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ORIGINAL ARTICLE

Basic Study

Integrin-linked kinase overexpression promotes epithelial-mesenchymal transition *via* nuclear factor-κB signaling in colorectal cancer cells

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Abstract

AIM: To investigate the effect of integrin-linked kinase (ILK) on proliferation, metastasis, and invasion of the colorectal cancer cell line SW480.

METHODS: In this study, the colorectal cancer cell line SW480 was stably transfected with ILK plasmids, and small interfering RNA (siRNA) was used to knockdown expression of nuclear factor (NF)-κB/p65. Methylthiazolium tetrazolium (MTT) assay was performed to measure proliferation, and the wound healing migration assay and matrigel invasion assay were used to test the metastasis and invasion ability of SW480 cells. To explore the epithelial-mesenchymal transition (EMT) process, embryonic development, and the invasion and metastasis of tumors, the protein level of E-cadherin, vimentin, snail, and slug was detected by western blot. Immunofluorescence was also used to detect E-cadherin expression. Western blot was used to determine the level of phosphorylated-inhibitor of kappa B (IκB)a, inhibitor of gamma B (IγB)a, and nuclear factor kappa B (NF-κB) expressions and to

explore the ILK signaling pathway.

RESULTS: Western blot results revealed that ILK expression significantly increased when ILK was overexpressed in SW480 cells ($P < 0.05$). Proliferation, metastasis, and invasion ability were improved in the vector-ILK group compared to the vector group ($P < 0.05$). Immunofluorescence results revealed that E-cadherin fluorescence intensity decreased after ILK was overexpressed ($P < 0.05$). Western blot results revealed that the protein expression of E-cadherin was reduced, while vimentin, snail, and slug were upregulated when ILK was overexpressed in SW480 cells ($P < 0.05$). In order to determine the role of the NF-κB signaling pathway in ILK overexpression promoted EMT occurrence, we overexpressed ILK in SW480 cells and found that levels of NF-κB/p65 and cytoplasmic phosphorylated-IκBa were increased and that cytoplasmic IκBa levels were decreased compared to the control group ($P < 0.05$). Furthermore, NF-κB/p65 knockout revealed that E-cadherin was increased in the overexpressed ILK group.

CONCLUSION: ILK overexpression improved the proliferation, metastasis, and invasion ability of SW480 cells, and this effect may be mediated by the NF-κB signaling pathway.

Key words: Colorectal cancer; Integrin-linked kinase; Epithelial-mesenchymal transition; Nuclear factor-κB; Overexpression

Core tip: In this study, the colorectal cancer cell line SW480 was stably transfected with integrin-linked kinase (ILK) plasmids, and the proliferation, metastasis, and invasion ability of the cells were tested. The results demonstrated that ILK overexpression improved the proliferation, metastasis, and invasion ability of cell line SW4802 and promoted the occurrence of the epithelial-mesenchymal transition in colorectal cancer cells. These effects may be mediated by the nuclear factor-κB signaling pathway.

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INTRODUCTION

Integrin-linked kinase (ILK) is a multifunctional receptor protein and a protein interaction integrin. Moreover, ILK can recruit other adapter molecules and regulate a variety of cellular processes *via* coupled signaling pathways, including cell growth, proliferation, apoptosis, survival, differentiation, migration, and invasion. Recent studies have shown that ILK is overexpressed and excessively activated in a number of human cancers[1,2]. It has been reported that the overexpression of ILK can enhance the rate of lung cancer cell migration, and it was shown that this enhancement was regulated by nuclear factor (NF)-κB-mediated matrix metalloproteinase (MMP)-9 expression[3,4]. Researchers have found that ILK was highly expressed in colorectal cancer tissues; and that ILK promoted tumor transfer and corrosion, which is mediated through the epithelial-mesenchymal transition (EMT) process[5,6]. However, the role and mechanism of ILK in colorectal cancer cells remains unclear. Some experts have reported that ILK overexpression can induce transcription factor snail and zinc finger E-box binding homeobox 1 (ZEB1) expression, resulting in the inhibition of E-cadherin expression[7-9]. The colorectal cancer cell line SW480 was used in this study, and ILK expression levels in this cell line were found to be relatively low. The present study aims to investigate the effect of ILK in colorectal cancer cell proliferation, invasion, and metastasis and to explore its underlying mechanism.

