Inhibition of hTERT in pancreatic cancer cells by pristimerin involves suppression of epigenetic regulators of gene transcription

DORRAH DEEB[1], XIAOHUA GAO[1], YONG BO LIU[1], YIGUAN ZHANG[2], JIAJIU SHAW[2], FREDERICK A. VALERIOTE[2] and SUBHASH C. GAUTAM[1]

[1]Departments of Surgery, Henry Ford Health System; [2]Drug Discovery Laboratories, Henry Ford Health System, Detroit, MI, USA

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Abstract. Previously we have shown that the inhibition of proliferation and induction of apoptosis in pancreatic ductal adenocarcinoma (PDA) cells by pristimerin (PM), a quinonemethide triterpenoid, was associated with the inhibition of human telomerase reverse transcriptase (hTERT) mRNA and hTERT protein. Herein we show that PM inhibits transcription factors and epigenetic processes that regulate hTERT expression. Treatment with PM inhibited transcription factors c-Myc, Sp1, NF-κB and kinases p-Akt and p-mTOR that regulate hTERT post-translationally. PM also downregulated DNA methyl transferases DNMT1 and DNMT3a and transcriptionally active chromatin markers, such as acetylated histone H3 (Lys9), acetylated histone H4, di-methyl H3 (Lys4) and trimethyl H3 (Lys9). In addition, chromatin immunoprecipitation (ChIP) analysis showed decrease in c-Myc and Sp1 transcription factors, but not repressive factors CTCF, E2F or Mad1 in the regulatory region of the hTERT promoter after treatment with PM. PM also reduced acetylated histone 3 and 4 and methylated H3 at hTERT promoter. Collectively, these results indicated that PM downregulates hTERT/telomerase through the inhibition of the genetic and epigenetic regulators of hTERT gene expression.

Introduction

Pancreatic ductal adenocarcinoma (PDA) is one of the most difficult to treat of all malignancies (1,2). Systemic gemcitabine alone or in combination with 5-fluorouracil, irinotecan and oxaliplatin (Folfirinox) is the current standard of care for advanced pancreatic cancer, providing short-term symptomatic improvement with minor impact on survival (3,4). Thus, there is a dire need for developing novel agents for palliative care of advanced pancreatic cancer.

Telomeres are nucleoprotein structures present at the end of chromosomes that are essential for chromosomal stability and prevention of end-to-end fusion (5). Shortening of telomeres triggers replicative senescence or apoptosis. Telomerase rebuilds and maintains telomere length by incorporating hexameric DNA repeats (TTAGGG) to the 3' flanking end of the telomeric DNA strands (6). Human telomerase is comprised of RNA template (hTERC) and the RNA dependent DNA polymerase (hTERT) (7,8). hTERC serves as a template for hTERT mediated telomere extension. In addition, hTERT associates with several proteins including a six protein complex called shelterin for proper functioning (9). Deregulated telomerase activity promotes tumorigenesis (10,11). hTERT expression and telomerase activity is elevated in PDA (12-14). Thus, reactivated hTERT/telomerase in PDA is a potential target for developing novel agents for the treatment of this malignancy.

Pristimerin (PM) is a quinonemethide triterpenoid present in various plant species. PM has shown potent antiproliferative and apoptosis-inducing activity against diverse types of cancer cells including pancreatic cancer cells (15-19). Antitumor effects of PM involve induction of apoptosis, generation of reactive oxygen species (ROS), mitochondrial dysfunction and inhibition of nuclear factor κB (NF-κB), Akt and MAP kinases (17-19). In a previous study, we showed that the inhibition of cell proliferation and induction of apoptosis in PDA cells by PM was associated with the inhibition of hTERT and its telomerase activity (20). In the present study, we investigated the role of epigenetic regulators of hTERT gene expression in mediating the antitumor activity PM. PM inhibited hTERT mRNA, native and phospho-hTERT protein and downregulated transcription factors and transcriptionally active chromatin markers that regulate hTERT transcription.

