Inhibition of JNK and prothymosin-alpha sensitizes hepatocellular carcinoma cells to cisplatin

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ABSTRACT

Cisplatin is a potent chemotherapeutic drug widely used for the treatment of human cancer. However, its efficacy against hepatocellular carcinoma (HCC) is poor for reasons that remain unclear. We show here that prothymosin-alpha (PTMA) is overexpressed in HCC cell lines. Silencing PTMA using short-hairpin RNA sensitizes HCC cells to cisplatin, while ectopic expression of PTMA induces cell resistance to the drug. Cisplatin inhibits both the JNK pathway and PTMA in a dose-dependent manner. Treatment with a JNK inhibitor also reduces PTMA protein stability and sensitizes HCC cells to cisplatin. Notably, the effects of PTMA silencing and JNK inhibition can be reversed by ectopic expression of PTMA. We show that PTMA silencing induces translocation of proapoptotic Bax to mitochondria and enhances cisplatin-induced cytochrome c release and caspase-9 activation. Conversely, ectopic expression of PTMA reverses these effects. Our results indicate that PTMA is positively regulated by JNK and protects HCC cells against cisplatin-induced cell death. The JNK/PTMA axis may thus represent a novel target for chemotherapy against HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is a complex liver disease associated with high mortality and prevalence worldwide [1]. HCC patients often respond poorly to current clinical treatments, including chemotherapy. Poor prognosis and high mortality rates are attributed in part to the difficulty in obtaining a diagnosis at an early stage. Alterations, up-regulations, and mutations in various genes and cellular signaling pathways have been shown to promote hepatocarcinogenesis and protect cancer cells against clinical treatment. We showed earlier that two anti-apoptotic proteins that are upregulated in human HCC [2,3]—HURP (hepatoma upregulated protein) and PTMA (prothymosin-alpha) [4,5]—represent important targets of sorafenib in HCC cells cultured in vitro [6,7].

PTMA has been described as a “thymic hormone” that may be used as a tumor biomarker and to trigger anticancer immune responses [8]. Recent evidence suggests that PTMA may play different roles depending on the cellular context. In the cell, PTMA controls the cell cycle, whereas in the extracellular space, this protein exerts immunomodulatory effects. PTMA expression and localization varies during hepatocyte proliferation and apoptosis in rat hepatocytes [9]. This protein was found to be highly expressed in human HCC [5]. In addition, high levels of PTMA and c-Myc were detected in various human tumors, including HCC [10–12]. c-Myc upregulates PTMA transcription [13], and c-Myc-binding sites were identified in both the proximal promoter and intron 1 of the PTMA gene [14–16]. We recently found that PTMA overexpression is negatively correlated with sorafenib sensitivity in HCC cells. The role of PTMA in the development of human HCC may involve regulation of PTMA at the transcriptional level by c-Myc [7]. In addition, we identified a sorafenib-responsive element in the PTMA promoter, and demonstrated that sorafenib inhibits PTMA expression and localization in vitro [17].

Abbreviations: DMSO, dimethyl sulfoxide; ERK, extracellular regulated protein kinase; PBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; HURP, hepatoma upregulated protein; JNK, c-Jun N-terminal kinase; Luc, luciferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PTMA, prothymosin-alpha; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative real-time reverse transcription-PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; VDAC, voltage-dependent anion channels.

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expression at the transcriptional level by inactivating the β-catenin/JNK pathway [6].

The mechanism underlying regulation of PTMA expression and the possibility that this protein may produce anti-apoptotic effects in HCC cells exposed to chemotherapy have not been investigated. Activation of mitogen-activated protein kinases (MAPKs) is believed to be a major factor in determining the fate of cancer cells in response to cisplatin. The role of MAPKs in response to cisplatin is complex as these proteins are able to induce apoptosis, but, depending on the cell type, may also suppress this process or be involved in other cellular pathways such as cell proliferation and differentiation [17]. As a member of the MAPK family, JNK modulates the activity of numerous proteins located in the mitochondria and the nucleus in response to cisplatin treatment, and also plays a major role in determining the fate of human tumors. The JNK signal pathway has been suggested to represent a double-edged sword in response to cisplatin, being a significant pro-apoptotic factor but also being associated with increased resistance to the drug [18]. We recently demonstrated that the JNK pathway and PTMA remain to be characterized in more details. In the present study, we show that PTMA is positively regulated by JNK and protects HCC cells against cell death induced by the chemotherapeutic drug cisplatin. The JNK/PTMA axis may thus represent a novel target for HCC treatment.

