Original Article
Silencing long noncoding RNA PVT1 inhibits tumorigenesis and cisplatin resistance of colorectal cancer

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Abstract: Long noncoding RNA plasmacytoma variant translocation 1 (PVT1) plays pivotal roles in tumorigenesis of many cancers, including colorectal cancer (CRC). However, the clinical significance and the biological functions of PVT1 in CRC remain largely unknown. In this study, we found that PVT1 was highly expressed in CRC tissues and cell lines compared with the corresponding non-cancerous samples and normal colon epithelial cells. Clinically, increased expression of PVT1 was positively correlated with tumor size, advanced histological stages, metastases, poor prognosis, and cisplatin resistance of CRC patients. In vitro studies showed that PVT1 silencing inhibited the proliferation, migration, invasion, and apoptosis escape of CRC cells. Knockdown of PVT1 in cisplatin-resistant CRC cells induced proliferation inhibition and apoptosis, whereas overexpression of PVT1 increased proliferation and decreased apoptosis of CRC cells. Mechanically, the levels of drug resistance-associated molecules, including multidrug resistance 1 and multidrug resistance protein 1, as well as the expression of anti-apoptotic Bcl-2 were significantly downregulated whereas the levels of pro-apoptotic Bax and cleaved caspase-3 were increased in PVT1-silenced cisplatin-resistant CRC cells. However, ectopic expression of PVT1 in CRC cells reversed the expressions of the molecules mentioned above. In addition, PVT1 overexpression in CRC cells significantly promoted cisplatin resistance in vivo. Collectively, these results demonstrated that PVT1 is a significant regulator in tumorigenesis and cisplatin resistance of CRC and provided evidence that PVT1 may be a promising target for CRC therapy.

Keywords: PVT1, colorectal cancer, tumorigenesis, cisplatin resistance

Introduction

Colorectal cancer (CRC) is the fourth most common diagnosed cancer and the second leading cause of cancer-related death worldwide [1]. The incidence of CRC has gradually increased due to environmental and lifestyle changes [2]. Despite great advances in radio- and chemotherapy combined with surgery, the five-year survival rate of CRC has not been improved owing to therapy failure [3]. Rapid growth, distant metastasis, and drug resistance are the major reasons for treatment failure of CRC [4]. Therefore, clarifying the underlying mechanisms and exploring new targets for CRC therapy are imperative.

Long noncoding RNAs (lncRNAs) are defined as transcripts comprising more than 200 nucleo-

tides in length without encoding proteins and previously believed to be transcriptional "noise" [5]. Increasing evidence revealed that lncRNAs involve in the physiological and pathological process of human diseases, including cancer [6, 7]. LncRNA plasmacytoma variant transcription 1 (PVT1) locates near Myc at human chromosome 8q24 [8] and plays oncogenic roles in a variety of cancers [9]. Upregulation of PVT1 contributes to the pathophysiology of ovarian and breast cancer [10]. PVT1 is much higher in gastric cancer patients and may be a novel predictor for gastric cancer [11]. PVT1 is significantly elevated in hepatocellular cancer tissues and patients with high expression of PVT1 has poor prognosis [12]. Guo et al. [13] found that the expression of PVT1 was higher in CRC tissues than that in normal tissues. Amplification of PVT1 is involved in poor prognosis of CRC via

Table 1. Correlation between PVT1 expression and clinicopathological characteristics in 112 CRC patients

Variables	Characteristics	PVT1 expression		P value
		High (n = 58)	Low (n = 54)	
Age (year)	< 60	25	25	0.206
	≥ 60	33	29	
Gender	Male	31	26	0.577
	Female	27	28	
Tumor size (cm)	≤ 5	26	37	0.018*
	> 5	32	17	
TNM stage	I/II	23	38	0.001*
	III/IV	35	16	
Lymph node metastasis	No	21	31	0.015*
	Yes	37	23	
Distant metastasis	No	28	40	0.007*
	Yes	30	14	

*$P < 0.05$.

apoptosis inhibition [14]. In addition, PVT1 can regulate the chemotherapy sensitivity in pancreatic cancer cells [15]. Overexpression of PVT1 promotes the development of cisplatin (DDP) resistance in gastric and ovarian cancer cells [16, 17]. However, the biological roles and the underlying mechanisms of PVT1 in CRC remain largely unknown.

In the present study, the expression of PVT1 was increased in CRC tissues and cell lines. Clinically, upregulation of PVT1 was positively associated with tumor size, advanced histological grades, metastases, poor prognosis, and cisplatin resistance of CRC patients. In vitro studies showed that knockdown of PVT1 inhibited proliferation, migration, invasion, and apoptosis resistance of CRC cells. PVT1 silencing promoted cisplatin sensitivity of cisplatin-resistant CRC cells, whereas overexpression of PVT1 enhanced cisplatin resistance of CRC cells. PVT1 involved in cisplatin resistance of CRC cells via upregulation of drug resistance-associated molecules, including multidrug resistance 1 (MDR1) and multidrug resistance protein 1 (MRP1), by blocking the intrinsic apoptotic pathway. Consistently, ectopic expression of PVT1 in CRC cells promoted cisplatin resistance in vivo. Overall, these findings demonstrated that upregulation of PVT1 promotes tumorigenesis and cisplatin resistance of CRC and PVT1 may be an effective target for CRC therapy.

Material and methods

Patients and tissue samples

A total of 112 pairs of matched cancer and adjacent non-cancerous tissues were obtained from the CRC patients who underwent surgical resection in the First Affiliated Hospital of Xinxiang Medical University between April 2006 and March 2011. All specimens were confirmed by pathological examinations. None of the patients received preoperative radio- or chemotherapy. All patients were followed-up to 2016 or until death. The clinicopathological features of the patients are summarized in **Table 1**. In addition, tumor tissues from 30 CRC patients with cisplatin-sensitive and 30 patients with cisplatin-resistant were collected. This study was approved by the ethics committee of Xinxiang Medical University, and written informed consent was provided for all patients.

