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Peroxiredoxin1 promotes cell proliferation, migration and invasion of colorectal cancer via p38MAPK signaling

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Abstract. – **OBJECTIVE: Peroxiredoxin1 (PRDX1), a class of thiol peroxidases, is a multifunctional protein. We aimed at analyzing the effect of PRDX1 on proliferation, apoptosis, migration and invasion of colorectal cancer and to investigate the potential mechanism.**

MATERIALS AND METHODS: Western blot and PCR were used to validate the silencing efficiency in SW480 cell by transfection of PRDX1-siRNA. The cell proliferation was detected by Cell Counting Kit-8 (CCK-8) test. Flow cytometry Annexin V/PI double staining was used to analyze cell apoptosis. Transwell and scratch test were used to detect the migration and invasion of cells. Signal pathway protein expression was analyzed by Western blot.

RESULTS: The expression of PRDX1 in SW480 cells could be reduced by siRNA effectively. The cell proliferation, migration and invasion were reduced significantly compared with control group after down-regulation of PRDX1 ($p<0.05$), while the cell apoptosis was enhanced significantly ($p<0.05$). The ratio of phospho-p38 mitogen-activated protein kinases (p-p38) /p38 mitogen-activated protein kinases (p38) was down-regulated after the down-regulation of PRDX1 ($p<0.05$). The ratio of phospho-c-Jun N-terminal protein kinase (p-JNK)/c-Jun N-terminal protein kinase (JNK) and phospho-extracellular regulated protein kinases (p-ERK)/extracellular regulated protein kinases (ERK) showed changes with no significant difference ($p>0.05$).

CONCLUSIONS: Down-regulation of PRDX1 in colorectal cancer SW480 cells could inhibit the cell proliferation, migration, invasion, and induce cell apoptosis. This is very likely to be achieved by activating the p38MAPK-signaling pathway.

Key Words:
PRDX1, Colorectal cancer, Proliferation, Apoptosis, Invasive ability, p38MAPK.

Abbreviation

Peroxiredoxin1 (PRDX1); Cell Counting Kit-8 (CCK-8); Phospho-c-Jun N-terminal protein kinase (p-JNK); c-Jun N-terminal protein kinase (JNK); Phospho-extracellular regulated protein kinases (p-ERK); extracellular regulated protein kinases (ERK); mitogen-activated protein kinase (MAPK); Colorectal cancer (CRC); Reactive oxygen species (ROS); Peroxiredoxins (PRDXs); Hydrogen peroxide (H_2O_2); Reverse transcriptase-polymerase chain reaction (RT-PCR); Reverse transcription (RT); Bicinchoninic acid (BCA); Dulbecco's Modified Eagle Medium (DMEM); Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); Polyvinylidene difluoride (PVDF); Tris-buffered saline-Tween (TBST); Standard deviation (SD); Least square difference (LSD); Mitogen activated protein kinase (MAPK).

Introduction

Colorectal cancer (CRC) is the second most widespread morbidity and mortality cancer in the world[1]. Surgical radical surgery with chemotherapy and radiotherapy, is the main treatment in colorectal cancer in clinics; the prognosis of advanced CRC is still poor[2,3]. Recently, the newest molecularly-targeted therapeutic agents showed advanced achievements in cell signaling mechanism in colorectal cancer. However, cell signaling transduction mechanisms in malignant evolution of colorectal need to further investigation in order to treat colorectal cancer individually. The occurrence of the colorectal cancer is a multi-step process with multi-factor, and it is closely related to the gene polymorphism, tumor-associated regulators and reactive oxygen species (ROS)[4]. Peroxiredoxins (PRDXs) are a ubiquitous fam-

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ily of antioxidant enzymes, acting as a hydrogen peroxide (H_2O_2) scavenger, molecular chaperone and immune modulator[5]. PRDX1 is an important member of this family, which is widely present in cytosol of prokaryotes and eukaryotes. As an antioxidant enzyme, PRDX1 participates in multiple ROS-related signaling pathways and affects the biological behavior of cells. Studies have shown that abnormal expression of PRDX1 has been observed in several human cancers, including breast, esophageal, lung and prostate cancers[6-8]. It is reported that PRDX1 was highly expressed in pancreatic ductal adenocarcinoma, and affected the invasion of pancreatic cancer cells by regulating the activity of p38MAPK signaling pathway[9]. However, there are few researches about the effect of PRDX1 on the invasion, migration and tumor formation in colorectal cancer. We aimed at investigating the possible molecular mechanism of PRDX1 in inhibiting cell proliferation, migration and invasion in colorectal cancer, which might provide a new prospective and effective way for the diagnosis and treatment of colorectal cancer.

