

Puerarin induces platinum-resistant epithelial ovarian cancer cell apoptosis by targeting SIRT1

Jianxiu Duan¹, Mingyuan Yin², Yaqin Shao³,
Jiao Zheng^{3*}  and Shengdan Nie^{3,*}

Abstract

Objective: Previous investigations indicated the anticancer activity of puerarin. The current study aimed to evaluate the effect and molecular mechanisms of puerarin in chemotherapy-resistant ovarian cancer cells.

Methods: We examined the effects of puerarin in platinum-resistant epithelial ovarian cancer cells *in vitro* and *in vivo*. We also analyzed the molecular mechanism underlying Wnt/ β -catenin inhibition and sirtuin 1 (SIRT1) regulation following puerarin treatment.

Results: Our study demonstrated that puerarin effectively inhibited cell growth *in vitro* and *in vivo* by increasing apoptosis in ovarian cancer cells. More importantly, puerarin sensitized cisplatin-resistant ovarian cancer cells to chemotherapy. Puerarin treatment decreased SIRT1 expression, which attenuated the nuclear accumulation of β -catenin to inhibit Wnt/ β -catenin signaling. In addition, SIRT1 overexpression diminished the effects of puerarin treatment on cisplatin-resistant ovarian cancer cells. Further analysis supported SIRT1/ β -catenin expression as a candidate biomarker for the disease progression of epithelial ovarian cancer.

Conclusions: Puerarin increased the apoptosis of platinum-resistant ovarian cancer cells. The mechanism is partly related to the downregulation of SIRT1 and subsequent inhibition of Wnt/ β -catenin signaling.

¹Clinical Trial Research Ward, Hunan Provincial People's Hospital, Changsha, Hunan Province, China

²Nursing Department, Hunan Provincial People's Hospital, Changsha, Hunan Province, China

³Agency for Clinical Trials of Drugs Office, Hunan Provincial People's Hospital, Changsha, Hunan Province, China

*These authors contributed equally to this work.

Corresponding author:

Jiao Zheng, Agency for Clinical Trials of Drugs Office, Hunan Provincial People's Hospital, Jiefang West Road 61, Furong District, Changsha, Hunan Province 410005, China.

Email: zhengjiao432@163.com



Keywords

Epithelial ovarian cancer, chemotherapy resistance, puerarin, platinum, sirtuin 1, Wnt/ β -catenin

Date received: 27 January 2021; accepted: 30 July 2021

Introduction

Acquired chemotherapy resistance is often observed in patients with epithelial ovarian cancer.¹ Initially, these patients usually respond well to chemotherapy but later experience recurrence and cancer-related motility due to chemotherapy resistance.² Platinum drugs are widely used in chemotherapy regimens for epithelial ovarian cancer. However, a large proportion of late-stage patients exhibit a poor response to platinum-based chemotherapy.³ Therefore, the development of new adjuvant therapies is urgently needed to improve the chemotherapy efficacy for ovarian cancer.

The canonical Wnt/ β -catenin pathway plays a pivotal role during the acquisition of chemoresistance in various malignant tumors.⁴ Constitutively activated Wnt signaling contributes to tumorigenesis and chemoresistance,⁵ whereas Wnt pathway inhibition suppresses cancer cell-related self-renewal and chemotherapy resistance.⁶ Further studies are needed to develop Wnt signaling inhibitors to improve chemotherapy sensitivity.

Traditional Chinese herbal medicines show significant advantages in cancer treatment, including prolonging the survival of patients.⁷ Generally, the active ingredients in herbal medicine also show high effectiveness in cancer treatment. Puerarin (7,4'-dihydroxy-8-C-glucosylisoflavone) is an isoflavonoid monomer extracted from the *Pueraria lobata* root.⁸ Adjuvant therapy with puerarin injection is widely used in vascular disease.⁹ Recent investigations

indicated the anticancer activity of puerarin,¹⁰ which was supported by the increased apoptosis of various tumor cell types. The current study aimed to evaluate the effects of puerarin on the chemotherapy sensitivity of epithelial ovarian cancer cells. The molecular mechanism by which puerarin treatment induces cell apoptosis was also studied.

Materials and methods

Cell culture and treatments

The human epithelial ovarian cancer cell lines SKOV3 and Caov-4 were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in McCoy's 5A medium with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and maintained at 37°C in a humidified atmosphere of 5% CO₂. Platinum-resistant SKOV-3 cells (SKOV-3/DDP) were generated by selecting subclones from cells treated with cisplatin (DDP, Sigma-Aldrich, St. Louis, MO, USA). Parental SKOV3 cells were treated with a DDP concentration gradient up to 100 μ M and then cultured in 100 μ M for a continuous 9 months. For puerarin treatments (#57652917, Sigma-Aldrich), a nonlinear regression model was used to evaluate the half maximal inhibitory concentration (IC₅₀) with GraphPad Prism 7.0 (San Diego, CA, USA).

Patients

We assessed the clinical significance of sirtuin 1 (SIRT1) and β -catenin expression in

374 patients with ovarian cancer with complete clinical and RNA sequencing data in The Cancer Genome Atlas (TCGA) database. The samples were classified as SIRT1 and β -catenin positive or negative expression based on the median value. There was no need for ethical approval or patient consent because this analysis used a publicly available database.

Cell counting kit-8 (CCK-8) analysis

Prepared cells were seeded at a density of 5000 cells/well in 96-well plates. Puerarin or 10 μ g/mL DDP was administrated as indicated. CCK-8 (Beyotime, Jiangsu, China) was used for cell proliferation following the manufacturer's recommendation. Relative cell growth was assessed with a microplate reader (Zenyth 3100, Anthos, UK) by measuring the absorbance at 450 nm. Independent experiments were performed in triplicate.

Xenografts models

Twelve 6-week-old female athymic BALB/c mice were purchased from the Shanghai Experimental Animal Center, Chinese Academy of Science, Shanghai, China. For xenografts, 1×10^6 SKOV-3/DDP cells were injected subcutaneously, and mice were maintained in pathogen-free conditions. Puerarin (30 mg/kg) or DDP (7 mg/kg) was administrated through the caudal vein. The xenograft nodules were measured with a caliper every other day, and their volume was calculated as the length \times width²/2. The mice were decapitated under anesthesia after 23 days of treatment. The volume of tumor grafts was less than 200 mm³. The protocols for the animal study were approved by The Institutional Animal Care and Use Committee of Hunan Provincial People's Hospital (2018SK50714).

