PIK3R3 induces epithelial-to-mesenchymal transition and promotes metastasis in colorectal cancer

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Abstract

Class IA phosphatidylinositol 3-kinase (PI3K) plays an essential role in the invasion and metastasis of cancer. However, the mechanisms and specific functions of PI3K isoforms in tumor invasion and metastasis are not fully understood. We evaluated the role of PIK3R3, a PI3K regulatory subunit encoded by the PIK3R3 gene, in colorectal cancer (CRC) invasion and metastasis. Clinic specimens and cell lines data shows the expression level of PIK3R3 is associated with CRC metastasis. Over-expression of PIK3R3 increases tumor migration and invasion in vitro, and promotes metastasis of CRCs in vivo. Furthermore, we investigates that over-expression of PIK3R3 depend on SNAI2 inducing significant epithelial-to-mesenchymal transition (EMT). Down-regulation of PIK3R3 reverses this process, which possibly contributes to the enhanced invasive and metastasizing abilities of CRC cells. In this study, we found that PIK3R3 plays an important role in CRC metastasis, and might be a potential and specific target for therapies against metastatic CRC.
Introduction

Metastasis is one of the biggest challenges in cancer therapy and is responsible for 90% of cancer patient deaths (1, 2). Targeting tumor metastasis process will improve the tumor therapy and give patients a longer overall survival(1). Activation of Class IA phosphatidylinositol 3-kinase(PI3K)/AKT signaling is necessary for many events involved in the metastatic pathway including escape of cells from the tumor environment, into and out of the circulation, blockage of apoptosis, activation of proliferation, and activation of angiogenesis(3-5). Targeting PI3K/AKT pathway by PI3K inhibitors can improve the outcome of cancer and many PI3K pathway inhibitors have been developed and are being evaluated in preclinical studies and in early clinical trials(3).

Class IA PI3Ks consist of a regulatory subunit and a catalytic subunit(6). Three mammalian genes, PIK3R1, PIK3R2, and PIK3R3, encode p85α (p85α, p55α, and p50α isoforms), p85β, and p55γ (also named p55PIK) regulatory subunits(7). The catalytic isoforms, p110α, p110β, and p110δ, are the products of three genes, PIK3CA, PIK3CB, and PIK3CD(8). PI3K signaling is activated in human cancers via several different mechanisms, including direct mutational activation or amplification of genes encoding key components of the PI3K pathway such as PIK3CA and AKT1, or loss of PTEN(3, 9). Several of PI3K subunits role in tumor development and metastasis have been investigated very well, including p110α, p110β, p85α and p85β(10-12). But now, the role and function of PIK3R3 in tumor metastasis still remain little known.

The PIK3R3, encoded by the PIR3R3 gene, can bind to the p110 catalytic subunit through the iSH2 domain (13). Compared with other regulatory subunits, PIK3R3 contains a unique NH2 terminal. We previously study found that the NH2 terminal of PIK3R3 mediated PIK3R3 specific functions different from other regulatory subunits, via to binding to some cell growth key proteins including the retinoblastoma protein(RB1) and proliferating cell nuclear antigen (PCNA)(14, 15). We have reported the PIK3R3 regulatory subunit is important for cell proliferation and tumor growth, and is over-expressed in some cancers (15-17). Zhang et al. also reported that the mRNA and protein levels of PIK3R3 in ovarian cancer were elevated compared
with those in normal ovarian epithelia, and that PIK3R3 knockdown induces ovarian cell apoptosis in vitro(18). Moreover, in our previously studies we investigated that blocking PIK3R3 inhibits cell cycle progression, induces cell differentiation and inhibits tumor angiogenesis(15, 16, 19). Although PIK3R3 is especially important in the tumorigenesis, cell cycle regulation, cell differentiation and angiogenesis, moreover, in our previous study, blocking PIK3R3 by PIK3R3 specific inhibitor Ad-N24 can prevent CRC liver metastasis in animal model(17), which showed PIK3R3 may played an important role in cancer metastasis, but the function and the mechanisms of PIK3R3 in tumor metastasis remains unknown.

Previous observations have indicated that epithelial-to-mesenchymal transition (EMT) is associated with tumor metastasis(20). EMT is a cellular and molecular process, whereby epithelial cells acquire mesenchymal, fibroblast-like properties and demonstrate reduced intercellular adhesion, increased cell motility, and invasion(21, 22).

