Silencing of prothymosin alpha sensitizes hepatocellular carcinoma cells to sorafenib by attenuating ERK and β-catenin signals and by activating the Bax/caspase-9 pathway

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\textbf{Abbreviations}: ChIP, chromatin immunoprecipitation; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; ERK, extracellular regulated protein kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; HURP, hepatoma upregulated protein; JNK, c-Jun N-terminal kinases; Luc, luciferase; MAPK, mitogen-activated protein kinase; 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.
ABSTRACT

Prothymosin alpha (PTMA) is highly expressed in various human tumors, including hepatocellular carcinoma (HCC). However, whether PTMA plays a role in this context remains unclear. In this study, we show that silencing PTMA using short hairpin RNA sensitizes HCC cells to the kinase inhibitor sorafenib. In contrast, ectopic expression of PTMA in HCC cells induces cell resistance to the kinase inhibitor. Our experiments indicate that sorafenib treatment moderately reduces PTMA mRNA and protein levels. While inhibitors targeting JNK, ERK or PI3K also reduce PTMA expression, we found that only ERK activation is suppressed by sorafenib. Treatment of HCC cells with the ERK inhibitor induces PTMA mRNA degradation, without affecting PTMA protein stability. Ectopic expression of MKK1/2-CA, a constitutive active ERK1/2 kinase, dramatically increases PTMA expression. Our results also show that cytotoxic doses of sorafenib cause dramatic reduction of β-catenin. Silencing β-catenin reduces PTMA level to the extent that sorafenib produces no further inhibition on PTMA protein level. Silencing PTMA significantly increases Bax/Bcl2 protein ratio and potentiates Bax translocation to mitochondria in response to sorafenib, a phenomenon associated with increased cytochrome c release from mitochondria and enhanced caspase-9 activation. These results indicate that PTMA acts as a survival protein that protects HCC cells against sorafenib-induced apoptosis and cell death, thus offering a new target for chemotherapy against HCC. In addition, the regulation of PTMA gene expression by MKK1/2-ERK1/2 and Wnt/β-catenin signaling appears to be critical for HCC cells to respond to sorafenib.
1. Introduction

Hepatocellular carcinoma (HCC) is a highly prevalent, treatment-resistant malignancy with a multifaceted molecular pathogenesis. HCC is the third highest cause of cancer-related deaths worldwide [1]. HCC patients often respond poorly to current drug therapies. In Taiwan, HCC is one of the most frequent and devastating malignancies, representing 83% of all cancer cases. Early diagnosis is difficult, leading to poor prognosis and high mortality rates.

Hepatocarcinogenesis is associated with alterations in several important cellular signaling pathways, including those involving RAF/MEK/ERK, phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), Wnt/β-catenin, insulin-like growth factor, hepatocyte growth factor/c-MET, and growth factor-regulated angiogenic signaling [2, 3]. Aberrant activation of Wnt signaling can be due to mutations in the β-catenin gene (CTNNB1), which is the second most frequent mutation observed in HCC, after mutations in the p53 tumor suppressor gene [4]. Advances in molecular oncology and rational drug design have led to the development of targeted therapies for a variety of hematological and solid tumors, including HCC [5].

Sorafenib was initially found to represent a potent inhibitor of Raf1 kinase in vitro [6, 7], and was later found to be a potent multi-kinase inhibitor targeting the RAF/MEK/ERK pathway as well as vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR)-β, KIT, FLT-3, RET and Wnt/β-catenin [8-10]. Sorafenib shows anti-oral-tumor activity by targeting MAPK signalling and by producing anti-angiogenic effects in rodent tumor xenograft models [9]. The multi-kinase inhibitor drug is effective as a single agent or in combination with other treatments in clinical trials of renal cell carcinoma (RCC), HCC, melanoma, and non-small-cell lung cancers (NSCLC) [11]. Cellular signaling of the Raf-1 and vascular endothelial growth factor (VEGF) pathways has been implicated in the molecular pathogenesis of HCC [12-15], thereby providing a rationale for using sorafenib in clinical settings. Two large, randomized, placebo-controlled, phase III clinical trials—the Sorafenib HCC Assessment Randomized Protocol (SHARP) and the Sorafenib Asia-Pacific (AP) trial—showed that sorafenib significantly enhances survival in patients with advanced HCC [2, 16, 17]. However, it remains unclear whether sorafenib inhibits kinase-regulated genes in HCC cells. Genes that are upregulated by the above mentioned kinases represent potential targets of sorafenib in HCC.

