ILK inhibition.

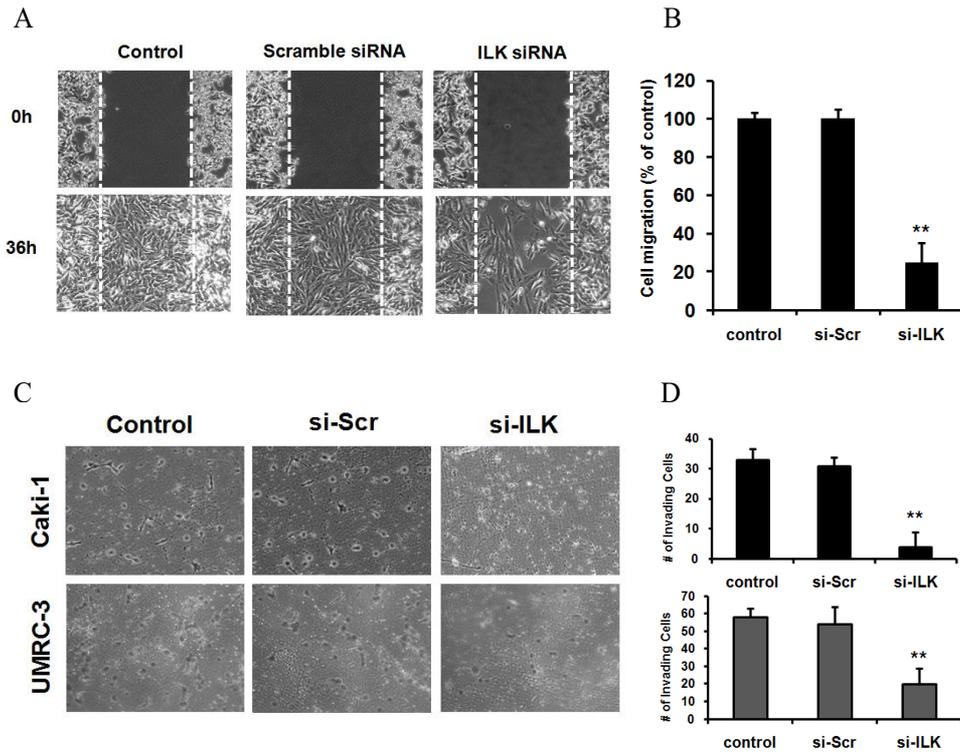


Figure 4. Changes in cell migration and invasion after knockdown of ILK in advanced RCC in vitro. After Caki-1 and UMRC-3 cells were transiently transfected with si-ILK or si-Scr, scratch and invasion assays confirmed that cell migration (A, B) and invasion (C-E) were reduced significantly, 20X magnification. * P < 0.01 vs. control, ** P < 0.001 vs. control

ILK regulates the formation of stress fibers 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. Rhodamine-phalloidin staining showed that ILK knockdown impeded stress fiber formation and organization compared with control cells (Fig. 5B). 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. 5C and D). 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 movement by suppressing the organization of stress fibers.