In this study, we analysis the association of PIK3R3 with colorectal cancer metastasis, we find PIK3R3 induces EMT in CRC cells and promotes CRC metastasis in vitro and in vivo. Furthermore, we elucidate the mechanism for PIK3R3 regulating CRC metastasis and inducing EMT process. Our data shows PIK3R3 may be also a potential and specific target for therapies against metastatic CRC, which may also gives a new sight for specific PI3K inhibitor development.

**Materials and methods**

**Cell Lines**

The CRC cell lines SW480, SW48, HT-29, HCT-116, LoVo and SW620 were purchased from the Type Culture Collection cell bank (Chinese Academy of Sciences, Beijing, P.R. China) in 2012. All cell lines were cultured at 37°C under a humidified atmosphere containing 5% CO2, and authenticated by short tandem repeat DNA profiling.
Reagents

Dulbecco’s modified Eagle’s medium (DMEM), Leibovitz Medium L-15 (L-15 medium), McCoy’s 5a medium, DMEM/F12, fetal bovine serum (FBS), and 0.25% trypsin were purchased from Hyclone. Antibodies against CDH1(E-cadherin), CDH2(N-cadherin), VIM(Vimentin), SNAI1(Snail) and SNAI2(Slug) were from Cell Signaling Technology, Inc. (USA). Antibodies against PIK3R3 and GAPDH were from Santa CruzBiotechnology (Santa Cruz, CA, USA). Lentivirus plasmids were purchased from Addgene; other chemical reagents were purchased from Good Time Biotech (Wuhan, P.R. China) or Promoter Company (Wuhan, P.R. China).

Plasmid generation and RNA interference (RNAi)

Full-length human PIK3R3 was isolated as described previously (23), and cDNA sub-cloned into pCDNA3 plasmids. Sequence-verified constructs were used in all experiments. The target sequence of the PIK3R3 short-interfering RNA (siRNA) was GGA CTT GCT TTA TGG GAA A. The si-h-PIK3R3-sense (5′-GGA CUU GCU UUA UGG GAA A dTdT-3′) and si-h-PIK3R3-antisense (3′-dTdT CCU GAA CGA AAU ACC CUU U-5′) sequences were purchased from RiboBio (Guangzhou, P.R. China), with siRNA used as the control. We cloned the same sequence as a short hairpin RNA (shRNA) into the pLKO1.0 plasmid for generation of lentiviruses.

Cell culture and lentivirus infections

The SW480 and SW620 cells were cultured in L-15 medium supplemented with 10% FBS. HT-29 and HCT-116 cells were cultured in McCoy’s 5a complete medium. LoVo cells were cultured in DMEM/F12 supplemented with 10% FBS. Lentiviruses were produced by co-transfecting HEK293T cells with lentiviral packaging plasmids (2.2 μg of pCMV-dR8.91, 0.25 ng of VSV-G/pMD2G, and 2.5 μg of Hairpin-pLKO.1). The supernatants were applied to LoVo cells at an equivalent titer. After 72 h, infected cells were selected with puromycin for 5 days, and at least 500 resistant clones from each group were pooled.
Transfection and monoclonal cell screening

Lipofectamine® 2000 reagent and Opti-MEM (Invitrogen) were used for cell transfection. After transfection, cells were incubated at 37°C/5% CO₂ for 24–48 h prior to testing for transgene expression. For stable transfection, cells were transfected using Lipofectamine® 2000; at 24 h post-transfection, 500 µg/mL G418 (Sigma-Aldrich, MO, USA) was applied. After 6 weeks, clones that overexpressed PIK3R3 were identified and collected. Cancer cells were transfected with siRNAs using Lipofectamine® RNAiMAX™ reagent (Invitrogen, Carlsbad, CA, USA) and incubated for 48 h at 37°C/5% CO₂. The inhibition efficiency of siRNAs on PIK3R3 was determined by western blotting.

Colony formation in soft agar

A soft agar assay was used to determine whether PIK3R3 increases anchorage-independent growth of tumor cells. SW480-control and SW480-PIK3R3 cells were incubated in DMEM supplemented with 1% (w/v) Bacto-agar, 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were then plated on a semi-solid medium (DMEM with 0.5% (w/v) Bacto-agar, 10% FBS, and penicillin-streptomycin); DMEM containing 10% FBS was added to wells after 2 days. After incubation for 14 days at 37°C/5% CO₂, colonies with a diameter greater than 50 µm were counted under a dissecting microscope at 400× magnification.