Cell culture

Human CRC cell lines (HT29, SW480, HCT116, RKO, and LoVo) and the normal colon epithelial cell line NCM460 were purchased from American Type Culture Collection (Manassas, VA, USA). Cisplatin-resistant LoVo/DDP and RKO/DDP cells were developed as previously described [16]. In brief, parental LoVo and RKO cells were subjected to persistent gradient exposure to cisplatin (Sigma-Aldrich, St. Louis, MO, USA) for 12 months, through increasing cisplatin concentration from 0.5 μg/mL until the cells acquired resistance to 10 μg/mL. Prior to each experiment, LoVo/DDP and RKO/DDP cells were cultured in drug-free Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) for 2 weeks. All the cells were cultured in DMEM supplemented with 10% of fetal bovine serum (FBS; Gibco), 100 U/mL of penicillin and 100 μg/mL of streptomycin (both from Sigma) in a humidified incubator with 5% CO_2 at 37°C.

Cell transfection and infection

Small interfering RNA specific for PVT1 (siPVT1: sense 5'-CCCAACAGGAGGACAGCUUTT-3' and antisense 5'-AAGCUGUCCUCCUGUUGGGTT-3')

Table 2. The primers used for qRT-PCR analyses

Genes	Primer sequences (5' to 3')	
PVT1	Forward	CAGCACTCTGGACGGAC
	Reverse	CAACAGGAGAAGCAAACA
MDR1	Forward	ACCAAGCGGCTCCGATACA
	Reverse	TCATTGGCGAGCCTGGTAGTC
MRP1	Forward	GGACCTGGACTTCGTTCTCA
	Reverse	CGTCCAGACTTCATCCG
GAPDH	Forward	ACCACAGTCCATGCCATCAC
	Reverse	TCACCACCCTGTTGCTGT A

and negative control siRNA (siNC) were synthesized by RiboBio Co. (Guangzhou, China). PVT1-overexpression lentiviral vector (LV-PVT1) and negative control lentiviral vector (LV-NC) were purchased from GenePharma (Shanghai, China). RKO, LoVo, RKO/DDP, and LoVo/DDP cells were transfected with 100 nM siPVT1 or siNC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The silence efficiency was analyzed by quantitative real-time PCR (qRT-PCR) assay 48 h after transfection. LoVo and RKO cells infected with LV-PVT1 and LV-NC at a multiplicity of infection of 200 PFU per cell. The stably-expressed cells were selected with G418 (500 mg/mL; Invitrogen) for 4 weeks.

qRT-PCR assay

Total RNA was extracted from tissues and cells using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from equal amounts of total RNA using the Prime Script™ RT reagent kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's protocol. Quantitative PCR was performed using SYBR Premix Ex Taq II (TaKaRa) on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, USA). The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primers used for PCR amplification are listed in **Table 2**.

Cell viability assay

Cell viability was measured by Cell Counting Kit-8 (CCK-8) assay. In brief, after the indicated oligonucleotides transfection or infection, the cells (1×10^3 cells/well) were seeded into 96-well plates (n = 5 for each time point) sup-

plemented 100 µL DMEM added 10% FBS with or without 10 µg/mL of cisplatin treatment. After incubation for 1, 2, 3, or 4 days, CCK8 (10 µL; Dojindo Laboratories, Kumamoto, Japan) was added to each well and cultured for 2 h. The optical density (OD) values were measured by using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm.

Cell apoptosis analysis by flow cytometry

Cell apoptosis was analyzed by flow cytometry using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). In brief, after the indicated treatments, the cells were collected and centrifuged. The cell pellets were resuspended in 1 × binding buffer at a concentration of 1×10^6 cells/mL. Cell suspension (100 µL) was incubated with 10 µL Annexin V-FITC for 15 min and counterstained with propidium iodide (PI) in the dark for 30 min. Apoptosis was analyzed by EPICS XL-MCL FACScan (Beckman Coulter, Brea, CA, USA) using the Cell-Quest software (Beckman Coulter).

Terminal transferase-mediated dUTP nick end labeling (TUNEL) assay

Cell apoptosis of the tumor tissues was detected by TUNEL assay using an In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. In brief, the sections of tumor tissues were incubated with the TUNEL reaction mixture at 37°C for 1 h in the dark. Labeled DNA was visualized with an anti-fluorescein antibody conjugated with peroxidase using 3,3'-diaminobenzidine (Sigma) as the chromogen. Sections were then washed and counterstained with hematoxylin (Sigma). For negative control, TdT was omitted from the reaction mixture. TUNEL-positive cells were imaged and mounted by a light microscope (Carl Zeiss, Berlin, Germany) and expressed as a percentage of the total cells.

Migration and invasion assays

Cell migration and invasion were determined by Transwell assays. For migration assay, the cells (2×10^4) with different treatments were seeded into the upper chamber of the Transwell inserts (8-µm pore size; Corning Costar, NY, USA) containing with serum-free DMEM. For

invasion assay, the members of the upper chamber were precoated with Matrigel (Sigma), and then, the cells were added into the upper chamber containing with serum-free DMEM. In the both assays, 500 µL DMEM with 10% FBS was added to the lower chamber for chemotaxis. After incubation for 24 h, the cells on the upper layer of the membrane were removed and those migrated or invaded through the membranes were fixed with methanol and stained with crystal violet (Sigma). Five randomly selected fields were observed under a fluorescence microscope (Olympus, Tokyo, Japan), and the average was calculated.

Western blot analysis

Proteins were extracted from the cultured cells with the indicated treatments using RIPA buffer (Beyotime Biotechnology, Shanghai, China). The lysates were centrifuged at 10,000×g for 20 min, and the supernatants were collected for western blot analysis. BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL, USA) was used to measure the protein concentration. Equal amounts of proteins were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membranes (Millipore Corp., Bedford, MA, USA). After blocking with 5% non-fat milk in Tris-buffered saline and Tween 20 for 1 h at room temperature, the membranes were incubated with primary antibodies against MDR1, MRP1, Bax, Bcl-2, cleaved (cl)-caspase-3, caspase-3, and GAPDH (all from Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. The membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma) and the blot signals were visualized by enhanced chemiluminescence kit (Santa Cruz, Dallas, TX, USA).

