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Original Research

Icariin inhibits autophagy and promotes apoptosis in SKVCR cells through mTOR signal pathway

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Abstract: Autophagy is a conserved biological process, which is regulated by mTOR pathway and is reported to be a self-protective process of cancer cells to counteract apoptosis. Icariin is an active flavonoid that is reported to inhibit autophagy. In this study, we investigated whether Icariin could induce a reduction of cell proliferation by inhibiting autophagy. SKVCR cells, which are resistant to vincristine, were used for the investigation. We used CCK8 test and flow cytometry assay to study the effects of Icariin on cell proliferation, cell apoptosis and cell circle. We performed transmission electron microscope (TEM), immunohistochemical assay and western blotting assay to study the level of autophagy after Icariin treatment. Finally, we investigated whether the mTOR pathway is a target of Icariin by using mTOR inhibitor rapamycin and detected autophagy and apoptosis via flow cytometry assay, TEM, immunohistochemical assay and western blotting assay. Decreased proliferation and increased apoptosis was observed after Icariin treatment in SKVCR cells, together with decreased level of autophagy. Application of rapamycin could reverse the anti-autophagic and pro- apoptotic effect of Icariin. Icariin can inhibit autophagy and promote apoptosis in SKVCR cells by activating mTOR signal pathway. Icariin attenuates tumorigenesis by inhibiting autophagy and inducing apoptosis.

Key words: Icariin; Autophagy; Apoptosis; SKVCR cell-lines; Multidrug resistance.

Introduction

Ovarian cancer is a kind of gynecological tumor. Radical resection followed by chemotherapy is the primary treatment in advanced ovarian cancer (1). Thus, the reactivity of cancer cells to cytotoxic drugs is a major determinant of the prognosis in patients with ovarian cancer, while multidrug resistance (MDR) is a critical risk factor for treatment failure (1, 2). The development of MDR depends on various complex processes, such as apoptosis, autophagy, DNA repair (1, 3-6).

Autophagy is a highly conserved biological process that maintains cellular viability by ensuring the degradation and recycling of cytosolic or aggregated proteins and defective organelles via lysosomal degradation pathway (7, 8). Autophagy can be triggered by internal and external stress such as cytotoxic drugs, irradiation, starvation, hypoxia or viral infection (9-11). Autophagy is reported to involve multiple steps in the development of cancer, such as cell motility (12, 13), cell invasion(14-16), cell viability, differentiation of cancer stem cell (17, 18), anoikis resistance (19, 20), transition of epithelial-to-mesenchymal cell (21, 22) and dormancy in tumor cells (23).

Apoptosis is a mechanism of programmed cell death, through which ectopic, aged, damaged or mutated cells are eliminated (24). Cross-talk between various intracellular and/or extracellular cell signals pathways regulate the balance between autophagy and apoptosis, which determines the cell fate (24, 25). Beclin-1 that binds to the Bcl-2 acts as an inhibitor of both autophagy and apoptosis. Studies have revealed the critical role of apoptosis in the development and maintenance of cell homeostasis (26, 27). Apoptosis and autophagy contribute to the sensitivity and resistance of tumors to various therapies (28). A further understanding of the mechanisms of interaction between autophagy and apoptosis would be valuable for the understanding of cancer biology.

mTOR is a conservative serine/threonine kinase, which comprises of two subunits, mTORC1 and mTORC2, and is involved in cell differentiation, proliferation, cycle regulation, motility and invasion (29, 30). mTORC1, and not mTORC2, is sensitive to stress and inhibited by rapamycin (31, 32). Various studies have showed an important role of mTORC1 in autophagy, and activation of mTOR would inhibit autophagy (33, 34). In tissues, with sufficient nutrient supplement, mTORC1 suppresses autophagy via ULK1 pathway. In tumor tissues, with glucose or amino acid insufficiency, mTOC1 activity is inhibited by AMPK, which results in the promotion of autophagy (33).

