JBUON 2019; 24(4): 1464-1469
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Silencing of PPMD1 inhibits proliferation of human colon cancer cells via induction of apoptosis and cell cycle arrest

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Summary

Purpose: *Accumulating reports have shown the oncogenic properties of PPMD1 (protein phosphatase, Mg^{2+}/Mn^{2+} dependent 1D) in different cancer types. This study was undertaken to explore the role and therapeutic potential of PPM1D in colon cancer.*

Methods: *HT-29 colon cancer cell line was used in this study. Expression analysis of PPMD1 was performed by qRT-PCR. Cell viability was determined by CCK-8 assay. DAPI, acridin orange/ethidium bromide (AO/EB) and propidium iodide (PI) staining assays were used for apoptosis detection. Cell cycle analysis was performed by flow cytometry. Protein expression was determined by western blot analysis.*

Results: *The results showed that the expression of PPMD1 was significantly upregulated in colon cancer by 3.2 to 4.8 fold. Silencing of PPMD1 caused significant decline in the proliferation rate of the HT-29 colon cancer cells that was due to induction of apoptosis as evidenced by DAPI, AO/EB and PI staining. Annexin V/PI showed a significant increase in the percentage of apoptotic of HT-29 cells upon silencing of PPMD1. The induction of apoptosis was also accompanied by increase in Bax and decrease in Bcl-2 expression. PPMD1 silencing also resulted in arrest of the HT-29 cells in the G2/M phase of the cell cycle which was also associated with upsurge of p21 and p53 and depletion of cyclin B1 expression levels. PPMD1 silencing also caused decrease in the viability of the HT-29 cells which was concomitant with suppression of MMP-2 and MMP-9 expression.*

Conclusion: *These findings suggest that PPMD1 has oncogenic properties in colon cancer and exhibit therapeutic implications in colon cancer treatment.*

Key words: *PPMD1, colon cancer, apoptosis, cell cycle arrest, invasion*

Introduction

PPM1D (protein phosphatase, Mg^{2+}/Mn^{2+} dependent 1D) has been reported to be dysregulated in several types of cancer and has been reported to exhibit therapeutic properties [1]. Belonging to the PPC2 family of serine/threonine protein phosphatase, has been shown to be upregulated in lung cancer, bladder cancer and pancreatic cancer to name a few [2-4]. PPM1D plays a diversity of roles in biological processes such as proliferation and apoptosis [5]. It exhibits oncogenic properties, and overexpression of PPMD in lung adenocarcinoma has been associated with poor prognosis and patient survival [6]. In yet another study, PPM1D has been shown to augment the tumorigenesis of

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Received: 30/12/2018; Accepted: 16/01/2019

mice mammary glands epithelia[7]. Its upregulated expression has also been reported in colon cancer [8], however, the role and therapeutic implications of PPM1D have not been investigated so far in this malignancy. This study was therefore undertaken to decipher the expression profile, role and therapeutic potential of PPMD1.

Colon cancer is the fourth principal cause of cancer-related mortality. It ranks as third common type of cancer and around 1.4 million new cases of colon cancer are reported annually [9]. In 2013, around 0.7 million deaths were reported to be attributed to colon cancer worldwide [10]. Although, the incidence of colon cancer has declined to some extent, it is believed it will increase by 60% till 2030 [11]. Late diagnosis and dearth of potent and safe chemotherapeutic drugs and therapeutic targets form a considerable obstacle in the treatment of colon cancer [12].

In this study, we examined the expression of PPMD1 in four different colon cancer cell lines and one normal cell line and explored its potential as therapeutic target.

