MiR-183-5p-PNPT1 Axis Enhances Cisplatin-induced Apoptosis in Bladder Cancer Cells*

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[Abstract] Objective: It has been reported that intrinsic apoptosis is associated with the progression of bladder cancer (BC). Recent evidence suggests that polyribonucleotide nucleotidyltransferase 1 (PNPT1) is a pivotal mediator involved in RNA decay and cell apoptosis. However, the regulation and roles of PNPT1 in bladder cancer remain largely unclear. **Methods:** The upstream miRNA regulators were predicted by in silico analysis. The expression levels of PNPT1 were evaluated by real-time PCR, Western blotting, and immunohistochemistry (IHC), while miR-183-5p levels were evaluated by qPCR in BC cell lines and tissues. *In vitro* and *in vivo* assays were performed to investigate the function of miR-183-5p and PNPT1 in apoptotic RNA decay and the tumorigenic capability of bladder cancer cells. **Results:** PNPT1 expression was decreased in BC tissues and cell lines. Overexpression of PNPT1 significantly promoted cisplatin-induced intrinsic apoptosis of BC cells, whereas depletion of PNPT1 potently alleviated these effects. Moreover, oncogenic miR-183-5p directly targeted the 3′ UTR of PNPT1 and reversed the tumor suppressive role of PNPT1. Intriguingly, miR-183-5p modulated not only PNPT1 but also Bcl2 modifying factor (BMF) to inhibit the mitochondrial outer membrane permeabilization (MOMP) in BC cells. **Conclusion:** Our results provide new insight into the mechanisms underlying intrinsic apoptosis in BC, suggesting that the miR-183-5p-PNPT1 regulatory axis regulates the apoptosis of BC cells and might represent a potential therapeutic avenue for the treatment of BC.
Key words: bladder cancer; polyribonucleotide nucleotidyltransferase 1; bcl2 modifying factor; mitochondrial outer membrane permeabilization; microRNA

Bladder cancer (BC) is the 9th most common malignancy worldwide, with closely 400 000 new cases diagnosed and 165 000 annual deaths[1]. Comparatively, 70%–80% of freshly confirmed BC patients present with non-muscle-invasive bladder cancer (NMIBC), while 10%–20% of these patients would ultimately develop muscle-invasive bladder cancer (MIBC)[2]. Despite intensive therapies, approximately half of patients with MIBC will have recurrent disease, and most of them would die from uncontrolled disease[3]. For patients with metastatic diseases, platinum-based combination chemotherapy is the present standard treatment, although the initial response rate is only 40%–70%[4]. Therefore, an improved understanding of BC progression at the molecular level and cisplatin resistance would have great clinical value.

MicroRNA (miRNA) is classified as a small noncoding RNA of 19–25 nucleotides, and is involved in various cell functions, including cell proliferation, differentiation, apoptosis, metabolism and cardiogenesis. Mechanistically, miRNAs repress gene expression by interacting with the 3′ UTR of the downstream messenger RNA (mRNA), leading to mRNA cleavage and the inhibition of mRNA translation[5].

In the initiation and progression of human cancers, miRNAs can be a tumor suppressor or a tumor inducer[6]. Increasing evidence implies that miRNAs are esteemed mediators of drug resistance in BC,

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highlighting its potential as anti-cancer agents in the treatment of chemotherapy-resistant BC.

Apoptosis is a form of programmed cell death, which can be initiated by one of two separate pathways. The extrinsic pathway requires the ligation of transmembrane death receptors, while the intrinsic (mitochondrial) pathway is executed by mitochondrial outer membrane permeabilization (MOMP) and the release of mitochondrial proteins[7]. The mitochondrial pathway centered on MOMP is controlled by pro- and anti-apoptotic factors delivered from the intermembrane space of the mitochondria, and this determines the balance between cell survival and death[8]. Predominant research on MOMP has focused on its role as a necessary step for cytochrome c release, caspase activation, and the execution phase of apoptosis. In recent years, numerous studies have revealed the crucial role of MOMP in cancer therapies[9, 10]. In particular, MOMP and Bcl-2 proteins can determine the responses of tumor cells to chemotherapy[11].

Polyribonucleotide nucleotidyltransferase 1 (PNPT1), a novel gene located in chromosome 2p16.1, encodes polynucleotide phosphorylase, which is an enzyme that modulates bacterial mRNA half-lives and conversely binds to 3′-adenines[12]. Localizing in the mitochondrial inner membrane, PNPT1 functions as a transporter to import chromosomally encoded RNAs into the mitochondrial matrix[13]. In addition, PNPT1 is involved in the transport of polycistronic mitochondrial transcripts and tRNAs[14]. Mutations of PNPT1 can lead to mitochondrial dysfunction and neurodevelopmental diseases[15], while heterozygous variants of PNPT1 can cause Leigh syndrome[16]. Strikingly, Liu *et al* recently revealed that the overexpression of PNPT1 can enhance apoptotic mRNA decay and cell death[17]. These results show that PNPT1 is of great importance in cancer cell survival and chemosensitivity.

In the present study, it was observed that PNPT1 was downregulated in BC, and was significantly associated with the clinical prognosis of patients with BC. Furthermore, PNPT1 was described as a new target of miR-183-5p, and was connected to this miRNA, in terms of regulating mitochondrial apoptosis. The present findings further revealed that miR-183-5p protects BC cells from apoptosis by targeting the Bcl2 modifying factor (BMF), a positive regulator of MOMP. In summary, the present results reveal the critical part of PNPT1 as an apoptosis regulator in BC, and discloses a new perspective for evolving innovative therapeutic strategies for chemo-resistant BC.

1 MATERIALS AND METHODS

1.1 Cell Culture

The BC cell lines (J82, T24, 5637, TCCSUP, RT4, EJ, UM-UC-3, SCaBER, SW780 and normal urothelial cell SV-HUC-1) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (China). Primary cultures of normal bladder urothelial cells (NBUCs) were established and cultured, as previously described[18]. SV-HUC-1 cells were cultured in F12K medium supplemented with 10% fetal bovine serum (FBS), while the other cell lines were cultured in Dulbecco's Modified Eagle's medium with 10% FBS (Gibco, USA).

1.2 Tissue Specimens

A total of 60 pairs of fresh BC tumor tissues and adjacent non-tumor adjacent tissues were surgically collected for qRT-PCR and Western blotting, and 196 paraffin-embedded tissues were collected from Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (China) for the immunostaining evaluation. The stage of the patients was determined according to the TNM staging system of the American Joint Committee on Cancer classification system. The clinicopathological characteristics of these BC patients are presented in table 1. The present study was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. A written informed consent was obtained from each patient.