Immunohistochemistry (IHC)

Formaldehyde-fixed xenografts were cut into 4- μ m-thick sections. The sections were subjected to antigen retrieval and endogenous peroxidase blockage. Primary antibodies for B-cell lymphoma 2 (Bcl-2, ab32124), Ki-67 (ab92472), SIRT1 (ab110304), β -catenin (ab16051) and control IgG (ab172730) were purchased from Abcam, Cambridge, MA, USA. IHC staining was performed with a Ventana Discovery XT automated staining system (Ventana Medical Systems, Inc., Tucson, AZ, USA). To obtain representative images, five fields at $\times 200$ magnification were assessed.

Flow cytometry analysis of cell apoptosis

All cells were harvested after the indicated treatment. The Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (C1063, Beyotime) was used following the supplier's recommendation. The cell apoptosis percentage was measured with a BD LSR II flow cytometer (FACScan; BD Biosciences, San Jose, CA, USA), and the data were analyzed with FlowJo software (Version 10, TreeStar, Ashland, OR, USA).

Clonogenic potency analysis

Single-cell suspensions of ovarian cancer cells were cultured in 12-well plates (200 cells/well) for 10 days. Puerarin (50 μ g/mL) or DDP (10 μ g/mL) was administrated as indicated. The cell clones were fixed with paraformaldehyde and stained with crystal violet (C0121, Beyotime). Colonies with more than 50 cells were counted.

Western blot assays

Total cell protein was extracted with modified RIPA lysis buffer (Beyotime). The nuclear and cytosolic cell fractions were extracted with the Nuclear and

Cytoplasmic Protein Extraction Kit (Beyotime). The protein concentration was measured with an Enhanced BCA Protein Assay Kit (Beyotime). An equal amount of protein was used for sodium dodecyl sulphate polyacrylamide gel electrophoresis and polyvinylidene fluoride membrane transfer. Primary antibodies for cleaved poly (ADP-ribose) polymerase (PARP, ab32064), Bcl-2 (ab32124), B-cell lymphoma-extra large (Bcl-xL, ab32370), B-cell associated X protein (Bax, ab182733), β -catenin (ab16051), cyclin D1 (ab16663), SIRT1 (ab110304), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ab181206), Lamin B1 (ab16048) and β -actin (ab8226) (all purchased from Abcam) were incubated with membranes. The immunoreactive bands were analyzed with an enhanced chemiluminescent substrate (Pierce, Rockford, IL, USA).

Cell transfection

Plasmids expressing human SIRT1 and a control empty vector were obtained from the Beyotime Institute of Biotechnology. Cells were transfected with plasmids using Lipofectamine 2000 (Beyotime) following the manufacturer's instructions.

Quantitative reverse transcription PCR (RT-qPCR)

Total mRNA was prepared with Trizol (Invitrogen, Carlsbad, CA, USA), and complementary DNA was reverse transcribed with a PrimeScript RT Reagent Kit (TaKaRa Bio, Tokyo, Japan). RT-qPCR assays were performed with a One-Step RT-PCR Kit Ver.2 (TaKaRa Bio). The quantity of mRNA was normalized to corresponding GAPDH levels. The primers were designed as follows: SIRT1 forward, 5'-TAGCCTTGTCAGATAAGGAAGG A-3' and reverse, 5'-ACAGCTTCACAGT CAACTTTGT-3' and GAPDH forward,

5'-TGTGGGCATCAATGGATTTGG-3' and reverse, 5'-ACACCATGTATTCCGG GTCAAT-3'.

Immunofluorescent staining

The transfected cells were attached to slides overnight with 4% paraformaldehyde. The slides were incubated with a β -catenin antibody (Abcam) overnight at 4°C. A FITC secondary antibody (Invitrogen) was added after washing with phosphate-buffered saline. Next, sections were stained with 0.1% 4', 6-diamidino-2-phenylindole to visualize the cell nuclei. The localization of β -catenin was examined with a confocal microscope (Leica, Wetzlar, Germany).

Statistical analysis

All assays were independently repeated at least three times. The results were presented as the mean \pm standard deviation. Comparisons were performed with one-way ANOVA or Student's t-test. Correlations between SIRT1/ β -catenin expression and clinical characteristics were assessed using GraphPad Prism. The prognostic value of the indicated markers was analyzed with Kaplan–Meier curves and the log-rank test. Statistical analysis was performed with IBM SPSS Statistics for Windows, Version 24 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a significant difference.

Results

Puerarin inhibits the proliferation of platinum-resistant ovarian cancer cells

First, we investigated the biological role of puerarin (Figure 1a) in human epithelial ovarian cancer cells. Ovarian cancer cells were treated with a puerarin concentration gradient for 48 hours, and then cell viability was determined by CCK-8 assays.

