Fisetin enhances cisplatin sensitivity in renal cell carcinoma via the CDK6/PI3K/Akt/mTOR signaling pathway

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Abstract. Cisplatin resistance is ubiquitous among patients with renal cell carcinoma (RCC). The present study assessed the role of fisetin in regulating cisplatin sensitivity and increasing the efficacy of chemotherapy for patients with RCC. Cell Counting Kit-8 and colony formation assays were used to assess the proliferation of RCC cells after fisetin and cisplatin treatment. The mRNA expression levels of cyclin-dependent kinase (CDK)6 were evaluated using reverse transcription-quantitative PCR. The expression levels of CDK6 and key proteins of the PI3K/Akt/mTOR signaling pathway were assessed using western blotting. The present study demonstrated that fisetin inhibited the proliferation and colony-forming ability of RCC cells, and induced apoptosis and cell cycle arrest in a dose-dependent manner. Additionally, fisetin enhanced the antineoplastic effects of cisplatin, as demonstrated by the increase in proliferation inhibition and apoptosis promotion after fisetin and cisplatin combination treatment. Furthermore, fisetin regulated the PI3K/Akt/mTOR signaling pathway through CDK6 inhibition, which enhanced cisplatin sensitivity. Overexpression of CDK6 neutralized the positive effects of fisetin on the improvement of cisplatin sensitivity in RCC cells. In conclusion, fisetin may enhance the sensitivity of RCC cells to cisplatin via the CDK6/PI3K/Akt/mTOR signaling pathway.

Introduction

Renal cell carcinoma (RCC) is a common cancer in the kidney that accounts for ~3% of all cancer cases worldwide (1). Currently, surgery is the main therapeutic approach for RCC; however, ~30% of patients with RCC develop distant metastasis following surgery (2). Although cisplatin is an effective therapeutic agent for certain types of cancer, RCC often exhibits resistance to cisplatin, and chemotherapy regimens with cisplatin alone are only effective in 4-6% of patients with RCC (3). Therefore, there is a need to improve the understanding of the underlying reasons for RCC chemoresistance and to identify new insights for RCC treatment.

Recent studies have reported that flavonoids have the power to affect critical biological activities during tumorigenesis (4-7). The naturally occurring flavonoid, 3,3',4',7-tetrahydroxyflavone, usually referred to as fisetin, occurs in vegetables and fruits, including apples, persimmons, kiwis, strawberries, grapes, onions and cucumbers. Fisetin displays pharmacological properties that include anti-inflammatory and antioxidant effects, and it has been reported to hinder the cell cycle in HT-29 human colon cancer cells (8). It has also been reported to produce anti-proliferative effects on prostate cancer (9). Moreover, fisetin suppresses tumor cells by regulating genes involved in apoptosis (10). Reports have revealed that fisetin can restrain the proliferation and metastasis of RCC cells by upregulating MEK/ERK and can increase the expression of 5-hydroxymethylcytosine to impede the viability and migration of RCC stem cells (11,12). In addition, the combination of fisetin and cisplatin has been reported to notably increase apoptosis of the A2780 ovarian cancer resistant cell line (13). Fisetin has also been reported to reverse cisplatin resistance in lung adenocarcinoma cells (14). However, there is a lack of research on the effect of fisetin on cisplatin resistance in RCC.

As serine/threonine kinases, the family of cyclin-dependent kinases (CDKs) are catalytically active upon binding to their respective regulatory subunits, cyclins, to regulate several key cellular processes, including cell cycle progression and transcription. However, when the kinase is abnormally activated, disordered cell cycle regulation can lead to uncontrolled cell proliferation, leading to development of cancer. Thus, CDKs represent a potent target for inhibitory cancer drugs (15). Among them, CDK6 has been reported to target and regulate RCC in multiple studies (16-19). Additionally, fisetin has been reported to regulate the cell cycle and restrain the expression of CDKs in cancer cells (8), and influence several signaling pathways, such as the MAPK and PI3K/Akt/mTOR signaling pathways (20). Furthermore, fisetin has been reported to promote autophagy by interfering with the mTOR signaling

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pathway (21). Therefore, we hypothesized that fisetin may also display antitumor effects in RCC and facilitate cisplatin sensitivity through the CDK6/PI3K/Akt/mTOR signaling pathway.

The present study determined the inhibitory role of fisetin on the development of RCC by evaluating cell proliferation, apoptosis and cell cycle arrest. Subsequently, the enhancement of cisplatin sensitivity was assessed using fisetin and cisplatin co-treatment in RCC cells.

Materials and methods

Cell culture. Human RCC Caki-1 (BFN60700344, ATCC, Shanghai) and 786-O (cat. no. BFN60700343, ATCC, Shanghai) cell lines, derived from two Caucasian males with different stages of RCC, were selected based on our previous research (11,22). The cell lines were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Nanjing KeyGen Biotech Co., Ltd.). The cells were maintained at 37˚C in an incubator (SANYO Electric Co., Ltd.) with 5% CO_2. Fisetin, with a purity of >98%, was obtained from Nanjing Best Biotechnology Co., Ltd (cat. no. D50546; 500 mg). The cisplatin-resistant cells were established by repeated subculturing with gradual increase of Cisplatin(Best Biotechnology Co., Ltd.; D50445-1 ml; 1, 2, 4, 6, 8 and 10 μM) over 6 months, and finally 10 μM cisplatin was used in the cisplatin and fisetin + cisplatin treatment group.

Cell transfection. CDK6 cDNA was cloned into pcDNA3.0 (Invitrogen™; Thermo Fisher Scientific, Inc.). A total of $1.5x10^5$ cells/well were seeded into 24-well plates 24 h before plasmid transfection. Transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. Caki-1 cells and 786-O cells were transfected with 4.5 μg pcDNA3.1-CDK6 or pcDNA3.1-entry at 37˚C for 48 h. An empty vector (pcDNA3.1-entry) was used as the negative control. At 48 h post-transfection, reverse transcription (RT)-quantitative (q)PCR was used to detect the efficiency.

