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Silencing PDZD8 Inhibits Bowel Cancer Cell Proliferation and Enhances Apoptosis

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Abstract

Bowel cancer is a rare malignant tumor of digestive tract, but its incidence has been increasing in recent years. PDZ domain-containing 8 proteins (PDZD8) is a capsid-stabilizing host factor, which has been reported to be involved in various human diseases such as murine leukemia virus (MLV), simian immunodeficiency virus (SIV) and HIV-1 infection. However, the functional importance of PDZD8 in human cancers has not been widely discussed. The present study aimed to investigate the significance of PDZD8 in bowel cancer development. PDZD8 expression in bowel cancer cell lines was detected by qRT-PCR. PDZD8 was knocked down in HCT 116 and RKO cells through infecting short hairpin RNA targeting PDZD8 (shPDZD8) lentivirus. The effects of PDZD8 depletion on HCT 116 and RKO cell phenotypes including proliferation, migration, apoptosis and cell cycle were detected by CCK8 assay, transwell and wound-healing assay and flow cytometry analysis. The results indicated that PDZD8 was highly expressed in bowel cancer cell lines. In addition, silencing PDZD8 in HCT 116 and RKO cell lines suppressed cell proliferation and migration, enhanced apoptosis as well as arrested cell cycle. In summary, knocking down PDZD8 might be a considerable therapeutic strategy for the treatment of bowel cancer.

Keywords: Bowel cancer; PDZD8; Cell proliferation; Cell apoptosis; Cell migration

Introduction

Bowel cancer is a rare malignant tumor of the gastrointestinal tract [1,2]. Despite this, the incidence of new bowel cancer cases has increased by an average of 2.3% per year in recent years [3]. Due to its rarity and heterogeneous clinical manifestations, bowel cancer is usually diagnosed at an advanced stage, which is related to poor treatment results, and to a large extent its outcomes are worse than other related malignancies such as colorectal cancer [3-5]. Although data have indicates that bowel cancer responds to treatment differently from colorectal cancer and requires a separate treatment method, few studies guide the best treatment [6,7]. Therefore, it is urgent to clarify the pathogenesis of bowel cancer in order to formulate more treatment strategies.

PDZ domain-containing 8 protein (PDZD8) is a capsid-stabilizing host factor, which is a critical component of the capsid-stabilizing activity in the cytoplasmic extracts [8]. In addition, PDZD8 has been previously identified as an HIV-1 cofactor [9]. Overexpression of PDZD8 has been reported to enhance murine leukemia virus (MLV), simian immunodeficiency virus (SIV), and HIV-1 infection [9]. In HIV-1 infection, PDZD8 mainly co-precipitates with HIV-1 Gag polyprotein, and depletion of the PDZD8 protein impairs HIV-1 infection [9]. Moreover, Henning et al. has reported that PDZD8 is a moesin interaction factor and a potential modulator of microtubule stability [10]. However, the relationship of PDZD8 and human cancers was unknown, let alone bowel cancer.

In this study, we provided evidence that PDZD8 is strongly expressed in bowel cancer cells. Knockdown of PDZD8 by short hairpin RNA (shRNA) diminished the abilities of bowel cancer cells to proliferation and migration, as well as enhanced cell apoptosis. Furthermore, silencing PDZD8 arrested cell cycle, resulting in a reduction of cell percentage in S phase and an increase in G2 phase. The identification of PDZD8 as a cancer-promoting factor reminded us that it might be a novel therapeutic target for bowel cancer treatment in the future.

Materials and Methods

Cell lines and cell culture

In this study, we selected human normal colorectal mucosal cell line FHC, human bowel cancer epithelial cell line DLD-1 and three bowel cancer cell lines SW480, RKO and HCT 116, which were provided by American type culture collection (ATCC) (https://www.atcc.org/). FHC, DLD-1, RKO and HCT 116 cell lines were cultured in 1640 medium with 10%FBS. Besides, SW480 cells were grown in L-15+10%FBS. They all were maintained a 37℃ incubator containing 5% CO_2. The medium was changed every 72h.

Lentivirus RNAi construction and infection

Based on the sequence of PDZD8, according to the RNAi sequence design principle, three RNAi target sequences were designed (shPDZD8-1, shPDZD8-2, shPDZD8-3), and the target with the best kinetic parameters was selected to enter the subsequent experiment. In addition, the recognized sequence Scramble: TTCTCCGAACGTGTCACGT was used as an RNAi negative control (Negative Control, NC).

