Original Articles

Silencing of LRRFIP1 reverses the epithelial–mesenchymal transition via inhibition of the Wnt/β-catenin signaling pathway

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ABSTRACT

The canonical Wnt/β-catenin signaling pathway has been shown to promote the epithelial–mesenchymal transition (EMT), which is a crucial process in multiple embryonic developmental processes and the progression of carcinomas. We recently provided evidence that leucine-rich repeat flightless-1-interacting protein 1 (LRRFIP1) promotes cancer metastasis and invasion. In the present study, we identified the signaling elements targeted by LRRFIP1 for promotion of the EMT in pancreatic and lung cancer. LRRFIP1 silencing reversed the EMT, as shown by increased expression of E-cadherin (an epithelial marker) and decreased expression of vimentin (a mesenchymal marker). Silencing of LRRFIP1 up-regulated phosphorylation of β-catenin and decreased its nuclear localization by targeting the β-catenin destruction complex. The expression of β-catenin and E-cadherin in the plasma membrane fraction was increased in LRRFIP1 silenced cancer cells, and the migration and invasion capabilities were strongly inhibited. In addition, this protein was highly expressed at the invasion front of malignant tissue collected from pancreatic cancer patients. Consequently, our data strongly suggested that LRRFIP1 played an important role in the invasion of carcinoma cells. Our data provide experimental evidence that LRRFIP1 is an attractive candidate for targeted therapy in human cancers.

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Introduction

Metastasis is the most important contributor to mortality in patients with cancer [1,2] and is the end product of a multistep cellular process. The pathogenesis of cancer metastasis, termed the invasion–metastasis cascade, includes invasion of cancer cells into surrounding tissue, intravasation, survival in the circulation, arrest at distant organ sites, extravasation, and growth of macroscopic secondary tumors in distant organs [3,4]. Accumulating evidence indicates that the epithelial–mesenchymal transition (EMT), an essential phenotypic conversion during embryonic development, is strongly associated with the invasion of carcinomas [5]. The EMT is characterized by changes in cell morphology during which epithelial cells acquire mesenchymal properties while losing cell–cell interactions and apicobasal polarity [6]. Cancer cells with the EMT phenotype exhibit enhanced motility and invasive abilities [7]. The initial step in the EMT is downregulation of E-cadherin by transcription factors, primarily Snail, Slug, Twist1, Twist2, zinc finger E-box binding homeobox (ZEB) 1, and ZEB2 [8]. The EMT can be triggered by different signals received from the tumor microenvironment, such as transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, Wnt, and Notch [9], and EMT-like changes are most common in spatially restricted growth areas, such as the advancing tumor margin (invasion front) [10].

The Wnt signaling pathway plays crucial roles in normal embryonic development and diseases of cell proliferation, including cancer [11–13], and includes canonical and noncanonical branches [14]. The canonical Wnt/β-catenin signaling pathway regulates cell proliferation and differentiation, two functions implicated in metastasis. Moreover, although noncanonical Wnt signaling generally acts in a tumor-suppressive manner, recent studies have shown that this pathway enhances cancer progression in the later stages of the disease [15]. Cytosolic β-catenin is the principal mediator of canonical Wnt/β-catenin signaling. In the absence of an extracellular Wnt ligand, the nonfunctional pool of cytoplasmic β-catenin is targeted for proteolysis by a large multiprotein assembly termed the “β-catenin destruction complex”; the core components of this complex include β-catenin itself, the Ser/Thr kinase glycogen synthase kinase (GSK-3β), the scaffolding protein Axin, and adenomatous...
polyposi coli (APC) [16]. Binding of Wnt ligand to its coreceptors Frizzled (Fz) and low-density lipoprotein (LDL) receptor-related protein (LRP) 5/6 activates disheveled (Dvl), which then inhibits GSK-
3β-mediated phosphorylation of β-catenin, and promotes stabilization and nuclear translocation of β-catenin. Nuclear translocation of β-catenin induces expression of downstream genes linked to the induction of EMT-like programs [17]. Accordingly, aberrant regulation of the Wnt signaling pathway has emerged as a prevalent theme in cancer biology.