Methods

Cell lines and transfections

The normal colon cell line CDD-18Co and colon cancer cell lines HT-29, RKO, SW948 and SW480 were procured from American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium in CO_2 incubator at 37°C with 98% humidity and 5% CO_2. Transfection of negative control (NC) and Si-PPMD1 were carried out with the help of Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) as per the manufacturer's protocol.

cDNA synthesis and quantitative RT-PCR

The total RNA was extracted from the normal and colon cancer cell lines using RNeasy kits (Qiagen, Inc., Valencia, CA, USA). The cDNA was synthesized using the Omniscript RT (Qiagen, Inc.) from 1 µg of RNA. The cDNA was used for the expression analysis by Taq PCR Master Mix kit (Qiagen, Inc.) as per the manufacturer's guidelines. The reaction mixture consisted of 20 µl containing 1.5 mM $MgCl_2$, 2.5 units Taq DNA Polymerase, 200 µM dNTP, 0.2 µM of each primer and 0.5 µg DNA. The cycling conditions were as follows: 95°C for 30 sec, followed by 32 cycles of 95°C for 30 sec and 55°C for 30 sec. GAPDH was used as internal control and the relative quantification ($2^{-\Delta\Delta Cq}$) method was used to evaluate the quantitative variation between the samples.

Cell viability and colony formation assay

The CCK-8 assay was used for the determination of the cell viability. In brief, the transfected HT-29 cells were seeded in 96-well plates and incubated at 37°C for 24 h and subjected to treatment 10 microtiters of CCK-8 solution. The cells were then again subjected to incubation for 2 h at 37°C in a humidifier (5% CO_2/95% O2). Optical density $(OD)_{450}$ was taken at different time intervals (0, 12, 24, 48 and 96 h) with the help of a microplate reader. In brief, 1×10^3 transfected HT-29 cells were plated in 6-well culture plates. The cells were subsequently incubated for 21 days at 37°C in CO_2 incubator and colonies were stained with 0.5 ml of 0.1% crystal violet solution for 1 h and counted. Colonies were counted from three independent experiments and the results were expressed as a percentage of colonies with NC.

Apoptosis assay

The transfected colon cancer HT-29 cells (0.6×10^6) were seeded in 6-well plates and incubated for 12 h. Following incubation, the HT-29 cells were subjected to incubation for 24 h at 37°C. As the cells sloughed off, 10 µl cell cultures were put onto glass slides and stained with DAPI, AO/EB and PI. The slides were covered with covers slips and examined with a fluorescent microscope. Annexin V/PI staining of the SW-948 cells was performed as previously described [13].

Cell invasion assay

Cell invasion was performed by employing Boyden chamber assay. Briefly, the colon HT-29 cancer cells at the density of 3×10^4 cells per well were added in 12% fetal bovine serum (FBS) and put in the upper chamber of transwells (8 µm) with Matrigel. Thereafter, Dulbecco's modified Eagle's medium containing 5% FBS was supplied to the lower chamber and the cells were incubated for 24 h. The non-invaded cells on the membrane's upper surface were removed. However, the cells that invaded to the lower surface were fixed in 100% methanol and finally stained with Giemsa. The cells that invaded to lower surface were counted under microscope (Olympus, Tokyo, Japan).

Western blotting

The HT-29 cells were cultured for 24 h and then harvested by centrifugation. The cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in RIPA lysis buffer. Bradford assay was used to determine the protein content. From each sample 40 µg of protein was subjected to SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to treatment with Tris buffered saline (TBS) and exposed to primary antibodies at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

Statistics

Experiments were carried out in triplicate and values are presented as mean ± standard deviation. GraphPad prism 7 was used for statistical analyses. Student's t-test was performed for comparison between two samples and $p<0.05$ indicated a statistically significant difference.

Results

PPM1D is overexpressed in colon cancer

The expression of PPM1D was carried out in one normal and four colon cancer cells with the help of quantitative RT-PCR. The results showed that, relative to the normal cells, the expression of PPM1D was significantly enhanced in colon cancer cells, been 3.2 to 4.8 fold higher (Figure 1A). The highest fold upregulation of PPMD1 was found in case of the HT-29 cells and therefore this cell line was taken forward for further experimentation. To unveil the role of PPM1D in colon cancer, its expression was silenced (Figure 1B) and the results revealed that PPM1D silencing resulted in significant decline in the proliferation rate and colony formation of the HT-29 cells (Figure 1C and 1D).