Cell motility and migration assays

The effects of PIK3R3 on the migratory and invasive capabilities of LoVo and SW480 cells were determined using a trans-well polycarbonate membrane (3422, Costa, USA) and the Boyden chamber assay. Briefly, 100,000 SW480 cells in 200 µL of serum-free DMEM were added to the upper portion of the inserts with 50,000 LoVo cells added to each well. The lower chambers were filled with DMEM supplemented with 10% FBS and incubated at 37°C/5% CO₂ for 48 h. The transwell membrane was fixed using 4% paraformaldehyde for 10 min at room temperature, washed thrice with phosphate-buffered saline (PBS), and then stained with 0.1% (w/v) crystal violet in 20%
(v/v) ethanol for 30 min. Cells that did not migrate were removed from the inserts using cotton swabs. Migrating cells were counted using a microscope, and quantified. For the invasion assay, the membrane was coated with matrigel (BD Catalog) before cells were seeded.

**RNA isolation and quantitative polymerase chain reaction (qPCR) assays**

RNA was extracted using TRIzol reagent (Invitrogen), and cDNA prepared from isolated RNA with a Superscript Reverse Transcriptase Kit (Transgene, Beijing, China) following the manufacturer’s instructions. We conducted qPCR assays on an ABI7300 instrument using the Super SYBR Green kit (Transgene, Beijing, China). Specific primer pairs were synthesized for the amplification and quantitation of the following gene expression products: PIK3R3 (5′-ATG TAC AAT ACG GTG TGG AGT ATG-3′ and 5′-GCT GGA GGA TCC ATT TCA AT-3′); CDH1 (5′-CGA GAG CTA CAC GTT CAC GG-3′ and 5′-GGG TGT CGA GGG AAA AAT AGG-3′); VIM (5′-GAC GCC ATC AAC ACC GAG TT-3′ and 5′-CTT TGT CGT TGG TTA GCT GGT-3′); SNAI1 (5′-CGA ACT GGA CAC ACA CAC ACA TAC AGT G-3′ and 5′-CTG AGG ATC TCT GGT TGT GGT-3′); SNAI2 (5′-GTA TGA TGA CTA CAG CGA-3′ and 5′-CTG AGG ATC TCT TGT GGT GGT-3′); ZEB1 (5′-CAT GAT GAA TGC GAG TCA-3′ and 5′-CTG AGG ATC TCT TGT GGT TGT-3′); and GAPDH (5′-CAT GAT GAA TGC GAG TCA-3′ and 5′-CTG AGG ATC TCT TGT GGT TGT-3′). LightCycler Data Analysis Version 3.1.102 (Roche, Mannheim, Germany) was used to analyze the obtained qPCR data. Relative quantification was done by normalizing the expression levels of the various genes to GAPDH, with all measurements carried out in duplicate.

**Western blotting**

Cells were lysed in NP-40 lysis buffer containing 10 nmol/L of phenylmethylsulfonyl fluoride and protease inhibitor. The lysate contents were measured using a bicinchoninic acid assay kit (Thermo, USA). We added an appropriate volume of 5× loading buffer to each sample such that its final concentration was 1×. Samples were boiled for 5 min at 95°C then subjected to sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE). Following SDS-PAGE, proteins were transferred to polyvinylidenedifluoride membranes for immunoblotting. Blots were probed with primary antibodies against AKT, p-AKT (s473), PIK3R3, GAPDH, CDH1, CDH2, VIM, SNAI1, and SNAI2. The appropriate secondary bodies were then used to determine the presence of various proteins in samples.

_Proliferation assays_

Proliferation was determined by cell counting and measurement of 5-bromo-2-deoxyuridine (BrdU) incorporation. Cell counting was conducted using a CASY TT-cell counter FACSCalibur from BD Biosciences according to the manufacturer’s instructions. For BrdU measurements, cells were seeded and treated in six-well plates, and then incubated with BrdU (final concentration of 10 µM) added to the culture medium for 30 min. The distribution of staining was calculated with ModFit LT software (Becton Dickinson, CA, USA).