Animal experiments

All animal experiments were approved by the Animal Research Committee of Xinxiang Medical University and were carried out in accordance with the International Guiding Principles for Animal Research. For xenograft tumors, six-week old male athymic nude mice (Institute of Zoology, Chinese Academy of Sciences, Beijing, China) received subcutaneous injection with uninfected or LV-NC- or LV-PVT1-infected RKO cells (5×10^6) into the right flanks. Tumor volume was calculated according to the following

formula: volume = (length × width^2)/2. Once the tumor size reached 60 mm^3, the mice implanted with the uninfected cells were treated with normal saline or cisplatin (5 mg/kg), and the animals implanted with LV-NC- or LV-PVT1-infected cells received cisplatin (5 mg/kg). Collectively, four groups, including control, cisplatin, LV-NC + cisplatin, and LV-PVT1 + cisplatin groups were divided in this study. Five mice were used for each group. Mice in the cisplatin-treated groups were given cisplatin by intraperitoneal injection twice per week, a total of six times. At six weeks after cell implantation, the animals were sacrificed and tumors were dissected out, measured, and weighed. The tumor tissues were sectioned and used for TUNEL analysis.

Statistical analysis

Statistical analyses were performed using SPSS 20.0 (Chicago, IL, USA). Data are expressed as mean ± SD. Student's *t*-test and one-way analysis of variance were used to determine the statistical significance between two groups or in multiple groups, respectively. Chi-squared tests were used to evaluate frequencies. The five-year survival curves were plotted with the Kaplan-Meier method and analyzed by the log-rank test. A value of *P* < 0.05 was considered as statistically significant.

Results

Upregulation of PVT1 is positively associated with progression, prognosis, and cisplatin-resistance of CRC

To explore the expression profiles of PVT1 in CRC, qRT-PCR analysis was performed in 112 pairs of CRC samples and adjacent non-cancerous tissues. The results showed that PVT1 was highly expressed in the cancer samples compared with the non-cancerous tissues (**Figure 1A**). Furthermore, the levels of PVT1 were much higher in the patients with advanced histological grades (III/IV) and in the cases with lymphatic and distant metastases (**Figure 1B** and **1C; Table 1**). The expression of PVT1 was also associated with tumor size but had no correlation with age and gender (**Table 1**). Meanwhile, the patients with low level of PVT1 had higher five-year survival rate than those with high expression of PVT1 (**Figure 1D**). In addition, PVT1 expression was significantly elevated in
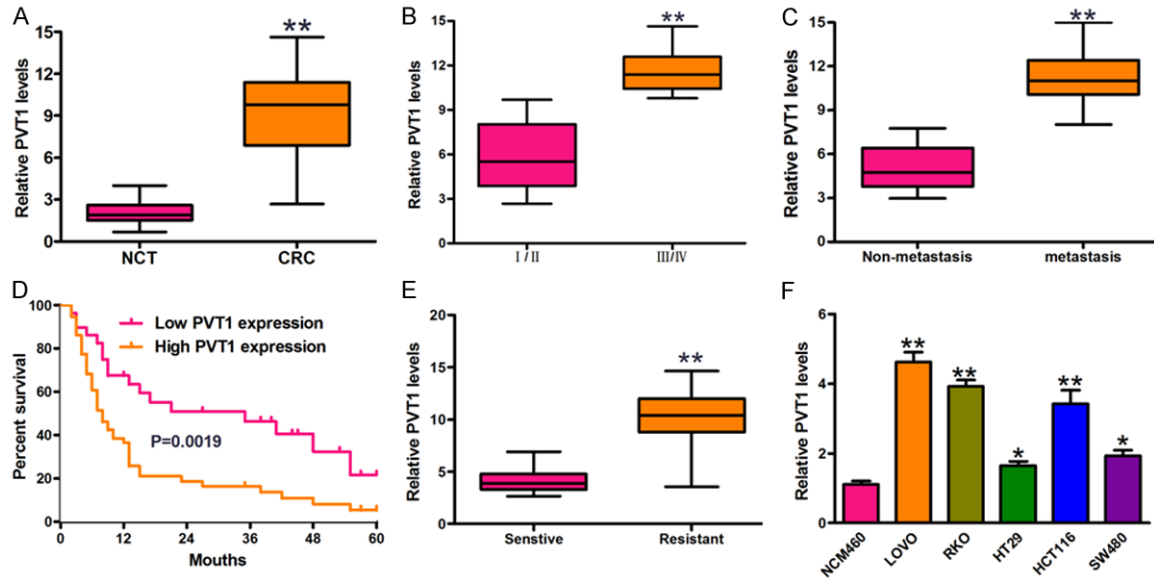
Figure 1. Upregulation of PVT1 in CRC tissues and cells and its clinical significance. (A) Relative expression of PVT1 in CRC samples (n = 112) and adjacent non-cancerous tissues (NCT; n = 112) was measured by qRT-PCR and normalized to GAPDH. (B and C) Comparisons of the levels of PVT1 in CRC patients with different tumor stages (B; I/II, n = 61; III/IV, n = 51) and in the cases with (n = 60) or without (n = 52) lymph node metastasis (C). (D) The five-year survival rate of the patients with high (n = 58) and low (n = 54) expression of PVT1 was plotted by Kaplan-Meier method. (E) The levels of PVT1 in CRC samples of the cisplatin-sensitive (n = 30) and cisplatin-resistant (n = 30) patients. (F) The expression of PVT1 in five CRC cell lines (HT29, SW480, HCT116, RKO, and LoVo) and in the normal colon epithelial cells (NCM460). All values are represented as mean ± SD of three replicates. *P < 0.05 compared with NCM460 cells in (F); **P < 0.01 compared with NCT group in (A), I/II stages in (B), non-metastasis group in (C), cisplatin-sensitive group in (E), and NCM460 cells in (F). NCT: non-cancerous tissues; CRC: colorectal cancer tissues.
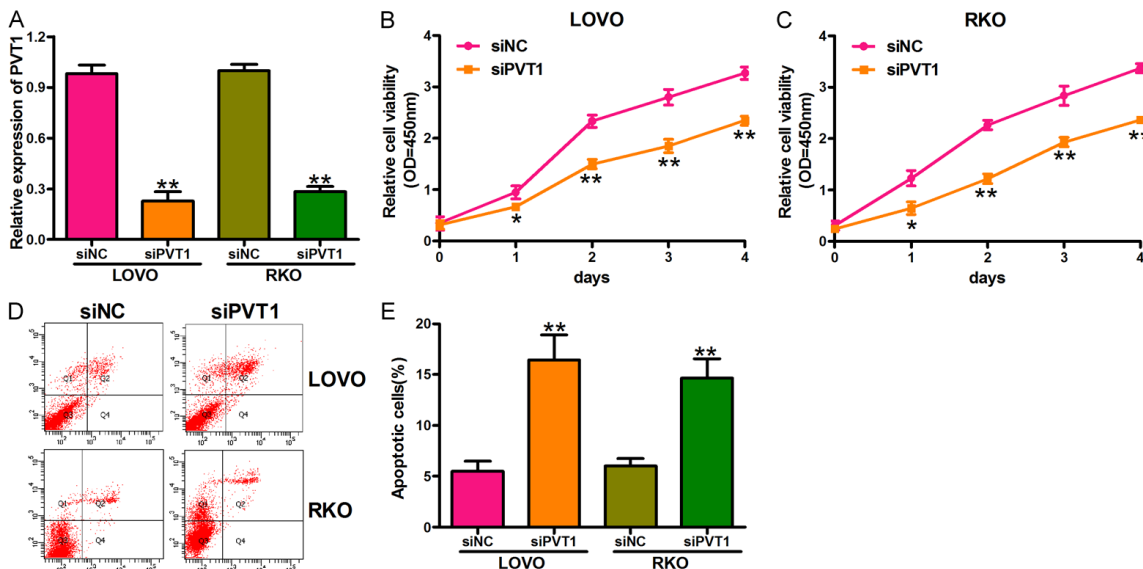

