α-catulin drives metastasis by activating ILK and driving an αvβ3 integrin signaling axis

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Running title: Alpha-catulin/ILK interaction promotes metastasis

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Abstract

α-catenin is oncoprotein that help sustains proliferation by preventing cellular senescence. Here we report that α-catenin also drives malignant invasion and metastasis. α-catenin was upregulated in highly invasive non-small cell lung cancer (NSCLC) cell lines, where its ectopic expression or shRNA-mediated attenuation enhanced or limited invasion or metastasis, respectively. α-catenin interacted with integrin-linked kinase (ILK), a serine/threonine protein kinase implicated in cancer cell proliferation, anti-apoptosis, invasion and angiogenesis. Attenuation of ILK or α-catenin reciprocally blocked cell migration and invasion induced by the other protein. Mechanistic investigations revealed that α-catenin activated Akt-NFκB signaling downstream of ILK, which in turn led to increased expression of fibronectin and integrin αvβ3. Pharmacological or antibody-mediated blockade of NFκB or αvβ3 was sufficient to inhibit α-catenin-induced cell migration and invasion. Clinically, high levels of expression of α-catenin and ILK were associated with poor overall survival in NSCLC patients. Taken together, our study demonstrates that α-catenin plays a critical role in cancer metastasis by activating the ILK-mediated Akt-NFκB-αvβ3 signaling axis.

PRÉCIS: Findings reveal a novel signaling axis of invasion and metastasis in lung adenocarcinoma that offers therapeutic targets to treat this aggressive disease.
Introduction

Lung cancer is the most common cause of cancer-related mortality (1). The high mortality rate in lung cancer patients is due to metastatic progression of the disease (2). The process of metastasis is highly complex and requires activation of the epithelial-mesenchymal transition, remodeling of the extra-cellular matrix, initiation of neo-angiogenesis and migration of cancer cells to distant organs (3-5). Currently, there are no effective drugs that can inhibit cancer metastasis. Thus, understanding the molecular mechanisms that cause cancer metastasis could identify new therapeutic targets.

α-Catulin is an 82-kDa protein that shares sequence homology with human vinculin and α-catenin (6). The N-terminal region of α-catulin contains binding sites for β-catenin, talin, and α-actin. The C-terminal region of α-catulin contains potential binding sites for the tight junction protein ZO-1 and the actin cytoskeleton (6, 7). The structure of α-catulin suggests that it may function as a cytoskeletal linker protein. In addition, previous studies have found that α-catulin is involved in the Rho signaling pathway through its interaction with Lbc, a Rho guanine nucleotide exchange factor (7). The Rho family has been shown to play important roles in focal adhesion formation and in the organization of the actin cytoskeleton (8). α-Catulin may also play a role in cancer because it can directly interact with IKK-β and activate nuclear factor kappa B (NF-κB) signaling, which promotes cancer cell migration and resistance to apoptosis (9). We recently reported that α-catulin is upregulated in both cancer cell lines and oral squamous cell carcinomas, and knocking down α-catulin induces cellular senescence (10).

ILK is a serine/threonine protein kinase that is usually located in focal adhesions and regulates many cellular processes, including proliferation, survival, differentiation,
migration and invasion and angiogenesis (11-13). ILK can regulate the phosphorylation of Akt at Ser473 in various cell types, and this phosphorylation is required for its full activation (14). By promoting Akt phosphorylation, ILK stimulates signaling pathways that regulate cell survival and migration (15-17). Other studies have also reported that high ILK expression promotes the epithelial-mesenchymal transition and leads to invasion and metastasis by stimulating Snail expression, which in turn suppresses E-cadherin expression (18). In clinical studies, overexpression of ILK is often a prominent feature of human malignancies, and it correlates with a poor outcome (19, 20). Thus, ILK is an attractive therapeutic target in human cancers.

Although α-catenin may function as a cytoskeletal linker protein and modulate the Rho signaling pathway, its functional role in cancer metastasis is still unclear. We found that overexpressing α-catenin in cancer cells remarkably increased cancer cell metastasis both in vitro and in vivo. We elucidated the molecular mechanisms by which α-catenin regulates tumor progression using a yeast two-hybrid genetic screen, and we identified ILK as an α-catenin-interacting protein. α-Catenin increases activation of NF-κB through an ILK-dependent pathway, which in turn increases fibronectin and integrin αβ3 protein expression. These signaling activation events promote cancer cell migration and invasion and metastasis.

