Anti-neoplastic effect of mangiferin on human ovarian adenocarcinoma OVCAR8 cells via the regulation of YAP

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Abstract. Ovarian cancer is the most malignant gynecologic neoplasm in women and has the worst prognosis of all cancer types in women based on the 5-year survival rates. A previous study indicated that mangiferin exerts an anti-neoplastic effect on human ovarian cancer cells by targeting Notch3. Additionally, it has been demonstrated that Notch signaling is a functionally important downstream effector of Yes-associated protein (YAP), therefore it was hypothesized that YAP may be involved in the antitumor effect of mangiferin. The present study aimed to further reveal the mangiferin-mediated inhibitory effect on ovarian cancer and investigate the molecular anticancer mechanism of mangiferin. Based on the *in vitro* data, accompanied with the significantly reduced cell proliferation of mangiferin-treated cells compared with mangiferin-treated YAP-overexpressed cells ($P<0.05$), YAP expression was identified to be substantially downregulated by mangiferin. In contrast, observations of the cell morphology and apoptotic percentages revealed that the antitumor effect of mangiferin may be reversed by YAP overexpression. Furthermore, decreased levels of migration and invasion were observed in mangiferin-treated cells, which may also be abrogated by YAP overexpression. Thus, these data further demonstrated that mangiferin inhibits metastasis by regulating YAP. Additionally, due to the frequent chemoresistance observed in cisplatin-based chemotherapy, the present study evaluated the cisplatin resistance in OVCAR8 cells and elucidated that mangiferin may sensitize the tumor cells to cisplatin; and this improved sensitization was also abolished by YAP overexpression. These results collectively indicated that YAP was not only closely associated with the anticancer effect of mangiferin, but also mediated drug resistance in tumor. Furthermore, the downregulation of downstream TEA domain transcription factor 4 expression was observed in the mangiferin-treated cells, further validating the inhibitory effect of mangiferin on YAP. In addition, OVCAR8 cell xenograft models revealed that through increasing the sensitivity of a tumor to cisplatin, mangiferin inhibited the growth of a tumor and increased the survival time of tumor xenograft mice. Based on these results, it was concluded that mangiferin may inhibit tumor cell growth and enhance cisplatin-sensitivity in OVCAR8 cells via the regulation of the YAP pathway. Altogether, by targeting YAP and enhancing the response to cisplatin treatment, mangiferin potentially functioned as a novel therapeutic agent in the treatment of ovarian cancer.

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Introduction

Ovarian cancer is the most malignant gynecological neoplasm occurring in women (1). According to the cancer statistic data for the United States in 2016, the mortality rate of ovarian cancer is the highest amongst all gynecological malignancies and is substantially higher compared with that of cervical cancer and endometrial cancer (1). Although surgery and postoperative chemotherapy greatly contribute to the survival of the patients, 70% of them will relapse due to drug resistance (2), highlighting the need for novel therapeutic regimens.

Accumulating evidence demonstrates that signaling pathways including the Hippo (3,4), Wnt/β-catenin (5,6) and

Notch (7,8) signaling pathways are involved in the pathogenesis of ovarian cancer. Among them, the Hippo pathway serves a pivotal role in regulating cell proliferation and apoptosis, therefore it has received substantial attention in research (9,10). In normal tissues, the Hippo pathway functions well in maintaining the size of an organ and tissue homeostasis (11). However, in a tumor, the deactivation of the Hippo pathway results in the nuclear translocation of Yes-associated protein (YAP) (12). As the key component of the Hippo pathway, YAP facilitates tumorigenesis (13-15). Additionally, YAP overexpression is associated with drug resistance (16). Conversely, the cytoplasmic retention of YAP by cytochalasin D or blebbistatin and the knockdown of YAP downstream transcriptional factors by short hairpin RNA may abrogate the drug resistance mediated by YAP (16,17). Concerning tumorigenesis and drug resistance, YAP has been recognized as a potential therapeutic target for the treatment of ovarian cancer.

Mangiferin, a naturally occurring glucosylxanthone, is an effective anti-neoplastic agent in malignant cancer types including prostate cancer (18), nasopharyngeal cancer (19), breast cancer (20,21) and lung cancer (22). One previous study demonstrated the anti-neoplastic effect of mangiferin in human lung carcinomas by inducing caspase-dependent apoptosis via the activation of nuclear factor-κβ and cyclin B1 (23). Additionally, the results of another previous study on mangiferin-treated OVCAR3 cells indicated the inhibitory role of mangiferin on cell proliferation through the regulation of Notch3 (24). Notably, Notch has been widely acknowledged as a key downstream gene regulated by the Hippo pathway (25). Therefore, it was hypothesized that YAP may be involved in the antitumor effects of mangiferin. However, until now to the best of our knowledge, no data on the YAP-mediated neoplastic activities of mangiferin in ovarian cancer have been reported. The present study aimed to reveal the pivotal role of the YAP pathway in the mangiferin-mediated antitumor effect in ovarian cancer.

Materials and methods

Reagents. Human ovarian adenocarcinoma OVCAR8 cells were purchased from American Type Culture Collection (Manassas, VA, USA). OVCAR8 cells are highly resistant to cisplatin (26), and respond poorly to high-dose platinum-based chemotherapy. Additionally, OVCAR8 cells are resistant to cadmium (26). Mangiferin was purchased from Shanghai PureOne Bioechnology (Shanghai, China; www.pureonebio.com), with a purity of >95%. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). MTT (cat. no. M5655), paraformaldehyde (PFA; cat. no. 16005), cisplatin (cat. no. 1134357), dimethyl sulfoxide (DMSO; cat. no. D2650), Annexin V-fluorescein isothiocyanate (FITC) Apoptosis detection kit (cat. no. APOAF), HEPES (cat. no. H3375), Triton X-100 (cat. no. H9284), 2 mmol/l sodium orthovanadate (cat. no. S6508), sodium fluoride (cat. no. S7920), 1 mmol/l edetic acid (cat. no. E9884), PMSF (cat. no. 78830), aprotinin (cat. no. A11530) and leupeptin were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Hoechst 33342 (cat. no. C1025) and a Bradford protein assay (cat. no. P0006) were purchased from Beyotime Institute of Biotechnology (Suzhou, China). NuPAGE® Bis-Tris gels (cat. no. NP0327BOX) were purchased from Thermo Fisher Scientific, Inc. Polyvinylidene difluoride (PVDF) membranes (cat. no. ISEQ00010) and electrochemiluminescence (ECL) reagents (cat. no. 345818) were purchased from EMD Millipore (Billerica, MA, USA). Mouse monoclonal β-actin antibody (cat. no. sc-47778; 1:5,000 dilution) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Matrigel (cat. no. 356234) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit polyclonal cleaved caspase-3 antibody (cat. no. 9661; 1:500 dilution) and rabbit polyclonal YAP antibody (cat. no. 4912; 1:1,000 dilution) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse monoclonal TEA domain transcription factor 4 (TEAD4) antibody (cat. no. ab58310; 1:1,000 dilution) and rabbit monoclonal cleaved poly(ADP-ribose) polymerase 1 (PARP1) antibody (cat. no. ab32138; 1:1,000 dilution) were purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) polyclonal antibody (cat. no. 115-035-003; 1:5,000 dilution) and HRP-conjugated goat anti-rabbit polyclonal IgG (cat. no. 111-035-003; 1:5,000 dilution) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Cell culture and MTT colorimetric assay. OVCAR8 cells were cultured in RPMI-1640 which contained 20% FBS, 10 μg/ml bovine insulin, 100 μg/ml streptomycin (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Thermo Fisher Scientific, Inc.) and 0.03% L-glutamine (Sigma-Aldrich; Merck KGaA).