Materials and methods

Reagents. Pristimerin (PM) was purchased from Sigma Chemicals (St. Louis, MO, USA). Antibodies against PARP-1,

Correspondence to: Dr Subhash C. Gautam, Departments of Surgery, Henry Ford Health System, One Ford Place, Detroit, MI 48202, USA
E-mail: sgautam1@hfhs.org

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p-Akt, p-mTOR, NF-κB (p65), Sp1, c-Myc and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-hTERT and p-TERT (Ser824) antibodies were obtained from Abcam Inc. (Cambridge, MA, USA). ChIP-validated antibodies anti-acetyl-histone H3 lysine 9, anti-acetyl-histone H4, anti-tri-methyl histone H3 lysine 9 and anti-di-methyl histone H3 lysine 4 were from Millipore (Billerica, MA, USA). Annexin V-FITC apoptosis detection kit II was obtained from BD Pharmingen (San Diego, CA, USA). CellTiter 96 AQueous One Solution Proliferation Assay System was from Promega (Madison, WI, USA). Stock solution of PM (100 mM) was prepared in DMSO and all test concentrations were prepared by diluting stock solution in tissue culture medium.

Cell lines. Panc-1 and MiaPaCa-2 PDA cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Both cell lines were grown in DMEM tissue culture medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 25 mM HEPES buffer. Cells were incubated at 37˚C in a humidified atmosphere consisting of 5% CO_2 and 95% humidity.

MTS assay. Tumor cells ($1x10^4$) in 100 μl of tissue culture medium were seeded into each well of a 96-well plate. After 24 h incubation to allow cells to adhere, cells were treated with PM at concentrations ranging from 0 to 5 μM. Cultures were incubated for additional 72 h and cell viability was then determined by the colorimetric MTS assay using CellTiter 96 AQueous One Solution Proliferation Assay System. This assay measures the bioreduction of tetrazolium compound MTS in the presence of electron-coupling reagent phenazine methosulfate by intracellular dehydrogenases. MTS and phenazine methosulfate were added to the culture wells, and cultures were incubated for 2 h at 37˚C. The absorbance, which is directly proportional to the number of viable cells in the cultures, was measured at 490 nm using a microplate reader.

Annexin V-FITC binding. Induction of apoptosis was assessed by the binding of Annexin V-FITC to phosphatidylserine, which is externalized to the outer leaflet of the plasma membrane early during induction of apoptosis. Briefly, Panc-1 and MiaPaCa-2 cells treated with PM (0-5 μM) for 24 h were resuspended in the binding buffer and 5 μl of Annexin V-FITC reagent and 5 μl of PI were added. After incubation for 30 min at room temperature in the dark, cells were analyzed by flow cytometry.

Measurement of hTERT expression. hTERT expression was measured by analyzing hTERT mRNA and hTERT protein. For hTERT mRNA, total cellular RNA was extracted with TRIzol reagent (Gibco) according to the manufacturer's recommendations. RNA (1 μg) was then reverse transcribed by Oligo(dT) primer and high fidelity reverse transcriptase (Boehringer Mannheim, Germany) to generate cDNAs. One μl of cDNA was used as the template for polymerase chain reaction (PCR) using hTERT primers: upper, 5'-TGTT TCTGGATTTGCAGGTG-3', and lower, 5'-GTTCTTGG CTTTCAGGATGG-3'; and GAPDH primers: upper, 5'-TCC CTC AAG, ATT, GTC AGC AA-3', and lower, 5'-AGA TCC ACA ACG GAT ACA TT-3'. The PCR conditions used were 33 cycles of denaturation (95˚C for 1 min), annealing (62˚C for 30 sec), and polymerization (72˚C for 1 min). The PCR products were separated on 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Gels were photographed and band densities were analyzed using the NIH/Scion image analysis software. The hTERT primers amplified a DNA fragment of 200 bp and the DNA fragment size amplified by GAPDH primers was 173 bp.

Western blotting. Cell lysates were prepared by detergent lysis [1% Triton-X 100 (v/v), 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 10% glycerol, 2 mM sodium vanadate, 5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 10 μg/ml 4-2-aminoethyl-benzenesulfinyl fluoride]. Lysates were clarified by centrifugation at 14,000 x g for 10 min at 4˚C, and protein concentrations were determined by Bradford assay. Samples (50 μg) were boiled in an equal volume of sample buffer (20% glycerol, 4% SDS, 0.2% bromophenol blue, 125 mM Tris-HCl (pH 7.5) and 640 mM 2-mercaptoethanol) and separated on 10% SDS-polyacrylamide gels. Proteins resolved on the gels were transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl with 0.05% Tween-20 (TPBS) and probed with protein specific primary antibodies followed by HRP-conjugated secondary antibody. Immune complexes were visualized with enhanced chemiluminescence detection system from Amersham Corp. (Arlington Heights, IL, USA). Protein bands were imaged and band densities analyzed using NIH/Scion image analysis software. The protein band densities were normalized to the corresponding β-actin band densities.