**Materials and Methods**

**Cell culture**

Four non-small cell lung cancer (NSCLC) cell lines with progressive levels of invasiveness (CL1-0, CL1-1, CL1-2, and CL1-5) were established and characterized as previously described (21). The NSCLC cell line A549 was obtained from the
American Type Tissue Collection and cultured in DMEM high glucose media supplemented with 10% FBS and antibiotics. All of the cell lines were incubated at 37°C in a humidified atmosphere with 5% CO₂.

**Confirmation of interaction by yeast-two hybrid**

The *Saccharomyces cerevisiae* strain AH109 was transformed using the yeast expression vector pAS2-1-α-catulin. Next, the transformed yeast cells were cultured in synthetic complete selective media lacking tryptophan overnight. The transformed yeast cells were transformed again with pACT2-ILK. Doubly transformed yeast cells were plated on yeast dropout media lacking tryptophan, leucine and histidine and supplemented with 5 mM 3-amino-1,2,4-triazole and 80 μg/mL X-gal; then, they were incubated for 5-7 days in a 30°C incubator. Positive colonies were plated on the same synthetic complete dropout media for amplification.

**Xenograft animal model**

For spontaneous metastasis assay, the cells (1×10⁶) were suspended in 100 μl HBSS and subcutaneously injected into the posterior flank of 8-week-old male NOD–SCID mice. The injected mice were euthanized after 12 weeks. The lungs were removed and fixed in 10% formalin. The number of lung tumor colonies was counted under a dissecting microscope. Representative lung tumors were removed, fixed, and embedded in paraffin. The embedded tissue was sectioned into 4-μm sections, and the sections were stained with hematoxylin-eosin for histological analysis.

For experimental metastasis assay, the cells (1×10⁶) were suspended in 100 μl HBSS and injected into the lateral tail vein of 8-week-old SCID mice. The injected mice were euthanized after 5 weeks. The lungs were removed and fixed in 10%
formalin. The number of lung tumor colonies was counted under a dissecting microscope. Representative lung tumors were removed, fixed, and embedded in paraffin. The embedded tissue was sectioned into 4-μm sections, and the sections were stained with hematoxylin-eosin for histological analysis.

**Survival analysis**

The public NSCLC cohort datasets used for validation were from the University of Michigan Cancer Center (22). A patient's risk score was calculated as the sum of the levels of expression of each gene. The patients were classified as having a high-risk gene signature or a low-risk gene signature, with the median risk score serving as the threshold value. The survival curves for both groups were obtained by the Kaplan-Meier method and were compared using the log-rank test. Multivariate Cox proportional hazard regression analysis was used to evaluate independent prognostic factors associated with patient survival, with the gene signature, age, sex and stage as the covariates. Both the log-rank test and the Cox test were two-sided, and a *P* value < 0.05 was considered to be statistically significant.

**Note:** Some materials and methods are available in supplementary information. Microarray data are available in the NCBI GEO repository under accession number GSE40141.

**Results**

**α-Catulin enhances lung cancer cell migration and invasion**

To investigate the expression level of α-catulin in several lung cancer cell lines, we
used quantitative reverse transcription (qRT)-PCR and Western blotting to measure the mRNA and protein expression of α-catulin in four lung cancer cell lines with progressive levels of invasiveness, including CL1-0, CL1-1, CL1-2, and CL1-5. The migratory/invasive ability of the four human lung cancer cell lines ranges from low in CL1-0 to high in CL1-5 (21), and the levels of migration and invasiveness are positively correlated with α-catulin expression (Fig. 1A). To confirm that α-catulin can regulate cancer cell migration and invasion, the CL1-0 cells were transduced with an α-catulin expression construct in a lentivirus vector, PLKO_AS2. The CL1-5 cells were infected with two different lentiviruses (shB01 and shC01) that produce specific short-hairpin RNAs (shRNAs) targeting α-catulin mRNA (bases 1401–1421 and 324–344, respectively). We found that α-catulin protein expression in the transduced CL1-0 cells increased up to 4-fold (Fig. 1B, left), but the proliferation rate was similar to that of the control cells (Supplementary Fig. S1A). After 2 days of infection and puromycin selection, we found that the α-catulin protein levels were reduced by approximately 80% in the shB01- or shC01-transduced CL1-5 cells (Fig. 1B, right), and the proliferation rate of these cells was reduced by 50% (Supplementary Fig. S1B). These cells were assayed for migration and invasion using a Transwell system. The results demonstrated that an increase of α-catulin in the CL1-0 cells promoted an approximately 3-fold increase in migration and invasion (Fig. 1C). In contrast, the migratory/invasive ability of the α-catulin-silenced CL1-5 cells was significantly decreased by more than 80% after normalization relative to the proliferation rate at the same time point (Fig. 1D). α-Catulin was also overexpressed in A549 lung cancer cells, and the results demonstrated that α-catulin promoted the migration and invasion of the A549 cells but did not influence cell proliferation (Supplementary Fig. S1C-F). Taken together, these results indicate that α-catulin expression levels were positively
associated with the migratory and invasive abilities of cancer cells.

