Original Article

Naringin inhibits cisplatin resistance of ovarian cancer cells by inhibiting autophagy mediated by the TGF-β2/smad2 pathway

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Background: Resistance to cisplatin (DDP) in patients with ovarian cancer (OC) poses a great challenge to improving the quality of life of patients. Past reports have revealed that naringin can induce apoptosis of OC cells and delay the occurrence of drug resistance in OC cells. However, the molecular role by which naringin inhibits DDP resistance in OC has not been definitively proven by researchers. The objective of this study is to investigate the effect of naringin on DDP resistance in OC cells and the specific mechanism of naringin mediating autophagy.

Methods: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry were selected to evaluate the role of naringin or DDP on the proliferation and apoptosis of human OC cells (SKOV3/A2780). The protein levels of Sequestosome 1 (SQSTM1/p62, hereinafter referred to as p62), microtubule-associated protein 1 light chain 3 (LC3), transforming growth factor-β2 (TGF-β2) and SMAD family member 2 (smad2) were detected with Western blotting assay. Immunofluorescence assay was also used to evaluate the level of LC3 in different groups of cells. Besides, functional analyses were performed *in vivo*.

Results: Naringin was shown to promote DDP sensitivity and apoptosis of human OC DDP-resistant cell line (SKOV3/A2780-DDP cells). Significantly increased p62 expression and reduced LC3 expression were found in naringin-treated cells. The autophagy agonist, rapamycin, reversed the effect of naringin on the resistance of SKOV3-DDP cells to DDP. Naringin inhibited levels of TGF-β2/smad2 pathway-related proteins, and regulated autophagy in SKOV3-DDP cells. *In vivo* experiments demonstrated that injection of naringin inhibited DDP resistance and autophagy in mice xenograft model.

Conclusions: In summary, naringin inhibits DDP resistance in OC cells by inhibiting autophagy mediated by the TGF-β2/smad2 pathway.

Keywords: Cisplatin (DDP); naringin; autophagy; transforming growth factor-β2/SMAD family member 2 pathway (TGF-β2/smad2 pathway); ovarian cancer (OC)

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Introduction

Ovarian cancer (OC), as a disease of the reproductive system, has caused great trouble to female survival and is also one of the major female diseases (1). Some research institutes predicted that the OC mortality rate would be 4.2/100,000 of all ages in the European Union in 2022 (2). Early-stage OC cannot be detected with the aid of current diagnostic tools, so OC patients are often treated in the late

stage (3). Nowadays, cytoreductive surgery combined with cisplatin (DPP) is the initial intervention mode for most patients (4). However, chemotherapy resistance is a major factor contributing to poor therapeutic efficacy, leading to tumor recurrence and metastasis, as well as patient mortality, which is the main cause for poor treatment outcomes of OC (5). Therefore, the search for novel DDP-based combination therapy has become a top priority.

Traditional Chinese medicine (TCM) has long been used for cancer treatment in Asian countries. TCM can not only improve the life quality and survival period of cancer patients, but also reduce chemotherapy resistance and chemotherapy-induced adverse effects (6,7). As a natural flavonoid drug, the Chinese herbal medicine, naringin, has been shown to have anti-cancer potential. It is involved in and regulates various activities and signal transduction processes in cancer development (8). Naringin is commonly used as a phosphatase and tensin homolog agonist to interfere with the biological action of OC cells (9). In animal experiments, naringin inhibits tumor growth (10). Importantly, naringin can reverse DDP resistance in OC cells (11). While the regulatory effect of naringin on OC has been previously demonstrated from multiple angles, the specific molecular mechanism by which naringin inhibits DDP resistance has not yet been reported.

DNA damage and inhibition of DNA synthesis are cytotoxic mechanisms of DDP in tumors, after which various signal transduction pathways in cancer cells, including autophagy, are activated. Autophagy induced by extracellular regulated kinase (ERK) or YES proto-oncogene 1 (YES1) can promote DDP resistance of OC cells (12,13). At the same time, TCM intervention in autophagy activity is also found to alter drug sensitivity of OC cells. Artesunate regulates drug-sensitive activity by interfering with autophagy in colon cancer cells (14). Solamargine also regulates cancer cell resistance via autophagy (15). In previous reports, naringin was also demonstrated to inhibit autophagy (16,17). Therefore, it is possible that naringin may inhibit DDP resistance by blocking autophagy in OC. This study focused on the effect of naringin on DDP resistance in OC cells and the specific mechanism of naringin mediating autophagy. We present this article in accordance with the MDAR and ARRIVE reporting checklists (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2156/rc).

Highlight box

Key findings

- Naringin inhibits cisplatin (DDP) resistance in ovarian cancer (OC) cells by inhibiting autophagy mediated by the transforming growth factor-β2 (TGF-β2)/SMAD family member 2 (smad2) pathway.

What is known and what is new?

- OC is a common cancer of the reproductive system, and DDP resistance is a major challenge to improve the quality of life of OC patients.
- Naringin can induce apoptosis of OC cells and delay the occurrence of drug resistance in OC cells.

What is the implication, and what should change now?