Prothymosin alpha (PTMA) is a small, highly acidic protein widely distributed in all mammalian tissues and found both intracellularly and extracellularly. The fact that
its primary structure is highly conserved in all mammalian species suggests that this protein has an essential function [18, 19]. The human form of PTMA has 110 amino acid residues (12.1 kDa). Cleavage of the N-terminal domain by lysosomal asparaginyl endopeptidase produces a 28-amino-acid active peptide called thymosin α1 [20]. New findings on the extracellular function of PTMA have yielded exciting insights into its cytokine-like functions, which involve stimulation of type I interferon via Toll-like receptor 4 [21]. PTMA plays essential roles in cell proliferation [22, 23], transcriptional regulation [24, 25], chromatin remodeling [26, 27], oxidative stress-response [28] and apoptosis [29, 30]. Furthermore, this protein is highly expressed in various tumor tissues such as breast cancer [31], hepatocarcinoma [32], lung cancer [33], neuroblastoma [34], bladder cancer [35], gastric [36] and upper urinary tract transitional cell carcinoma [37]. Accordingly, PTMA is used as a cancer prognostic marker, including for cancers of the breast, stomach, lung, prostate, bladder, head-and-neck, and thyroid [31, 33, 36, 38-41].

A cell-death-regulatory pathway consisting of PTMA and the tumor suppressor PHAP (putative HLA-DR-associated protein), with each protein playing a distinctive role in regulating apoptosome formation and activity, has been identified previously [29]. Inhibition screening studies performed using small molecules revealed that PTMA-mediated inhibition of apoptosome formation occurs through blockage of its interaction with Apaf-1 [42].

Alterations in several important cellular signaling pathways are associated with hepatocarcinogenesis. Genes upregulated by these signaling pathways are potential candidates to prevent the anti-apoptotic effects and chemoresistance observed in HCC therapy. We previously demonstrated that HURP (hepatoma upregulated protein), a protein overexpressed in human hepatocellular carcinoma [43], represents an important target of sorafenib in HCC cells cultured in vitro [44]. Early-stage clinical trials investigating the efficacy of up to 60 compounds against HCC are currently in progress. These studies may considerably change the management strategy for HCC, and combination therapies may be developed for patients with advanced stage of the disease. So far, sorafenib is the only targeted therapy that produces significant beneficial effects in HCC patients.

In the present study, we found that PTMA is upregulated in HCC cells and that this protein may represent a novel target of sorafenib. Notably, silencing of PTMA enhances sorafenib-induced caspase activation, Bax translocation to mitochondria, and cytochrome c release into the cytosol. We also demonstrated that sorafenib inhibits PTMA expression through inactivation of p-ERK and β-catenin, two putative positive regulators of the PTMA gene.
2. Materials and methods

2.1. Cell cultures and reagents

Primary liver Chang liver cells and hepatocellular carcinoma cells (Huh7, J7, SK-Hep1, and Mahlavu) 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). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ (v/v) in air. The reagents used included antibodies against caspase-3, caspase-9, Akt, p-Akt, p-JNK, p-P38, β-catenin (Cell Signaling, Danvers, MA, USA), PTMA, JNK, ERK, p-ERK, P38, IκB-α, P65, Elk-1, survivin, Mcl-1, Bcl-X₁, Bcl-2, Bax, Bad, cytochrome c, GAPDH, α-tubulin, lamin A/C, VDAC, HA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Flag (Sigma-Aldrich, St. Louis, MO, USA). Kinase inhibitors against NF-κBi (Bay11-7082), JNKi (SP600125), P38i (SB203580) (Santa Cruz Biotechnology), ERKi (U0126) (Calbiochem, CA, USA), and PI3Ki (Wortmannin; Cell Signaling) were also used. Sorafenib (Bayer HealthCare AG, Berlin, Germany) was kindly provided by Dr. T.-C. Chang (Chang Gung Memorial Hospital, Taoyuan, Taiwan). The other chemicals were purchased from Sigma-Aldrich.

2.2. Plasmids and transfection

The pLKO-AS3w-puro expression vector was purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). The pLKO-AS3w-eGFP-puro plasmid was used as a negative control. The DNA sequence containing PTMA’s open-reading frame (ORF) was cloned from Mahlavu cells by using specific primers (forward primer with XhoI site, 5’-GCCTCGAGATGTCAGACGCAGCCGTAGACACC-3’; reverse primer with XbaI site, 5’-TTTCTAGACTAGTCATCCTCGGTGCTTCTCTG-3’), and ligated with the pGEM-T Easy vector (Promega, Madison, WI, USA) using the T4 DNA ligase. PTMA ORF was released from the pGEM-T Easy vector using the restriction enzymes XhoI and XbaI, and was inserted into the pCMV-Flag expression vector, resulting in pCMV-FlagPTMA. The FlagPTMA sequence was isolated from pCMV-FlagPTMA using specific primers (forward primer with NheI site, 5’-GCTAGCATACGACTACGACGACCGTACCACCC-3’; reverse primer with PmeI site, 5’-GTGTTAACATTTAGGTGATGACACTATAG-3’), and ligated with the pGEM-T Easy vector (Promega). FlagPTMA sequence was released from the pGEM-T Easy vector using the restriction enzymes NheI and PmeI, and was inserted in the pLKO-AS3w-puro expression vector, resulting in pLKO-FlagPTMA. MKK1/2CA plasmids (constitutively active MKK1/2 vectors) were kindly provided by Dr. Yun-Wei Lin (National Chiayi University, Chiayi, Taiwan; originally obtained from
Dr. Jia-Ling Yang, National Tsing Hua University, Taiwan). Plasmid construction and preparation was performed according to standard protocols [45]. Cells were transfected with plasmids using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the supplier. Transfected cells were incubated for 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 previously described [46]. The primers used were the following: PTMA, forward, 5’-CGAAATCACCACCAAGGACT-3’; reverse, 5’-GTCGGTCTTCTGCTTCTTGG-3’; and GAPDH, forward, 5’-TCTCTGACACCCACCAACTGCTT-3’; reverse, 5’-GAGGGGGCCATCCACGTCTT-3’. All unknown samples and controls were done in triplicate. Relative quantification was calculated by the ΔΔCt method and normalized against GAPDH as described before [47]. Namely, the ΔCt for each candidate was calculated as ΔCt (candidate) = [Ct (candidate) - Ct (GAPDH)]. The relative abundance of the candidate gene X was shown as 2^{ΔCt(X) - ΔCt(GAPDH)}.