2. Materials and methods

2.1. Cell culture and reagents

Hepatocellular carcinoma cells (Huh7, J7, SK-Hep1, and Mahlavu) were obtained from the American Cell Type Collection (ATCC; Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml; Gibco), and streptomycin (100 mg/ml; Gibco). Cells were cultured at 37°C in a humidified atmosphere of 5% CO2 (v/v) in air. Commercial antibodies that react against Bad, Bid, pJNK, PAKT, AKT (Cell Signaling, Danvers, MA, USA), PTMA, survivin, Mcl-1, Bcl-Xc, Bcl-2, Bax, Bim, GAPDH, VDAC, JNK, pP38, P38, pERK, ERK, and IkB-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used according to guidelines provided by the manufacturer. Chemical inhibitors against NF-κB (Bay11-7082), JNK (SP600125), P38 (SB203580) (Santa Cruz Biotechnology), MEK/ERK (U0126) (Calbiochem, Billerica, MA, USA), and PI3K (Wortmannin; Cell Signaling) were also used.

2.2. Plasmids and cell transfection

The pcDNA3 plasmid was used as a negative control. pcDNA3-PTMA was constructed as previously described [7]. Plasmid construction and preparation was performed according to standard procedures [19]. HCC cells were transfected with plasmids using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the supplier. Transfected cells were incubated 48 h for overexpression of the plasmids.

2.3. Quantitative real-time reverse transcription-PCR (qRT-PCR)

qRT-PCR, or in short qPCR, was performed on total RNA extracted with Trizol (Invitrogen) and 200 nM of primers as before [20]. The primers used were as follows: PTMA, forward, 5'-GAGGGGCAACGCTCCTTCTTCT-3'; reverse, 5'-GAGGGGCAACGCTCCTTCTTCT-3'; GAPDH, forward, 5'-TCTTGCACACCAACTCTGTT-3'; reverse, 5'-GAGGGGCAACGCTCCTTCTTCT-3'. All samples and controls were prepared in triplicate. Relative quantification was calculated using the ΔΔCt method and normalized against GAPDH as described earlier [21]. Namely, ΔCt was calculated as ΔCt (candidate) = Ct (candidate) – Ct (GAPDH). Relative abundance of the candidate gene X was shown as 2^ΔΔCt (X).

2.4. Western blot analysis

Whole cell protein extracts were prepared for immunoblotting as before [22]. Protein concentration was determined using the Bradford assay and the BioRad dye reagent (BioRad, Hercules, CA, USA). Proteins (50 μg) from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membranes, prior to incubation with antibodies according to the instructions of the manufacturer. Protein signals were revealed using enhanced chemiluminescence according to specifications of the supplier (Pierce, Rockford, IL, USA). Protein band intensity was quantified using a scanning densitometer (Personal Densitometer SI; Amersham Biosciences, Sunnyvale, CA, USA).

2.5. Gene silencing using short-hairpin RNA

pLK0.1 plasmid expressing shRNA to silence PTMA (TRCN0000135421) was purchased from the National RNAi Core Facility (Taipei, Taiwan). Luciferase shRNA (TRCN0000072244) was used as a negative control. Unless indicated otherwise, transient transfection was performed by adding 2 μg/well of shRNA plasmid along with 5 μl/well of Lipofectamine into cell suspensions kept in six-well plates (1.5 × 10^6 cells/well) as described earlier [21]. Stable clones expressing shRNA plasmids via lentivirus as vector were established in HCC cells.

2.6. Preparation of mitochondria and cytosol fractions

Cells were washed once with PBS, prior to lysis in 3.5 mM Tris-HCl, 2 mM NaCl, 0.5 mM MgCl2 using a homogenizer with a motor-driven Teflon pestle. Cell homogenates were immediately mixed with nine volumes of 0.35 M Tris-HCl, 0.2 M NaCl, 50 mM MgCl2, and centrifuged for 3 min at 1600 × g to pellet unbroken cells, debris, and cell nuclei. The supernatant was centrifuged under the same conditions. The final supernatant was partitioned in tubes and centrifuged at 13,000 g for 5 min. The supernatant (cytosolic protein) was collected. Mitochondrial pellets were washed once with 35 mM Tris-HCl, 20 mM NaCl, 5 mM MgCl2, and resuspended in lysis buffer (1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail; BD Biosciences, San Jose, CA, USA) and incubation for 15 min. The entire purification process was performed at 4°C.