**α-Catulin enhances lung cancer cell metastasis in vivo**

To investigate whether α-catulin can enhance lung cancer cell metastasis in vivo, we generated lung tumor metastasis models by subcutaneously injecting control, α-catulin-overexpressing CL1-0, or α-catulin knockdown CL1-5 cells into non-obese diabetic (NOD)-severe combined immunodeficiency (SCID) mice and measured the number of lung nodule formations (Fig. 2A). Following subcutaneous injection, the α-catulin-overexpressing cells induced a similar incidence of tumor formation compared to the controls, with each group having 4 tumors resulting from a total of 4 inoculations. Interestingly, twelve weeks after subcutaneous injection, the mice that were injected with α-catulin-overexpressing cells displayed a significantly elevated incidence of metastatic tumors in the lung compared with the controls (Fig. 2B and C). At this time point, the mice that were injected with α-catulin-overexpressing cells had larger tumors than the mice that were injected with control cells (Supplementary Fig. S2A). In contrast, we found that the mice that were subcutaneously injected with the shC01-infected CL1-5 cells had a significantly decreased number of lung nodules compared to those injected with the shLuci-infected control cells (Fig. 2D). The incidence of tumor formation from shC01-infected CL1-5 cells was similar to the incidence in the control cells, with each group having 8 tumors resulting from a total of 10 inoculations. However, the average tumor size in the mice injected with the shC01-infected CL1-5 cells was notably smaller (Supplementary Fig. S2B). We also evaluated the metastatic potential for lung colonization following tail vein injections (Fig. 2E). Five weeks after the tail vein injections, the number of lung nodule formations increased up to 4-fold in the mice injected with the
α-catulin-overexpressing cells (Fig. 2F) and significantly decreased in the mice inoculated with the α-catulin-depleted cells (Fig. 2G and H). The average lung weight from the mice that were injected with the shC01-infected CL1-5 cells also decreased compared to the lungs from the control mice that were injected with the shLuci-infected CL1-5 cells (Supplementary Fig. S2C). This series of assays demonstrated that by increasing the migratory and invasive behavior of lung cancer cells, α-catulin promoted cancer cell metastasis in vivo.

α-Catulin enhances lung cancer cell migration and invasion through interaction with ILK

To dissect the mechanism of action of α-catulin in cancer cell migration and invasion, we carried out a yeast-two hybrid screen using full-length α-catulin as bait to identify its interaction partners. One of the key interacting proteins that we identified was integrin-linked kinase (ILK) (Fig. 3A). To confirm the binding of ILK to α-catulin, protein lysates were immunoprecipitated with anti-α-catulin or anti-ILK antibodies and then visualized by Western blotting. The results demonstrated that endogenous α-catulin could interact with ILK (Fig. 3B). Next, we wanted to determine which domain of ILK interacted with α-catulin. Deletion mutants of FLAG-ILK (Fig. 3C) and full-length α-catulin-GFP were cotransfected into CL1-0 cells, and the protein lysates were immunoprecipitated with an anti-FLAG antibody and visualized by Western blotting with an anti-α-catulin antibody. As shown in Figure 3D, the full-length and the 1-170 amino acid region of ILK interacted with α-catulin. ILK has been found to play an important role in inducing the epithelial-mesenchymal transition and in promoting cancer cell migration and invasion (18). We also found that the protein expression level of ILK ranges from low in CL1-0 to high in CL1-5. (Supplementary Fig. S3). Thus,
we wanted to test whether the interaction between ILK and α-catulin mediated α-catulin-driven cancer cell migration and invasion. We used specific shRNAs to silence ILK expression in α-catulin-overexpressing CL1-0 cells (Supplementary Fig. S4A). These cells were assayed for migration and invasion using a Transwell system. The results revealed that α-catulin overexpressing CL1-0 cells exhibited increased cell migration and invasion, and depletion of ILK expression diminished this phenomenon (Fig. 3E). Surprisingly, we found that α-catulin also plays a key role in ILK-mediated cancer cell migration and invasion. We knocked down the expression of α-catulin in ILK overexpressing CL1-5 cells, which led to a decrease in the migratory/invasive ability induced by ILK expression (Fig. 3F, Supplementary Fig. S4B).

