Therapeutic Effects of Punicalagin Against Ovarian Carcinoma Cells in Association With β-Catenin Signaling Inhibition

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Aim: The aim of this study was to investigate the effects of punicalagin, a polyphenol isolated from *Punica granatum*, on human A2780 ovarian cancer cells in vitro.

Methods: The viability of human A2780 ovarian cells was evaluated using Cell Counting Kit-8 assay. Cell cycle was detected with flow cytometry analysis. The protein expression levels of Bcl-2, Bax, β-catenin, cyclin D1, survivin, tissue inhibitor of metalloproteinase (TIMP)-2, and TIMP-3 were measured using Western blot analysis. Matrix metalloproteinase (MMP)-2 and MMP-9 activity was determined with gelatin zymography. Wound healing assay was used to determine cell migration.

Results: Punicalagin inhibited the cell viability of A2780 cells in a dose- and time-dependent manner, and the cell cycle of A2780 cells was arrested in G1/S phase transition. The treatment also induced apoptosis as shown by the up-regulation of Bax and down-regulation of Bcl-2. On the other hand, punicalagin treatment increased the expressions of TIMP-2 and TIMP-3, decreased the activities of MMP-2 and MMP-9, and inhibited cell migration. In addition, the β-catenin pathway was suppressed as shown by the down-regulations of β-catenin and its downstream factors including cyclin D1 and survivin.

Conclusions: Punicalagin may have cancer-chemopreventive as well as cancer-chemotherapeutic effects against human ovarian cancer in humans through the inhibition of β-catenin signaling pathway.

Key Words: Punicalagin, Ovarian cancer, β-catenin, Cell cycle, Cell migration, Apoptosis

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Ovarian cancer (OC) is the most lethal gynecological malignancy and the fifth leading cause of death from cancer in women worldwide.[1,2] It is usually diagnosed at a late stage, with a 5-year survival rate of less than 25%.[3] Surgical resection and chemotherapy are still the major therapeutic strategies for OC. However, the efficacy of chemotherapy is limited by chemoresistance and adverse effects. So, developing effective and secure therapies for the prevention of OC has become an important approach to reduce cancer burden. One such candidate is botanical products. Substantial research has found that many epigenetic and genetic factors can significantly influence the genesis and development of OC.

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The authors declare no conflicts of interest.

Author contributions: Jian-ming Tang, Jie Min, and Li Hong designed the experiments; Jian-ming Tang, Jie Min, Ming Hu, Yang Li, and Jiang Yang performed the experiments; Jian-ming Tang, Jie Min, Li Hong, Bing-shu Li, Sha-sha Hong, and Cheng Liu analyzed the data; Jian-ming Tang wrote the manuscript; and Jian-ming Tang and Jie Min are the co-first authors.

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Among them, the activation of canonical Wnt/β-catenin signaling plays a pivotal role in intracellular signal transduction. Wnt/β-catenin target genes, such as c-myc, cyclin D1, and survivin, regulate cell proliferation and apoptosis, thereby mediating cancer initiation, invasion, and progression.

Pomegranate (*Punica granatum*) is an ancient fruit with extensive regard as an article of medicine and is now being recognized as a potential chemopreventive and anticancer agent. Increasing body of evidence has underscored the cancer preventive efficacy of pomegranate both in vitro and in vivo animal models, such as breast cancer, skin cancer, prostate cancer, lung cancer, and colon cancer.[4–9] Punicalagin (PUN; 2,3-hexahydroxydiphenoyl-gallagyl-D-glucose, Figure 1) is the major bioactive component of pomegranate peel and has been proven to have antioxidant, anti-inflammatory, antiviral, antiproliferation, and anticancer properties.[5,10–15] Punicalagin has been shown to induce apoptosis in human promyelocytic leukemia HL-60 cells, HT-29 and HCT116 colon cancer lines, colon adenocarcinoma Caco-2 cells, and U87MG glioma cells.[7,16,17] Previous researches had revealed that PUN provoked the effects on various tumor cell lines: up-regulate the expression levels of Bax,[6,18] Bad,[18] cleaved-PRAP,[6,16,17] and cytochrome C[7]; promote the activation of caspase-3[7] and caspase-9[17]; and down-regulate the expression of Bcl-2,[6,17] Bcl-X_L,[6,7,18] and cell cycle proteins such as cyclins A,[7,17] B1,[7,17] D1,[6] D2,[6] and E[6] and then regulate the proliferation and apoptosis of cancer cells.