_Immunohistochemistry (IHC)_

Sections of paraffin-embedded tissues were dewaxed and heated in a pressure cooker. Endogenous peroxidase activity was blocked using H₂O₂. Sections were incubated with normal goat serum buffer for 30 min and then with a polyclonal goat anti-PIK3R3 IgG (diluted 1:100) for 1 h at room temperature. Sections were then incubated with rabbit anti-goat horse radish peroxidase conjugate secondary antibody before a DAB kit (BosterBio, Wuhan) was used to develop the color reaction. Sections were counterstained with hematoxylin and eosin (HE), and dehydrated through xylene and alcohol before mounting. Images were acquired with a Leica2000 fluorescence microscope.

_Metastatic models_

We determined the effects of PIK3R3 on metastasis using a liver metastasis assay. Our study was performed in accordance with institutional guidelines, and was approved by the Animal Experimentation Committee of the Central Institute for
Experimental Animals, Tongji Hospital. We bred female BALB/c NOD mice and used them once they reached 4–5 weeks old. Colon cancer cells were harvested with 0.25% trypsin-EDTA solution. The collected cells were washed and suspended in serum-free medium at a concentration of $2 \times 10^5$ cells/mL. Experimental liver metastases were generated by an intrasplenic injection of $1 \times 10^5$ cancer cells (50 μL of cell suspensions) and splenectomy. Mice were sacrificed 6 weeks later and liver metastases enumerated immediately without fixation. The numbers of metastatic livers clones in each liver, and small foci were compared in the two groups.

Statistical analysis

All data were expressed as the mean ± the standard error of the mean. Between-group and among-group comparisons were conducted using Student’s $t$-tests and analysis of variance, respectively. Differences were considered statistically significant when the $p$-value was less than 0.05.

Results

**PIK3R3 enhances cell proliferation and anchorage-independent growth of CRC cells**

In our previous study, blocking PIK3R3 by PIK3R3 specific inhibitor Ad-N24 can prevent CRC liver metastasis in animal model (17). To confirm the relationship of expression levels of PIK3R3 with cancer metastasis, we detected the expression level of PIK3R3 in five different metastatic ability CRC cell lines (HCT-116, HT-29, SW480, SW48, SW620, and LoVo), in this five cell lines, SW620 and LoVo were derived from metastases. Data showed, in SW620 and LoVo cells, both mRNA and protein levels of PIK3R3 were higher than the other three derived from primary tumors cell lines (Figure 1A). Analysis by IHC in 2 CRC liver metastasis samples showed that the expression level of PIK3R3 was high in liver metastases (Figure 1B). Then, we generated PIK3R3 over-expression and knockdown cell lines in HT-29 and LoVo cells. Stable PIK3R3 were generated and confirmed by western blotting (Figure 1C, 1D). Results from the BrdU incorporation assays are presented in Figure 1E. The
DNA incorporation rate in cells over-expressing PIK3R3 was 75 ± 5% compared with 48 ± 4.5% \((P=0.003)\) in control cells.

Then we detected the anchorage-independent growth of CRC cells after changing the expression level of PIK3R3, as shown in Figure.1, the HT-29 cells over-expressing PIK3R3 \((97.5 ± 5.7 \text{ colonies/well})\) formed more colonies than control cells \((52.5 ± 15.7; P= 0.001; \text{Figure.1F})\). While, PIK3R3 knockdown suppressed colony formation in LoVo cells \((48.5 ± 9.3 \text{ vs. } 73.5 ± 8.7 \text{ colonies/well, PIK3R3 knockdown vs. control cells; } P= 0.002; \text{Figure.1G})\).

**PIK3R3 enhances CRC cell mobility, migration and invasion**

The ability of a cancer cell to undergo mobility and migration allow it to change position within the tissues, this process allow cancer cells leave from primary tumor(21). To investigate the effects of PIK3R3 on cell mobility and migration, we seeded HT-29 or SW48 cells in modified Boyden chambers and assessed cell migration 48 h later. Cells high PIK3R3 levels showed an increase in migration both in HT-29 and SW48 cell lines (Figure.2A, 2B). The effects of PIK3R3 on cell migration were also examined via scratch-wound assays in HT-29 and SW48 cells. After 24 and 48h, cells over-expressing PIK3R3 migration more distance than vector control cells (Figure.2C, 2D).