2.7. Analysis of cell viability and apoptosis

Cells were treated with cisplatin in culture medium for three days unless indicated otherwise. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [23]. Percentage of viable cells was calculated as the ratio of OD_{570nm} values of treated cells divided by the OD_{570nm} values of control cells. To evaluate apoptosis, sub-G1 cells were quantified as before [24]. Stained nuclei were analyzed using the BD FACScan Flow Cytometer (Becton & Dickinson, San Jose, CA, USA) with 10,000 events/determination. The LYSYS II software was used to assess cell cycle distribution. Unless indicated otherwise, samples were prepared in quadruplicate and three independent experiments were performed.
2.8. Statistical analysis

Data were reported as means ± standard deviation (SD). Three independent experiments were performed unless indicated otherwise. Statistical significance (p value) was calculated with a two-tailed Student’s t test for single comparison. The symbols *, **, and *** denote p < 0.05, p < 0.01 and p < 0.001, respectively.

3. Results

3.1. PTMA silencing sensitizes HCC cell lines to cisplatin-induced apoptosis

To assess the role of PTMA in cisplatin-treated HCC cells, we exposed HCC cell lines (Huh7, SK-Hep1, Mahlavu, and J7) to a lethal concentration of cisplatin (10 μM) and examined the level

![Image](https://via.placeholder.com/150)

Fig. 1. PTMA silencing down-regulates PTMA, up-regulates Bax, and enhances cisplatin-induced apoptosis and cell death in HCC cells. (A) The antiapoptotic protein PTMA is severely down-regulated by cisplatin in HCC cell lines. While Bcl-2, Bcl-XL, and Mcl-1 were not affected, survivin protein levels increased in HCC cells exposed to cisplatin. Quantified PTMA protein ratio is shown in the lower panel. (B) Bax apoptotic protein is moderately up-regulated by cisplatin. Quantified Bax protein ratio is shown in the lower panel. (C) Enhancement of cisplatin-induced caspase-9, caspase-3 and PARP cleavage following PTMA knockdown. Mahlavu cells stably expressing either shPTMA or shLuc plasmids via the lentivirus vector were treated with the indicated concentrations of cisplatin for 24 h. Fifty μg of total protein extract was used in immunoblotting experiments to detect caspase-9, caspase-3 and full-length PARP (FL PARP) as well as cleaved PARP. (D) Enhancement of cisplatin-induced sub-G1 cell accumulation following PTMA silencing. Cell sensitivity was assessed using the MTT assay. IC50 (the concentration of cisplatin that inhibits 50% of cell growth) and sensitization factor (SF50) are shown. SF50 was calculated by dividing the IC50 of control shLuc cells by that of shPTMA cells. Results are expressed as means ± SD for experiments performed in triplicate.
of anti-apoptotic proteins (Fig. 1A) and pro-apoptotic proteins (Fig. 1B) using Western blot analysis. While PTMA protein levels were reduced following treatment with cisplatin, the level of Bax increased in these cells. Compared with the shLuc control, silencing of PTMA using shRNA enhanced cisplatin-induced activation of caspase-3 and 9 and cleavage of the PARP substrate in Mahlavu cells, which express abundant PTMA (Fig. 1C). Flow cytometry analysis showed that apoptotic, sub-G1 Mahlavu cells increased in a time-dependent manner and concentration-dependent manner following treatment with cisplatin (Fig. 1D). Notably, PTMA silencing further increased cisplatin-induced accumulation of sub-G1 cells (Fig. 1D). In the MTT cell viability assay, PTMA silencing also enhanced cisplatin-induced cell death, producing a sensitization factor (SF50) of 1.71 (Fig. 1E; SF50 = IC50 shLuc/IC50 shPTMA). These results indicate that PTMA silencing enhances cisplatin-induced apoptosis and cell death in HCC cells.