Table 1 The correlation between PNPT1 expression and clinicopathological features

Feature	*n*	PNPT1 expression		*P*
		Low	High	
Sex				0.714
Male	121	90	31	
Female	75	54	21	
Age (years)				0.435
≤60	92	69	23	
>60	104	75	29	
Tumor grade				0.518
Low	117	84	33	
High	79	60	19	
Tumor size				0.284
<3 cm	143	108	35	
≥3 cm	53	36	17	
T classification				0.010
Ta, T1	130	88	42	
T2–T4	66	56	10	
Total number of patients	196	144	52	

1.3 RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted from tissues and cells using the Trizol kit (Invitrogen, USA), and the cDNA was synthesized according to manufacturer's instructions. Real-time PCR (RT-PCR) was performed on the StepOne Plus real-time PCR system (Life Technologies, USA). GAPDH was utilized as an internal control. The total miRNA was extracted

from cultured cells and tissues using the mirVana miRNA Isolation Kit (Ambion, USA). The cDNA was synthesized out of 5 ng of total RNA using the Taqman miRNA reverse transcription kit (Applied Biosystems, USA). The miR-183-5p expression level was evaluated using the miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems, USA). The miRNA expression was determined based on the threshold cycle (Ct), and the relative expression level was determined using $2^{-[(Ct\ of\ miR\text{-}183\text{-}5p)-(Ct\ of\ U6)]}$ after normalization with the U6 small nuclear RNA expression. The sequences of primers are presented in table 2.

Table 2 QRT-PCR primer sequences

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
PNPT1	GCGAGCACTATGGAGTAGCG	GCAGTGTCACCTGACTGTACTA
GAPDH	AAGGTGAAGGTCGGAGTCAAC	GGGGTCATTGATGGCAACAATA
MiR-183-5p	CGCGTATGGCACTGGTAGAA	AGTGCAGGGTCCGAGGTATT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
7SL	ATCGGGTGTCCGCACTAAGTT	CAGCACGGGAGTTTTGACCT
ACTB	AAGGCCAACCGCGAGAAGAT	ACAGCCTGGATAGCAACGTACA
TUBA	TCTGTGAAACTGGTGCTGGA	AGTGACCACGGGCATAGTTGTT
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG

QRT-PCR: quantitative real time polymerase chain reaction; PNPTI: polyribonucleotide nucleotidyltransferase

1.4 Western Blotting

The proteins were extracted and analyzed by performing the standard Western blotting procedure. The protein lysates were separated by SDS-PAGE gels, and transferred onto a PVDF membrane (Millipore, Germany). After blocking for one h, the membranes were incubated with the primary antibodies for one h at room temperature. The antibodies were as follows: anti-PNPT1 (Cat. no. ab96176, Abcam, USA), anti-cytochrome c (Cat. no. ab133504, Abcam, USA), anti-COX Ⅳ (Cat. no. ab202554, Abcam, USA), anti-cleaved Caspase-3 (Cat. no. ab214430, Abcam, USA), anti-BMF (Cat. no. ab9655, Abcam, USA), and anti-Bcl2 (Cat. no. ab32124, Abcam, USA) antibodies. Finally, the Western blot bands were captured through the gel imaging system (Bio-Rad, USA).

1.5 Immunohistochemical Staining

Paraffin blocks of formalin-fixed surgical specimens were prepared in sections, and immunohistochemically stained. After the sections were dewaxed and rehydrated, antigen retrieval was performed using 10 μmol/L citrate buffer solution (pH=6.0). The primary antibodies were as follows: anti-PNPT1 (Cat. no. ab96176, Abcam) and anti-ki67 antibody (Cat. no. ab92742, Abcam). Then, the sections were developed for 2 min with the enzyme substrate 3,3′-diaminobenzidine chromagen (DAB, DAKO), and counterstained with Mayer's hematoxylin. The assessment of immunoreactivity was dependent on the semiquantitative analysis. The surgical specimen staining patterns were scored as follows: 0 point, no staining; 1 point, weak staining; 2 points, moderate staining; 3 points, strong staining. The percentage of positive cells was scored as follows: 0 point, negative; 1 point, 1%–10%; 2 points, 11%–50%; 3 points, 51%–80%; 4 points, >80%. For statistical purposes, the staining intensity score and the proportion of positive tumor cells were multiplied to obtain the final score, in which ≤3 points indicated a low expression, while 4–12 points indicated a high expression.

1.6 Assessment of Apoptosis

Cells were trypsinized, washed with PBS, and fixed in ice-cold 70% (v/v) ethanol at 4°C for one h. After washing with cold PBS twice, the cells were stained with PE-annexin V (BD Pharmingen, USA), 7-AAD (BD Pharmingen, USA), or DiIC1 (5) (BD Pharmingen, USA), and 50 mg/mL of propidium iodide (Sigma-Aldrich, USA), according to the manufacturer's instructions. Finally, the stained cells were analyzed using FACSCalibur (BD Biosciences, USA).

1.7 Plasmids, Lentiviral Infection and Transfection

The cDNA of the PNPT1 gene was amplified by PCR, and cloned into the GV358 lentiviral vector (GeneChem, China). The oligo of PNPT1 shRNAs was synthesized and inserted to the GV248 vector (GeneChem, China). Stable cell lines that expressed PNPT1 (PNPT1) or PNPT1-shRNAs (PNPT1-Ri1 and PNPT1- Ri2) were selected and fixed with 0.5 mg/mL of puromycin for 10 days. The miR-183-5p mimics, negative control, and anti-miR-183-5p inhibitor (miR-183-5p-in) were purchased from RiboBio Co. Ltd. (China). The miRNA or miRNA inhibitor was transfected with the Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions.

1.8 Dual Luciferase Reporter Assay

The luciferase reporter vectors for the wild-type or mutant PNPT1/BMF 3′ UTR, which contained the miR-183-5p binding site, were constructed in the pMIR-REPORT luciferase system (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The dual luciferase assay (Promega, USA) was carried out at 48 h after transfection according to manufacturer's instructions (Promega, USA). Three

independent experiments were realized, and the data were presented as mean±standard deviation (SD).

1.9 Sphere Formation Assay

A total of 5×10^2 cells were seeded in 6-well ultra-low cluster plates for 10–12 days. Then, the spheres were cultured in DMEM/F12 serum-free medium supplemented with 2% B27 (Cat. no. 17504044, Invitrogen, USA), 20 ng/mL of EGF (Cat. no. AF-100-15, PeproTech, China), and 20 ng/mL of bFGF (Cat. no. 100-18B, PeproTech, China). After 10–12 days, the number of cell spheres (tight, spherical and non-adherent masses of >50 μm in diameter) was counted, and the images of these spheres were captured under an inverse microscope. Sphere formation efficiency=Colonies/Input cells×100%.

1.10 Animal Experiments

The present study was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. For the tumor formation assay, 3×10^6 cells were suspended in 5 μL of PBS, and these were directly and subcutaneously injected into BALB/c-nu mice. The tumor progression was tracked using the *In Vivo* Optical Imaging System (*In Vivo* FX PRO, Bruker Corporation, USA). At 30th day after the injection of BC cells, these mice were euthanized, and the tumors were excised and imaged. Then, the tumor volumes were calculated from the digital caliper raw data using the formula, $(L\times W^2)/2$, in which, L represented the long diameter of the tumor, W represented the short diameter of the tumor. All experiments were conducted before the disease burden resulted in decreased appetite, loss of body weight, or increased sensitivity to touch.