2.4. Western blot analysis
Whole cell protein extracts were prepared for immunoblotting as described [48]. 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, and the membranes were incubated with antibodies according to the instructions of the manufacturer. The signal on the membranes was revealed using enhanced chemiluminescence according to the specifications of the supplier (Pierce, Rockford, IL, USA). The intensity of the protein bands was determined using a scanning densitometer (Personal Densitometer SI: Amersham Biosciences, Sunnyvale, CA, USA).

2.5. Silencing of selected genes using short-hairpin RNA
pLKO.1 plasmids expressing shRNA were purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Luciferase shRNA (TRCN0000072244) was used as a negative control. Transient transfection was done by adding 2 μg/well (unless indicated otherwise) of shRNA plasmids along with 5 μl/well of Lipofectamine (Invitrogen) into cell suspensions kept in six-well plates (1.5 × 10^4 cells/well) as described before [47]. The plasmids used included PTMA (TRCN0000135421), CTNNB1 (TRCN0000314991), and Elk-1 (TRCN0000237873).
Stable clones expressing shRNA plasmids via lentivirus as vector were established in HCC cells.

2.6. Fractionation of mitochondria and cytosol

Cells were washed once with PBS, prior to lysis in 3.5 mM Tris-HCl, 2 mM NaCl, 0.5 mM MgCl₂ using a Thomas homogenizer with a motor-driven Teflon pestle. The cell homogenate was immediately mixed with nine volumes of 0.35 M Tris-HCl, 0.2 M NaCl, 50 mM MgCl₂, and spun for 3 min at 1,600g to pellet unbroken cells, debris, and nuclei. The supernatant was centrifuged under the same conditions. The final supernatant was partitioned in tubes and spun at 13,000g for 5 min. The supernatant (cytosolic protein) was collected. The mitochondrial pellets were washed once with 35 mM Tris-HCl, 20 mM NaCl, 5 mM MgCl₂, prior to lysis 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. Fractionation of nuclei and cytosol

Cells were washed once with PBS, and resuspended in extraction buffer (10 mM KCl, 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, PMSF), followed by incubation on ice for 15 min. Cells were broken by pipetting up-and-down 30 times, followed by centrifugation at 13,000g for 1 min, and the supernatant was harvested (cytosolic proteins). Pellets (containing nuclei) were lysed in lysis buffer as above, and incubated for 15 min at 4 °C.

2.8. Analysis of cell viability and apoptosis

Cells were treated with sorafenib in culture medium for 3 days unless indicated otherwise. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described previously [49]. Percentage of viable cells was calculated as the ratio of OD₅₇₀ nm values for treated cells divided by the OD₅₇₀ nm values for control cells in quadruplicate. To evaluate apoptosis, sub-G₁ cells were measured as described [50]. 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. Three independent experiments were performed, unless indicated otherwise.

2.9. Analysis of PTMA protein stability

Cells (5 × 10⁵) plated on 60-mm tissue culture dishes were grown for 24 h, and
cycloheximide (CHX) was then added at 100 μg/ml. At various times following addition of CHX, cells were harvested and lysed in lysis buffer, followed by Western blot analysis with PTMA antibody. Protein band intensity was determined as above.

2.10. Statistical analysis

The data were reported as mean values ± 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. Silencing PTMA enhances sorafenib-induced cytotoxicity in HCC cells

To study the role of PTMA in sorafenib-treated HCC cells, we first examined the expression levels of PTMA mRNA and proteins in various HCC cell lines. Compared to Chang liver cells, HCC cell lines (except Mahlavu) showed higher levels of PTMA mRNA as determined by q-PCR (Fig. 1A). Notably, Huh7 cells expressed 7 times more PTMA mRNA than Chang liver cells while SK-Hep1 and J7 showed a 2-fold increase (Fig. 1A). Furthermore, Huh7 and Mahlavu cells respectively showed a 2- and 3-fold increase of PTMA protein compared to Chang liver cells (Fig. 1B). SK-Hep1 and J7 cells showed PTMA protein levels similar to Chang liver cells (Fig. 1B). These results indicate that PTMA mRNA and protein levels are increased to different extents in HCC cell lines.