OVCAR8 cells at a logarithmic growth phase were seeded in a 96-well plate ($3x10^4$ cells per well) and incubated at 37˚C for 24 h. For the cell proliferation experiments examining the inhibitory effect of mangiferin via the YAP pathway, OVCAR8 cells were treated with mangiferin (25 μg/ml) at 37˚C for 24 h following transfection by Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) with either an empty vector (pcDNA3; Addgene, Inc., Cambridge, MA, USA) or YAP overexpression plasmids for 12, 24, 36 and 48 h. For the experiments examining cisplatin sensitivity, OVCAR8 cells in three groups were respectively treated at 37˚C for 6 h with cisplatin (1 μg/ml) or cisplatin (1 μg/ml) and mangiferin (25 μg/ml) combined, following transfection by Lipofectamine 2000 with either an empty vector (pcDNA3) or YAP overexpression plasmids at 37˚C for 48 h. Subsequently, cells in each well were treated with 0.05 mg MTT (10 μl of 5 mg/ml) and incubated at 37˚C for 4 h. Subsequent to incubation, the supernatant was removed. For the suspended cells, following centrifugation at 200 x g for 5 min at 4˚C, the medium was discarded and 150 μl DMSO was added to each well. The plate was thoroughly agitated by hand shaking for 10 min. Finally, the absorbance was measured at a wavelength of 570 nm using a spectrophotometer (Model 3550 Microplate Reader; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was determined via the following equation: Cell viability (%)=[optical density (OD) 570 nm (drug)/OD 570 nm (control)] x100%. Experiments were repeated three times, and data are representative of replicate experiments.

Cell colony in soft agar assay. Cells were plated in triplicate wells in 6-well plates for 14 days for a flat colony formation

assay. For the soft agar assay, $2x10^3$ cells were plated in complete medium (RPMI-1640 medium plus 10% FBS) in addition to 0.6% agar in triplicate wells in 6-well plates. Subsequent to transfection with either an empty vector (pcDNA3) or YAP overexpression plasmids, mangiferin (25 μg/ml) was added to the medium. Medium was replaced every 48 h and visible colonies were counted using light microscopy after 21 days.

Cell morphology and Hoechst 33342 staining. OVCAR8 cells were seeded into a 6-well culture plate at a density of $4x10^5$ cells/well and cultured for 24 h. Cells were treated with mangiferin (25 μg/ml) subsequent to the transfection with either the empty vector (pcDNA3) or YAP overexpression plasmids. The cell morphology was observed under phase contrast microscopy (x400 magnification). Hoechst 33342 staining was applied to further detect viable cells. In brief, cells were fixed with 4% PFA for 30 min at room temperature, and then cells were washed twice with PBS. Hoechst 33342 (5 μg/ml) was added and incubated for 15 min at 37˚C, and then the cells were washed and analyzed immediately with a fluorescence microscope (Olympus Corporation).

Annexin V-FITC/propidium iodide (PI) flow cytometry. OVCAR8 cells were treated with mangiferin (25 μg/ml) for 24 h following transfection with either an empty vector (pcDNA3) or YAP overexpression plasmids. Then, the detached and adherent cells were collected at 300 x g at 4˚C for 5 min. The cells were labelled for 15 min at room temperature using an Annexin V-FITC and PI apoptosis detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. In detail, the cells were washed twice with PBS and centrifuged at 300 x g at 4˚C for 5 min. The cells were resuspended with binding buffer and then the Annexin V and PI staining solution was added. At least $1x10^5$ cells were analyzed on a FACS Aria II cytometer (BD Biosciences). Data analyses were performed using FlowJo Software version 9.7.1 (FlowJo LLC, Ashland, OR, USA). Experiments were repeated three times, and data are representative of replicate experiments.

Wound healing assay. The migration of cells in the logarithmic growth was examined through a wound healing assay. Cells were cultured in a 12-well plate at a density of $5x10^4$ cells/well until a confluent monolayer was formed. A 200 μl pipette tip was used to scratch the wells and the initial with of the scratch was ~100 μm. Then, the wells were rinsed with phosphate buffered saline (PBS) and cultured in RPMI-1640 medium with 10% FBS at 37˚C for 24 h. The cells were treated with 25 μg/ml mangiferin at 37˚C for 24 h subsequent to transfection with either an empty vector (pcDNA3) or YAP overexpression plasmids. Images were obtained through light microscopy (Olympus CKX31; Olympus Corporation, Tokyo, Japan). The cell migration was measured by Image J software (version 1.48; National Institutes of Health, Bethesda, MD, USA) with a contrasting quantification of pixels in the area of the scratch at 24 h. Migration inhibition rate (%)=(1-experimental group pixels/control group pixels) x100%.

Matrigel cell invasion assays. A Matrigel cell invasion assay was performed using Transwell cell culture chambers (8-mm pore size; EMD Millipore). Transwell membranes were firstly coated with 100 μl Matrigel matrix (1 mg/ml; BD Biosciences). OVCAR8 cells ($5x10^4$ cells/well) were added to serum-free RPMI-1640 medium and placed in the upper chamber of the Transwell insert and incubated at 37˚C for 24 h with 25 μg/ml mangiferin subsequent to transfection with either an empty vector (pcDNA3) or YAP overexpression plasmids. RPMI-1640 containing 20% FBS was added to the lower chamber as a chemoattractant. After 24 h incubation at 37˚C, the upper surface of the chambers was scraped using a cotton swab. The cells on the lower surface were fixed with 4% PFA in PBS for 30 min at 37˚C. Subsequent to this, the chambers were rinsed with PBS and cells in the lower chamber were manually counted and analyzed under a light microscope at a x200 magnification.

