PRDX2 Performs the Oncogenic Functions in Ewing's Sarcoma

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Abstract

Background: Ewing's sarcoma (ES) is the second most common malignant primary bone tumor in children and adolescents, characterized by malignant proliferation of small round cells. The survival rate of this disease continues to be low. Peroxiredoxin2 (PRDX2) is a multifunctional peroxidase family member with anti-oxidation, involvement in intracellular signaling, chaperones, and tumor. But the function and underlying mechanism of PRDX2 in ES is still unknown. Herein we investigated the role and mechanism of PRDX2 in the development of ES, and tested its potential for the treatment of ES.

Methods: We explored the function of PRDX2 on ES through knocking down the expression of NKAP in A673 and RDES cells by siRNA interference (siPRDX2). We examined the effects of siPRDX2 on cell motility and apoptosis using CCK8, colony formation, transwell, gelatin zymography, flow cytometry, PI/Hoechst33342 double dye and western blot assays. In addition, western blot was used to analyze the activation of the AKT/mTOR signaling pathway.

Results: Here we showed that downregulation of PRDX2 strongly inhibited the motility of A673 and RDES cells. Interestingly, siPRDX2 induced cell apoptosis. Furthermore, the expression of anti-apoptotic protein Bcl2 in siPRDX2 group was significantly decreased, while the expression of pro-apoptotic protein Bax and cleaved Caspase-9 was strongly increased. Finally to identify the molecular mechanisms involved, we examined related proteins of the AKT/mTOR signaling pathway and found that siPRDX2 significantly inhibited the phosphorylation of AKT and the expression of Cyclin D1.

Conclusion: These observations suggest that siPRDX2 inhibited the ES cells motility and induced apoptosis in which the AKT/mTOR signaling pathway involved. The enhanced understanding to this molecular mechanism has provided a strong basis for the development of novel therapeutic strategies for ES.

Introduction

Ewing's sarcoma (ES) is an important member of the Ewing's sarcomafamily of tumors (ESFT)[1]. ES has the characteristics of high malignancy, short course, high metastasis rate and rapid recurrence[2]. About 80% of ES cases occur in patients around the age of 20. Although the treatment is constantly updated, the 5-year survival rate of this disease has been low[3]. Especially for patients with tumor metastasis, the survival rate is less than 30%, and this data has not changed during the past 30 years[4]. Therefore, a better understanding of the mechanism of its development will help us to find new diagnostic and therapeutic options.

Peroxiredoxin2 (PRDX2) is the one of Peroxiredoxins (PRDXs), a family of antioxidant enzymes first discovered in yeast[5]. PRDX2 acts as antioxidant (clearing reactive oxygen), signal transduction and molecular chaperone to play an important role in the normal life activities of organisms[6]. In vitro tests, PRDX2 from Anopheles stephensi protects mosquito cells from damage caused by ROS from Plasmodium infection[7]. In vivo PRDX2 has high affinity for H_2O_2 which can act as a second intracellular

messenger. Hence PRDX2 plays an important regulatory role in the concentration of H_2O_2 indirectly regulating intracellular signaling[8]. PRDX2 is present in some cancer cells and neurological disorders such as Alzheimer's disease, Niemann-Pick's disease and Down's syndrome[8, 9, 10, 11]. It is well known that the production of ROS in neurological disorders is accompanied by an increase in the expression of antioxidant proteins including PRDX2, which is beneficial to the body to protect neuronal cells from oxidative stress[12]. Considering that PRDX2 may have an anti-apoptotic effect, some researchers explore the function of PRDX2 in tumor and the results show that PRDX2 is involved in tumor formation and progression[5]. However, the function of PRDX2 in ES remains unclear.

To explore the function of PRDX2 on A673 and RDES cells, we knocked down the expression of PRDX2 by siRNA interference. The function analysis revealed that siPRDX2 hindered cell motility, and induced cell apoptosis. We further revealed its underlying mechanisms and found siPRDX2 inhibited activation of the AKT/mTOR signaling pathway. In a word, our study provided a novel perspective to treat for ES and the potential utility of PRDX2 inhibitors for ES therapy.