β-Catenin is also a component of the cadherin-based adherens junction complexes formed at cell–cell adhesion sites [18,19]. Association of E-cadherin with β-catenin and α-catenin is crucial for stable cell–cell adhesion, and binding of β-catenin to these partners is regulated by tyrosine phosphorylation of β-catenin [20]. Moreover, sequestration of β-catenin at the membrane facilitates stable cell–cell adhesion, and binding of β-catenin to these parts—association of E-cadherin with β-catenin and α-catenin is crucial for canonical and noncanonical Wnt signaling pathways in carcinoma [21]. Based on these mechanisms, β-catenin is an integral structural component of cadherin-based adherens junctions and a key nuclear effector of Wnt/β-catenin signaling in the nucleus [23]. Imbalances in the structural and signaling properties of β-catenin often result in disease and deregulated growth associated with cancer and metastasis [24].

The leucine-rich repeat flightless-1-interacting protein 1/GC-rich binding factor 2 (LRRFIP1/GCF2) gene encodes a 752-amin acid protein with a molecular weight of 160 kDa [25]. Previous reports have indicated that LRRFIP1 interacts with several molecules in the cytoplasm and regulates the cytoskeleton and migration [26,27]. LRRFIP1 was revealed to interact with Dvl and positively regulate canonical and noncanonical Wnt signaling pathways in carcinoma cells [26]. In addition, LRRFIP1 has been implicated in invasion and metastasis [28]. Therefore, we hypothesized that LRRFIP1 also plays an essential role in cancer cell EMT and invasion.

It was also indicated that activation of β-catenin-dependent canonical Wnt signaling promotes EMT and metastasis in pancreatic cancer models [29]. In addition, our preliminary data showed high expression of LRRFIP1 at the invasion front of pancreatic cancer tissues in immunohistochemical studies. Hence, we investigated the role of LRRFIP1 in the EMT and invasion of pancreatic cancer cells. Because we focused on early events in cancer progression and metastasis, we analyzed the canonical Wnt signaling pathway, evaluating changes in the localization of β-catenin in relation to LRRFIP1 expression.

Materials and methods

Cell lines and treatments

Five human pancreatic cancer cell lines (BxPC-3, PANC-1, MIA PaCa-2, PK-8, and PK-1) and the AS59 lung cancer cell line were used in our study. BxPC-3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). PANC-1 was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Three additional pancreatic cancer cell lines (MIA PaCa-2, PK-8, and PK-1) were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The cell lines were obtained directly from these institutions, which characterized and authenticated the cell lines by short tandem repeat analysis, and all cell lines were passaged in our laboratory for less than 6 months after receipt.

The morphology of cells was visualized with a phase-contrast microscope (Axion Vert.A1, Carl Zeiss, Germany).

Antibodies and reagents

Mouse anti-LRRFIP1 monoclonal antibodies were obtained from BD Biosciences (Bedford, MA, USA); mouse anti-E-cadherin monoclonal antibodies were purchased from Zymed Laboratories (South San Francisco, CA, USA); rabbit monoclonal antibodies against human E-cadherin, vimentin, β-catenin, phospho-β-catenin (Ser33/37), and GSK-3β were purchased from Cell Signaling Technology (Danvers, MA, USA); and rabbit anti-Dvl, anti-APC, and anti-Axin polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human TGF-β1 and TNF-α were purchased from Wako Pure Chemicals (Osaka, Japan). Recombinant human Wnt-3a was purchased from R&D Systems (Minneapolis, MN, USA).

Human samples

Human tissue specimens were obtained with consent from patients who received a diagnosis of pancreatic cancer (n = 5) at Tohoku University Hospital. Specimens were fixed in 10% formalin and embedded in paraffin. Tissue specimens were encoded to protect patient confidentiality and processed under protocols approved by the Ethics Committee of Tohoku University Graduate School of Medicine.