MATERIALS AND METHODS

Materials

Transfection reagent lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States), PVDF film (Millipore, Bedford, MA, United States), anti-ILA antibody (Cell Signaling Technology, Danvers, MA, United States), anti-E-cadherin antibody and anti-Vimentin antibody (Santa Cruz Biotechnology, Dallas, TX, United States), anti-Slug antibody (Abcam, Cambridge, MA, United States), and anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO, United States).

Construction of ILK overexpressed SW480 cell line

The human colorectal cancer cell line SW480 was obtained from the Cell Bank, Chinese Academy of Medical Sciences (Shanghai, China) and cultured in Leibovitz L-15 medium (Gibco, Grand Island, NY, United States) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, United States) and antibodies. Cells were cultured in an incubator containing 5% CO_2 at 37 ℃ and were passaged for 2-3 d until 85% confluence was achieved.

Human ILK gene coding sequence was obtained by polymerase chain reaction (PCR) amplification and connected the target gene to the pcDNA3.1 vector. Sequencing detection revealed no mutation in the target gene. In a six-well plate, 2×10^5 cells were seeded into each well. After 1 d of culture, cells were transfected, and transfection reagent lipofectamine 2000 was applied to transfect 2 μg/mL of

overexpressed ILK plasmids (pcDNA3.1-ILK) or empty vector. After 48 h of transfection, cells were placed into a selective medium (G418, 800 mg/mL) for 3-4 wk. G418-resistant clones were filtered and amplified after reverse transcriptase (RT)-PCR and western blot confirmation.

Cell proliferation experiment

Cells were cultured in 96-well plates (2×10^3 cells/well) for 24, 48, and 72 h. Then, 20 μL of MTT was added into each well and cultured in an incubator for another 2 h. At the end of the culture period, the liquid in the well was discarded, 200 μL of dimethylsulfoxide (DMSO) was added, and optical density (OD) was measured with a microplate reader at 450 nm.

Wound healing assay

Cells were cultured in six-well plates, and the culture medium was discarded when it reached approximately 80% confluence. Then, the monolayer was scraped using the tip of a 200-μL pipette, cells were washed three times with phosphate buffered saline (PBS), and a photograph was taken under a microscope. Subsequently, cells were maintained in serum-free medium for another 6 h. Then, a photograph was taken and the migration rate was computed.

The culture medium was discarded when cells reached 60% confluence. Cells were washed three times with PBS and cultured in an incubator for another 24 h. After trypsinization, cells were collected in a tube, centrifuged, and resuspended in FBS free culture medium to a final concentration of 1×10^5 cells/mL. The transwell chamber was placed onto a 24-well plate containing 800 μL of culture medium with 20% FBS. Then, cell suspensions were injected into the devices through the inlet channels. The plate was incubated for 24 h in 5% CO_2 at 37 ℃. After incubation, the transwell chamber was taken out, washed with PBS, and the upper layer of the chamber was cleaned with a cotton swab. The chamber was fixed with formalin for 20 min, dyed with hematoxylin for 5 min, and washed with PBS. Then, the number of cells were observed and counted with a microscope.

siRNA interference

NF-κBp65 small interfering RNA (siRNA) 5′-CCUCCUU UCAGGAGAUGAATT-3′; scramble control 5′-UUCUCCGA ACGUGUCACGUTT-3′. After transfection with lipofectamine 2000 for 48 h, cells were obtained and analyzed.

Western blot

Cells were lysed for cytosol-nuclear isolation; and nuclear and cytoplasmic lysates were collected. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and proteins were transferred onto a polyvinylidene (PVDF) membrane. Primary antibodies used for incubation were as follows: membrane anti-ILK antibody (diluted 1:1000), anti-E-cadherin antibody (diluted 1:100), anti-NF-KBp65 antibody (diluted 1:1000), anti-inhibitor of kappa B (IkB)a antibody (diluted 1:1000), anti-phosphorylated IkBa antibody (diluted 1:1000), anti-vimentin antibody (diluted 1:1000), anti-E-cadherin antibody (diluted 1:500), anti-snail antibody (diluted 1:500), anti-slug antibody (diluted 1:1000), and anti-β-actin antibody (diluted 1:5000). Then, membranes were treated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Specific protein bands were detected using an enhanced chemiluminescence assay kit (Santa Cruz Biotechnology).

Immunofluorescence

Cells were cultured on round coverslips and fixed with 4% paraformaldehyde for 30 min. Then, 0.1% Triton X-100 was used for permeabilization. Cells were incubated in fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit antibodies for 90 min, washed with secondary antibodies, and fixed by 4′,6-diamidino-2-phenylindole (DAPI). A laser scanning confocal microscope (FV1000S-SIM/IX81; Olympus, Tokyo, Japan) was used to observe staining.