Silencing of PPM1D triggers apoptosis in HT-29 colon cancer cells

To decipher the reason behind the decrease in the proliferation of the Si-PPM1D transfected HT-29 cells, different apoptosis assays, such DAPI, AO/EB and PI staining, were performed (Figure 2A). In case of AO/EB assay it was observed that the orange red color of the nuclei increased as the expression of PPM1D was silenced. In DAPI staining assay, the DAPI-positive cells were higher in Si-PPM1D transfected cells. PI staining also revealed increase in the number of PI-positive cells. All these results indicated that PPM1D silencing triggered apoptosis in the HT-29 colon cancer cells. Annexin V/PI staining revealed that the apoptotic cell percentage increased from 5.4% in NC to 64.9% Si-PPM1D cells (Figure 2B). The induction of apoptosis was further confirmed by expression analysis of Bax and Bcl-2 proteins by western blotting which showed that PPM1D silencing caused increase ($p<0.05$) in the expression of Bax, while the expression of Bcl-2 was decreased ($p<0.05$) (Figure 2C).

Silencing of PPM1D triggers G2/M arrest of HT-29 colon cancer cells

The effects of PP1MD silencing were also investigated on the distribution of the HT-29 cells in different phases of the cell cycle. The results

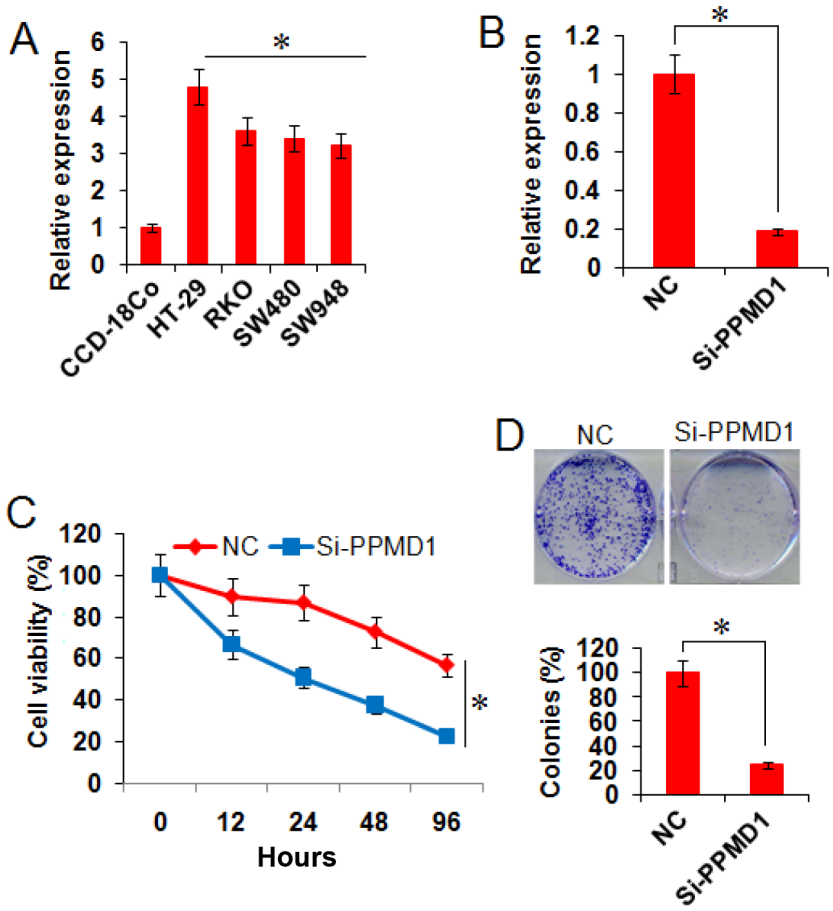

Figure 1. A: Expression of PPMD1 in normal and colon cancer cell lines. **B:** Expression of PPMD1 in NC and Si-PPMD1 transfected HT-29 cells. **C:** CCK-8 assay showing the viability of NC and Si-PPMD1 transfected HT-29 cells. **D:** Colony formation of the NC and Si-PPMD1 transfected HT-29 cells. The experiments were performed in triplicate and expressed as mean ± SD (*$p<0.05$).