In this study, we investigated the effect of PUN on A2780 ovarian carcinoma cells. We demonstrated that PUN-induced A2780 cell apoptosis through the inhibition of β-catenin signaling pathway caused G1/S phase transition arrest and apoptosis-related protein alterations. In addition, PUN can suppress the invasion ability of A2780 cells through the inhibition of cell migration and the alteration of protein expression levels of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs).

MATERIALS AND METHODS

Chemical and Reagents

A2780 cells were obtained from the China Center for Type Culture Collection (Wuhan, China). Punicalagin (>98% high performance liquid chromatography purity) was purchased from the Chengdu Must Bio-tech Co, Ltd (Chengdu, China). Cell Counting Kit (CCK)-8 was purchased from the MultiSciences Biotech Co, Ltd (Hangzhou, China). Dulbecco modified Eagle medium (DMEM) and phosphate-buffered saline were purchased from Jenom Biotech Co, Ltd (Hangzhou, China). Trypsin/EDTA solution and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA). Bicinchoninic acid (BCA) protein assay kit and Cell Cycle Analysis Kit were purchased from the Beyotime Institute of Biotechnology (Suzhou, China). MMP Gelatin Zymography Assay Kit was purchased from the Applygen Technologies, Inc (Beijing, China). Antibodies to β-catenin, survivin, cyclin D1, Bcl-2, Bax, β-actin, and TIMP-3 were obtained from the Abcam plc (Cambridge, UK). Antibodies to TIMP-2 were purchased from the Santa Cruz Biotechnology, Inc (Dallas, TX).

Cell Culture

A2780 cells were cultured in DMEM supplemented with heat-inactivated 10% FBS and 1% antibiotics (100-IU penicillin and 100-μg/mL streptomycin) in a humidified incubator at 37°C and 5% CO_2. Logarithmically growing cells were used in all the subsequent experiments.

Cell Viability Assay

Cells were seeded into 96-well plates at a density of 5000 cells/well and then incubated for 24 hours. Then, cells were treated with PUN (0, 3, 6, 12, and 24 μM) for another 12, 24, and 48 hours. After that, cell viability was measured using the CCK-8 method, in which 10 μL of the CCK-8 reagent was added, and the cells were placed into an incubator with conditions of 5% CO_2 and 37°C for approximately 2 hours. Finally, the optical density at 450 nm was detected by using a PerkinElmer Victor3 1420 Multilabel Counter (PerkinElmer, Inc, Waltham, MA). Optical density values represent cell viability.

Cell Cycle Analysis

Cells were seeded into 6-well plates with 2×10^5 cells/well for 24 hours of culture for cell cycle analysis. After synchronization and treatment for another 24 hours with PUN (0, 3, 6, and 12 μM), the cells were harvested and then fixed with precooled 70% ethanol at 4°C overnight. Fixed cells were washed with phosphate-buffered saline and then stained with the Cell Cycle Analysis Kit for 30 minutes at 4°C. Stained cells were determined using a FACSCalibur system (BD, Franklin Lakes, NJ) to examine cell cycle distribution, followed using the MODFIT software for data analysis.