Immunohistochemistry

Mouse monoclonal antibodies targeting human LRRFIP1 and E-cadherin were used for immunohistochemistry. A Histofine Kit (Nichirei Biosciences), which uses the streptavidin–biotin amplification method, was employed for immunohistochemical analysis. The antigen–antibody complex was visualized with 3,3’-diaminobenzidine solution (1 mmol/L 3,3’-diaminobenzidine, 50 mmol/L Tris–HCl buffer [pH 7.6], and 0.006% H2O2). The sections were then counterstained with hematoxylin, dehydrated using a graded series of alcohols, mounted, and observed under a microscope (BZ-9000, KEYENCE, Tokyo, Japan).

Quantitative analysis of immunohistochemical samples

Immunostained sections were analyzed using a HistoFAX image cytometer (TissueGnostics, Vienna, Austria), and the specific diaminobenzidine density in pancreatic duct cells was analyzed using HistoQuest software (TissueGnostics).

siRNA transfection

LRRFIP1 was knocked down using specific siRNA oligonucleotides (Stealth RNAi siRNA Duplex Oligoribonucleotides; Life Technologies). Stealth RNAi Negative Control Duplexes (Life Technologies) were used as the negative control. The siRNA LRRFIP1 sequences used in this study were as follows: siRNA1, sense (5’-ggaaucaagacaccu cuagcaga-3’); siRNA2, sense (5’-cagaucaucgaauccaugucgu-3’). The siRNA control sequence in this study was 5’-ggaaucaagacaccucaagaaag-3’.

Immunofluorescence

For immunofluorescent staining, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and rinsed with 3× phosphate-buffered saline (PBS) for 5 min before blocking in 5% normal goat serum and 0.3% Triton X-100 in PBS for 1 h at room temperature. The cells were incubated with primary antibodies at 4 °C overnight, washed, and then incubated with secondary antibodies for 2 h at room temperature. Fluorescence images were obtained using a confocal laser-scanning microscope (CLSM; Czi, Nikon, Japan). Isotype-matched mouse or rabbit IgG was used as a negative control with the same dilutions as the primary antibodies.

Cell proliferation, migration, and invasion assays

Proliferation assays were performed at 0, 24, 48, and 72 h after treatment in quadruplicate using CellTiter 96 Aqueous One Solution Cell Proliferation Assays (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

Migration assays were performed using Radius 24-well cell migration assay plates (Cell Biolabs, San Diego, CA, USA). Digital images of the gap closure were obtained using a phase-contrast microscope (Carl Zeiss). The migration area was analyzed using ImageJ software, and the data were presented as the average percent closure ± SE.

For isolation of total protein, cells were lysed in radio-immunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). For nuclear extracts, cells were lysed in NE-PER extraction reagent (Thermo Scientific) according to the manufacturer’s protocol. Following primary antibody incubation, membranes were incubated with HRP-conjugated secondary antibodies, and signals were detected using the Clarity Western ECL Substrate (Bio-Rad). Protein bands were visualized using an ImageQuant LAS 4000 mini system (GE Healthcare, Buckinghamshire, UK). Densitometry analysis was performed using ImageJ software, and band intensities were normalized to those of GAPDH [30].

Western blotting

For isolation of total protein, cells were lysed in radio-immunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). For nuclear extracts, cells were lysed in NE-PER extraction reagent (Thermo Scientific) according to the manufacturer’s protocol. Following primary antibody incubation, membranes were incubated with HRP-conjugated secondary antibodies, and signals were detected using the Clarity Western ECL Substrate (Bio-Rad). Protein bands were visualized using an ImageQuant LAS 4000 mini system (GE Healthcare, Buckinghamshire, UK). Densitometry analysis was performed using ImageJ software, and band intensities were normalized to those of GAPDH [30].
Quantitative real-time PCR

RNA was extracted using NucleoSpin RNA II (Takara Bio, Kyoto, Japan) and analyzed by Nanodrop (Thermo Scientific); RNA (1 μg) was transcribed into cDNA using PrimeScript RT Master Mix (Perfect Real Time; Takara Bio) according to the manufacturer's instructions. Quantitative PCR was performed using a StepOnePlus Real-Time PCR system (Life Technologies) with SYBR Premix ExTaq II (Tli RNaseH Plus) and ROX plus (Takara Bio). Relative quantification of mRNA within the samples was performed using the 2^{-ΔΔCt} method, and the results were averaged and normalized against the expression of GAPDH as an internal control in each sample. All reactions were performed in quadruplicate, and the experiments were conducted at least three times. The sequences of the primer pairs used in this study are shown in Supplemental Table S1.