Chomatin immunoprecipitation (ChIP) assay. ChIP analysis of transcriptionally active chromatin markers interacting with hTERT promoter was performed using the EZ-ChIP kit (Upstate Biotechnology) according to the instructions included in the kit. ChIP-validated antibodies used were: anti-acetyl-histone H3 lysine 9, anti-acetyl-histone H4, anti-tri-methyl histone H3 lysine 9 and anti-di-methyl histone H3 lysine 4. ChIP-purified DNA from control cells (untreated) and cells treated with PM (0-5 μM) for 48 h was amplified by PCR using hTERT promoter primers: forward, 5'-TCCCCTTCACGTCCGGCATT-3'; reverse, 5'-AGCGGAGAGAGGTCGAATCG-3'. The PCR products were separated on 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The hTERT primers amplified a DNA fragment of 200 bp.

Statistical analysis. Data are presented as means ± SD. The differences between control and treatment groups were analyzed using Student's t-test and differences with $p<0.05$ were considered statistically significant.

Results

Pristimerin reduces viability and induces apoptosis in PDA cells. To measure the effect of PM on viability of PDA cells, Panc-1 and MiaPaCa-2 cells were treated with PM for 72 h at concentrations ranging from 0.0 to 5.0 μM. At the end of the treatment, viability of cultures was determined by MTS assay.