Materials And Methods

Cell culture and transfection

Human ES cell lines A673 and RDES were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were grown under the following standard conditions: RPMI-1640 medium (HYCLONE, USA) containing 10% FBS, 37 °C and 5% CO_2. In brief, siRNA for PRDX2 (siPRDX2) was mixed with the diluted Lipofectamine 2000 based on the manufacturer's instruction. After incubated for 20 min at room temperature, the mixture was added into the culture plate for 6 h incubation with 5% CO_2 at 37 °C. Then the cells were treated with complete medium at 37 °C in a CO_2 incubator for 48 h.

Quantitative real-time PCR analysis (qRT-PCR)

Total mRNA was isolated from cells using an RNA isolation kit (CWBIO, Beijing, China). Then the total RNA was reversely transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA). ABI Prism 7300 sequence detector (Applied Biosystems) and SYBR Green reagent were performed for qRT-PCR and normalized to the expression of β-actin mRNA. Relative expression was calculated using the ΔΔCt method for each sample. Primers were as follows: β-actin sense, 5' CCCGAGCCGTGTTTCCT 3', and antisense, 5' GTCCCAGTTGGTGACGATGC 3'; PRDX2 sense, 5' CCTTCAAAGAGGTGAAGCTG 3', and antisense, 5' GTTGCTGAACGCGATGAT 3'. The assay was performed three times.

Western blot assay

A673 and RDES cells transfected with siPRDX2 were lysed with RIPA buffer (CWBIO, Beijing, China). Protein was separated by gel electrophoresis using SDS-PAGE and blotted onto PVDF membrane. The membrane was probed with primary antibodies against PRDX2, Bcl-2, Bax, cleased-Caspase3, AKT, p-AKT, CyclinD1 and Tubulin (Solarbio Science & Technology, Beijing, China) overnight at 4 °C. Then the PVDF

was probed with secondary antibodies for 1 h at room temperature. Finally protein bands were detected using an ECL reagent, normalized to the expression of Tubulin. Western blot assay was performed in triplicate.

CCK8 assay

In brief, cells were planted into 96-well plates at a density of 1×10^3 per well. After cultured for 24 h, 10 µl of CCK8 solution (Solarbio Science & Technology, Beijing, China) was added into each well and after incubated for 1.5 h at 37 °C, OD value was detected at 450 nm. Each experiment included triplicate measurements.

Colony formation assay

2×10^2 cells transfected with siPRDX2 were seeded into a 35mm dish and cultured in a cell culture incubator for 2 to 3 weeks. After formed visible clones, the cells were fixed with 4% paraformaldehyde solution for 20 minutes. Then the clones were stained with 0.1% crystal violet for 30 minutes and were taken a photograph and counted. Clone formation assay was repeated in three independent experiments.

Transwell assays

For the invasion assay, 10 µl Matrigel (BD, USA) that melt overnight was added to the upper chamber for 4-6 h, while 500 µl DMEM medium without serum was added to the lower chamber for 30 mimutes. About 1×10^5 cells transfected with siPRDX2 were planted in the upper chamber, while 500 µl complete medium was added to the lower chamber. After cultivating overnight, the invasive cells were fixed. Then five fields of view were randomly selected and photographed under the microscope. For the migration assay, the transwell chambers were coated with uncoated filters, and same procedures as those for the invasion assay were followed. Each transwell assay included triplicate measurements.

Gelatin zymography assay

After transfection for 48 h, 2.5×10^6 cells were planted into a 6 cm dish with 2 ml DMEM medium without serum. The proteins were separated by SDS-PAGE. The gel was stained with 0.25% Coomassie Blue R-250 for 4 hours and decolorized by a decolorizing solution from dark blue background to show clear bands. The assay was performed in triplicate.

Flow cytometry assay

Annexin V-FITC Apoptosis Detection kit I (Thermo Fisher Scientific, Shanghai, China) was used to the analysis of cell apoptosis. Transfected cells were collected and washed with PBS buffer twice. Then Annexin V / FITC mix and PI dye were added to cell suspension in dark for 15 minutes. Analysis of cell apoptosis was performed using flow cytometry. Flow cytometry assay was performed three times.