Figure 2. Knockdown of PVT1 suppressed proliferation and apoptosis resistance of CRC cells. LoVo and RKO cells were transfected with siPVT1 or siNC for 48 h. (A) The inhibitory efficiency of siPVT1 transfection on the expression of PVT1 was measured by qRT-PCR assay. (B and C) Cell viability was measured by CCK8 assay at the indicated time points in LoVo (B) and RKO (C) cells. (D) Flow cytometry assay was performed to detect cell apoptosis. (E) The percentage of apoptotic cells was calculated in (D). All values are represented as mean ± SD of three replicates. *P < 0.05, **P < 0.01 compared with siNC group.
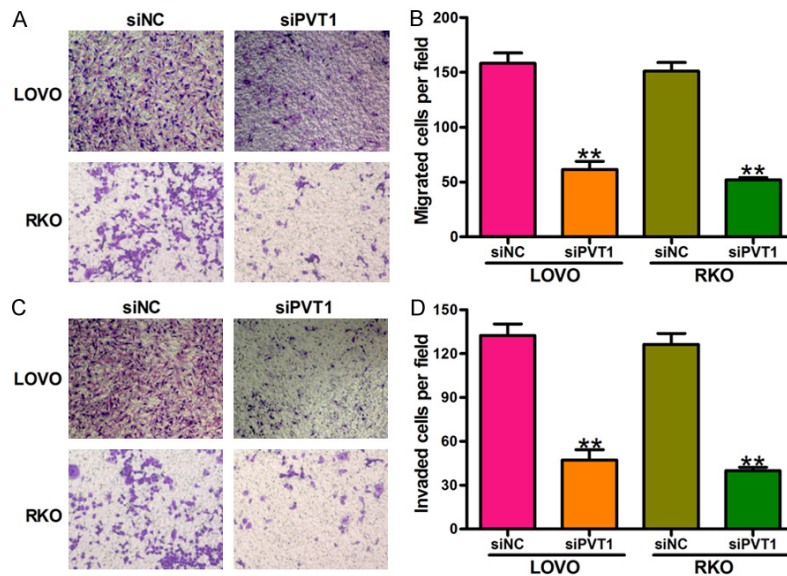
Figure 3. Silencing of PVT1 inhibited the migratory and invasive abilities of CRC cells. LoVo and RKO cells were transfected with siPVT1 or siNC for 48 h. (A and C) Transwell assays were performed to evaluate the migration (A) and invasion (C) of siPVT1- or siNC-transfected LoVo and RKO cells. (B and D) The number of migrated (B) and invaded (D) cells was calculated. All values are represented as mean ± SD of three replicates. **$P < 0.01$ compared with siNC group.

to significant elevation of the apoptotic LoVo and RKO cells (**Figure 2D** and **2E**). These results demonstrated that PVT1 silencing inhibits proliferation and induces apoptosis of CRC cells.

PVT1 depletion inhibits migration and invasion of CRC cells

To address the roles of PVT1 in the regulation of CRC cell migration and invasion, Transwell assays were performed. The results showed that knockdown of PVT1 significantly suppressed the migration of LoVo and RKO cells (**Figure 3A** and **3B**). Similarly, the invasive ability of LoVo and RKO cells was also reduced by siPVT1 transfection (**Figure 3C** and **3D**). These data suggested that PVT1 silencing suppresses migratory and invasive abilities of CRC cells.

PVT1 knockdown enhances the cisplatin sensitivity of cisplatin-resistant CRC cells

To explore the association of PVT1 and cisplatin-resistance in CRC cells, two cisplatin-resistant cell lines, LoVo/DDP and RKO/DDP, were established. Consistent with the results in CRC tissues, PVT1 expression was much higher in LoVo/DDP and RKO/DDP cells than that in LoVo and RKO cells, respectively (**Figure 4A**). To determine the effects of PVT1 knockdown on the proliferation and apoptosis of LoVo/DDP and RKO/DDP cells, siPVT1 and siNC were transfected into LoVo/DDP and RKO/DDP cells. As shown in **Figure 4B**, siPVT1 transfection effectively inhibited the expression of PVT1 in LoVo/DDP and RKO/DDP cells. The results of CCK8 assay showed that the viability of cisplatin- or (siNC + cisplatin)-treated LoVo/DDP and RKO/DDP cells had no significant difference with the control cells. However, cisplatin significantly reduced the viability of PVT1-depleted LoVo/DDP and RKO/DDP cells (**Figure 4C** and **4D**). In addition, cisplatin enhanced the apoptosis of siPVT1-transfected LoVo/DDP and RKO/

the tumors derived from cisplatin-resistant patients compared with those from cisplatin-sensitive patients (**Figure 1E**). To further investigate the association between PVT1 and CRC, we examined the levels of PVT1 in five CRC cell lines. As shown in **Figure 1F**, PVT1 expression was much higher in CRC cells than that in the normal colon epithelial cells. LoVo and RKO cells which had the highest PVT1 level were selected for the subsequent experiments. These results indicated that upregulation of PVT1 is positively associated with tumor size, advanced histological grades, metastases, poor outcomes, and cisplatin resistance of CRC.