Western blot analysis. OVCAR8 cells were transfected with either an empty vector (pcDNA3) or YAP overexpression plasmids using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) for 24 h at 37˚C, and then incubated with 25 μg/ml mangiferin for a further 24 h. Adherent and floating cells were collected. The cell pellets were resuspended in a lysis buffer and lysed at 4˚C for 15 min. The lysis buffer consisted of 50 mmol/l HEPES (pH 7.4), 1% Triton X-100, 2 mmol/l sodium orthovanadate, 100 mol/l sodium fluoride, 1 mmol/l edetic acid, 1 mmol/l PMSF, 10 mg/l aprotinin and 10 mg/l leupeptin. Centrifugation at 12,000 x g for 15 min at 4˚C was performed and followed by the determination of supernatant protein content using a Bradford protein assay (cat. no. P0006; Beyotime Institute of Biotechnology). Equal quantities (10 μg) of the total protein were separated using 4-12% NuPAGE® Bis-Tris gels (Thermo Fisher Scientific, Inc.) and were transferred onto PVDF membranes (EMD Millipore). The membranes were soaked in blocking buffer (5% bovine serum albumin; Sigma-Aldrich; Merck KGaA). Then the proteins of interest were detected using primary antibodies (incubated overnight at 4˚C) and secondary antibodies (incubated for 1 h at room temperature), which was finally visualized using ECL (EMD Millipore). Experiments were repeated three times. Data are representative of replicate experiments and analyzed using ImageJ software (version 1.44; National Institutes of Health, Bethesda, MD, USA).

Luciferase assay. OVCAR8 cells were transfected with either an empty vector (pcDNA3) or YAP overexpression plasmids by Lipofectamine 2000 for 24 h at 37˚C, and then incubated with 25 μg/ml mangiferin for another 24 h. Cells were grown in RPMI-1640 medium supplemented with 20% FBS and puromycin (Thermo Fisher Scientific, Inc.) for selection. A total of $5x10^4$ cells in 24-well plates were transfected at 37˚C for 24 h with 0.5 μg synthetic 8xGTIIC TEAD luciferase promoter plasmids (Addgene, Inc.) and 0.1 ug Renilla luciferase control reporter (Promega Corporation, Madison, WI, USA), using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). Single (5'-TGTGGAATGTGT-3') and tandem (5'-TGTGGAATGTGTGGAATGTGT-3') TEAD4 binding sites were cloned upstream of the CMV promoter of the pGL4-hRluc vector. A luciferase assay was performed 24 h after transfection using a Dual-Luciferase Reporter Assay system (Promega Corporation) according to the manufacturer's protocol. Luminescent signaling was detected using a

GloMax-96 Microplate Luminometer (Promega Corporation) according to the manufacturer's protocol.

Plasmids and lentivirus preparation. The polymerase chain reaction (PCR) products for YAP with a restriction enzyme cutting site (EcoRI and XbaI) and pcDNA3 (Addgene, Inc.) with the same sticky ends were obtained and ligated by T4 DNA ligase (Tiangen Biotech, Co., Ltd., Beijing, China). In detail, the RNA of OVCAR8 cells were extracted using a Qiagen RNeasy kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol and reverse transcribed to cDNA using a reverse transcription system (Promega Corporation), which was used as the template. For the reverse transcription protocol, RNA was incubated at 70˚C for 10 min, centrifuged at 1,000 x g at 4˚C for 20 sec and then placed on ice. Then with the random primers provided in the kit, dNTP mixture, AMV reverse transcriptase, $MgCl_2$, buffer and nuclease free water were added to the microcentrifuge tube. The reaction mixture was incubated at room temperature for 10 min, then incubated at 42˚C for 15 min. The sample was heated at 95˚C for 5 min, then incubated at 5˚C for 5 min. The YAP gene was amplified using PCR from cDNA using the following primers: Forward (containing an EcoRI restriction site as underlined), 5'-GAA TTCGAGGCAGAAGCCATGG-3' and reverse (containing a XbaI restriction site as underlined), 5'-TAGAGCTCTATA ACCATGTAAGAAAGCT-3'. The thermocycling conditions were as follows: 95˚C for 180 sec to open the template, then 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 60 sec for 35 cycles. Further extension was performed at 72˚C for 10 min, and then maintained at 4˚C. Then the PCR products were electrophoresed using 1% agarose (Sigma-Aldrich; Merck KGaA), stained with ethidium bromide (10 mg/ml; Tiangen Biotech, Co., Ltd.), visualized under ultraviolet light, and then purified using a gel purification kit (Tiangen Biotech, Co., Ltd.), according to the manufacturer's protocols. All restriction enzymes and the T4 DNA ligase were purchased from Tiangen Biotech, Co., Ltd. The insertion of YAP in pcDNA3 was performed and confirmed by sequencing.

The PCR product of YAP (as described above) was cloned into pTY linkers. Third-generation vectors were used in this experiment. A total of 2 μg YAP lentiviral vectors were transiently transfected into $1x10^5$ OVCAR8 cells using Lipofectamine 2000 at 37˚C for 48 h. Briefly, OVCAR8 cells, cultured in DMEM medium plus 10% FBS, were co-transfected with 2 μg vector plasmids, including a helper construct, envelope plasmid, tat plasmid and pTY linker containing YAP. Then the viral supernatant was harvested at 48 h, filtered through a 0.45-μm filter, subjected to ultracentrifugation (113,000 x g at 4˚C for 2 h) for a 100-fold concentration and stored at -80˚C. Then, the lentiviral supernatant was thawed at 37˚C and diluted in 0.9% saline (Sichuan Kelun Pharmaceutical, Co., Ltd., Chengdu, Sichuan, China) and polybrene (8 μg/ml final concentration; Sigma-Aldrich; Merck KGaA) to produce a dose of $1.6x10^7$ transducing units in a 50 μl injection volume. The virus was injected intravenously into the BALB/c nude mice with a 30-gauge needle on a 1-cc syringe. A total of 3 consecutive injections were administered at 3-day intervals (n=6).

Tumor volume and survival assay in vivo. A total of $5x10^6$ OVCAR8 cells were injected into BALB/c nude female mice (n=60 in total and n=20 per group; 5-6 weeks old; 16-18 g body weight; purchased from the Affiliated Laboratory Animal Center of Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital, Chengdu, China). All animals were maintained at 26˚C at 40-60% humidity in a 12 h light/dark cycle with *ad libitum* access to water and food, which was in accordance with the individually ventilated cages requirements at the Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital. Prior to the initiation of daily treatment, the tumors were allowed to grow to a size of 100-550 mm^3. No mouse bearing multiple tumors was observed in the present study. During the present study, the maximum body loss in an animal due to cachexia was 11.3%, and the longest diameter exhibited by a single subcutaneous tumor was 1.2 cm, which is smaller compared with the Institutional Animal Care and Use Committee Guidelines (2.0 cm) of the American Association for Laboratory Animal Science (https://www.aalas.org/), therefore it was confirmed that the tumor burden did not exceed the recommended dimensions. Subsequent to tumor formation by OVCAR8 cells, mice were randomly divided into three groups: i) A cisplatin group, where cisplatin (10 mg/kg) was intraperitoneally administered; 2) A cisplatin and mangiferin plus empty virus transfection group, where cisplatin (10 mg/kg) and mangiferin (50 mg/kg) were intraperitoneally administered; 3) A cisplatin and mangiferin plus YAP overexpression lentivirus transfection group, where cisplatin (10 mg/kg) and mangiferin (50 mg/kg) were intraperitoneally administered. The treatment lasted for a further two weeks. All experiments were ethically approved by the ethics committee of Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital.