PI/Hoechst33342 double dye assay

Transfected cells (3×10^3) were planted into 96-well plates. After incubation for 24 h, cells were stained with 1X binding buffer containing dyes of Hoechst33342 and PI in dark for 10-15 minutes. Finally cells were photographed by fluorescence microscope. This assay included triplicate measurements.

Statistical analysis

Experimental results in this study were performed using SPSS 18.0 software. The comparison between two groups was analyzed using Student's t-test. One-way ANOVA with the Turkey-Kramer post hoc test was used for multiple groups. Date was expressed as mean ± SD, and differences with P value of less than 0.05 were considered statistically significant.

Results

A673 and RDES cell lines were successfully transfected with siPRDX2

To investigate the function of PRDX2 in ES cells, we selected A673 and RDES cell lines as subjects and constructed cell lines transfected with siPRDX2 (siPRDX2 group) by siRNA interference. A673 and RDES cell lines added transfection reagents were as a control group (NC group).

RNA and protein of the NC group and the siPRDX2 group were collected. The results of qPCR and western blot assays shown in Figs 1A, 1B, indicated that the expression levels of PRDX2 in mRNA and protein were significantly down-regulated in the siPRDX2 group ($P<0.05$).

Knockdown of PRDX2 inhibited the motility of A673 and RDES cells

The effect of siPRDX2 on the proliferation of A673 and RDES cells was examined by CCK8 and colony formation assays. As shown in Figs 2A, 2B, the proliferation of A673 and RDES cells was significantly decreased after knocking down PRDX2. Clone formation assay showed similar results that the number and percentage of clones of A673 and RDES cells decreased in siPRDX2 group compared with NC group after knockdown of PRDX2 (Figs 2C, 2D) ($P<0.05$).

Subsequently, we assessed the effect of knockdown of PRDX2 on the invasion and migration ability of A673 and RDES cells by transwell assay, and gelatin zymography assay was used to detect the effect of PRDX2 knockdown on the activity of MMP9. The results showed in Figs 2E, 2F that the number of invaded and migrated cells in siPRDX2 group significantly declined in comparison to that of control cells($P<0.05$). As shown in Fig. 2G, expression of MMP9 was reduced from 1.26±0.06 or 1.31±0.07 to 0.27±0.08 or 0.21±0.05 in A673 and RDES cells respectively in siPRDX2 group compared with NC group ($P<0.05$). In summary, siPRDX2 significantly inhibited the motility of A673 and RDES cells.

Knockdown of PRDX2 induced the apoptosis of A673 and RDES cells

To further explore the effect of PRDX2 knockdown on the apoptosis of A673 and RDES cells, we performed flow cytometry, PI/Hoechst33342 double dye and western blot assays. Flow cytometry results

shown in Figs 3A, 3B, indicted that the cell apoptosis percentage of A673 and RDES cells after knockdown of PRDX2 was significantly increased compared with the NC group ($P<0.05$). As shown in Figs 3C, 3D, PI/Hoechst33342 double dye results showed the similar result. Quantification results of PI/Hoechst33342 double dye assay shown in Fig. 3E, indicated that rate of apoptosis in siPRDX2 group was significantly increased from 3.27% or 1.63% to 13.38% or 15.04% in A673 and RDES cells respectively ($P<0.05$). Western blot results showed that the expression of anti-apoptotic protein Bcl2 was decreased, while the expression of pro-apoptotic protein Bax and cleaved-caspase 9 was increased in A673 and RDES cells (Figs 3F, 3G, $P<0.05$). Taken together, siPRDX2 significantly induced the apoptosis of A673 and RDES cells.

Knockdown of PRDX2 inhibited the activation of AKT/mTOR signaling pathway in A673 and RDES cells

As we all know, the AKT/mTOR signaling pathway is the hub involved in a variety of physiologic functions linking growth factors, metabolism, proliferation, survival, apoptosis[13, 14]. Hence we detected the effect of siPRDX2 on the AKT/mTOR signaling pathway to illustrate the mechanism of PRDX2 acting on the ES cells. As shown in Figs 4A, 4B, after knockdown of PRDX2, AKT phosphorylation level was significantly inhibited, and the expression level of cell proliferation-related protein Cyclin D1 was decreased in A673 cell ($P<0.05$). Western blot results showed the similar result in RDES cells (Figs 4C, 4D, $P<0.05$), which suggested that activation of the AKT/mTOR signaling pathway was inhibited by siPRDX2 and the AKT/mTOR signaling pathway might be involved in the functional role of PRDX2 in ES.