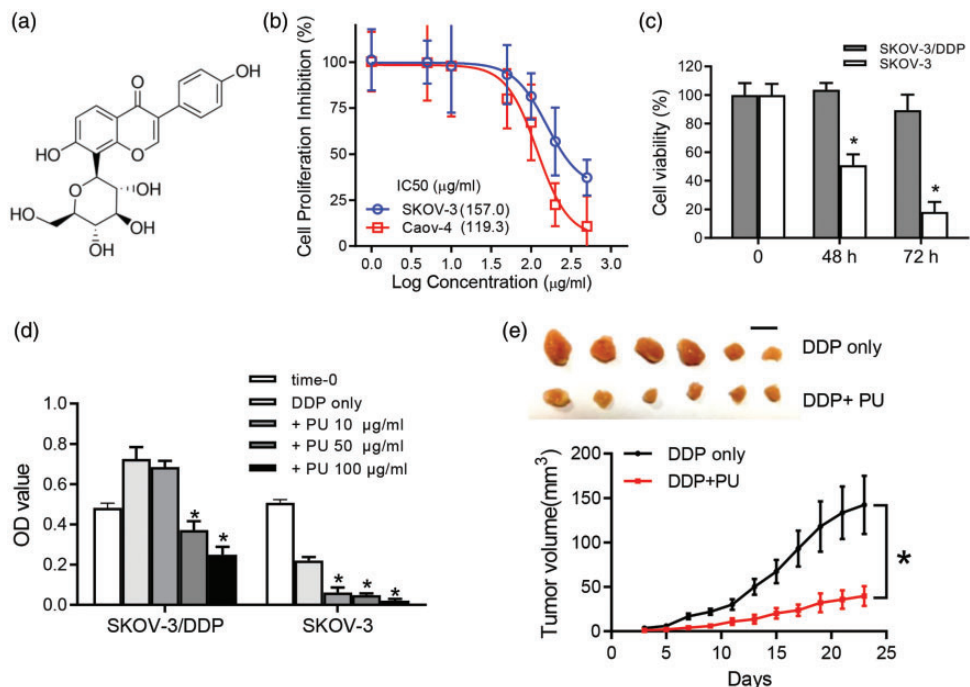


Figure 1. Puerarin inhibits the proliferation of platinum-resistant ovarian cancer cells. (a) The chemical structure of PU. (b) The ovarian cancer cell lines SKOV-3 and Caov-4 were treated with a PU gradient for 48 hours. A nonlinear regression model was used to evaluate the IC₅₀ with GraphPad Prism 7.0. (c) Cell viability analysis was performed with SKOV-3/DDP and parental cells treated with DDP (10 $\mu\text{g/ml}$) for 48 or 72 hours. Cell viability inhibition was measured by CCK-8 assays. (d) SKOV-3/DDP and parental cells were treated with DDP and PU (0, 10, 50 and 100 $\mu\text{g/ml}$) for 48 hours. Cell proliferation inhibition was measured with CCK-8 assays. (e) Xenograft growth of SKOV-3/DDP cells was recorded (bottom panel). Puerarin and DDP were administered with an intravenous infusion every other day. The xenografts were harvested after 23 days of treatment (top panel). Bar = 10 mm. Data represent the mean \pm SD, * $P < 0.05$. PU, puerarin; IC₅₀, half maximal inhibitory concentration; DDP, cisplatin, CCK-8, cell counting kit-8; OD, optical density; SKOV-3/DDP, platinum-resistant ovarian cancer cells.

Our results demonstrated that the IC₅₀ of puerarin in SKOV-3 and Caov-4 cells was 157.0 $\mu\text{g/ml}$ and 119.3 $\mu\text{g/ml}$, respectively (Figure 1b). Next, SKOV-3/DDP cells were treated with puerarin. The platinum drug resistance ability was confirmed with CCK-8 assays, which showed increased survival of SKOV-3/DDP cells compared with parental cells ($P < 0.05$, Figure 1c). Significant cell growth inhibition was observed when SKOV-3/DDP cells were treated with 100 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$ puerarin combined with DDP for 48 hours ($P < 0.05$, Figure 1d). Moreover,

combination treatment with DDP and 10 $\mu\text{g/ml}$ puerarin inhibited the proliferation of parental SKOV-3 cells ($P < 0.05$, Figure 1d). Furthermore, the *in vivo* effect of puerarin was evaluated in xenograft models. SKOV-3/DDP cells were implanted subcutaneously into 6-week-old female athymic nude mice. Then, the mice were administered intraperitoneal injections of DDP and puerarin (0.1 mg/kg) or DDP alone every 2 days. A significant inhibition of tumor growth was observed in the combined treatment group (Figure 1e). In addition, the mean tumor volumes in the

puerarin-treated group were significantly lower than those in the control group on day 23 ($P < 0.05$, Figure 1e).

Puerarin increases the apoptosis of platinum-resistant ovarian cancer cells

Further analysis was performed to determine the mechanism underlying the inhibitory effects of puerarin in platinum-resistant ovarian cancer cells. IHC staining was performed with xenografts, which showed decreased levels of Bcl-2 in puerarin-treated tumors compared with the control group. In contrast, no significant decrease was observed in Ki-67 expression (Figure 2a). Moreover, flow cytometry assays indicated that 50 $\mu\text{g}/\text{mL}$ puerarin treatment increased the apoptosis of SKOV-3 cells ($P < 0.05$, Figure 2b). More importantly, the combined treatment induced a higher percentage of apoptotic cells than the control group (Figure 2b). In addition, puerarin treatment significantly increased the early and late-stage apoptosis of SKOV-3 and SKOV-3/DDP cells ($P < 0.01$, Figure 2b). The clonogenic potency was evaluated following treatment with puerarin, which showed that puerarin treatment decreased the number of SKOV-3 and SKOV-3/DDP cell clones ($P < 0.05$, Figure 2c). Moreover, western blot assays supported the increased pro-apoptotic activity in puerarin-treated cells. Specifically, the results showed elevated cleaved PARP and decreased Bcl-2, Bcl-xL and Bax levels (Figure 2d). Collectively, our results indicated that puerarin increased the apoptosis of platinum-resistant ovarian cancer cells.

Puerarin inhibits Wnt/ β -catenin signaling to sensitize platinum-resistant ovarian cancer cells

Previous studies demonstrated that Wnt/ β -catenin signaling contributed to platinum resistance in ovarian cancer cells, especially

by decreasing apoptotic cell death.¹¹ Next, we focused on the mechanisms by which puerarin treatment regulates the Wnt/ β -catenin pathway in platinum-resistant ovarian cancer cells. Decreased β -catenin and cyclin-D1 levels were observed after 50 $\mu\text{g}/\text{mL}$ puerarin treatment for 72 hours (Figure 3a). More importantly, puerarin treatment decreased the nuclear accumulation of β -catenin (Figure 3b). We further screened the genes related to Wnt/ β -catenin signaling and found decreased SIRT1 mRNA expression after puerarin treatment ($P < 0.05$, Figure 3c). Western blot assays also supported that puerarin treatment decreased SIRT1 protein levels (Figure 3d). IHC staining of β -catenin and SIRT1 was performed with the xenografts of SKOV-3/DDP cells, which showed that puerarin treatment decreased β -catenin and SIRT1 expression (Figure 3e). These results demonstrated that puerarin treatment inhibited Wnt/ β -catenin signaling, which was correlated with SIRT1 downregulation.