Materials and Methods

Cell Culture

Colorectal cancer cells, SW480, (Shanghai Cell Bank of Chinese Academy of Sciences, Shanghai, China) were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) Gibco (Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) Gibco (Grand Island, NY, USA), penicillin-streptomycin (100 IU/ml-100 μg/ml) at 37°C in a humid atmosphere (5% CO_2, 95% N_2).

Cell Transfection

PRDX1 siRNA sequence and negative control sequence (NC siRNA) (Invitrogen, Carlsbad, CA, USA) were transfected to SW480 cells according to the specification by Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). Cells were seeded in the 6-well plates for 24 h before transfection, and the cell density was adjusted to 4×10^5/ml. Cells were washed with Dulbecco's modified eagle Medium (DMEM)/F12 (no serum, no antibiotics) before transfection. PRDX1 siRNA mixture/NC siRNA and Lipofectamine® 2000 were diluted by DMEM/F12 (no serum, no antibiotics) according to the specification and then transferred to the SW480 cells, respectively. Cells were divided into control group, NC siRNA group, and PRDX1 siRNA group.

RT-PCR

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay was used to analyze the expressions of PRDX1 mRNA. Total RNA was extracted using the TRIzol kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. The concentration of total RNA was measured by ultraviolet spectroscopy. Then, reverse transcription (RT) was carried out according to the specification of RT Kit (Biosystems, Foster City, CA, USA). The following primers were used for the specific amplification of GAPDH: forward, 5'-TGAACGGGAAGCTCACTGG-3', reverse, 5'-GCTTCACCACCTTCTTGATGTC-3', PRDX1: forward, 5'-AATGCAAAAATTGGG-TATCCTGC-3', reverse, 5'-CGTGGGACACA-CAAAAGTAAAGT-3'. PCR reaction system was cDNA 5 μl, Forward Primer 0.5 μl, Reverse Primer 0.5 μl, 10 × EasyTaq Buffer 2 μl, d NTPs 1 μl, EasyTaq DNA Polymerase 1 μl, refilled ddH2O to 20 μl (Biosystems, Foster City, CA, USA). The reaction was performed 35 cycles at 95°C for 10 min, 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 10 min; the reaction was terminated at 4°C. PCR products were measured with Bio-Rad Imager (Bio-Rad, Hercules, CA, USA). The mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method. GAPDH mRNA was used as the internal reference mRNA.

CCK-8 Assay

CCK-8 kit (Olympus, Tokyo, Japan) was used to detect cell proliferation. The collection of the logarithmic growth phase of the transfected or untransfected cells was collected and plated. Next, it was cultured for 1-7 days. The proliferation of cells was detected by CCK-8 test with continuous determination of absorbance at 450 nm. Growth curve of each group of cells was drawn, respectively.

Clonal Formation Assay

Clonal formation experiments were performed to further detect of cell proliferation. The cells in logarithmic phase were cultured in the 6-well plates at a density of 500 cells per well. The cells were incubated at 37°C with 5% CO_2 and saturated humidity in the culture incubator for 2-3 weeks. The culture process was terminated when visible clone could be seen. The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4, Solarbio, Beijing, China), fixed with methanol for 5 min and then stained with GIMSA (Solarbio, Beijing, China) staining fluid for 10-30 min. The

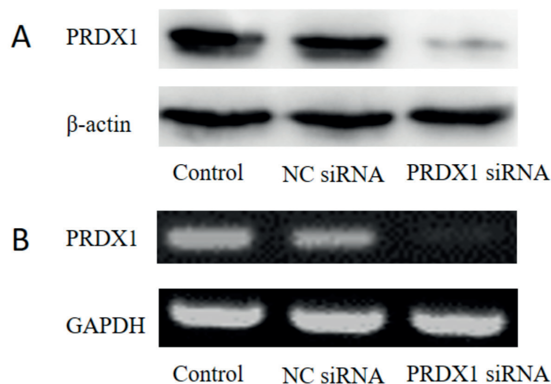

Figure 1. PRDX1 expression analysis in each group. ***(A)*** The expression of PRDX1 protein measured by Western blot. ***(B)*** The expression of PRDX1 mRNA detected by PCR.

plates were rinsed in distilled water and dried in air. The cloned cells were directly counted with the naked eye; clones with more than 50 cells were counted on a microscope (×100, Bio-Rad, Hercules, CA, USA).