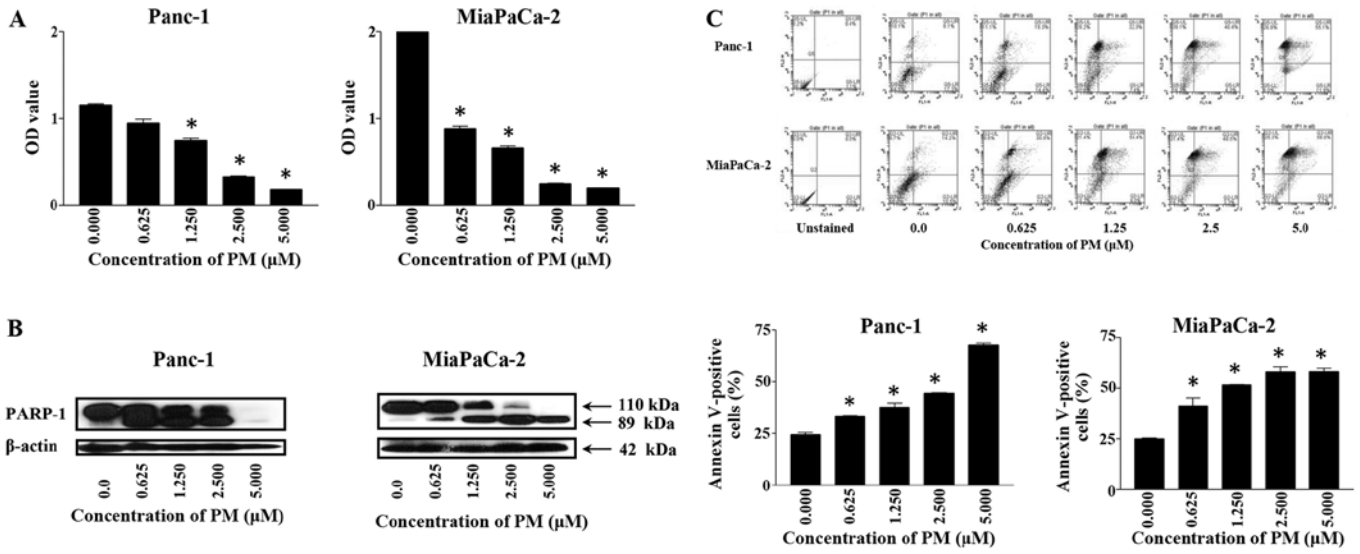

Figure 1. Pristimerin (PM) reduces viability and induces apoptosis in PDA cells. (A) Panc-1 or MiaPaCa-2 ($1x10^4$) cells were seeded in each well of a 96-well plate. Twenty-four hours later, cells were treated with PM at concentrations ranging from 0 to 5 μM for 72 h in triplicate. Cell viability was measured by MTS assay using CellTiter AQueous Assay System. (B) Cleavage of PARP-1. Panc-1 and MiaPaCa-2 cells treated with PM (0-5 μM) for 24 h were analyzed for cleavage of PARP-1 by western blotting. (C) Annexin V-FITC binding. Panc-1 and MiaPaCa-2 cells were treated with PM at 0-5 μM for 24 h. Cells were then reacted with 5 μl of Annexin V-FITC and 5 μl PI for 30 min and percentage of Annexin V-FITC binding cells was determined by flow cytometry. Upper panel, flow cytograph of a representative experiment; lower panel, bar graph representing mean ± SD of three experiments. *P<0.05.

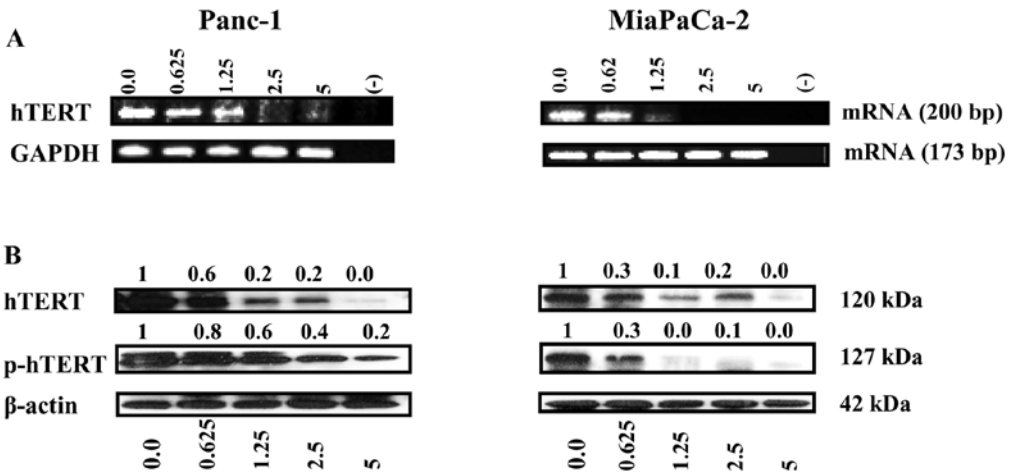

Figure 2. Pristimerin (PM) inhibits hTERT mRNA and hTERT protein in PDA cells. (A) Effect on hTERT gene expression. Panc-1 and MiaPaCa-2 cells were treated with PM (0-5 μM) for 48 h and total cellular RNA was prepared using TRIzol reagent. Cellular RNA (1 μl) was reverse transcribed using oligo(dT) primer and high fidelity reverse transcriptase. cDNA (1 μl) was amplified using hTERT or GAPDH primers. Amplified products were separated on 2% DNA agarose gel. Gels were stained with ethidium bromide and amplified DNA fragments were identified by base pair sizes. (B) Effect on hTERT protein. MiaPaCa-2 and Panc-1 cells were treated with PM as above and cell lysates were analyzed for hTERT and p-hTERT protein by western blotting. Values above blots represents change in mRNA or protein expression after treatment with PM compared to untreated control considered as 1.0. Each experiment was repeated at least two times.

As shown in Fig. 1A, treatment with PM significantly reduced the viability of both cell lines (p<0.05). In the case of Panc-1 cells, the reduction in viability ranged from 18 to 84% (e.g., 18, 36, 72 and 84% at 0.0625, 1.25, 2.5 and 5 μM, respectively). PM reduced the viability of MiaPaCa-2 cells more potently at lower concentrations than in Panc-1 cells. For example, viability of MiaPaCa-2 was inhibited 58 and 68% at 0.0625 and 1.25 μM PM, which increased to 88-90% inhibition at 2.5-5 μM (p<0.05).