Cell invasion is an intrinsic cellular pathway whereby cells respond to extracellular stimuli to migrate through and modulate the structure of their extracellular matrix (ECM)(21). In cancer, cell invasion allow neoplastic cells to enter lymphatic and blood vessels for dissemination into the circulation, and then undergo metastatic growth in distant organs (24, 25). To investigate the effect of PIK3R3 on cell invasion, we employed the trans-well assay for detection. The membrane was coated with matrix-gel (BD Catalog) before cells were seeded. Data showed the HT-29 and SW48 cells overexpressing PIK3R3 migrated through membranes exhibiting higher rates compared with the control cells (Figure.2E, 2F).

**Knockdown PIK3R3 inhibits CRC cell migration and invasion**

To confirm the effects of PIK3R3 on CRC cell migration and invasion, we silenced PIK3R3 by transfecting PIK3R3 siRNA in SW48 and LoVo cells, PIK3R3 silencing
significantly decreased LoVo and SW48 cell migration (Figure.3A, 3B). In invasion assay, knockdown of PIK3R3 abrogated the invasion compared with control group both in LoVo and SW48 cells (Figure.3C, 3D).

**PIK3R3 promotes CRC growth and metastasis in vivo**

To confirm the effects of PIK3R3 on tumor metastasis in vivo, we injected HT-29 cells over-expressing PIK3R3 into mice; control mice were injected with an empty vector. In the control group, no visible metastases formed in any of the mice; however, we did observe liver metastases in two mice from the experimental group (Figure.4A, Table 1). Metastatic nests were found in all mice from the PIK3R3 over-expression group, and in three mice from the control group. Using HE staining, we found that the metastatic foci in the PIK3R3 over-expression group were larger than those in the controls (Figure.4B). Our IHC results showed that PIK3R3 was expressed in metastatic nests and tiny foci of the experimental group; however, this staining was absent when examining the control group (Figure.4C).

**PIK3R3 mediates EMT in CRC**

The EMT is considered to be a significant step in the invasive cascade(20). We showed that the HT-29 cell line stably over-expressing PIK3R3 had a mesenchymal phenotype compared with the control cell line(Figure.5A). Cells over-expressing PIK3R3 became fibroblast-like in appearance, exhibited decreased levels of the epithelial marker CDH1, but an increase in the expression levels of the mesenchymal markers CDH2 and VIM(Figure.5B).SNAI1 and SNAI2 were the key transcription factor in EMT progression, our data showed the expression of SNAI2 was enhanced with PIK3R3 over-expression, while there was no significant change in SNAI1 expression(Figure.5B).To verify these findings at the protein level, molecular analyses involving qPCR assays were conducted on HT-29 cells(Figure.5C).

To further confirm the relationship between PIK3R3 and EMT, several markers were analyzed by western blotting in the stable LoVo PIK3R3 knockdown cell line. Silencing of PIK3R3 induced an increase of CDH1, and a decrease of VIM, CDH2, and SNAI2 (Figure.5D).The qPCR results revealed that CDH1 mRNA levels were elevated, while VIM and SNAI2 levels were reduced; SNAI1 and ZEB1 levels
remained unaltered (Figure.5E).

**PIK3R3 depend on SNAI2 regulating CDH1 expression and promoting EMT**

Signaling pathways involved in EMT are complex, but the hallmark of EMT in cancer is the down-regulation of CDH1, which is also thought to be a repressor of invasion and metastasis (22, 26). Several transcription factors have been implicated in the transcriptional repression of CDH1, including zinc finger proteins, SNAI1, SNAI2, ZEB1/ZEB1, and so on (26-28). We constructed the CDH1 promoter vector pGL-E-cad promoter, and transfected the vector into HT-29 PIK3R3 over-expression stable cells and LoVo PIK3R3 siRNA stable cells, data showed when PIK3R3 up-regulated, the CDH1 was transcriptional activated, when down-regulated PIK3R3, CDH1 was transcriptional repressed. The same results were got in SW48 cells (Figure.6A). Than we constructed a mutated CDH1 promoter vector pGL-E-cad promoter\textsuperscript{mu}, which contained the SNAI2 binding domain mutation, then we transfected the vector into HT-29 PIK3R3 over-expression stable cells or LoVo PIK3R3 siRNA stable cells, data showed there had no changes of relative luciferase activation upon PIK3R3 expression level changing (Figure.6B). To further confirm PIK3R3 regulating CDH1 expression, we knockdown SNAI2 in HT-29 PIK3R3 over-expressing cells, data showed CDH1 transcriptional activity was reversed (Figure.6C). We further detected the protein level changes after down-regulation of SNAI2 in HT-29 PIK3R3 over-expressing cells, which showed down-regulation of SNAI2 reversed PIK3R3 induced EMT (Figure.6D).