**α-Catulin-promoted activation of the Akt-NF-κB signaling pathway is ILK dependent**

Previous studies have shown that Akt Ser473 could be directly phosphorylated by ILK, which results in Akt activation (14). Activation of Akt leads to IκB-α degradation and activates the NF-κB signaling pathway. Thus, we sought to elucidate whether the Akt-NF-κB signaling pathway is involved in α-catulin-regulated cancer cell migration and invasion. As shown in Figure 4A, compared with the control cells, overexpression of α-catulin in the CL1-0 cells dramatically increased the phosphorylation of Akt at Ser473, IκB-α at Ser32 and NF-κB at Ser536. IκB is an inhibitor of NF-κB activation. The phosphorylation of IκB-α at Ser32 is required for its degradation, which in turn leads to the phosphorylation of NF-κB at Ser536 and promotes NF-κB translocation into the nucleus (23). Conversely, when we silenced α-catulin expression in the CL1-5 cells, the phosphorylation of Akt at Ser473, IκB-α at Ser32 and NF-κB at Ser536
decreased (Fig. 4B). Knockdown of ILK in the α-catulin overexpressing cells diminished α-catulin-induced NF-κB activation (Fig. 4C). Interestingly, knockdown of α-catulin in the ILK overexpressing cells also inhibited the activation of the Akt-NF-κB signaling pathway (Fig. 4D). Next, we wanted to test whether NF-κB activation was involved in α-catulin-induced cancer cell migration and invasion. We used an IκB kinase inhibitor, Bay 11-7082, to treat the α-catulin overexpressing cells and found that inhibition of NF-κB effectively decreased α-catulin-induced cell migration and invasion (Fig. 4E).

α-Catulin activates an integrin-ILK-NF-κB positive feedback loop to promote cell migration and invasion

To identify the molecular mechanisms by which α-catulin affects cancer cell migration and invasion, we used a cDNA microarray to analyze the genes regulated by overexpression of α-catulin. A MetaCore software analysis of global genes revealed that cytoskeleton remodeling was the major pathway that was significantly altered in the α-catulin overexpressing cells (Fig. 5A). Several genes involved in the reorganization of the cytoskeleton were markedly upregulated in the α-catulin overexpressing cells (Fig 5B). In addition, many NF-κB target genes were also increased in the α-catulin overexpressing cells, suggesting that α-catulin indeed activates the NF-κB pathway (Supplementary Table S1). The main function of ILK is to connect integrins to the cytoskeleton and to regulate cell-extracellular matrix (ECM) interactions, cytoskeletal organization and cell signaling, with important roles in cancer metastasis (11, 24). Interestingly, we found that the ECM-integrin-ILK-GTPase signaling pathway, including fibronectin 1 (FN1), type IV collagen, integrins, cdc42 and ROCKs, is the predominant axis that is altered in α-catulin overexpressing cells.
We also checked the expression levels of alpha-catulin in other pair cell lines (CL 1-1, CL 1-5F4 and PC9, PC9/IR) and found the expression of CTNNAL1 was positively correlated with those of ILK/FN1/ITGB3 and their invasive ability (Supplementary Fig. S5) (21, 25). Overexpression of α-catulin increased FN1 and integrin αvβ3 expression, whereas knockdown of α-catulin decreased expression (Fig. 5C). FN1 is a known NF-κB target gene (26). Our results demonstrated that ILK-Akt-NF-κB signaling is required for α-catulin-promoted cancer invasiveness. Therefore, we further examined whether α-catulin induction of FN1 and integrins is dependent on ILK-NF-κB signaling. The results showed that silencing ILK in the α-catulin-overexpressing CL1-0 cells effectively blocked α-catulin-enhanced FN1 and integrin αvβ3 expression (Fig. 5D, left). In contrast, knockdown of α-catulin in the ILK overexpressing cells diminished ILK-activated FN1 and integrin αvβ3 upregulation (Fig. 5D, right). Next, we found that the α-catulin overexpressing cells treated with the NF-κB inhibitor Bay 11-7082 had a decreased expression of FN1 and integrin αvβ3 (Fig. 5E). Furthermore, we treated the α-catulin overexpressing cells with IgG or an integrin αvβ3 neutralizing antibody and found that the anti-αvβ3 antibody significantly decreased cell migration and invasion (Fig. 5F). These data suggest a positive feedback loop in which, upon α-catulin overexpression, ILK-NF-κB signaling activation increases FN1 and integrin αvβ3 expression, which further modulates ECM-integrin-ILK-mediated cytoskeletal reorganization to promote cell migration and invasion.