Discussion

It is well known that higher levels of ROS in most tumors contribute to tumor progression compared to normal tissues[15] and PRDX2 is an important member of the ROS scavenging system[16]. As shown in previous study, Hypoxia-inducible factor (HIF) controls the transcription of genes which plays an important role in the pathogenesis of cancer and PRDX2 is a direct HIF target gene and that PRDX2 expression is induced by prolonged hypoxia[17]. In fact, it has been reported that PRDX2 has proliferative and anti-apoptotic properties and thus may induce carcinogenic changes[18]. In colon cancer, the dysfunction of PRDX2 promotes 5-FU-induced apoptosis[19]. PRDX2 is highly expressed in patients with osteosarcoma who have poor response to induction chemotherapy[6]. In addition, PRDX2 depletion increases the sensitivity of osteosarcoma cells to chemotherapeutic drugs[20]. What's more, PRDX2 depletion significantly promotes cell death of cancer cells treated with DNA damaging agents[21]. It's in agreement with our results that siPRDX2 induces cells apoptosis by flow cytometry and PI/Hoechst33342 double dye assays. In colorectal cancer, the expression of PRDX2 in HCT116 cells in siPRDA2 group is significantly lower than that of NC group. In addition, the invasive capabilities of HCT116 cells are significantly declined in PRDX2 siRNA explants than those of NC siRNA explants[22]. But in melanoma, silencing of PRDX2 contributes to enhanced proliferation, motility and metastasis of melanoma cells[23]. In this study, the proliferation of ES cells was significantly decreased after knocking down PRDX2 by CCK8 and colony formation assays. Furthermore, siPRDX2 inhibited the invasion and migration of A673 and RDES cells by transwell assay, which hinted that PRDX2 may promote ES metastasis. In squamous

cervical carcinoma, it is characterized by overexpression of PRDX2 and NCF2[24]. In pancreatic cancer, the studies of gemcitabine-sensitive proteomics in KLM1 and resistant KLM1-R cells show a significant up-regulation of PRDX2 in KLM1-R cells[25]. These all hint that PRDX2 acts as an oncogene in carcinoma. Here we detected PRDX2 inhibited the motility and induced apoptosis in ES cell. These results indicated that PRDX2 was involved in the progression of carcinoma cells and may play an oncogene function in ES.

Despite the effort of PRDX2 on ES cells, the accurate mechanism by which PRDX2 plays an important role in the development of ES remains unidentified. The PI3K / Akt / mTOR pathway is a prototype survival pathway that is constitutively activated in many types of cancer[26]. Additionally the PI3K / Akt / mTOR pathway is an attractive therapeutic target in cancer because it acts as a meeting point for many growth stimuli and controls the cellular processes that contribute to cancer development and maintenance through its downstream substrates. Furthermore, activation of the Akt/mTOR pathway confers resistance to many types of cancer treatment[26]. As described in previous studies, in colon cancer cells, silencing of PRDX2 promotes 5-FU-induced apoptosis via the PI3K/AKT signal pathway[19]. Recently, PRDX2 gene deficiency reduces p-AKT expression and PI3K/AKT pathway inhibition promotes cells apoptosis. Here, we found that the dysfunction of PRDX2 inhibited the activation of AKT / mTOR signaling pathway and induced cells apoptosis. These results indicated that the AKT / mTOR signaling pathway contributes to siPRDX2 induced cells apoptosis.

Conclusion

Collectively, our research indicates that PRDX2 functions as an oncogene in the development of ES, and the AKT / mTOR signaling pathway is involved in this process. These findings lay the foundation for PRDX2 as a novel prognostic marker and therapeutic target for the diagnosis and treatment of ES.

