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Original Paper

PIK3R3 Promotes Metastasis of Pancreatic Cancer via ZEB1 Induced Epithelial-Mesenchymal Transition

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Key Words
Pik3r3 • Pancreatic cancer • Epithelial-mesenchymal transition • ZEB1 • Metastasis

Abstract
Background/Aims: PIK3R3 is a regulatory subunit of phosphatidylinositol 3-kinase (PI3K) which plays an essential role in the metastasis of several types of cancer. However, whether PIK3R3 can promote the metastasis of pancreatic cancer (PC) is still unclear. In this study, we characterized the role of PIK3R3 in metastasis of PC and underlying potential mechanisms. ***Method***s: RT-PCR, western blot, immunofluorescence (IF) and immunohistochemistry (IHC) were applied to investigate the expression of genes and proteins in different cell lines and tissues. To assess the function of PIK3R3 and related mechanisms, the cells with RNAi-mediated knockdown or overexpression were used to perform a series of *in vitro* and *in vivo* assays. ***Results:*** PIK3R3 was significantly overexpressed in pancreatic cancer tissues, especially in metastatic cancer tissues, as well as in pancreatic cancer cells. Functional assays suggested that overexpression or knockdown of PIK3R3 could respectively promote or suppress the migration and invasion of PC cells *in vitro* and *in vivo*. Further mechanism related studies demonstrated that ERK1/2-ZEB1 pathway-triggered epithelial-mesenchymal transition (EMT) might be responsible for the PIK3R3-induced PC cell migration and invasion. ***Conclusion:*** PIK3R3 could promote the metastasis of PC by facilitating ZEB1 induced EMT, and could act as a potential therapeutic target to limit PC metastasis.

Introduction

Pancreatic cancer (PC) is the fifth leading cause of cancer-related death worldwide, with a 5-year relative survival of only 8% and a median survival of 6 months [1]. The morbidity of this cancer is persistently increased over the past decades; however, the survival rate is not significantly improved [2]. The only available therapy for PC is radical resection which

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prolongs survival and offers a potential cure [3]. However, the surgical resection rate is less than 20% due to the frequent occurring of local advance and distant metastasis [4]. Therefore, investigating the potential mechanisms of PC invasion and metastasis may contribute to the exploration of novel therapeutic strategy [5, 6].

Epithelial-mesenchymal transition (EMT) and MMPs were widely reported to be responsible for the invasion and metastasis of PC cells [7, 8]. EMT is an essential phenotypic conversion from epithelial to mesenchymal phenotype during embryonic development, and is significantly correlated with cancer invasion and metastasis [9]. The conversion of EMT is promoted by transcription factors (such as SNAIL, SLUG, TWIST1, ZEB1, and ZEB2) which can be triggered by several endogenous and exogenous factors via specific signaling pathways [10-12].

PIK3R3 is a regulatory subunit of phosphatidylinositol 3-kinase (PI3K) [13]. Several studies have reported that PIK3R3 is abnormally overexpressed in some types of cancer, such as lung cancer, colorectal cancer, and gastric cancer [14-16]. Data obtained from clinical samples suggested that overexpression of this molecule is significantly associated with the progression and prognosis of certain cancers, and functional assays demonstrated that PIK3R3 could promote the proliferation, migration, and invasion of some cancers *in vitro* and vivo [17-19]. Some studies have mentioned the role of PIK3R3 in PC [20, 21]. However, the function and mechanism of PIK3R3 in PC is still unclear, especially the function of PIK3R3 in tumor metastasis. Therefore, in the present study we assessed PIK3R3 expression in PC tissues and cell lines, and further investigated the role and mechanisms of PIK3R3 in PC cell metastasis.