The CTNNAL1 plus ILK 2-gene signature can predict survival in NSCLC patients

The clinical characteristics of 177 patients with NSCLC from the Michigan cohort are summarized according to the risk associated with their CTNNAL1 plus ILK 2-gene signature (Supplementary Table S2). The Kaplan-Meier survival curves for the high-
and low-risk groups in the Michigan cohort were separable and showed a significant poor survival rate only in the group exhibiting both high levels of ILK and CTNNAL1 (log rank test, $p=0.019$) (Fig. 6A–C). To show that inclusion of the 2-gene signature in the clinical model could be used to predict survival independently of the cancer stage, we conducted a multivariate Cox regression. From the initial set of 4 variables (age, gender, stage, and 2-gene signature), the final model contained only the stage and the 2-gene signature (Table 1).

**Discussion**

Elucidating complex oncogenic signaling pathways is important for identifying novel therapeutic targets for clinical treatment. Here, our data demonstrate that α-catulin plays a critical role in lung cancer metastasis. Expression of α-catulin was correlated with cell migratory/invasive ability *in vitro* and cancer metastasis *in vivo* in NSCLC. Moreover, we demonstrated that α-catulin promoted the migration and invasion of NSCLC cells through the ILK/NF-κB/integrin network. α-Catulin directly interacted with ILK, which in turn activated the ILK/Akt/NF-κB signaling pathway. This led to increased expression of the NF-κB downstream genes fibronectin and integrin αvβ3, which sequentially activated NF-κB signaling and resulted in cancer cell migration, invasion and metastasis (Fig. 6D). The 2-gene signature (ILK plus CTNNAL1) was even more strongly associated with clinical outcomes. Most importantly, blocking expression of α-catulin, ILK, NF-κB and integrin αvβ3 prevented cancer cell migration and invasion.

The interactions between the extracellular matrix proteins, integrins and the actin cytoskeleton are important for cell migration (27). The ILK–PINCH–parvin (IPP) complex functions as an integrin signaling adaptor. The IPP complex can act as a
structural module that connects integrins to the actin cytoskeleton, and it can also act as a signaling platform that modulates various cellular processes, including cell migration (11). ILK is a central component for the assembly of the IPP complex, and it contributes both its adaptor properties and its kinase activity to directly activate several signaling pathways downstream of the integrins (12). Here, we found that α-catenin interacted with multiple ankyrin (ANK) repeat domains of ILK that are required for ILK localization to focal adhesions and for ILK signaling. The interaction between α-catenin and ILK enhanced the phosphorylation of Akt at Ser473. Knockdown of α-catenin or ILK decreased Akt activation. Previous studies have demonstrated that the ANK domain of ILK also interacts with PINCH1 (28) and the ILK-associated protein (ILKAP) (29). Deletion of the ANK domain of ILK or knockdown of PINCH1 expression could disrupt ILK localization to focal adhesions and decrease Akt Ser473 phosphorylation (30). ILKAP is a type-2C protein phosphatase that binds to ILK and negatively regulates ILK signaling. These observations support the idea that ILK activity can be regulated by protein-protein interactions. In addition, α-catenin is considered to be a cytoskeletal linker protein (6) due to its structural similarity to vinculin, which links the actin cytoskeleton to integrins (31). These results suggest that α-catenin is an integrin signaling adaptor and plays a critical role in regulating integrin-mediated cellular functions via binding to ILK.