3.2. Ectopic expression of PTMA protects cells against cisplatin-induced apoptosis

Compared with GFP overexpression used as control, overexpression of FlagPTMA (a PTMA fusion protein containing the Flag tag) reduced cisplatin-induced activation of caspase-3 and -9, and cleavage of PARP in J7 and SK-Hep1 cell lines, which both
express low PTMA protein level (Fig. 2A). While the amount of sub-G1 cells increased in a dose-dependent manner following treatment with cisplatin, FlagPTMA overexpression significantly reduced sub-G1 cell accumulation (Fig. 2B). Reduction of cisplatin-induced cell viability was reversed by FlagPTMA overexpression in J7 cells, producing a resistance factor (RF50) of 3.38 (Fig. 2C; RF50 = IC50 Flag PTMA/IC50 GFP). Similarly, the effects of cisplatin on sub-G1 cells and cell viability were reversed by FlagPTMA overexpression in SK-Hep1 cells (Fig. 2D and E).

3.3. Cisplatin enhances PTMA protein degradation via inhibition of JNK

To assess the mechanism underlying the down-regulation of PTMA by cisplatin, we monitored protein stability in Mahlavu cells pre-treated with cycloheximide, an inhibitor of translation initiation. PTMA protein degradation rate was enhanced by cisplatin treatment, producing a regression slope of −0.93 for cisplatin-treated cells vs. −0.56 for control PBS-treated cells (Fig. 3A and B). In contrast, steady-state PTMA mRNA levels were not affected by cisplatin treatment (Fig. 3C). In fact, mRNA degradation rate was only slightly reduced in cells pre-treated with actinomycin D, an inhibitor of mRNA synthesis, and incubated in media containing cisplatin (Fig. 3D, slope of −3.343 for cisplatin-treated cells vs. −4.800 for PBS-treated cells).

To determine the signaling pathway involved in PTMA protein degradation, we examined the kinase pathways affected by cisplatin in Mahlavu cells. Antibodies that react against phosphorylated kinases were used to assess kinase activation. While several kinases such as NFκB (IκB-α), P38, ERK and AKT were unaffected or their level of phosphorylation increased following treatment with cisplatin, JNK was transiently inhibited by the drug (Fig. 4A). Treatment with kinase inhibitors revealed that PTMA protein levels were inhibited by JNK, MEK/ERK, and PI3K inhibitors (Fig. 4B). These results suggest that the down-regulation of PTMA observed in cisplatin-treated HCC cells may occur via inhibition of the JNK pathway. To confirm the functional role of JNK, we treated Mahlavu cells with the JNK inhibitor and observed that PTMA protein degradation was indeed induced in the presence of cycloheximide (Fig. 4C and D).

3.4. Ectopic expression of PTMA rescues the effects of JNK inhibitor on cisplatin sensitivity

The significance of the JNK pathway in regulating cisplatin sensitivity was examined using the MTT cell viability assay. While JNK inhibition sensitized Mahlavu cells to cisplatin compared to control DMSO (Fig. 4E, SF50 = 1.56), ectopic expression of PTMA restored cell viability, producing a RF50 of 2.40 (Fig. 4E). Inhibition of JNK activity by cisplatin (Fig. 4F) and reduction of PTMA protein level by JNKi (Fig. 4G) were observed in Huh7 cells. Sensitization of Huh7 cells to cisplatin by JNKi could be restored by PTMA overexpression (Fig. 4H, RF50 = 3.75). Similarly, inhibition of JNK activity by cisplatin (Fig. 4I) and PTMA protein levels by JNKi (Fig. 4J) were observed in J7 cells. Sensitization of J7 cells to cisplatin by JNKi was also restored by PTMA overexpression (Fig. 4K, RF50 = 4.44). Exogenous expression of FlagPTMA as well as endogenous PTMA protein level is shown in Fig. 4L. These results show that JNK mediates cisplatin-induced PTMA down-regulation and that ectopic expression of PTMA rescues the effects of JNK inhibition on HCC cell sensitivity to cisplatin.