SIRT1 attenuates the chemosensitivity of puerarin-treated platinum-resistant ovarian cancer cells

To further confirm the role of SIRT1 in puerarin-treated ovarian cancer cells, SKOV-3/DDP cells were infected with a lentivirus expressing SIRT1. RT-qPCR assays confirmed increased mRNA levels of SIRT1 in SKOV-3/DDP cells. Puerarin failed to downregulate SIRT1 mRNA expression in transfected cells (Figure 4a). Similarly, western blot assays supported that puerarin treatment failed to decrease β -catenin protein levels in SIRT1-overexpressing cells (Figure 4b). The nuclear translocation of β -catenin was measured in SIRT1-overexpressing cells and parental cells, which showed that SIRT1 overexpression increased the nuclear accumulation of β -catenin (Figure 4c). Immunofluorescence analysis was performed to assess the

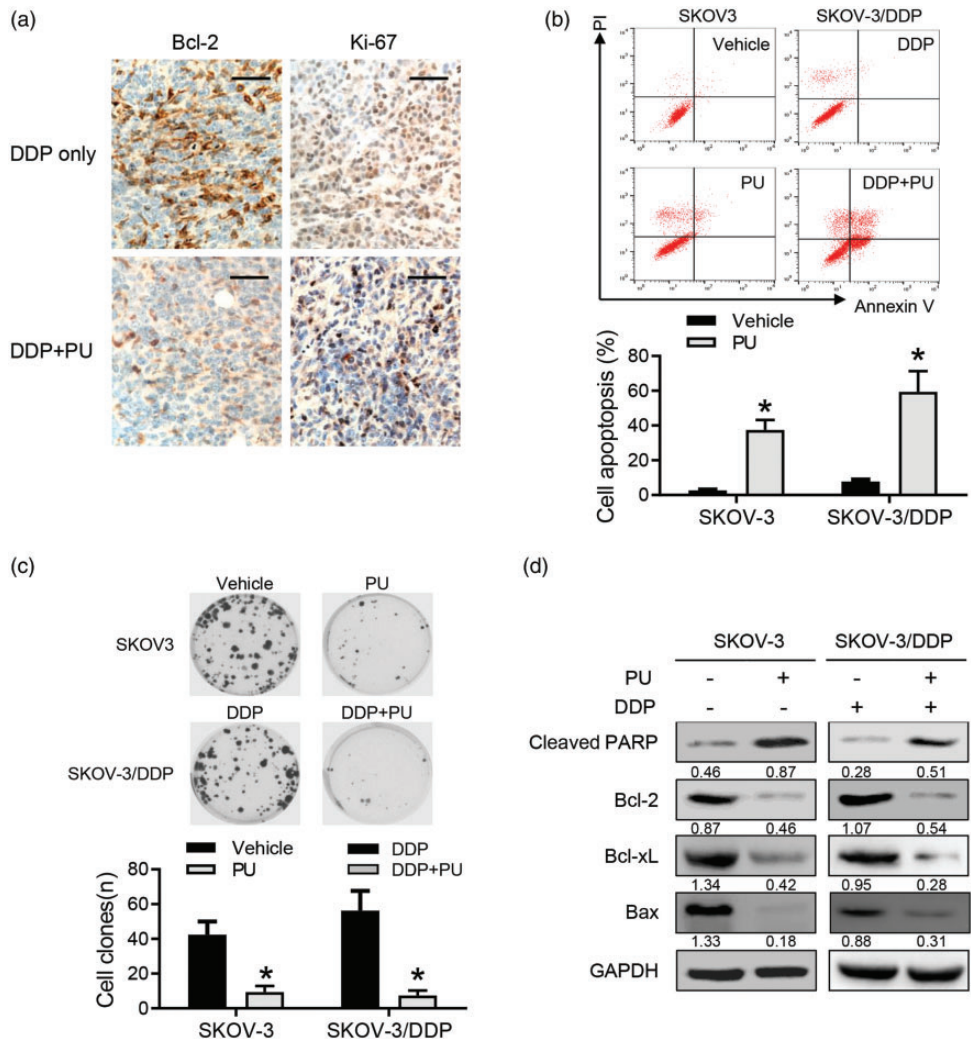


Figure 2. Puerarin increases the apoptosis of platinum-resistant ovarian cancer cells. (a) The xenografts from DDP- or DDP + PU-treated SKOV-3/DDP cells were harvested for IHC staining of Bcl-2 and Ki-67. Scale bars, 50 μ m. (b) Ovarian cancer cells were prepared with different treatments: SKOV-3, vehicle or PU (50 μ g/mL); SKOV-3/DDP, DDP or DDP + PU. Cell apoptosis was measured by flow cytometry with PI and Annexin V staining. Statistical analysis of cell apoptosis was performed with different treatments. * $P < 0.01$. (c) The clonogenic potency of SKOV-3 and DDP resistant cells was evaluated after treatment with puerarin (50 μ g/mL). (d) Western blot assays were performed for apoptosis-related proteins (cleaved PARP, Bcl-2, Bcl-xL and Bax) in PU-treated SKOV-3 or SKOV-3/DDP cells. GAPDH was used as the loading control, * $P < 0.05$.

DDP, cisplatin; PU, puerarin; IHC, immunohistochemistry; Bcl-2, B-cell lymphoma 2; PI, propidium iodide; PARP, poly (ADP-ribose) polymerase; Bcl-xL, B-cell lymphoma-extra large; Bax, B-cell associated X protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SKOV-3/DDP, platinum-resistant ovarian cancer cells.