Western Blot

After being digested from plates, after treatment with PUN (0, 3, 6, and 12 μM), then, total protein was extracted from A2780 cells using RIPA buffer containing phenylmethanesulfonyl fluoride. To measure protein concentrations, a BCA assay kit (Beyotime, China) was used according to the manufacturer's instructions. After adding protein loading buffer (200 mM of DL-Dithiothreitol, 40 mM of Tris/HCl, 40% glycerol, 4% sodium dodecyl sulfate (SDS); pH, 6.8; 0.032% bromophenol blue) and denaturing at 95°C for 5 minutes, 30 μg of the total protein was separated from these samples by 10% SDS–polyacrylamide gel electrophoresis and then transferred into activated polyvinylidene fluoride membranes. After blocking in 5% skimmed milk at 37°C for 1 hour, the membranes were blotted with appropriate primary antibodies at 4°C overnight, followed by fluorescence-labeled secondary antibodies (IRDye700 and IRDye800, goat antimouse/rabbit, 1:10000) for 1 hour at 37°C. The primary antibodies information is as follows: anti–β-catenin (1:5000), anti-survivin (1:5000), anti–cyclin D1 (1:10000), anti–Bcl-2 (1:200), anti-Bax (1:1000), anti–β-actin (1:1000), anti–TIMP-2 (1:200), and anti–TIMP-3 (1:1000). Signals were detected with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

Wound Healing Assay

A2780 cells were seeded in 2-cm Petri dish at a concentration of 1×10^5 cells/mL and were grown overnight. A wound was then made in the cells by scratching on the cell

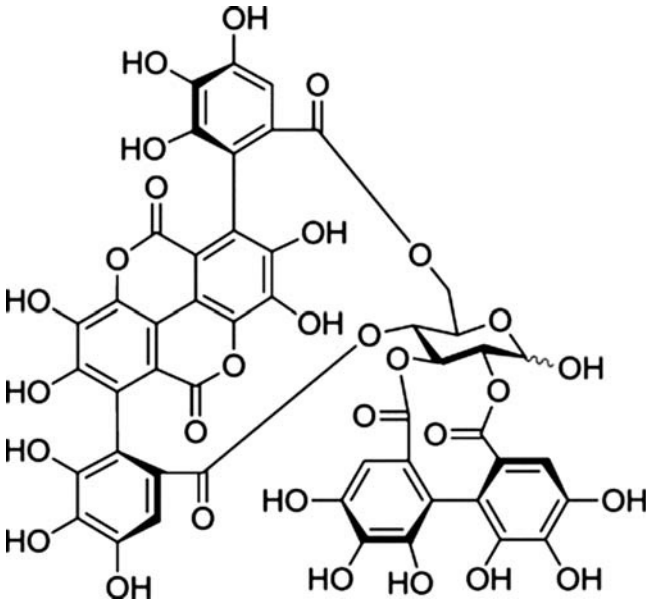

FIGURE 1. The chemical structures of PUN.[13]

layer with a sharp tip followed by further 24-hour incubation with PUN (6 μM) under the serum-free condition. The gap created by the scraping with or without treatment was then measured under a microscope to provide an indication of the wound healing capability of the cells.

MMP Gelatin Zymography

After PUN (0, 3, 6, and 12 μM) treatment, the culture medium was collected. Samples were analyzed by BCA assay, and equal protein amounts were mixed with equal amounts of nonreduced sample buffer and electrophoresed on 10% SDS-polyacrylamide gels containing 1-mg/mL gelatin as a protease substrate. Sodium dodecyl sulfate was removed through a 30-minute incubation in 2.5% Triton X-100, and gels were incubated in 20-mM glycine, pH of 8.3, 10-mM $CaCl_2$, and 1-μM $ZnCl_2$ at 37°C overnight. The gels were stained with Coomassie Blue to visualize zones of gelatinolytic activity. Gelatinase-dependent proteolysis was detected as a clear area in a light-blue field. After scanning the experiment results, the graphic analysis software Quantity One (by American Bio-Rad Corp) was used to carry out gray analysis on the specific bands.

Statistical Analysis

All statistical analyses were performed with SPSS 19.0 (IBM Corporation, Armonk, NY), and data are presented here as mean (SD). The data were further subjected to analysis of variance. Differences between the 2 groups were determined using Student *t* test, and multiple means were compared by Tukey test. *P* values less than 0.05 were considered statistically significant.