The body weight of the mice was measured daily prior to and following the mangiferin treatment. Then, 5 mice in each group were sacrificed, and 5 mice in each group were raised for a survival assay. For the sacrificed mice, the subcutaneous tumors were removed and weighed, while the volume of the tumors was determined in 3 dimensions with venire calipers according to the following formula: Tumor volume=length x width x depth x0.5. After 20 days of treatment, mice were sacrificed by cervical dislocation, and subcutaneous tumor masses were determined.

Statistical analysis. All data were expressed as the mean ± standard error of the mean from at least three independent experiments. Data analysis was performed using GraphPad Prism 5.0 software (Graphpad Software, Inc., La Jolla, CA, USA), and $P<0.05$ was considered to indicate a statistically significant difference. One-way analysis of variance followed by Bonferroni's post-hoc test, two-way analysis of variance followed by Bonferroni's post-hoc test and a Student's t-test (paired) were performed to determine the statistical significance. All experiments were performed at least in triplicate and repeated at least three times, and the data are representative of replicate experiments.

Results

Mangiferin inhibits cell proliferation via the regulation of YAP. One previous study revealed that mangiferin may inhibit the proliferation and induce the apoptosis of ovarian cancer cells through the regulation of Notch3, which is a

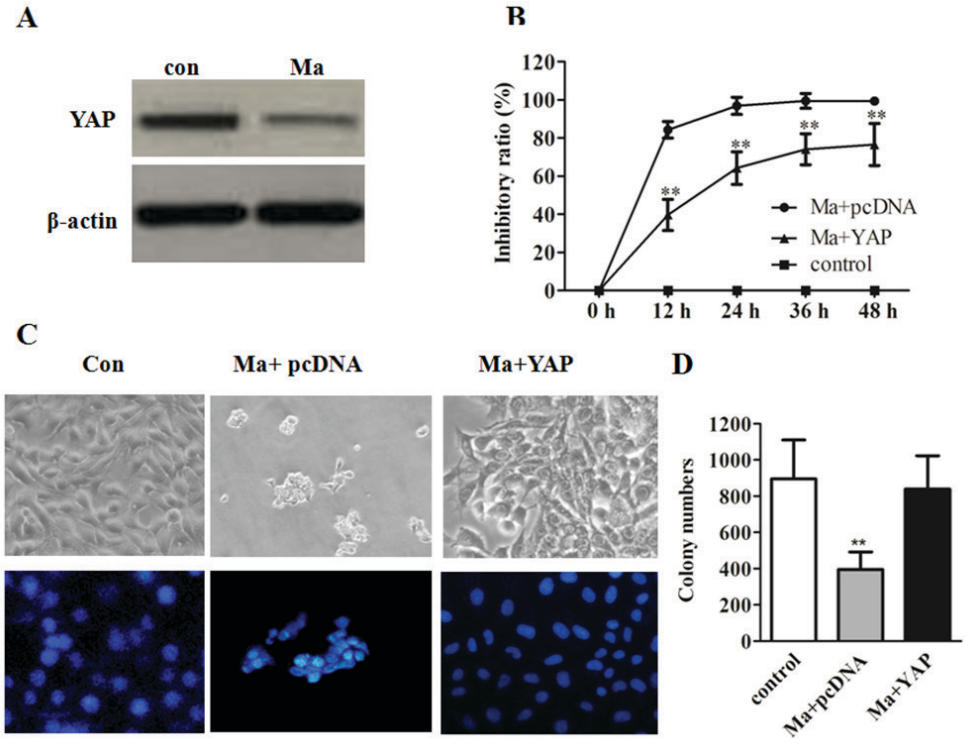

Figure 1. Mangiferin inhibits YAP-dependent cell proliferation. (A) Representative western blot analysis images of OVCAR8 cells treated with dimethyl sulfoxide control or mangiferin. (B) Inhibitory ratio of OVCAR8 cells treated with mangiferin combined with either an empty vector (pcDNA3) or a YAP overexpression plasmid. (C) Representative phase contrast and fluorescent pictures of OVCAR8 cells (left), OVCAR8 cells treated with mangiferin plus either empty vector (pcDNA3; middle) or YAP overexpression plasmid (right). (D) Quantified numbers of cell colonies. **P<0.01 vs. the Ma+pcDNA group. Data are presented as the mean ± standard error of the mean from 3 independent experiments. YAP, Yes-associated protein; Ma, mangiferin; Con, control.

downstream effector of YAP (24). Thus, it was hypothesized that YAP may also be involved in the antitumor efficacy of mangiferin. To verify this hypothesis and elucidate the mechanism of mangiferin inhibiting cell proliferation, YAP protein expression was assessed. As presented in Fig. 1A, a comparatively decreased expression of YAP was observed in mangiferin-treated cells. To further validate that YAP was involved in mangiferin-inhibited cell proliferation, a time course study for the mangiferin-treated group and YAP-overexpressed mangiferin-treated group (abbreviated as the YAP-overexpressed group) was performed. As predicted, compared with the mangiferin-treated group, the inhibitory rate of the YAP-overexpressed group was significantly decreased (P<0.01; Fig. 1B). In addition, based on cell fluorescent staining and cell morphology images, OVCAR8 cells treated with mangiferin demonstrated shrinkage of the cytoplasm and a condensed nucleus. However, a greater number of viable cells were observed in YAP overexpressed cells, which indicated that mangiferin may mediate cell apoptosis via the inhibition of YAP (Fig. 1C). Additionally, the quantified cell colony numbers revealed a significantly higher number of colonies in the YAP-overexpressed group compared with the mangiferin-treated group (P<0.01) further substantiated that YAP may facilitate cell growth by counteracting the effects of mangiferin (Fig. 1D).

Mangiferin induces apoptosis via the regulation of YAP. As demonstrated in Fig. 2A, increased caspase-3 cleavage and PARP cleavage were produced in mangiferin-treated cells, compared with in the mangiferin-treated YAP-overexpressed cells. To further demonstrate that mangiferin may induce apoptosis through the inhibition of YAP, cells were stained with Annexin V and PI, and the apoptotic cell percentages were analyzed using flow cytometry. As presented in Fig. 2B, there were more early apoptotic cells (Annexin V^+/PI^-) and late apoptotic cells (Annexin V^+/PI^+) in the mangiferin-treated group compared with the YAP-overexpressed cells (Fig. 2B). Quantified apoptotic percentages further revealed that mangiferin-induced apoptosis was significantly inhibited by the overexpression of YAP (P<0.05; Fig. 2C).