PTMA expression in Mahlavu cells appeared to be different from the other HCC cell lines examined. The abundant PTMA protein level and low PTMA mRNA level observed in Mahlavu cells suggest that the PTMA protein may be more stable in these cells. To investigate the role of PTMA, a protein found previously to have anti-apoptotic activity [29], we expressed short hairpin RNA (shPTMA) against this protein in Mahlavu cells and treated the cells with sorafenib to induce cell death and apoptosis. While PTMA mRNA level decreased by more than 80% in shPTMA-expressing cells (Fig. 2A), PTMA protein level was downregulated by around 50% (Fig. 2B). Using the MTT assay, we found that HCC cells showed significantly increased sensitivity to sorafenib following silencing of PTMA, with a sensitization factor (SF50) calculated at 1.51 (Fig. 2C; SF50 was calculated by dividing the IC50 of control shLuc cells by that of shPTMA cells). Caspase-9 and caspase-3 activation as well as PARP cleavage were induced by sorafenib (20 μM) in Mahlavu cells (Fig. 2D, compare lanes 1 and 2). While caspase activation was not significantly affected by PTMA silencing (Fig. 2D, lane 1 vs. lane 3), sorafenib-induced caspase
activities were enhanced in shPTMA-expressing cells (Fig. 2D, compare lanes 2 and 4). We also noted that the PTMA protein level was reduced by sorafenib treatment (Fig. 2D, lane 1 vs. 2 and lane 3 vs. 4). To verify that sorafenib-induced apoptosis is potentiated by PTMA knockdown, we examined the level of sub-G1 cells using flow cytometry. Sorafenib-induced sub-G1 cells were significantly increased by shPTMA in a time-dependent manner, with the highest increase being observed at 72 hrs (Fig. 4E; 10 μM). Sorafenib-induced sub-G1 cells also significantly increased in a dose-dependent manner (10 vs. 20 μM sorafenib), with the most dramatic difference being observed after 24 hrs of treatment (Fig. 2E). These results suggest that PTMA silencing enhances the cytotoxic and pro-apoptotic effects of sorafenib in HCC cells.

3.2. Ectopic expression of PTMA protects HCC cells against sorafenib treatment

To examine the role of PTMA in modulating the cytotoxic effect of sorafenib, we expressed PTMA (FlagPTMA fusion) in J7 and SK-Hep1 cells (Fig. 3A) since these cells expressed the lowest levels of PTMA protein among the HCC cell lines tested (Fig. 1B). Using a range of sorafenib doses that induced a sensitive response, we observed that PTMA overexpression protected the cells against sorafenib-induced cell death, producing resistance factor (RF₅₀) of 1.69 and 2.06 for J7 and SK-Hep1 cells, respectively (Fig. 3B). PTMA overexpression slightly reduced caspase activation in J7 cells (Fig. 3C, left panel). Surprisingly, sorafenib used at cytotoxic doses (10 and 20 μM) for as long as 72 hrs did not activate caspases in SK-Hep1 cells (Fig. 3C, right panel). However, slightly higher levels of full-length PARP were found in PTMA-overexpressing SK-Hep1 cells compared to control GFP-expressing cells (Fig. 3C, right panel). The protective effects of PTMA against sorafenib-induced sub-G1 cell accumulation were observed in both HCC cell lines (Fig. 3D, 10 and 20 μM).

3.3. Sorafenib inhibits PTMA expression in HCC cells by enhancing PTMA mRNA degradation

The results presented above suggest that sorafenib inhibits PTMA activity. To verify the underlying mechanism, we examined PTMA mRNA and protein levels in Mahlavu cells following sorafenib treatment. Both PTMA mRNA and protein levels were reduced in a dose-dependent manner in cells exposed to sorafenib (Fig. 4A and B). The degradation rate of PTMA protein in sorafenib-treated Mahlavu cells appeared to be similar to that observed for the DMSO control (Fig. 4C). In this experiment, the cells were pre-incubated with the protein synthesis inhibitor cycloheximide (CHX) for various periods of time, followed by evaluation of PTMA protein level. PTMA was moderately stable, with 50% remaining in both groups after 24 hrs of CHX treatment (Fig. 4C). Linear regression of PTMA protein levels over
time showed similar degradation rates in both groups (Fig. 4C, \( R^2 \) of 0.97 and 0.98). We also examined PTMA mRNA degradation following treatment of the cells with the transcription inhibitor actinomycin D (Fig. 4D). PTMA mRNA levels decreased to a larger extent in sorafenib-treated cells than in the DMSO control (Fig. 4D). Linear regression analysis revealed a 2.87-fold difference between the two slopes (Fig. 4D, -1.957 vs. -5.614), approaching the ratio of PTMA protein between DMSO and sorafenib treatments (1.0 vs. 0.4 in Fig. 4B, compare lanes 1 and 3). Taken together, these results show that sorafenib may inhibit PTMA expression in HCC cells by enhancing PTMA mRNA degradation.