Fig. 3. Cisplatin attenuate PTMA expression through protein degradation. (A) Suppression of PTMA protein expression by cisplatin. Mahlavu cells were pre-incubated with cycloheximide (CHX), an inhibitor of protein synthesis, followed by the indicated incubation in cisplatin-containing media. (B) Increased degradation rate of PTMA protein in cisplatin-treated Mahlavu cells. Protein level was normalized to GAPDH. Linear regression of the kinetic pattern of PTMA protein level is shown. (C) Lack of inhibition of PTMA mRNA expression by cisplatin. PTMA mRNA level was evaluated by qPCR. The difference in mRNA level was compared to PBS control. Results are expressed as means ± SD for experiments performed in triplicate. P < 0.05, **P < 0.01. (D) Slight reduction in the degradation rate of PTMA mRNA in cisplatin-treated Mahlavu cells. Cells were pre-incubated with actinomycin D (Act. D), an inhibitor of RNA synthesis, followed by the indicated incubation. Results are shown as linear regression of the kinetic pattern of PTMA mRNA level.
Fig. 4. Downregulation of PTMA by cisplatin is mediated by the JNK signaling pathway. (A) Transient decrease of JNK signaling in cisplatin-treated Mahlavu cells. Cells were treated with cisplatin (10 μM) for the indicated time. NF-κB activity is shown by the level of its inhibitor IκB. Fold change of phosphorylated kinase is calculated after normalization to total kinase. (B) Reduction of PTMA protein level by JNK, MEK/ERK, and PI3K inhibitors in Mahlavu cells. Cells were treated with the indicated concentrations of inhibitors (see Materials and methods) for 4 h. Identification of kinase pathway by using specific kinase inhibitors, including NF-κB (Bay11-7082), JNK (SP600125), p38 (SB203580), ERK (U0126), and PI3K (Wortmannin). DMSO, used to dissolve the inhibitors, was used as control. Relative protein level (each lane was first normalized to GADPH) was calculated against the DMSO control (lane 1). (C) Suppression of PTMA protein expression by JNK inhibitor (JNKi). Mahlavu cells were pre-incubated with cycloheximide (CHX), followed by the indicated incubation in JNKi-containing media. (D) Increased degradation rate of PTMA protein in JNKi-treated Mahlavu cells. Linear regression of the kinetic pattern of PTMA protein level is indicated. (E) Sensitization of Mahlavu cells to cisplatin by JNKi treatment and reversal by PTMA overexpression. Cell viability was assessed using the MTT assay. Differences are calculated as averages of three experiments. Sensitization factor (SF50) and resistance factors (RF50) are shown. Symbols are the same as in Fig. 1. (F) Decrease of JNK signaling in cisplatin-treated Huh7 cells. (G) Reduction of PTMA protein level by JNK, MEK/ERK, and PI3K inhibitors in Huh7 cells. (H) Sensitization of Huh7 cells to cisplatin by JNKi treatment and reversal by PTMA overexpression. Symbols are the same as for panel E. (I) Decrease of JNK signaling in cisplatin-treated J7 cells. (J) Reduction of PTMA protein level by JNK, MEK/ERK, and PI3K inhibitors in J7 cells. (K) Sensitization of J7 cells to cisplatin by JNKi treatment and reversal by PTMA overexpression. (L) Expression of endogenous and exogenous PTMA in Mahlavu, J7, and Huh7 cells. These data are references for the cell viability experiments (panels E, H and K). The results shown are expressed as means ± SD for experiments performed in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001.
3.5. Ptma silencing enhances translocation of proapoptotic Bax to mitochondria, and cisplatin-induced cytochrome c release and caspase-9 activation

To assess the effects of PTMA on cisplatin-induced apoptosis, we examined the expression of anti-apoptotic and pro-apoptotic proteins in Mahlavu cells following PTMA silencing. While the steady-state level of survivin and Bcl-XL increased in cisplatin-treated cells, Mcl-1 and Bcl-2 protein levels were only moderately reduced (Fig. 5A, lanes 1–3). Except for Bcl-XL, the level of these proteins increased following PTMA silencing (Fig. 5A, compare lanes 1 and 4). Silencing of PTMA produced only a minor effect on these anti-apoptotic proteins (Fig. 5A, compare lanes 1–3 with lanes 4–6). Pro-apoptotic proteins such as Bad and Bax were induced by cisplatin. Silencing of PTMA only slightly affected cisplatin-induced regulation of these proteins (Fig. 5A, compare lanes 1–3 with lanes 4–6). Like most anti-apoptotic proteins, Bax level approximately doubled following down-regulation of PTMA in cells not treated with cisplatin. Unexpectedly, cisplatin-induced mitochondrial translocation of Bax (Fig. 5B, compare lanes 1–3 with lanes 7–9) was enhanced by PTMA silencing (compare lanes 7–9 with lanes 10–12). Furthermore, cytosolic levels of cytochrome c increased following cisplatin treatment (Fig. 5B, lanes 1–3). On the other hand, release of cytochrome c from mitochondria was enhanced by PTMA silencing (Fig. 5B, compare lanes 1–3 with lanes 4–6). In addition, cisplatin-induced cleavage of caspase-9 was enhanced by PTMA silencing. Thus, induction of cisplatin-induced apoptotic signals following PTMA silencing includes mitochondrial translocation of Bax (Fig. 5C), cytochrome c release from mitochondria (Fig. 5D), and activation of caspase-9 (Fig. 5E). It has been well documented that caspase-9 activation is dependent on Bax associated cytochrome c release from mitochondria [25].