RESULTS

PUN Decreases the Viability of A2780 Cells

To evaluate the effects of PUN on ovarian carcinoma, A2780 cells were treated with increasing concentrations of PUN for 12, 24, and 48 hours, and then, the viability of the cells was assessed by the CCK-8 assay. As revealed in Figure 2, after PUN treatment, the viability of A2780 cells was significantly decreased in a dose- and time-dependent manner.

G1/S Phase Transition Is Arrested by PUN in A2780 Cells

To further investigate how PUN caused the growth inhibition of A2780 cells, the cell cycle distribution was conducted by propidium iodide staining using fluorescence activated cell sorting analysis after treatment with increasing concentrations (0, 3, 6, and 12 μM) of PUN. As shown in Figure 3A, the number of cells in the G1 phase increased after PUN treatment for 24 hours compared with controls.

PUN Alters the Protein Expression Levels of Bcl2 and Bax in A2780 Cells

To elucidate the mechanisms underlying the induction of apoptosis by PUN in A2780 cells, the mitochondrial features of the intrinsic apoptotic pathway were analyzed. Pro-apoptotic members of the Bcl-2 family, including Bax, are required for the induction of mitochondrial dysfunction during apoptosis. The protein expression levels of Bax and Bcl-2 were assessed by Western blotting. The results indicated that the treatment of A2780 cells with increasing doses of PUN for 24 hours enhanced the expression of Bax and down-regulated the expression of antiapoptotic Bcl-2 (Fig. 3B).

β-Catenin Signaling Pathway Is Down-regulated by PUN

The activation of β-catenin signaling pathway constitutes an important event in the promotion of cell growth and

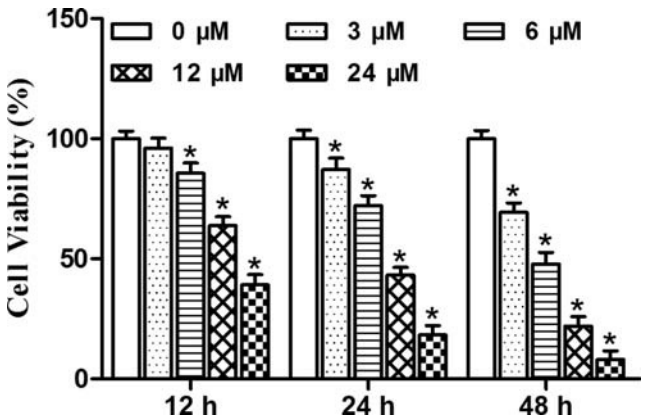

FIGURE 2. Punicalagin inhibits the viability of A2780 cells in vitro. A2780 cells were treated with increasing concentrations of PUN (0, 3, 6, 12, and 24 μM) for 12, 24, and 48 hours, and CCK-8 assay was subsequently used to evaluate the cell viability. The number of viable cells was proportional to the absorbance at 450 nm. Treatment with PUN induced a dose- and time-dependent inhibition of cell viability. *$P < 0.05$ vs 0-μM PUN.