Materials and Methods

Patients

25 pairs of primary cancer and adjacent tissues were obtained from patients diagnosed with PC at Pancreas Center, The First Affiliated Hospital of Nanjing Medical University from 2013 to 2015, as well as 25 metastatic cancer tissues. 25 metastatic cancer tissues were obtained from metastatic sites (patients without radical operation), and primary cancer tissues were obtained from the primary sites (patients with radical operation). Primary cancer and adjacent tissues were obtained from radical resection specimens, and metastatic cancer tissues were got from biopsy specimens of distant metastases. All tissues applied in this study were fixed in formalin, embedded paraffin, and further used to IHC. Our study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University.

Immunohistochemistry (IHC) Staining and Assessment

IHC was performed according to a standard immunoperoxidase staining procedure [22] . Two experienced pathologists who were blinded to enrolled patients viewed the tissue sections independently. The methods for IHC assessment applied in our previous study were used here [23]. All antibodies used here were all purchased from Abcam (Cambridge, MA, USA).

Cells and Cell Culture

The normal human pancreatic ductal cell line hTERT-HPNE and all pancreatic cancer cell lines were available in our laboratory. hTERT-HPNE cell line was cultured based on the recommendation of ATCC (Rockville, MD, USA). All PC cell lines mentioned above were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL).

Stable transfection of PC cells

Recombinant lentivirus for PIK3R3 overexpression and knockdown were purchased from Genepharma, Shanghai, China. Human PIK3R3 coding sequencer was amplified and cloned into a pGLV3/H1/GFP+Puro vector to further generate PIK3R3 overexpression lentivirus. Biologically active short hairpin RNAs (ShRNA) were applied to generate PIK3R3 knockdown lentivirus. The ShRNA target sequence for human PIK3R3 used in this study were shown as follows: 5'-GGGAGGAGGTAAATGACAAAT-3', 5'-GGGAATTAAGAATGAGGATGC-3',

5'-GGTTTGTTGAGGATATCAATC-3'. Mia PaCa-2 and BxPC-3 cells were respectively transfected with PIK3R3 overexpression and knockdown lentivirus. Puromycin was used to select the stably transfected cells. The efficiency of PIK3R3 overexpression and knockdown were detected by qRT-PCR and Western blot.

Quantitative real time reverse transcription polymerase chain reaction

Trizol reagent was used to extract total RNA from different cells. PrimeScript RT Master Mix was applied to reverse-transcribe total RNA into cDNA. The Step One Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and FastStart Universal SYBR Green Master were used to perform the qRT-PCR amplification. All relevant experiments here were conducted based on the instructions provided by manufacturer. Primer Premier 5 and Oligo 6 were respectively applied to design and check the specific primers for human PIK3R3, ZEB1, and β-ACTIN. The sequences of primers are as followed: PIK3R3 (sense, 5'-CTTGCTCTGTGGTGGCCGAT-3' and antisense, 5'-GACGTTGAGGGAGTCGTTGT-3'), ZEB1 (sense, 5'-GATGATGAATGCGAGTCAGATGC-3' and antisense, 5'-ACAGCAGTGTCTTGTTGTTGT-3'), Beta-Actin (sense, 5'-AGCGAGCATCCCCCAAAGTT-3' and antisense, 5'-GGGCACGAAGGCTCATCATT-3'). The relative expression of certain mRNA was normalized by formula $2^{-\Delta\Delta Ct}$ (Ct means the cycle threshold).

Western blotting

Total protein was extracted by using a Total Protein Extraction Kit which containing PMSF, protease inhibitors, and phosphatase inhibitors. Extracted protein was mixed with 5× SDS, and further degenerated in boiling water for 5min. Western blot analysis was performed according to the standardized methods. Anti-PIK3R3 (ab97862), E-Cadherin (ab133597), Vimentin (ab137321), SNAIL (ab53519), SLUG (ab85936), ZEB1 (ab124512), ZEB2 (ab138222) antibodies were all purchased from Abcam (Cambridge, MA, USA). Anti-ERK1/2 (#4695) and p-ERK1/2 (#4370) antibodies were obtained from Cell Signaling Technology (Danfoss, MA, USA).