Fig. 5. Cisplatin-induced caspase-9 activation is enhanced by PTMA silencing and is associated with expression and mitochondrial translocation of Bax in Mahlavu cells. (A) Regulation of Bcl-2 and Bax following cisplatin and PTMA silencing in Mahlavu cells. Other apoptotic regulators (survivin, Mcl-1, Bcl-XL) were not affected by shPTMA. Relative protein level (with each lane first normalized to GAPDH) was calculated against shLuc control (lane 1). (B) Increase in mitochondrial Bax and cisplatin-induced mitochondrial release of cytochrome c by shPTMA in Mahlavu cells. Caspase-9 cleavage was enhanced by shPTMA. α-Tubulin and VDAC are used as cytosol and mitochondria markers, respectively. (C) Quantification of mitochondrial Bax in shPTMA-expressing cells. (D) Quantification of cytosolic cytochrome c in shPTMA cells. (E) Quantification of caspase-9 cleavage in shPTMA-expressing cells. Results are expressed as means ± SD for experiments performed in triplicate. *P < 0.05, **P < 0.01.
believe that PTMA silencing enhances Bax translocation and that this process plays a critical role in inducing caspase-9 activation in Mahlavu cells.

3.6. Ectopic expression of PTMA inhibits Bax translocation to mitochondria and induces cytochrome c release and caspase-9 activation

To confirm the role of PTMA in regulating apoptosis, we performed similar experiments in J7 cells which express minimal amount of PTMA. While Bcl-2 protein level was induced by PTMA overexpression, other anti-apoptotic proteins were not affected by PTMA overexpression (Fig. 6A, compare lanes 1–3 with lanes 4–6). Cisplatin-induced Bad and Bax protein levels remained unchanged following FlagPTMA overexpression compared to GFP control. Conversely, cisplatin-induced mitochondrial translocation of Bax protein was reduced by ectopic expression of PTMA (Fig. 6B, compare lanes 7–9 with lanes 10–12). Furthermore, cisplatin-induced cytosolic cytochrome c level and caspase-9 activation were reduced by PTMA overexpression in these cells (Fig. 6B). Quantification of the apoptotic proteins is shown in Fig. 6C–E. Taken together, our results demonstrate that PTMA plays an important role in regulating cisplatin-induced apoptosis in HCC cells via pathways that have not previously described.

4. Discussion

Using loss-of-function and gain-of-function assays, we report here that PTMA plays a key role in regulating cisplatin sensitivity in HCC cells. Overexpression and silencing of PTMA respectively protects and sensitizes HCC cell lines to cisplatin, supporting the concept that PTMA represents an anti-apoptotic protein [7].

Fig. 6. Cisplatin-induced caspase-9 activation is down-regulated by ectopic expression of PTMA in J7 cells. (A) Reduction of Bax and induction of Bcl-2 by PTMA overexpression (FlagPTMA) in J7 cells. (B) Reduction of cisplatin-induced mitochondrial localization of Bax and cisplatin-induced mitochondrial release of cytochrome c by PTMA overexpression (FlagPTMA). (C) Quantification of mitochondrial Bax in FlagPTMA-expressing cells. (D) Quantification of decreased cytosolic cytochrome c in FlagPTMA-expressing cells. (E) Quantification of caspase-9 in FlagPTMA-expressing cells. Results are expressed as mean ± SD for experiments performed in triplicate. *P < 0.05, **P < 0.01.
Compared to other known anti-apoptotic proteins, PTMA is the most consistently down-regulated protein in HCC cells treated with a cytotoxic concentration of cisplatin. However, unlike sorafenib for which a significant correlation has been observed between the IC$_{50}$ produced by the drug and PTMA level observed in HCC cell lines [6], the correlation between the IC$_{50}$ of cisplatin and PTMA level observed in the present study is relatively low. Nevertheless, our results suggest that PTMA clearly regulates cisplatin sensitivity in isogenic HCC cells. The contribution of other known anti-apoptotic proteins in regulating cisplatin sensitivity appears to be minimal since their expression levels were not affected by cisplatin in the HCC cell lines studied here (see Fig. 1). Clinical evidence also indicates that PTMA represents an important marker that may help to predict therapy outcome. A recent study which examined immunohistochemistry of tumor tissues from 226 HCC patients who underwent curative hepatectomy showed that PTMA protein expression may represent a novel predictor of early recurrence and recurrence-free survival in HCC patients [26].