RNA extraction and qRT-PCR

The total RNA was collected using TRIzol reagent (Sigma, St. Louis, MO, USA), which was then used to synthesize cDNA with

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Promega M-MLV Kit (Promega Corporation, Madison, Wisconsin, USA). Real-time quantitative PCR system was performed with SYBR Green Mastermixs Kit (Vazyme, Nanjing, Jiangsu, China). The internal control was GAPDH and the relative expression of mRNA was calculated according to the $2^{-\Delta\Delta Ct}$ method. The sequences of primers used in qRT-PCR were as follows: (Table 1)

Table 1: The sequences of primers used in qRT-PCR.

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
PDZD8	ATCATCAAGCGCAAGCACAC	GTAAGTGCCCATTGTTGTATATGGA
GAPDH	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA

Western blot assay

The total proteins were extracted and quantified with BCA protein assay kit (Thermo Fisher Scientific, Cat. #A53227). After that, the proteins were segregated by 10% SDS-PAGE and transferred into PVDF membranes for Western blot assay. Then, the membranes were blocked and incubated with primary antibodies and second antibodies at room temperature for 2 h. Finally, the ECL+plusTM Western blotting system kit was used for color rendering and X-ray imaging was carried out. The primary antibodies used in western blotting were as follows: PDZD8 (Rabbit, Wuhan Sanying, #25512-1-AP) and GAPDH (1:30000, Mouse, Proteintech, #60004-1-lg). The secondary antibody used in western blotting was Goat Anti-Mouse (1:3000, Beyotime, Beijing, China) and Goat Anti-Rabbit (1:3000, Beyotime, Beijing, China).

CCK8 assay

After infecting lentiviruses shPDZD8 and shCtrl, RKO and HCT 116 cells were digested and resuspended. 100 μL cell suspensions was added in a 96-well plate (2000 cell/well). Next day, 10 μL CCK-8 was added into the well 2~4 h before the termination of the culture. After 4 h, the 96-well plate was placed on a shaker and oscillated for 2-5 min, and the OD value was measured for 5 days by the microplate reader at 450 nm. The experiment was repeated three times.

Cell migration detection

After infecting lentiviruses shPDZD8 and shCtrl, the migration levels of RKO and HCT 116 cells were detected through performing transwell assay and wound-healing assay. For transwell assay, the upper chamber was incubated with 100 μL serum-free medium for 1-2 h, and then cells were seeded into each chamber. At the same time, 600 μL medium with 30% FBS was added in the lower chamber. After that, the upper chamber was transferred to the lower chamber and incubated for 40 h. 400 μL Giemsa were added for cells staining. Finally, the cells were dissolved in 10% acetic acid and the value of OD570 was detected. The experiment was repeated three times and the migration ability of cells was determined.

For wound-healing assay, 100 μL RKO and HCT 116 cell suspension were cultured in a 96-well plate at the density of 5×10^4 cells/well. On the next day, the low-concentration serum medium was supplemented, and a scratch tester was used to align the center of the 96-well plate and gently upward push to form scratches. The cells were then washed with serum-free medium and 0.5% FBS was added. Finally, the cells were cultured in an incubator with 5% CO_2 at 37℃. According to the degree of healing, the plate was scanned at the appropriate time and the migration area was analyzed with Cellomics (Thermo, USA). The experiment was repeated three times.

Detection of cell apoptosis and cell cycle by fluorescence activated Cells Sorting (FACS)

After infecting lentivirus, RKO and HCT 116 cells were inoculated in a 6-well plate (2 mL/well). When the cell confluence reached 85%, the cell suspension was centrifuged and the supernatants were discarded. Then, the cells were washed with 4℃ pre-cooled D-Hanks (pH=7.2~7.4). Next, 10 μL Annexin V-APC (eBioscience, San Diego, CA, USA) was added for staining in the dark. The cell apoptosis level was measured and the apoptotic rate was analyzed with FACSCalibur (BD Biosciences, San Jose, CA, USA). Considering cell cycle, RKO and HCT 116 cells were plated in 6-cm dishes (5 ml/well). The cell suspension was processed as above. Then the cells were washed with 4℃ pre-cooled PBS and ethanol, and stained with solution PI. The changes of cell cycle were detected by FACSCalibur (BD Biosciences, San Jose, CA, USA). Each experiment was repeated three times.