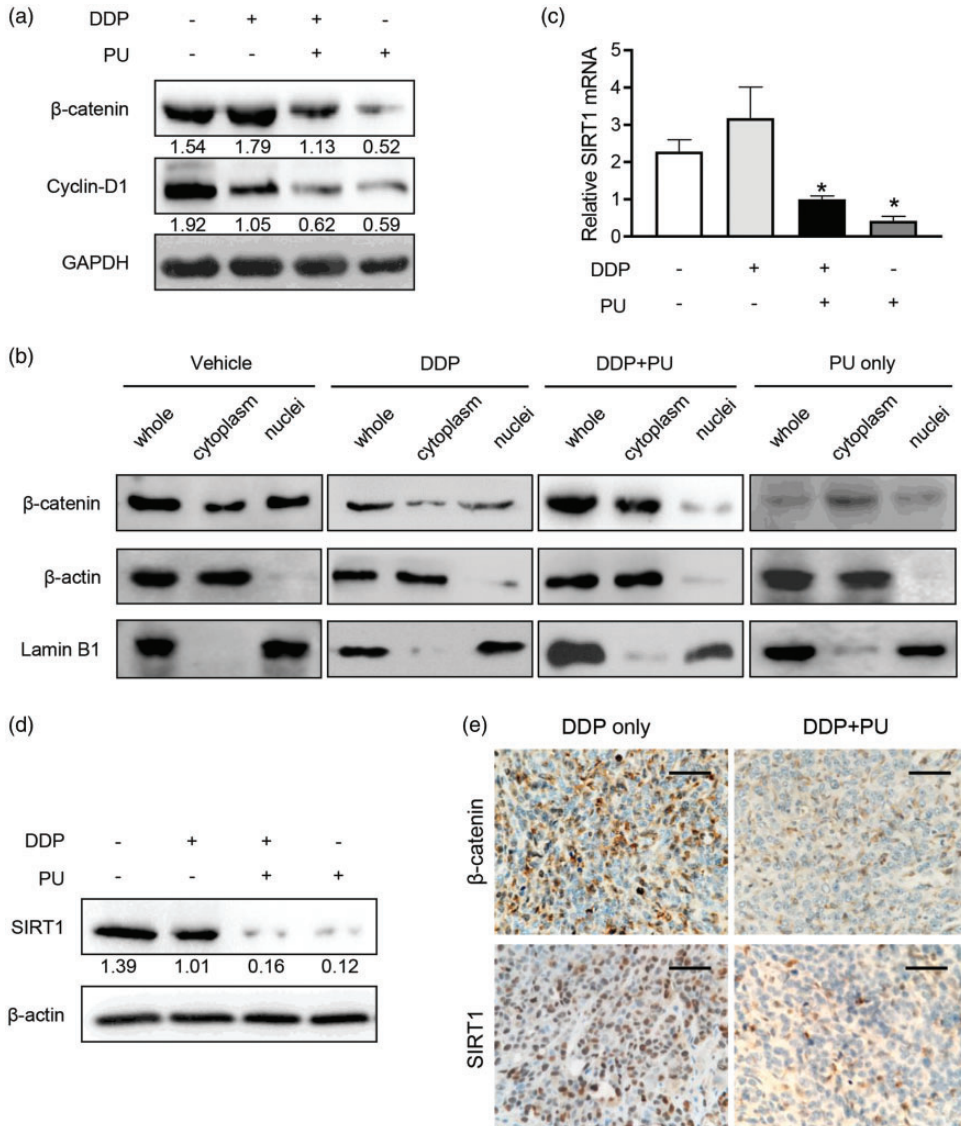


Figure 3. Puerarin inhibits Wnt/ β -catenin signaling to sensitize platinum-resistant ovarian cancer cells. (a) Western blot assays were performed for the expression of β -catenin and cyclin D1 in SKOV-3/DDP cells. Different treatments were administrated as indicated. GAPDH, loading control. (b) β -catenin localization in SKOV-3/DDP cells with different treatments (vehicle, DDP only, DDP+PU or PU only) was studied using western blot assays. Lamin B1, nuclear control; β -actin, cytoplasmic control. (c) RT-qPCR assays were performed for SIRT1 mRNA expression in SKOV-3/DDP cells treated as indicated. GAPDH was used as the control. (d) Western blot assays were performed for the protein levels of SIRT1 in SKOV-3/DDP cells treated as indicated. GAPDH, loading control. (e) The xenografts from DDP- or DDP + PU-treated SKOV-3/DDP cells were harvested for IHC staining of β -catenin and SIRT1. Scale bars, 50 μ m. * $P < 0.05$. DDP, cisplatin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PU, puerarin; RT-qPCR, quantitative reverse transcription PCR; SIRT1, sirtuin 1; IHC, immunohistochemistry; SKOV-3/DDP, platinum-resistant ovarian cancer cells.