Transwell Assay

The effect of cell invasion was detected with transwell chamber (8 µm pore size; Costar, Switzerland). 1 mg/ml BD Matrigel Matrix (Becton Dickinson, Franklin Lakes, NY, USA) was coated on inserts. Cells suspended in 200 µl of fresh medium were added to inserts and cultured for 24 h. Then, the surface of the cell membrane was removed with a cotton swab; cells in lower layer were fixed and stained with 0.1% crystal violet (Solarbio, Beijing, China). The number of cells was scored in randomly 5 chosen fields under a microscope (×100, Bio-Rad, Hercules, CA, USA).

Scratch test

The cells in logarithmic phase were cultured in the 6-well plates at a density of 3×10^5 cells per well. The plates were scratched with 10 µl tip after cell adherent with the density of about 80%. Next, we extracted the medium of each well; the cells were washed with phosphate buffered saline (PBS) and added with fresh medium. Scratches were photographed at 0 h, 24 h, 48 h to record cell confluence.

Western Blot Analysis

Protein was collected after treatment with each group. Protein concentration was detected with bicinchoninic acid (BCA) Thermo Fisher Scientific (Waltham, MA, USA) method. The proteins were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto polyvinylidene difluoride (PVDF) Merck (Darmstadt, Germany) membranes for 2 h. Then, they were sealed for 1 h with Tris-buffered saline and Tween 20 (TBST-20) solution with 5% skim milk powder after washed, incubated overnight with dilutions of the primary antibodies, and incubated for 1 h with secondary antibodies after washed rendered the color with ECL substrate (Sigma-Aldrich, St. Louis, MO, USA). The results were analyzed by gel electrophoretic image analyzer with β-actin as internal control.

Statistical Analysis

Data were described as mean ± standard deviation (SD). Statistical analysis was performed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Comparison between multiple groups was analyzed by one-way ANOVA with the least square difference (LSD) post-hoc test method. $p<0.05$ was considered statistically significant.

Results

PRDX1 Expression Analysis

The expression of PRDX1 protein and mRNA in each group was detected by Western blot and PCR. As shown in Figure 1, the expression of PRDX1 protein and mRNA in NC siRNA group was not significantly different from that in control group, but the expression of PRDX1 in PRDX1 siRNA group was significantly lower than that in control group ($p<0.05$).

Silencing PRDX1 Expression Inhibits SW480 Cell Proliferation

Cells were continuously cultured for 1-7 days after transfection, and the growth curve was plotted (Figure 2B). The results showed that cells growth curve were “S-type” in control group and negative control group (NC siRNA). The activity of cells in PRDX1siRNA group was significantly lower than that in the control group ($p<0.05$), while from day 3, the activity of silenced PRDX1 expression cells was slightly lower compared with the other two control groups. Cell proliferation was significantly inhibited from day 5. There was no significant difference between normal control group and NC siRNA group, while the difference between PRDX1 siRNA group and control group was statistically significant from 5 day. Cloning