1.11 Statistical Analysis

The SPSS 19.0 software (China) was used to analyze all data through Pearson's chi-square and Student *t*-test. The Kaplan-Meyer method was used to generate the survival curves, and log-rank test was performed to compare the survival rates. $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Decreased PNPT1 Expression Correlates with BC Aggressiveness

The expression of PNPT1 was initially evaluated in BC tissues and cells to determine the impact of PNPT1 on the progression of BC. In 83.3% (50/60) of BC patients, the mRNA expression of PNPT1 in BC tissues decreased, when compared to matched normal tissues (fig. 1A). The Western blotting revealed that the expression of PNPT1 in BC was significantly lower, than that in non-tumor adjacent tissues (fig. 1B). Furthermore, compared to normal urothelial cells and NBUCs, PNPT1 was markedly downregulated in all 9 BC cell lines (figs. 1C and 1D).

Next, the relationship between the clinicopathological features of BC and PNPT1 expression was evaluated. The PNPT1 expression in 196 archival formalin-fixed, paraffin-embedded human BC samples was assessed by immunohistochemistry (fig. 1E), and a negative relationship between the expression levels of PNPT1 and the clinical stage (T classification) was found ($P=0.010$, table 1). The Kaplan-Meier analysis revealed that the overall survival and the recurrent-free survival were positively associated with the PNPT1 expression ($P=0.016$ and $P<0.001$, respectively; fig. 1F). In addition, the multivariate analysis revealed that the PNPT1 expression was an independent prognostic factor for BC patients (table 3).

2.2 PNPT1 Promotes mRNA Decay and Cisplatin-induced Apoptosis in BC Cells

In order to identify the biological role of PNPT1 in BC cells, it was determined whether MOMP is required for mRNA decay in cisplatin-treated BC cells. The results indicated that MOMP, as assessed by the mitochondrial release of cytochrome c and mitochondrial depolarization by DiIC1(5) staining, was detected after the addition of cisplatin (fig. 2A and 2B). Furthermore, the results indicated that PNPT1 was released during MOMP (fig. 2A), and that PNPT1 ectopic expression was promoted, while the silencing of the PNPT1 expression inhibited the mRNA decay of 4 housekeeping genes after cisplatin treatment (fig. 2C and 2D). Strikingly, it was found that the ectopic expression of PNPT1 significantly increased the cisplatin-mediated cell death (Annexin V staining, fig. 2E) and enhanced the cisplatin-induced apoptosis in BC cells as shown by 7-AAD staining and subdiploid DNA assays (fig. 2F and 2G). Furthermore, the ectopic PNPT1 significantly decreased the clonogenic survival of BC cells after cisplatin treatment (fig. 2H). In addition, the PNPT1 overexpression greatly increased the caspase-3 cleavage and activation by cisplatin (fig. 2I). These *in vitro* results support the role of PNPT1 in promoting mRNA decay and caspase-independent death, and in enhancing cisplatin-induced apoptosis in BC cells.

2.3 PNPT1 Inhibits Progression of BC

Next, the role of PNPT1 in BC cell sensitivity to cisplatin was determined. The MTT assay revealed that PNPT1 significantly increased the tumor cell sensitivity to cisplatin, while the knockdown of PNPT1 conferred the cisplatin resistance and attenuated the cisplatin-induced apoptosis (fig. 3A). Furthermore, the effect of PNPT1 on the tumorigenic activity of BC cells was analyzed. It is noteworthy that PNPT1-silenced cells formed larger, and approximately 1.5- or 2.0-fold more spheres than vector control cells, while PNPT1 overexpressing cells formed smaller and fewer spheres than the vector control cells (fig. 3B). In order to verify the role of PNPT1 in BC progression

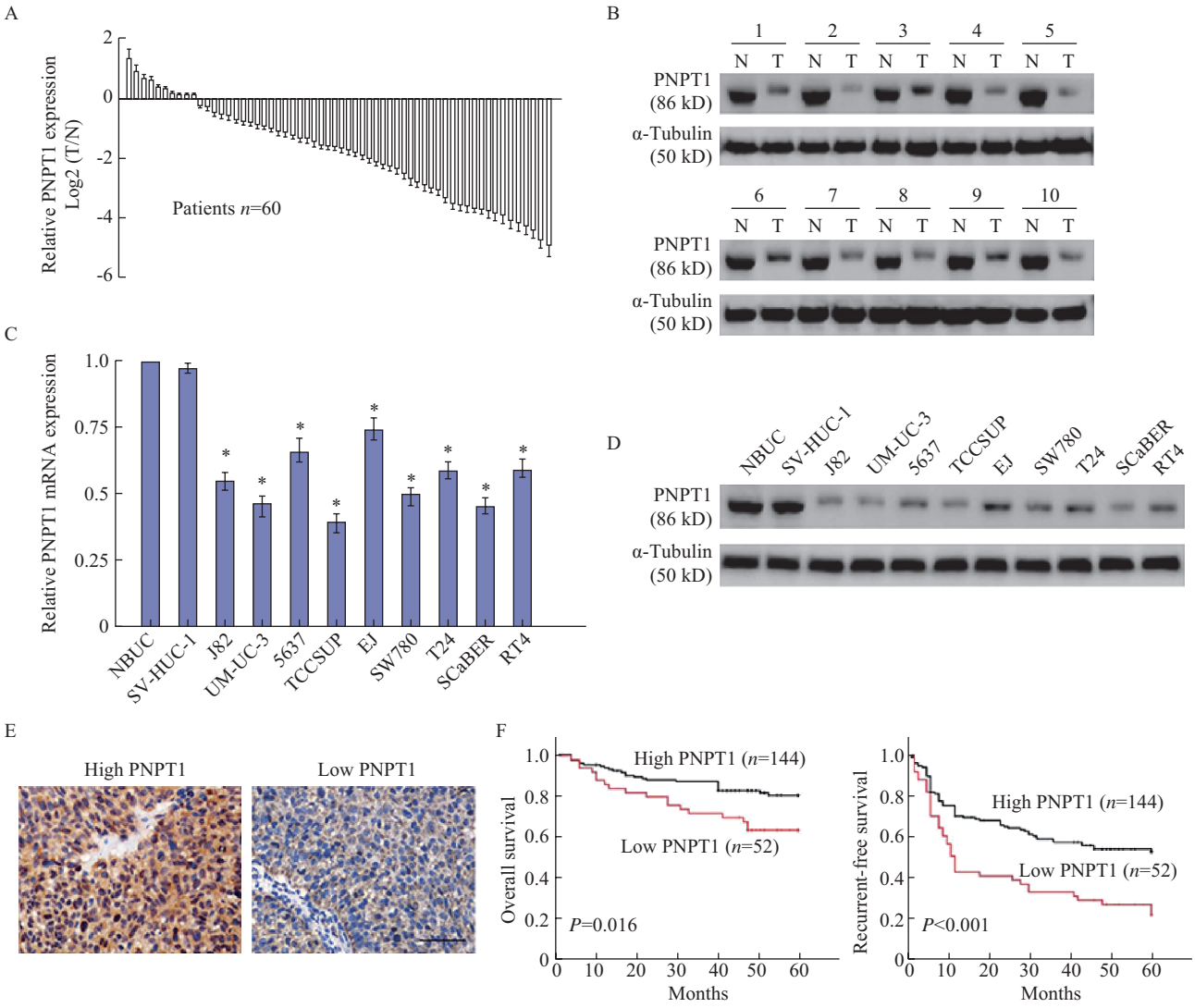

Fig. 1 Down-regulation of PNPT1 expression in bladder cancer, and its correlation with prognosis