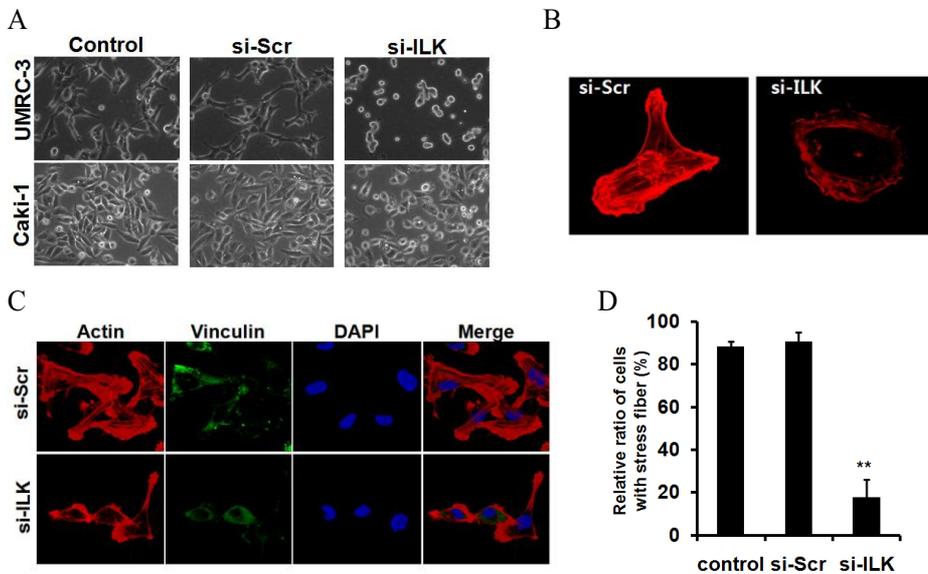
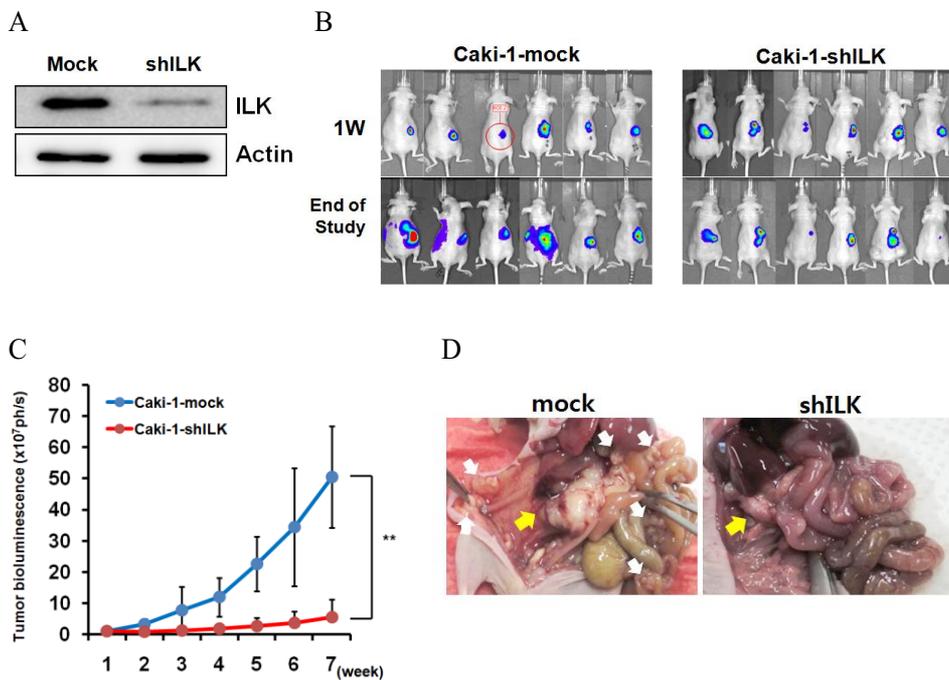


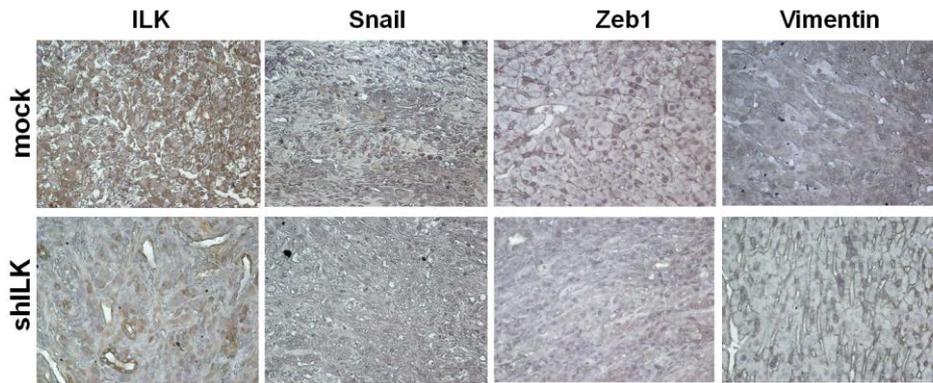
Figure 5. ILK regulates stress fiber formation and focal adhesions in RCC cells. Replating after transient transfection revealed morphologic changes in cells transfected with si-ILK, 20X magnification (A). Staining with rhodamine-phalloidin showed that ILK knockdown significantly suppressed stress fiber formation (B) and reduced focal adhesions (green) (C, D, 40X magnification). * P < 0.01 vs. control, ** P < 0.001 vs. control

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 (Fig 6D). Immunohistochemical staining showed that ILK was successfully knocked down *in vivo*, and also showed that Snail, Zeb-1, and vimentin were downregulated 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 in RCC.