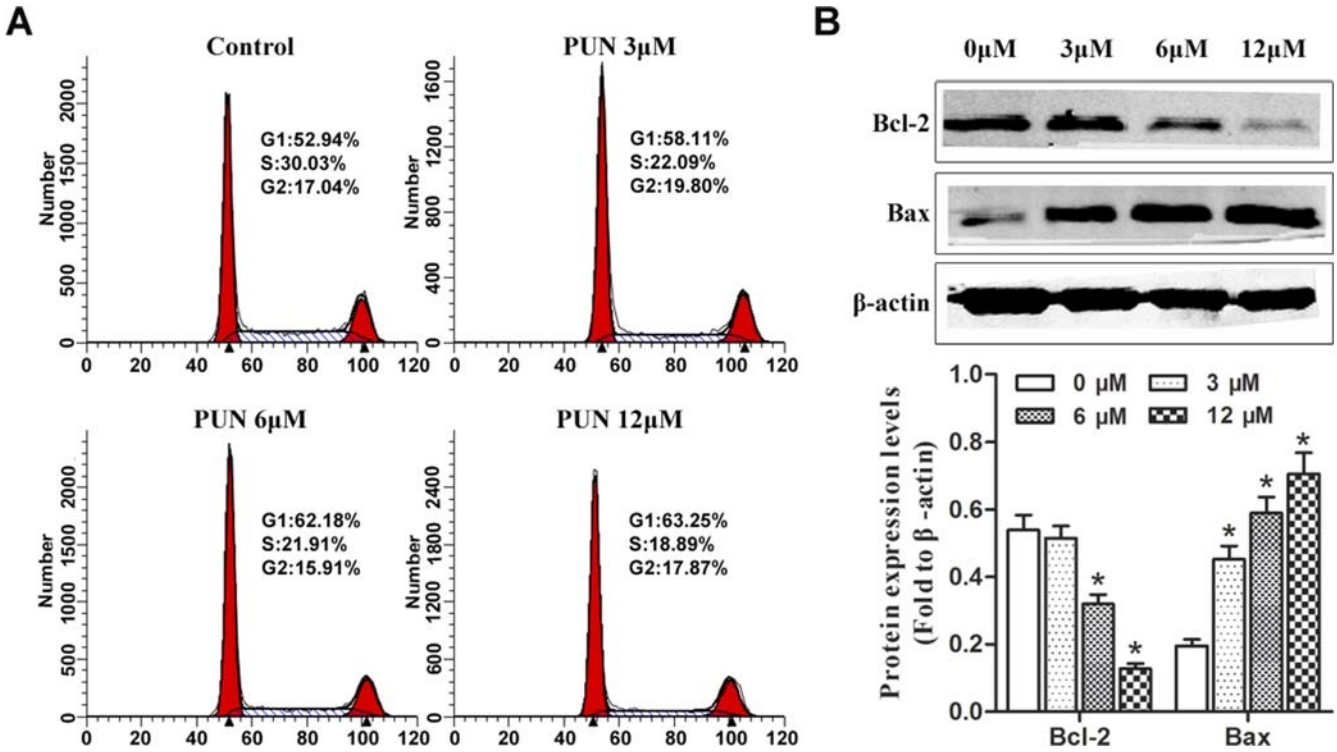

FIGURE 3. Cell cycle distribution (A) and changes of apoptosis-related protein levels (B) in A2780 cells after treatment with increasing concentrations of PUN (0, 3, 6, and 12 μM) for 24 hours. Each experiment was performed thrice, and representative data are shown. *$P < 0.05$ vs 0-μM PUN.

the carcinogenesis of OC. It was interesting to determine whether the PUN could affect the expression of β-catenin in A2780 cells. So, A2780 cells were treated with 0-, 3-, 6-, and 12-μM PUN for 24 hours, and then we determined the changes in the levels of β-catenin and its downstream factors including cyclin D1 and survivin by Western blot. As shown in Figure 4, a significant reduction of β-catenin, cyclin D1, and survivin was observed in the treated cells compared with their untreated counterparts.

PUN Inhibits the Migration Progress of A2780 Cells and Alters the Expression of MMPs and TIMPs

The effects of PUN on migration progression were also evaluated via wound healing assay. The size of the region representing the wound site was significantly larger in the presence of PUN (6 μM) than controls (Fig. 5A). This indicated that cells not treated with PUN had better would healing