Statistical analysis

All data are presented as mean ± SD. Using SPSS 13.0 software (SPSS, Chicago, IL), data were compared by one-way analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant.

RESULTS

Overexpression of ILK in SW480 cells increased ILK protein expressions

To verify the low expression levels of ILK in stably transfected SW480 cells, there were three transfection treatment groups: SW480, pcDNA3.1, and pcDNA3.1-ILK. After 24 h of transfection, ILK expression level was detected by western blot. Results revealed that ILK expression was significantly increased in the pcDNA3.1-ILK group compared with the vector group (Figure 1A). In addition, grayscale analysis revealed that ILK expression increased in the pcDNA3.1-ILK group, compared with the vector group (Figure 1B), and this difference was statistically significant ($P < 0.05$).

Overexpression of ILK enhances proliferation of SW480

MTT assay was used to detect cell proliferation and validate whether the increase in ILK expression affected the proliferation of SW480 cells. The results (Figure 2) revealed that there was no statistical difference between the vector and control groups. However, the proliferation rate was much higher at 48 and 72 h in the vector-ILK group than in the vector group; and the difference was statistically significant ($P < 0.05$).

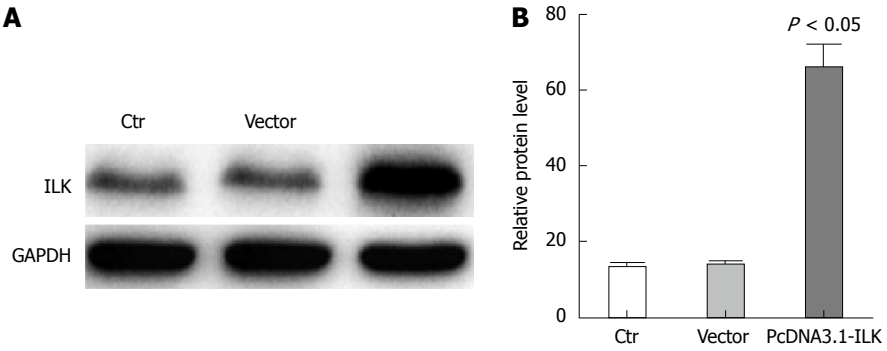


Figure 1 Overexpression of integrin-linked kinase in SW480 cells increased integrin-linked kinase protein expression. A: Integrin-linked kinase (ILK) expression in the control, vector, and PCDNA3.1-ILK groups were detected by western blot, with GAPDH as an internal control. This experiment was repeated three times; B: Statistical analysis revealed that ILK expressions significantly increased in the PcDNA3.1-ILK group compared with the vector group. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

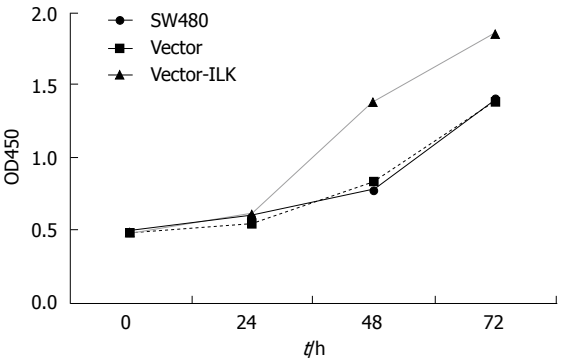


Figure 2 Proliferation rate of SW480 cells due to integrin-linked kinase overexpression.

Wound healing assay

Wound healing assay is an easily performed experiment that can detect migration ability, invasiveness, and metastasis of cells (Figures 3 and 4). Six and 12 h of culture after the scrape was made, the cells migrated much faster in the vector-ILK group than in the control group ($P < 0.05$).

Matrigel invasion assay

In the transwell method, cells can be induced to migrate from low nutrient culture medium to the high nutrient culture medium, allowing for the detection of the invasion ability of cells (Figures 5 and 6). The number of cells that migrated to the other side of the chamber was slightly higher in the vector group than in the control group, but the difference was not statistically significant ($P < 0.05$). More cells migrated to the other side of the chamber in the vector-ILK group ($P < 0.05$).