**Discussion**

It was originally believed that PIK3R3 was a regulatory subunit of PI3K (13, 29). Some studies have showed that PIK3R3 is over-expressed in ovarian cancers and other cells (18, 30). Statistical evidence suggested that PIK3R3 is related to tumor malignance (30). We previously study have reported the PIK3R3 regulatory subunit is important for cell proliferation and tumor growth via to the NH\textsubscript{2} terminal of PIK3R3 binding to some cell growth key proteins including RB1 and PCNA (14, 15, 17). Moreover, in our previous study, blocking PIK3R3 by PIK3R3 specific inhibitor...
Ad-N24 can prevent CRC liver metastasis in animal model (17), but the role of PIK3R3 in cancer metastasis still remained unclearly.

In this study, we found that the PIK3R3 expression level in CRC tissues was higher than that in normal colon mucosa, especially during metastasis. Two cell lines derived from metastases showed higher PIK3R3 levels, suggesting that PIK3R3 is related to tumorigenesis and metastasis. Further supporting this notion, we generated stable cell lines where PIK3R3 was overexpressed or knocked down. The anchorage-independent growth of CRC cells was significantly promoted by PIK3R3 over-expression. We observed migration and invasion between cells with different PIK3R3 levels; PIK3R3 induced more cells to infiltrate through the trans-well membrane. These results indicated that PIK3R3 promotes the migration and invasion of CRC cells. Knockdown of PIK3R3 inhibited these processes, demonstrating that these effects are mediated by PIK3R3. The effects of PIK3R3 on CRC metastasis were studied in a hepatic metastasis mouse model. We saw that PIK3R3 induced the CRCs to form more and larger colonies in the liver indicating that PIK3R3 promotes CRC metastasis by positively regulating cell migration and invasion.

EMT had been implicated the key step in the progression of tumors toward metastasis and invasion (21, 26, 31). Cancer cells undergoing EMT lost proper target recognition and avoided apoptosis(26). Epithelial cells undergoing EMT rearranged their cytoskeletons and developed features of mesenchymal cells, losing cell-cell adhesion structures and polarity(32). In EMT changes, mesenchymal cell specific marker proteins including CDH2 and VIM were up-regulated and tight- or adherens-junction proteins such as CDH1 were down-regulated (20, 26). In this study, we found that up-regulating PIK3R3 induced CRC cells EMT, when we knockdown PIK3R3 in cells, this manner was reversed. This showed that PIK3R3 promoted CRC metastasis may mediate by inducing CRC cells EMT.

CDH1 was a cell–cell adhesion molecule, which formed epithelial adherent junctions and sequestrate CTNNB1(33, 34). CDH1 lost expression was often linked to a higher incidence of metastasis and tumor recurrence (34, 35). The loss of CDH1 and the resulting suppression of cell–cell adhesion had been regarded as a crucial step in the
epithelial–mesenchymal transition (EMT) process(34). Generally, loss of CDH1 expression in human tumors was most commonly caused by methylation of its promoter, or up-regulation of the transcriptional repressors SNAI1, SNAI2 and ZEB1, which targeted the CDH1 promoter(33). In our study, we noticed that PIK3R3 over-expression can decrease CDH1 transcriptional activation, moreover, we found that PIK3R3 up-regulated the transcription of CDH1 mediated by SNAI2, and that SNAI2 was down-regulated in cells where PIK3R3 was knocked down. This finding demonstrated that SNAI2 is required for PIK3R3-induced EMT.

Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) had been reported affecting microenvironment, inflammation, and EMT and was associated with longer survival among patients with mutated-PIK3CA colorectal cancer, but not among patients with wildtype PIK3CA cancer(36, 37). In this study, our results demonstrate that PIK3R3 is expressed in CRC cells, and that its activation leads to phenotypic changes associated with tumor progression and metastasis. Although we showed that treatment-targeting PIK3R3 inhibits tumor growth, the roles of PIK3R3 in CRC metastasis indicate novel functions for this molecule in tumorigenesis. The expression of PIK3R3 in CRC cells serves as a “predictor” for PIK3R3-targeted anti-neoplastic regimens. Comprehensive understanding of the expression and functions of PIK3R3 in CRC, and their associated biological markers, will facilitate the development of better-targeted therapies against CRC. But there still have minor defect in this study, we did not clear the mechanism of PIK3R3 regulating SNAI2 expression, which should be the main work in our future study.