Mangiferin suppresses migration and invasion. To further investigate the migration capability of tumor cells and to address the association between mangiferin-inhibited cell migration and YAP, a wound healing assay was performed and the images of the cell migration were captured through microscopy (Fig. 3A and B). After 24 h of treatment, cell fusion was observed in the mangiferin-treated YAP-overexpressed group, with a significant increase in the fold increase of migrated cells compared with the mangiferin-treated group (P<0.01). Conversely, a scratch was clearly observed in the mangiferin-treated group at 24 h. These data indicated that mangiferin may effectively suppress tumor cell migration, and that this suppression may be reversed by YAP overexpression. Additionally, a cell invasion assay was performed. As revealed in Fig. 3C, the data suggested that YAP was able to significantly reverse the mangiferin-suppressed invasion capability (P<0.05), further validating the involvement of

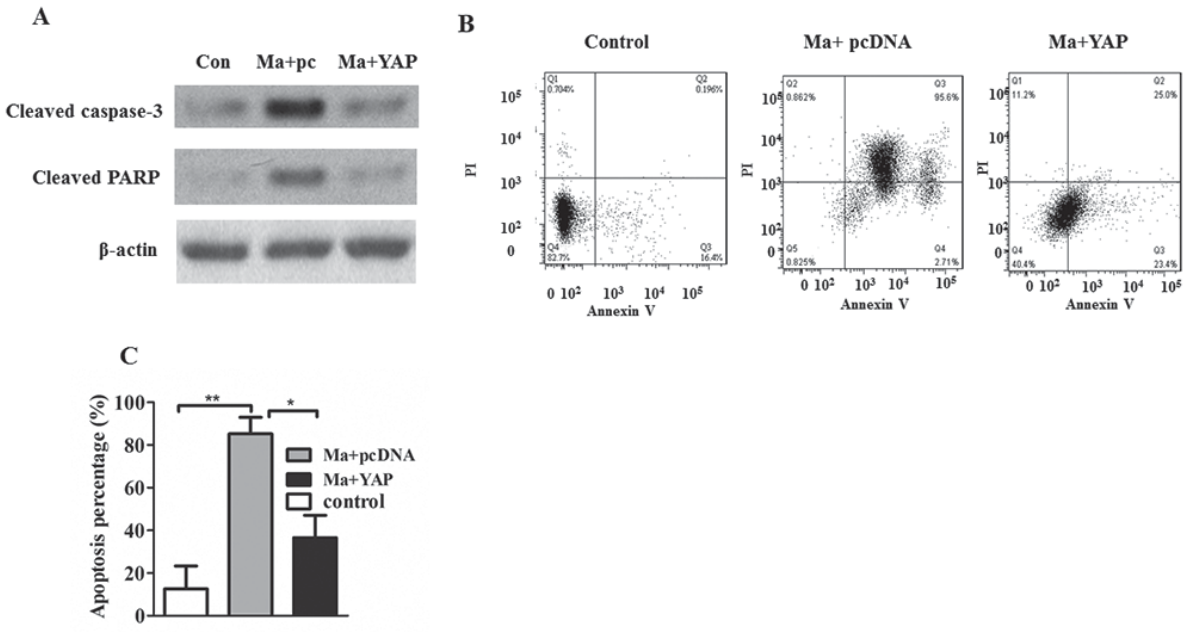

Figure 2. Mangiferin induces apoptosis via the inhibition of YAP. (A) Representative western blot analysis images of key proteins (caspase-3 and PARP) of apoptosis. (B) Flow cytometric analysis of apoptosis. (C) Quantified apoptotic cell percentages. *P<0.05 and **P<0.01 vs. the Ma+pcDNA group. Data are presented as the mean ± standard error of the mean from 3 independent experiments. YAP, Yes-associated protein; Ma, mangiferin; PARP, poly(ADP ribose) polymerase; PI, propidium iodide.

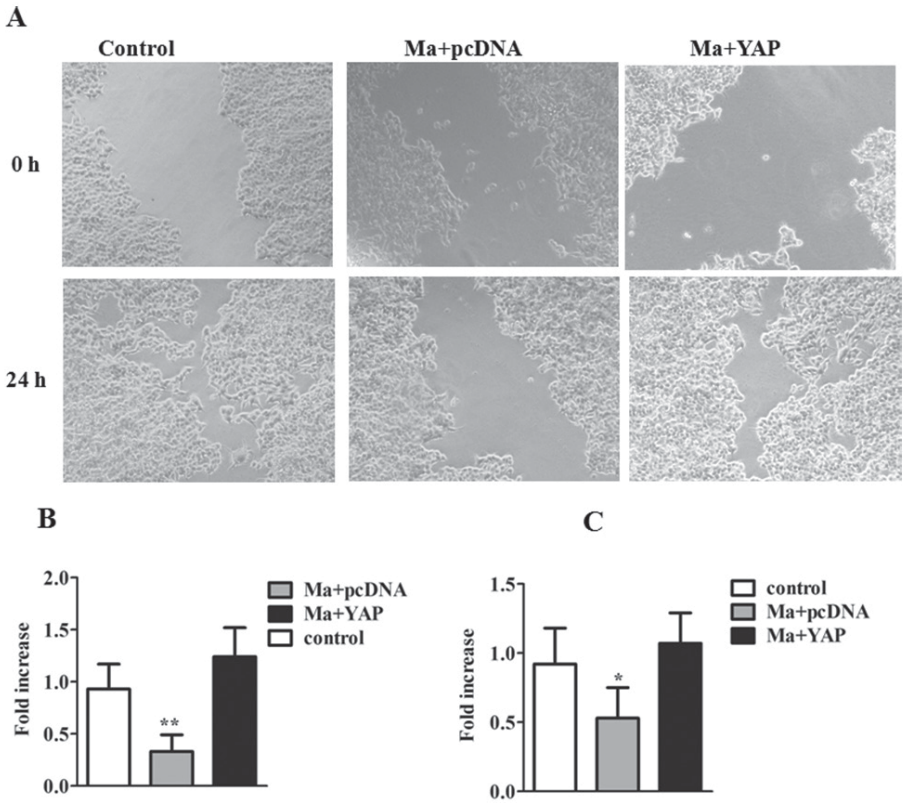

Figure 3. Mangiferin inhibits migration and invasion. (A) Wound healing images of cell treated with mangiferin in addition to either an empty vector (pcDNA; left), cells treated with mangiferin plus either empty vector (pcDNA; middle) or YAP overexpression plasmid (right). Quantified (B) cell migration and (C) invasion capability. *P<0.05 and **P<0.01 vs. the Ma+pcDNA group. Data are presented as the mean ± standard error of the mean from 3 independent experiments. YAP, Yes-associated protein; Ma, mangiferin.