A: the PNPT1 mRNA expression in 60 pairs of bladder cancer and adjacent non-tumor tissues; B: the protein expression of PNPT1 in 10 pairs of randomly selected tumor tissues (T) and adjacent non-tumor tissues (N); C and D: the PNPT1 mRNA and protein expression in normal bladder urothelial cells (NBUCs), human uroepithelial cells (SV-HUC-1), and bladder cancer cell lines (J82, T24, 5637, TCCSUP, RT4, EJ, UM-UC-3 and SW780); E: representative images for the immunohistochemistry of PNPT1 in bladder cancer specimens. Scale bar: 100 μm; F: overall and recurrent-free survival curves in relation to the PNPT1 status in 196 bladder cancer patients. $^{*}P<0.05$ *vs.* NBUC

Table 3 Multivariate analysis of the overall survival (OS) and recurrent-free survival (RFS) of patients with bladder cancer

Prognostic variables	OS		RFS	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Gender (male *vs.* female)	1.018 (0.537–1.931)	0.956	1.093 (0.692–1.727)	0.702
Age (>60 *vs.* ≤60 years)	1.010 (0.556–1.833)	0.975	1.197 (0.802–1.785)	0.379
Tumor grade (high *vs.* low)	1.578 (0.859–2.901)	0.142	1.018 (0.699–1.672)	0.726
Tumor size (≥3 cm *vs.* <3 cm)	1.724 (0.829–3.587)	0.145	1.661 (1.004–2.747)	0.048
T classification (T2–4 *vs.* T1, Ta)	2.551 (1.354–4.806)	0.004	1.219 (0.779–1.908)	0.386
PNPT1 (high *vs.* low)	0.366 (0.197–0.618)	0.002	0.422 (0.276–0.647)	0.001

Total number of patients: 196; number of deaths: 47; number of recurrences: 101

in vivo, a xenograft murine model was used, in which BC cells that stably expressed PNPT1 or PNPT1-Ri vectors were subcutaneously implanted into the flanks of mice. The *in vivo* study revealed that the tumors that overexpressed PNPT1 were significantly smaller and lighter than control cells (fig. 3C–3E). For the immunohistochemistry staining, it was found that PNPT1 significantly suppressed the expression of Ki67 (fig. 3F). Collectively, these results illustrate that PNPT1 plays a significant role in suppressing BC

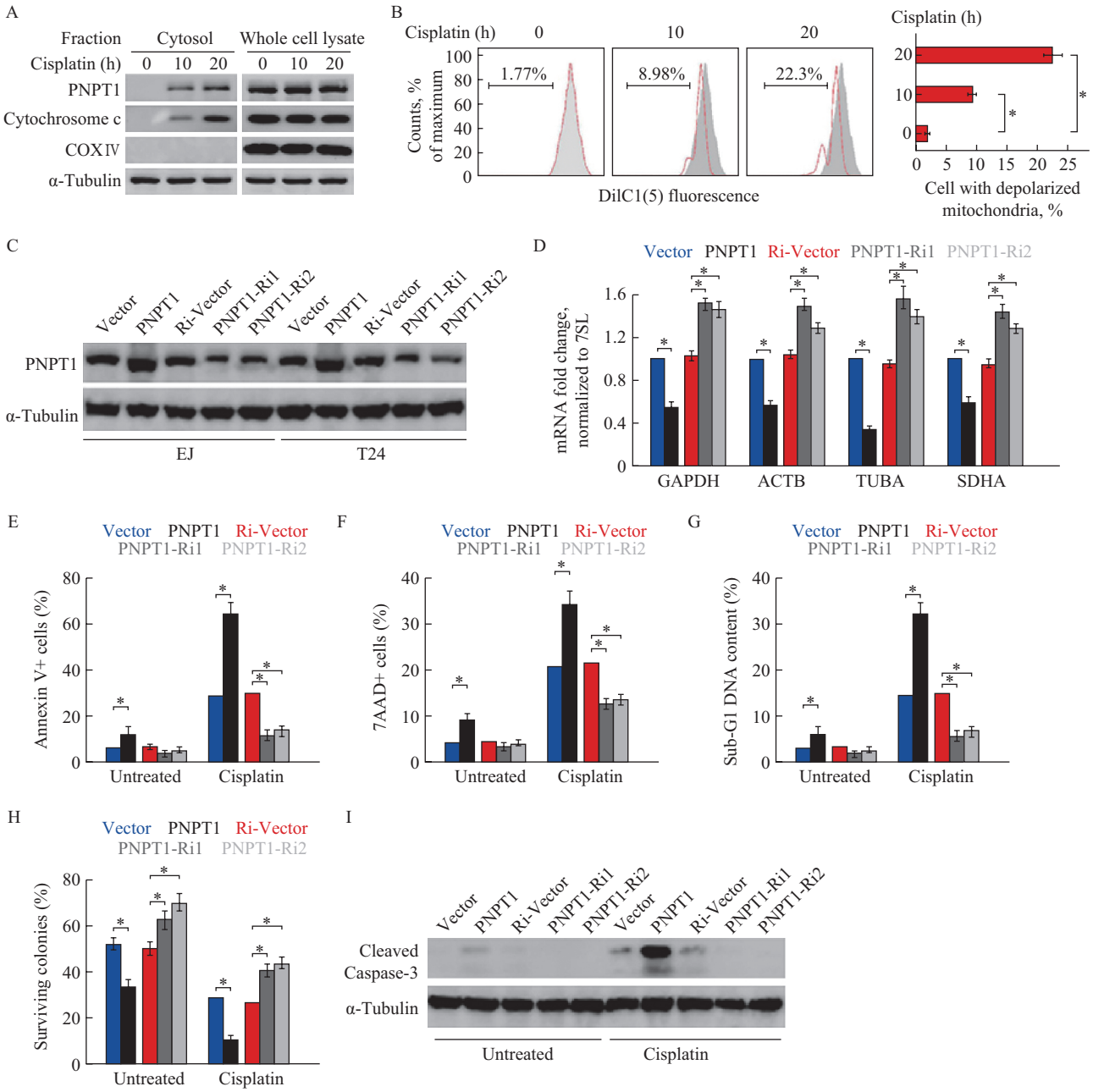

Fig. 2 PNPT1 promotes mRNA decay and cisplatin-induced apoptosis in bladder cancer cells

A and B: Cisplatin-treated EJ cells were analyzed by immunoblot for PNPT1 expression and cytochrome c release (A), and DiIC1(5) (BD Pharmingen, USA) staining for mitochondrial depolarization (B) (left, representative experiment; right, mean data); C: Western blot analysis of the PNPT1 expression in EJ and T24 cells that stably expressed and silenced PNPT1, and α-tubulin was used as the loading control; D: The quantitative real-time PCR analysis for the expression levels of GAPDH, ACTB, TUBA and SDHA in the indicated cells; E–I: EJ cells, which were transfected to overexpress PNPT1 or PNPT1-shRNAs, were treated with or without cisplatin, and analyzed for cell death by Annexin V staining (E), 7-AAD staining (F), sub-diploid DNA content (G), cell survival by clonogenic survival assay (H), and caspase activation by immunoblot for caspase-3 cleavage (I). The graphs present the mean±standard deviation (SD) of three technical replicates. The data are representative of at least three independent experiments, $^{*}P<0.05$

progression *in vivo*.