Declarations

Acknowledgements

Authors' contributions

RX, ZF, YA participated in the study design, performed data analysis and data interpretation. RX wrote the manuscript. RX, ZF, YA participated in data collection assisted in literature search and provided critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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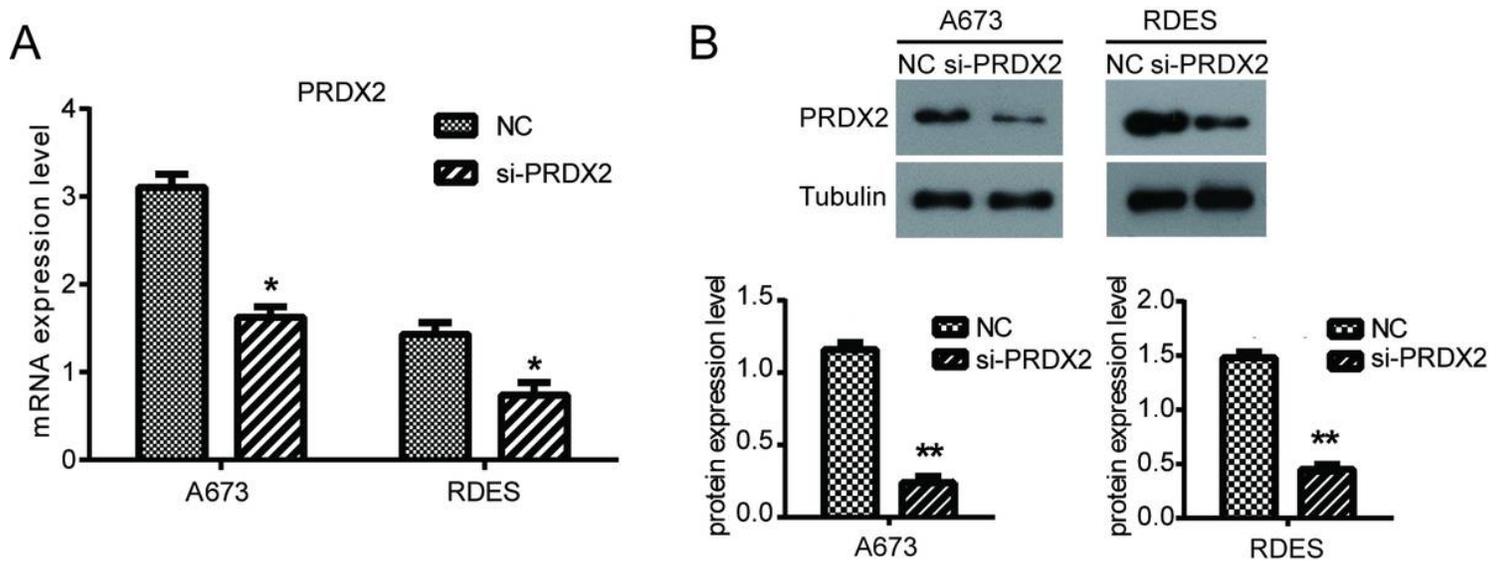
Figures