Knockdown of PVT1 suppresses proliferation and promotes apoptosis of CRC cells

To investigate the functions of PVT1 in the proliferation and apoptosis of CRC cells, siPVT1 and siNC were transfected into LoVo and RKO cells. As shown in **Figure 2A**, qRT-PCR analyses confirmed that PVT1 expression was significantly repressed in LoVo and RKO cells transfected with siPVT1. The viability of siPVT1-transfected LoVo and RKO cells was markedly decreased compared with those cells with siNC transfection (**Figure 2B** and **2C**). Flow cytometry assay showed that siPVT1 transfection led
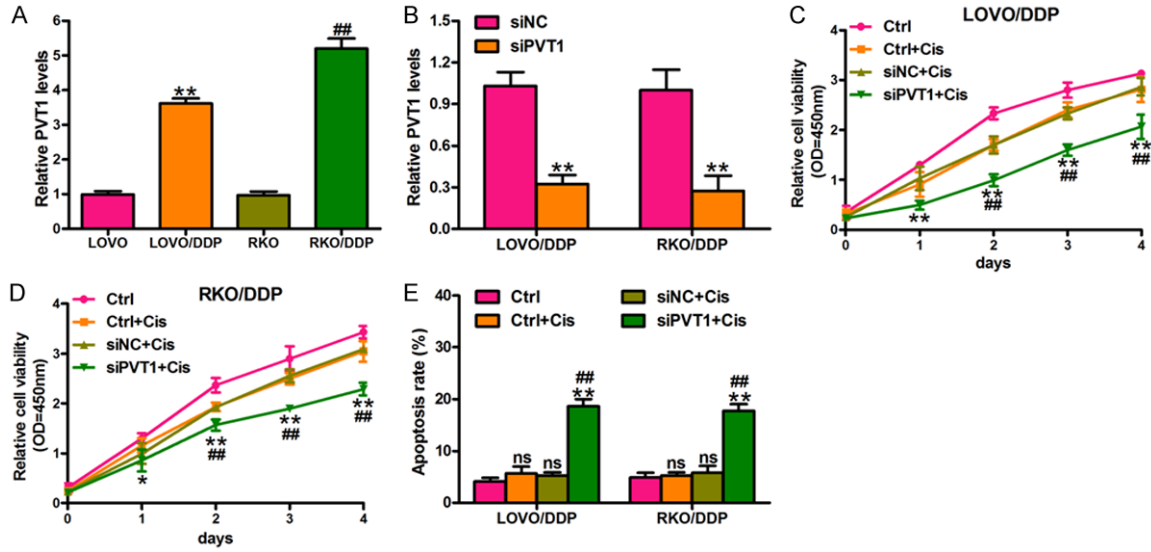
Figure 4. PVT1 depletion enhanced the cisplatin sensitivity of cisplatin-resistant CRC cells. (A) The differential expressions of PVT1 between LoVo and RKO cells and the cisplatin-resistant LoVo/DDP and RKO/DDP cells. (B) The inhibitory efficiency of siPVT1 transfection on the expression of PVT1 in LoVo/DDP and RKO/DDP cells. (C-E) LoVo/DDP and RKO/DDP cells were treated with vehicle (control) or 10 μg/mL of cisplatin or the cells were pre-transfected with siNC or siPVT1, followed by vehicle or cisplatin treatment. CCK8 assays were performed to measure cell viability (C and D) and flow cytometry was conducted to assess cell apoptosis (E). All values are represented as mean ± SD of three replicates. *$P < 0.05$, **$P < 0.01$ compared with LoVo cells in (A), siNC group in (B), and Ctrl group in (C-E); ##$P < 0.01$ compared with RKO cells in (A), and (Ctrl + Cis) or (siPVT1 + Cis) group in (C-E). Ctrl: control; Cis: cisplatin; ns: no significance.
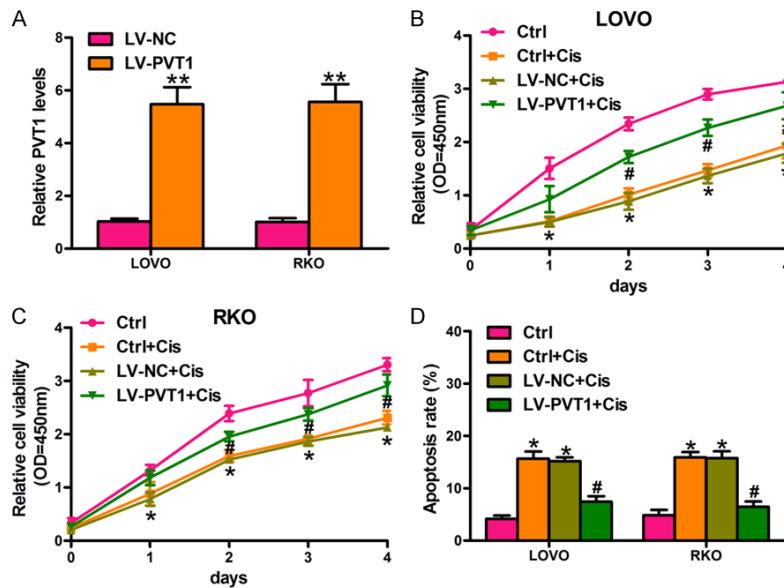

Figure 5. PVT1 overexpression enhanced the cisplatin resistance of CRC cells. (A) PVT1 expression in LoVo and RKO cells infected with LV-PVT1 and LV-NC was measured by qRT-PCR assay. (B-D) LoVo and RKO cells were treated with vehicle (control) or 10 μg/mL of cisplatin or the cells were pre-infected with LV-NC or LV-PVT1, followed by vehicle or cisplatin treatment. The viability of the cells was measured by CCK8 assay (B and C) and the apoptosis of the cells were evaluated by flow cytometry assay (D). All values are represented as mean ± SD of three replicates. *$P < 0.05$, **$P < 0.01$ compared with LV-NC group in (A) and Ctrl group in (B-D); #$P < 0.05$ compared with (Ctrl + cisplatin) or (LV-NC + cisplatin) group in (B-D). Ctrl: control; Cis: cisplatin.