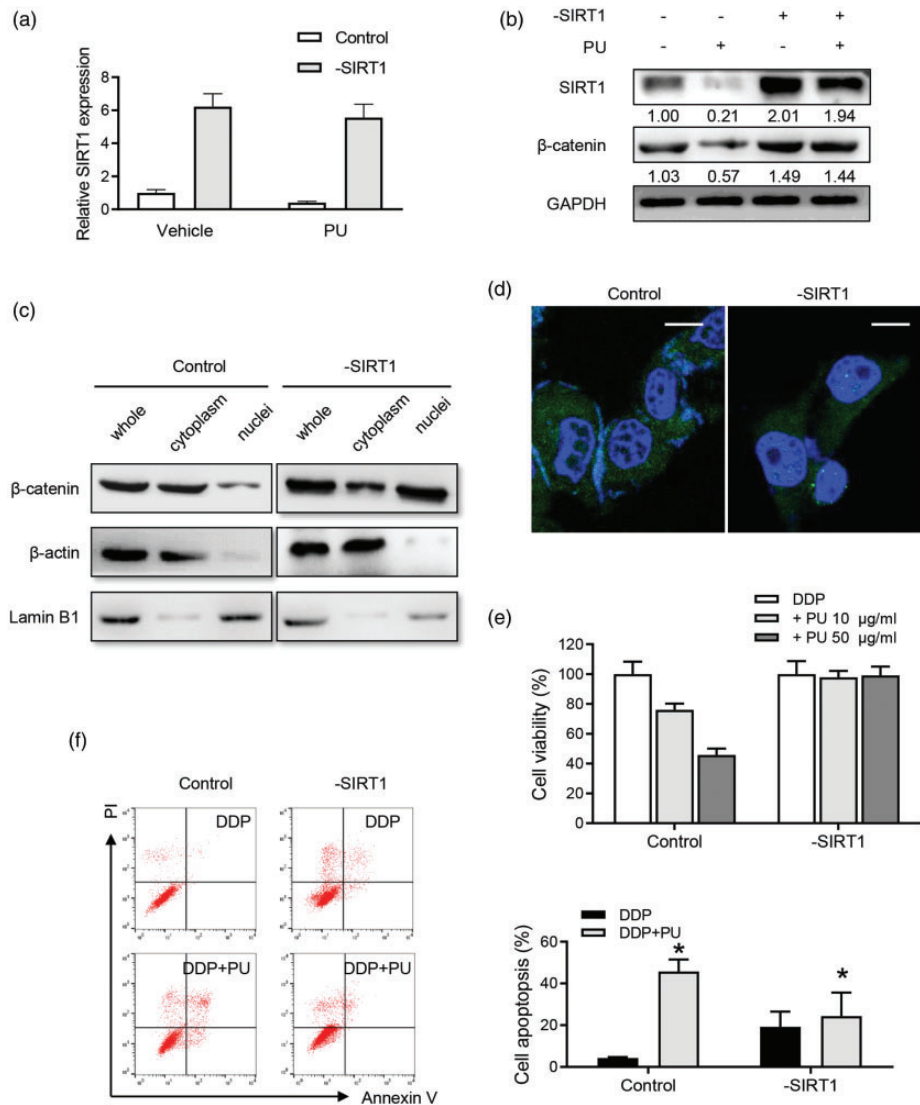


Figure 4. SIRT1 attenuates the chemoresistance of puerarin-treated platinum-resistant ovarian cancer cells. (a) RT-qPCR assays were performed for SIRT1 mRNA levels in transfected SKOV-3/DDP-control or -SIRT1 (SIRT1 overexpressing) cells. GAPDH was used as a control. (b) Western blot assays were performed for the protein levels of SIRT1 in infected SKOV-3/DDP cells. GAPDH, loading control. (c) The nuclear translocation of β -catenin was measured in SIRT1-overexpressing cells and parental cells. Lamin B1, nuclear control; β -actin, cytoplasm control. (d) Immunofluorescence analysis was performed to determine the translocation of β -catenin in infected cells. Scale bars, 10 μ m. (e) Transfected SKOV-3/DDP cells were treated with DDP and PU (10 or 50 μ g/mL) for 48 hours. CCK-8 assays were performed for cell viability. (f) Transfected cells were treated with DDP and PU (50 μ g/mL) for 48 hours. Flow cytometry assays were performed to determine the proportion of apoptotic cells. Cell apoptosis percentage was compared among different treatment groups. * $P < 0.05$.

SIRT1, sirtuin 1; RT-qPCR, quantitative reverse transcription PCR; DDP, cisplatin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PU, puerarin; CCK-8, cell counting kit-8; SKOV-3/DDP, platinum-resistant ovarian cancer cells; PI, propidium iodine.

translocation of β -catenin in transfected cells, and the results also demonstrated that SIRT1 overexpression increased the nuclear accumulation of β -catenin following puerarin treatment (Figure 4d). CCK-8 assays were performed to analyze cell proliferation after DDP treatment. The combined puerarin treatment failed to inhibit the proliferation of SIRT1-overexpressing ovarian cancer cells compared with the control cells (Figure 4e). Moreover, flow cytometry analysis indicated no significant increase in the apoptosis of SIRT1-overexpressing cells compared with the control cells (Figure 4f). Our data indicated that SIRT1 played a critical role in the puerarin treatment-induced platinum sensitivity of ovarian cancer cells.

SIRT1/ β -catenin expression predicts the disease progression of patients with epithelial ovarian cancer

We further analyzed the clinical significance of SIRT1/ β -catenin expression using TCGA database. Our analysis showed no significant correlation between SIRT1 or β -catenin mRNA levels and age (Figure 5a). Similarly, no significant difference was observed between different histological grades (Figure 5b). We divided the subjects into two groups based on the median values of marker expression: SIRT1⁺/ β -catenin⁺ and others. The group with SIRT1⁺/ β -catenin⁺ expression showed worse disease-free survival than other groups ($P=0.011$, Figure 5c) but no significant difference in overall survival (Figure 5d). Our findings indicated that SIRT1⁺/ β -catenin⁺ expression predicted the disease progression of patients with ovarian cancer.

Discussion

Epithelial ovarian cancer is a common cancer in women worldwide. Most patients

are diagnosed with advanced disease because of its insidious onset,¹² resulting in limited survival. Chemotherapy resistance is a significant obstacle that impairs the effectiveness of ovarian cancer therapy, especially for patients with advanced stages. Chemoresistance is a major contributor to the mortality of patients with ovarian cancer.¹³ Thus, further therapeutic strategies to restore chemotherapy sensitivity may improve patient survival. Here, we provided evidence that puerarin resensitizes ovarian cancer cells to cisplatin chemotherapy by inhibiting Wnt/ β -catenin signaling, which was related to the downregulation of SIRT1.

Aberrant canonical Wnt/ β -catenin signaling activation contributes to the development and progression of epithelial ovarian cancer. Mutations in several factors are observed in ovarian cancer, such as catenin β 1, axin 1 or adenomatous polyposis coli, which are associated with the hyperactivity of Wnt/ β -catenin signaling.^{14,15} Moreover, canonical Wnt/ β -catenin signaling maintains the self-renewal ability of ovarian cancer cells to promote chemotherapy resistance.^{16,17} Thus, Wnt/ β -catenin pathway blockade represents a promising strategy for cancer therapy.¹⁸ Our study indicated that Wnt/ β -catenin signaling activation also contributed to the platinum-based chemotherapy resistance of epithelial ovarian cancer cells. Specifically, increased nuclear accumulation of β -catenin was observed in cisplatin-resistant ovarian cancer cells. Combined treatment with puerarin and cisplatin increased the apoptotic percentage of ovarian cancer cells. More importantly, puerarin treatment sensitized cisplatin-resistant cells to chemotherapy, providing a novel mechanism to reverse chemotherapy resistance. Notably, the canonical Wnt/ β -catenin pathway is critical for the renewal of normal tissue, and long-term treatment with specific inhibitors induces various side effects.¹⁹ Therefore,