For cells with high-energy metabolism, such as tumor cells or those with insufficient nutrients, autophagy serves as a critical source of amino acids, nucleotides and fatty acids (28, 31, 33). Studies have indicated that autophagy induces self-protectivity in cancer cells to counteract apoptosis, and autophagy may play an important role in the treatment responsiveness to chemotherapy (1, 4). Thus, inhibition of autophagy may be a potential therapeutic target in cancer. SKVCR cells are a subset of parental ovarian cancer cell SKOV3 with resistance to vincristine, which is widely used to study the mechanism of MDR (35, 36). Icariin is an active

flavonoid, which is extracted from of *Epimedium brevicornum Maxim* (37-39). Icariin was reported to inhibit autophagy via Bcl-2-dependent pathway (37). Liu et al. has reported that Icariin shows anti-cancer activity in multidrug resistance cancer cells (40). In this study, we used SKVCR cell-line to determine whether Icariin inhibits autophagy via mTOR signal pathway to promote apoptosis in SKVCR cell-lines.

Materials and Methods

Reagents and antibodies

Icariin was purchased from Sigma. (St. Louis, MO, U.S.A.). Icariin was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, U.S.A.) to a stock solution at a concentration of 400 μg/mL in -20˚C. Stock solution was diluted with Earle's balanced salt solution or MEM medium to a final concentration of 40 μg/mL or 80 μg/mL. DMSO was added in the vehicle control group. a-MEM medium was prepared according to studies previously reported [14]. Apoptosis detection kit (V13242), including Propidium iodide and Annexin V-FITC were obtained from Invitrogen. DAPI (AR1177) was purchased from Boster (Wuhan, China) primary antibodies, including caspase 3 (#9668S), LC3IB (#2775S), beclin 1 (#4122S), bax (#2772S), bcl-2 (#2872S) and monoclonal mouse anti-GAPDH antibody (#51332S) was obtained from Santa Cruz. Horseradish peroxidase-(HRP)-conjugated polyclonal goat anti-mouse and anti-rabbit (Santa Cruz, CA, USA) antibodies were used as the secondary antibodies in this study.

Cell culture

SKVCR cell-line (multidrug-resistant phenotype) was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in a-MEM (10% fetal bovine serum, 100 U/mL of penicillin/streptomycin) at 37 ˚C, 5% CO_2. a-MEM medium containing 2.0 mg/mL icariin were used to maintain the drug-resistant phenotype of SKVCR.

Electron microscopy

Electron microscopy was performed according to the previous study (41). After treatment, 2% glutaraldehyde in 0.1 M Na-phosphate buffer (PB) at pH 7.4 was used to fix SKVCR cells for one hour. The cells were washed in phosphate buffer three times and 1% OsO_4 in 0.1 M cacodylate buffer at pH 7.4 were used for post fixation for one hour. The cells were washed in distilled water and incubated with 50% ethanol for 10 min and block stained with 2% uranyl acetate in 70% ethanol for two hours. The cells were further dehydrated in ethanol and embedded in epoxy resin. Ultrathin sections were doubly stained with uranyl acetate and lead citrate and observed under an H7000 electron microscope (Hitachi, Tokyo, Japan). The features of double- or multiple-membrane structures surrounding the undigested cytoplasmic constituents and single membrane structures containing cytoplasmic components, respectively identified Autophagosomes and autolysosomes.

Western blotting

SKVCR cells were washed by phosphate buffered saline (PBS) and lysed in radio immunoprecipitation assay lysis buffer (150 mM NaCl, 1% (v/v) NP-40, 20 mM Tris HCl, 1% (v/v) Triton X-100, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.1% (w/v) SDS, protease inhibitor phosphatase inhibitor). Whole-cell lysates were centrifuged at 12,000 rpm for 10 min. BCA protein assay kit was used for protein quantification. Electrophoresis was performed in 10–12% SDS polyacrylamide gel, and the protein were transferred onto a PVDF membrane (Bio-Rad, U.S.A.) at 90 V for two hours. The membranes were incubated in 5% BSA and then incubated with primary antibodies at 4˚C, overnight. The membranes were washed in PBS, and then incubated with a secondary antibody at 25˚C for one hour. Image-Pro Plus 6.0 quantified the specific protein band intensities.

Cell cycle assay

SKVCR cells were disposed as previously described. Seventy-two hours later, the cells were collected and washed in PBS and were fixed with 100% ethanol. The fixed cells were washed with PBS twice. The cells were stained with solution containing 100 μg/mL PI and 50 μg/mL RNase (Sigma, USA) at 37˚C for 20 min in the dark. Cell clumps were removed using a nylon mesh sieve, and the stained cells were analyzed on a flow cytometry (BD). Data was analyzed using the CELL Quest and matched Mod Fit LT software.