DDP cells compared with those cells with siNC transfection or treated with cisplatin alone or the control cells (**Figure 4E**). These findings indicated that PVT1 knockdown attenuates cisplatin resistance of the cisplatin-resistant CRC cells.

Overexpression of PVT1 promotes cisplatin resistance of CRC cells

We next sought to determine the effects of PVT1 overexpression on cisplatin resistance in CRC cells. Infection of LV-PVT1 into LoVo and RKO cells led to significant upregulation of PVT1 expression (**Figure 5A**). Cisplatin- or (LV-NC + cisplatin)-treated LoVo and RKO cells had much lower viability than that of the control cells. The viability of cisplatin-treated LoVo and
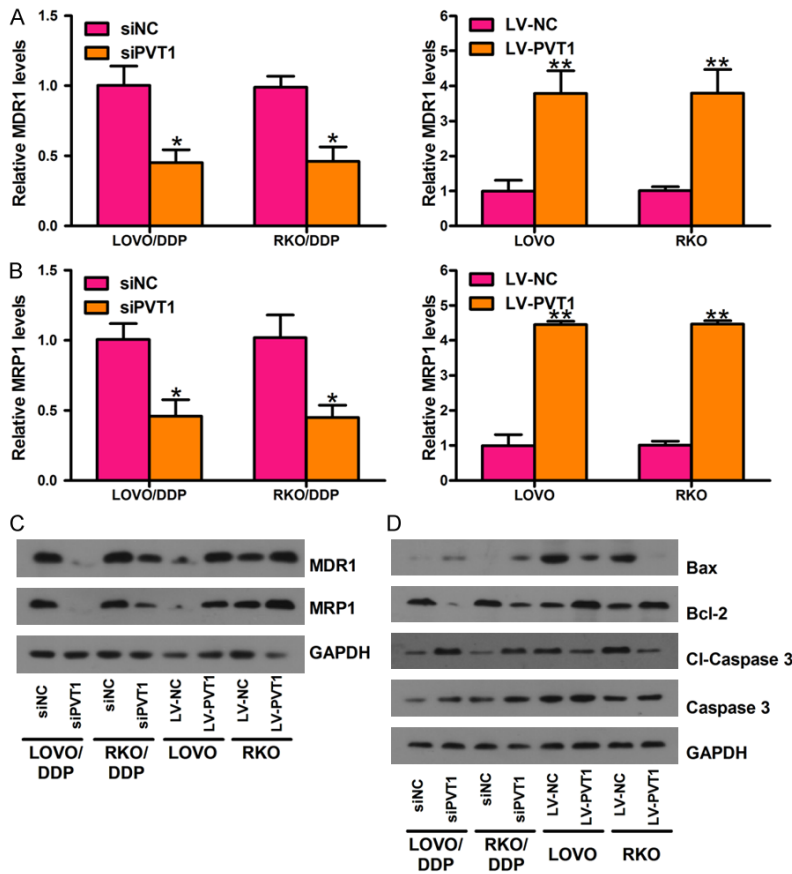
Figure 6. PVT1 promoted cisplatin resistance via inhibition of intrinsic apoptotic pathway in CRC cells. LoVo/DDP and RKO/DDP cells were transfected with siPVT1 or siNC, and LoVo and RKO cells were infected with LV-PVT1 or LV-NC. (A and B) mRNA expressions of MDR1 (A) and MRP1 (B) were detected by qRT-PCR assays. (C and D) Representative western blot results of MDR1 and MRP1 (C), and Bax, Bcl-2, caspase-3, and cl-caspase-3 (D). GAPDH was used as the normal control. All values are represented as mean ± SD of three replicates. *P < 0.05 compared with siNC group in (A and B; left panel); **P < 0.01 compared with LV-NC group in (A and B; right panel).

MRP1 were significantly downregulated in siPVT1-transfected LoVo/DDP and RKO/DDP cells but were markedly upregulated in LoVo and RKO cells with LV-PVT1 infection (**Figure 6A** and **6B**). Western blot analyses showed the similar results with the qRT-PCR assay (**Figure 6C** and Supplementary Figure 1). To investigate the mechanisms by which PVT1 enhances the cisplatin resistance in CRC cells, we measured the protein expression of intrinsic apoptotic-associated proteins. As shown in **Figure 6D** and Supplementary Figure 1, PVT1 depletion in LoVo/DDP and RKO/DDP cells enhanced the pro-apoptotic protein levels (Bax and cl-caspase-3) but decreased the anti-apoptotic Bcl-2 protein expression. However, PVT1 overexpression in LoVo and RKO cells reversed the protein expressions mentioned above. All these results demonstrated that PVT1 promotes cisplatin resistance via inhibition of intrinsic apoptotic pathway in CRC cells.

RKO cells with LV-PVT1 infection was significantly increased compared with the cells with LV-NC infection (**Figure 5B** and **5C**). The result of flow cytometry revealed that cisplatin markedly enhanced the apoptosis of uninfected or LV-NC-infected LoVo and RKO cells but did not influence that of the cells with PVT1 overexpression (**Figure 5D**). These results suggested that PVT1 overexpression enhances cisplatin resistance in CRC cells.