Immunofluorescent assay

Cells were seeded on the 12-well Cell Culture Plates and cultured for 24h. Cells were washed with PBS and fixed in stationary liquid for 15 minutes at room temperature. And then, cells were washed with PBS for three times after discarding stationary liquid. Blocking Buffer for immunocytofluorescence was added to the plate for 1h at room temperature. Primary antibodies were added to the plate and incubated at 4°C overnight, followed by incubation with Cy™ 3 labeled secondary antibodies (Jackson Laboratory) for 1h at room temperature. Nuclei was further stained with DAPI for 5min at room temperature. A fluorescence microscopy was applied to photograph Fluorescence images. Anti- E-Cadherin and Vimentin antibodies were all purchased from Abcam (Cambridge, MA, USA).

Migration and Invasion assays

In vitro cell invasion and migration ability were respectively evaluated by using Matrigel-coated and non-Matrigel-coated transwell filters (BD Biosciences) as described in previous study [24]. The cells for migration assays were harvested after 24h, and the cells for invasion were harvested 48h.

Animal studies

All female BALB/c nude mice aged 5 weeks used in this study were purchased from Model Animal Research Center of Nanjing University. To assess the prometastatic function of PIK3R3 *in vivo*, fresh cancer cells (1×10^6 per mice) were implanted into the pancreas under anesthesia which was induced by 1% pentobarbital sodium. After four weeks of tumor implantation, all nude mice were sacrificed. Primary and metastatic tumors were obtained from the mice to photograph images, and then, fixed in formalin for further paraffin embedding.

Statistical analysis

Independent experiments were performed three times to generate respective data in each figure. The Mann–Whitney U-test was applied to compare PIK3R3 expression in pancreatic cancer and paired adjacent tissues. The comparison between two groups was done by independent Student's t-test. SPSS 20.0 software were used to perform all the analyses. All data were expressed as mean ± SD. Differences were considered statistically significant at $P < 0.05$.

Results

Aberrantoverexpression of PIK3R3 in PC tissue and cell lines

IHC analyses suggested that the expression of PIK3R3 in primary and metastatic cancer tissues was significantly higher than that in adjacent tissues, while PIK3R3 expression in metastatic tissues was the highest (Fig. 1A). Such results suggested that PIK3R3 might be associated with PC metastasis.

Then, the expression levels of PIK3R3 in seven pancreatic cancer cell lines were evaluated using qRT-PCR and Western blot, including AsPC-1, Mia PaCa-2, SW1990, BxPc-3, Capan-2, CFPAC-1, and PANC-1 (Fig. 1B). Compared to the normal human pancreatic ductal cell hTERT-HPNE, almost all cancer cell lines expressed increased levels of PIK3R3. And then, Mia PaCa-2 (highest) and BxPc-3 (lowest) were respectively selected to establish PIK3R3 knockdown and overexpression cell lines. Stable PIK3R3 knockdown and overexpression cell lines were established and further confirmed via qRT-PCR and Western blot (Fig. 1C).