- Our findings implied that naringin plays an essential role in OC resistance, and indicates that naringin can block autophagy to reverse DDP resistance in OC cells with TGF-β2/smad2-related factors. Combining naringin with DDP may serve as an effective adjutant therapy for OC.

Methods

Cells and reagents

Human OC cells (SKOV3 and A2780) were purchased from the Chinese Academy of Sciences (Beijing, China). Human OC DDP-resistant cell line (SKOV3-DDP and A2780-DDP) was purchased from Jinyuan Biotechnology (Shanghai, China). Roswell Park Memorial Institute (RPMI) 1640 culture-medium was purchased from Thermo Fisher Scientific (cat. No.11875119, Gibco, NY, USA). Fetal bovine serum (FBS) was also purchased from Thermo Fisher Scientific (cat. No.10100, Gibco). Penicillin Streptomycin Liquid was obtained from Beijing Solaibo Technology Co., LTD. (cat. No. P1400, Beijing, China). Naringin (cat. No. HY-N0153), DDP (cat. No. HY-17394) and SRI-011381 (cat. No. HY-100347) were respectively purchased from MedChemExpress (New Jersey, USA). Rapamycin (cat. No. 553210) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies of transforming growth factor-β2 (TGF-β2) (ab238249), phosphorylation SMAD family member 2 (p-smad2) (ab280888), SMAD family member 2 (smad2) (ab40855), Sequestosome 1 (SQSTM1/p62, hereinafter referred to as p62) (ab56416) and horseradish peroxidase (HRP) conjugated secondary (ab6721, 1b6789) were obtained from abcam (Cambridge, UK). The antibody of microtubule-associated protein 1 light chain 3 (LC3) (cat. No. 4108S) was purchased from Cell Signaling Technology (Boston, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT (Sigma, St. Louis, MO, USA) was dissolved into a 5 mg/mL solution using phosphate buffered saline (PBS) and stored at 4 ℃ for use. The cell count was adjusted to a concentration of 5×10^4/mL, seeded in a 96-well plate with 100 µL per well, and treated with DDP (0, 1, 2, 4, 8, 16, or 32 µg/mL) for 48 h in cytoxicity assays. In addition, cells were also treated with 18 µg/mL DDP, 20 µmol/L naringin (11) and 10 nmol/L rapamycin for 48 h. Then, 10 µL of MTT solution (5 mg/mL, 0.5% MTT) was added to each well for a continued culture for 4 h. The cell viability was measured at 490 nm by enzyme-labeled assay, and the half maximal inhibitory concentration (IC50) value of DDP was calculated by fitting the equation.

Apoptosis assay

The Alexa fluor 488 Annexin V/dead cell apoptosis Kit (Thermo Fisher Scientific) was used for performing flow cytometry. To analyze the apoptosis of OC cells, samples were fixed using 70% ethanol at 4 ℃ for 4 h and incubated with Annexin V-fluorescein isothiocyanate (FITC)-propidium iodide (PI) for 5 min in the dark. The apoptosis level was evaluated by flow cytometry (Becon Dickinson FACS Calibur, NY, USA).

Immunofluorescence assay

Cells were added onto EZ slides (Millipore, Boston, MA, USA) and treated with 4% paraformaldehyde (Sigma) for 0.5 h. Subsequently, the cells were permeabilized with 0.5% Triton X-100 (Sigma). The samples were incubated with LC3 antibody (1:1,000) overnight at 4 ℃, washed three times with phosphate buffer for 5 min each time, and incubated with the secondary antibody (1:200) at room temperature for 2 h in the dark. Nuclei were stained with 4',6-diamidino-2'-phenylindole (DAPI) for 5 min at room temperature, and BX53 Fluorescence Microscope was used for imaging (Olympus, Tokyo, Japan).

Western blot

Total protein was extracted with radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein isolation. The isolated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA), and first incubated with antibodies p62 (1:1,000), LC3 (1:1,000), TGF-β2 (1:1,000), smad2 (1:1,000), p-smad2 (1:1,000) and GAPDH (1:1,000) overnight at 4 ℃. Following three times of washing with phosphate buffer (5 min each time), the membrane was then incubated with the secondary antibodies (1:5,000) at room temperature in the dark for 2 h. Immunoblots were visualized using an enhanced chemiluminescence kit. ImageJ software was used to analyze the protein expression gray value.

Xenograft model of OC cells

To establish a tumor model of OC, SKOV3-DDP cells (1×10^6 cells) were subcutaneously implanted into 18 BALB/c nude mice [Hangzhou Medical College Laboratory Animal Center, China. Production License: SCXK(Zhe)2019-0002; License number: SYXK(Zhe)2019-0011]. The mice were grouped using a random number table method (n=6). After the tumor volume reached over 50 mm^3, the model mice are intraperitoneally injected with 2 mg/kg naringin and/or 3 mg/kg DDP every day for 10 consecutive days. Tumor volume was evaluated with slide calipers (0.5 × length × width × height) at 5-day intervals up to day 20. Later, the mice were euthanized, and samples were collected, photographed, and weighed. The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Center of Laboratory Animals (ZJCLA) (Approval No. ZJCLA-IACUC-20040156), in compliance with institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration.