Statistical analysis

All data were analyzed by GraphPad Prism 6 (San Diego, CA, USA) and presented as the mean ± SD. Student's t-test (for comparisons of two groups) was used to evaluate the statistical significance, and the value of P less than 0.05 was considered to be significantly different.

Results

PDZD8 is abundantly expressed in bowel cancer cells

In order to clarify the significance of PDZD8 in bowel cancer, we first investigated the patterns of PDZD8 in human normal colorectal mucosal cell line FHC, human bowel cancer epithelial cell line DLD-1 and three bowel cancer cell lines SW480, RKO and HCT 116 by qRT-PCR assay. As exhibited in Figure 1A, the relative mRNA levels of

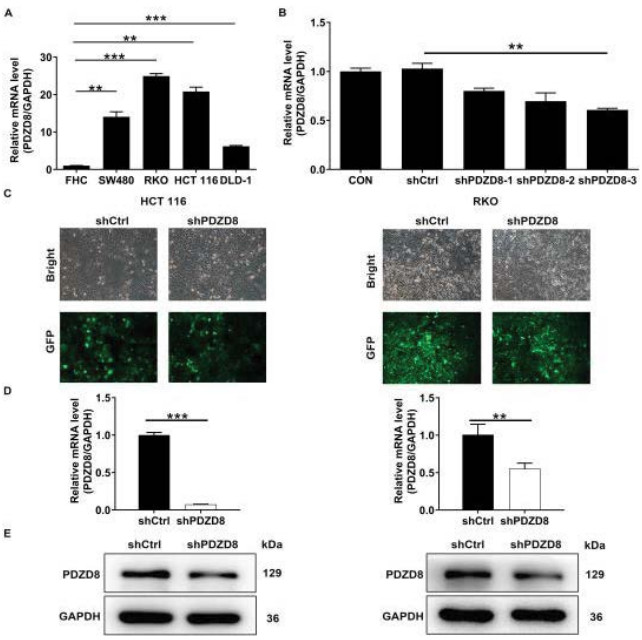

Figure 1: PDZD8 was upregulated in bowel cancer cell lines and PDZD8 knockdown cell models were constructed. (A) The mRNA expression of PDZD8 in human normal colorectal mucosal cell line FHC, human bowel cancer epithelial cell line DLD-1 and three bowel cancer cell lines SW480, RKO and HCT 116 was detected by qRT-PCR. (B) The knockdown efficiencies of shPDZD8-1, shPDZD8-2 and shPDZD8-3 were evaluated by qRT-PCR. (C) The fluorescence expression in cells was observed after 72 h-infection. Magnification times: 200×. (D) After infection, the PDZD8 mRNA level in RKO and HCT 116 cell lines was analyzed by qRT-PCR. (E) The expression of PDZD8 protein in RKO and HCT 116 cell lines after infection was detected by western blot.

Results were presented as mean ± SD. ** P < 0.01, *** P < 0.001.

PDZD8 were higher in bowel cancer cell lines in comparison with FHC cells, especially in RKO and HCT 116 cell lines. Thus, RKO and HCT 116 cell lines were employed for further study.

HCT 116 and RKO cell models with PDZD8 depletion are created

In this section, we aimed to bring to light the functional roles of PDZD8 in bowel cancer cells. Let nature take its course, we knocked down PDZD8 in HCT 116 and RKO cell models through infecting three short hairpin RNA targeting PDZD8 (shPDZD8-1, shPDZD8-2, shPDZD8-3). The most optimal interference target was shPDZD8-3, screened by qRT-PCR experiment, and the knockdown efficiency reached 41.00% ($P < 0.01$) (Figure 1B). Subsequently, shPDZD8-3 was infected into HCT 116 and RKO cell lines, and the infection and knockdown efficiencies were evaluated. Through detecting the green fluorescence, generated by the GFP inside the cells, we observed > 80% infection efficiencies in both cell lines (Figure 1C). Moreover, the knockdown efficiencies of PDZD8 were tested by qRT-PCR and western blot analysis. As shown in Figure 1D, the knockdown efficiencies of PDZD8 were 92.53% ($P < 0.001$) and 44.49% ($P < 0.01$) in HCT 116 and RKO cells, respectively. Similarly, the results of western blot assay evidenced decreased PDZD8 protein level in both cell lines upon infecting shPDZD8-3 (Figure 1E). Taken together, these data provided a reference for the successful establishment of PDZD8 knockdown cell models.