Whether PM induces apoptosis in PDA cells was investigated next. Induction of apoptosis was measured by the cleavage PARP-1 and Annexin V-FITC binding by western blotting and flow cytometry, respectively. As shown in Fig. 1B, treatment with PM for 24 h caused the cleavage of PARP-1. The cleavage of PARP-1 was detectable at 0.625 μM PM by the appearance of the 89 kDa split product in both cell lines. The cleavage of PARP-1 was more pronounced at higher concentrations of PM, especially in MiaPaCa-2 cells. The induction of apoptosis was confirmed by the increased binding of Annexin V-FITC after treatment of cells with PM for 24 h. As shown in Fig. 1C (upper and bottom panels), 25-20% of untreated Panc-1 and MiaPaCa-2 cells bound

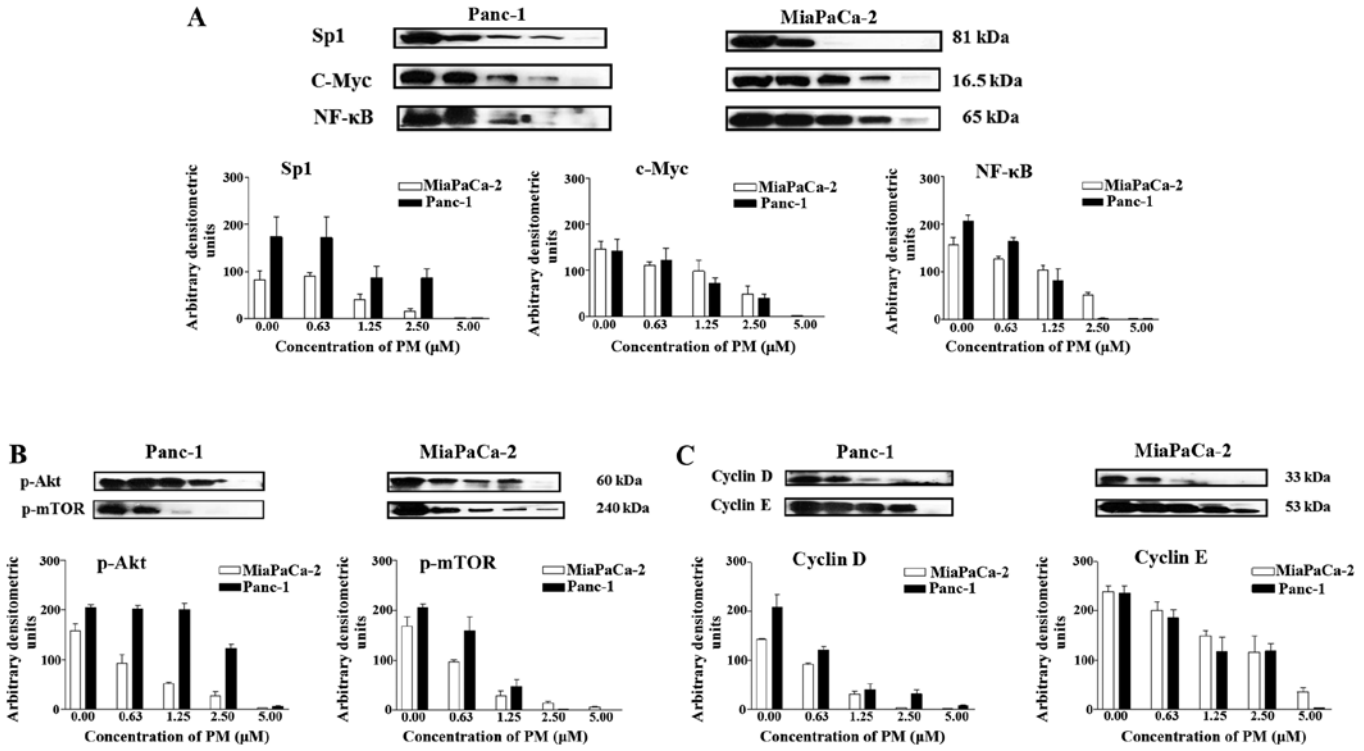

Figure 3. Pristimerin (PM) downregulates proteins that regulate hTERT and the cell cycle. Panc-1 and MiaPaCa-2 cells were treated with PM (0-5 μM) for 48 h and cell lysates were analyzed for transcription factors c-Myc, Sp1 and NF-κB that regulate transcription of hTERT (A) and protein kinases p-Akt and p-mTOR that post-translationally phosphorylate and regulate the telomerase activity of hTERT (B) and cyclins D and E (C) that regulate cell cycle progression by western blotting. Bar graphs represent mean ± SE of densitometric values of blots from two to three experiments.