Statistical analysis

The data were processed with GraphPad Prism software (version 8.0) and analyzed as mean ± standard deviation (SD). One-way analysis of variance with Bonferroni correction post-hoc was performed for the comparison of multiple independent variables. Each experiment was repeated at least three times. This study followed the principle of blind method, and neither the experimental operator nor the data analyst knew the specific experimental grouping. P values <0.05 were considered statistically significant.

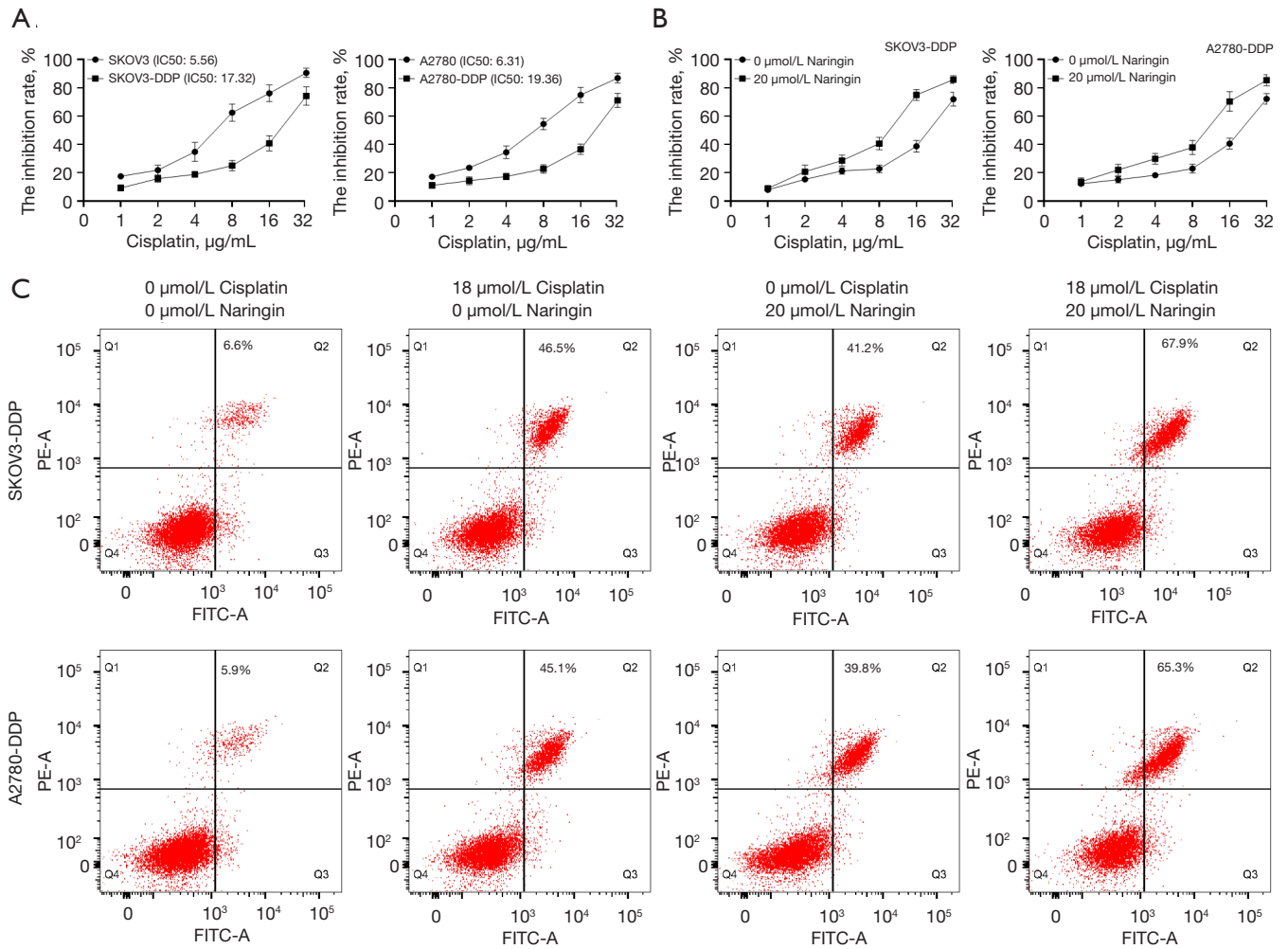

Figure 1 Naringin changed drug sensitivity of DDP OC cell lines. (A) MTT was selected to measure the activity of SKOV3, A2780, SKOV3-DDP and A2780-DDP to cisplatin. (B) MTT was selected to measure the cisplatin sensitivity of drug resistant cell lines treated with or without naringin. (C) Apoptosis level was evaluated in different treatment groups. Each experiment was repeated at least three times. SKOV3, human ovarian cancer cell; IC50, half maximal inhibitory concentration; SKOV3-DDP, human ovarian cancer cisplatin-resistant cell line; DDP, cisplatin; A2780, human ovarian cancer cell; A2780-DDP, human ovarian cancer cisplatin-resistant cell line; FITC-A, fluorescein isothiocyanate-protein A; PE-A, phycoerythrin-area; OC, ovarian cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Results