2.4 MiR-183-5p Inhibits BC Cell Apoptosis by Directly Targeting PNPT1

It was found that PNPT1 may be a potential target of miR-183-5p by in silico study using three bioinformatic algorithms (fig. 4A and 4B). In the Western blot analysis, it was found that the inhibition of miR-183-5p promoted the protein expression of PNPT1, while the overexpression of miR-183-5p had an opposite effect (fig. 4C). Next, luciferase reporter assay was performed on the 3′ UTR constructs with a binding site-mutation or the wild-type. As shown in fig. 4D, the overexpression of miR-183-5p in BC cells suppressed the wild-type luciferase activity, while the mutant PNPT1 3′ UTR construct did not have this effect. Furthermore, the depletion of miR-183-5p had

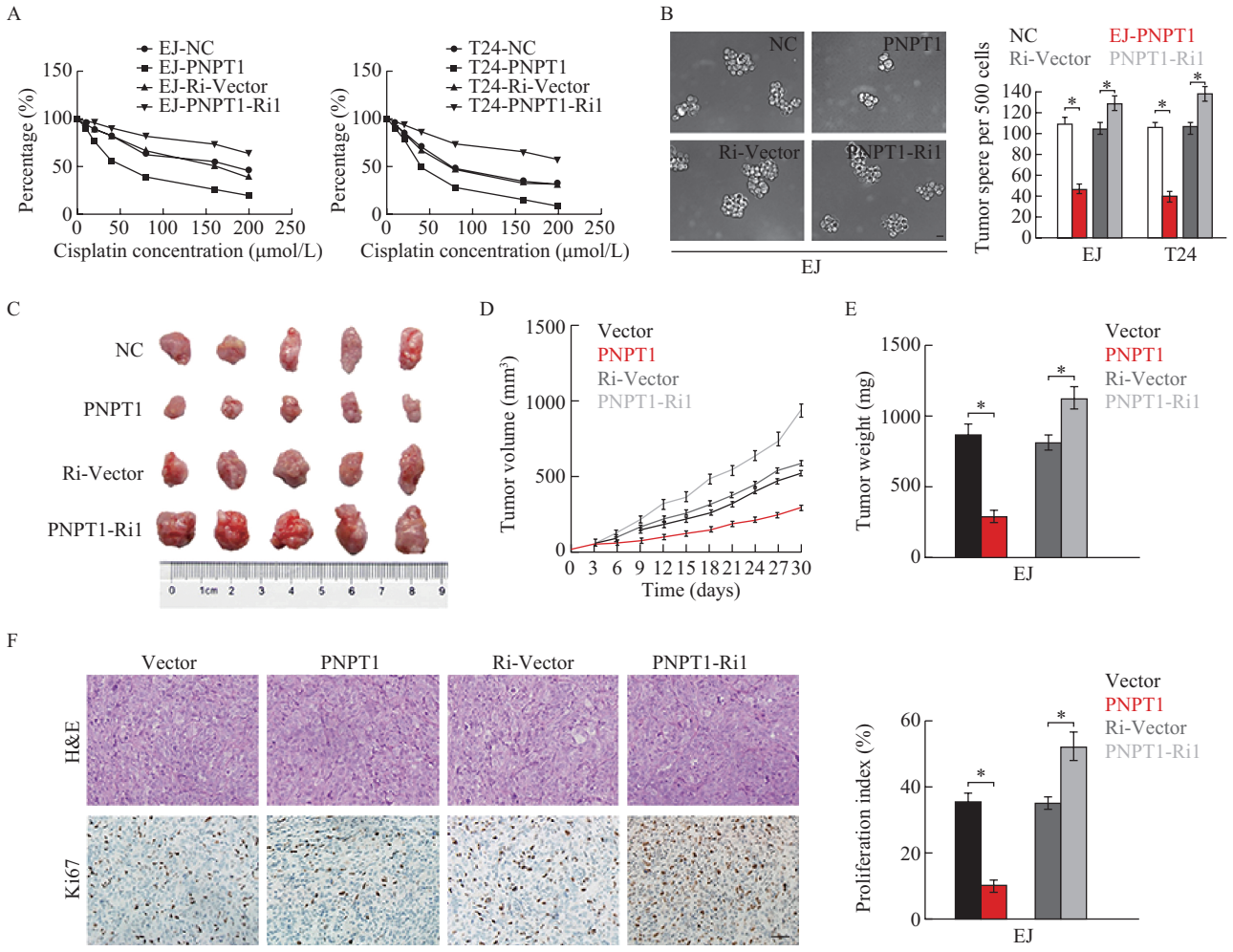

Fig. 3 PNPT1 suppresses the tumorigenicity of bladder cancer

A: the MTT assays for cell viability after 48 h of treatment with cisplatin for the indicated cells; B: the representative micrographs (left) and quantification (right) of colonies formed by the indicated cells, as determined by tumor sphere formation assay. Scale bar: 50 μm; C: representative images of tumors obtained from the xenograft model in nude mice; D: The tumor volumes were measured on the indicated days; E: the tumor weights of all mice in each group; F: The immunohistochemical staining revealed the expression of Ki67 in the indicated tissues. Scale bar: 100 μm. The data are presented as the mean±standard deviation (SD) of three independent experiments. $^{*}P<0.05$

a counter-effect, enhancing the direct targeting of miR-183-5p. The further analysis revealed that the expression of miR-183-5p was elevated in all 9 BC cell lines (fig. 4E), and that in 45 freshly frozen BC samples, the mRNA levels of miR-183-5p and PNPT1 were negatively correlated (fig. 4F).

In order to further clarify the function of miR-183-5p-induced cell apoptosis by inhibiting PNPT1, PNPT1-mt (with the mutant 3′ UTR) and PNPT1-wt (with the wild-type 3′ UTR) were transfected into overexpressed miR-183-5p cells. The Western blot analysis revealed that PNPT1 was significantly upregulated after the transfection of PNPT1-mt into miR-183-5p overexpressing cells (fig. 5A). The ectopic expression of PNPT1 reversed the effects of miR-183-5p on the modulation of mRNA decay and cisplatin-induced apoptosis, but this was not affected by the transfection of PNPT1-wt (fig. 5B–5G). Overall, these results indicate that miR-183-5p inhibits mRNA decay and cisplatin-induced apoptosis through the inhibition of PNPT1.