ILK can promote EMT occurrence

We over-expressed ILK in SW480 cells to investigate whether EMT occurrence and ILK expression in colorectal cancer are correlated. Using immunofluorescence staining, we found that E-cadherin fluorescence intensity was significantly reduced in the overexpressed ILK group (Figure 7B) compared with the no-load group (Figure 7A, $P < 0.05$). As explained, mutual adhesion between cells decreased, and EMT may have occurred. Therefore, EMT occurrence in colorectal cancer may be mediated by ILK. The transcription factors vimentin, snail, and slug have regulatory roles in cell EMT occurrence. To further validate our results, we overexpressed ILK in SW480 cells and used western blot for protein detection. We found that the expression of vimentin, snail, and slug was increased, while expression of E-cadherin was decreased (Figure 7C). In grayscale analysis, vimentin, snail, slug, and E-cadherin expression differences were statistically significant ($P < 0.05$, Figure 7D). Experimental results revealed that EMT occurrence in SW480 cells was promoted by ILK.

NF-κB signaling pathway mediated the ILK-induced EMT occurrence

In order to investigate whether ILK overexpression-induced EMT occurrence was directly or indirectly regulated *via* the NF-κB signaling pathway, we overexpressed ILK in SW480 cells and found using western blot that NF-κBp65 and cytoplasmic phosphorylated p-IκBa expression levels were significantly higher and that cytoplasmic IγBa expression was reduced in the overexpressed ILK group compared with the control group ($P < 0.05$, Figure 8A and B). In addition, we carried out siRNAp65 interference treatments on SW480 cells and simultaneously overexpressed ILK. By western blot, we found that E-cadherin expression in the vector-ILK cell line increased in the siRNAp65 treated group compared with that in the ILK overexpression group and the empty vector transfection group; and the difference was statistically significant ($P < 0.05$, Figure 8C and D). Taken together, these results demonstrate that the NF-κB signaling pathway mediated ILK-induced EMT occurrence.

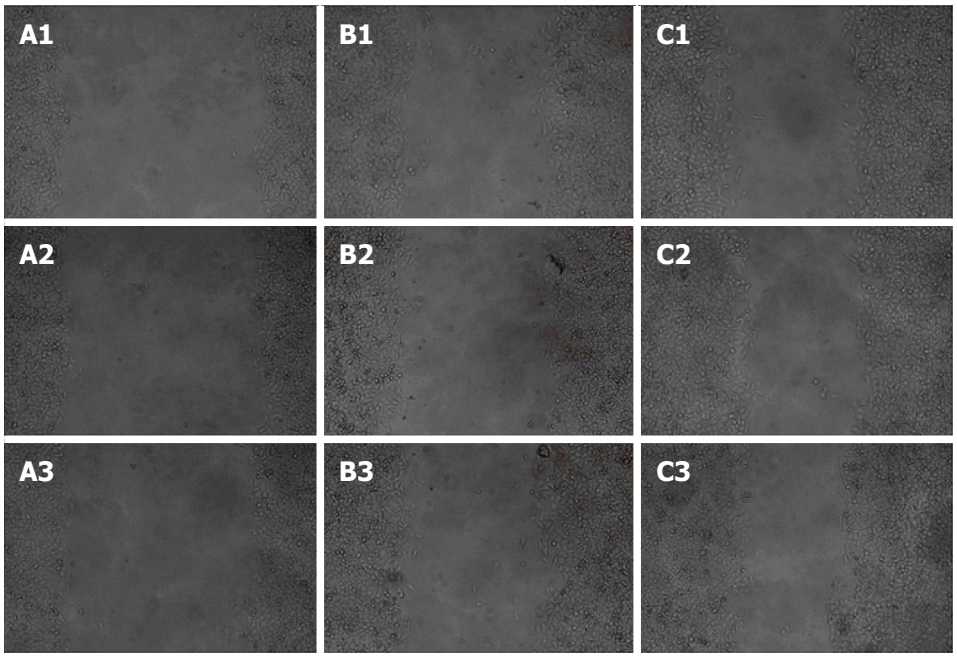


Figure 3 Wound healing assay. A1, A2, and A3 show the migration condition of cells in the control group after the scrape was made at 0, 6, and 12 h, in which only a small amount of cells migrated after 12 h; B1, B2, and B3 show the migration condition of the vector group, in which only a small amount of cells migrated after 12 h. C1, C2, and C3 show the migration condition of the vector-integrin-linked kinase group, in which a number of cells migrated into the center.