PVT1 promotes cisplatin resistance of CRC cells by inhibiting intrinsic apoptotic pathway

We subsequent determined the expression of drug resistance-associated molecules, including MDR1 and MRP1, by qRT-PCR and Western blot analyses. The mRNA levels of MDR1 and

Ectopic expression of PVT1 enhances cisplatin resistance of CRC cells in vivo

To confirm the effect of PVT1 in tumor growth and cisplatin resistance in vivo, a mouse xenograft model was established. The nude mice were subcutaneously injected with RKO cells uninfected or infected with LV-NC or LV-PVT1, followed by cisplatin treatment. As shown in **Figure 7A**, the results showed that cisplatin- and (LV-NC + cisplatin)-treated mice displayed much smaller tumor size than the control animals. However, PVT1 overexpression significantly attenuated the inhibition of cisplatin treatment on tumor growth. The tumor weight and tumor volume showed the similar results (**Figure 7B** and **7C**). TUNEL assay showed that cisplatin treatment led to more apoptotic cells
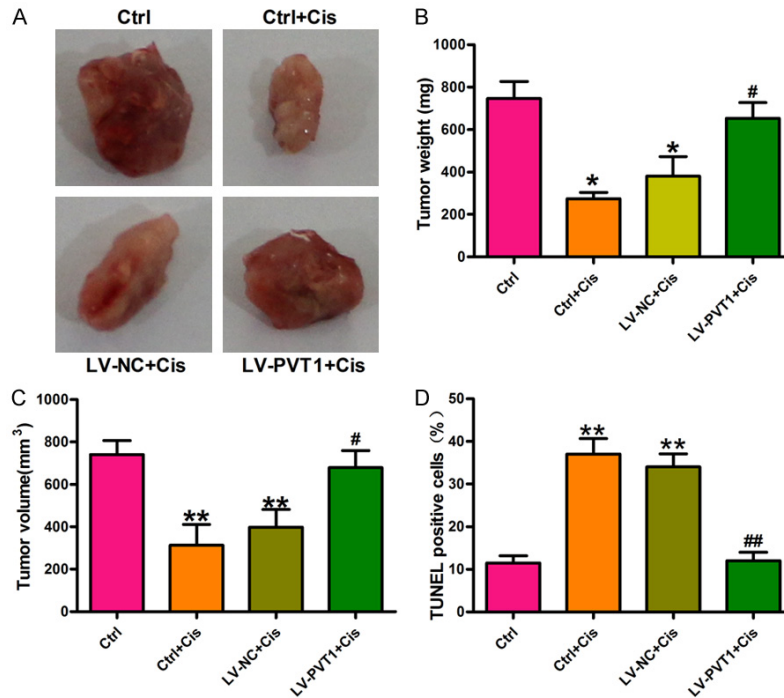
Figure 7. PVT1 overexpression in CRC cells enhanced cisplatin resistance in vivo. Male six-week-old nude mice were inoculated subcutaneously into right hind flanks with uninfected or LV-NC- or LV-PVT1-infected RKO cells. Once the tumor size reached 60 mm³, the mice were intraperitoneally injected with normal saline or cisplatin (5 mg/kg) twice per week, a total of six times. The mice implanted with uninfected cells and injected with normal saline were used as control. All animals were scarified six weeks after tumor cell implantation and the tumors were collected, photographed, weighted, and measured. The tumor tissues were sectioned for TUNEL assay. (A) Representative photos of tumors formed in nude mice. (B and C) The tumor weight (B) and tumor volume (C) of subcutaneous implantation models of CRC. (D) The apoptosis of tumor tissues was detected by TUNEL assay. All values are represented as mean ± SD of three replicates. $*P < 0.05$, $**P < 0.01$ compared with compared with Ctrl group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with (Ctrl + cisplatin) or (LV-NC + cisplatin) group. Ctrl: control; Cis: cisplatin.

silencing inhibited proliferation and induced apoptosis of CRC cells. Fourth, knockdown of PVT1 suppressed migratory and invasive abilities of CRC cells. Fifth, PVT1 depletion attenuated cisplatin resistance of cisplatin-resistant CRC cells. Sixth, overexpression of PVT1 enhanced cisplatin resistance of CRC cells. Seventh, PVT1 promoted cisplatin resistance by inhibiting intrinsic apoptotic pathway in CRC cells. Lastly, PVT1 overexpression in CRC cells enhanced cisplatin resistance in vivo. Collectively, PVT1 is a positive regulator in progression and drug resistance of CRC and targeting PVT1 may be an effective scheme for CRC therapy.

CRC is one of the most frequent malignancies with high morbidity and mortality globally [1]. Thus, focusing on exploration of the underlying mechanisms and finding new therapeutic strategies directed against specific targets have become increasingly desirable. Several reviews have focused on the functional roles of lncRNAs in multiple human cancers, such as breast cancer [18], gastric cancer [11], hepatocellular cancer [19], prostate cancer [20], CRC [21], etc. Upregulation of lncRNA PVT1 has been found in many cancers, including breast cancer, ovarian cancer, hepatocellular cancer, gastric cancer, bladder cancer, and CRC [13, 22]. PVT1 plays clinical significant roles in several cancers. For example, in non-small cell lung cancer, increased expression of PVT1 is significantly correlated with histological grade and lymph node metastasis and PVT1 is an independent prognostic marker [23]. PVT1 overexpression is a poor prognostic biomarker of small cell lung cancer [24]. High expression of PVT1 correlates with poor overall survival of cervical cancer patients [25]. In addition, high

of the tumors derived from the uninfected or LV-NC-infected RKO cells. However, PVT1 overexpression significantly reduced the apoptosis caused by cisplatin (**Figure 7D**). These results suggested that PVT1 overexpression promotes cisplatin resistance of CRC cells in vivo.

Discussion

In this study, we demonstrated that PVT1 promotes tumorigenesis and cisplatin resistance of CRC. Key findings were as follows: First, PVT1 was upregulated in CRC tissues and cells. Second, high expression of PVT1 was positively associated with tumor size, advanced tumor stages, lymph node and distant metastases, and cisplatin resistance, but inversely correlated with prognosis of CRC patients. Third, PVT1

expression of PVT1 indicates poor prognoses of gastric and hepatocellular cancers [11, 12]. Furthermore, amplification of PVT1 is involved in poor prognosis of CRC [14]. Consistently, in the present study, PVT1 expression was markedly elevated in CRC tissues and upregulation of PVT1 was positively associated with tumor size, advanced histological grades, and metastases, but negatively correlated with outcomes of CRC patients, suggesting clinical significance of PVT1 in CRC.

PVT1 plays tumor-promoting roles in cancer progression. Reportedly, PVT1 knockdown inhibits proliferation and increases apoptosis of ovarian and breast cancer cells [10]. PVT1 promotes cell proliferation, cell cycling, and the acquisition of stem cell-like properties in hepatocellular cancer cells by stabilizing NOP2 [12]. PVT1 depletion inhibits the migration and invasion of lung cancer cells [23, 24]. Knockdown of PVT1 significantly decreases cell proliferation, migration, and invasion, but increases apoptosis of cervical cancer cells [25]. Moreover, Takahashi et al. [14] indicated that CRC cells transfected with PVT1 siRNA show significant loss of proliferation and invasion capabilities. In accordance with these results, we here found PVT1 expression was much higher CRC cell lines than that in the normal colon epithelial cells. Silencing of PVT1 using siRNA technology led to significant decrease in proliferation, migration, and invasion, and increase in apoptosis of LoVo and RKO cells. These results indicated that PVT1 promotes aggressive behaviors of CRC.