The level of apoptotic Bax was also induced by cisplatin in all HCC cell lines tested in the present study (Fig. 1). Other known apoptotic proteins (Bad, Bim, and Bid) were not affected by cisplatin in these cells. Mitochondrial translocation of Bax was enhanced by PTMA silencing in Mahlavu cells which constitutively express a high level of this protein. In contrast, ectopic expression of PTMA in J7 cells, which express relatively low PTMA protein level, reduced the mitochondrial fraction of Bax protein (Fig. 5). These results suggest that the overall level of apoptotic Bax protein and its mitochondrial fraction are upregulated by cisplatin. The change in Bax level and cellular location in HCC cells in response to cisplatin stress correlates with cytochrome c release from mitochondria and apoptosome activation (caspase-9). Activation of downstream caspase-3, apoptotic sub-G1 cells and cell growth inhibition by cisplatin was also enhanced by PTMA silencing, while PTMA overexpression reversed these cellular processes (see Figs. 1 and 2). To our knowledge, we demonstrate for the first time the functional role of PTMA in regulating Bax and caspase-dependent apoptosis.

In this study, we found that inhibition of JNK, MEK and PI3K by specific kinase inhibitors consistently down-regulated PTMA expression in HCC cell lines (Mahlavu, Huh7, and J7). Only the JNK signal was inhibited by cisplatin in these cells (see Fig. 4), suggesting that cisplatin may inhibit PTMA via the JNK pathway. We also observed that PTMA protein degradation was faster following treatment with either cisplatin or the JNK inhibitor compared to control. However, the level of PTMA mRNA was not affected by these treatments (Figs. 3 and 4). These results indicate that PTMA regulates cisplatin-induced apoptosis in HCC cells by regulating JNK at the post-translational level. We have previously described the cellular mechanism in which sorafenib targets PTMA gene transcription by inhibiting ERK kinase-regulated Myc/Max and beta-catenin-regulated JNK/AP-1/TCF4 in HCC cells [6,7]. Like sorafenib, cisplatin down-regulates PTMA expression in HCC cells. Unlike sorafenib, however, cisplatin down-regulates PTMA expression in a post-translational manner via the JNK pathway (see the model presented in Fig. 7).

In the present study, we also examined the possible involvement of PTMA reduction by cisplatin, which led to enhanced mitochondrial translocation of pro-apoptotic Bax (Fig. 7). The same cellular response was observed earlier in HCC cells in response to sorafenib [7], indicating that this cellular response may be critical to both treatments. Using cell viability assay, we observed that PTMA silencing produces a SFR of 1.71 in response to cisplatin while sorafenib produces a SFR of 1.51 [7]. In our experiments, both drugs produced similar effects in cultured HCC cells when used within the micromolar concentration range, suggesting that a combination of either drug and PTMA silencing may show similar efficacy in inhibiting HCC cell growth. The mechanism identified for cisplatin-induced apoptosis is also responsible for the activities of other platinum-containing anti-cancer agents, such as carboplatin, which is also used in cancer treatment (data not shown).

Our finding that the PTMA/JNK signaling pathway is involved in response to cisplatin is likely to have clinical implications. Sorafenib was described as a multi-kinase inhibitor that targets various pathways including RAF/MEK/ERK, vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR)-beta, KIT, FLT-3, RET and Wnt/beta-catenin [27–29]. Cellular signaling of the Raf-1 and vascular endothelial growth factor (VEGF) pathways has been implicated in HCC pathogenesis [30–33], thereby providing a rationale for the clinical use of sorafenib. However, limited survival benefits have been observed since sorafenib has been used as a standard therapy for advanced HCC [34,35]. Our results indicate that targeting or silencing PTMA may enhance the effects of cisplatin and sorafenib in HCC. The PTMA-based response of HCC cells to cisplatin appears to be similar to that of sorafenib, which may lead to the potential use of cisplatin in advanced HCC. However, the microenvironment of HCC should be taken into account while evaluating the efficacy of these drugs in clinical settings.

Conflict of interest

The authors declare no conflict of interest.

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