Annexin V-FITC. After treatment with PM, the percentage of Annexin V-FITC-binding Panc-1 cells ranged from 33 to 68% at 0.625-5 μM PM. The percentage of Annexin V-FITC-binding also increased in MiaPaCa-2 cells from 45% at 0.0625 μM to 60% at 5 μM PM. Together, the cleavage of PARP-1 and an increase in Annexin V-FITC-binding demonstrated induction of apoptosis by PM in PDA cells.

PM inhibits hTERT gene expression. The inhibition of hTERT/telomerase leads to cellular senescence and/or apoptosis. We thus determined the effect of PM on the expression hTERT mRNA and hTERT protein. The effect on hTERT gene expression was measured by analyzing hTERT mRNA by RT-PCR. Treatment with PM resulted in significant to complete inhibition of hTERT mRNA in both cell lines at 1.25-5 μM PM without affecting the expression of GAPDH (Fig. 2A). As shown in Fig. 2B, PM also reduced both the native and phosphorylated hTERT (p-hTERT) levels in both cell lines. Together, these data showed inhibition of hTERT expression in PDA cells by PM.

PM inhibits cellular proteins that regulate hTERT expression. hTERT plays a major role in cell proliferation and inhibition of apoptosis by maintaining telomere length. Thus, we examined the effect of PM on proteins that regulate hTERT gene transcription, post-translational modification of hTERT and cell division. PM inhibited the transcription factors Sp1, c-Myc, and NF-κB (p65) which control hTERT gene expression in a dose-dependent manner with complete inhibition occurring at 5 μM PM (Fig. 3A). PM also inhibited p-Akt and p-mTOR that modify hTERT post-translationally in both cell lines (Fig. 3B). In addition, depending on the concentration, treatment with PM (0 to 5 μM) partially to completely inhibited cyclin D1 and cyclin E (Fig. 3C). Overall, these data showed that PM inhibits proteins that regulate hTERT expression, post-translational modifications of hTERT and cell cycle progression.

PM inhibits epigenetic regulators of hTERT expression. Promoter methylation and histone modifications play a critical role in hTERT expression. Whether PM targets effectors of epigenetic pathways of hTERT gene expression was evaluated. First, we analyzed the effect of PM on DNA methyltransferases responsible for DNA methylation. PM caused significant decrease in DNA methyltransferases DNMT1 and DNTM3α in both cell lines at the lowest concentration of 0.625 μM with complete inhibition at higher concentrations (Fig. 4A).

In addition to DNA methylation, histone modifications (e.g., histone acetylation and histone methylation) play pivotal roles in hTERT transcription, therefore, we determined the effect of PM on histone acetylation and methylation. For histone acetylation, effect of PM on cellular levels of transcriptionally active acetylated histone H3 at lysine 9 (ac-H3K9) and acetylated histone H4 (ac-H4) was analyzed. Treatment with PM significantly to completely inhibited ac-H3K9 and ac-H4 in both cell lines (Fig. 4B). PM also affected histone methylation as histone markers dimethyl-H3 lysine 4 (di-me-H3K4) and trimethy-H3 lysine 9 (tri-me-H3K9) were drastically reduced in cells treated with PM (Fig. 4B).

The preceding findings demonstrated the inhibition of transcription factors and transcriptionally active histone