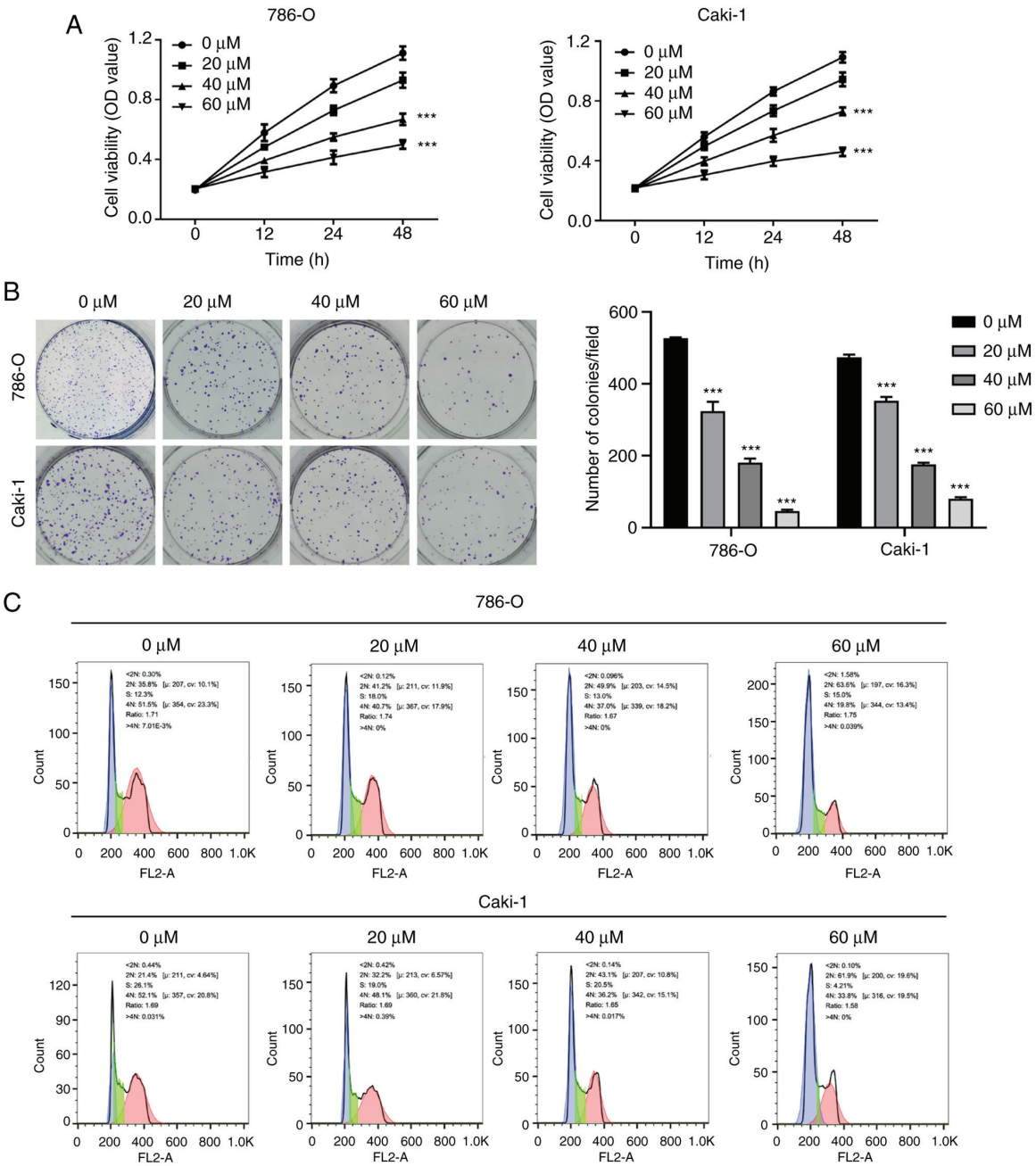
Cell Counting Kit-8 (CCK-8) assay. The CCK-8 assay kit (Beyotime Institute of Biotechnology) was used to assess RCC cell proliferation. A total of $5x10^3$ cells/well (100 μl/well) were seeded in a 96-well plate and were routinely cultured for 24 h. Subsequently, 100 μl fisetin-containing medium was added to each well to adjust the final concentration of fisetin to 20, 40 and 60 μM. These concentrations were chosen based on previous studies (23,24). After incubation for 0, 12, 24 and 48 h at 37˚C, 10 μl CCK-8 solution was added to each well and maintained for 2 h at 37˚C. Finally, the optical density values were read at 450 nm.

Colony formation assay. RCC cells were placed at a concentration of 1,000 cells/well in a 6-well plate and cultured at 37˚C. After 14 days, RCC cells were washed and fixed in 4% paraformaldehyde, followed by staining with leuco crystal violet (Wuhan Servicebio Technology Co., Ltd.). After washing and drying, the number of colonies >0.3 mm in diameter was counted by naked eye.

Cell cycle analysis. A total of $5x10^6$ cells were collected and washed with cold PBS, followed by overnight fixation with 70% ethanol at 4˚C. Cells were centrifuged at 300 x g for 10 min at 4˚C, washed with cold PBS and then the supernatant was discarded. RNase A (100 μl) was then added to resuspend the cells and maintained at 37˚C for 30 min. PI solution (400 μl) was added and thoroughly mixed with the cells, and the mixture was placed in the dark at 4˚C for 30 min. Flow cytometry (Attune NxT flow cytometer, Thermo Fisher. Scientific, Inc.; FlowJo V10.10.0, link: https://www.flowjo.com/) was then used to analyze cells in different cell cycle phases.

Western blot analysis. RIPA buffer (Nanjing Best Biotechnology Co., Ltd.) supplemented with 1% PMSF (Nanjing Best Biotechnology Co, Ltd.) was used to isolate total proteins from RCC cells. The concentration of proteins was detected using the BCA kit (Beyotime Institute of Biotechnology), and then SDS-PAGE was used to separate the proteins (50 micrograms per lane. Due to the wide range of molecular weights of proteins involved in this study, the gel concentrations were divided into the following: 6% for more than 200 kDa; 8% for 100-200 kDa; 10% for 40-60 kDa; 12% was used for 20 to 40 kDa), which were transferred to PVDF membranes (MilliporeSigma). Blocking was performed using 5% skim milk powder at room temperature for 1 h. Overnight at 4˚C, the membranes were incubated with primary antibodies against cyclin B1 (4138, Cell Signaling Technology, Inc.; dilution: 1:1,000), p21 (2947, Cell Signaling Technology, Inc.; 1:1,000), p27 (3686, Cell Signaling Technology, Inc.; dilution: 1:1,000), Bax (41162; Cell Signaling Technology, Inc.; dilution: 1:1,000), Bcl2 (sc-7382, Santa Cruz Biotechnology, Inc.; dilution: 1:800), Cleaved caspase -3 (9664, Cell Signaling Technology, Inc.; 1:1,000), Cleaved caspase -9 (20750, Cell Signaling Technology, Inc.; dilution: 1:1,000), CDK6 (sc-7961, Santa Cruz Biotechnology, Inc.; dilution: 1:800), phosphorylated (p)-Akt (sc-377556, Santa Cruz Biotechnology, Inc.; 1:800), Akt (sc-5298, Santa Cruz Biotechnology, Inc.; dilution: 1:800), p-PI3K (ab278545, Abcam; dilution: 1:200), PI3K (sc-365290, Santa Cruz Biotechnology, Inc.; dilution: 1:800), p-mTOR (sc-293133, Santa Cruz Biotechnology, Inc.; 1:800), mTOR (sc-517464; Santa Cruz Biotechnology, Inc.; dilution: 1:800) and β-actin (sc-81178, Santa Cruz Biotechnology, Inc.; dilution: 1:800). After three washes with TBST (2% Tween), membranes were hybridized with HRP-conjugated secondary antibodies (ab131368, ab99697, ab190369; all Abcam; dilution: 1:1,000) for 90 min at room temperature. Finally, protein bands were detected using the ECL Assay Kit (Shanghai Yeasen Biotechnology Co., Ltd.). Semi-quantitative analysis of WB bands was performed using ImageJ (V 1.8.0, link: https://imagej.net/software/imagej/).