3.4. Sorafenib downregulates PTMA by inhibiting ERK and β-catenin pathways

To evaluate the possibility that PTMA down-regulation by sorafenib may be due to kinase inhibition, we investigated kinase activity in Mahlavu cells treated with 10 μM sorafenib (Fig. 5). Only ERK activation (phosphorylated ERK) was inhibited by sorafenib in a time-dependent manner (Fig. 5A, left panel). The level of inhibition of various kinases following a short exposure of the cells to sorafenib was also examined (Fig. 5A, right panel). The concentrations of inhibitors used were all effective in inhibiting the target kinase following treatment of the cells for 4 hrs (Fig. 5B), indicating that the lack of kinase inhibition shown in Fig. 5A was not due to loss of inhibitor activity. PTMA protein level was suppressed by about 50% following treatment with JNK, ERK, and PI3K inhibitors (Fig. 5C). Similarly, these kinase inhibitors significantly decreased PTMA mRNA levels (Fig. 5D). These results suggest that suppression of PTMA by sorafenib may occur through inhibition of ERK in HCC cells.

To verify the role of ERK in sorafenib-induced down-regulation of PTMA, we examined the effects of the ERK inhibitor on the stability of PTMA mRNA and protein (detected in Fig. 4). The degradation rate of PTMA mRNA was enhanced 2.36-fold by the ERK inhibitor (Fig. 6A, slope -1.371 vs. -3.229). In contrast, the degradation rate of PTMA protein was similar for both the ERK inhibitor and control DMSO (Fig. 6B). To confirm the role of ERK in regulating PTMA expression in response to sorafenib, we expressed M KK1/2-CA (a constitutively active ERK1/2 kinase) in Mahlavu cells prior to sorafenib treatment. Ectopic expression of M KK1/2-CA caused a dramatic increase of PTMA (Fig. 6C, compare lanes 1 and 3). While p-ERK was highly suppressed by sorafenib in cells expressing the pcDNA3 vector control, the level of p-ERK remained relatively high in the cells expressing M KK1/2-CA (Fig. 6C, lane 2 vs. lane 4). These results demonstrate that the ERK pathway mediates the regulation of PTMA expression in response to sorafenib.

Besides, sequence analysis of the PTMA promoter and regulatory regions
revealed a putative binding site for Elk-1, a transcription factor activated by ERK [51]. However, knockdown of Elk-1 using shRNA did not affect PTMA protein level (Fig. 6D).

3.5. Sorafenib downregulates PTMA by inhibiting the β-catenin pathway

Aberrant activation of Wnt signaling can be due to mutations in the β-catenin gene (CTNNB1), which represent the second most frequent mutation observed in HCC [4]. Sorafenib modulates Wnt/β-catenin signaling in experimental models that harbor the CTNNB1 class signature [52]. Whether this pathway regulates PTMA expression in HCC cells remains unclear. Here, we observed a 50% reduction of PTMA expression following β-catenin/CTNNB1 knockdown in Mahlavu cells (Fig. 6E, compare lanes 1 and 3). While sorafenib treatment reduced β-catenin/CTNNB1 by 80%, the drug reduced PTMA to 40% of control (Fig. 6E). However, β-catenin/CTNNB1 knockdown did not further decrease PTMA protein level in sorafenib-treated cells (Fig. 5E, compare lanes 2 and 4). In addition, the ERK inhibitor did not affect nuclear localization of β-catenin in our experiments (Fig. 6F). These results suggest that down-regulation of PTMA by sorafenib in Mahlavu cells may involve the β-catenin pathway, while being independent of the ERK pathway.

3.6. Silencing PTMA potentiates caspase activation by sorafenib: enhanced mitochondrial translocation of Bax and cytochrome c release

To assess the mechanism of caspase activation following PTMA silencing, we investigated the changes in the regulatory proteins responsible for caspase activation. Among the anti-apoptotic proteins examined in Mahlavu cells, survivin and Mcl-1 protein levels were reduced in a dose-dependent manner by sorafenib, whereas Bcl-XL remained essentially unchanged (Fig. 7A, lanes 1-3). Bad protein level was upregulated by 10 μM sorafenib, but was not affected by shPTMA. Most protein patterns were similar for both shLuc and shPTMA treatments (Fig. 7A, compare lanes 1-3 and lanes 4-6). Interestingly, while Bax and Bcl-2 levels increased with the dose of sorafenib in shLuc control, both protein levels dramatically increased in shPTMA-expressing cells (Fig. 7A, lane 1 vs. lane 4). However, Bax and Bcl-2 levels did not increase further in the presence of sorafenib. Average Bax/Bcl-2 ratios increased in shPTMA-expressing cells compared to control shLuc (Fig. 7A, right panel). To define the fraction of mitochondrial Bax, we prepared cell fractions for the same experimental samples. Representative results indicated that the endogenous level of Bax was located preferentially in the mitochondria in shPTMA cells compared to control shLuc cells (36% vs. 7%) (Fig. 7B, compare lanes 10 and 7). We observed a sorafenib-dose-dependent increase of mitochondrial Bax (Fig. 7B,
compare lanes 7, 8 and 9). However, the mitochondrial translocation of Bax induced by sorafenib in shPTMA cells was not higher than that seen in the untreated control (Fig. 7B, compare lanes 10, 11 and 12). These differences were statistically significant (Fig. 7C). Intrinsical, high level of mitochondrial Bad protein was detected in control (55%) compared to shPTMA condition (39%) (Fig. 7B, compare lanes 7 and 10). While the Bad protein level was reduced by 10 μM sorafenib, it was not further reduced by 20 μM sorafenib. However, such regulation was not affected by shPTMA (Fig. 7B, compare lanes 7-9 and 10-12). The role of Bad protein in this study may be minimal. The significance of the mitochondrial translocation of Bax was supported by the release of mitochondrial cytochrome c into the cytosol. While a slight increase of cytosolic cytochrome c was induced by sorafenib in shLuc cells, cytosolic cytochrome c dramatically increased in shPTMA-treated cells (Fig. 7B and D). Activation of caspase-9 by sorafenib was also enhanced by shPTMA (Fig. 7B and E).