Cell Proliferation and apoptosis assays

To analyze cell proliferation, cells were cultured in a 96-well plate in a-MEM medium which had 2.0 mg/ml icariin for 72 hours. Cell proliferation were detected by the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay kit (Roche, USA) according to manufacturer's instructions.

To analyze an apoptosis detecting kit was used to measure cell apoptosis in cells pre-labelled with PI and Annexin V–FITC (Invitrogen, Burlington, Canada) according to manufacturer's instructions. Samples were analyzed by a flow cytometry and the results were recorded by matched Cell Quest software (Becton Dickinson, San Jose, CA, USA).

Immunohistochemical analysis

The cells were fixed and processed for paraffin-embedded slides. After antigen retrieval, 3% hydrogen peroxide was used to inactivate endogenous peroxidase, and then the sections were blocked with 1% BSA. The sections were incubated with primary antibodies. The slides were washed with PBS and incubated with biotinylated–labeled secondary antibody, and then conjugated with HRP-labeled streptavidin (Dako, Glostrup, Denmark). Substrate diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) were used as the chromogen. Photographs were taken using BioRad Lasersharp MRC500 scanning confocal microscopy system.

Statistics analysis

All the statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 20.0 software. The data is presented as the mean ± SEM. Paired or unpaired Student's *t*-test was used in cases of standardized expression data. Statistically significance was considered at $P \leq 0.05$.

Results

A. Cell proliferation of SKVOR cell by CKK8 assay in the first three days in control group, 40 μg/mL group and 80 μg/mL group; B. Cell cycle profile of SKVOR cell assessed by flow cytometry using PI staining to determine the DNA content in control group, 40 μg/mL group and 80 μg/mL group; C. Cell apoptosis of SKVOR cell by flow cytometry in control group, 40 μg/mL group and 80 μg/mL group.

Role of Icariin in SKVCR cells apoptosis and proliferation

CCK8 test and flow cytometry assay were performed to study the effects of Icariin on the apoptosis and proliferation of SKVCR cells. In CCK8 test, a significant decreased cell proliferation was observed in 80 μg/mL (OD_{450}: 0.436 ± 0.055) and 40 μg/mL groups (OD_{450}: 0.664 ± 0.05) compared with the control group (OD_{450}: 1.061 ± 0.104) ($P < 0.05$, Figure 1A) after 72 hours. Flow cytometry assay revealed that Icariin treatment altered cell cycle progression. As shown in Figure 1B, Icariin treatment increased cells in the G1 phase (80 μg/mL group, 71.90 %; 40 μg/mL group, 61.21 %) compared with the control group (52.95 %, Figure 1B) and showed a corresponding decrease in cell numbers in the S phase (control group, 52.95 %; 40 μg/mL group, 25.17 %; 80 μg/mL group, 13.23 %), but no change in G2 phase (control group, 18.11 %; 40 μg/mL group, 13.62 %; 80 μg/mL group, 14.87 %) (Figure 1B). Additionally, in 80 μg/mL group the percentage of late cell apoptosis (38.3%, right lower quadrant, Figure 1C) and early cell apoptosis (5.4%, right upper quadrant, Figure 1C) were markedly increased. Bcl-2, Bax and Caspase-3 are the specific hallmarks of apoptosis. Protein levels of Bax and Caspase-3 were significantly increased followed by Icariin treatment.

Bcl-2 expression was decreased as compared to the normal control cells (Figure 2C). These results indicated that Icariin treatment inhibited proliferation of SKVCR cells and induced an obvious increase in apoptosis by inducing G1/S phase arrest.