Cell apoptosis measurement. RCC cell apoptosis was assessed by applying the Annexin V-FITC kit (BioLegend, Inc.). Briefly, $1x10^5$ cells were suspended in 500 μl binding buffer, then 5 μl Annexin V-FITC was added and incubated for 10 min at 4˚C. Subsequently, 5 μl PI solution was added and incubated for another 15 min at 25˚C. Flow cytometry (Attune NxT flow cytometer, Thermo Fisher. Scientific, Inc.; FlowJo V10.10.0) was then performed to count the number of apoptotic cells.



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RT-qPCR. A total of 1x10^7 cells were lysed in 1 ml TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and mixed with 0.2 ml chloroform. Subsequently, 0.5 ml isopropanol was added to the supernatant after centrifugation at 12,000 x g for 8 min at 4˚C. The mixture was centrifuged at 12,000 x g for 10 min at 4˚C. The precipitate was collected, washed with 75% ethanol and dissolved in diethyl pyrocarbonate water. cDNA was then synthesized using the 1st Strand cDNA Synthesis Kit (Shanghai Yeasen Biotechnology Co., Ltd.) according to the manufacturer's protocol. The cDNA levels were then detected using the Applied Biosystems® 7500 (Thermo Fisher Scientific, Inc.) with the qPCR SYBR Green Master Mix (Shanghai Yeasen Biotechnology Co., Ltd.). Pre-denaturation was performed at 95˚C for 30 sec. Denaturation was performed at 95˚C for 10 sec and annealing at 60˚C for 30 sec for 40 cycles. GAPDH was used as an internal reference. The primers were as follows: CDK6, forward (F) 5'-CGACTGACACTCGCA GCC-3' and reverse (R) 5'-AGTCCAGAATCATTGCACCTG AG-3' and GAPDH, F 5'-TCATTTCCTGGTATGACA ACG A-3' and R 5'-GGTCTTACTCCTTGGAGGC-3'. Gene expression was calculated using GADPH and the $2^{-\Delta\Delta Cq}$ method (25).

Statistical analysis. All of the data in the present study were analyzed using GraphPad 8(Dotmatics) and are presented as the mean ± standard deviation. To compare groups, one-way ANOVA or the Student's unpaired t test were used. Tukey's HSD (Honestly Significant Difference) test was used for post hoc testing. $P<0.05$ was considered to indicate a statistically significant difference. Each individual experiment was performed in triplicate.