2.5 MiR-183-5p Targets MOMP Regulator BMF

As shown in fig. 6A, it can be observed that BMF, a key regulator of MOMP, can be a miR-183-5p potential target through the use of bioinformatics algorithms. The Western bot analysis revealed that the overexpression of miR-183-5p reduced the BMF expression in BC cells, while the silencing of miR-183-5p enhanced the BMF expression (fig. 6B), indicating that in BC cells, miR-183-5p negatively regulates BMF. In addition, the luciferase assay revealed that the upregulation of miR-183-5p inhibited the reporter activity driven by the 3′ UTR of BMF, and not by the mutant 3′ UTR of BMF in BC cells, while the silencing of miR-183-5p improved this (fig. 6C). Furthermore, the miR-183-5p overexpression attenuated the mitochondrial release of cytochrome c and mitochondrial depolarization, while the inhibition of miR-183-5p led to a counter-

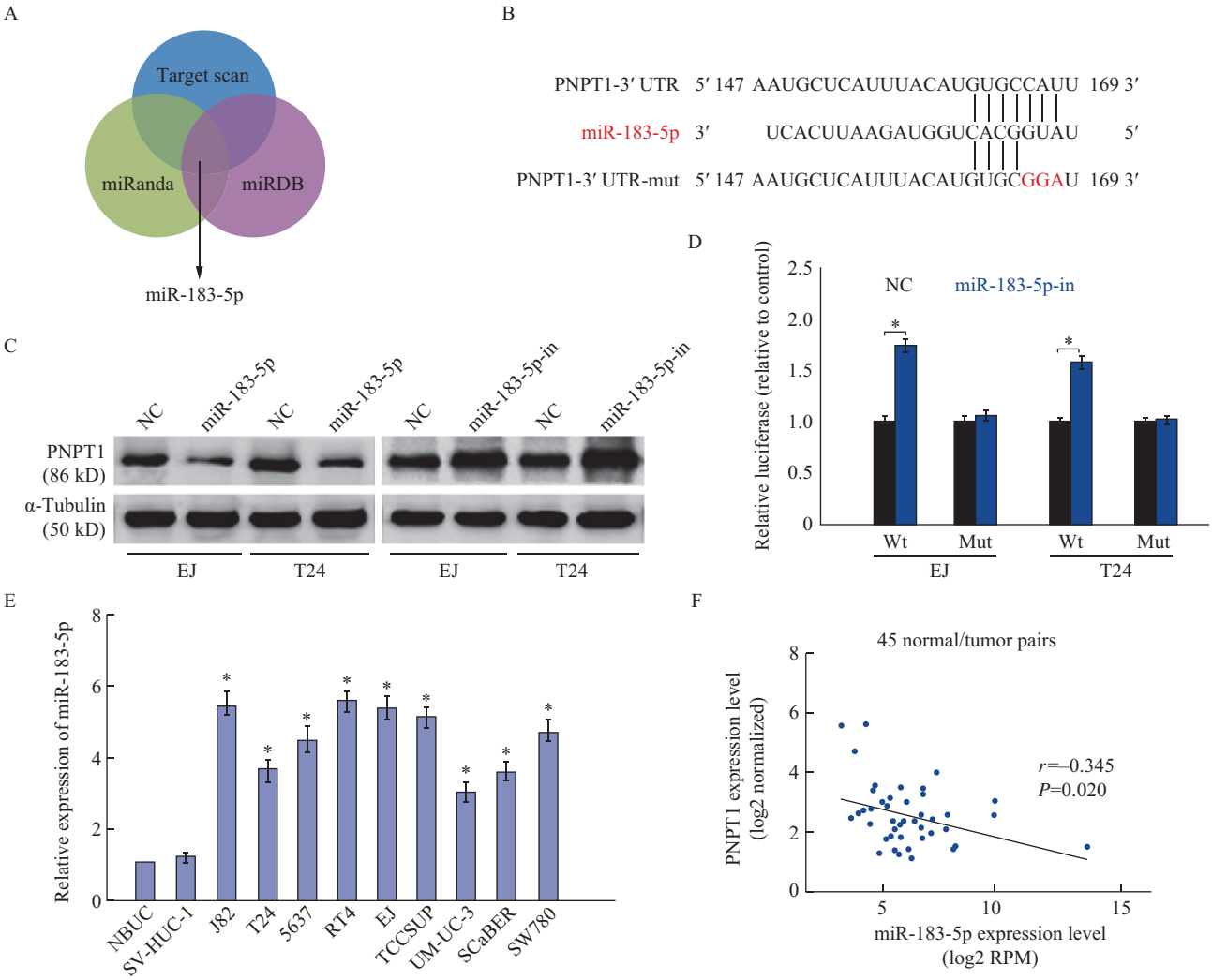

Fig. 4 Regulation of PNPT1 by miR-183-5p in bladder cancer

A: The Venn diagram shows that miR-183-5p is commonly predicted by miRanda, miRDB and TargetScan; B: The schematic diagram shows the same binding site of miR-183-5p at position 162–168 of the PNPT1 3′ UTR; C: The Western blot analysis shows the PNPT1 expression in the indicated cells, and α-Tubulin was used as the loading control; D: Bladder cancer cells were transfected with the luciferase reporter construct that contained the wild-type (Wt) or mutated (Mut) PNPT1 3′ UTR, together with the miR-183-5p mimic or miR-183-5p inhibitor oligomer. The luciferase activity was determined at 24 h after transfection; E: the quantitative real-time PCR analysis of the miR-183-5p expression in NBUCs, SV-HUC-1, and the bladder cancer cell lines; F: the correlation between miR-183-5p and PNPT1 protein expression in 45 cases of bladder cancer tissues (P=0.020). The error bars represent the mean±standard deviation (SD) of three independent experiments. $^*P$<0.05

effect (fig. 6B and 6D). Overall, these findings indicate that miR-183-5p directly targets BMF, resulting in the inhibition of MOMP.

2.6 Clinical Correlation of MiR-183-5p with PNPT1 and BMF in Human BC Tissues

In order to further determine the clinical significance of miR-183-5p-induced PNPT1 and BMF downregulation, and the subsequent cell apoptosis in BC tissues, the miR-183-5p expression and expression levels of PNPT1 and BMF were examined. It was found that the miR-183-5p expression was inversely correlated with the expression of PNPT1 and BMF (fig. 7A). Overall, these present findings suggest that the overexpression of miR-183-5p inhibits the cisplatin-induced apoptosis by inhibiting PNPT1 and BMF, resulting in the tumor progression of BC (fig. 7B).

3 DISCUSSION

The main results of the present study revealed that PNPT1 contributes to mRNA decay and cisplatin-induced apoptosis in BC. Meanwhile, miR-183-5p inhibits MOMP and mitochondrial apoptosis in BC cells by directly targeting PNPT1 and BMF, resulting in the progression of BC. Overall, these results reveal a new pathway that requires the miR-183-5p-PNPT1 axis for BC development and chemosensitivity.

Extensive research has shown that the majority of chemotherapies destroy cancer cells via the apoptotic cell death pathway, and that evasion from apoptosis