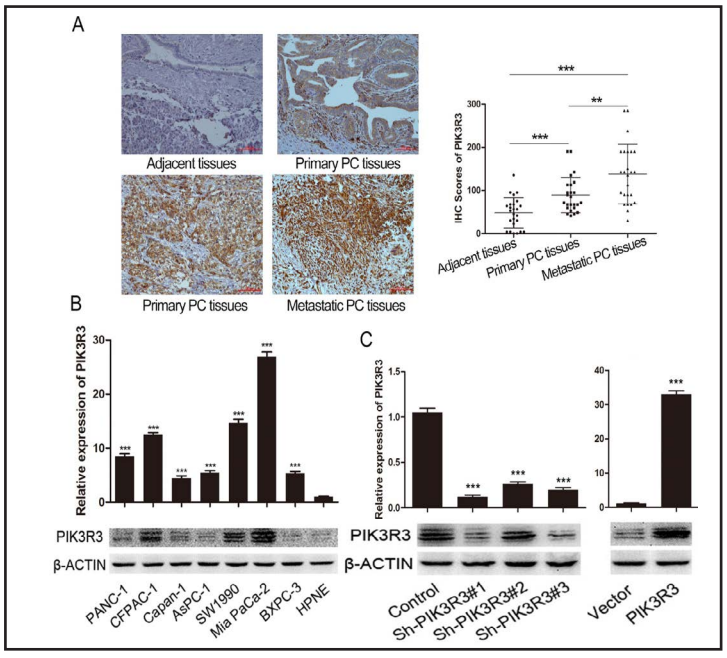

Fig. 1. The expression of PIK3R3 in PC. (A) Higher expression of PIK3R3 was observed in primary cancer tissues and metastatic tissues. (B) The expression of PIK3R3 was detected in PC cell lines. (C) The expression of PIK3R3 in stable knockdown and overexpression cell lines were confirmed by qRT-PCR (Upper) and Western blot (Lower). ** P<0.01 and *** P<0.001.

PIK3R3 promotes the migration and invasion of PC cells

Matrigel-coated and non-Matrigel-coated transwell filters were applied to evaluate the migration and invasion of these cells *in vitro*. As shown in (Fig. 2A), the numbers of

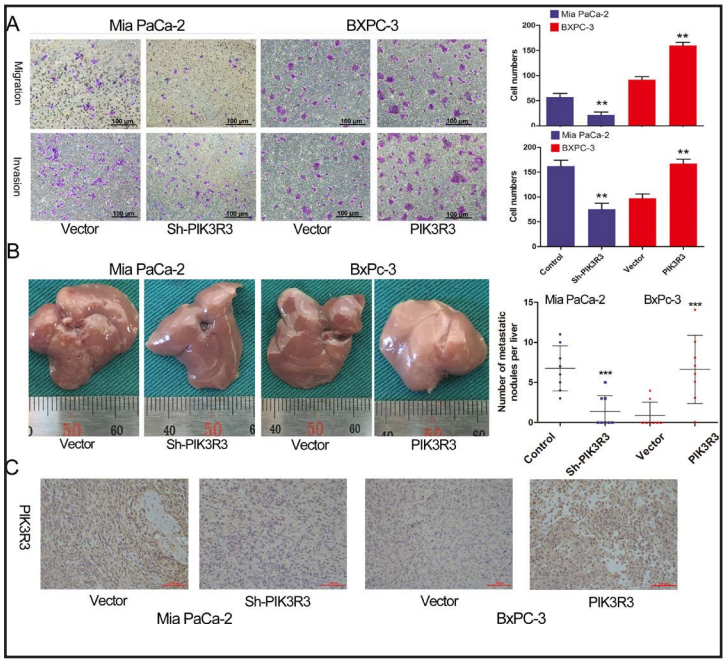

Fig. 2. PIK3R3 enhanced the migration and invasion of PC cells. (A) PIK3R3 knockdown and overexpression respectively inhibited and promoted the migration and invasion of PC cells in vitro. (B) The effects of PIK3R3 on PC cell migration and invasion was investigated in vivo. (C) The expression of PIK3R3 in PC cell xenografts was detected by IHC. ** P<0.01. and *** P<0.001.

migratory cells were significantly increased in PIK3R3 overexpression cells comparing to control cells, as well as invasive cells. However, the tendency of cell migration and invasion in PIK3R3 knockdown cells was contrary.

To further confirm the role of PIK3R3 in PC cell migration and invasion *in vivo*, we established an orthotopic pancreatic tumor model by implanting cancer cells in pancreas of the nude mice for 4 weeks. Metastatic nodules in liver were observed in 8 of 8 Mia PaCa-2-Control cell implanted mice, 3 of 8 Mia PaCa-2-Sh-PIK3R3 cell implanted mice, 2 of 8 BxPc-3-Vector cell implanted mice, and 7 of 8 BxPc-3-PIK3R3 cell implanted mice. Furthermore, the numbers of metastatic nodules per liver were significantly higher in PIK3R3 high expression cells (Mia PaCa-2-Control cells *VS.* Mia PaCa-2-Sh-PIK3R3 cell; BxPc-3-PIK3R3 cells *VS.* BxPc-3-Vector cells) (Fig. 2B). The expression of PIK3R3 in tissues obtained from different mice models was also confirmed by IHC (Fig. 2C). Taken together, all results in this aspect revealed that PIK3R3 could promote the migration and invasion of PC cells in both vitro and vivo.