In situ proximity ligation assay (PLA)

Cultures, treated as indicated, were immediately fixed in 4% paraformaldehyde on ice for 15 min and subjected to in situ PLA using a Duolink Detection kit (Olink Bioscience, Uppsala, Sweden) according to the manufacturer's instructions. Cells were fixed, nonspecific binding sites were blocked, and immunostaining was performed using anti-mouse primary antibodies and anti-rabbit primary antibodies. Subsequently, species-specific secondary antibodies conjugated with oligonucleotides (PLA probes) were used according to the manufacturer's protocol. Cell images were obtained using a confocal microscope (Nikon). The fluorescent signal from each detected pair of PLA probes was visualized as a distinct individual dot. A negative control without primary antibodies was also performed. Representative results are shown from experiments repeated at least three times.

Preparation of membrane fractions

Cells were scraped from culture dishes and collected by centrifugation at 230 × g for 5 min at 4°C. The cells were suspended in suspension buffer (10 mM Tris–HCl [pH 7.4] containing 250 mM sucrose and 1 mM EGTA) with protease cocktail (Sigma-Aldrich) and then lysed by nitrogen cavitation at 750 psi for 15 min at 4°C in a pressure vessel (Parr, Moline, IL). Homogenates were centrifuged at 10,000 × g for 10 min at 4°C. Supernatants were ultracentrifuged at 100,000 × g for 40 min at 4°C, and the resulting pellets were resuspended. Suspensions were layered on top of 38% (w/v) sucrose and centrifuged at 100,000 × g for 40 min at 4°C. The turbid layer at the interface was recovered, suspended in suspension buffer, and centrifuged at 100,000 × g for 40 min at 4°C to obtain the plasma membrane fraction. Protein concentrations were measured by the Lowry method using DC protein assay reagent (Bio-Rad).

Statistical analysis

Quantitative data (mean ± SE, two or three experiments) were analyzed using the paired Student's t-test for statistical significance. Differences were considered significant when p < 0.05.

Results

Effects of LRRFIP1 silencing on EMT-related gene expression

Previous reports have indicated that LRRFIP1 was implicated in the regulation of the cytoskeleton, adhesion, and migration. To assess the effects of LRRFIP1 on EMT-related gene expression, siRNA specifically targeting LRRFIP1 was transfected into PANC-1 and A549 cells. As shown in Fig. 1A, expression of E-cadherin was increased in LRRFIP1-knockdown PANC-1 and A549 cells. Additionally, expression of vimentin was decreased in LRRFIP1-knockdown cells. Furthermore, immunofluorescence microscopy suggested that silencing of LRRFIP1 in PANC-1 and A549 cells caused a shift in the

![Fig. 1. Silencing of LRRFIP1 induced reversal of the EMT in cancer cells. (A) Western blotting analysis of E-cadherin and vimentin in LRRFIP1-knockdown PANC-1 and A549 cells. GAPDH served as a loading control. (B) Confocal immunofluorescent analysis of E-cadherin and vimentin in LRRFIP1-knockdown PANC-1 and A549 cells. E-cadherin (green, top panel), LRRFIP1 (red), and vimentin (green, bottom panel) are shown. Nuclei were stained with DAPI (blue). Scale bar, 10 μm.](image-url)
expression of mesenchymal markers (vimentin) to epithelial markers (E-cadherin) (Fig. 1B).

**Expression of LRRFIP1 and EMT makers in pancreatic cancer cell lines**

The expression levels of LRRFIP1 and EMT markers (e.g., E-cadherin and vimentin) were analyzed in five pancreatic cancer cell lines (PANC-1, MIA PaCa-2, BxPC-3, PK-8, and PK-1) (Fig. 2). Three pancreatic cancer cell lines (BxPC-3, PK-8, and PK-1) expressed high levels of E-cadherin and low levels of vimentin. In contrast, the other pancreatic cancer cells (PANC-1 and MIA PaCa-2) expressed low levels of E-cadherin and high levels of vimentin. The expression of LRRFIP1 in BxPC-3, PK-8, and PK-1 cells was remarkably lower than that in PANC-1 and MIA PaCa-2 cells. Therefore, high expression of LRRFIP1 was suggested to associate with high expression of mesenchymal markers (e.g., vimentin) but low expression of epithelial markers (e.g., E-cadherin) in pancreatic cancer cells.