Chemotherapy is a major therapeutic strategy for the patients with advanced stage of CRC. Cisplatin is widely used for chemotherapy of various types of cancers, including CRC [26, 27]. However, chronic cisplatin exposure of CRC cells leads to cisplatin resistance and chemotherapy failure [28]. Some lncRNAs are reported to involve in the development of drug resistance in many cancers. For instance, lncRNA AK126698 regulates cisplatin resistance of A549 cells through the Wnt pathway [29]. LncRNA TUG1 mediates methotrexate resistance in CRC via miR-186/CPEB2 axis [30]. LncRNA MALAT1 is associated with oxaliplatin resistance by regulating EZH2 in CRC [31]. PVT1 plays a central role in cisplatin resistance of cervical cancer cells [25]. Furthermore, in gastric and ovarian cancers, PVT1 is overexpressed in both cisplatin-resistant tissues and cells and PVT1 knockdown reverses the cisplatin resistance whereas PVT1 overexpression enhances cisplatin resistance of gastric and ovarian cancer cells [16, 17]. Additionally, the expression of the well-known genes involved in drug resistance, including MDR1 and MRP1, are much higher in cisplatin-resistant gastric cancer cells than that in cisplatin-sensitive cells [16]. In line with these results, in the present study, increased expression of PVT1 was found in cisplatin-resistant CRC tissues and cells. Silencing of PVT1 in LoVo/DDP and RKO/DDP cells inhibited proliferation and apoptosis resistance and decreased the expression of MDR1 and MRP1, whereas ectopic expression of PVT1 promoted cell proliferation, inhibited apoptosis, and increased the levels of MDR1 and MRP1 in LoVo and RKO cells. In addition, PVT1 overexpression in RKO cells promoted cisplatin resistance in vivo. These results demonstrate that PVT1 promotes the development of cisplatin resistance in CRC.

Many chemotherapeutic agents exert their anticancer activities by inducing apoptosis [32, 33]. Increasing evidences are available to support the hypothesis that apoptosis failure is an important reason for the development of drug resistance [34]. The key proteins such as Bax, Bcl-2, and caspase-3 play important roles in intrinsic apoptosis pathway and are found to be involved in the multidrug resistance of CRC cells [35]. PVT1 knockdown in cisplatin-resistant ovarian cancer cells increased the expression of caspase-3 [17]. In addition, PVT1 has been reported to exert anti-apoptotic activity in CRC cells [14]. In this study, we found that PVT1 silencing upregulated the expression of pro-apoptotic proteins (Bax and cl-caspase-3) and downregulated the anti-apoptotic Bcl-2 expression in LoVo/DDP and RKO/DDP cells, whereas overexpression of PVT1 reversed the expression of the proteins mentioned above in LoVo and RKO cells, suggesting that PVT1 enhances cisplatin resistance of CRC cells by inhibiting intrinsic apoptosis signaling.

In summary, we demonstrated that PVT1 was a positive regulator in tumorigenesis and cisplatin resistance of CRC. Clinically, PVT1 was significantly associated with tumor size, advanced tumor stages, metastases, and poor prognosis of CRC patients. In vitro experiments showed that PVT1 knockdown inhibited proliferation,

migration, invasion, and apoptosis escape of CRC cells. Knockdown of PVT1 in cisplatin-resistant CRC cells suppressed cell proliferation and apoptosis resistance while overexpression of PVT1 promoted the proliferation and inhibited apoptosis of CRC cells. PVT1 enhanced cisplatin resistance by inhibiting intrinsic apoptosis signaling in CRC cells. PVT1 overexpression promoted cisplatin resistance in vivo. Overall, these findings provided insight into the effective strategy for CRC therapy by targeting PVT1.

Disclosure of conflict of interest

None.

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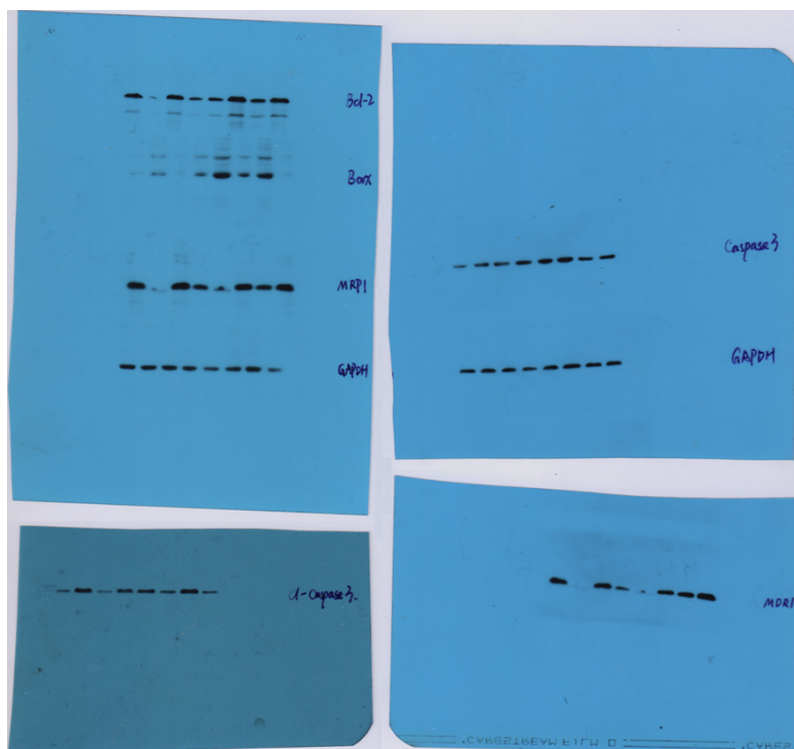
Supplementary Figure 1. The original films of western blot results corresponding to **Figure 6C** and **6D**.