YAP in mangiferin-inhibited migration and invasion events in OVCAR8 cells.

Mangiferin results in the downregulation of YAP. To further investigate the molecular mechanism of mangiferin, a luciferase

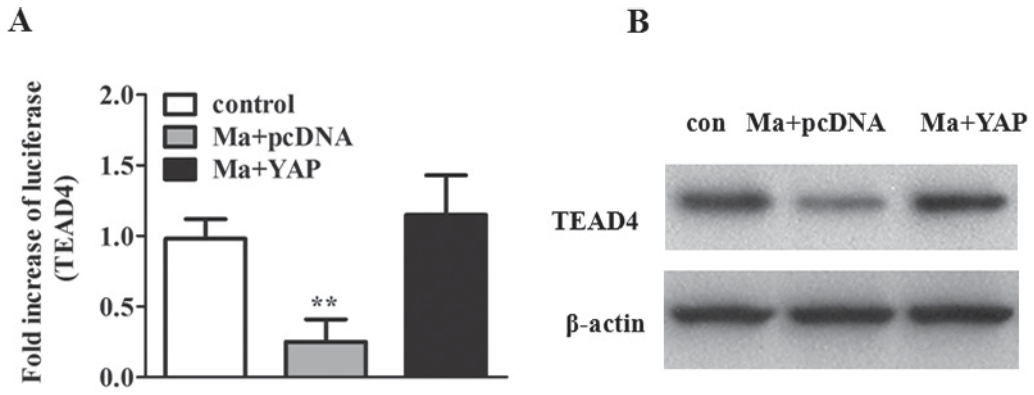

Figure 4. Mangiferin inactivates TEAD4. (A) Luciferase assay of TEAD4. (B) Representative western blot analysis images of TEAD4. **P<0.01 vs. the Ma+pcDNA group. Data are presented as the mean ± standard error of the mean from 3 independent experiments. YAP, Yes-associated protein; Ma, mangiferin; TEAD4, TEA domain transcription factor 4.

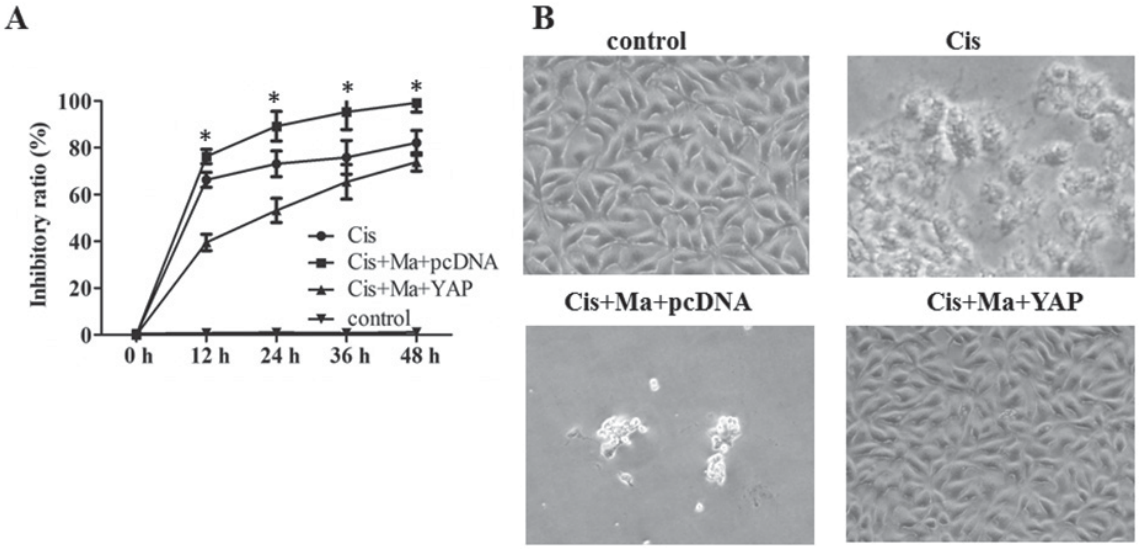

Figure 5. Mangiferin increases cell sensitivity to cisplatin. (A) Inhibitory ratio and (B) representative phase contrast images of OVCAR8 cells untreated, OVCAR8 cells treated with cisplatin, mangiferin plus either empty vector (pcDNA) or YAP overexpression plasmid. *P<0.05, Cis+MA+pcDNA group compared with all other groups. Data are presented as the mean ± standard error of the mean from 3 independent experiments. YAP, Yes-associated protein; Ma, mangiferin; Cis, cisplatin.

assay was performed. As presented in Fig. 4A, mangiferin reduced TEAD4-dependent luciferase activity, whereas YAP overexpression resulted in the significant activation of TEAD4-dependent luciferase activity (P<0.01). Furthermore, western blot analysis of TEAD4 was performed. It was revealed that TEAD4 protein levels were decreased upon mangiferin treatment and elevated in YAP-overexpressed cells (Fig. 4B).

Mangiferin enhances the sensitivity of OVCAR8 cells to cisplatin. To address the effect of YAP on the enhanced chemotherapy sensitivity induced by mangiferin, the proliferation of YAP-overexpressing OVCAR8 cells in the presence of mangiferin and cisplatin was assessed. As presented in Fig. 5A, cisplatin combined with mangiferin was able to inhibit cell proliferation, which may be abrogated by YAP overexpression. To further validate this hypothesis, cell morphology was observed under phase contrast microscopy. As presented in Fig. 5B, compared with the cisplatin-treated group, fewer viable cells were observed in the mangiferin and cisplatin combined-treated cells, whereas a greater number of viable cells were observed in the YAP-overexpressed OVCAR8 cells. Altogether, these results suggested that mangiferin enhanced the sensitivity of OVCAR8 cells to cisplatin via the inhibition of YAP.