PIK3R3 promotes EMT and ZEB1 expression of PC cells

As mentioned in introduction, EMT was a metastatic mechanism for several cancers. Therefore, we formulated a hypothesis that whether PIK3R3 could promote PC cell migration and invasion via EMT.

We firstly assessed the EMT biomarkers in different PC cells by using Western blot and immunofluorescence, including E-Cadherin and Vimentin. As shown in Fig. 3A and 3B, Western blot and immunofluorescence data suggested that E-Cadherin protein level was decreased in BxPc-3-PIK3R3 cells in comparison with BxPc-3-Vector cells; however, E-Cadherin protein was not detected in Mia PaCa-2 related cells. Compared to control cells, Vimentin was down-regulated in Mia PaCa-2-Sh-PIK3R3 cells and up-regulated in BxPc-3-PIK3R3. Furthermore, the tendency of E-Cadherin and Vimentin expression in xenografts were similar to that in cancer cells (Fig. 3C).

Although PIK3R3 could promote EMT in PC cells, the mechanisms involved in this process was absolutely unknown. EMT was commonly regulated by several transcription factors, such as SNAIL, SLUG, ZEB1, and ZEB2. Thus, these transcription factors were considered as mediators for the PIK3R3 induced EMT.

To test this hypothesis, we firstly evaluated the association between PIK3R3 expression and SNAIL, SLUG, ZEB1, and ZEB2 expression based on the data provided by TCGA (The Cancer Genome Atlas, http://cancergenome.nih.gov/). The analytic data suggested that PIK3R3 expression was positively associated with SNAIL, ZEB1, and ZEB2 expression (Fig. 3D). However, only ZEB1 was up- or down-regulated in PIK3R3 overexpression or knockdown cells (Fig. 3E). These results indicated that PIK3R3 may induce EMT by increasing ZEB1 expression.

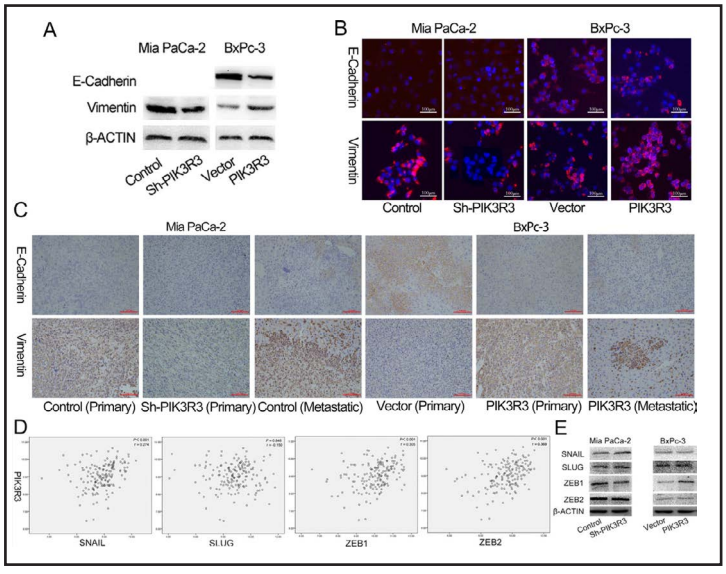

Fig. 3. PIK3R3 promoted the EMT process of PC cells. (A, B) Altered expression of E-Cadherin and Vimentin was detected in PIK3R3 knockdown and overexpression PC cells. (C) The results of IHC results confirmed the PIK3R3 mediated EMT biomarker expression. (D) PIK3R3 expression was positively associated with SNAIL, ZEB1, and ZEB2. (E) Only ZEB1 was regulated by PIK3R3.