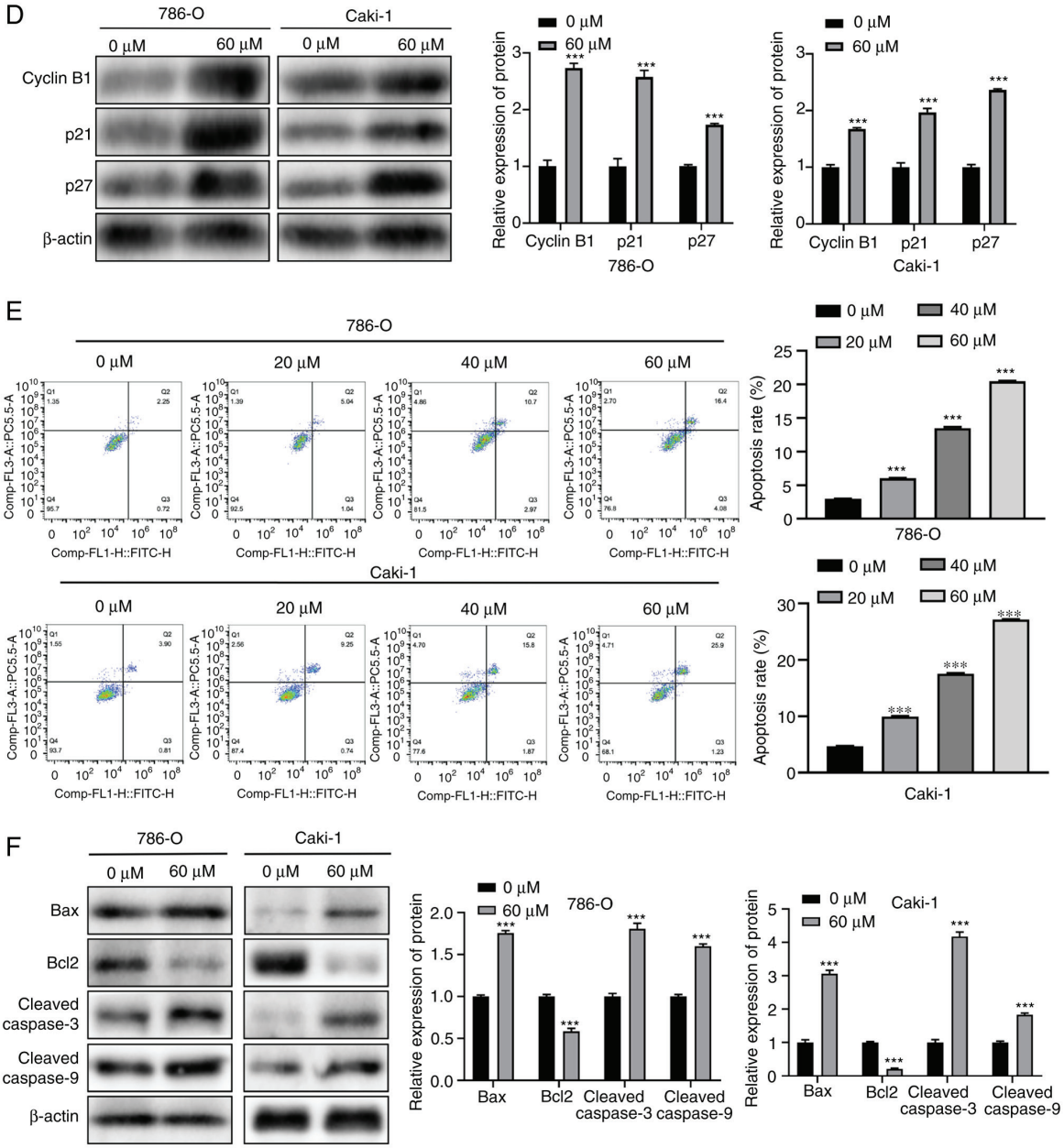
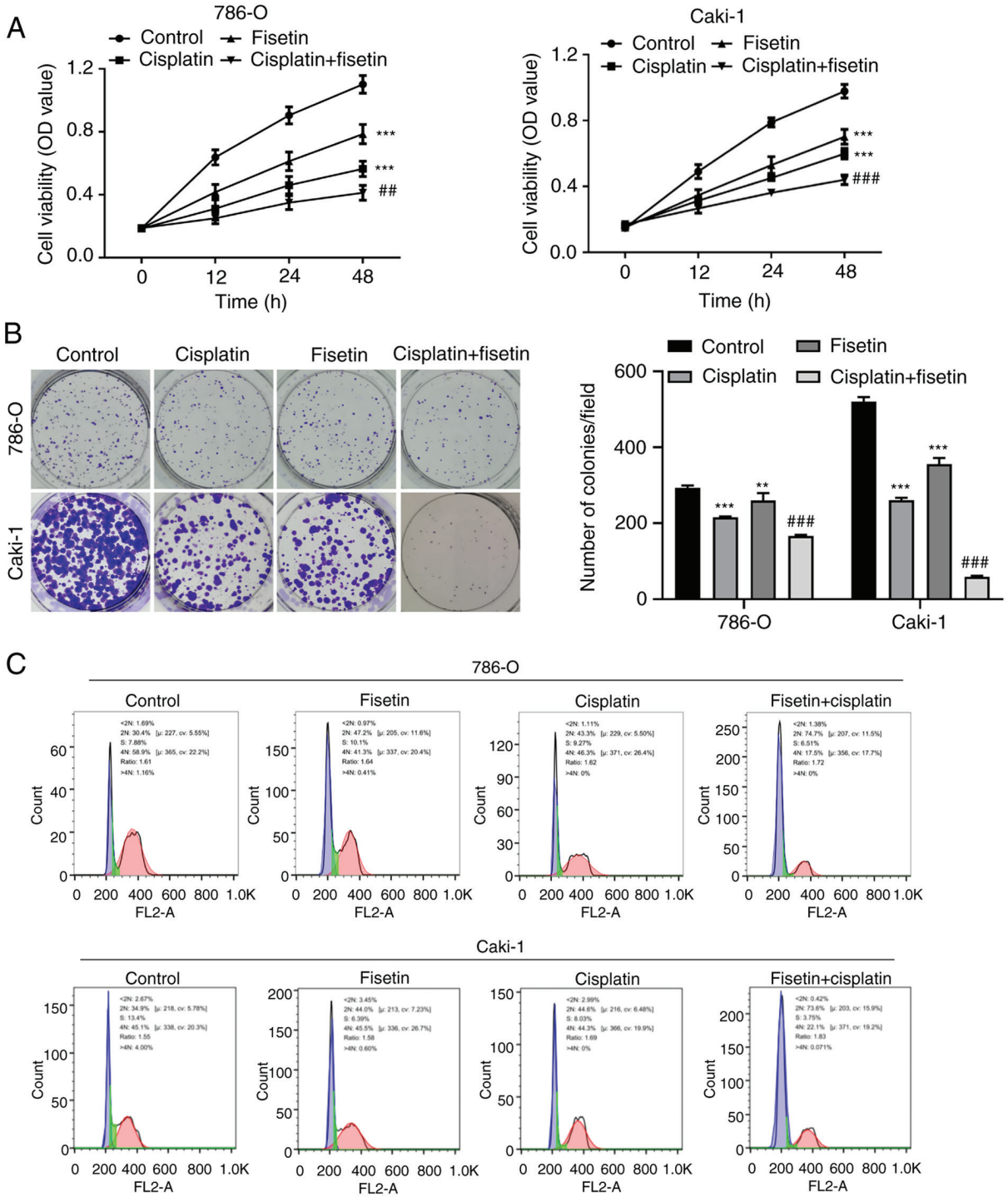


Figure 1. Fisetin decreases renal cell carcinoma cell proliferation and promotes apoptosis. (A) Cell Counting Kit-8 assay was used to assess the effects of different doses of fisetin on the proliferation of 786-O and Caki-1 cells at 0, 12, 24 and 48 h. Fisetin exhibited the greatest inhibitory effect at 60·M. (B) Colony formation assay was used to assess the effects of different doses of fisetin on the proliferation of 786-O and Caki-1 cells. The effects of fisetin were dose-dependent and the fewest colonies were formed at 60·M. (C) Cell cycle arrest assessed using flow cytometry. The number of cells in G_1 phase notably increased with the increase of fisetin concentration, whereas the number of cells in G_2/M phase was markedly reduced in a dose-dependent manner. (D) Expression levels of cell cycle-related proteins assessed using western blotting. Fisetin significantly promoted the expression of cyclin B1, p21 and p27. (E) Caki-1 and 786-O cell apoptosis was assessed using flow cytometry. The apoptosis rate was significantly elevated with an increase in fisetin dosage. (F) Expression levels of apoptosis marker proteins assessed after fisetin treatment using western blotting. Fisetin significantly upregulated Bax and cleaved-caspase-3/9 in both 786-O and Caki-1 cells, whereas the expression levels of Bcl2 were significantly decreased following fisetin treatment. OD, optical density. ***P<0.001 vs. 0 μM.

Results

Fisetin inhibits the proliferation of RCC cells and promotes apoptosis. The effects of fisetin on the development of RCC cells were assessed using different concentrations of fisetin. First, the CCK-8 assay was used to evaluate the viability of the 786-O and Caki-1 cells. The results revealed that fisetin significantly inhibited the proliferation of 786-O and Caki-1 cells in a dose-dependent manner (Fig. 1A). Furthermore, tumor colony formation was significantly inhibited by fisetin treatment, and the strongest inhibitory effects were achieved in response to 60 μM fisetin (Fig. 1B). Moreover, with an increase in fisetin concentration, the cells exhibited a G_1 phase block, indicating that fisetin could deter the proliferation of 786-O and Caki-1 cells and the proliferation inhibition was most marked in response to 60 μM fisetin (Fig. 1C). The expression levels of cell cycle-related proteins cyclin B1, p21 and p27 were significantly increased in the fisetin treatment group compared with those in the control group (Fig. 1D), with the cell cycle arrest in the G_2/M phase. Apoptosis was also observed in the



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786-O and Caki-1 cells. The findings revealed that fisetin significantly increased the proportion of apoptotic cells and promoted RCC cell apoptosis, in comparison with non-treated cells (Fig. 1E). Furthermore, the expression levels of the apoptotic activators Bax and cleaved-caspase 3/9 were significantly increased, whereas the expression levels of the apoptotic inhibitor Bcl2 were significantly decreased after fisetin treatment compared with those in the control group (Fig. 1F). These findings indicated that fisetin impeded RCC cell proliferation and enhanced apoptosis. Additionally, the effects of fisetin on RCC cell development were dose-dependent.