Mangiferin decreases tumor volume in vivo. To evaluate the effect of YAP on the anti-neoplastic properties of mangiferin and mangiferin-increased tumor sensitivity to cisplatin in an *in vivo* tumor xenograft model system, OVCAR8 cells were xenografted into nude mice. As presented in Fig. 6A, the xenograft tumor size decreased significantly in the empty vector-transfected mangiferin and cisplatin combined-treated group following a 20-day treatment, compared with that of the cisplatin monotherapy group (P<0.05). However, in the YAP-overexpressed mangiferin and cisplatin combined-treated

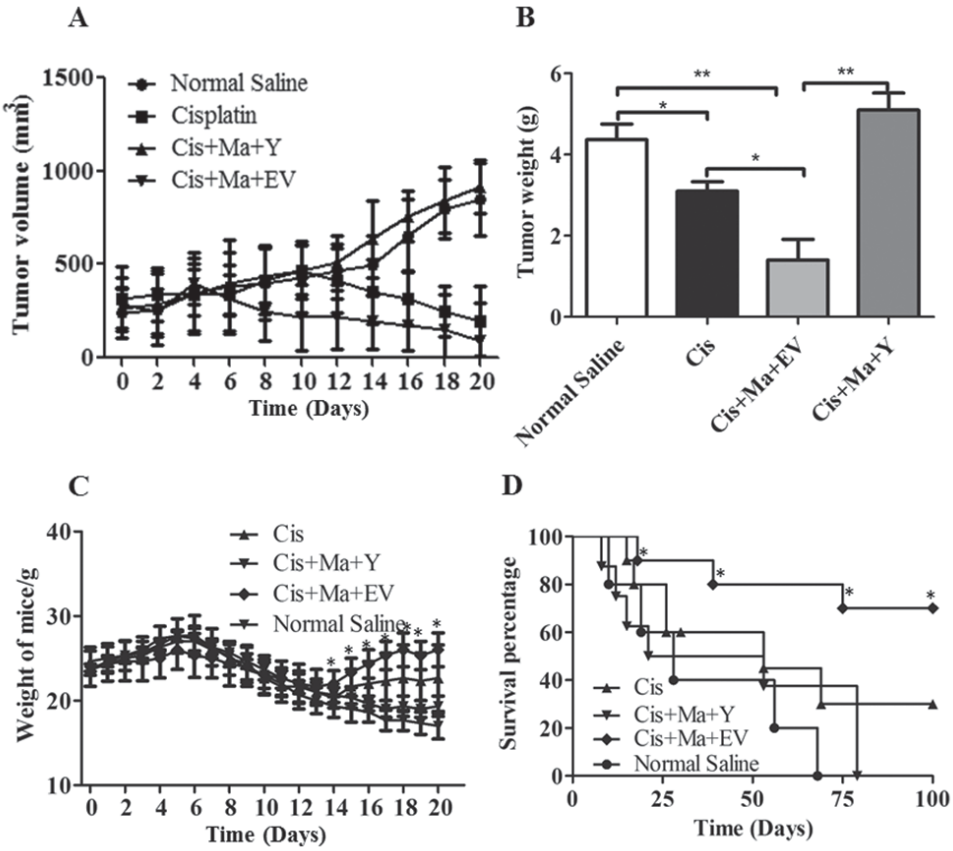

Figure 6. Mangiferin inhibits tumor growth *in vivo*. (A) Tumor volumes. (B) Tumor weights of each group (n=5). *P<0.05 and **P<0.01 with comparisons shown by lines. (C) Body weights of OVCAR8 cell xenograft mice (n=5). P<0.05, Cis+MA+EV group compared with all other groups. (D) Survival curve of the OVCAR8 cell xenograft mice (n=5). P<0.05, Cis+MA+EV group compared with all other groups. Y, Yes-associated protein; M/Ma, mangiferin; Cis, cisplatin; EV, empty virus.

group, the xenograft tumor size was increased. Similarly, the empty vector and mangiferin and cisplatin-treated mice also demonstrated significantly decreased xenograft tumor weights compared with the cisplatin monotherapy group (P<0.05; Fig. 6B), suggesting that mangiferin may increase the sensitivity of ovarian tumor types to cisplatin. In accordance with the decreased tumor size, the body weight of the mice in the mangiferin and cisplatin-treated group also demonstrated a significant increase compared with the cisplatin monotherapy group (P<0.05; Fig. 6C). Furthermore, the survival days for the YAP-overexpressed mangiferin and cisplatin-treated mice were 15, 17, 26, 53 and 69 days. The survival days for mice treated with mangiferin and cisplatin were 36, 39, 75, 100 and 100. But for the control mice treated with cisplatin alone, the survival days were 15, 33, 53, 79 and 100 days. Therefore, judging from the survival data, mangiferin in addition to cisplatin treatment may increase the life span of ovarian cell xenograft mice compared with any other groups (Fig. 6D). Altogether, the *in vivo* results suggested that mangiferin increased cisplatin sensitivity via inhibition of the YAP pathway.

Discussion

As the majority of patients are diagnosed at an advanced stage, ovarian cancer is the fifth leading cause of mortality amongst women with gynecological malignancies (1). At the advanced stages of ovarian cancer, extensive intraperitoneal metastases are often observed, partially contributing to the high mortality of this disease (2,27). At the beginning of standard treatment, the majority of patients are sensitive to primary or interval cytoreductive surgery and platinum-based chemotherapy. However, the majority will relapse despite completing surgery and chemotherapy, and develop drug resistance (28). Therefore, it is of great urgency to develop alternative therapeutic agents for the treatment of ovarian cancer. Abundantly isolated from different parts of *Mangifera indica L* (mango tree), mangiferin has been identified to be a valuable compound with manifold uses in immunomodulatory, antidiabetic, hepatoprotective, analgesic, antioxidative, antiaging, antitumor, anti-bacterial and antiviral effects (29-33). A previous study demonstrated that mangiferin reduced the Notch3 protein levels in ovarian cancer (24). Additionally, YAP is an ovarian cancer oncogene and promotes ovarian cancer cell tumorigenesis (4,34,35), and the Notch signaling pathway is generally acknowledged as the downstream target of YAP (25). The Notch signaling pathway is a key determinant of embryogenesis, and comparative upregulations of several elements of the Notch signaling pathway and a number of Notch targeted genes were observed with the activation of YAP (25,36,37). Due to the robust binding of TEAD4 and YAP to the promoter regions of Notch2 and SRY-Box 9 (Sox9), C2 and Sox9 were validated

to be the direct transcriptional targets of YAP and TEAD complexes (25,38). In other words, Hippo/YAP is able to directly regulate the Notch pathway genes to control Notch signaling. Therefore, based on these studies, it was hypothesized that mangiferin may induce apoptosis through the regulation of YAP in ovarian cancer. In the present study, it was elucidated that mangiferin is able to suppress the expression of YAP, inhibit cell proliferation, limit migration and invasion capabilities and induce apoptosis in ovarian cancer cells. However, these antitumor effects of mangiferin may be abrogated through the overexpression of YAP. Additionally, luciferase assay data further verified that mangiferin served an antitumor function via the downregulation of YAP. Furthermore, data on cell proliferation, cell morphology, xenografted tumor volume and weight, mice body weight and mice survival collectively suggested that mangiferin is able to increase chemotherapy sensitivity *in vitro* and *in vivo* through the inhibition of YAP. Nevertheless, the present study only investigated the YAP-mediated antitumor effect of mangiferin in ovarian cancer OVCAR8 cells, which was a defect of the present study. Thus, follow-up studies using other cell lines are required to further validate the mediation effect of YAP on mangiferin-treated ovarian cancer. Aside from the expression of YAP and Notch signaling in ovarian cancer cells, there are previous studies reporting on their expression in clinical samples of ovarian cancer (4,7,8,34,35,39). Specifically, in the ovarian carcinoma samples from patients with ovarian cancer, YAP was upregulated and associated with patient prognosis. The present study aimed to provide solid evidence for the therapeutic effect of mangiferin on ovarian cancer, and expound the involved molecular mechanisms.