Naringin inhibited the drug resistance of SKOV3-DDP and A2780-DDP cell lines

To determine the sensitivity and resistance of DDP resistant cells to DDP, SKOV3/SKOV3-DDP and A2780/A2780-DDP cells were treated with different doses of DDP. SKOV3-DPP (IC50 =17.32 μg/mL) and A2780-DDP (IC50 =19.36 μg/mL) cells were shown to have higher IC50 values than SKOV3 (IC50 =5.56 μg/mL) and A2780 (IC50 =6.31 μg/mL) cells (*Figure 1A*). Further, the OC resistant cell lines were treated with naringin. Compared with the untreated OC resistant cell lines, naringin-treated cell lines were more sensitive to DDP (*Figure 1B*). Since the IC50 value of both OC cells was close to 18 μg/mL, 18 μg/mL DDP was defined for cell treatment in subsequent experiments. In addition, we observed that DDP and naringin individually increased the apoptosis level of both OC cells. Moreover, the combination of DDP and naringin showed a more significant promoting effect on apoptosis (*Figure 1C*).

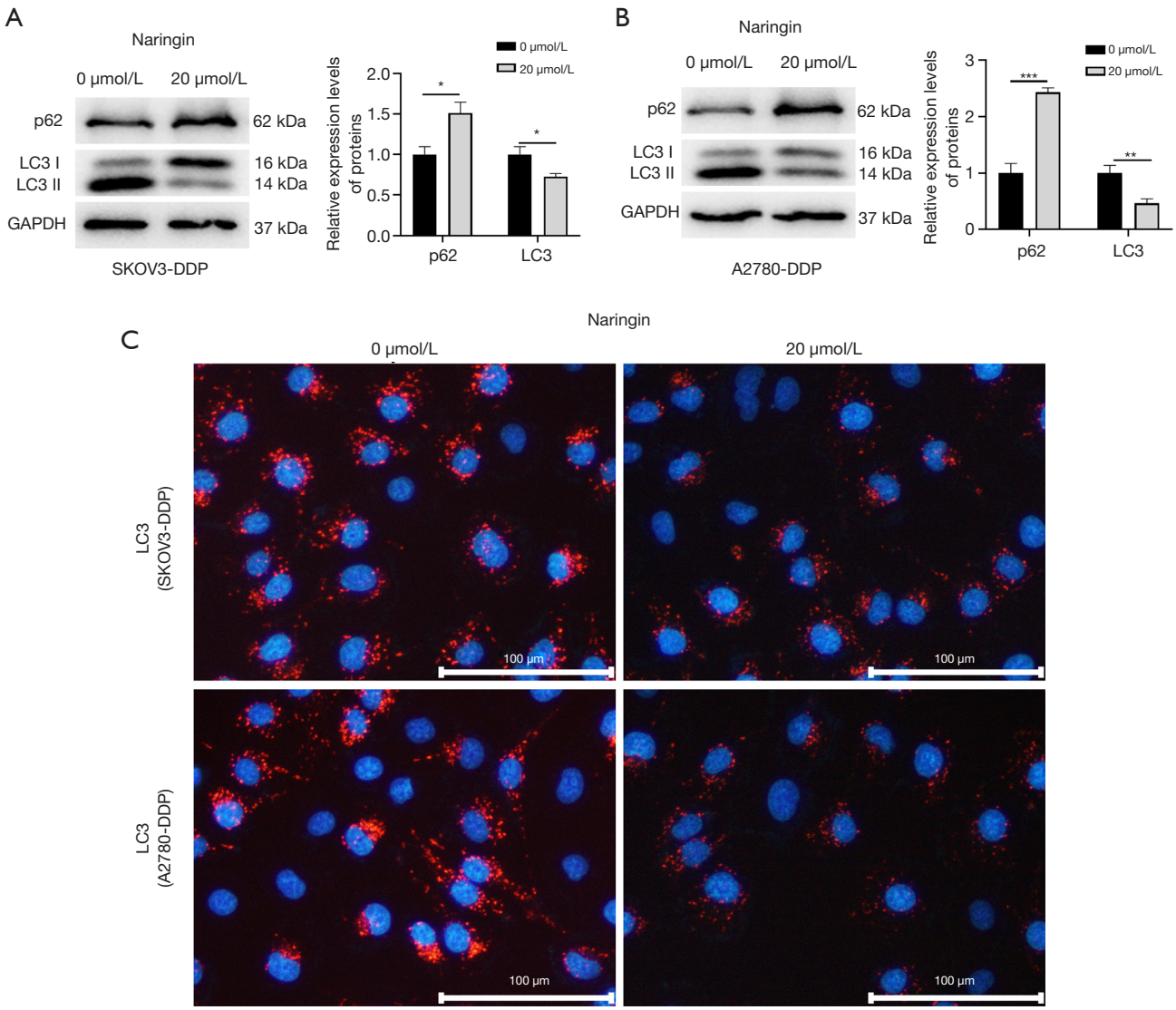

Figure 2 Naringin regulated the autophagy of DDP cells. (A) LC3 and p62 protein levels were evaluated in cells treated with or without naringin (SKOV3-DDP). (B) LC3 and p62 protein levels were evaluated in cells treated with or without naringin (A2780-DDP). (C) Immunofluorescence assay was used for evaluation of LC3 in different cells, with LC3 in pink and DAPI in blue. Each experiment was repeated at least three times. *, P<0.05; **, P<0.01; ***, P<0.001. SKOV3-DDP, human ovarian cancer cisplatin-resistant cell line; DDP, cisplatin; p62(SQSTM1), sequestosome 1; LC3, microtubule-associated protein 1 light chain 3; A2780-DDP, human ovarian cancer cisplatin-resistant cell line; DAPI, 4',6-diamidino-2'-phenylindole.

These results suggest that naringin can enhance the sensitivity of OC resistant cells to DDP, and the efficacy can be enhanced when used in combination with DDP.

Naringin inhibited autophagy in OC resistant cell lines

When SKOV3-DDP and A2780-DDP cells were treated with naringin, the level of p62 was upregulated while the level of LC3 was inhibited (*Figure 2A,2B*). Immunofluorescence assay further confirmed that naringin could reduce the proportion of LC3 positive cells (*Figure 2C*). Subsequent studies further clarified whether naringin regulates DDP resistance in OC by inhibiting autophagy.