Fisetin enhances cisplatin sensitivity in RCC cells. The role of fisetin in cisplatin drug resistance was assessed and it was demonstrated that although fisetin or cisplatin treatment alone could inhibit 786-O and Caki-1 cell proliferation, the combination of these two drugs displayed significantly stronger anti-proliferative effects, in comparison with the control (Fig. 2A). Therefore, it was hypothesized that fisetin may enhance cisplatin sensitivity in RCC cells. To evaluate this hypothesis, the ability of the cells to form tumorspheres after combination treatment was assessed. The results demonstrated that the number and size of tumorspheres were markedly

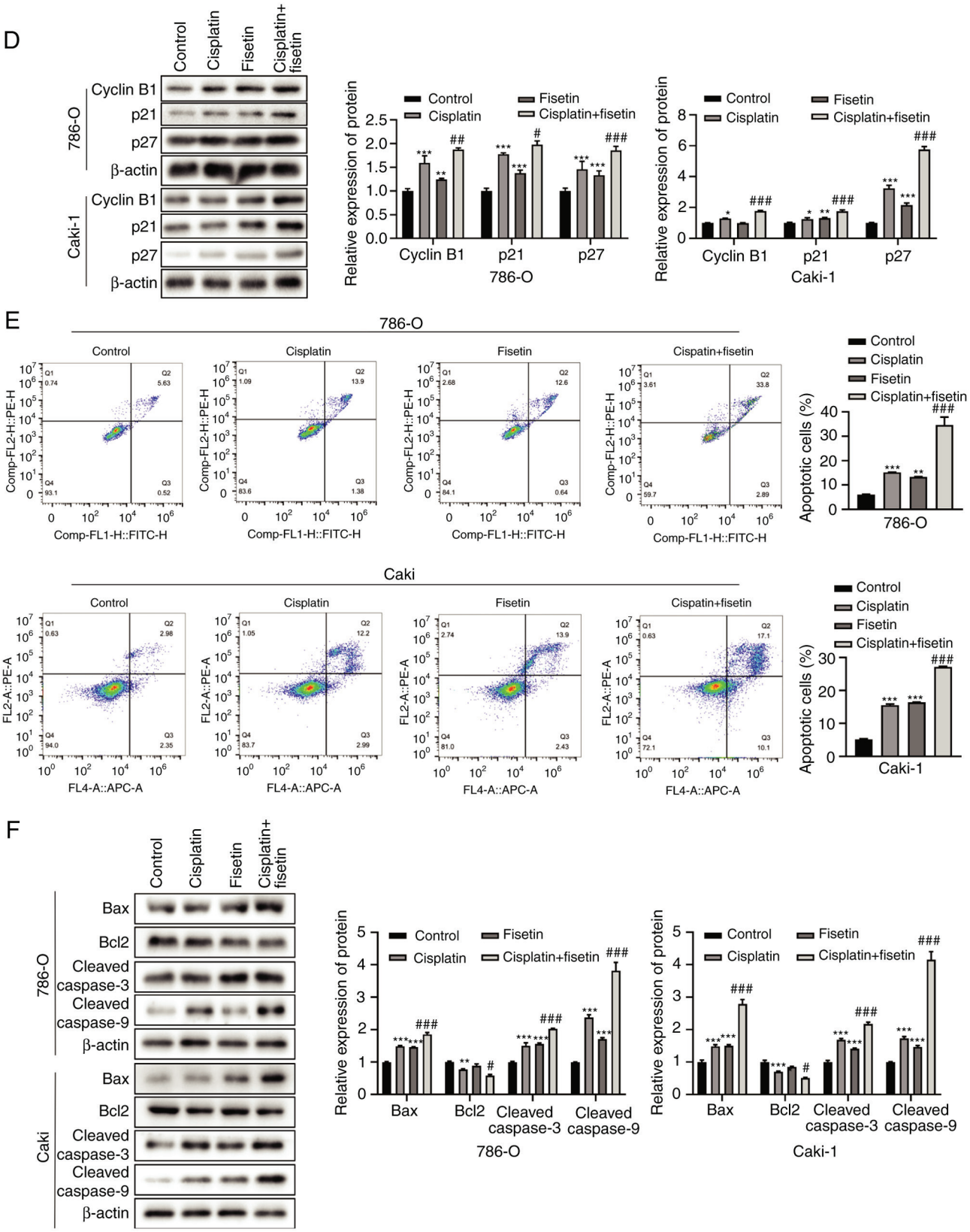


Figure 2. Fisetin enhances cisplatin sensitivity in 786-O and Caki-1 RCC cells. (A) Effects of fisetin and cisplatin combination treatment on RCC cell proliferation. Fisetin or cisplatin alone significantly inhibited cell proliferation; however, the combination of these two drugs demonstrated a stronger inhibitory effect. (B) Colony formation assays after fisetin and cisplatin combination treatment. Combination therapy demonstrated marked proliferation suppression compared with cisplatin or fisetin alone treatment. (C) Effects of fisetin and cisplatin alone, compared with the combination, on the cell cycle progression of 786-O and Caki-1 cells. Fisetin and cisplatin in combination notably increased the proportion of cells in G_1 phase and decreased that in G_2/M phase. (D) Change in the expression levels of cell cycle-related proteins when fisetin and cisplatin were used in combination. The expression levels of cyclin B1, p21 and p27 exhibited the greatest increase following fisetin + cisplatin treatment. (E) Effects of fisetin and cisplatin alone and combined on apoptosis. Combination treatment resulted in the greatest increase in the proportion of apoptotic 786-O and Caki-1 cells. (F) Effects of combined fisetin and cisplatin treatment on apoptosis-associated protein expression. Fisetin + cisplatin upregulated Bax, cleaved-caspase 3/9, and downregulated Bcl2 to a larger extent than fisetin or cisplatin alone. RCC, renal cell carcinoma; OD, optical density. *P<0.05, **P<0.01, ***P<0.001, vs. Control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, vs. cisplatin.