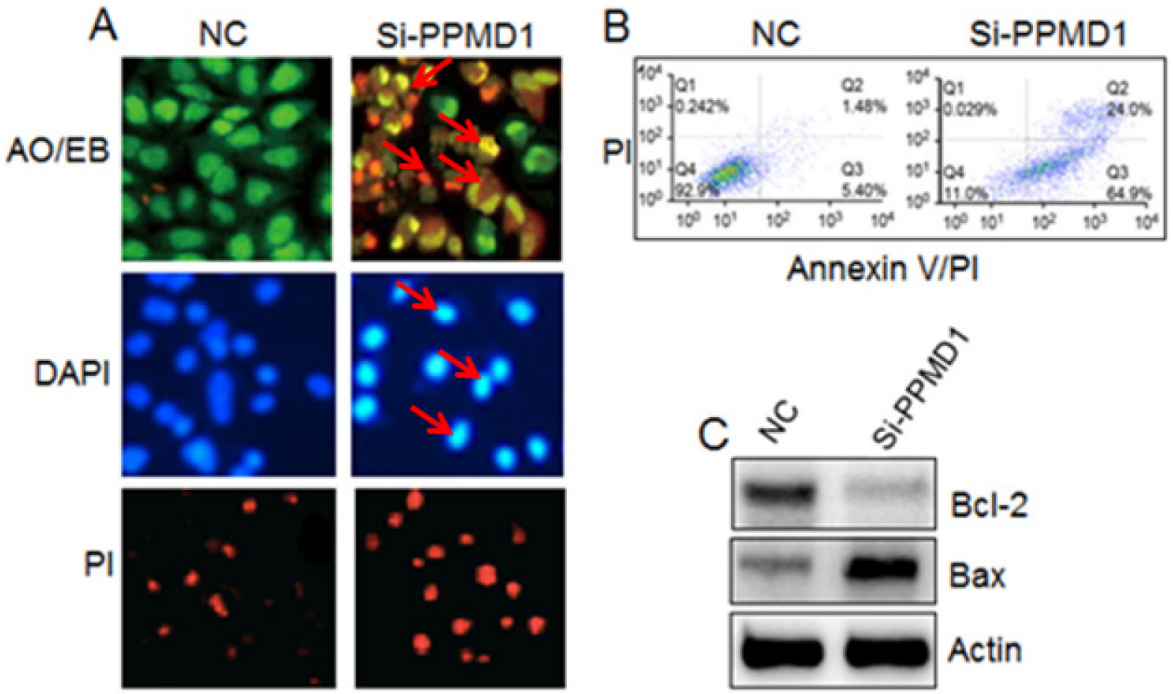

Figure 2. A: AO/EB, DAPI and PI staining of NC and Si-PPMD1 transfected HT-29 cells showing that PPMD1 silencing triggers apoptosis in the HT-29 colon cancer cells (arrows show apoptotic cells). **B:** Annexin V/PI staining of NC and Si-PPMD1 transfected HT-29 cells showing that suppression triggers apoptosis in the HT-29 colon cancer cells. **C:** Western blotting protein expression of Bcl-2 and Bax in NC and Si-PPMD1 transfected HT-29 cells showing that PPMD1 silencing increases Bax and decreases Bcl-2 expression. The experiments were performed in triplicate.