Ectopic expression of PTMA was induced in J7 cells, which express a minimal level of PTMA. Ectopic expression of PTMA (FlagPTMA) did not significantly alter the changes seen in sorafenib-induced survivin, Mcl-1, or Bcl-XL levels. Bax levels were induced 1.8- and 1.7-fold in control GFP cells treated with 10 μM and 20 μM sorafenib, respectively (Fig. 8A, lanes 1-3). While Bax levels were induced 1.6-fold by 10 μM sorafenib in PTMA-expressing cells, Bax was not induced by 20 μM of the drug (Fig. 8A, lanes 4-6), indicating that sorafenib-induced Bax levels can be suppressed by PTMA overexpression. Like Mahlavu cells, J7 cells showed a dose-dependent increase of Bcl-2 following sorafenib treatment (Fig. 8A, lanes 1-3), but the drug induced little increase of Bcl-2 in PTMA-expressing cells (Fig. 8A, lanes 4-6). We further noted that endogenous Bax levels were not affected by PTMA overexpression in J7 cells, whereas the levels of endogenous Bcl-2 considerably increased in PTMA-expressing cells (Fig. 8A). Average Bax/Bcl-2 ratios were significantly reduced by PTMA overexpression (Fig. 8A, right panel). Mitochondrial Bax localization and cytochrome c release were also investigated in sorafenib-treated J7 cells. A slight increase of mitochondrial Bax was detected in FlagPTMA cells compared to control GFP cells (52% vs. 45%) (Fig. 8B, compare lanes 10 and 7), whereas lower amounts of mitochondrial Bax was induced by sorafenib (20 μM) in FlagPTMA cells compared to control GFP (56% vs. 64%) (Fig. 8B, compare lanes 12 and 9). However, these differences were not statistically significant (Fig. 8C). Furthermore, while more endogenous cytochrome c was found in the cytosol of FlagPTMA cells than in control GFP cells (9% vs. 2%) (Fig. 8B, compare lanes 4 and 1; Fig. 8D), no more cytosolic cytochrome c was induced by sorafenib (20 μM) in J7 cells (35% vs. 39%) (Fig. 8B, compare lanes 6 and 3). Activation of caspase-9 by
sorafenib was also reduced by PTMA overexpression in J7 cells (Fig. 8B, compare lanes 1-3 and lanes 4-6; Fig. 8E). The role of Bad protein probably negligible as revealed by evidence that mitochondrial Bad protein was not induced by sorafenib and not affected by PTMA overexpression (Fig. 8B, compare lanes 7-9 and 10-12). Taken together, these results suggest that PTMA may regulate sorafenib-induced caspase activation through regulation of mitochondrial translocation of Bax and cytochrome c release.

3.7. PTMA expression correlates with chemoresistance in HCC cell lines and reduced survival in HCC patients

To verify the importance of PTMA in regulating cell sensitivity to sorafenib, we compared the PTMA protein levels in HCC cell lines with the IC_{50} of sorafenib treatment (Fig. 9A). We observed a high correlation between these two factors (R^2 = 0.91). Using a bioinformatic analysis of database originally designed to identify human cancer genes in a mouse model [53], we analyzed the expression profile of microarray samples containing 91 human HCC patients from predefined subclasses (National Cancer Institute/NIH; GEO accession: GSE1898) [54]. The calculated data showed significantly higher PTMA expression levels in the HCC subgroup associated with poor survival (subgroup A; n = 40) compared with the one associated with good survival (subgroup B; n = 51). We also found a positive correlation between high PTMA gene expression and poor prognosis in human HCC patients (Fig. 9B).