The Hippo/YAP pathway is an evolutionarily and functionally conserved signaling network, serving a pivotal function in controlling organ size by stimulating cell proliferation and reducing apoptosis (40,41). As an important protein in the Hippo pathway, YAP may be translocated into the nucleus where it binds with TEAD. The binding of YAP with TEAD consequently contributes to cell proliferation, apoptosis, evasion and the amplification of stem cells (13,15). Previous studies on the Hippo/YAP pathway demonstrate that the overexpression of YAP is present in malignant cancer types including liver cancer, breast cancer, colorectal cancer and prostate carcinomas with advanced proliferation, metastasis and poor survival rate (42-45). According to previous evidence, YAP served a critical role in the cell growth and tumorigenesis of ovarian cancer *in vitro* and *in vivo* (34). In the present study, although tumor cell proliferation was greatly inhibited and apoptosis was substantially induced by mangiferin treatment, the antitumor effect of mangiferin was significantly abrogated by YAP overexpression ($P<0.05$). Therefore, mangiferin served an inhibitory role in cell proliferation and an inductive role in apoptosis through the suppression of YAP. In addition, as the most important nuclear transcription factor downstream of YAP gene, the activity and regulation of TEAD are crucial to YAP function (25,38). Therefore, to ascertain the influence of YAP activity on mangiferin, a luciferase assay was performed in mangiferin-treated cells and mangiferin-treated YAP overexpression cells. As expected, compared with YAP-overexpressing cells treated with mangiferin, a significant lower activity of TEAD4-dependent luciferase was observed in the mangiferin-treated cells. The inactivation of TEAD4-dependent luciferase activity demonstrated that mangiferin treatment suppressed the expression of YAP.

Mangiferin may influence not only tumor cell proliferation and apoptosis, but also the migration and invasion capability of ovarian cancer cells. A study on breast cancer cells indicated that mangiferin may significantly weaken cell invasion and inhibit cell migration in a dose-dependent manner (20). As reported by Gayani *et al* (18), mangiferin was also determined to be a potential anti-invasive agent in prostate cancer cells through an invasion assay. In addition, a study by Takeda *et al* (46) revealed that mangiferin inhibited spontaneous metastasis and tumor growth through *in vivo* experiments on a mouse metastatic melanoma model. Based on the potential suppressive effect of mangiferin on the migration and invasion capabilities of breast cancer, prostate cancer and melanoma, it was hypothesized that mangiferin may possess a similar function in ovarian cancer. Thus, cell migration and invasion studies were performed. As expected, the results of the present study revealed a substantial gap in the cells treated with mangiferin. To further study the mechanism of mangiferin-mediated inhibition of metastasis, the present study focused on the YAP pathway. As predicted, cell fusion was observed in YAP overexpressed cells treated with mangiferin, indicating that mangiferin mediated cell migration through the YAP pathway. A cell invasion assay using Matrigel also proved the inhibitory function of mangiferin on ovarian cancer cells through the downregulation of YAP. However, the absence of single-cell movement analyses is a limitation of the present study.

Aside from the confirmed therapeutic effect of mangiferin monotherapy in malignant tumor types, the question arises on whether mangiferin may contribute to tackling the high rates of chemoresistance in tumor chemotherapy. Platinum-based chemotherapeutic agents are widely deemed to be primary anticancer drugs and are clinically used for ovarian cancer treatment (47). Although high-grade serious ovarian cancer types initially respond well to platinum-based chemotherapy, these patients ultimately suffer from relapse and progression to chemotherapy resistance (48). Drug resistance is a serious and frequent problem in ovarian cancer which requires a more comprehensive understanding of the resistance mechanism and a better solution. In the present study, it was demonstrated that the dysregulation of the Toll-like receptor 4 (TLR4)-interleukin 6 (IL6)-Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway was closely associated with the development of a diverse range of human solid tumor types (49-51), highlighting the importance of this pathway as a therapeutic target to treat the persistent disease of high-grade serious ovarian cancer. Interestingly, IL6 is able to trigger the activation of downstream YAP through glycoprotein 130, a co-receptor of IL6, which ultimately resulted in the nuclear translocation of YAP (52). Therefore, as a participative effector of the TLR4-IL6-JAK/STAT3 pathway, YAP expression may also be involved in chemotherapy resistance-associated relapse. A study by Jeong *et al* (53) demonstrated that the activation of YAP was tightly associated with drug resistance to paclitaxel in the treatment of ovarian cancer. Additionally, increased chemoresistance was associated with the elevated expression of YAP. A study by Xia *et al* (34) proved that

YAP increased the resistance of ovarian cancer cell lines to cisplatin and taxol. However, another previous study sufficiently demonstrated that mangiferin was able to increase the chemotherapeutic sensitivity of a tumor, the mechanism of which remains obscure (24). Hence, it was proposed that mangiferin may mediate improved drug sensitivity by the inhibition of YAP. As predicted, subsequent to genetic engineering studies on ovarian cancer cells, it was clearly identified that mangiferin was able to inhibit the expression of YAP, thereby increasing the sensitivity of a tumor to cisplatin. Therefore, YAP served as an important factor counteracting mangiferin in chemotherapy resistance. This conclusion was further confirmed by the cell inhibitory curve and cell morphology. In an OVCAR8 xenografted murine model, the *in vivo* data further demonstrated that mangiferin substantially improved the chemosensitivity of ovarian tumor types to cisplatin and inhibited tumor growth. Additionally, migratory tumor types did not occur in other parts of the murine body during the 100-day observation period. Thus, it was further indicated that not only the tumor growth was influenced by mangiferin, but also tumor migration and invasion were suppressed following mangiferin treatment.

In summary, as a molecular targeted therapeutic agent of YAP, mangiferin may be a valuable potential novel drug for the treatment of human ovarian cancer. Further investigations on the molecular mechanism of mangiferin in the near future will ensure that the present study will result in mangiferin being used as a novel therapeutic drug by inhibiting cell growth, metastasis and enhancing the tumor response to cisplatin treatment for ovarian cancer treatment.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YW and RT designed the study. SD, YY and WH performed the cytological experiments. TL, HW and XL performed the animal experiments. XH performed the statistical analysis. SD and TL were major contributors to the figures and writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal handling was in performed accordance with the Ethics Committee of Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital (Sichuan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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