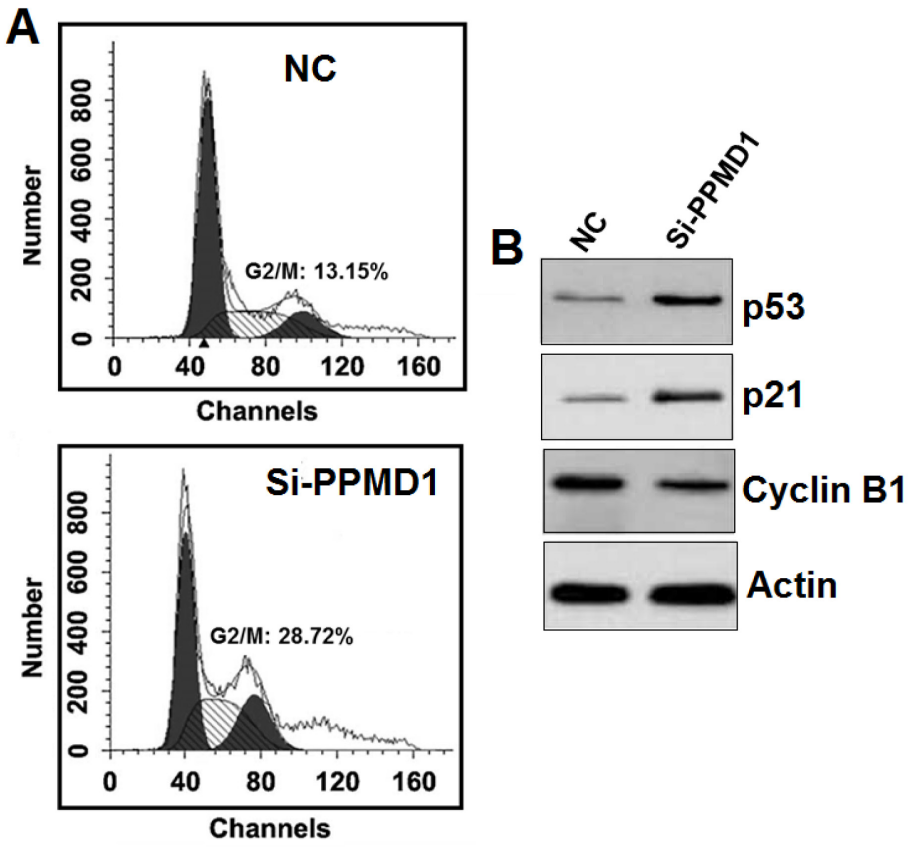

Figure 3. A: Cell cycle analysis of NC and Si-PPMD1-transfected HT-29 cells performed by flow cytometry, showing PPMD1 silencing triggers G2/M arrest of the HT-29 cells. **B:** Western blotting: p53, p21 and Cyclin B1 expression in NC and Si-PPMD1-transfected HT-29 cells showing that PPMD1 silencing causes inhibition of Cyclin B1 expression and upregulation of p21 and p53 expression. The experiments were performed in triplicate.

revealed that PPM1D caused remarkable increase in the G2/M phase of the cell cycle from 13.1% in NC to around 28.72% in the Si-PPMD1 cells, indicative of G2/M arrest ($p<0.05$) (Figure 3A). The G2/M arrest was further confirmed by examining the expression of the cell cycle related proteins. It was found that PPMD1 silencing increased the expression of p21 and p53 ($p<0.05$) while the expression of cyclin B1 decreased ($p<0.05$;Figure 3B).

Silencing of PPM1D inhibits invasion of HT-29 cells

The effects of PPM1D silencing were examined on the invasion of the HT-29 cells by transwell assay. The results showed that upon silencing of PPM1D, the invasion of HT-29 cells decreased significantly by 63% upon PPM1D silencing ($p<0.05$;Figure 4A). Western blot analysis revealed also that PPM1D silencing resulted in decrease in the expression of MMP-2 and 9 (Figure 4B).