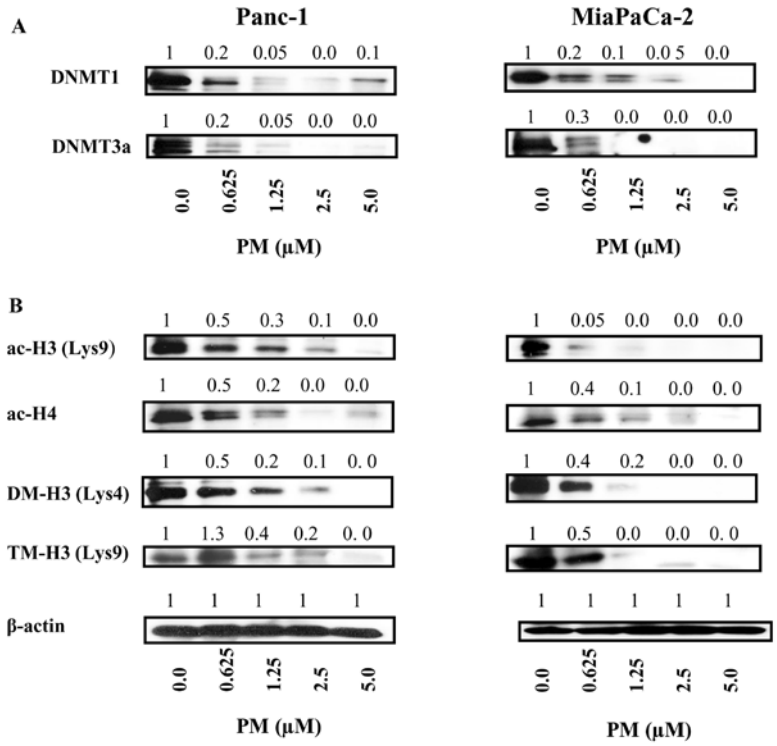

Figure 4. Effect of pristimerin (PM) on DNA methyl transferases (DNMTs) and transcriptionally active chromatin markers. (A) Panc-1 and MiaPaCa-2 cells were treated with PM (0-5 μM) for 48 h and cellular levels of DNMT1 and DNMT3a DNA methyl transferases were analyzed by western blotting. (B) For effect on transcriptionally active chromatin markers, tumor cells were treated with PM as above and cell lysates were analyzed for acetylated histone H3 (Lys9), acetylated histone H4, di-methyl H3 (Lys4) and tri-methyl H3 (Lys9) by western blotting. Values above blots represent change in protein expression after treatment with PM compared to untreated control considered as 1.0. The experiment was repeated two times.

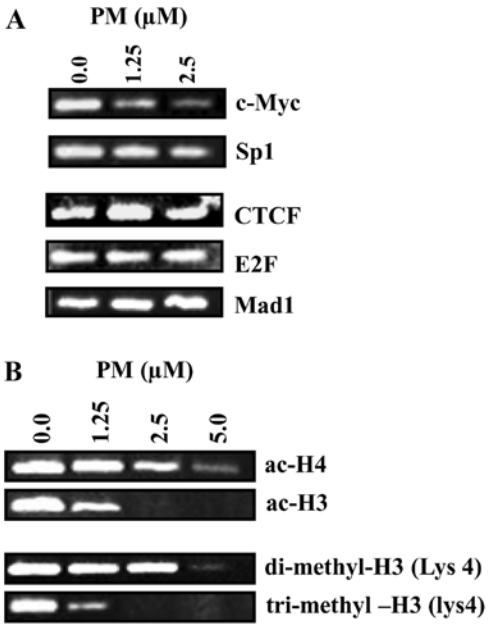

Figure 5. Pristimerin (PM) alters levels of transcription factors and active chromatin markers in hTERT promoter. Panc-1 and MiaPaCa-2 cells were treated with PM (0-5 μM) for 48 h. DNA interacting chromatin was immunoprecipitated using ChIP-validated antibodies to transcription factors c-Myc and Sp1 or repressive factors, CTCF, E2F and Mad1 (A) or active chromatin markers acetyl-histone H3 lysine 9, acetyl-histone H4, tri-methyl histone H3 lysine 9 and di-methyl histone H3 lysine 4 (B). ChIP-purified DNA from control cells (untreated) and cells treated with PM was amplified by PCR using hTERT promoter primers: forward, 5'-TCCCCTTCACGTCCGGCATT-3'; reverse, 5'-AGCGGAGAGAGGTCGAATCG-3'. The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The hTERT primers amplified a DNA fragment of 200 bp.

markers by PM. Whether PM impacts transcription factors and histone modifications at hTERT promoter was analyzed next. For this, we analyzed changes in levels of positive (c-Myc and Sp1) and repressive transcription factors (CTCF, E2F and Mad1) and transcriptionally active histones (ac-H4, ac-H4, DM-H3 and TM-H3) in the regulatory region of hTERT promoter by ChIP assay after treatment with PM. As shown in Fig. 5A, treatment with PM partially to significantly reduced the level of c-Myc and Sp1 in hTERT promoter. In contrast, repressive factors CTCF, E2F and Mad1 were not affected by PM. Furthermore, ChIP analysis of histone modifications at hTERT promoter showed decrease in ac-H3 and ac-H4 at 2.5-5 μM PM and significant to complete reduction in DM-H3 and TM-H3 at 1.25-5 μM PM (Fig. 5B). These data indicated that inhibition of hTERT expression by PM involves inhibition of the transcription factors and transcriptionally active chromatin markers that upregulate hTERT gene expression.