Knockdown of PDZD8 inhibits proliferation of bowel cancer cells

Having established HCT 116 and RKO cell models with PDZD8 depletion, we focused on the effects of PDZD8 knockdown on cell phenotypes of HCT 116 and RKO. First, the data from CCK8 assay pointed out that silencing PDZD8 obviously impaired the abilities of cell proliferation. In detail, the fold changes of the OD value were -1.7 and -1.3 in HCT 116 and RKO cells, respectively ($P < 0.001$, Figure 2A). These data denoted that PDZD8 knockdown could inhibit the proliferation of human bowel cancer cells.

Knockdown of PDZD8 inhibits migration of bowel cancer cells

Here, we performed transwell and wound-healing experiments to reveal the alterations of migration capability upon silencing PDZD8. Transwell assay demonstrated that, after the infection of lentivirus, compared with shCtrl group: In HCT 116 cells, the migration rate of cells in shPDZD8 group were decreased by 98%. In RKO cells, the migration rate of cells in shPDZD8 group was decreased by 79% ($P < 0.001$, Figure 2B). On the other hand, the appearance of Figure 2C taken at 24 h after wounding indicated again provided evidence regarding the damaged bowel cancer cell migration abilities after PDZD8 down regulation. Therefore, it could be concluded that PDZD8 knockdown inhibited the migration of human bowel cancer cells.

Knockdown of PDZD8 induces apoptosis and arrests cell cycle of bowel cancer cells

In addition proliferation and migration, we also wondered the influence of PDZD8 loss on cell apoptosis and cell cycle through flow cytometry analysis. As observed in Figure 3A, PDZD8 knockdown promoted the cell apoptosis of HCT 116 and RKO cells, and the fold changes were 2.0 and 1.8, respectively. Besides, cell percentage exhibited a restraint in S phase and an augment in G2 phase (Figure 3B). In general, all above data confirmed that the knockdown of PDZD8 could promote apoptosis and arrest cell cycle of human bowel cancer cells.

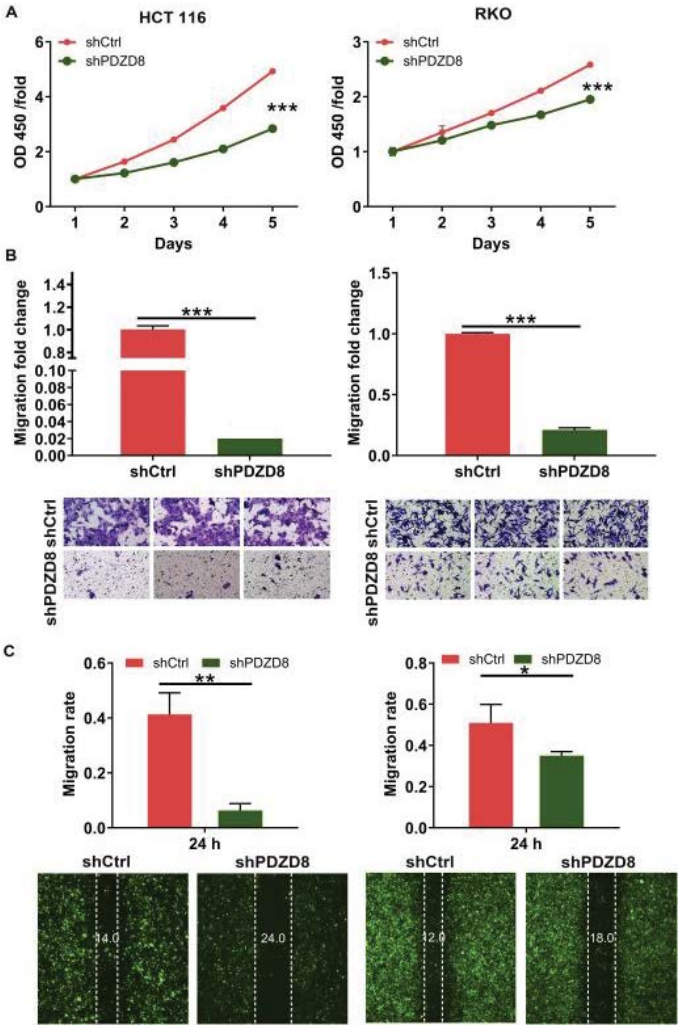

Figure 2: PDZD8 knockdown inhibited cell proliferation and migration. (A) The changes of cell proliferation were evaluated in RKO and HCT 116 cell lines after infection by CCK8 assay. (B, C) After infection, the migration abilities of RKO and HCT 116 cell were assessed by transwell assay (B) and wound-healing experiment (C).