4. Discussion

In this study, we found a functional role for PTMA in potentiating the efficacy of sorafenib therapy in HCC cells. The importance of PTMA was revealed based on the observation that silencing PTMA increases sorafenib-induced apoptosis while PTMA overexpression reverses this effect. These results were observed in both Mahlavu and J7 cells. Moreover, we obtained similar results in SK-Hep1 and Huh7 cells (data not shown). Our results suggest that PTMA protects HCC cells against sorafenib. Furthermore, endogenous PTMA expression levels correlated with the cell response to sorafenib in HCC cell lines (Fig. 9A, R^2 = 0.91). Genome-wide analysis of HCC patients using publicly available datasets also revealed that higher PTMA levels are found in patients with poor survival, further supporting the importance of PTMA in sorafenib therapy against HCC.

We also detected alterations in the expression and localization of mitochondrial components such as Bax and Bcl-2 in HCC cells following PTMA knockdown or overexpression. Silencing PTMA using shRNA elicited a dramatic increase of mitochondrial Bax, sorafenib-induced cytochrome c release, and caspase-9 activation
in Mahlavu cells (Fig. 7). While 45% of Bax was located in the mitochondria in J7 cells in the absence of exogenous stress, only a slight increase in the level of mitochondrial Bax was found in these cells. Interestingly, while mitochondrial Bax was slightly induced by sorafenib (10 μM) in J7 cells, with or without PTMA overexpression, significant increase of cytochrome c release was found in the cells with or without PTMA overexpression (Fig. 8C and D). Mitochondrial Bax and cytosolic cytochrome c were not further induced by high concentration of sorafenib (20 μM) in J7 cells, suggesting that 40-70% translocation to mitochondria may represent the maximal level observed in this context. Bax mitochondrial translocation to 40% could be induced by shPTMA alone or in combination with sorafenib in Mahlavu cells (Fig. 7C). In this sense, PTMA may regulate Bax translocation and produce a pro-apoptotic state, depending on the level of Bax in HCC cell lines. In these cell systems, a proportional association was found between sorafenib-induced cytochrome c release and caspase-9 activation. Interestingly, both intrinsic and induced cytochrome c release from mitochondria were regulated by PTMA in the cell systems studied. However, only sorafenib-induced caspase-9 activation was regulated by PTMA, suggesting that caspase-9 activation involves additional factors such as physical binding of PTMA to apoptosome activator [29, 42]. This observation may be explained by biochemical evidence obtained from in vitro experiments. Wang and colleagues first demonstrated the existence of a death regulatory pathway consisting of PTMA and PHAP, with each playing a distinctive role in regulating apoptosome formation and activity [29]. Subsequent studies revealed that inhibition of apoptosome formation by PTMA is due to a blockade of the interaction between PTMA and Apaf-1, which constitutes a key component of apoptosome activation [42]. Taken together, we suggest that PTMA regulates caspase-9/apoptosome activation in two stages: by direct interaction with the activating component Apaf-1 and by indirect interaction with mitochondrial proteins such as Bax and Bcl-2 (see Fig. 9C). Although it is currently unclear how PTMA regulates mitochondrial translocation of Bax, the experimental evidence shown in this study reveals the existence of an additional pathway in which PTMA regulates cytochrome c release and thus caspase-9/apoptosome activation in HCC cells.

Regulation of mitochondria-mediated apoptosis may be more complex. An expanding family of Bcl-2-related proteins share sequence homology and cluster according to four conserved Bcl-2 homology (BH1-4) domains, which control the ability of these proteins to form dimers and function as regulators of apoptosis. Pro-apoptotic Bid and Bad, for example, possess the minimal death domain BH3, and the phosphorylation of Bad connects survival signals to the Bcl-2 family. These proteins also reveal the existence of coordination between opposing pathways.
regulating proliferation and cell death [55]. Mutation of Bad shows that the BH3 domain is critical for binding to the pocket of the antagonist Bcl-XL and for its death agonist activity [56]. In our study, while the expression level of Bad was regulated by sorafenib, the patterns of expression were not significantly affected by either knockdown or overexpression of PTMA in HCC cells (Fig. 7A and 8A). However, endogenous Bad level appeared to be slightly suppressed by PTMA overexpression in J7 cells. The overall contribution of minimal changes of Bad and Bcl-XL induced by PTMA for apoptosome activation may be negligible. Nevertheless, measurement of cytosolic and membrane distribution of the Bad protein is needed to clarify its function in apoptosis. Furthermore, Bad is rapidly phosphorylated on two serine residues in response to the survival factor IL-3 [57]. These serines represent canonical 14-3-3 binding sites, and phosphorylated Bad appears to be the inactive form incapable of binding Bcl-XL and that remains sequestered in the cytosol in association with 14-3-3 [57]. Further studies are needed to verify the role of Bad in regulating sorafenib-induced apoptosome activation and apoptosis by PTMA. Taken together, it appears that regulation of Bax distribution by PTMA may play an important role in controlling cytochrome c release and apoptosis in response to sorafenib.