Figure 1

A673 and RDES cell lines were successfully transfected with siPRDX2 (A) qRT-PCR assay demonstrated siRNA efficiently inhibited the expression of PRDX2 in both A673 and RDES cells. (B) Western blot verified the inhibition of PRDX2 expression in protein level. * represents P<0.05, ** represents P<0.01 compared with NC.
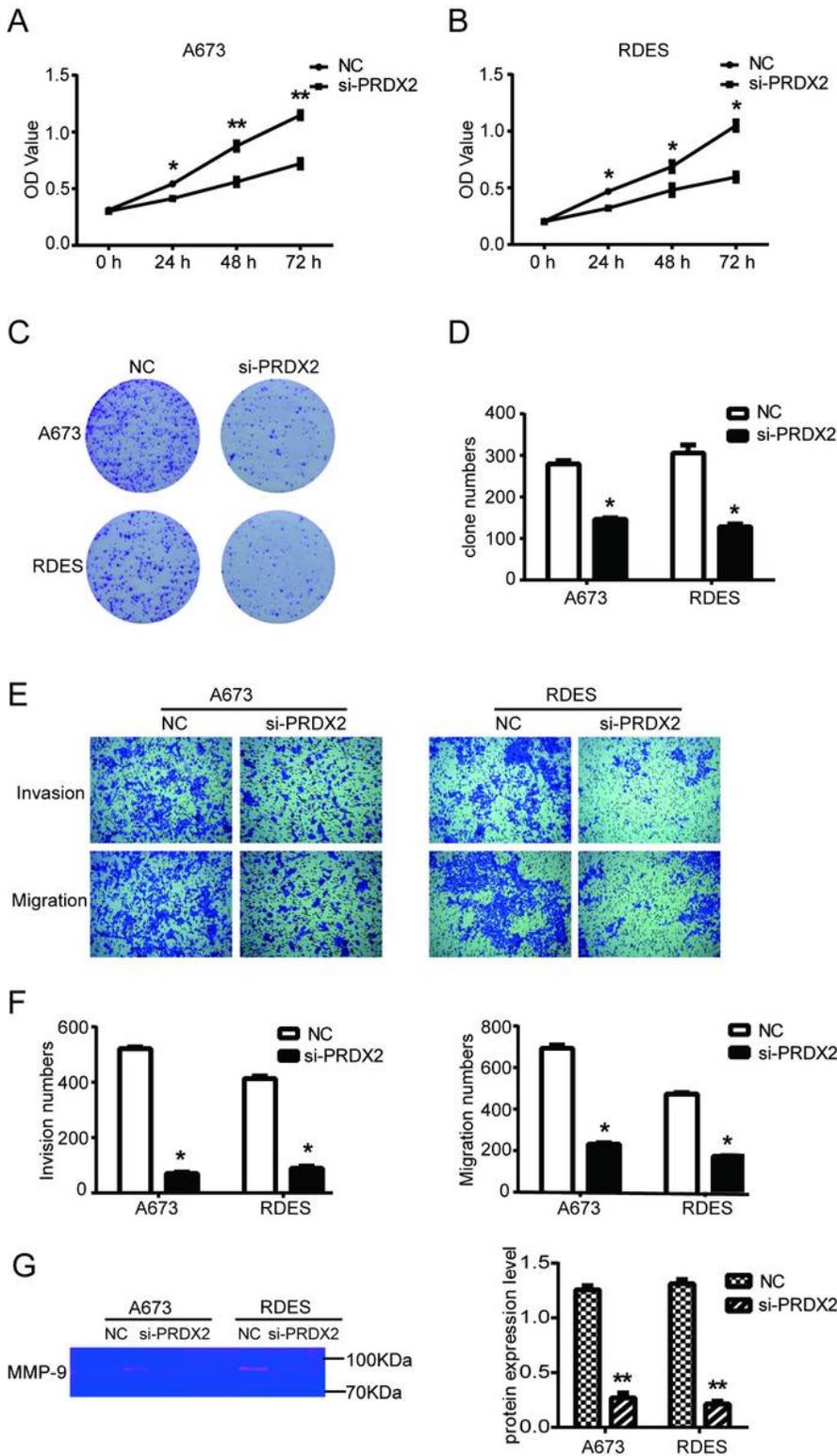
Figure 2