E



F

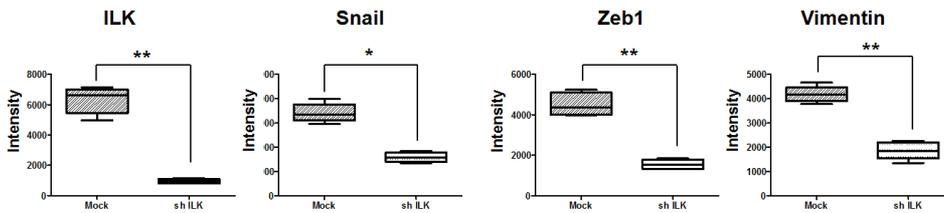


Figure 6. ILK knockdown inhibits tumor progression and distant metastasis in vivo. Orthotopic RCC model and bioluminescence imaging tracing showed that inhibition of ILK induced primary tumor regression and inhibited invasion and metastases. (A) Western blot of shILK transfected Caki-1 cells showing significant knockdown of ILK compared to mock transfected cells. (B) In vivo analysis of cell growth showed significant growth inhibition of Caki-1-shILK compared to caki-1-mock cells. (C) Xenograft model showing metastasis (white arrows) and original implanted tumor (yellow). Mouse with Caki-1-shILK did not develop metastases while those with Caki-1-Mock developed multiple metastases. (E and F) Immunohistochemical staining showed that EMT markers were downregulated by knockdown of ILK in orthotopic xenografts, 20X magnification. ** P <0.001 vs. control

IV. DISCUSSION

In RCC, the development of novel therapies that suppress cancer metastases is an urgent therapeutic need 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. As well, we showed that ILK may promote EMT by regulating the transcription factors Snail and ZEB1, as well as cell movement by inducing focal adhesions and actin organizations. In addition, inhibition of ILK suppresses both migration and invasion in RCC by impeding the formation of stress fibers and focal adhesion plaques. 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 has been 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. Our study also revealed that ILK is related to proliferation in RCC cells, but is not a central driver of tumor proliferation according to *in vitro* experiments. Nevertheless, remarkable suppression of primary tumor size was observed in orthotopic xenografts containing shILK transfectants, suggesting that inhibition of ILK suppresses tumor progression by blocking cell movement and angiogenesis rather than direct suppression of tumor proliferation in RCC.

Metastases require the interaction of tumor cells with the ECM, which is mediated by the formation of elongated, integrin β 1-containing adhesion plaques [30]. The development of such plaques requires the prior assembly of integrin

β 1-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-3 β [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]. Vimentin, the intermediate filament protein, is an important marker of the EMT and a requisite regulator of mesenchymal cell migration [33]. 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 [33, 34]. 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.

V. CONCLUSION

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

1. Chow WH, Devesa SS, Warren JL, Fraumeni JF Jr. Rising incidence of renal cell cancer in the United States. *JAMA* 1999;281:1628-1631.
2. Gupta K, Miller JD, Li JZ, Russell MW, Charbonneau C. Epidemiologic and socioeconomic burden of metastatic renal cell carcinoma (mRCC): a literature review. *Cancer Treat Rev* 2008;34:193-205.3.
3. Ljungberg B, Campbell SC, Choi HY, Jacqmin D, Lee JE, Weikert S, Kiemeny LA. The epidemiology of renal cell carcinoma. *Eur Urol* 2011;60:615-21
4. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 2009;15:232-9.
5. Pàez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Viñals F, Inoue M, Bergers G, Hanahan D, Casanovas O. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 2009;15:220-31.
6. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 2003;15:740–6.
7. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005;17:548–58.
8. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* 2006;172:973-81.
9. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009;119:1420-8.
10. Tse JC and Kalluri R. Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment. *J Cell Biochem* 2007;101:816–829.
11. Yang J and Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell* 2008;14:818–829.
12. Weinberg RA. Twisted epithelial-mesenchymal transition blocks senescence. *Nat Cell Biol* 2008;10:1021–1023.

13. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008;133:704–715.
14. Hannigan G, Troussard AA, Dedhar S. Integrin-linked kinase: a cancer therapeutic target unique among its ILK. *Nat Rev Cancer* 2005;5:51-63.
15. Yamaji S, Suzuki A, Kanamori H, Mishima W, Yoshimi R, Takasaki H, Takabayashi M, Fujimaki K, Fujisawa S, Ohno S, Ishigatsubo Y. Affixin interacts with α -actinin and mediates integrin signaling for reorganization of F-actin induced by initial cell-substrate interaction. *J Cell Biol* 2004;165:539–551.
16. Yamaji, S. et al. Possible role of ILK-affixin complex in integrin-cytoskeleton linkage during platelet aggregation. *Biochem Biophys Res Commun* 2002;297:1324–1331.
17. Oloumi A, McPhee T, Dedhar S. Regulation of E-cadherin expression and β -catenin/Tcf transcriptional activity by the integrin-linked kinase. *Biochim Biophys Acta* 2004;1691:1–15.
18. Deng JT, Van Lierop JE, Sutherland C, Walsh MP. Ca^{2+} -independent smooth muscle contraction. A novel function for integrin-linked kinase. *J Biol Chem* 2001;276:16365–16373.
19. Tan C, Cruet-Hennequart S, Troussard A, Fazli L, Costello P, Sutton K, Wheeler J, Gleave M, Sanghera J, Dedhar S. Regulation of tumor angiogenesis by integrin-linked kinase (ILK). *Cancer Cell* 2004;5:79–90.
20. Persad S, Attwell S, Gray V, Delcommenne M, Troussard A, Sanghera J, Dedhar S.. Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. *Proc Natl Acad Sci* 2000;97:3207–3212.
21. Marotta A, Tan C, Gray V, Malik S, Gallinger S, Sanghera J, Dupuis B, Owen D, Dedhar S, Salh B.. Dysregulation of integrin-linked kinase (ILK) signaling in colonic polyposis. *Oncogene* 2001;20:6250–6257.
22. Ito R, Oue N, Zhu X, Yoshida K, Nakayama H, Yokozaki H, Yasui W. Expression of integrin-linked kinase is closely correlated with invasion and metastasis of gastric carcinoma. *Virchows Arch* 2003;442:118–123.
23. Ahmed N, Riley C, Oliva K, Stutt E, Rice GE, Quinn MA. Integrin-linked

- kinase expression increases with ovarian tumour grade and is sustained by peritoneal tumour fluid. *J Pathol* 2003;201:229–237.
24. Dai DL, Makretsov N, Campos EI, Huang C, Zhou Y, Huntsman D, Martinka M, Li G. Increased expression of integrin-linked kinase is correlated with melanoma progression and poor patient survival. *Clin Cancer Res* 2003;9:4409–4414.
 25. McDonald PC, Fielding AB, Dedhar S. Integrin-linked kinase--essential roles in physiology and cancer biology. *J Cell Sci* 2008;121:3121-32.
 26. Matsui Y, Hadaschik BA, Fazli L, Andersen RJ, Gleave ME, So AI. Intravesical combination treatment with antisense oligonucleotides targeting heat shock protein-27 and HTI-286 as a novel strategy for high-grade bladder cancer. *Mol Cancer Ther* 2009;8:2402-11.
 27. Weber GF. Why does cancer therapy lack effective anti-metastasis drugs? *Cancer Lett* 2013;328:207-11.
 28. Steeg PS, Theodorescu D. Metastasis: a therapeutic target for cancer. *Nat Clin Pract Oncol* 2008;5:206-19.
 29. Engelman Mde F, Grande RM, Naves MA, de Franco MF, de Paulo Castro Teixeira V. Integrin-linked kinase (ILK) expression correlates with tumor severity in clear cell renal carcinoma. *Pathol Oncol Res.* 2013;19:27-33.
 30. Shibue T, Brooks MW, Weinberg RA. An integrin-linked machinery of cytoskeletal regulation that enables experimental tumor initiation and metastatic colonization. *Cancer Cell* 2013;24:481-98.
 31. Zheng H, Kang Y. Multilayer control of the EMT master regulators. *Oncogene* 2013 Apr 22. doi: 10.1038/onc.2013.128. [Epub ahead of print]
 32. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol* 2004;6:931-40.
 33. Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, Tiron C, Lorens JB, Ivaska J. Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. *Oncogene* 2011;30:1436-48.
 34. Mendez MG, Kojima S, Goldman RD. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. *FASEB J* 2010;24:1838-51.