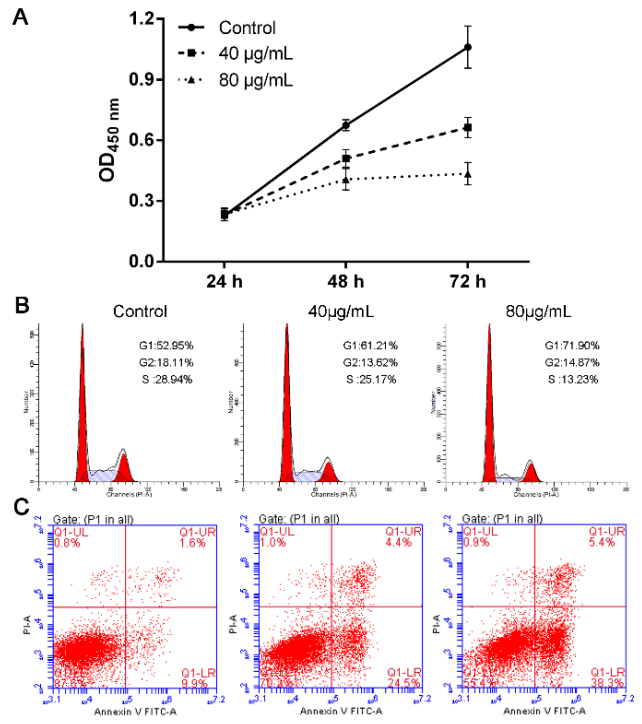

Figure 1. Icariin treatment effects SKVCR cell apoptosis and proliferation**.** A. Cell proliferation of SKVOR cell by CKK8 assay in the first three days in control group, 40 μg/mL group and 80 μg/mL group; B. Cell cycle profile of SKVOR cell assessed by flow cytometry using PI staining to determine the DNA content in control group, 40 μg/mL group and 80 μg/mL group; C. Cell apoptosis of SKVOR cell by flow cytometry in control group, 40 μg/mL group and 80 μg/mL group.

Icariin treatment inhibits autophagy in SKVCR cells.

Icariin was reported to inhibit autophagy. Thus, to determine the underlying mechanism by which Icariin treatment increases apoptosis in SKVCR cells, we detected the level of autophagy after Icariin treatment. After Icariin treatment, a significant decrease of LC3B in Icariin treatment groups was observed compared with control group, using immunofluorescence assay (Figure 2B). By using TEM, a reduction in number of autophagosome was observed in SKVCR cells followed by Icariin treatment as compared to the control group (Figure 2A). We also detected the autophagosomal marker microtubule-associated protein light chain 3 (LC3)-I, LC3 II and Beclin1 by western blotting assay. LC3 plays a critical role in autophagy. The conversion of LC3 I to LC3II is considered as a marker of the autophagosome formation. Beclin1 is involved in the initiation of the formation of the autophagasome in autophagy. In Icariin-treated SKVCR cells, a decreased conversion of LC3 I to LC3II was observed, and the protein level of Beclin1 was decreased as compared to control group

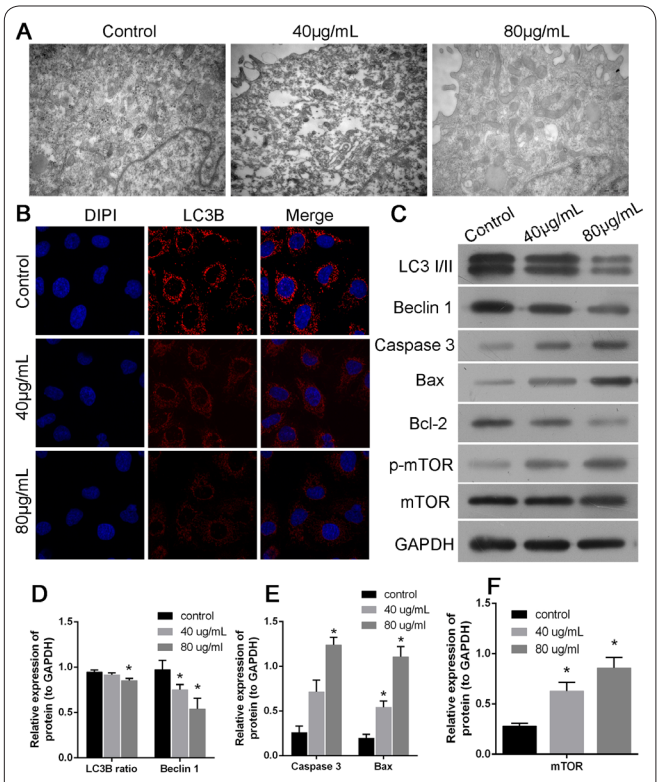

Figure 2. Icariin treatment inhibits autophagy in SKVCR cells. A. Image of autophagosomes detected by TEM; B. Expression of LC3B detected by immunofluorescence assay in control group, 40 μg/mL group, and 80 μg/mL group; C, D, E, F. Protein expression pattern of Bax, Caspase-3, Bcl-2, LCI/II, mTOR, p-mTOR and Beclin1 in control group, 40 μg/mL group and 80 μg/mL group.

cells (Figure 2C, D, E, F).