**Effects of LRRFIP1 silencing on migration and invasion in pancreatic cancer cells**

To confirm the epithelial features of LRRFIP1-knockdown cancer cells, migration and invasion assays were performed. In the migration assay, the migration area was significantly decreased in cells transfected with LRRFIP1 siRNA (Fig. 3A). Additionally, the number of invading cells was significantly decreased in cells transfected with LRRFIP1 siRNA relative to cells transfected with control siRNA (Fig. 3B). These data suggested that LRRFIP1 expression regulated the migration and invasion of cancer cells.

**LRRFIP1 interacted with the β-catenin destruction complex**

To determine whether the Wnt/β-catenin signaling pathway was functional in PANC-1 and A549 cells, cultures were stimulated with 100 ng/mL Wnt-3a. After 48 h, the stimulated cultures contained higher levels of β-catenin protein in their whole cell lysates than PANC-1 and A549 cells treated only with vehicle (Fig. 4A). Additionally, Wnt-3a stimulated the nuclear translocation of β-catenin.
Fig. 4. LRRFIP1 interacted with the β-catenin destruction complex. (A) Detection of β-catenin by western blotting analysis of whole cell lysates in PANC-1 and A549 cells induced by stimulation with Wnt-3a (100 ng/mL) for 48 h. GAPDH served as loading controls. (B) Quantitative real-time PCR of Slug, Twist, and ZEB1 in PANC-1 and A549 cells induced by stimulation with Wnt-3a for 48 h. (C and D) LRRFIP1 interacted with the β-catenin destruction complex (disheveled, APC, Axin, GSK-3β, and β-catenin) in PANC-1 (top panel) and A549 (bottom panel) cells. PLA signals (red) and phalloidin (green) are shown. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. Data are expressed as the mean ± SE (*p < 0.05).
in PANC-1 and A549 cells. Furthermore, the mRNA expression levels of EMT-associated transcriptional factors, such as Slug, Twist, and ZEB1, were elevated in PANC-1 and A549 cells induced with 100 ng/mL Wnt-3a (Fig. 4B). These data demonstrated that the Wnt/β-catenin pathways regulating EMT were functional in PANC-1 and A549 cells.

To examine whether endogenous LRRFIP1 interacted with Dvl in PANC-1 and A549 cells, Duolink in situ PLAs were performed. As demonstrated in Fig. 4C, strong fluorescence signals were detected in the presence of specific antibodies, indicating that LRRFIP1 and Dvl interacted in these cells. To confirm the specificity of the signals, PLAs were performed for LRRFIP1-knockdown PANC-1 or A549 cells. The fluorescent signals were significantly decreased by silencing of LRRFIP1 expression (Fig. 4C). To investigate the interaction between LRRFIP1 and each component of the β-catenin destruction complex, further experiments were performed. APC, Axin, GSK-3β, and β-catenin were all found to colocalize with LRRFIP1 (Fig. 4D). These data strongly suggested that LRRFIP1 interacted with the β-catenin destruction complex and participated in the regulation of β-catenin stabilization.

Silencing of LRRFIP1 led to recruitment of β-catenin and E-cadherin to the plasma membrane