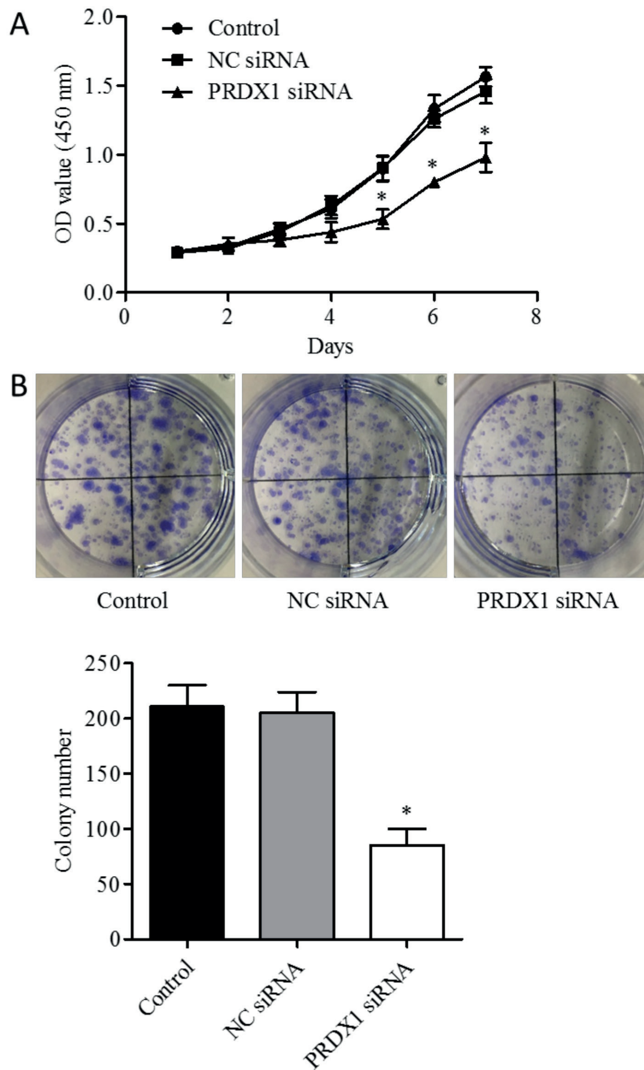

Figure 2. Effect of PRDX1 on proliferation of colorectal cancer cell SW480. ***(A)*** Growth curve of cells in each group. ***(B)*** Colony formation assay for detection of cell proliferation of each group. Bars indicated mean ± SD (N=3). *$p<0.05$ *vs.* control group.

formation experiments showed that down-regulation of PRDX1 could significantly reduce the cloning formation rate (Figure 2B). We investigated the effect of silenced PRDX1 expression on cell apoptosis by flow cytometry (Figure 3). The results showed that there was no significant difference between control group and NC siRNA group (5.9%, 6.8%, $p>0.05$), while the apoptosis rate of PRDX1 siRNA group was significantly upregulated (21.8%, $p<0.05$ *vs.* Control), indicating that the down-regulation of PRDX1 could promote colorectal cancer cell SW480 apoptosis.

Down-Regulation of PRDX1 Inhibits the Invasion and Migration of Colorectal Cancer cell SW480

Cell migration ability was observed by scratch test (Figure 4A). The relative migration rate of the control group and NC siRNA group at 48 h cells were 69.7% and 72.3% respectively; there was no significant difference ($p>0.05$). The migration rate of PRDX1 siRNA group was only 35.7% at 48 h, which was statistically significant compared with the other two groups ($p<0.05$). Transwell assay was used to detect the cell invasion ability (Figure 4B). The results were consistent with the scratch test. The numbers of cells invading the lower surface in control group and NC siRNA group were 63.7 and 62.3, respectively ($p>0.05$). However, the number of cells in PRDX1 siRNA group was 25.3, which was statistically significant compared with the other two groups ($p<0.05$).

Silencing of PRDX1 Expression Affects p38 MAPK Signaling of Colorectal Cancer Cell SW480

The expression of p38 MAPK signaling protein in different treatment of SW480 cells was detect-

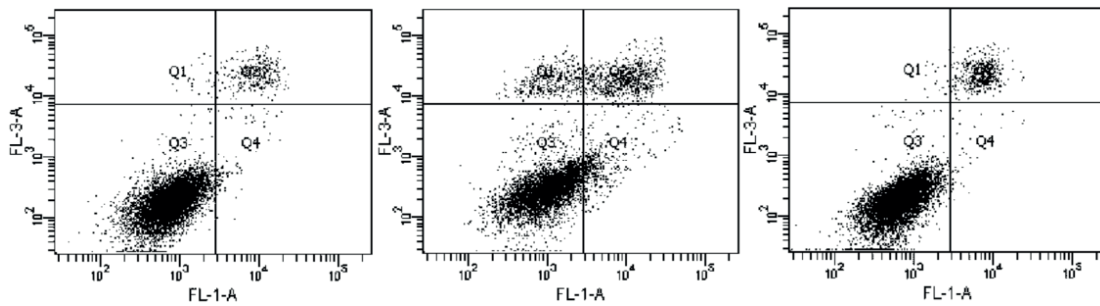

Figure 3. Effect of PRDX1 on apoptosis of colorectal cancer cell SW480.

ed by Western blot (Figure 5). The results showed that there was no significant difference p-p38/p38 ratio between the control group and NC siRNA group ($p>0.05$), the ratio of p-p38/p38 decreased compared with the control group and NC siRNA group after down-regulation of PRDX1 ($p<0.05$). Results of other two signal pathways JNK and ERK1/2 detection showed that there was no significant difference in p-JNK/JNK and p-ERK/ERK, although ratio changed. Thus, we conjec-