The mammalian target of rapamycin (mTOR) participates in cell growth and autophagy, and activation of mTOR terminates autophagy. In order to detect whether Icariin regulates autophagy via mTOR pathway, we examined the levels of mTOR and p-mTOR by western blotting. Elevation of p-mTOR levels was observed in Icariin treated groups as compared to the control group (Figure 2C). These results showed that Icariin treatment induced apoptosis in SKVCR cells by inhibiting autophagy, and mTOR may be target pathway for Icariin.

Icariin increased apoptosis in SKVCR cells by inhibiting autophagy and targeting mTOR pathway

To validate the hypothesis that Icariin increased SKVCR apoptosis by inhibiting autophagy and targeting mTOR pathway, we used the inhibitor of mTOR rapamycin to detect whether rapamycin (abbreviated Rapa) could reverse the effect of Icariin on autophagy and apoptosis in SKVCR cells. Flow cytometry assay revealed that in Rapa+Icariin group, the percentage of early cell apoptosis (29.0%, right lower quadrant, Figure 3A) and late cell apoptosis (4.8%, right upper quadrant, Figure 3A) were markedly decreased as compared to Icariin group (early cell apoptosis, 40.5%, right lower quadrant; late cell apoptosis, 5.9%, right upper quadrant; Figure 3A) and control group (early cell apoptosis, 6.9%, right lower quadrant; late cell apoptosis, 1.7%, right upper quadrant; Figure 3A). In Rapa + Icariin group, decreased number of cells in the G1 phase (67.51 %) was observed as compared to the Icariin group (73.70 %) and control group (50.64 %), respectively, and a corresponding decrease in cell numbers in the S phase in Rapa+Icariin group (control group, 27.16 %; Icariin group, 9.55 %; Rapa+Icariin group, 16.55 %) was observed. However, no changed in G2 phase (control group, 22.20 %; Icariin group, 16.76 %; Rapa+Icariin group, 15.95 %) (Figure 3B) was observed. In Rapa+Icariin group, protein levels of Bax and cleaved Caspase-3 were significantly decreased, and increased Bcl-2 expression was found (Figure 3C, G) as compared to Icariin group. The ratio of protein level of LC3 I/II was significantly lower in Rapa+Icariin group than that in Icariin group, and Beclin1 expression was higher in Rapa+Icariin group as compared to Icariin group (Figure 3C, F). A reduction of p-mTOR level was found in Rapa+Icariin group as compared to Icariin group (Figure 3C, H). Immunofluorescence assay revealed a significant increase of LC3B in Rapa+Icariin treatment groups as compared to Icariin group (Figure 3D). application of rapamycin reversed the Icariin treatment-induced reduction of autophagosome in SKVCR cells (Figure 3E). These results indicated that Icariin treatment induced apoptosis of SKVCR cells by inhibiting autophagy, and mTOR is a target pathway of Icariin.

Discussion

In this study, we investigated the effect of Icariin on SKVCR cells proliferation, and detected the underlying mechanism. We found that, Icariin treatment could significantly decrease SKVCR cells proliferation by increasing cell apoptosis. Icariin could inhibit autophagy in SKVCR cells, and mTOR signal pathway is a target of icariin.

For ovarian cancer, MDR in post-surgery chemotherapy is a critical problem for both patients and physicians (42, 43). Studies on the mechanism of MDR have provided some evidence that p-glycoprotein, cancer stem cell and non-coding RNA are involved in the development of MDR (44, 45). Recently, autophagy has been reported to be a novel mechanism of MDR (46, 47). Autophagy is a highly conservative cellular process, during which the cytoplasmic components are degraded in a lysosomal-dependent manner and recycled (28, 34). Autophagy can be activated when cells are exposed to chemotherapy drugs (7) and facilitate the elimination