Further studies should examine PTMA levels in various types of cancers to confirm if the findings reported here can be verified in other cancer cells. In addition, the molecular mechanism underlying the upregulation of PTMA remains unclear. The formation and progression of HCC is associated with alterations in several surviving signaling pathways, including the RAF/MEK/ERK pathway, phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway, β-catenin/Wnt pathway and growth factor-regulated angiogenic signaling [2, 3]. In our study, PTMA was found to be upregulated in HCC cell lines. PTMA gene regulation and possible ways how it may be inhibited by sorafenib in HCC cells is proposed in Fig. 9C. Our results indicate that the ERK and Wnt/β-catenin pathways, possibly acting independently, are important for regulating PTMA gene transcription. PTMA protein stability was less affected by sorafenib treatment. Other authors have also provided evidence that sorafenib modulates Wnt/β-catenin signaling in 642 HCC cases and 21 liver cancer cell lines of the human CTNNB1 class [52]. In the latter study, Wnt activation was observed in 49.1% of HCC patients, and was further classified as belonging to either the CTNNB1 class (21.5%) or Wnt-TGFβ class (27.6%). Sorafenib decreased Wnt signaling and β-catenin protein in a sub-population of HCC cell lines, including HepG2 (CTNNB1 class), SNU387 (Wnt-TGFβ class), and Huh7 (lithium-chloride-pathway activation). The suppressive effect of sorafenib on CTNNB1 class-specific Wnt pathway activation was validated in HepG2 xenografts in nude mice, and was accompanied by decreased tumor volume and increased
survival of the treated animals [52]. Consistent with the effects on ERK-dependent signaling pathway, it has been demonstrated that sorafenib inhibits cell proliferation and induces apoptosis in various HCC cell lines and tumor xenografts [58]. In addition, sorafenib significantly inhibits breast tumor-initiating cells and mammary xenograft tumor formation by inhibiting RAF1-MEK-ERK-β-catenin activation [59]. Thus, ERK and β-catenin activation may act sequentially or separately on PTMA gene expression and may be suppressed by sorafenib in HCC cells, depending on the genetic context of the cells.

The inhibitory effects of sorafenib on PTMA gene expression occurring at the mRNA or protein levels remain uncharacterized. Our experimental evidence reveals that treatment with sorafenib or the ERK inhibitor enhances the degradation of PTMA mRNA in HCC cells, whereas the PTMA protein remains unaffected (Fig. 4, 6A and B). Furthermore, silencing of Elk-1, a MAPK-activated transcription factor [60], appears not to affect intrinsic or sorafenib-regulated PTMA expression in Mahlavu cells. These results suggest that ERK-mediated regulation of PTMA expression may control mRNA stability.

Overexpression of PTMA resulted in increased mRNA and protein levels for the endogenous p53 target genes, hdm2 and p21, and induced cell death in various cell lines such as the non-cancerous HEK293. PTMA also increased transcriptional activity of p53 in mammalian reporter gene assays in various cell lines, including HEK293 and the HCC HepG2. PTMA also enhances exogenous p53 reporter gene assays in lung cancer H1299 cells (p53-null). Silencing PTMA through RNA interference decreased p53 transcriptional activity [61]. In contrast, the results presented here appear to be contradictory to this report. While HepG2 shows normal p53, the HCC cell lines used in this study (e.g., Mahlavu, Huh7) display abnormal p53 gene or protein levels [62]. The integrity of cellular p53 may be important in the regulatory effect of PTMA on apoptosis. The contradictory observations regarding PTMA regulation of apoptosis in different cell lines may be partly explained by p53-dependent or -independent pathways.

By directly blocking Bax mitochondrial translocation and activation, nucleophosmin helps human HCC cells evade cytotoxic drug effects independently of p53-mediated cell death. Furthermore, silencing of nucleophosmin significantly sensitized HCC cells to anticancer therapies [63], suggesting that nucleophosmin is a potential target for combination with other therapies for HCC, particularly those that harbor inactivated p53 gene. Similarly, by blocking Bax mitochondrial translocation and activation, PTMA may help human HCC cells (p53 mutated) evade cytotoxic drug effects. These observations reveal that silencing the “anti-apoptotic protein” (such as PTMA or nucleophosmin) in combination with cancer therapeutic agents
may represent a way to enhance apoptosis in HCC cells. Our findings are of clinical significance since PTMA up-regulation and p53 mutations are usually found in advanced human cancers, including HCC.

These results also have important implications to understand the role of PTMA in sorafenib-treated HCC cells. When HCC formation and progression require alterations in specific genes such as oncogenes, drugs that selectively block their products might slow tumor growth. Many clinical trials have evaluated the use of tyrosine kinase inhibitor drugs as first-line therapies for HCC, including those testing a combination of drugs with sorafenib (e.g., erlotinib) or comparing existing therapies with sorafenib (e.g., linifanib). For patients who do not respond to sorafenib, tyrosine kinase inhibitors such as brivanib, everolimus, and monoclonal antibodies (e.g., ramucirumab) are being tested as second-line therapies [64]. The observation that PTMA plays a critical role in HCC progression or drug resistance is an important finding in the present study. Compounds such as sorafenib that potently interfere with signaling pathways and effectors (like PTMA) that are required for HCC progression may be used to treat selected human patients and improve current therapies against HCC.

Conflict of interest

The authors declare no conflict of interest.

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