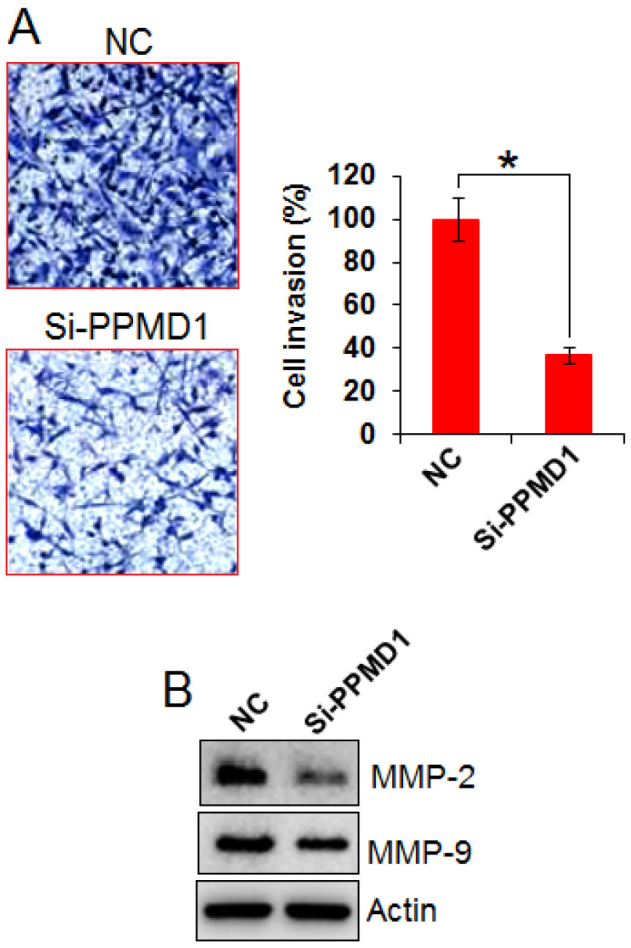

Figure 4. A: Cell invasion of the NC and Si-PPMD1-transfected HT-29 cells showing that PPMD1 silencing inhibits the HT-29 cell invasion, and **B:** Western blotting: MMP-2 and MMP-9 expression in NC and Si-PPMD1-transfected HT-29 cells: PPMD1 silencing causes decrease in the expression of MMP-2 and MMP-9. The experiments were performed in triplicate and expressed as mean±SD (*$p<0.05$).

Discussion

Colon cancer is one of the disastrousmalignancies. The clinical outcome of colon cancer is unsatisfactory and treatment strategies have a number of flaws. The currently available chemotherapeutic agents create considerable adverse effects, have moderate therapeutic potential and therapeutic targets are lacking [14]. The present study was designed to investigate the role and therapeutic potential of PPM1D in colon cancer. PPM1D has been reported to regulate tumorigenesis of different cancer types. Herein, we observed that PPM1D was significantly overexpressed in colon cancer and its suppression could decrease the proliferation rate of the colon HT-29 cells. Several previous studies also support the present findings, for example, PPM1D silencing has been reported to inhibit the growth of lung cancer and ovarian cancer cells [4,15]. Investigation of the molecular mechanisms underlying the decrease in the proliferation rate of the HT-29 cells was carried out by DAPI, AO/EB and PI staining and all of these assays showed that PPM1D silencing triggered apoptotic cell death of the HT-29 cells.

Bax and Bcl-2 are important marker proteins for apoptosis. The increase in the Bax/Bcl-2 expression ratio has been shown to favor apoptosis [16]. Herein, we observed that silencing of PPM1D enhanced the expression of Bax and decreased the expression of Bcl-2, favoring apoptosis. Moreover, PPM1D has also been reported to play a vital role in cell cycle regulation [17]by activating of check points Chk1 and Chk2 [17,18]. In this study, the results showed that silencing PPM1D causes arrest of the HT-29 cells at the G2/M cell cycle phase. Cyclins are important cell cycle regulators [19] and in this study we found that PPM1D silencing in HT-29 cells caused decrease in the expression of cyclin B1 and upsurge of p21 and p53. Previous studies have also indicated the role of PPM1D in the activation of p53 [20]. PPM1D has been shown to regulate the invasion of glioma cells [21]. Herein, the results of the Boyden chamber assay also showed that PPM1D silencing resulted in decreased invasion of HT-29 cells.

Conclusion

The findings of this study revealed that PPM1D is upregulated in colon cancer cells and silencing of PPM1D decreases the proliferation of HT-29 colon cancer cells via induction of apoptosis and cell cycle arrest. In addition, PPM1D silencing also reduces the invasion of the HT-29 cells. Taken to-

gether, PPM1D may have important therapeutic implications in the treatment of colon cancer.

Acknowledgements

This project was funded by the Hunan Provincial Natural Science Foundation(2017 JJ3230) and the Hunan Provincial Department of Education Science Research Project(17C1233).

Conflict of interests

The authors declare no conflict of interests.

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