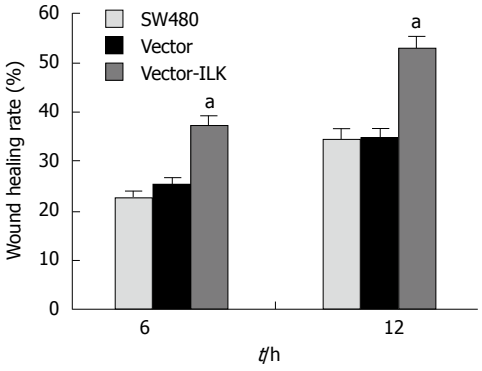


Figure 4 Wound healing rate in the three groups. $^{a}P < 0.05$ *vs* the vector group.

DISCUSSION

ILK is a vinculin serine/threonine kinase that is highly expressed in malignant tumors[10]. Some researchers have found that the role of ILK in different tumor types is not the same. In progressive pediatric tumors, breast cancer, and rhabdomyosarcoma tumors, ILK has been linked with tumor suppression[3,11,12], whereas in colon cancer, pancreatic cancer, melanoma, prostate cancer, and glioblastoma, ILK plays a role in the promotion of tumor metastasis and erosion[13-15]. Currently, many experts have reported that ILK has carcinogenic effects in colorectal cancer. Furthermore, pathological results have shown that high ILK expression levels are related to colorectal cancer staging, lymph node metastasis, and survival of patients[16,17]. For example, Li *et al*[6] reported that patients with colorectal cancer who have high ILK expression levels have a shorter survival time compared to patients with colorectal cancer who have low ILK expression levels. However, how ILK influences the migration of colorectal cancer cells and its mechanism remains unclear[6,18,19]. In this study, ILK was overexpressed in the cell line SW480 to detect the proliferation, migration, and invasion ability of cells. Then, immunofluorescence was used to detect E-cadherin levels (a biomarker of EMT) and to explore the possible mechanisms underlying the effects of ILK.

Tumor metastasis and recurrence caused by tumor cell invasion or metastasis are the leading causes of death among cancer patients[20,21]. A metastatic tumor manifests when cancer cells leave the tumor lesion, invade the adjacent tissue, grow, and proliferate in that area[22,23]. A portion of the tumor cells invades the lymphatic system, blood vessels, and body cavity and enters tissues far from the tumor lesion; resulting in the formation of a secondary tumor. In this study, we found that enhancing ILK expression levels in SW480 enhanced the proliferation ability of cells. Wound healing and matrigel invasion assays revealed that enhanced ILK expression levels improved metastasis and invasion of colorectal cancer cells. These results were consistent with some clinical reports in which patients with colorectal cancer who had high ILK expression levels were with poor prognosis.

Many theories can be used to explain the invasion and metastasis of tumors. Among these theories, EMT is a very classical theory[24,25]. EMT is a molecular program in which an epithelial cell loses its intercellular

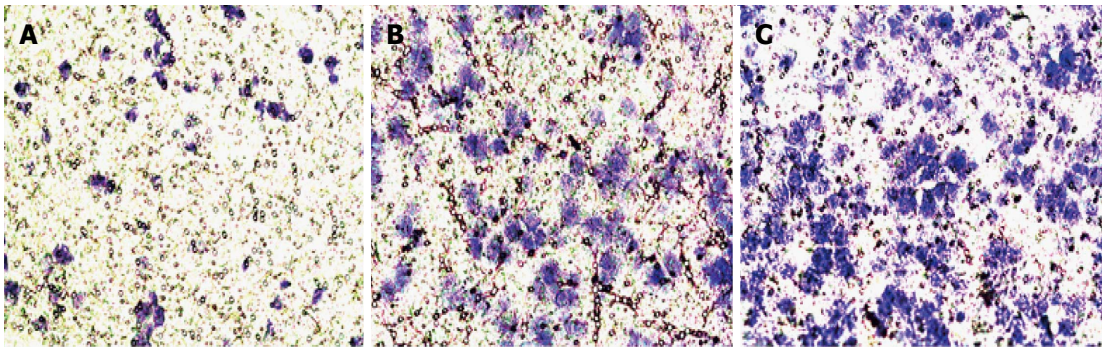


Figure 5 Matrigel invasion assay. A: The control group, only a small amount of cells migrated to the other side of the chamber; B: The vector group, only a small amount of cells migrated to the other side of the chamber; C: The vector-ILK group, a number of cells migrated to the other side of the chamber.