Discussion

Although the antiproliferative and apoptosis-inducing activity of pristimerin (PM) has been shown in tumor cell lines, including pancreatic cancer cell lines (14-19), the molecular mechanism of the anticancer effects of PM has not been fully delineated. Telomerase, the enzyme that rebuilds and maintains telomere length, plays a vital role in cell proliferation and prevention of apoptosis. Deregulated telomerase activity promotes tumorigenesis and provides unlimited proliferative advantage to the cancer cells. On the other hand, inhibition of hTERT, the gene that codes for the catalytic subunit of

telomerase results in lack of telomerase activity and consequently the inhibition of cell proliferation, cellular senescence or apoptotic cell death.

In a previous study, we showed that inhibition of cell proliferation and induction of apoptosis by PM correlated with the inhibition of hTERT and its telomerase activity in PDA cells, suggesting that inhibition of telomerase is part of the mechanism by which PM inhibits proliferation of PDA cells (20). Since hTERT gene expression is heavily regulated epigenetically, the present study was undertaken to determine the effect of PM on the epigenetic regulators of hTERT gene transcription. First though, we reevaluated the effect of PM on the viability and expression of hTERT in PDA cells. Indeed, new data confirmed our previous findings that inhibition of proliferation and induction of apoptosis in PDA cells by PM is associated with the inhibition of hTERT mRNA as well as production and phosphorylation of hTERT protein. These findings are in agreement with other reports showing that the inhibition of hTERT telomerase activity is necessary for the antiproliferative and apoptosis-inducing activity of natural compounds (21). However, whether PM binds and degrades RNA template or causes shortening of telomeres remains to be determined.

A number of factors and molecules that regulate hTERT transcription have been identified. The hTERT core promoter contains binding sites for transcription factors, such as Sp1, c-Myc, NF-κB and STAT-3 (22,23). Inhibition of these transcription factors would likely impact transcription of hTERT gene. PM inhibited Sp1, c-Myc and NF-κB in PDA cells, indicating that diminished hTERT expression and protein production by PM is at least partly attributed to the inhibition of these transcription factors. Post-translationally, phosphorylation of hTERT by protein kinase B/Akt and mTOR is required for nuclear import and activation of hTERT telomerase activity (20,24). PM inhibited both p-Akt and p-mTOR, indicating that inhibition of post-translational modifications by PM also contributes to the inhibition of hTERT/telomerase.

As stated before hTERT gene is heavily regulated through the epigenetic mechanisms. Contrary to the prevalent view that hypermethylation of gene promoters typically inhibits their transcription; hypermethylation of hTERT promoter is associated with increased hTERT expression (25,26). Epigenetic processes that regulate gene expression include DNA methylation, chromatin remodeling and modulation of the activity of enzymes and factors associated with these processes.

Promoter DNA methylation catalyzed by DNMTs plays an important role in hTERT transcription. DNMT1, a maintenance methyltransferase, maintains hypermethylation of hTERT promoter, whereas DNMT3a and DNMT3b are responsible for *de novo* activity (27). PM inhibited DNMT1 and DNMT3a in Panc-1 and MiaPaCa-2 cells, thereby accounting for demethylation of hTERT promoter and inhibition of hTERT expression. Besides DNA methylation, histone acetylation and methylation also play critical roles in the transcription of hTERT gene. The histone modifications result in the loosening of chromatin which allows binding of the activators and/or repressors of gene transcription at gene promoters (28). PM inhibited cellular levels of transcriptionally active acetylated histones ac-H3 and ac-H4. PM also inhibited the active di-methyl-H3 lysine 4 and inactive chromatin marker trimethyl-H3K9. The decrease in transcription factors and transcriptionally active chromatin markers suggested that PM may also alter the levels of transcription factors and chromatin structures at the regulatory region of hTERT promoter.

ChIP analysis showed decrease in c-Myc and Sp1 transcription factors that upregulate the expression of hTERT without affecting the repressive factors CTCF, E2F and Mad1. PM also reduced the levels of transcriptionally active chromatin markers ac-H3 and ac-H4, DM-H3 and TM-H3 in hTERT promoter. These data demonstrated that downregulation of transcription factors and active chromatin markers plays a role in the inhibition of hTERT expression by PM in pancreatic cancer cells.

Acknowledgements

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