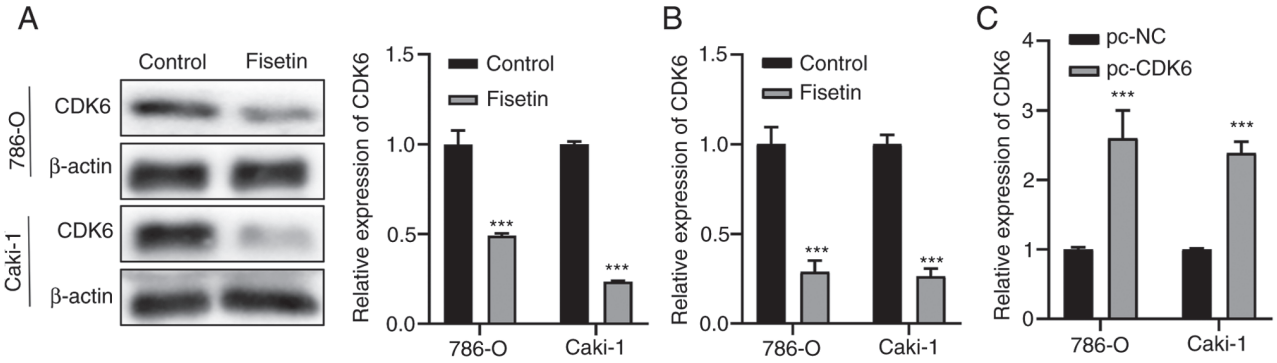


Figure 3. Fisetin enhances cisplatin sensitivity via CDK6 in renal cell carcinoma cells. Expression levels of CDK6 (A) protein and (B) mRNA were significantly decreased in the fisetin treatment group in both 786-O and Caki-1 cells. (C) Reverse transcription-quantitative PCR used to assess the transfection efficiency of pc-CDK6. CDK6, cyclin-dependent kinase 6; NC, negative control; pc, pcDNA. ***P<0.001, vs. control.

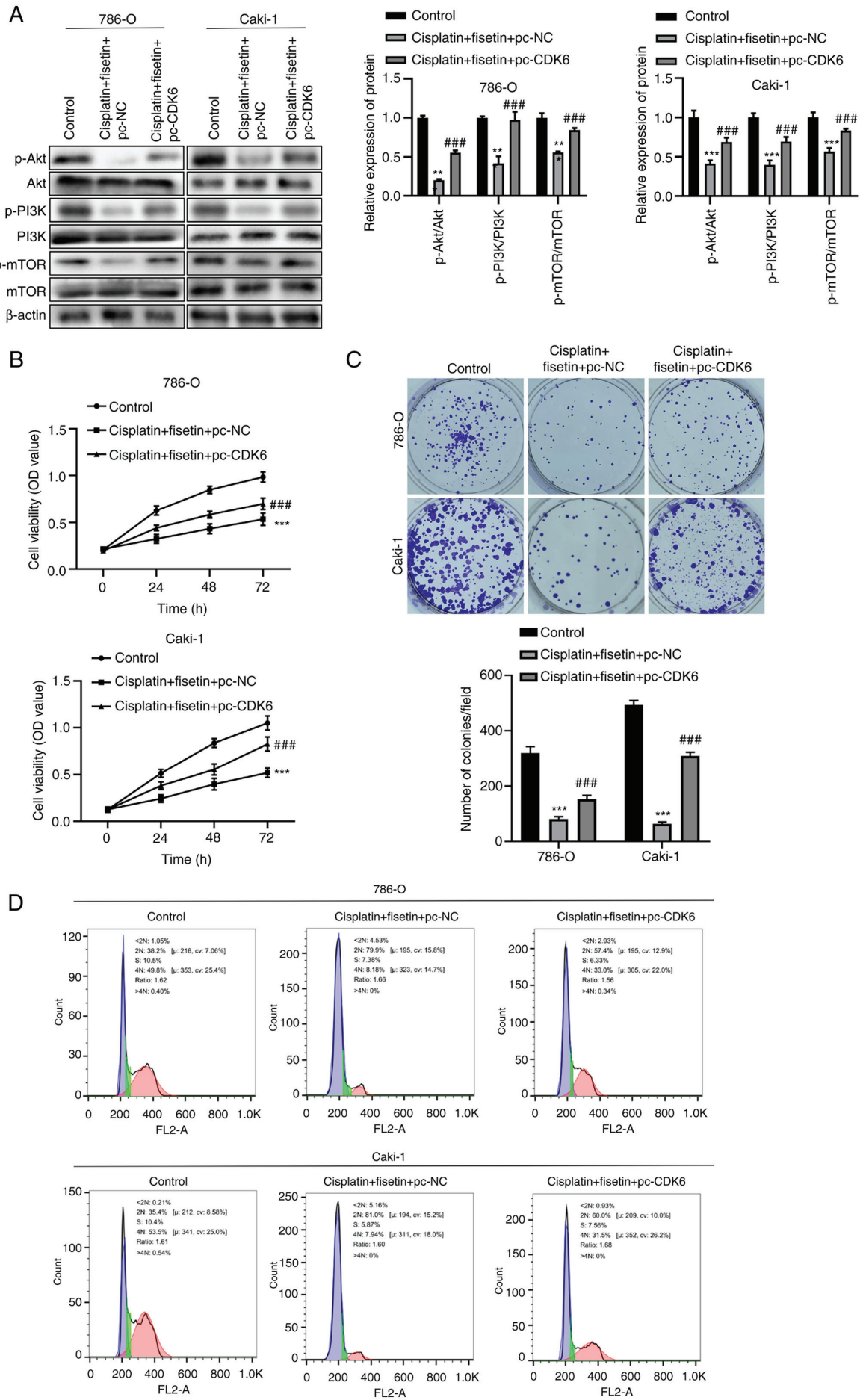
decreased when cells were treated with cisplatin supplemented with fisetin (Fig. 2B). Furthermore, an augmented inhibitory effect was also demonstrated in the cell cycle of the two RCC cell lines, as shown in Fig. 2C, where the proportion of cells in the G_1 phase notably increased in the cisplatin + fisetin combination treatment group compared with cisplatin treatment group. The expression levels of cycle-related proteins cyclin B1, p21 and p27 were also significantly increased when treated with this drug combination compared with cisplatin treatment group (Fig. 2D), inducing the cell cycle arrest in the G_2/M phase. Fisetin also significantly enhanced the inhibitory impacts of cisplatin on 786-O and Caki-1 cell apoptosis; the proportion of apoptotic cells was significantly higher in the group treated with cisplatin combined with fisetin compared with that treated with cisplatin or fisetin alone (Fig. 2E), as were the expression levels of apoptosis activators, Bax and cleaved-caspase 3/9 (Fig. 2F). Conversely, the expression levels of the apoptotic inhibitor Bcl2 were significantly reduced when cisplatin was combined with fisetin compared with cisplatin (Fig. 2F). Fisetin and cisplatin in combination enhanced the chemosensitivity of RCC cells and thus demonstrated marked antitumor effects on the development of RCC cells.