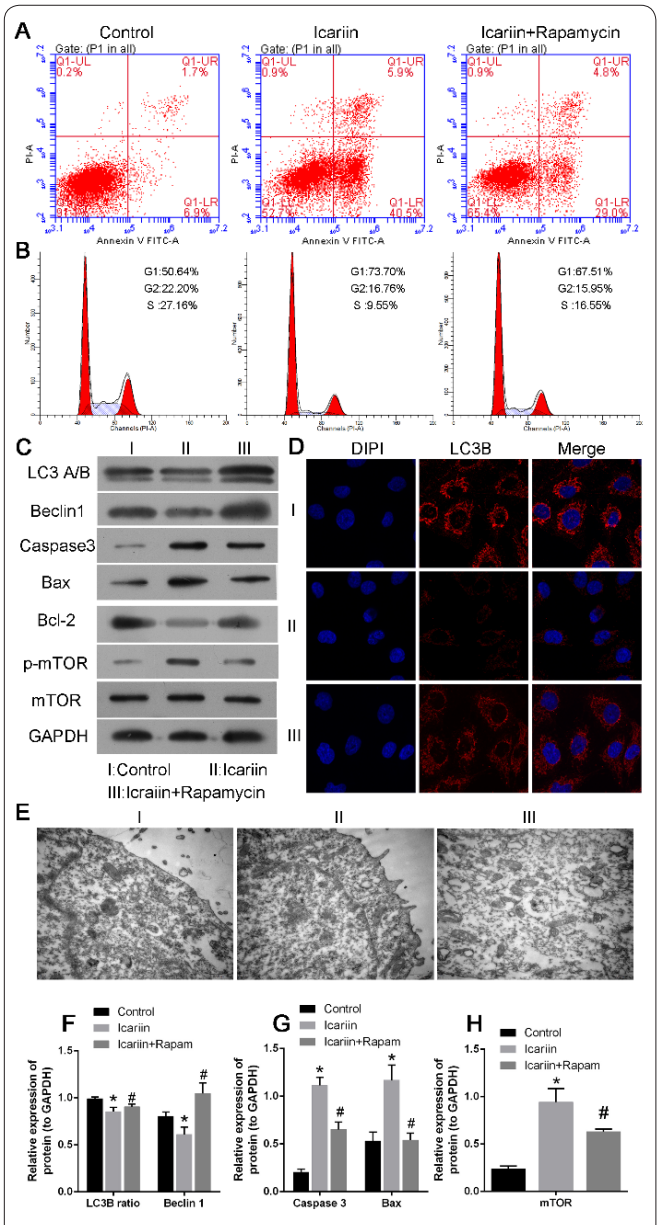

Figure 3. Icariin increased SKVCR cells apoptosis by inhibiting autophagy and targeting mTOR pathway. A. Cell apoptosis of SKVOR cell by flow cytometry in control group, Icariin group and Rapa+ Icariin group; B. Cell cycle profile of SKVOR cell assessed by flow cytometry in control group, Icariin group and Rapa+ Icariin group; C. Expression of Bax, Caspase-3, Bcl-2, LCI/II, mTOR, p-mTOR and Beclin-1 in control group, Icariin group and Rapa+ Icariin group. D. Expression of LC3B detected by immunofluorescence assay in control group, Icariin group and Rapa+ Icariin group. E, F, G, H. Images of autophagosome detected by TEM in control group, Icariin group and Rapa+ Icariin group.

of toxic components. Additionally, in cancer cells with high-energy metabolism, autophagy could provide amino acids, nucleotides and fatty acids to maintain cell survival and proliferation (7, 8, 26). Therefore, autophagy contributes to the development of MDR, and inhibition of autophagy may restore the responsiveness to cytotoxic drugs in resistant cancer cells. In our study, we found that Icariin could significantly decrease SKVCR cell proliferation by increasing cell apoptosis. Liu et al. evaluated the potential MDR reversal activity of semi-synthesized Icariin derivatives. It was reported that the inhibitory ability of Icariin is attributed to the inhibition of the P-gp efflux function and down regulation of P-gp gene expression (40). Our study is consistent with the results of Liu et al. Further investigations are required to understand the potential underlying mechanism of Icariin's anti-cancer effect.