ERK1/2-ZEB1mediates PIK3R3 induced EMT

We knocked down ZEB1 expression in BxPc-3-PIK3R3 cells to further investigate the role of ZEB1 by transiently transfecting ZEB1 SiRNA. The efficiency of ZEB1 SiRNA was shown in Fig. 4A. After silencing ZEB1 expression, the PIK3R3 overexpression-induced EMT phenotype (E-Cadherin and Vimentin expression) of BxPc-3 cells was reversed (Fig. 4B and 4C). Furthermore, the migration and invasion of BxPc-3-PIK3R3 cells were also decreased following the knockdown of ZEB1 (Fig. 4D). These results demonstrated that ZEB1 may mediate PIK3R3-induced EMT phenotype and aggressive property.

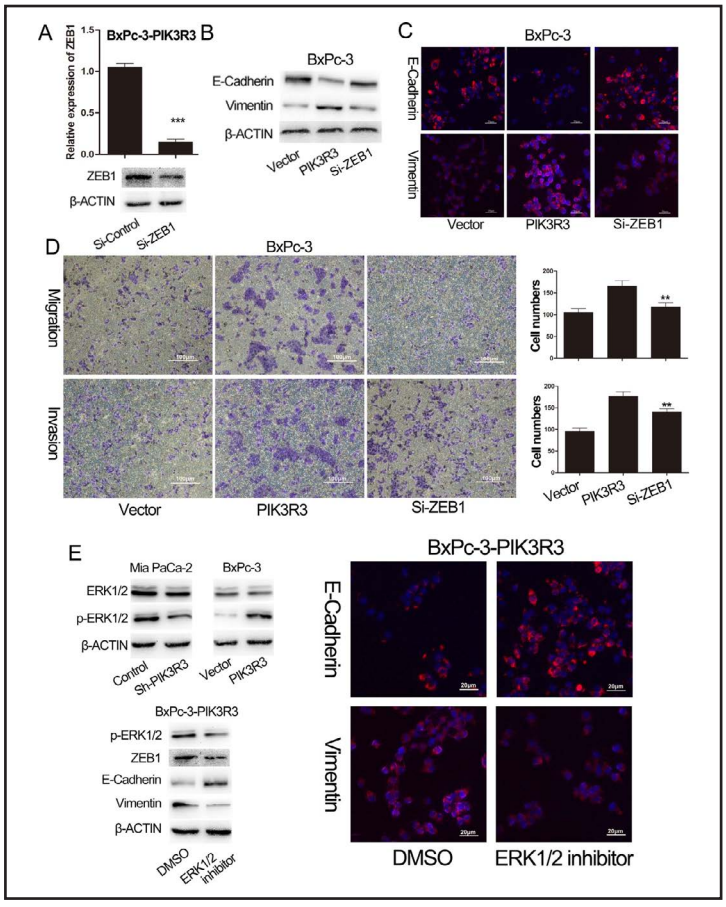

Fig. 4. ERK1/2-ZEB1 mediated the PIK3R3 induced cancer cell EMT, migration, and invasion. (A) The interference efficiency of Si-RNA for ZEB1 in PIK3R3-overexpressed cells was confirmed. (B-D) ZEB1 might mediate PIK3R3-regulated EMT phenotype and aggressive property. (E) PIK3R3 promoted ZEB1 up-regulation was mediated by the increase of ERK1/2 phosphorylation . ** P<0.01.

Furthermore, ERK1/2-ZEB1 pathway was widely reported to regulate the process of cancer cell EMT [25]. Therefore, we investigated the phosphorylation levels of ERK1/2 in PIK3R3 overexpression and knockdown cells. The ERK1/2 phosphorylation was respectively increased and decreased in overexpression and knockdown cells (Fig. 4E). Moreover, the inhibition of ERK1/2 by specific inhibitor (FR180204, 0.3mM) in BxPc-3-PIK3R3 cells resulted in the downregulation of ZEB1 expression, as well as the alternation of EMT phenotype (Fig. 4F).