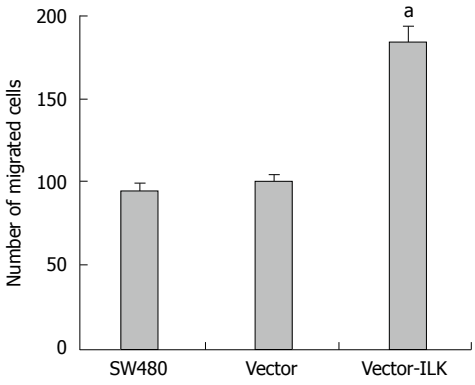


Figure 6 Number of cells that migrated to the other side of the chamber. $^{a}P < 0.05$ *vs* the vector group.

adhesion and acquires a migratory mesenchymal phenotype. E-cadherin expression is affected by this change, and the shape of cells are also converted from an epithelial state to a mesenchyma state; changing the motility of cells[26,27]. EMT plays an important role in the early stage of embryonic development, the invasion and metastasis of advanced tumors, and fibrosis after chronic inflammation[28]. Studies have found that cancer cells overexpress EMT-related genes and that these cells also have an initial cancer metastasis function. After EMT is induced in cells, epithelial cells lose their polarity. At the same time, cell adhesion ability also declines, cells relatively disperse, and migration ability is enhanced[29]. These changes become the main form of local tumor cell invasion and distant metastasis. In colorectal cancer experiments *in vitro*, ILK was overexpressed in SW480 cells. By western blot, we found that E-cadherin expression was significantly reduced, while vimentin expression was significantly increased; and by immunofluorescence staining, we found that E-cadherin in the overexpressed ILK group was significantly reduced ($P < 0.05$). It was further demonstrated in colorectal cancer that the increase in ILK expression levels and decrease in E-cadherin expression were linked. Our results showed that ILK can decrease the expression of E-cadherin; thereby promoting EMT, which leads to tumor metastasis and invasion. However, the expression of snail, slug, and other E-cadherin inhibitors was increased. Our results also confirmed that ILK can promote the EMT process in colorectal cancer *via* upregulation of the expression of snail and slug.

This study found that activation of the NF-κB signaling pathway may directly or indirectly regulate target proteins. Furthermore, activation of NF-κB is related to an aggressive phenotype, and its target proteins include snail and slug. Moreover, the NF-κB pathway plays a crucial role in EMT. Some experts have reported that ILK regulated melanoma angiogenesis *via* the NF-κB/interleukin (IL)-6 pathway[21]. Our results revealed that ILK overexpression activated the NF-κB signaling pathway, increased NF-κB/p65 levels, increased cytoplasmic levels of phosphorylated p-IκBa, and reduced cytosolic IγBa ($P < 0.05$). In colorectal cancer, we confirmed that the NF-κB signaling pathway participated in the overexpression of ILK *in vitro*, which induced EMT occurrence. In addition, we used siRNAp65 knockout experiments to successfully demonstrate that ILK inhibited the expression of E-cadherin partly by activation of the NF-κB signaling pathway. In conclusion, our results confirmed that the overexpression of ILK induces EMT occurrence, which promotes the invasion and metastasis of colorectal cancer *in vitro*. Moreover, this was partly mediated by the NF-κB signaling pathway; which also shows that the NF-κB pathway plays an important role in EMT in colorectal cancer.

In conclusion, this *in vitro* colorectal cancer study confirmed that overexpression of ILK can promote EMT occurrence in colorectal cancer cells, which was partly regulated *via* the NF-κB signaling pathway. As previously described, ILK plays an important role in promoting EMT occurrence in colorectal cancer cells and provides a new therapeutic target for the treatment of colorectal cancer. However, the mechanism of EMT regulatory factor expressions remains unclear and further research is needed.