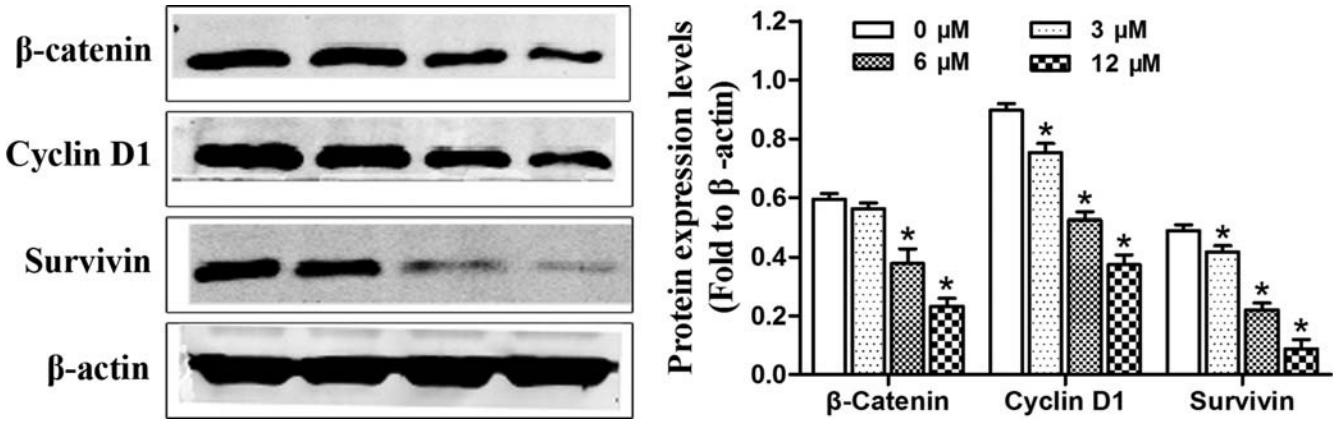

FIGURE 4. Changes in β-catenin signaling-associated protein in A2780 cells after treatment with increasing concentrations of PUN (0, 3, 6, and 12 μM) for 24 hours. Each experiment was performed thrice, and representative data are shown. *$P < 0.05$ vs 0-μM PUN.