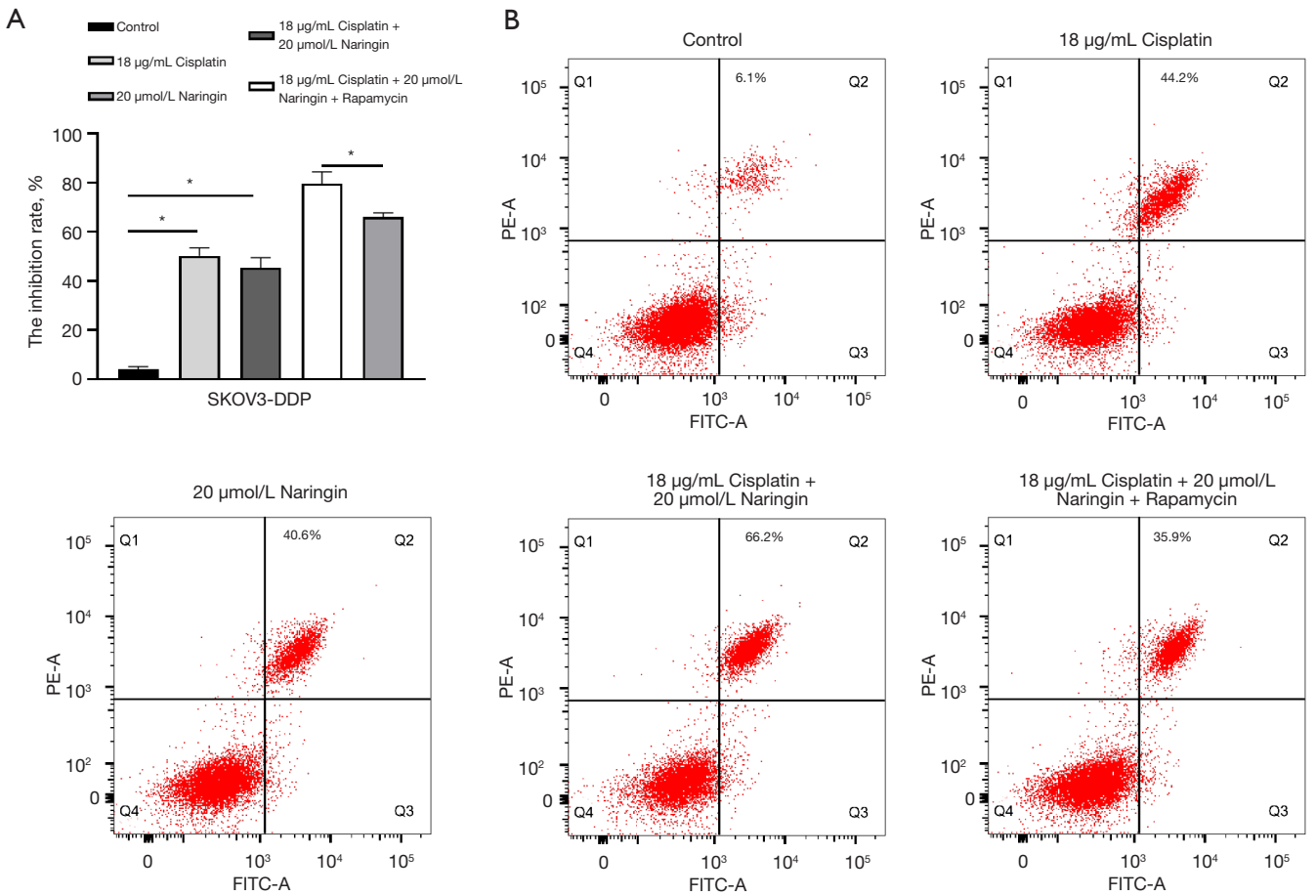

Figure 3 Naringin down-regulated cisplatin resistance by inhibiting autophagy in OC cell lines. (A) MTT was selected to measure the inhibition rate of cisplatin, naringin and rapamycin to SKOV3-DDP cells. (B) The apoptosis rate of different treatments was evaluated. Each experiment was repeated at least three times. *, P<0.05. SKOV3-DDP, human ovarian cancer cisplatin-resistant cell line; DDP, cisplatin; FITC-A, fluorescein isothiocyanate-protein A; PE-A, phycoerythrin-area; OC, ovarian cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Naringin inhibited the autophagy of OC cell lines to inhibit DDP resistance

To confirm whether naringin regulates DDP resistance in OC by inhibiting autophagy, the autophagy agonist, rapamycin, was used. Compared to the use of naringin or DDP alone, the combination of naringin and DDP enhanced the inhibition of SKOV3-DDP cells by DDP, while rapamycin reversed this effect (*Figure 3A*). Flow cytometry results also revealed higher levels of apoptosis following combined treatment with DDP and naringin as compared to treatment with DDP alone (*Figure 3B*). The cell apoptosis in the combined treatment group was inhibited by rapamycin (*Figure 3B*).

Naringin regulated the activity of OC cell autophagy via inhibiting the TGF-β2/smad2 pathway

Next, the effect of naringin on the TGF-β2/smad2 signal axis was further investigated. The level of TGF-β2 and p-smad2 in SKOV3-DDP cell lines was inhibited by naringin (*Figure 4A*). The TGF-β agonist, SRI-011381, promoted the level of TGF-β2/smad2-related factors (*Figure 4A*). In order to clarify whether the autophagy of OC resistant cell lines would be inhibited after the TGF-β2/smad2 signaling axis was blocked, Western blot and immunofluorescence assay were used. The results showed that the level of LC3 in SKOV3-DDP cells was downregulated by naringin, and such inhibitory effect was