Discussion

In this study, we found that PIK3R3 was overexpressed in PC tissues, especially in metastatic tissues. PIK3R3 overexpression could promote the migration and invasion of PC cells via ZEB1 upregulation induced EMT, while PIK3R3 knockdown could inhibit this phenomenon. Thus, PIK3R3 might be regarded as a potential therapeutic target to establish novel therapy strategies.

Previous studies suggested that the PIK3R3 is important in the regulation of several cell biological behaviors, such as proliferation, differentiation, cell cycle regulation, apoptosis, migration, and invasion [16, 26, 27]. More specifically, PIK3R3 could stimulate DNA synthesis and cell-cycle progression via its interaction with proliferation cell nuclear antigen (PCNA) [26]. PIK3R3 deficiency could lead to the decrease of proliferation of GC cells and induce

G0/G1 cell cycle arrest by decreasing retinoblastoma protein (Rb) phosphorylation, and the expression of cyclin D1 and PCNA [15]. Blocking PIK3R3 signaling by Tat transactivator protein (TAT)-N24 could suppress the proliferation and induce the differentiation of leukemia cells [27]. Apoptosis induced by parquet was associated with decreased PIK3R3 expression and caspase activation [28]. Furthermore, PIK3R3 could promote cell migration and invasion in various types of cancer via different mechanisms [29], for example, this protein could enhance the migration and invasion of colorectal cancer by SLUG triggered EMT [19]. Similar to above-mentioned results, our data demonstrated that PIK3R3 could promote the migration and invasion of PC cells for the first time by experiments both *in vitro* and *in vivo*, and this function of PIK3R3 might be depending on the ZEB1 induced EMT.

EMT has been identified as a crucial step in the metastasis and invasion of malignancies [30, 31] EMT is characterized by the up-regulation of mesenchymal cell-specific marker proteins including N-Cadherin and Vimentin, and the down-regulation of epithelial cell-specific marker proteins such as E-Cadherin [32]. In our study, our result suggested that mesenchymal cell-specific biomarker Vimentin expression were respectively increased and decreased in PIK3R3 overexpression and knockdown cells; however, epithelial cell-specific biomarkers expression E-Cadherin showed the opposite trend. These result indicated that PIK3R3 was a promotive factor for EMT of PC cells.

EMT is commonly activated by several embryonic and mesenchymal transcriptional factors. Some transcriptional factors could bind to the promoter region of E-Cadherin to suppress its expression, and further trigger the EMT conversion and metastasis of tumor cells, including SNAIL, SLUG, ZEB1 and ZEB2 [33, 34]. Among them, ZEB1 (characterized by the presence of 2 zinc finger clusters) is a well investigated transcriptional factor to act as a driver of EMT and cancer progression toward metastasis and invasion [35]. In our study, we have recognized ZEB1 as a downstream protein of PIK3R3 by evaluating the correlation between above-mentioned transcriptional factors and PIK3R3 expression based on the data obtained from TCGA, and assessing their expression in stable PIK3R3 overexpression and knockdown cells. Downregulation of ZEB1 in stable PIK3R3 overexpression cells could partially rescue EMT, migration, and invasion of pancreatic cancer cells. Obviously, the role of ZEB1 in cancer progression was further confirmed in this aspect. Furthermore, we also suggested that ERK1/2 participated in PIK3R3 mediated ZEB1 expression.

Conclusion

In conclusion, this is the first study to report that PIK3R3 was overexpressed in PC samples, and PIK3R3 could promote the migration and invasion of PC cells via enhancing ZEB1 mediated EMT. These results suggest that PIK3R3 may serve as an oncogene in PC pathogenesis and PIK3R3-targeted therapy for PC might be possible in future.

Disclosure Statement

The authors declare that they have no competing interests.

Acknowledgements

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