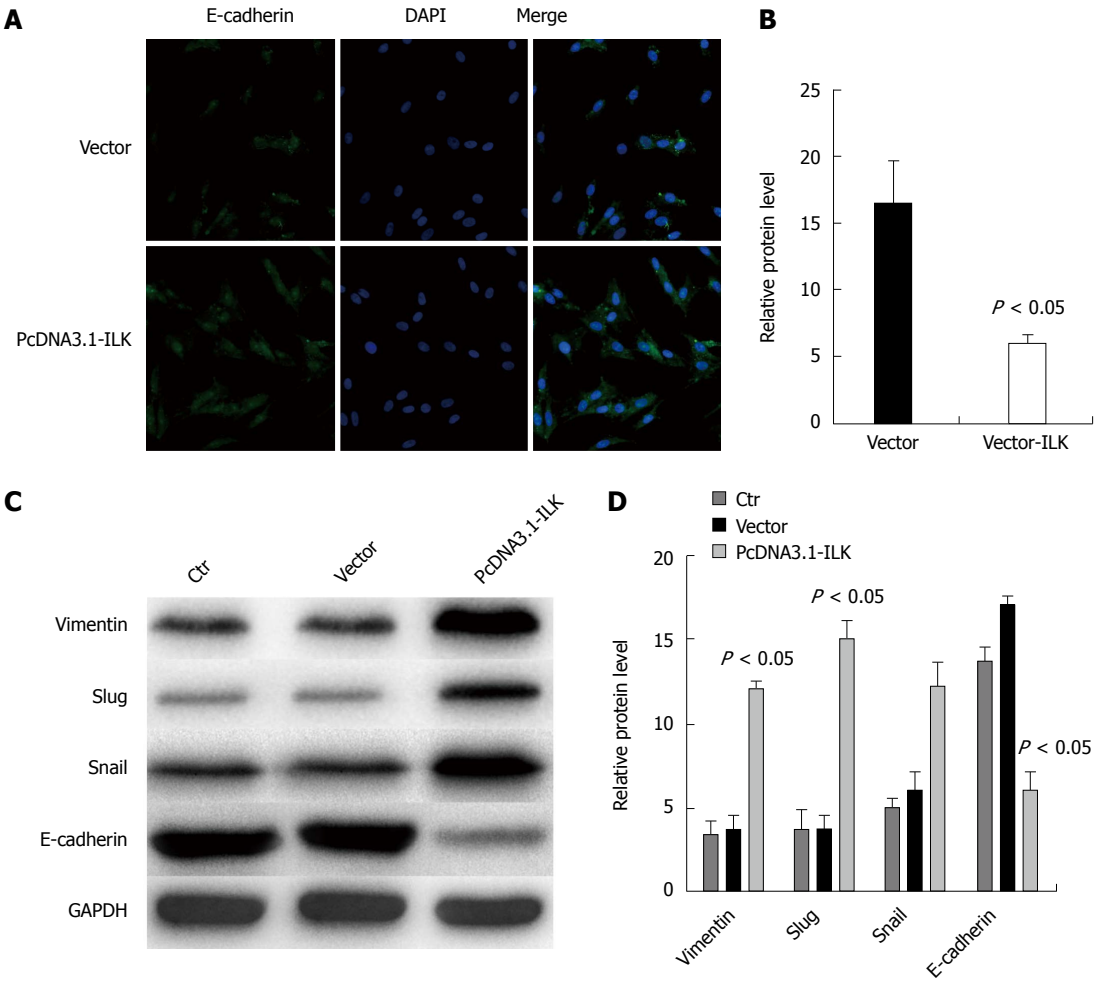
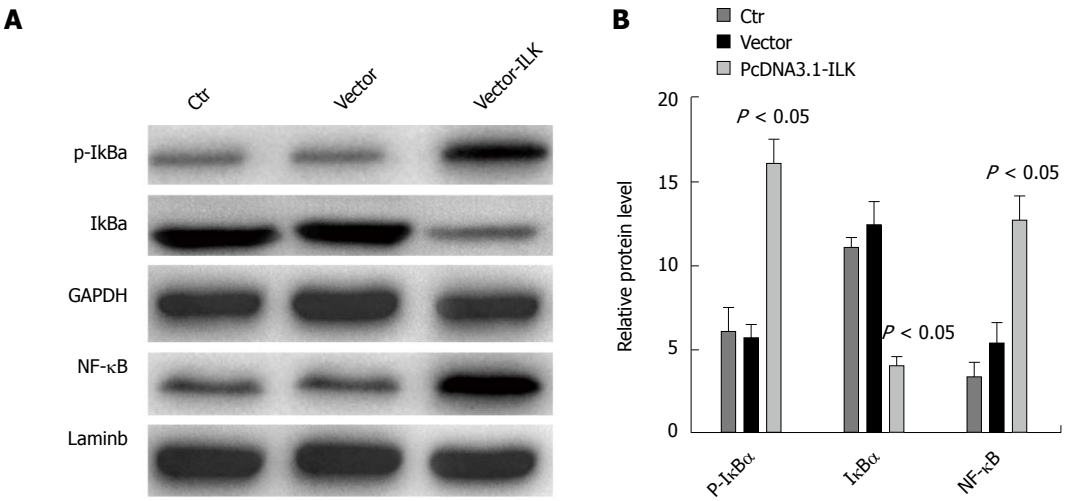


Figure 7 Integrin-linked kinase can promote epithelial-mesenchymal transition occurrence. A: Immunofluorescence revealed that E-cadherin fluorescence intensity significantly decreased in the pcDNA3.1-ILK group compared with the vector group; B: The difference between the vector group and pcDNA3.1-ILK group was statistically significant; C: Western blot revealed that vimentin, slug, and snail expression was increased, while E-cadherin expressions was reduced in the pcDNA3.1-ILK group; compared with the vector group. This experiment was repeated three times; D: The difference between the vector group and pcDNA3.1-ILK group was statistically significant. ILK: Integrin-linked kinase.