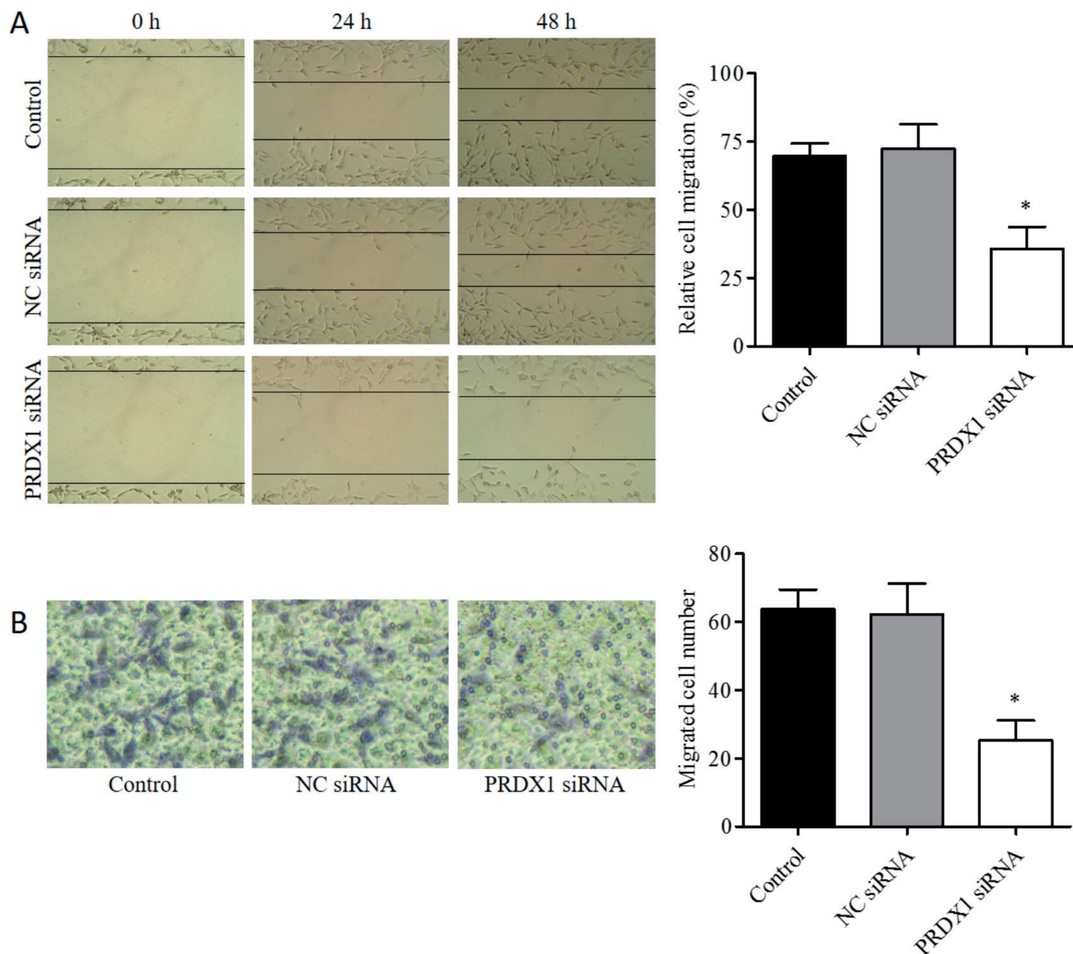

Figure 4. Effects of down-regulation of PRDX1 on the invasion and migration of colorectal cancer cell SW480. ***(A)*** Scratch tests were performed to analyze the cell migration ability; the histogram showed the statistical results of cell relative mobility at 48 h (n=3). ***(B)*** Transwell assay was used to detect the cell invasion ability, the histogram showed the statistical results of invasion ability (n=3). *$p<0.05$ *vs.* control group.