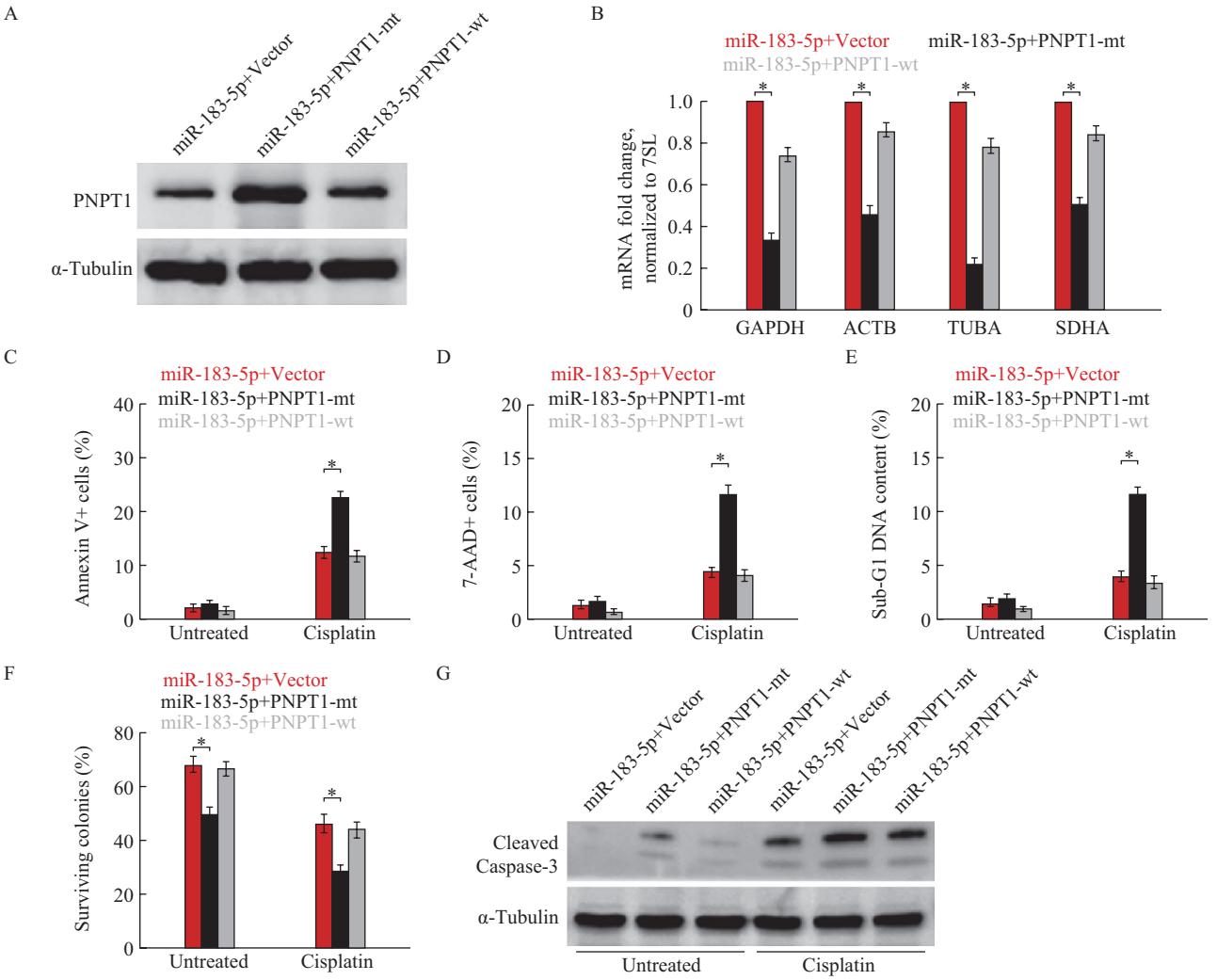

Fig. 5 PNPT1 mediates the miR-183-5p-inhibited apoptosis of bladder cancer cells

A: the Western blot analysis of the PNPT1 expression levels in the indicated cells; B: the quantitative real-time PCR analysis for the expression levels of GAPDH, ACTB, TUBA and SDHA in the indicated cells; C–G: analysis of cell death by Annexin V staining (C), 7-AAD staining (D), sub-diploid DNA content (E), cell survival by clonogenic survival assay (F), and caspase activation by immunoblot for caspase-3 cleavage (G) in the indicated cells. The graphs present the mean±standard deviation (SD) of three technical replicates. The data are representative of at least three independent experiments, $^{*}P<0.05$

is the acquired ability of tumor cells escaping from the cytotoxic effect of chemotherapeutic drugs[19]. Since mitochondria are the main regulators of apoptosis, defects in mitochondrial machinery have been conceivably linked to cancer cell survival and chemosensitivity. For example, Suzuki *et al* reported that the depletion of mitochondrial genome resulted in the resistance to TNF-induced apoptosis in human myelogenous leukemia cells[20]. In another study, Park *et al* reported that mtDNA-depleted hepatoma cells are resistant to hydrogen peroxide and ROS-inducing agents, which may be due to the elevated expression of manganese superoxide dismutase and antioxidant enzymesglutathione peroxidase[21]. In pancreatic cancer, Sancho *et al* reported that a subpopulation of CD133+ CSCs with low mitochondrial mass and increased metabolic plasticity was resistant to mitochondrial targeting drug treatment[22]. Collectively, these findings suggest that targeting mitochondrial defects can potentially restore the cancer cell sensitivity to drugs.

Recently, PNPT1 has been involved in the mediation of multiple physiological processes, including mitochondrial homeostasis maintenance, mtRNA import, cellular senescence, and chronic inflammation[23]. However, the clinical significance and biological role of PNPT1 in human cancers remain unknown. The present study revealed that the overexpression of PNPT1 markedly facilitated the apoptotic mRNA decay and suppressed the cisplatin resistance, while the knockdown of PNPT1 dramatically inhibited the apoptotic mRNA decay and enhanced the cisplatin resistance of BC cells. Furthermore, the PNPT1 expression was significantly correlated with the clinical stage of BC patients, suggesting that decreasing the PNPT1 expression can be a crucial step in BC progression. To the best of our