Reference


Table 1  Numbers of liver metastases and micrometastasis after intrasplenic injection.

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Figure Legends

Figure 1. PIK3R3 enhances cell proliferation and anchorage-independent growth of CRC cells. (A) Various levels of PIK3R3 expression were detected in CRC cell lines by Western-blotting and real-time PCR analyses. (B) Evaluation of PIK3R3 expression in human colorectal cancer tissues. The expression of PIK3R3 was assessed using IHC. High levels of PIK3R3 expression were observed when they were compared with the adjacent normal colorectal epithelium. (C) Over-expression of PIK3R3 in stable cell lines was confirmed by western blot. (D) Knockdown of PIK3R3 in stable cell lines was confirmed by western blot. (E) BrdU assay showed that PIK3R3 promoted DNA synthesis. The scatter plot of BrdU assay by cytometry showed the increasing ratio of S-phage cells (left) in PIK3R3 over-expression cells and quantified data was shown on the right. (F) HT-29 with or without PIK3R3 over-expression cells were plated in soft agar, and colonies were counted 14 days later. HT-29 cells with PIK3R3 over-expression formed more colonies than controls. \( p<0.05 \) vs. control cells. (G) LoVo with or without PIK3R3 knockdown cells were plated in soft agar, and colonies were counted 14 days later. Knockdown of PIK3R3 decreased colony formation in LoVo cells. \( p<0.05 \) vs. control cells. The results above were reproducible in three independent experiments.

Figure 2. PIK3R3 enhances CRC cell mobility, migration and invasion. (A) In transwell migration assays, migrated HT-29 cells were stained with crystal violet. PIK3R3 over-expression significantly increased cell migration at 48 h. (B) In transwell migration assays, migrated SW48 cells were stained with crystal violet. PIK3R3 over-expression significantly increased cell migration at 48 h, quantified data was shown on the right. (C) PIK3R3 expression increased CRC cell migration ability in HT-29. Confluent HT-29 cells cultured in 96-well plates were carefully wounded using sterile pipette tips. The cells migration was photographed under a phase contrast.
microscope. The distance of cells migrating into the initial wound area was measured and expressed as mean±SEM, \( p<0.05 \) vs. the control group. (D) PIK3R3 expression increased CRC cell migration ability in SW48. The distance of cells migrating into the initial wound area was measured and expressed as mean±SEM, \( p<0.05 \) vs. the control group. (E) In transwell invasion assays, invaded HT-29 cells were stained with crystal violet. PIK3R3 over-expression significantly increased cell invasion at 48 h, quantified data was shown on the right. (F) SW48 transwell invasion assays was shown, and quantified data was shown on the right. The results above were reproducible in three independent experiments.

Figure 3. Knockdown PIK3R3 inhibits CRC cell migration and invasion. (A) Knockdown PIK3R3 in LoVo cells, transwell migration assays, migrated LoVo cells were stained with crystal violet. Quantified data was shown on the right. (B) Knockdown PIK3R3 in SW48 cells, transwell migration assays, migrated SW48 cells were stained with crystal violet. Quantified data was shown on the right. (C) Knockdown PIK3R3 in LoVo cells, transwell invasion assays, invaded LoVo cells were stained with crystal violet. Quantified data was shown on the right. (D) Knockdown PIK3R3 in SW48 cells, transwell invasion assays, invaded SW48 cells were stained with crystal violet. Quantified data was shown on the right. Cells was counted and expressed as mean±SEM, \( p<0.05 \) vs. the control group. All experiments were performed at least thrice independently.

Figure 4. PIK3R3 promotes CRC growth and metastasis in vivo. In the HT-29 model, PIK3R3 promoted the metastasis and macrometastasis of HT-29 cells. HT-29 cells were injected intrasplenically into female BABL/C NOD mice, and mice were killed 6 weeks later. Tumor islets were observed in livers by staining sections with H&E staining. (A) Typical views of liver presenting macroscopic metastases at week 6. Arrows denote metastatic niche. (B) H&E staining of metastatic liver tumor tissues of nude mouse models. (C) PIK3R3 staining of metastatic liver tumor tissues of nude mouse models. All experiments are detected and analyzed in triplicates.