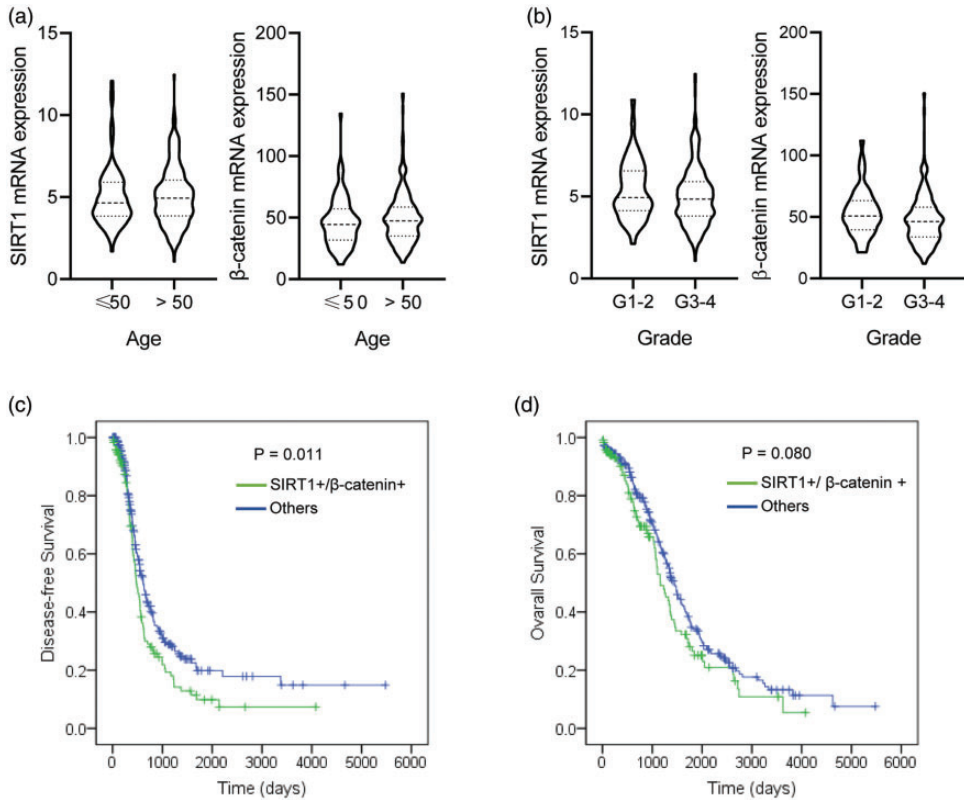


Figure 5. SIRT1/ β -catenin expression predicts the disease progression of patients with epithelial ovarian cancer: (a) SIRT1 and β -catenin mRNA levels were compared between different age groups using patient data from TCGA (≤ 50 years vs. > 50 years). (b) SIRT1 and β -catenin mRNA levels were compared between different histological grades (Grade 1–2 vs. Grade 3–4). (c, d) Kaplan–Meier analysis was performed for the disease-free survival (c) and overall survival (d) of the SIRT1/ β -catenin+ and other groups ($P = 0.011$ and $P = 0.080$, respectively).

SIRT1, sirtuin 1; TCGA, The Cancer Genome Atlas.

the safety of puerarin in a clinical setting requires further assessment, especially during the long-term treatment with cytotoxic regimens for chemotherapy.

Puerarin is widely used in the treatment of vascular disease, diabetes and Parkinson's disease.^{20,21} Moreover, puerarin administration dose-dependently and time-dependently induced cell apoptosis, which supported its use as an anticancer agent for various tumors.^{22,23} Previous investigations and our study indicated that puerarin increased mitochondria-mediated

apoptosis to inhibit cancer cell proliferation *in vitro* and *in vivo*,²⁴ which was the predominant contributor to ovarian cancer cell death. Previous studies supported that nuclear factor kappa B (NF- κ B) and c-Jun N-terminal kinase signaling pathways were involved in puerarin-induced cell apoptosis.^{10,25} Our study indicated that puerarin treatment inhibited the Wnt/ β -catenin pathway in ovarian cancer. SIRT1 regulation contributed predominantly to the effects of Wnt/ β -catenin inhibition-induced cell apoptosis. Notably, puerarin protected

proximal tubular cells from nephrotoxicity caused by platinum-based chemotherapy,^{26,27} which supported the safety of the clinical use of puerarin in cancer treatment. However, puerarin showed poor oral absorption and bioavailability because of its low water solubility and liposolubility.²² Thus, further investigation is needed to determine the appropriate dosage of puerarin for clinical applications.

SIRT1 is a member of the NAD⁺-dependent histone deacetylase family. Elevated SIRT1 expression is observed in various tumors, such as breast, colon and prostate cancers.^{28,29} However, its function for chemotherapy sensitivity is dependent on the tumor type and microenvironment.³⁰ Previous studies indicated that SIRT1 maintained cell proliferation under oxidative and genotoxic stress conditions.³¹ Epithelial ovarian cancer cells exposed to hypoxic conditions also showed SIRT1 overexpression, which was correlated to NF- κ B signaling pathway activation.³² Previous studies also indicated that SIRT1 worked as a protein deacetylase to promote c-myc deacetylation, which increased β -catenin transcriptional activity.^{33,34} Furthermore, SIRT1 contributed to the deacetylation of β -catenin. The epigenetic modification of β -catenin was crucial for its nuclear translocation and Wnt signaling activation.^{35,36} Moreover, canonical Wnt signaling was reported as a key player in modulating the fate of ovarian cancer cells, including inducing apoptosis.³⁷ Previous studies indicated that significantly decreased SIRT1 expression was observed in puerarin-treated cancer cells.^{22,38} We also found that elevated SIRT1 expression contributed to the cisplatin resistance of ovarian cancer cells, which was correlated to the activation of Wnt/ β -catenin signaling. Furthermore, we provided evidence of SIRT1 expression as a candidate prognostic biomarker for disease progression with the

TCGA database analysis. Our results indicated a poor response to chemotherapy in patients with ovarian cancer and elevated SIRT1/ β -catenin expression.

In conclusion, our study demonstrated that puerarin effectively inhibited cell growth *in vitro* and *in vivo* by increasing the apoptosis of epithelial ovarian cancer cells. More importantly, puerarin sensitized cisplatin-resistant ovarian cancer cells to platinum-based chemotherapy. Puerarin treatment decreased SIRT1 expression in ovarian cancer cells, which reduced the expression and nuclear accumulation of β -catenin to inhibit Wnt signaling. Future clinical trials are required to confirm the clinical use of puerarin for patients with ovarian cancer.


Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Science and Technology Innovation Project of Hunan (2018SK50714).

ORCID iD

Jiao Zheng  <https://orcid.org/0000-0003-0595-5626>

References

1. Christie EL and Bowtell DDL. Acquired chemotherapy resistance in ovarian cancer. *Ann Oncol* 2017; 28: viii13–viii15.
2. Eisenhauer EA. Real-world evidence in the treatment of ovarian cancer. *Ann Oncol* 2017; 28: viii61–viii65.
3. Fang F, Cardenas H, Huang H, et al. Genomic and epigenomic signatures in ovarian cancer associated with re-sensitization to platinum drugs. *Cancer Res* 2018; 78: 631–644.