ABSTRACT (IN KOREAN)

상피세포의 중간엽세포로의 이행 과정 조절을 통한 신세포암의 침윤 및 전이 현상의 억제에서 인테그린 연계 인산화 효소의 역할에 대한 연구

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한 경 석

신장암은 기존의 고식적인 항암제와 방사선 요법에 거의 반응하지 않는 치료내성을 가지고 있는 암으로, 진행성 신장암의 경우 예후가 매우 불량하다. 그 동안 신장암에는 10% 정도의 반응률을 보이는 면역요법 이외에 이렇다 할 효과적인 치료가 없었으나, 최근 개발된 종양의 혈관생성을 억제하는 치료들이 신장암에 효과를 보이기 시작하면서, 현재는 진행성 신장암 환자들의 기대여명을 의미 있게 증가시킬 수 있는 효과적인 일차 치료제로서 사용되고 있다. 하지만, 이러한 혈관생성억제 치료는 40-60%의 높은 반응률에도 불구하고, 비교적 짧은 시간 내에 거의 모든 환자들에서 혈관생성억제 치료에 내성이 발생하게 되며, 그 이후로는 더욱 급격한 속도로 진행하게 되는 암을 막을 방법이 현재로서는 없는 실정이다. 진행성 신장암 환자에서 치료 중에도 불구하고 급격하게 병의 경과를 악화시키는 가장 큰 요인은 폐나 간과 같은 생명을 유지하는데 필수적인 장기로 원격 전이가 계속해서 발생하고 이러한 전이 병변의 급격한 진행은 결국 장기 부전으로 인한 사망에 이르는 직접적인 원인이 된다. 폐전이는 신장암 환자에서 가장 빈번하게 발생하는 원격 전이로 대부분의 환자들은 폐전이로 인한 폐부전으로 사망한다.

인테그린 연계 인산화효소는 인테그린과 상호 작용하는 serine/threonin 활성화효소로, 세포 표면의 인테그린과 세포 내의 액틴 세포골격을 연결하는 핵심적인 역할을 하면서 이를 통해 세포 외 환경의 변화에 따라 세포의 움직임을 조절하는 매우 중요한 단백질로, 여러 암에서 발현이 증가해 있음이 관찰되었다. 인테그린 연계 활성화효소 단백질의 발현 증가는 세포의 형질을 중간엽 세포와 같이 변화시키면서 상피세포의 중간엽세포로의 변이 같은 현상 즉 세포의 이동성 및 침윤성을 증가시키게 된다. 원발 종양에서 중간엽세포로의 변이를 거친 암세포는 세포의 이동성과 침윤성이 급격히 증가하면서 혈관을 통한 혈행성 암전이가 발생하게 되는데, 인테그린 연계 인산화효소는 이러한 과정에서 매우 중요한 역할을 하는 것으로 알려져 있다. 본 연구에서는 신장암 세포에서 인테그린 연계 인산화효소가 암의 병기와 전이성이 증가할수록 발현이 급격히 증가함을 보여 주었으며, 저병기의 신장암에서 유래된 세포주에서 인테그린 연계 인산화효소의 활성화가 세포의 형태를 중간엽 세포의 형태로 변환시키며 이는 Snail과 Zeb-1과 같은 전사 인자의 활성화와 관계됨을 보여주었다. 한편, 인테그린 연계 인산화효소의 발현 억제는 세포의 생존이나 세포 주기의 변화에 의미 있는 영향을 미치지 않는 것으로, 세포의 이동 및 침윤이 현저히 감소함을 보여 주었으며, 세포 골격의 재배치의 억제를 통한 focal adhesion이 감소됨을 보여주었다. 한편, 신장암 동물 모델을 통해서 인테그린 연계 인산화효소가 억제되는 경우, 의미 있게 침윤과 전이가 억제되며 종양의 성장이 또한 억제되는 것을 보여주었다. 이로부터, 인테그린 연계 인산화효소는 신장암에서 침윤과 전이를 억제할 수 있는 중요한 치료 표적이 됨을 보여 주었다.

핵심되는 말 : 신세포암, 인테그린-연계 활성화효소, 전이, 침윤, 상피세포의 중간엽 세포로의 변이

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