Figure 5. PIK3R3 mediates EMT in CRC. (A) HT-29 cells with and without PIK3R3 overexpression were photographed under a phase contrast microscope. A
mesenchymal phenotype was observed in the PIK3R3-overexpressed HT-29 cells compared with the control cells. (B) PIK3R3 and several markers involved in the EMT process, such as, CDH1, CDH2, VIM, SNAI2 and SNAI1 were detected by western blot analysis in the HT-29 PIK3R3-overexpressed and control cells. (C) PIK3R3 and several markers involved in the EMT process, such as, CDH1, VIM, SNAI2, SNAI1 and ZEB1 were detected by quantitative real-time PCR in the HT-29 PIK3R3-overexpressed and control cells. All experiments are detected and analyzed in triplicates. (D) PIK3R3 and several markers involved in the EMT process, such as, CDH1, CDH2, VIM, SNAI2 and SNAI1 were detected by western blot analysis in the LoVo PIK3R3-downexpressed and control cells. (E) CDH1, VIM, SNAI2, SNAI1 and ZEB1 were detected by quantitative real-time PCR in the LoVo PIK3R3-downexpressed and control cells. All experiments are detected and analyzed in triplicates.

Figure 6. PIK3R3 depend on SNAI2 regulating CDH1 expression and promoting EMT. (A) HT-29 over-expressing PIK3R3 cells, SW48 over-expressing PIK3R3 cells, LoVo knockdown PIK3R3 cells and SW48 knockdown PIK3R3 cells were transfected the CDH1 promoter vector pGL-E-cad promoter, relative luciferase activation was detected. (B) HT-29 over-expressing PIK3R3 cells, SW48 over-expressing PIK3R3 cells, LoVo knockdown PIK3R3 cells and SW48 knockdown PIK3R3 cells were transfected the mutated CDH1 promoter vector pGL-E-cad promoter mu, relative luciferase activation was detected. (C) Inhibition of SNAI2 reverses the CDH1 transcriptional activation caused by PIK3R3. Stable overexpression PIK3R3 or control HT-29 cells were transfected si-SNAI2 or si-Con, then the relative luciferase activation of CDH1 promoted was detected. (D) Inhibition of SNAI2 reverses the EMT caused by PIK3R3. Stable overexpression PIK3R3 or control HT-29 cells were transfected si-SNAI2 or si-Con, the SNAI2, CDH1 and VIM were detected by western blot. All experiments are detected and analyzed in triplicates.
**Figure 1**

A. Graph showing PIK3R3 mRNA fold change compared with HCT-116.

B. Images of normal tissue, primary tumor, and liver metastasis stained with different colors.

C. Western blots showing PIK3R3 and GAPDH expression levels in HT-29 cells.

D. Western blots showing PIK3R3 and GAPDH expression levels in LoVo cells.

E. Flow cytometry scatter plots showing BrdU incorporation in HT-29 cells with different treatments.

F. Images showing the number of colonies per well for different treatments.

G. Images showing the number of colonies per well for different treatments.
Figure 2

A  Migration(HT-29)

B  Migration(SW48)

C  Invasion(HT-29)

D  HT29(Scratch assay)

E  SW48(Scratch assay)

F  Invasion(SW48)
Figure 3

A

Si-Con  
Si-PIK3R3  

Migration (LoVo)  

B

Si-Con  
Si-PIK3R3  

Migration (SW48)  

C

Si-Con  
Si-PIK3R3  

Invasion (LoVo)  

D

Si-Con  
Si-PIK3R3  

Invasion (SW48)
Figure 4

A

Vector

PIK3R3

Metastasis niche

B

Vector

PIK3R3

100X

400X

100X

400X

HE staining

C

Vector

PIK3R3

100X

400X

100X

400X
Figure 5

A. Vector and PIK3R3

B. Morphology (HT-29)

C. Relative mRNA level

D. Si-Con and Si-PIK3R3

E. LoVo
**Figure 6**

**A**

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Blot images for PIK3R3, SNAI2, CDH1, VIM, GAPDH for HT-29.
Molecular Cancer Therapeutics

PIK3R3 induces epithelial-to-mesenchymal transition and promotes metastasis in colorectal cancer

Guihua Wang, Xi Yang, Chuan Li, et al.

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