Results were presented as mean ± SD. *P < 0.05, ** P < 0.01, *** P < 0.001.

Discussion

Bowel cancer is a rare disease, accounting for less than 0.6% of all new cancer cases [1,11]. Nevertheless, the incidence of bowel cancer has increased across recent years [2,4,12]. On the other hand, bowel cancer is a heterogeneous group of malignant tumors, requiring specialized and individualized methods [13]. At present, the treatment of small bowel adenocarcinoma has made a lot of progress, but it still fails to bring patients a better quality of life [14]. Therefore, it is urgent to clarify the pathogenesis of bowel cancer in order to formulate more treatment strategies.

Previous studies demonstrated that the host protein PDZD8 could interact with the HIV-1 Gag protein. In this way, elevating PDZD8 enhanced the risk of HIV-1 infection. In contrast, PDZD8 knockdown led to approximately 10-fold reduction in infection of cells by HIV-1 and murine leukemia virus vectors [8,9]. Furthermore, downregulation of PDZD8 impairs the ability of cytoplasmic lysates of human cells to stabilize HIV-1 CA-NC complexes in vitro. More interestingly, PDZD8 knockdown accelerates the disassembly of

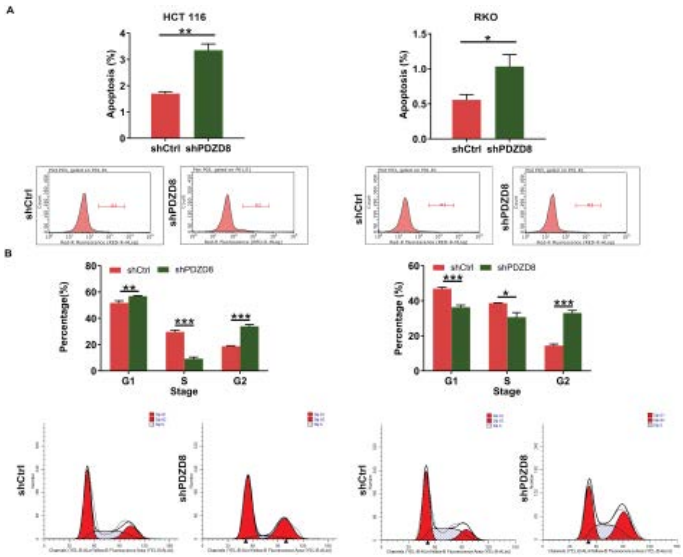

Figure 3: The effects of PDZD8 knockdown on cell apoptosis and cell cycle. (A) The effects of PDZD8 knockdown on RKO and HCT 116 cell apoptosis were examined by flow cytometry. (B) The effects of PDZD8 knockdown on RKO and HCT 116 cell cycle were determined by flow cytometry.

Results were presented as mean ± SD. *P < 0.05, ** P < 0.01, *** P < 0.001.

the HIV-1 capsids in infected cells, thereby blocking infection prior to reverse transcription. On the other hand, early post-entry events were also affected by PDZD8 downregulation [8]. Therefore, PDZD8 was proposed as a potential target for intervention in HIV-1 infection [15]. In this paper, we examined the expression levels and functional roles of PDZD8 in bowel cancer cells. The findings demonstrated that PDZD8 expressed at a higher pattern in bowel cancer cells. It was noteworthy that the knockdown of PDZD8 inhibited cell proliferation and migration, induced apoptosis and caused cell cycle arrest. Thus, we proposed that it might be a promising therapeutic candidate for bowel cancer treatment.

Finally, although our current research provides important and constructive findings, the exact mechanism of PDZD8's action in bowel cancer cells remains unknown. Also, we have not yet determined the relationship between PDZD8 expression and the prognosis of bowel cancer patients. In addition, more studies are needed to support the promotion role of PDZD8 in bowel cancer. These knowledges will, in turn, provide a theoretical basis for new strategies for the diagnosis and treatment of bowel cancer.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

Ang Li and XiaohongYang designed this research. Jie Yuan and Jianping Liu operated experiments. Xiang Li and Xiaochao Fu conducted the data procession and analysis. Jie Yuan completed the manuscript which was reviewed by XiaohongYang. All the authors have confirmed the submission of this manuscript.

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