In addition to serving as the adaptor connecting integrin to the actin cytoskeleton, ILK is also a serine/threonine protein kinase. Glycogen synthase kinase 3β (GSK-3β) and Akt are the two major downstream substrates of ILK in cancer cells (32). ILK directly phosphorylates GSK-3β at Ser9 and induces the dissociation of GSK-3β from its substrate β-catenin, leading to the stabilization and activation of β-catenin (33). ILK also activates Akt through phosphorylation of Akt at Ser473, which regulates genes essential for survival, proliferation and migration (16, 34). Moreover, ILK promotes
melanoma, ovarian and bladder cancer cell migration through the phosphorylation of GSK-3β and AKT (35–37). However, we found that in NSCLC cell lines, the interaction between α-catenin and ILK induced cell migration and invasion through the phosphorylation of Akt, but not GSK-3β (Fig. 3 and Supplementary Fig. S6). These data indicate that ILK might act through different downstream signaling pathways in different tissues. Consistent with this, there are several studies demonstrating that ILK kinase function appears to be cell type-dependent. Inhibition of ILK activity in breast cancer cells resulted in a decrease in Akt phosphorylation but had no effect on non-transformed cell types, including normal breast epithelial cells and mesenchymal cells (38). In cultured sympathetic neurons, the ILK inhibitor decreased NGF-induced stimulation of GSK-3β but had no effect on the phosphorylation of Akt and resulted in reduced depolarization-induced dendrite formation (39).

Our data demonstrated that the interaction of α-catenin and ILK in NSCLC cells was associated with NF-κB activation via Akt phosphorylation. Previous studies have indicated that Akt can phosphorylate IKK α/β at Ser176/178, which in turn leads to IκB-α degradation and activation of the NF-κB signaling pathway (23, 40). Moreover, α-catenin has been found to interact with IKK α/β (9). Thus, we suggest that α-catenin may link ILK/AKT with IKK α/β to promote NF-κB activation. Interestingly, we also found that α-catenin increased the expression of FN1 and integrin αvβ3, which act upstream of ILK signaling. Anti-integrin αvβ3 neutralizing antibody blocks α-catenin-mediated cell migration and invasion. Additionally, we found that treatment with FN1 increased NF-κB activation in CL1-0 cells, and this activation was blocked by an ILK inhibitor (Supplementary Figure S7). It has been previously shown that α-catenin plays an important role in fibronectin-mediated wound repair and proliferation (41). Thus, following α-catenin-induced upregulation of FN1 and integrin
"αβ3 expression via the interaction with ILK, FN1 then binds to integrins and activates the α-catulin/ILK complex in a positive feedback loop.

Currently, there are no efficient drugs that inhibit tumor progression in NSCLC patients. Identifying the key pathways regulating tumor growth, angiogenesis and metastasis may be helpful for developing more effective chemotherapeutics. Recent studies indicated that NF-κB was involved in cancer metastasis by regulating various cancer-related genes (42–44). Thus, the molecular mechanisms that are involved in NF-κB activation in cancer cells become more important. Our results support the hypothesis that the α-catulin/ILK complex is a key upstream regulator of NF-κB signaling. Inhibiting α-catulin, ILK, NF-κB, or integrin αβ3 prevented cancer cell migration and invasion. In addition, α-catulin is known to have oncogenic potential through its interaction with IKKβ and induction of NF-κB activity (9) and by preventing cellular senescence (10). Moreover, inhibitors of ILK (27), NF-κB (45) and integrin (46) have been used in animal model studies and clinical trials. Therefore, the α-catulin/ILK signaling pathway in NSCLC might provide new opportunities for therapeutic intervention, and the development of α-catulin inhibitors may be beneficial for cancer therapy.

We have found that silencing α-catulin in cancer cells decreased cell proliferation in vitro and in vivo (10). In this study, although α-catulin overexpression did not alter the cell proliferation rate, it promoted tumor growth in vivo (Supplementary Fig. S1A, S2A). Several studies indicate that tumor growth is not only determined by cancer cells themselves but also by the tumor microenvironment and angiogenesis (47, 48). To stimulate angiogenesis, tumors increase the production of many angiogenic factors, such as fibroblast growth factor and vascular endothelial cell growth factor (VEGF) (49, 50). Here, we found that several angiogenic factors, including angiopoietin-1, FGF2, Interleukin-8, neuregulin-1, placenta growth factor, VEGFA and VEGFC, were
upregulated in α-catulin overexpressing cells (Supplementary Table S3). Taken together, α-catulin may promote tumor growth in vivo by enhancing tumor angiogenesis.

In conclusion, our data strongly suggest that the α-catulin/ILK/NF-κB/integrin αvβ3 signaling pathway is associated with cell migration and invasion in NSCLC. Fibronectin and integrin αvβ3 may regulate the activity of α-catulin and ILK in a positive feedback loop. Thus, this novel signaling axis may be a potential therapeutic target for treating NSCLC metastasis.

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References


Table 1. Multivariate Cox regression analysis of the two-gene signature for predicting survival in lung cancer patients.