4. Nusse R and Clevers H. Wnt/beta-catenin signaling, disease, and emerging therapeutic modalities. *Cell* 2017; 169: 985–999.
5. Zhan T, Rindtorff N and Boutros M. Wnt signaling in cancer. *Oncogene* 2017; 36: 1461–1473.
6. Zhong Z and Virshup DM. Wnt signaling and drug resistance in cancer. *Mol Pharmacol* 2020; 97: 72–89.
7. Li FS and Weng JK. Demystifying traditional herbal medicine with modern approach. *Nat Plants* 2017; 3: 17109.
8. Liu G, Liu Z and Yuan S. Recent advances in methods of puerarin biotransformation. *Mini Rev Med Chem* 2016; 16: 1392–1402.
9. Liu H, Zhang X, Zhong X, et al. Puerarin inhibits vascular calcification of uremic rats. *Eur J Pharmacol* 2019; 855: 235–243.
10. Ahmad B, Khan S, Liu Y, et al. Molecular mechanisms of anticancer activities of puerarin. *Cancer Manag Res* 2020; 12: 79–90.
11. Nagaraj AB, Joseph P, Kovalenko O, et al. Critical role of Wnt/beta-catenin signaling in driving epithelial ovarian cancer platinum resistance. *Oncotarget* 2015; 6: 23720–23734.
12. Webb PM and Jordan SJ. Epidemiology of epithelial ovarian cancer. *Best Pract Res Clin Obstet Gynaecol* 2017; 41: 3–14.
13. Van Zyl B, Tang D and Bowden NA. Biomarkers of platinum resistance in ovarian cancer: what can we use to improve treatment. *Endocr Relat Cancer* 2018; 25: R303–R318.
14. Fujikura K, Akita M, Ajiki T, et al. Recurrent mutations in APC and CTNNB1 and activated wnt/beta-catenin signaling in intraductal papillary neoplasms of the bile duct: a whole exome sequencing study. *Am J Surg Pathol* 2018; 42: 1674–1685.
15. Wang J, Shibayama Y, Zhang A, et al. (Pro) renin receptor promotes colorectal cancer through the Wnt/beta-catenin signalling pathway despite constitutive pathway component mutations. *Br J Cancer* 2019; 120: 229–237.
16. Chen MW, Yang ST, Chien MH, et al. The STAT3-miRNA-92-wnt signaling pathway regulates spheroid formation and malignant progression in ovarian cancer. *Cancer Res* 2017; 77: 1955–1967.
17. Teeuwssen M and Fodde R. Wnt signaling in ovarian cancer stemness, EMT, and therapy resistance. *J Clin Med* 2019; 8: 1658.
18. Dzobo K, Thomford NE and Senthebane DA. Targeting the versatile Wnt/beta-catenin pathway in cancer biology and therapeutics: from concept to actionable strategy. *OmicS* 2019; 23: 517–538.
19. Krishnamurthy N and Kurzrock R. Targeting the Wnt/beta-catenin pathway in cancer: update on effectors and inhibitors. *Cancer Treat Rev* 2018; 62: 50–60.
20. Zhou YX, Zhang H and Peng C. Puerarin: a review of pharmacological effects. *Phytother Res* 2014; 28: 961–975.
21. Yi T, Huang J, Chen X, et al. Synthesis, characterization, and formulation of poly-puterarin as a biodegradable and biosafe drug delivery platform for anti-cancer therapy. *Biomater Sci* 2019; 7: 2152–2164.
22. Liu X, Li S, Li Y, et al. Puerarin inhibits proliferation and induces apoptosis by upregulation of miR-16 in bladder cancer cell line T24. *Oncol Res* 2018; 26: 1227–1234.
23. Ye G, Kan S, Chen J, et al. Puerarin in inducing apoptosis of bladder cancer cells through inhibiting SIRT1/p53 pathway. *Oncol Lett* 2019; 17: 195–200.
24. Chen T, Chen H, Wang Y, et al. In vitro and in vivo antitumour activities of puerarin 6''-O-xyloside on human lung carcinoma A549 cell line via the induction of the mitochondria-mediated apoptosis pathway. *Pharm Biol* 2016; 54: 1793–1799.
25. Liu X, Zhao W, Wang W, et al. Puerarin suppresses LPS-induced breast cancer cell migration, invasion and adhesion by blockade NF-kappaB and Erk pathway. *Biomed Pharmacother* 2017; 92: 429–436.
26. Ma X, Yan L, Zhu Q, et al. Puerarin attenuates cisplatin-induced rat nephrotoxicity: The involvement of TLR4/NF-kappaB signaling pathway. *PLoS One* 2017; 12: e0171612.
27. Li X, Zhu Q, Zheng R, et al. Puerarin attenuates diabetic nephropathy by promoting autophagy in podocytes. *Front Physiol* 2020; 11: 73.
28. Karbasforooshan H, Roohbakhsh A and Karimi G. SIRT1 and microRNAs: The

- role in breast, lung and prostate cancers. *Exp Cell Res* 2018; 367: 1–6.
29. Ferrer CM, Lu TY, Bacigalupa ZA, et al. O-GlcNAcylation regulates breast cancer metastasis via SIRT1 modulation of FOXM1 pathway. *Oncogene* 2017; 36: 559–569.
 30. Jin X, Wei Y, Liu Y, et al. Resveratrol promotes sensitization to Doxorubicin by inhibiting epithelial-mesenchymal transition and modulating SIRT1/beta-catenin signaling pathway in breast cancer. *Cancer Med* 2019; 8: 1246–1257.
 31. So D, Shin HW, Kim J, et al. Cervical cancer is addicted to SIRT1 disarming the AIM2 antiviral defense. *Oncogene* 2018; 37: 5191–5204.
 32. Qin J, Liu Y, Lu Y, et al. Hypoxia-inducible factor 1 alpha promotes cancer stem cell-like properties in human ovarian cancer cells by upregulating SIRT1 expression. *Sci Rep* 2017; 7: 10592.
 33. Yao Y, Hua Q, Zhou Y, et al. CircRNA has_circ_0001946 promotes cell growth in lung adenocarcinoma by regulating miR-135a-5p/SIRT1 axis and activating Wnt/beta-catenin signaling pathway. *Biomed Pharmacother* 2019; 111: 1367–1375.
 34. Huang J, Zhou L, Chen J, et al. Hyperoside attenuate inflammation in HT22 cells via upregulating SIRT1 to activities Wnt/beta-catenin and sonic hedgehog pathways. *Neural Plast* 2021; 2021: 8706400.
 35. Bartoli-Leonard F, Wilkinson FL, Langford-Smith AWW, et al. The interplay of SIRT1 and Wnt signaling in vascular calcification. *Front Cardiovasc Med* 2018; 5: 183.
 36. Chen X, Huan H, Liu C, et al. Deacetylation of beta-catenin by SIRT1 regulates self-renewal and oncogenesis of liver cancer stem cells. *Cancer Lett* 2019; 463: 1–10.
 37. Binju M, Amaya-Padilla MA, Wan G, et al. Therapeutic inducers of apoptosis in ovarian cancer. *Cancers (Basel)* 2019; 11: 1786.
 38. Wang ZK, Chen RR, Li JH, et al. Puerarin protects against myocardial ischemia/reperfusion injury by inhibiting inflammation and the NLRP3 inflammasome: The role of the SIRT1/NF-kappaB pathway. *Int Immunopharmacol* 2020; 89: 107086.