The function of LRRFIP1 in the Wnt/β-catenin signaling pathway was determined by testing the effects of gene silencing on GSK-3β and the phosphorylation of β-catenin. Western blot analysis indicated that LRRFIP1 knockdown in PANC-1 or A549 cells induced an increase in the levels of both GSK-3β and phosphorylated β-catenin. Because phosphorylated β-catenin is targeted for degradation [31], these data suggested that Wnt/β-catenin signaling was strongly suppressed by silencing of LRRFIP1 (Fig. 5A). Moreover, the quantity of β-catenin in the nucleus was decreased (Fig. 5B), suggesting that knockdown of LRRFIP1 inhibited the Wnt/β-catenin signaling pathway via interaction with the β-catenin destruction complex. In LRRFIP1-knockdown PANC-1 cells, expression of β-catenin and E-cadherin was increased in the plasma membrane fraction (Fig. 5C). These data suggested that the increase in total β-catenin levels reflected an increase in the E-cadherin-associated membrane fraction of β-catenin (Fig. 5C). To further support this finding, we examined the subcellular distribution of β-catenin by immunofluorescence staining. As shown in Fig. 5D, β-catenin staining was exclusively localized to the cell membrane in LRRFIP1-knockdown PANC-1 cells.

E-cadherin gene promoter was negatively regulated by LRRFIP1 silencing

We tested whether transcription factors known to directly inhibit the E-cadherin gene promoter were affected by LRRFIP1 silencing. The mRNA expression levels of Snail, Slug, and Twist were consistently decreased in LRRFIP1-knockdown PANC-1 and A549 cells (p < 0.05; Fig. 6). These data suggested that LRRFIP1 stimulated the

![Fig. 5. Silencing of LRRFIP1 inhibited the Wnt/β-catenin signaling pathway. (A) Levels of GSK-3β and phosphorylated β-catenin in LRRFIP1-knockdown PANC-1 and A549 cells determined by western blotting with anti-GSK-3β and anti-phospho-β-catenin (S33/S37)-specific antibodies. GAPDH served as a loading control. (B) Nuclear fractions from LRRFIP1-knockdown PANC-1 cells were processed for western blotting with anti-β-catenin antibodies. Histone H1 served as loading controls. (C) Plasma membrane fractions or whole cell lysate of β-catenin and E-cadherin in LRRFIP1-knockdown PANC-1 cells. β-Actin and GAPDH served as a loading control. (D) Localization of β-catenin in LRRFIP1-knockdown PANC-1 cells. Scale bar, 10 μm.](image-url)
Fig. 6. Silencing of LRRFIP1 suppressed the expression of Snail, Slug and Twist mRNA. Expression levels of Snail, Slug, and Twist mRNAs were determined by Quantitative real-time PCR analysis in LRRFIP1-knockdown PANC-1 and A549 cells. Data are expressed as the mean ± SE (*p < 0.05).

Fig. 7. Expression of LRRFIP1 and E-cadherin in human pancreatic adenocarcinoma samples. The expression levels of LRRFIP1 and E-cadherin were analyzed in pancreatic adenocarcinoma samples by immunohistochemical staining. (A) Strong staining of E-cadherin in normal tissues and strong staining of LRRFIP1 in the cancer invasion front and central cancerous region compared to normal tissues. PD indicates pancreatic ducts. (B) The staining intensities of LRRFIP1 and E-cadherin were determined using a HistoFAXS image cytometer and comparative analysis of staining intensities was performed. Data are expressed as the mean ± SE (*p < 0.05). The photographs were obtained at 20× magnification.
EMT primarily through Snail, Slug, and Twist in PANC-1 and A549 cells.

**LRRFIP1 expression was highest at the invasion front of pancreatic cancer tissues**

Because low E-cadherin expression has been associated with poor clinical outcomes in several types of cancers [32,33], we conducted experiments to investigate the spatial relationship between LRRFIP1 and E-cadherin in pancreatic cancer tissues. Immunohistochemical staining was performed in normal tissues, central regions of cancer tissues, and at the invasion front for both LRRFIP1 and E-cadherin (Fig. 7A). Comparative analysis of staining intensities revealed that E-cadherin expression was significantly lower at the leading edge than at the center of the cancerous area (Fig. 7B). In contrast, LRRFIP1 expression was highest at the invasion front among these three areas. The inverse relationship between the expression levels of LRRFIP1 and E-cadherin was suggested in pancreatic cancer tissues.