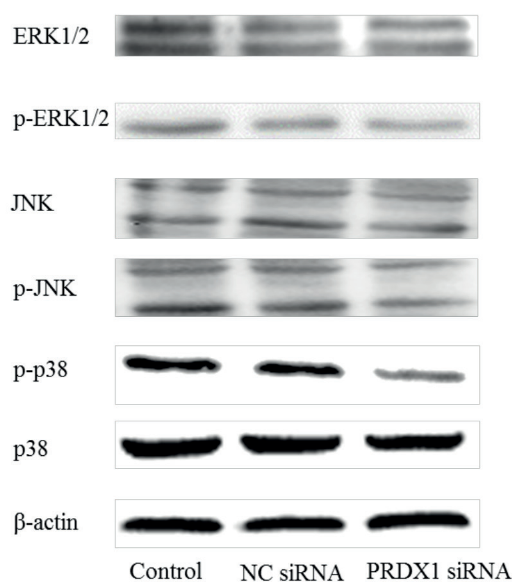

Figure 5. Effects of PRDX1 expression on p38MAPK protein expression.

tured that down-regulation of PRDX1 might be mainly related to the p38 MAPK signal pathway.

Discussion

Evidence suggests that PRDX1 may play an important role in various tumors proliferation and aberrant expression of PRDX1 serves as a potential therapeutic target and/or a valuable prognostic factor in various tumors, such as liver cancer[9], breast cancer[10], and colorectal cancer[11]. This study demonstrated that down-regulation of PRDX1 leads to the inhibition of colorectal cancer cell proliferation. These results suggested that PRDX1 could be a potential therapy target for CRC. The proposed molecular pathway for the inhibitory effects of down-regulation of PRDX1 was p38MAPK signal pathway.

Colorectal cancer is still one of the most high frequency cancers worldwide. The mortality rate of CRC remains high despite significant advances in diagnosis and treatment. It is essential to improve the understanding of proteins and molecular pathways that affect cell proliferation and survival functions in colorectal cancer. PRDX1 belongs to a family of peroxiredoxins which can effectively remove intracellular ROS[12,13]. Various studies have been carried out to investigate the potential role of PRDX1 in cancers, but the results were controversial[14]. PRDX1 was firstly regarded as a tumor suppressor in the study which showed that the down-regulation of PRDX1 promoted the tumor invasion and poor clinical outcome in esophageal squamous cell carcinoma[15]. However, accumulated studies found the expression of PRDX1 was enhanced in numerous types of cancers, including breast cancer[16], lung cancer[17] and gallbladder cancer[18]. Numerous reports proved that silencing PRDX1 inhibited cell proliferation and led to cell apoptosis in cancer cells. We also observed that down-regulating PRDX1 using specific siRNA could reduce the abilities of cell proliferation and colony formation.

Apoptosis is defined as the evolutionary conserved mechanism in the balance of cell proliferation tissue homeostasis. Excessive cell growth and insufficient apoptosis are often associated with CRC development and progression. So, the modulation of apoptosis has an immense potential in CRC therapeutic intervention[19]. It has been identified that PRDX1 is a conserved protein highly expressed during proliferation[20]. In nucleus, oligomeric PRDX1 directly associates with p53 or transcription factors, thus affecting their bioactivities upon gene regulation, which in turn induce or suppress cell death. In cytoplasm, PRDX1 has anti-apoptotic potential through direct or indirect interactions with several ROS-dependent (redox regulation) effectors[21] In this study, down-regulation of PRDX1 induced apoptosis of CRC cell SW480. The results confirmed that down-regulation of PRDX1 could be used as a potential target in the treatment of CRC.

Mitogen activated protein kinase (MAPK) chain is one of the important pathways in eukaryotic signaling networks and plays an important role in gene expression regulation and cytoplasmic activity in eukaryotic signaling networks[22,23]. The p38MAPK pathway regulates multiple physiologic and pathologic processes and activates apoptosis in response to oxidative-stress in several cancers[24-26]. It is reported that p38MAPK was high-expressed and correlated with proliferation and apoptosis index in CRC, which means p38MAPK pathway plays an important role in the occurrence and development of CRC. In this study, we found that the ratio of p-p38/p38 decreased after down-regulation of PRDX1, while the ratio of p-JNK/JNK and p-ERK/ERK was no significant difference after down-regulation of PRDX1. Taken together, our findings strongly suggest that down-regulation of PRDX1 might be mainly related to the p38MAPK signal pathway.

Conclusions

We showed the anti-proliferative, apoptotic, anti-invasion and anti-migration effects of down-regulation of PRDX1 in CRC cell SW480.

The effects might correlate with down-regulation of p38MAPK pathway. Our observation suggests that down-regulation of PRDX1 could be a promising regimen in the therapy against intestinal tumorigenesis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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