Knockdown of PRDX2 inhibited the motility of A673 and RDES cells (A and B) CCK-8 assay detected the proliferation of A673 and RDES cells. (C and D) Cell proliferation also detected by clone formation assay. (E and F) Transwell assay verified siPRDX2 inhibited the invasion and migration of both A673 and RDES cells. (G) The effect of siPRDX2 on MMP9 expression was detected by gelatin zymography assay. * represents P<0.05, ** represents P<0.01 compared with NC.
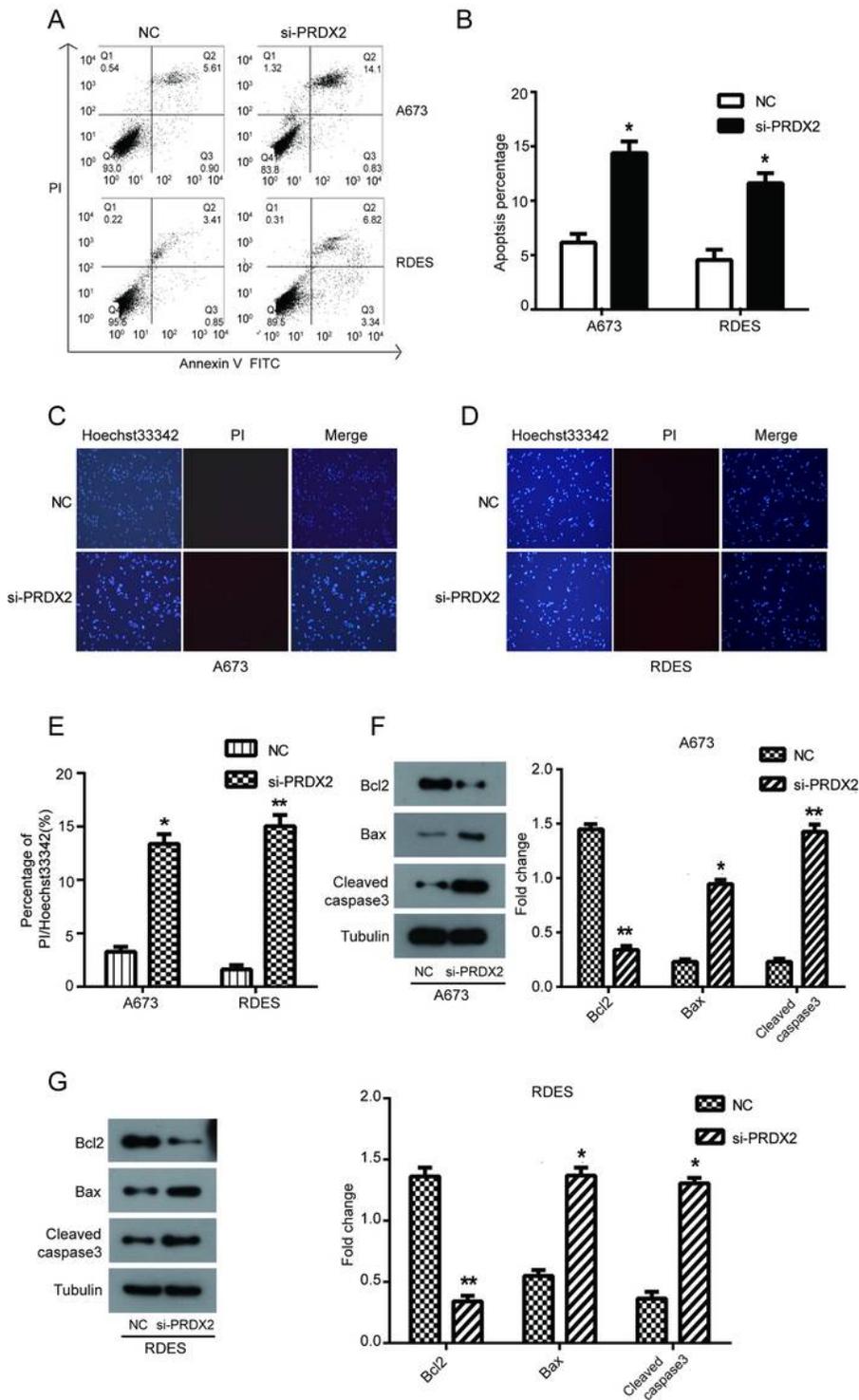
Figure 3