Fisetin enhances cisplatin sensitivity through CDK6 in RCC cells. To elucidate the mechanisms underlying the effects of fisetin, the targets of fisetin were assessed and it was demonstrated that the protein expression levels of CDK6 were significantly decreased after treating 786-O and Caki-1 cells with fisetin compared with those in the control group (Fig. 3A). Subsequently, the mRNA expression levels of CDK6 were assessed using RT-qPCR and the results demonstrated that the mRNA expression levels of CDK6 were also significantly decreased by fisetin treatment compared with those in the control group (Fig. 3B). Therefore, we hypothesized that fisetin enhanced the sensitivity of cisplatin to RCC cells via the inhibition of CDK6 expression. RT-qPCR results demonstrated that the expression levels of CDK6 in both cell lines were significantly increased post-transfection with pcDNA3.1-CDK6 compared with those in the pc-negative control cells (Fig. 3C).

Fisetin enhances cisplatin sensitivity in RCC cells via the PI3K/Akt/mTOR signaling pathway. To assess our hypothesis, CDK6 was overexpressed in 786-O and Caki-1 cells. The findings revealed that the expression levels of p-Akt, p-PI3K and p-mTOR were significantly reduced when cells were treated with cisplatin and fisetin compared with cisplatin but were significantly increased after CDK6 overexpression (Fig. 4A). These results suggested that fisetin may inhibit the PI3K/Akt signaling pathway by targeting CDK6, thereby enhancing the sensitivity of RCC cells to cisplatin. Treatment with fisetin combined with cisplatin significantly inhibited cell proliferation, whereas overexpression of CDK6 partially reversed the proliferation inhibition caused by fisetin (Fig. 4B). Moreover, fisetin and cisplatin treatment significantly reduced tumor colony formation compared with control and CDK6 overexpression significantly undermined the inhibitory effect caused by fisetin compared with cisplatin + fisetin + pc-NC group (Fig. 4C). In addition, combination treatment notably arrested the cell cycle in the G2 phase for a longer time and could not normally enter the M phase, whereas overexpression of CDK6 counteracted the effects of cisplatin and fisetin combination (Fig. 4D). Treatment with fisetin and cisplatin also significantly enhanced the expression levels of cyclin B1, p21 and p27 compared with control group, and overexpression of CDK6 significantly decreased the expression levels of these three proteins compared with cisplatin + fisetin + pc-NC group (Fig. 4E), decreasing the proportion of cells in G_2/M phase. Furthermore, the cisplatin + fisetin treatment group demonstrated a significant increase in apoptosis compared with control group, whereas overexpression of CDK6 significantly reduced the apoptosis-inducing effect caused by this drug combination compared with cisplatin + fisetin + pc-NC group (Fig. 4F). Cisplatin + fisetin treatment also significantly increased the expression levels of the proapoptotic proteins Bax and cleaved-caspase 3/9, and it significantly reduced the expression levels of the anti-apoptotic protein Bcl2 compared with control group, whereas overexpression of CDK6 partially reversed these effects compared with cisplatin + fisetin + pc-NC group (Fig. 4G).

Discussion

RCC is a common kidney neoplasm that ranks second in urinary tumor mortality, accounting for >90% of kidney cancer cases worldwide (26). Chemotherapy is widely regarded as the most effective treatment method for RCC because of its simplicity and fast reaction (27,28). Nevertheless, cancer cells can



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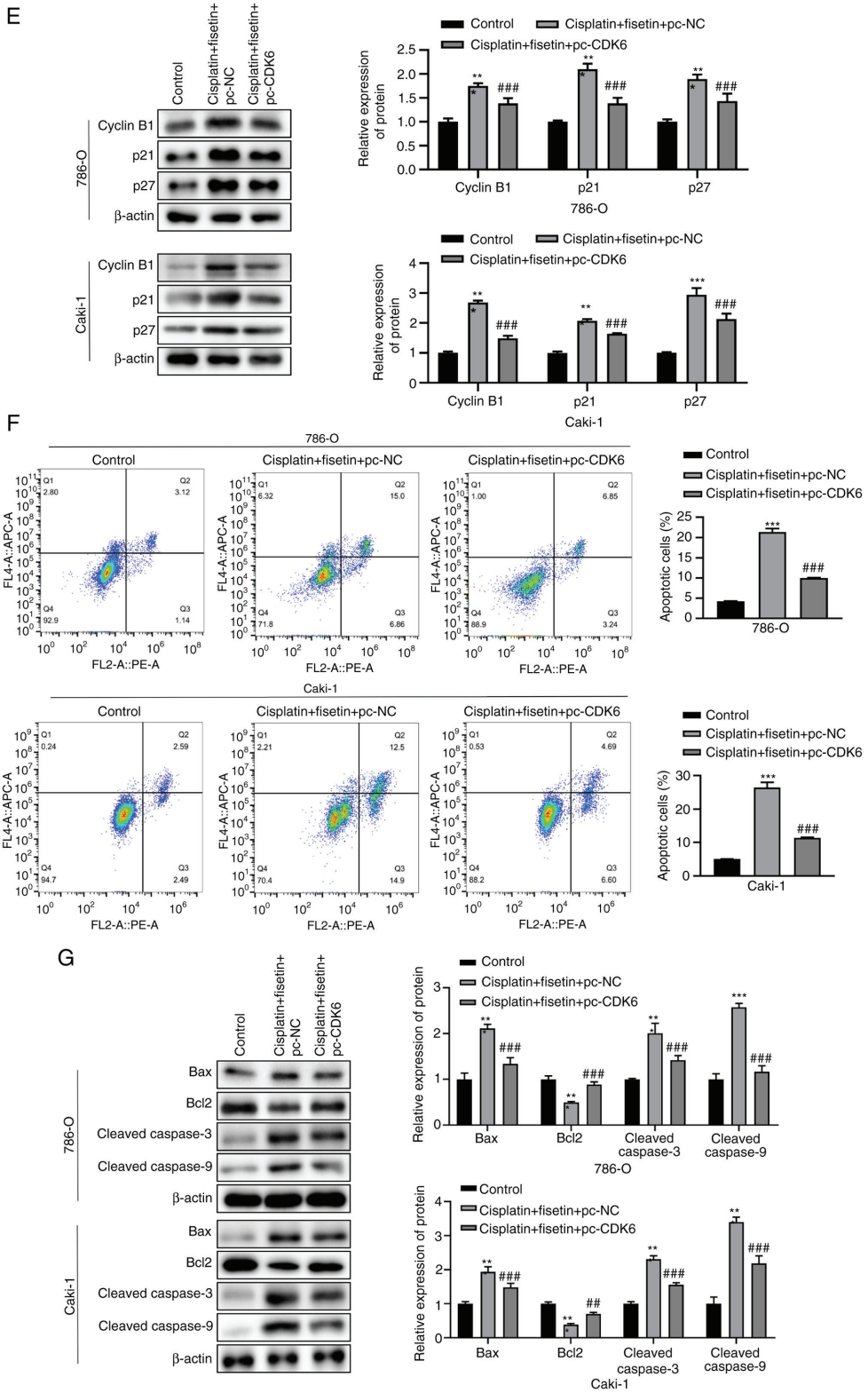