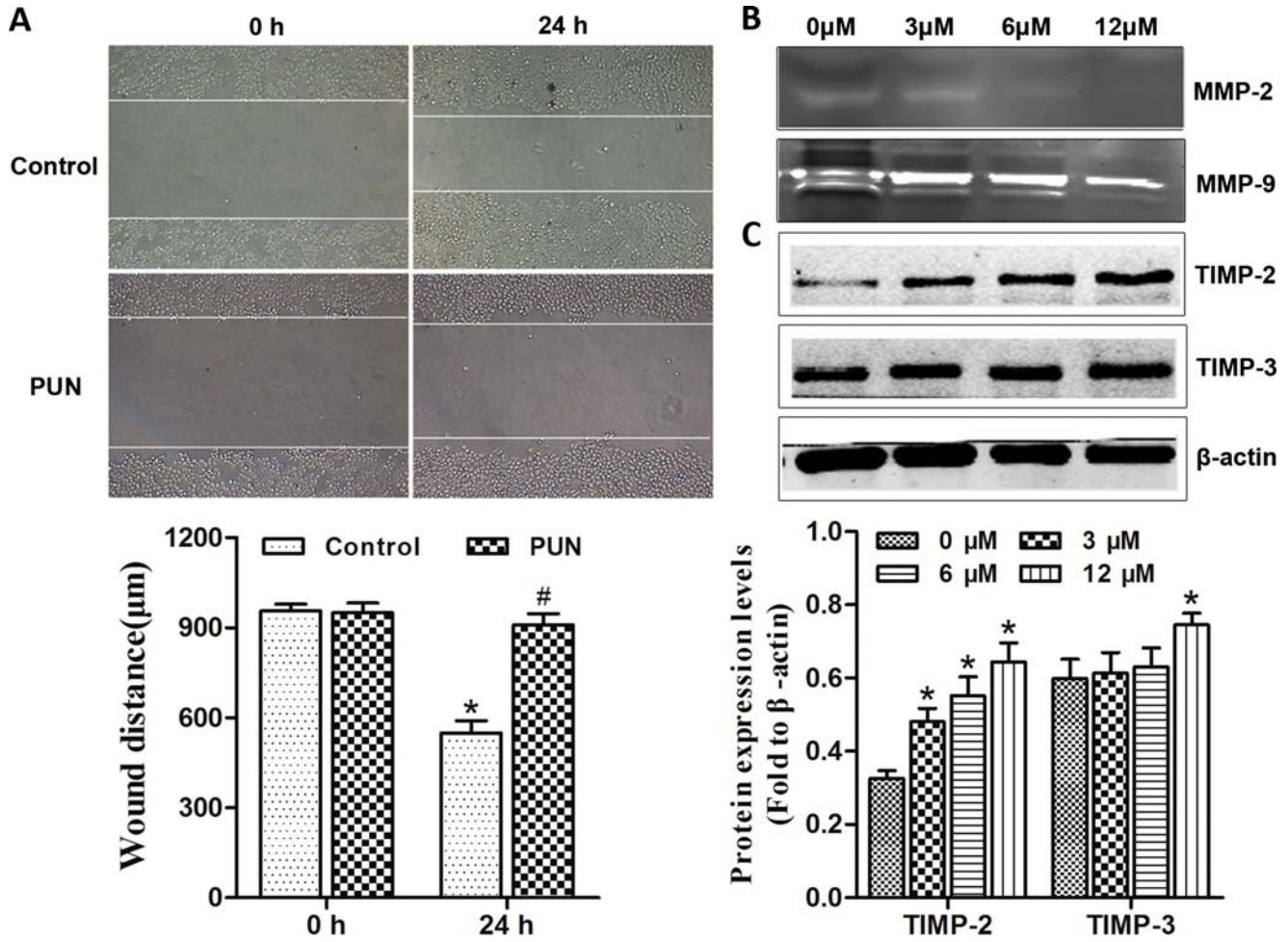

FIGURE 5. The effects of PUN on cell migration and the expressions of MMP-2, MMP-9, TIMP-2, and TIMP-3 in A2780 cells. A, Wound healing assay: a uniform scratch was made, and the cells were cultured in DMEM without FBS for 24 hours; the extent of closure was photographed at 0 and 24 hours (original magnification, ×100). Punicalagin significantly inhibits the migration of A2780 cells. B, MMP gelatin zymography: the MMP-2 and MMP-9 expressions were detected in A2780 cells after treatment with increasing concentrations of PUN (0, 3, 6, and 12 μM) for 24 hours by MMP gelatin zymography. Our results showed that both MMP-2 and MMP-9 were decreased. C, The expressions of TIMP-2 and TIMP-3 were analyzed by Western blotting in A2780 cells after PUN treatment (0, 3, 6, and 12 μM). Both TIMP-2 and TIMP-3 were down-regulated after PUN treatment. Each experiment was performed thrice, and representative data are shown. *$P < 0.05$ vs 0-μM PUN.

capacity than cells that were treated with PUN. In addition, the MMP-2 and MMP-9 activities were higher in PUN-treated cells than in controls (Fig. 5B). The MMP inhibitors, TIMP-2 and TIMP-3, were up-regulated in PUN groups (Fig. 5C). It suggests that PUN could interfere with the invasion capabilities of A2780 cells, probably through disrupting their migration and altering the expression of MMPs and TIMPs.

DISCUSSION

Currently, surgery and subsequent chemotherapy remain the primary treatments for OCs and have improved the median survival in patients with OC for the last 2 decades. However, they do not always achieve fully satisfactory outcome because of drug resistance in patients with OC. Thus, it is crucial to find a new strategy to reverse drug resistance in patients with OC. In the recent years, the anticancer properties of botanical products have been attracting increasing attention for their favorable therapeutic effects and safety. In this study, we show that PUN, a major bioactive component of the pomegranate peel, effectively targets A2780 OC cells in vitro. Importantly, PUN significantly suppresses the proliferative and invasive properties of A2780 cells and inhibits the Wnt/β-catenin signaling of A2780 cells.