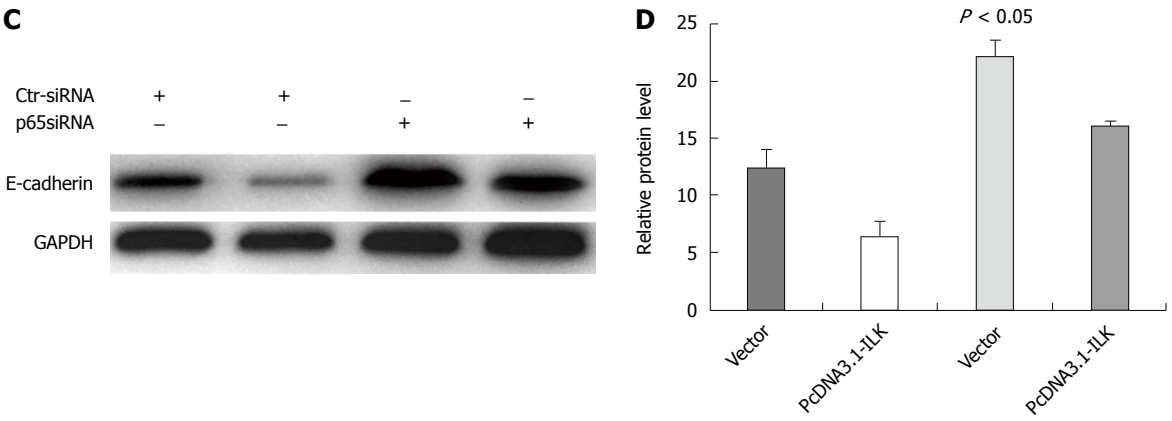


Figure 8 Nuclear factor-κB signaling pathway mediated the integrin-linked kinase-induced epithelial-mesenchymal transition occurrence. A: Western blot detection of p-IκBa, IγBa, and NF-κB expression; B: Statistical analysis, in which the difference between the vector group and vector-ILK group was statistically significant; C: After treatment with siRNA, E-cadherin expression was detected in cells; D: Statistical analysis: the difference between the vector-ILK cell line in the siRNAp65 treated group and control siRNA group was statistically significant. This experiment was repeated three times. ILK: Integrin-linked kinase; NF-κB: Nuclear factor-κB; siRNA, small interfering RNA.

COMMENTS

Background

Integrin-linked kinase (ILK) is a multifunctional receptor protein and a protein interaction integrin. Moreover, ILK can recruit other adapter molecules and regulate a variety of cellular processes *via* coupled signaling pathways, including cell growth, proliferation, apoptosis, survival, differentiation, migration, and invasion. Recent studies have shown that ILK is overexpressed and excessively activated in a number of human cancers.

Research frontiers

Some researchers have found that the role of ILK in different tumors is not the same. In progressive pediatric tumors, breast cancer, and rhabdomyosarcoma tumors, ILK can play a role in tumor suppression. However, in colon cancer, pancreatic cancer, melanoma, prostate cancer, and glioblastoma, ILK plays a role in promoting tumor metastasis and erosion. Currently, many experts have reported that ILK has carcinogenic effects in colorectal cancer. Furthermore, pathological results have shown that high ILK expression levels are related to colorectal cancer staging, lymph node metastasis, and survival of patients.

Innovations and breakthroughs

Overexpression of ILK promotes epithelial-mesenchymal transition (EMT) occurrence in colorectal cancer cells, which was partly regulated *via* the nuclear factor (NF)-κB signaling pathway.

Applications

ILK plays an important factor in the process of promoting EMT occurrence in colorectal cancer cells and provides a new therapeutic target for the treatment of colorectal cancer.

Peer-review

This *in vitro* colorectal cancer experiment confirms that overexpression of ILK can promote EMT occurrence in colorectal cancer cells, which was partly regulated *via* the NF-κB signaling pathway. ILK plays an important factor in the process of promoting EMT occurrence in colorectal cancer cells and may provide a new therapeutic target for the treatment of colorectal cancer.

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