**Discussion**

A recent comprehensive genetic analysis of human breast and colon cancer identified 189 genes, including *LRRFIP1*, as new candidate oncogenes or tumor-suppressor genes [34]. However, the role played by LRRFIP1 in cancer has not yet been clearly defined. In the present study, we found that LRRFIP1 was highly concentrated at the invasion front of malignant tissue collected from patients with pancreatic cancer. Therefore, we tested the hypothesis that LRRFIP1 contributes to the malignancy of cancer tissue. Suppression of LRRFIP1 expression was strongly associated with epithelial features in terms of EMT markers such as E-cadherin and vimentin. Using a multidisciplinary approach, we found that LRRFIP1 stimulated the EMT through regulation of the canonical Wnt/β-catenin signaling pathway. Therefore, we provided evidence that LRRFIP1 played an important role in the initiation of cancer invasion.

The EMT program, one of the central mechanisms that induces tumor metastasis and tissue invasion, is a process through which epithelial cells lose their polarity and are converted to a mesenchymal phenotype [35–37]. E-cadherin is a suppressor of cell invasion and metastasis, and its downregulation is a key event during the EMT in cancer. In the present study, we demonstrated LRRFIP1 silencing in cancer cells strongly accelerated E-cadherin expression. Additionally, LRRFIP1 suppressed cells exhibited weak migration and invasion properties. Cancer cells with low LRRFIP1 expression also exhibited low expression of vimentin (a mesenchymal marker), but high expression of E-cadherin (an epithelial marker). These data suggested that silencing of the *LRRFIP1* gene may prevent cancer cell invasion by interfering with the EMT program.

**LRRFIP1 is thought to target the canonical Wnt/β-catenin signaling pathway, as supported by our previous report in which we found that LRRFIP1 interacts with Dvl [26]. Stimulation of the Wnt/β-catenin signaling pathway has been shown to downregulate E-cadherin [38]. In fact, the progression of several types of cancer is associated with hyperactivation of the Wnt signaling cascade. Generally, oncogenic activation of the Wnt/β-catenin pathway is triggered by inactivation of the destruction complex, resulting in high levels of β-catenin expression and translocation of Wnt target genes [39–41]. In the present study, functional analysis by the PLA method revealed that LRRFIP1 colocalized with all major components of the β-catenin destruction complex (Dvl, APC, Axin, GSK-3β, and β-catenin). Additionally, we found that β-catenin phosphorylation was an essential step in the destruction pathway. In PANC-1 and A549 cells, LRRFIP1 silencing led to decreases in the levels of GSK-3β expression and β-catenin phosphorylation. Collectively, these data suggested that knockdown of LRRFIP1 expression suppressed canonical Wnt/β-catenin signaling by targeting the β-catenin destruction complex. However, whether LRRFIP1 interferes directly with the formation of this complex remains unknown.

The Wnt/β-catenin-dependent EMT program is mediated by translocation of β-catenin to the nucleus and activation of specific transcription factors [8]. Additionally, GSK-3β has been characterized as the main kinase responsible for the subcellular localization and protein stability of Snail [42,43]. Similarly, we showed that LRRFIP1 silencing dramatically reduced the expression of Snail, Slug, and Twist in PANC-1 and A549 cells. Because these transcription factors reduce the expression of the epithelial marker E-cadherin [44], LRRFIP1 silencing has the potential to reverse the EMT in cancer cells. Silencing of LRRFIP1 as a Wnt pathway-related gene is associated with translocation of β-catenin from the nucleus to cadherin-based cell–cell connections located close to the plasma membrane, which enhances cell–cell adhesion and reduces mobility [45].

In summary, our results demonstrated that repression of endogenous LRRFIP1 caused almost complete reversal of EMT markers and inhibited migration and invasion in cancer cells. Silencing of LRRFIP1 up-regulated phosphorylation of β-catenin and decreased its nuclear localization by targeting the β-catenin destruction complex. The expression of β-catenin and E-cadherin in the plasma membrane fraction was increased in LRRFIP1 silenced cancer cells, and the migration and invasion capabilities were strongly inhibited. Our discoveries highlight the potential application of anti-LRRFIP1 inhibitors for therapeutic intervention and the prevention of metastasis in various tumor types.

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**Conflict of interest**

The authors disclose no potential conflicts of interest.

**Appendix: Supplementary material**

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.05.023.

**References**


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