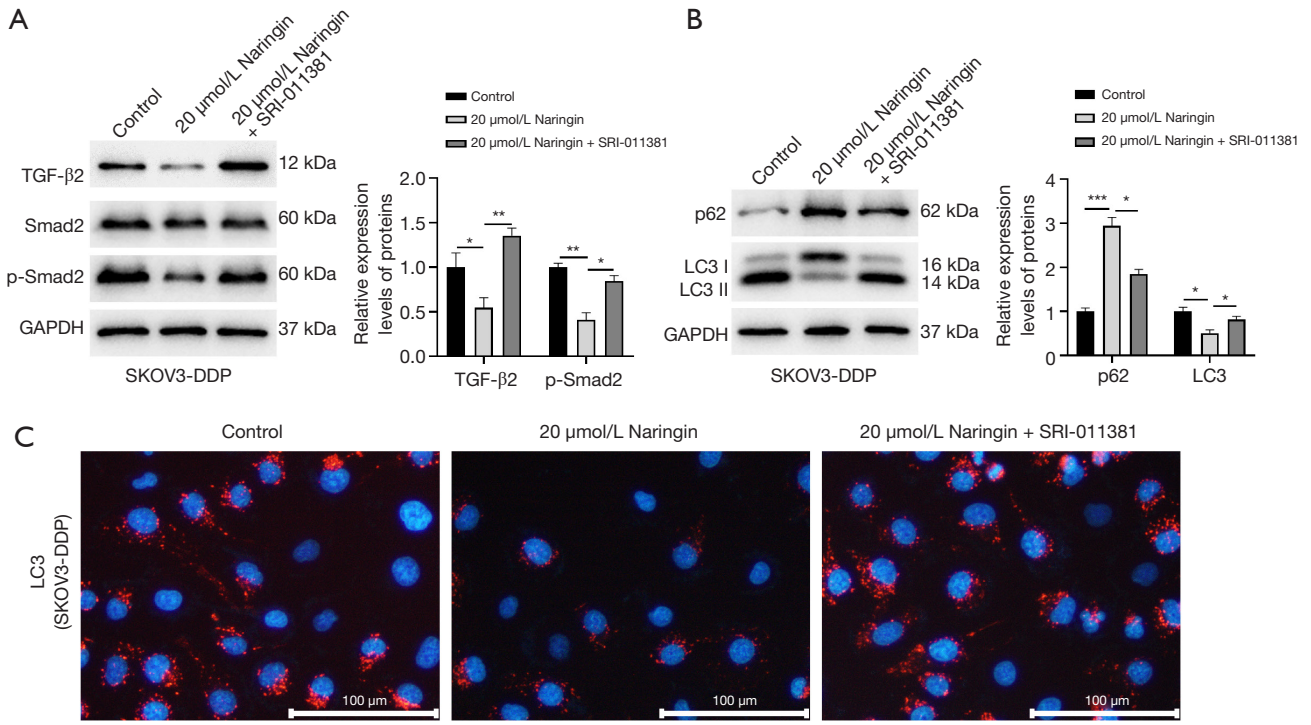

Figure 4 Naringin blocked the TGF-β2/smad2 related proteins to regulate autophagy of OC cells. (A) TGF-β2, p-smad2 and smad2 levels were evaluated. (B) p62 and LC3 levels were tested. (C) LC3 puncta in different groups of cells were detected with immunofluorescence assay, with LC3 in pink and DAPI in blue. Each experiment was repeated at least three times. *, P<0.05; **, P<0.01; ***, P<0.001. SKOV3-DDP, human ovarian cancer cisplatin-resistant cell line; DDP, cisplatin; TGF-β2, transforming growth factor-β2; smad2, SMAD family member 2; p-smad2, phosphorylation SMAD family member 2; p62(SQSTM1), sequestosome 1; LC3, microtubule-associated protein 1 light chain 3; OC, ovarian cancer; DAPI, 4',6-diamidino-2'-phenylindole.

reversed after treatment with SRI-011381 (*Figure 4B*,*4C*). p62 level was higher in naringin-treated cells. Compared with naringin-treated cells, cells after the combined treatment of SRI-011381 and naringin showed lower expression of p62 (*Figure 4B*).

In vivo verification of the inhibition of drug resistance and autophagy in OC by naringin

The conclusion drawn from *in vitro* experiments that naringin could regulate autophagy and reverse DDP resistance in OC resistant cell lines was further verified *in vivo*. The SKOV3-DDP cell line was used for construction of a mouse tumor xenograft model. After the tumor model was successfully established, the model mice were treated with DDP. There were no significant changes in the tumor volume and weight after DDP treatment (*Figure 5A*,*5B*), suggesting that the mice were resistant to DDP. When being treated with naringin and DDP, the tumor volume and weight decreased significantly (*Figure 5A*,*5B*). Protein levels revealed naringin blocked autophagy in the tumor tissues of mice (*Figure 5C*). The levels of TGF-β2 and p-smad2 in the tumor tissues also decreased significantly (*Figure 5D*).

Discussion

Surgery combined with DDP is a common treatment option for patients with advanced OC. However, some OC patients may develop resistance to DDP during treatment, leading to tumor recurrence or even death (18). In the studies of DDP resistance in OC cells, many TCMs, including naringin, have exhibited remarkable properties in reversing DDP resistance (19,20). Compared with other chemotherapy drugs, TCMs has lower toxicity and is more suitable. At the same time, due to the complexity of components in TCMs, the regulatory mechanism of DDP resistance in OC cells deserve attention. In the present study, naringin enhanced