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51.
Figure Legends

Figure 1. α-Catulin promotes lung cancer cell migration and invasion in vitro. A, the endogenous α-catulin mRNA and protein levels in lung cancer cell lines were determined by qRT-PCR and Western blotting. GAPDH and β-actin were used as loading controls. B, α-catulin was overexpressed in CL1-0 cells by pLKO_AS2 α-catulin, and it was knocked down in CL1-5 cells by the stable expression of pLKO.1 shB01 and pLKO.1 shC01 against α-catulin. RT-PCR and Western blotting were used to determine α-catulin expression. C, migration and invasion assays with α-catulin overexpressing and control CL1-0 cells. The relative migration and invasion abilities were normalized relative to the control cells. D, migration and invasion assays for α-catulin-depleted and control CL1-5 cells. The data are presented as the mean ± SDs of three independent assays.

Figure 2. α-Catulin enhances lung cancer cell metastasis in vivo. A, a diagram of the lung metastasis experiment. Lung cancer cells were subcutaneously injected into NOD/SCID mice, and the tumor nodules were counted in the animals’ lungs. B, representative images of metastatic lung nodules (upper) and histological staining (H&E) of lung sections from mice injected with control or α-catulin overexpressing CL1-0 cells. C and D, quantitation of the metastatic lung nodules from mice injected with α-catulin-overexpressing CL1-0 or α-catulin-depleted cells. The number of lung metastatic nodules in individual mice was counted under a dissection microscope after 12 weeks. E, a diagram of the lung metastasis experiment. Lung cancer cells were injected into the tail vein of NOD/SCID mice and the tumor nodules in the animals’ lungs were counted. F and G, quantitation of metastatic lung nodules from mice injected with either α-catulin-overexpressing CL1-0 or α-catulin knockdown cells.
The number of lung metastatic nodules in individual mice was counted under a dissection microscope after 35 days. H, representative images of metastatic lung nodules (upper) and histological staining (H&E) of lung sections from mice injected with control (sh-Luci) or α-catulin-depleted CL1-5 (sh-C01) cells.

**Figure 3.** α-Catulin enhances lung cancer cell migration and invasion through its interaction with ILK. A, yeast two-hybrid analysis of the interaction between ILK and α-catulin. Interactions were detected based on the ability of the co-transformants to grow in the absence of histidine and exhibit β-galactosidase activity. B, protein lysates from CL1-5 cells were immunoprecipitated with IgG, α-catulin or ILK antibodies and visualized by immunoblotting with anti-α-catulin and anti-ILK antibodies. C, a diagram of the full-length and various deletion constructs of ILK. D, co-immunoprecipitation of endogenous α-catulin and various FLAG-ILK constructs. After transfection with different FLAG-ILK constructs, immunoprecipitation was performed with an anti-FLAG antibody. The immunoprecipitates (IP) and 10% of the whole cell extract (WCE) were fractionated by SDS-PAGE, and the immunoblots were probed with anti-α-catulin or anti-FLAG antibodies. E, knockdown of ILK reduced α-catulin-promoted cell migration and invasion. CL1-0 cells were infected with pLKO_AS2, pLKO_AS2 α-catulin or pLKO_AS2 α-catulin combined with sh-ILK virus. The relative migratory and invasive abilities were normalized relative to those of the control cells. F, knockdown of α-catulin inhibited ILK-induced cell migration and invasion. The experiment was replicated 3 times. The data are presented as the mean ± SDs of three independent assays.

**Figure 4.** The interaction between α-catulin and ILK plays an important role in the
AKT signaling pathway. A, CL1-0 cells were transfected with GFP or α-catenin-GFP constructs. Protein expression was assessed by Western blotting. B, CL1-5 cells were infected with lentiviruses carrying shB01 and shC01 targeting α-catenin or the negative control luciferase (shLuci) and analyzed by Western blotting. C, CL1-0 cells were transfected with GFP or α-catenin-GFP and then infected with shILKA01 and shILKB01 lentiviruses targeting ILK. The protein expression was assessed by Western blotting. D, CL1-5 cells were transfected with FLAG or ILK-FLAG plasmids and then infected with shB01 and shC01 lentiviruses. The protein expression was assessed by immunoblotting. E, pLKO_AS2 and pLKO_AS2 α-catenin-infected CL1-0 cells were treated with DMSO or NF-κB inhibitor (BAY). The relative migratory and invasive abilities were normalized to those of the control cells. The experiment was replicated 3 times. The data are presented as the mean ± SDs of three independent assays.