Figure 4. Fisetin enhances cisplatin sensitivity in RCC cells via the PI3K/Akt/mTOR signaling pathway to inhibit cell proliferation, delay the cell cycle and promote apoptosis. (A) Expression levels of key proteins in the PI3K/Akt/mTOR signaling pathway assessed following fisetin and cisplatin treatment. Fisetin and cisplatin significantly decreased the protein expression levels of p-Akt, p-PI3K and p-mTOR, but CDK6 overexpression increased the levels of p-Akt, p-PI3K and p-mTOR. (B) RCC cell proliferation after CDK6 overexpression. Fisetin and cisplatin reduced 786-O and Caki-1 cell proliferation, whereas the effects were undermined by CDK6 overexpression. (C) Number of colonies formed significantly increased after CDK6 overexpression. Fisetin + cisplatin treatment inhibited RCC cell-forming colonies, whereas CDK6 overexpression demonstrated the opposite effect. (D) Number of cells remaining in G_1 phase notably increased, whereas they decreased in G_2/M phase after the combination of fisetin and cisplatin treatment. CDK6 overexpression counteracted effects of fisetin and cisplatin. (E) Overexpression of CDK6 neutralized the effects of fisetin and cisplatin on the expression levels of cell cycle-associated proteins. The expression levels of cyclin B1, p21 and p27 were significantly reduced after CDK6 overexpression. (F) Proportion of apoptotic RCC cells notably reduced after CDK6 overexpression, which undermined the proapoptotic role of fisetin and cisplatin. (G) Overexpression of CDK6 neutralized the effects of fisetin and cisplatin on the expression levels of apoptosis-associated proteins. CDK6 significantly downregulated Bax, cleaved-caspase 3 and cleaved-caspase 9 in both 786-O cells and Caki-1 cells, whereas the expression levels of Bcl2 were significantly increased after CDK6 overexpression. RCC, renal cell carcinoma; pc, pcDNA; NC, negative control; OD, optical density; p, phosphorylated. *P<0.05, **P<0.01, ***P<0.001, vs. Control; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, vs. cisplatin + fisetin + pc-NC.

lack sensitivity to chemotherapy and the gradual development of drug resistance has presented challenges for RCC treatment. As a result, understanding the underlying mechanisms of drug resistance is essential in developing effective treatment regimens.

Cisplatin is a highly effective chemotherapy drug. Even though cisplatin has been demonstrated to be effective in the treatment of numerous malignancies (29,30), RCC is insensitive to cisplatin therapy (22), as shown by the low response rate to cisplatin alone. Therefore, more research into the mechanism of cisplatin resistance is required.

Fisetin is a naturally occurring flavonoid (31) with antioxidant, antidiabetic, anti-inflammatory (32), anticancer (21,33) and neuroprotective properties (34). Fisetin promotes the apoptosis of several cancer cell types by constraining COX-2 (35), impeding the Wnt/EGFR signaling pathway (36), strengthening caspase-3 cascade reactions, boosting the caspase-3/8 dependent pathway, and amplifying the activity of Ca^{2+} and caspase-3-dependent endonuclease (37). Fisetin has been shown to serve a role in RCC development. Fisetin has been reported to reduce A-498, ACHN and 786-O cell proliferation in a concentration-dependent manner, and to induce the cell cycle to arrest in the G_2/M phase (33). Moreover, fisetin restrains RCC cell metastasis by suppressing a disintegrin and a metalloprotease 9, cathepsin S and cathepsin B, and increasing the expression of activated ERK (11). However, it remains unknown as to whether fisetin can influence cisplatin resistance in RCC cells.

The findings from the present study indicated that fisetin caused a reduction in the proliferation of RCC cell lines in a concentration-dependent manner. Fisetin also restrained RCC cell proliferation by inducing cell cycle arrest. Following fisetin treatment, the proportion of cells in the G_1 phase increased, whereas the G_2/M fraction decreased detected by flow cytometry and WB. The expression levels of cyclin B1, p21 and p27 were also increased when cells were treated with fisetin. Apoptotic induction was also observed in RCC cells treated with fisetin, and this process was concentration-dependent. Fisetin upregulated the expression levels of Bax and cleaved-caspase 3/9, whereas the expression levels of Bcl2 were decreased. Furthermore, fisetin and cisplatin in combination enhanced the antitumor effects. Fisetin combined with cisplatin demonstrated a greater induction of proliferation inhibition and apoptosis in RCC cells, coupled with a notable rise in the expression levels of cyclin B1, p21 and p27, and the activation of caspase-3 and -9. Furthermore, it was demonstrated that the expression levels of CDK6 were inhibited when RCC cells were exposed to fisetin. Moreover, fisetin increased the expression levels of p-PI3K, p-Akt and p-mTOR, thereby upregulating the activity of the PI3K/Akt/mTOR signaling pathway. Overexpression of CDK6 was shown to undermine the effects of fisetin on cisplatin sensitivity in RCC cells and to counteract the antitumor effects of the fisetin-cisplatin combination. Taken together, the results of the present study demonstrated that fisetin enhanced cisplatin sensitivity through the CDK6/PI3K/Akt/mTOR signaling pathway in RCC. The findings provide insight into overcoming chemotherapy resistance in RCC and highlight that combination therapy may be a promising strategy for RCC treatment in the future.

Nevertheless, certain limitations exist in the present work. The efficiency of fisetin and cisplatin combination therapy in the treatment of tumor-bearing mice has not been defined yet. Whether this combination regimen has the potential to be used clinically and the response rate among patients with RCC need to be further assessed. As for the molecular mechanism, whether fisetin affects CDK6 expression through direct binding or indirect regulation should be explored in future work. In addition, the present study demonstrated that the PI3K signaling pathway may be involved in the antitumor effect of fisetin, and whether other tumor-related signaling pathways also affect fisetin-mediated cisplatin sensitivity should be the subject of follow-up studies with animal experiments performed to verify the effectiveness demonstrated *in vitro*.

In conclusion, increasing concentrations of fisetin inhibited RCC cell proliferation, and promoted cell cycle arrest and cell apoptosis. Moreover, fisetin reduced cisplatin resistance and increased its ability to kill RCC cells. Finally, fisetin and cisplatin in combination demonstrated greater antitumor effects than fisetin and cisplatin alone via regulation of the CDK6/PI3K/Akt/mTOR signaling pathway.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

TJ, YL, YJ and YX discussed the concept of the paper, investigated the background of the paper, performed the data analysis and confirm the authenticity of all the raw data. TJ, YL and YJ wrote the manuscript, and YX revised and improved the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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