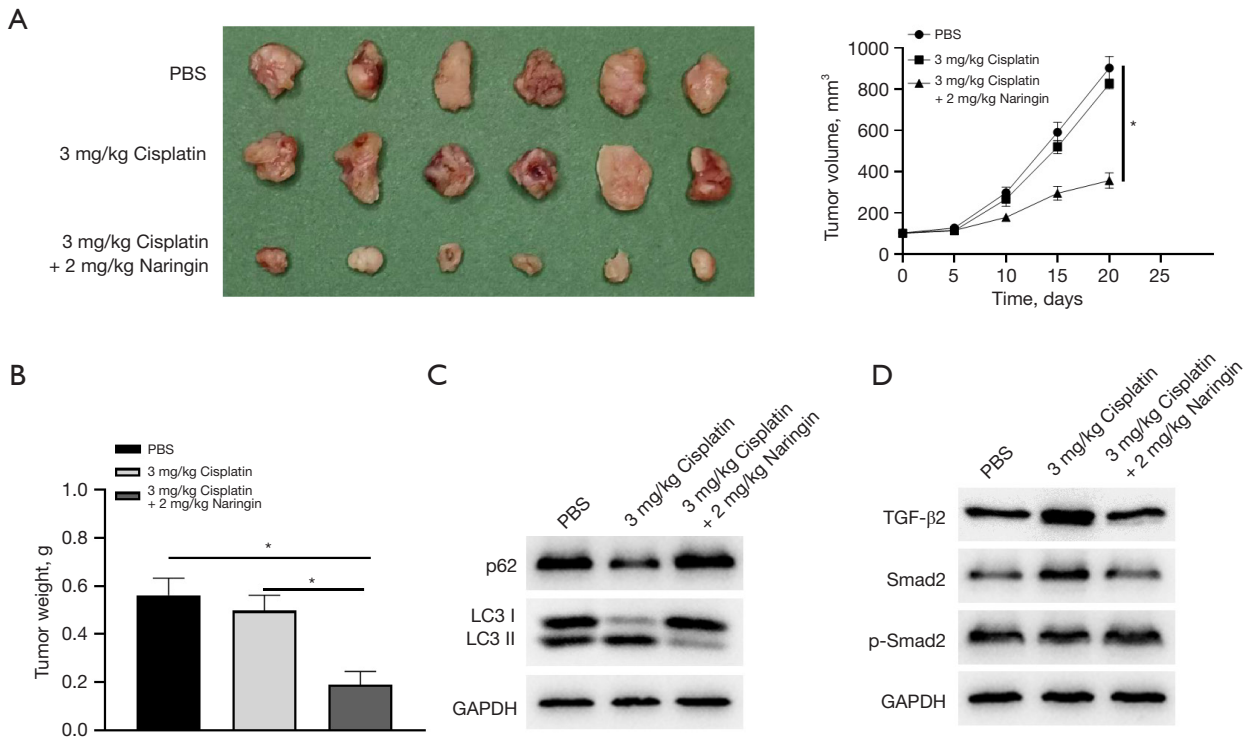

Figure 5 *In vivo* verification of the inhibition of drug resistance and autophagy in OC by naringin. (A) The tumor volume was evaluated with slide calipers. (B) The weight of tumor after cisplatin and naringin treatment. (C) The levels of p62 and LC3 in different groups of tumor tissues. (D) The levels of TGF-β2, p-smad2 and smad2. Each experiment was repeated at least three times. *, P<0.05. PBS, phosphate buffered saline; p62(SQSTM1), sequestosome 1; LC3, microtubule-associated protein 1 light chain 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF-β2, transforming growth factor-β2; smad2, SMAD family member 2; p-smad2, phosphorylation SMAD family member 2; OC, ovarian cancer.

the activity of DDP resistant cells to DDP. Naringin-treated OC cells were less resistant to DDP. Combined treatment with naringin and DDP resulted in higher levels of apoptosis of DDP resistant cells. Further, our results demonstrated that naringin can regulate the DDP activity of OC cells via blocking autophagy activity.

TCMs are often used in combination with chemotherapy agents in the treatment of OC by virtue of their low toxicity. Moreover, there are diverse molecular mechanisms of DDP-based combination therapy for OC patients. Some researchers found that bitter melon may enhance the DDP activity in OC cells by inducing ferroptosis and activating the AMP-activated protein kinase (AMPK) signaling pathway (21). Shikonin was found to enhance DDP sensitivity by inducing ferroptosis and reactive oxygen species (ROS) activity in OC cells (22). In the present study, naringin was found to inhibit DDP resistance in OC. Previous studies on the relationship between naringin and DDP in OC have rarely suggested that naringin may reverse DDP resistance by regulating factor-kB or Wnt/β-catenin pathways (11,19). This study investigated whether the change of DDP resistance in OC is caused by autophagy.

Autophagy is a lysosome-mediated degradation in all eukaryotic cells (23). Autophagy plays completely different roles at different stages of cancer development. There is increasing evidence that autophagy has complex effects on cancer (24,25). It has been found that the chemoresistance of OC cells to certain chemotherapy drugs is caused by inducing autophagy. Paclitaxel resistance could be promoted via inducing autophagy (26). DDP resistance was also related to autophagy in OC (27). According to the results of this study, the autophagy agonist, rapamycin, could promote drug resistance in SKOV3-DDP cells, and naringin may also inhibit OC resistance by inhibiting autophagy. The naringin-treated OC cells showed significantly increased p62 expression and reduced LC3 expression. Previous