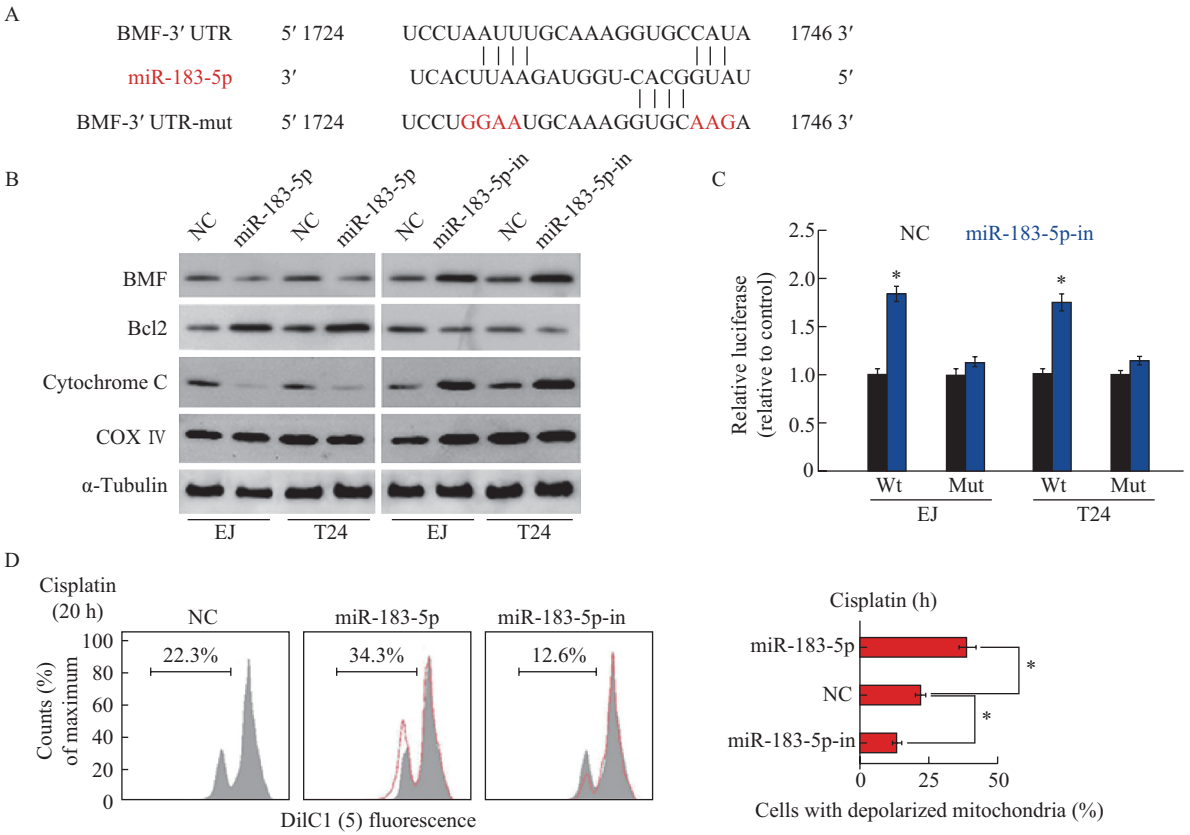

Fig. 6 MiR-183-5p targets both PNPT1 and Bcl2 modifying factor (BMF) in bladder cancer cells

A: the predicted miR-183-5p target sequences in the 3′ UTRs of the BMF gene; B: The Western blot analysis for the expression levels of BMF, Bcl-2 and cytochrome c in the indicated cells, and α-Tubulin and COX IV were used as the loading controls; C: Bladder cancer cells were transfected with the luciferase reporter construct that contained the wild-type (Wt) or mutated (Mut) BMF 3′ UTR, together with the miR-183-5p mimic or miR-183-5p inhibitor oligomer. The luciferase activity was determined at 24 h after transfection; D: the analysis of mitochondrial depolarization by DiIC1(5) staining in the indicated cells. The bar graphs present the statistical analysis of three independent experiments ($^{*}P<0.05$ *vs*. NC).

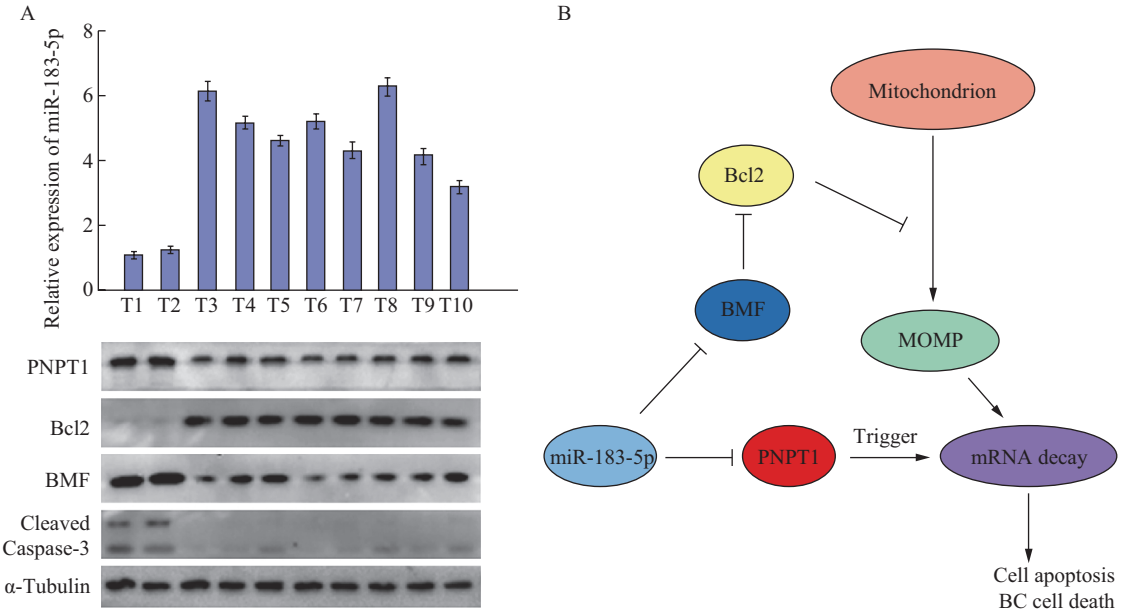

Fig. 7 Clinical relevance of miR-183-5p with PNPT1 and Bcl2 modifying factor (BMF) in bladder cancer

A: correlation of the miR-183-5p expression with PNPT1, BMF, Bcl-2 and cleaved caspase-3 in 10 bladder cancer tissues. U6 was used as the control for RNA loading. The miR-183-5p expression levels were normalized to that of the miR-183-5p expression of sample one. Each bar represents the mean±standard deviation (SD) of three independent experiments; B: schematic illustration of the mechanistic model for the role of miR-183-5p in mediating the mitochondrial apoptotic pathway in bladder cancer

knowledge, the present study is the first to demonstrate the relationship between the PNPT1 expression of and development and progression of cancer.

The importance of miRNAs in MOMP and tumor-associated mitochondrial apoptosis has recently emerged. For instance, the overexpression of miR-125b significantly enhances the cytotoxicity of doxorubicin to breast cancer cells. Concordantly, the treatment of miR-125b plus doxorubicin results in loss of mitochondrial membrane potential and MOMP[24]. In line with this study, Fiori *et al* reported that miR-663 regulates the apoptosis of non-small cell lung cancer by controlling MOMP through targeting PUMA/BBC3 and BTG2[25]. Furthermore, miR-183-5p has been reported to function as an oncogene in BC[26, 27], although the underlying mechanism remains not fully understood.

The present study supports the emerging mitochondrial role of miR-183-5p by identifying PNPT1 as a novel regulatory molecule with pivotal involvement in tumor-related mitochondrial apoptosis. Thus, a novel mechanism for the modulation of mitochondrial apoptosis and BC chemosensitivity was identified.

Given the central roles of Bcl-2 in the mitochondrial apoptotic pathway, targeting the Bcl-2 family of proteins can be a promising strategy to sensitize cancer cells to be vulnerable to chemotherapy. For instance, Li *et al* reported that mTOR inhibitors induce the suppression of MCL-1 and the overexpression of PUMA, which facilitates the release of apoptotic regulators and enhances the antitumor efficacy of BH3 mimetics in triple-negative breast cancer[28]. Similar results have been reported by Zhang *et al*, suggesting that miR-34 enhances the sensitization against gemcitabine-mediated apoptosis by targeting Slug/PUMA in pancreatic cancer cells[29]. In addition, Cardenas *et al* reported that the adipocyte-induced upregulation of Bcl-xl is correlated to acquired chemoresistance in ovarian cancer cells[30]. Importantly, the present study identified BMF, a BCL-2 modifying factor during MOMP, as a target of miR-183-5p, and as a targeting gene to reduce chemoresistance in BC cells. Hence, these present findings show that miR-183-5p acts as an onco-miRNA by promoting MOMP and apoptotic mRNA decay in BC.

In conclusion, the present study is the first to describe the correlation between the miR-183-5p-PNPT1 axis-mediated mitochondrial apoptosis and BC progression. The present results discover the vital role of the miR-183-5p-PNPT1 axis in regulating mRNA decay and cell apoptosis. PNPT1 may present as a potential therapeutic route in the treatment of BC.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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