Figure 5. Integrin αvβ3 is the downstream target of the α-catenin-ILK signaling pathway, which mediates cancer cell migration and invasion. A, pathway analysis of differential global gene expression in α-catenin-overexpressing A549 cells. MetaCore software with GeneGo Map Folders was used to analyze the pathways containing the universal genes that were differentially expressed in α-catenin-overexpressing A549 cells. B, the expression levels of genes involved in the cytoskeleton remodeling pathway were individually normalized to GAPDH. The data were presented as log2 ratios of the normalized expression in α-catenin-overexpressing to the expression in control cells. C, the expression of fibronectin and integrins in α-catenin-overexpressing CL1-0 and α-catenin-depleted CL1-5 cells was assessed by RT-PCR (left) and Western blotting (right). GAPDH and β-actin were used as loading controls. D, Western blotting
analyzed the expression of fibronectin and integrins in α-catenin-overexpressing CL1-0 cells (left) and ILK-overexpressing CL1-5 cells (right). α-Catenin-overexpressing CL1-0 cells were infected with shILKA01 and shILKB01 lentiviruses targeting ILK. ILK-overexpressing CL1-5 cells were infected with shCATUB01 and shCATUC01 lentiviruses targeting α-catenin. E, pLKO_AS2 and pLKO_AS2 α-catenin-infected CL1-0 cells were treated with different doses of NF-κB inhibitor (BAY). RT-PCR was used to determine mRNA expression. F, pLKO_AS2 and pLKO_AS2 α-catenin-infected CL1-0 cells were treated with IgG or anti-integrin αvβ3 neutralizing antibody. The relative migratory and invasive abilities were normalized to those of the control cells. The data are presented as the mean ± the SDs of three independent assays.

**Figure 6.** The two-gene signature of CTNNAL1 and ILK significantly correlates with poor overall survival in NSCLC patients. Survival analysis of 177 patients from the Michigan NSCLC cohort based on (A) CTNNAL1 (B) ILK and (C) CTNNAL1 and ILK gene expression using the Kaplan–Meier method. D, α-catenin increased activation of NF-κB through an ILK dependent pathway, which in turn increased fibronectin and integrin αvβ3 protein expression. This positive feedback signaling loop results in increased cancer cell migration and invasion and metastasis.
Figure 1

A

Relative mRNA expression level of α-Catulin

CL1-0 CL1-1 CL1-2 CL1-5

α-Catulin

1.0 1.8 3.8 6.9

β-actin

B

RT-PCR

CL1-0 CL1-5

PLKO_αAS2 α-Catulin

1.0 27.5 1.0 0.1 0.2

GADPH

WB

α-Catulin

1.0 3.3 1.0 0.4 0.6

β-actin

C

PLKO_αAS2 α-Catulin

Migration Invasion

D

sh-Luci sh-B01 sh-C01

Migration Invasion

Relative migration ability %

p<0.05 p<0.05

Relative invasion ability %

p<0.01 p<0.01 p<0.01
Figure 2

A

B

C

D

E

F

G

H

PLKO AS2 α-Catulin

Numbers of metastatic nodules

p<0.05

Numbers of metastatic nodules

sh-Luci sh-C01

p<0.05

p<0.05

p<0.01
Figure 5

A

-Log (p Value) vs Pathway fold

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<th>Pathway fold</th>
<th>pValue</th>
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<td>59/102</td>
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<td>2. ESR1 regulation of G1/S transition</td>
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<td>3. TGF, WNT and cytoskeletal remodeling</td>
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<td>4. IL-1 signaling pathway</td>
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<td>5. Apoptosis and survival_BAD phosphorylation</td>
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Relative expression

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C

CL1-0

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CL1-5

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CL1-0

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CL1-5

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E

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<tr>
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F

Relative migration ability

Relative invasion ability

- p<0.001
- p<0.01
Figure 6

A. CTNNAL1

- Low-risk signature (n=88)
- High-risk signature (n=89)

Probability of overall survival

P=0.17

Months

B. ILK

- Low-risk signature (n=88)
- High-risk signature (n=89)

Probability of overall survival

P=0.52

Months

C. CTNNAL1 and ILK

- Low-risk signature (n=88)
- High-risk signature (n=89)

Probability of overall survival

P=0.019

Months

D. Diagram showing the interaction between CTNNAL1, ILK, AKT, and other molecules in the context of migration and invasion.
α-catenin drives metastasis by activating ILK and driving an avβ3 integrin signaling axis

Chen-Hsien Liang, Szu-ying Chiu, I-Ling Hsu, et al.

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