studies have focused on the influence of naringin on autophagy (28,29). Naringin can also inhibit autophagy via phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) (16). The aforementioned research all reveals that naringin can block the autophagy of OC cells and thus inhibit DDP resistance.

The specific molecular mechanism of naringin in regulating autophagy in OC cells was further investigated. TGF-β could induce autophagy via the smad in pancreatic cancer (30). TGF-β/smad2 also activates autophagy in hepatocellular carcinoma (31). Some TCMs have also been found to regulate autophagy by blocking the TGF-β/smad related factors. Curcumin regulates oxidative stress and autophagy via inhibiting TGF-β/smad related factors (32). Quercetin reduces autophagy via TGF-β/smads signaling (33). Additionally, naringin is considered to regulate the TGF-β/smad axis (34). In our study, we identified that naringin could also inhibit TGF-β2/smad2 signal transduction. In addition, TGF-β pathway agonists also reversed the inhibition of autophagy by naringin in SKOV3-DDP cells. *In vivo* experimental results also revealed that naringin could inhibit the TGF-β2/smad2 signal transduction and autophagy-related factors in OC tumor tissues. These results strengthened the evidence that naringin could inhibit autophagy via the TGF-β2/smad2 signaling pathway.

Conclusions

In conclusion, the current research demonstrates that naringin plays an essential role in OC resistance, and indicates that naringin can block autophagy to reverse DDP resistance in OC cells with TGF-β2/smad2-related factors. It is worth noting the certain limitations of this study. Firstly, although the effect of naringin on the DDP resistance in OC was investigated, the specific therapeutic effect of naringin on OC patients and the possible adverse effects of naringin remain to be studied clinically. Secondly, naringin was revealed to regulate DDP resistance in OC through autophagy, and more possible molecular mechanisms including ferroptosis also need to be investigated.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the MDAR and ARRIVE reporting checklists. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2156/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2156/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2156/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Center of Laboratory Animals (ZJCLA) (Approval No. ZJCLA-IACUC-20040156), in compliance with institutional guidelines for the care and use of animals.

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