Knockdown of PRDX2 induced the apoptosis of A673 and RDES cells (A and B) Flow cytometry assay detected the percentage of apoptotic cells was increased in siPRDX2 group. (C-E) Increasing the percentage of apoptotic cells in siPRDX2 group also was verified by PI/Hoechst33342 double dye assay. (F and G) Western blot assay detected the expression of apoptosis-associated proteins Bcl2, Bax and cleaved Caspase 9. * represents $P<0.05$, ** represents $P<0.01$ compared with NC.
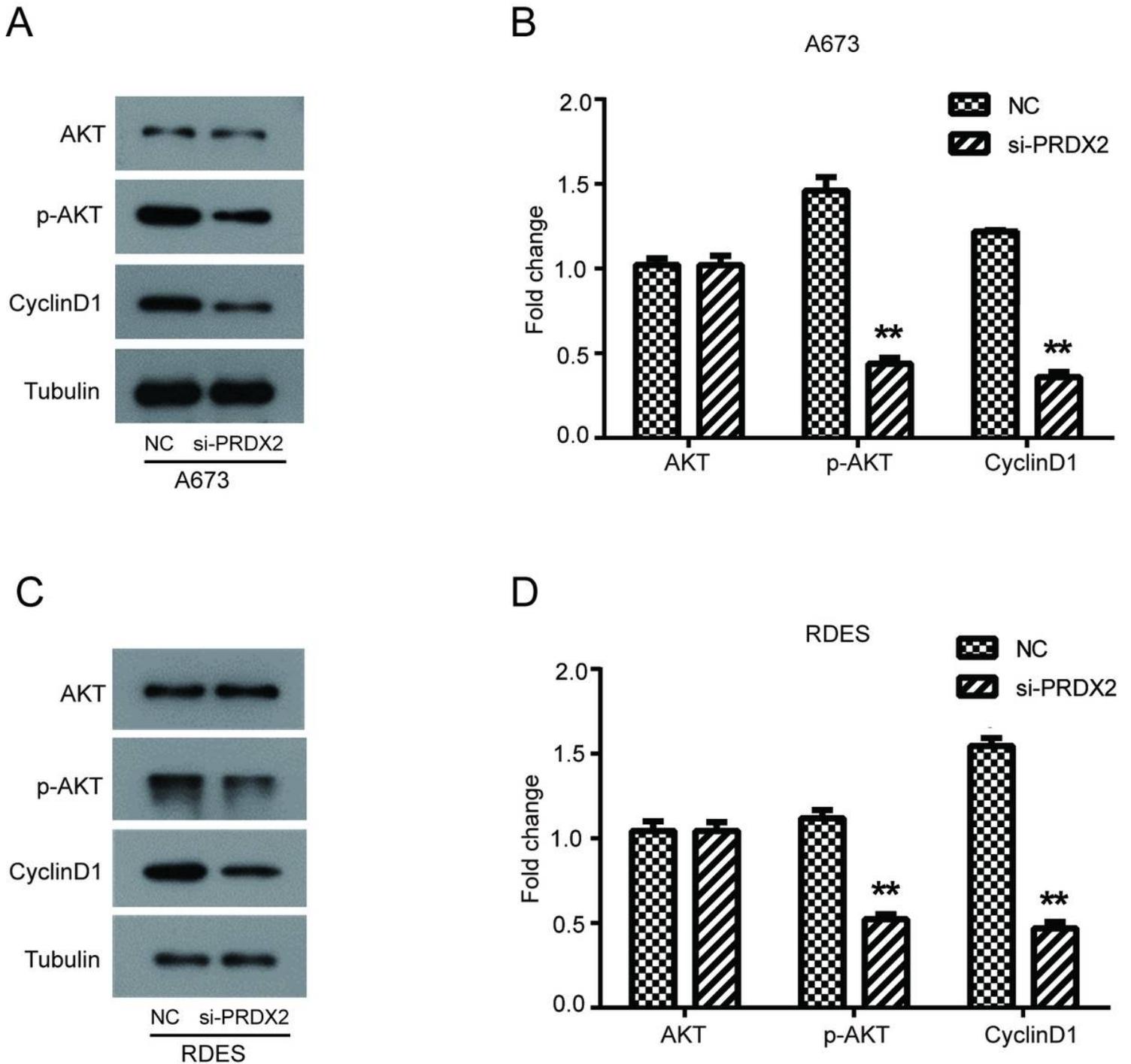
Figure 4

Knockdown of PRDX2 inhibited the activation of AKT/mTOR signaling pathway in A673 and RDES cells (A-D) Western blot image and quantification assays indicated that siPRDX2 reduced the phosphorylation of AKT, as well as the expression of the downstream effector CyclinD1. * represents P<0.05, ** represents P<0.01 compared with NC.