In this study, SKVCR cells showed a decreased autophagy and increased apoptosis as well as decreased cell proliferation as compared to negative control groups following Icariin treatment. Liang et al. demonstrated that autophagy level is higher in SKVCR than SKOV3 (35, 36). Our results are in accordance with study by Liang et al.; decreased expression of Beclin-1 and ratio of LC3I/LC3II was observed. Autophagy and apoptosis are found to be two associated physiological processes. Proteins that are involved in autophagy, including Beclin-1, ATG3 and AMBRA1, have been found to interact with apoptosis-related proteins such as Bcl-2 (48, 49). Isolated Beclin-1 promotes autophagy, when binding to Bcl-2, Beclin-1 and Bcl-2, and inhibit both autophagy and apoptosis (50, 51). During nutrient starvation, Bcl-2 is phosphorylated by JNK1, and the heterodimers of Bcl-2 and Beclin-1 is severed (51). Phosphorylated Bcl-2 also interacts with Bax, which forms homodimers to promote apoptosis in order to form Bcl-2-Bax heterodimers to serve as the anti-apoptotic complex (52). Caspase-3 activated by Bax homodimers acts as an executor of apoptosis (53). In this study, it was observed that the level of anti-apoptotic proteins Bcl-2 decreased, whereas the level of pro-apoptotic proteins Bax and Caspase 3 increased following Icariin treatment. These results indicated that Icariin could inhibit cell proliferation and induce cell apoptosis by regulating autophagy.

Several studies have been performed to study the correlation between autophagy and cell cycle progression. G0, G1, S, G2 and M-phase comprise the whole cell cycle, and G0, G1, S and G2 are collectively referred to as interphase (54). In our study, Icariin treatment could inhibit autophagy and induced an increase in percentage of cells in G1 phase and decrease in number of cells in S phase of SKVCR cells, thereby suggesting that Icariin treatment regulates cell cycle by inducing G1 phase cell cycle arrest. This result provides further evidences that Icariin treatment decreased cell proliferation.

mTOR is a serine-threonine kinase, which plays an important role in cell cycle regulation, cell differentiation, cell proliferation, cell motility and invasion. mTOR pathway comprises of mTORC1 and mTORC2. mTORC1 is sensitive to alternation of energy and stress, and rapamycin is its inhibitor, while mTORC1 acts as a negative regulator of autophagy (30, 33). mTOR suppress autophagy by phosphorylating ULK1 in nutrient-rich conditions, while during limited glucose or amino acid supply, AMPK indirectly inhibit mTOC1 activity to promote autophagy (55, 56). Furthermore, phosphorylation of Beclin-1 by ULK1 activates autophagy. In this study, it was observed that Icariin treatment increases the level of p-mTOR; however, application of rapamycin reversed this effect. These results indicated that Icariin induces reduction of autophagy by promoting mTOR pathway.

Icariin treatment activated mTOR pathway and, in turn, increased the level of p-mTOR. When administered with mTOR inhibitor rapamycin, the anti-cancer effect of Icariin was revered, as demonstrated by an increase in the level of Beclin-1, ratio of LC3I/LC3II and quantity of autophagosome. Tang et al. demonstrated that Icariin facilitates PI3K/Akt-mediated mTOR/4EBP1 activation and treatment with Icariin 30 μM alone could significantly increase the expression of p-mTOR, p-p70S6K and p-4EBP1. The phosphorylation of mTOR, p70S6K and 4EBP1 by Icariin was prevented by the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (39). Li et al. also reported that Icariin inhibited autophagy potentially by suppressing the activation of AMPK/mTOR pathway (38). Increase in p-mTOR levels was observed in Icariin treatment groups as compared to control group (Figure 2C). These results indicated that mTOR is a target pathway of Icariin.

The study suggested that Icariin could inhibit autophagy and increase apoptosis in SKVCR cells, and mTOR is the potential target through which Icariin inhibit autophagy. These findings showed that Icariin may serve as a novel therapeutic approach to resolve MDR in cancer treatment by inhibiting autophagy. Future studies, however, are required to study the mechanism of Icariin-mediated inhibition of autophagy in SKVCR cells.

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Interests of conflict

The authors declare that they have no competing interests.

Authors' contribution

SYJ and HC designed and conducted the experiments; SJD and SYJ collected and analyzed the data; SYJ and HC drafted the manuscript; DYF revised the draft; SYJ provided the administrative support.

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