PUN inhibits the proliferation of A2780 cells in a dose- and time-dependent manner in our study. Previous studies have reported the antiproliferation and proapoptosis effects of PUN on several cancer cell lines through the alterations of apoptosis-related protein expression and cell cycle arrest.[4,6–8,14,16,17,19] Besides, pomegranate fruit extract (PFE) was reported to

up-regulate the proapoptotic proteins (Bax and Bak) and down-regulate the antiapoptotic proteins (Bcl-XL and Bcl-2) on human prostate cancer PC3 cells.[6] On the other hand, PUN decreases the expression of Bcl-2 and increases the expressions of activated caspase-9 and PARP in human U87MG glioma cells.[17] Similarly, PUN induces apoptosis in human colon cancer cell lines via the intrinsic pathway with the release of cytochrome-c into the cytosol, activation of initiator caspase-9 and effector caspase-3, and down-regulation of Bcl-XL.[7] In our study, PUN significantly decreased the expression of antiapoptotic protein Bax and increased the expression of proapoptotic protein Bcl-2 in A2780 cells. In addition, PFE can decrease the expression of cyclins D1, D2, and E and cyclin-dependent kinases 2, 4, and 6 in human prostate cancer PC3 cells[6] and human lung cancer A549 cells and cell cycle arrest in the G1 phase.[8] Differently, PUN down-regulates cyclins A and B1 and up-regulates cyclin E in human colon adenocarcinoma Caco-2 cells and cell cycle arrest in the S phase.[7] In human U87MG glioma cells, PUN provokes the effects of the up-regulation of cyclin E and the down-regulation of cyclins A and B and the cell cycle arrest in the G2/M phase.[17] In our study, PUN inhibits the β-catenin signaling pathway and decreases the expression of cyclin D1. In controlled cell growth, cyclin D1 is indispensable in the phosphorylation of Rb and its release from E2F, which results in the progression of the cell cycle and cellular proliferation. The cell cycle distribution shows that the PUN-treated A2780 cells were arrested in the G1/S phase. These results indicate that PUN may have different effects on cell cycle progression of different cancer cells.

Many epigenetic and genetic factors can significantly influence the genesis and development of OC. Previous researches[8,14] about lung cancer A549 cells revealed that the inhibition of MAPK, PI3K/AKT, and NF-kB/p65 signaling, as well as the down-modulation of Ki-67 and PCNA protein levels, by PUN suggests that the decrease in growth and the viability of lung cancer cells are results of decreased cellular proliferation. Furthermore, PUN increases AMPK and $p27^{T198}$ phosphorylation and then induces autophagic cell death of U87MG cells.[17] There is evidence that pomegranate juice significantly suppresses TNF-α–induced COX-2 protein expression, NF-kB binding, and AKT activation in several cancer cell lines. In addition to these signaling pathways, the Wnt/β-catenin pathway also contributes to tumorigenesis and progression.[1,2,20–23] Substantial researches revealed that the Wnt/β-catenin pathway was related to OC, specifically its important role in chemoresistance and its potential role as a target for chemosensitization. Wnt/β-catenin target genes regulate cell proliferation and apoptosis, thereby mediating cancer initiation and progression, and can be divided into 2 groups: a "stemness/proliferation group" that is active early in tumor progression and an "EMT/dissemination group" that is expressed in late-stage tumors.[1,21] The Wnt/β-catenin pathway has been shown to be a therapeutic molecular target for OC. In this study, we found that PUN decreases the expression of β-catenin and its downstream proteins including cyclin D1 and survivin, which are the obbligato factors of cell growth and proliferation.

Beyond all the previously mentioned roles, PFE was reported to inhibit UV-mediated expressions of MMPs (1, 2, 3, 7, 8, 9, 11, and 12) and the decrease of TIMP-1 and attenuate UV-induced oxidative stress and, in turn, stress-induced molecular pathways associated with a high risk of carcinogenesis in EpiDerm.[5,18,24] Matrix metalloproteinases and TIMPs are the crucial factors of tumor invasion. We suppose whether PUN has potential inhibitory effect on tumor cell invasion. In our research, PUN significantly inhibited the migration of A2780 cells after treatment for 24 hours. In addition, the activities of MMP-2 and MMP-9 were decreased, and the protein expression levels of TIMP-2 and TIMP-3 were increased after PUN treatment.

In summary, we have identified a novel activity for PUN in human OC A2780 cells, namely, the ability to induce proliferation suppression, cell cycle arrest, and invasion inhibition in association with β-catenin signaling inhibition. Therefore, PUN may be useful in the development of adjuvant therapies to treat OC.

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