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Saikosaponin-d suppresses cell growth in renal cell carcinoma through EGFR/p38 signaling pathway

C. CAI‡, H. ZHANG‡, Y. OU‡, Y. JIANG, D. ZHONG, H. QI*, Q. DANG*

Department of Urology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, P.R. China

**Correspondence: 2668241842@qq.com, dangqiang0128@163.com*
‡Contributed equally to this work.

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The study aimed to explore the effect of Saikosaponin-d (SSd) and its underlying mechanism on cell growth inhibition as well as induction of apoptosis and cell cycle arrest in renal cell carcinoma (RCC). MTT assay and colony formation assay were employed in this study, with the results indicating that RCC cells proliferation was inhibited by SSd at different doses. Analysis by flow cytometry revealed that RCC cell proliferation inhibitory effect of SSd was achieved by inducing apoptosis and cell cycle arrest at G0/G1 phase via up-regulation of p53. As compared to the control group, SSd can significantly inhibit the growth of 769-P and 786-O cell lines and induce apoptosis and cell cycle arrest. The mechanism exploration demonstrated that inhibiting the activation of EGFR/p38 signaling pathways was the molecular basis of SSd's biological effects such as inducing apoptotic death, inhibiting cell growth as well as up-regulating p53 expression in human RCC cells. In conclusion, our data suggest that SSd may serve as a promising intervention for chemopreventive or chemotherapeutic treatment for patients with RCC.

Key words: Saikosaponin-d, RCC, cell growth, EGFR/p38

As one of the world's most common malignant tumor, Renal cell cancer (RCC) contributes about 2~3% of the malignancies in adult population, with an increase of 2~3% every decade in terms of its morbidity [1, 2]. The major treatments for RCC include partial nephrectomy (<4cm in the largest dimension) and nephrectomy (>4cm in the largest dimension) if, of course, the patients are "lucky enough" to be detected and diagnosed in the early stage. Nevertheless, postoperative recurrence or metastasis, which accounts for about 30~40% of the total cases, is still a major concern for these patients, leading to at least a hundred thousand annual deaths [3, 4]. Medical interventions available for RCC patients with metastasis are chemotherapy, radiotherapy, and immunotherapy and so on. However, the effect has been proved being limited, creating an urgent demand for a new solution with great safety and efficacy in RCC treatment.

Saikosaponin-d (SSd) is an extract from Bupleurum falcatum L, usually used as a common drug material in traditional Chinese medicine (TCM) for anti-inflammatory and anti-infectious purposes [5, 6]. According to some reports, this saponin derivative was found to have some pharmacological activities similar to sterides such as pain-relieving, anti-inflammation, immunoregulation, anti-viral function, and hepatocyte protection [7]. Its anti-neoplastic effect, however, was reported until recently by the studies that focused on the fields of thyroid cancer [8], human non-small cell lung cancer (NSCLC) [9] and liver cancer [10]. The action mechanism and targets or pathways of SSd are still unclear and subject to further exploration.

Being a member of the ErbB receptor tyrosine kinase family, epidermal growth factor receptor (EGFR) serves as an essential factor in multiple cellular processes involving with cell proliferation, survival, and cell migration [11-13]. The over-expression of EGFR has been reported in cases with clear-cell RCC tumors in recent studies and the association between EGFR expression and cancer progression has been confirmed [14-16]. According to the pre-clinical study of Ming Feng Chena, SSd is found to carry a proliferation and migration inhibiting function in HSC-T6 cells in rat models via the pathway that regulates the phosphorylation of p38 and ERK1/2 [17]. In this study, we attempted to look into the effect of SSd on cell proliferation and apoptosis through

EGFR/p38 pathway in RCC, which was the first try in related research areas.

Materials and methods

Human RCC cell lines (769-P and 786-O) provided by Medical School of Xi'an Jiaotong University were employed for cell culture. Buffer used to preserve the cells was prepared with RPMI-1640 containing 10% fetal bovine serum (FBS), 50 μg/ml streptomycin, 100 IU/ml penicillin and 2 mM of L-glutamine. The cells were cultured at 37°C under a humidified condition with 5% CO_2.

Chemicals. SSd purchased from Shfeng (Shanghai, China) was dissolved in 50 mM of DMSO and then preserved at -20°C for further use; anti-human p53, c-Fos, β-Actin and GAPDH antibodies were obtained from Santa Cruz Biotechnology; and Cell Signaling Technology was the provider for antibodies against EGFR, p-EGFR, p-ERK, MEK, p-MEK, p38 and p-p38; Recombinant Human EGF, AG1478 and SB203580 were purchased from Peprotech, Selleckchem and Medchem Express, respectively; Nanjing KeyGen Biotech was the manufacturer for Annexin V-FITC Apoptosis Assay kit and Cell Cycle Assay Kit.

MTT assay (a tetrazolium-based assay) was employed for cell viability determination using 96-well plates, with 50 μl of media which contains one thousand cells in each well. The cells were treated respectively with different doses of SSd or with buffer not containing SSd (0 μM of SSd) serving as the control group before incubation for several times; after the addition of 0.5 mg/ml of MTT, the cells were incubated at 37°C for another hour; the supernatant was removed and the cells were dissolved with DMSO; finally, a 96-well microplate reader was used for colorimetric analysis at 490 nm [18]. The procedure was carried out in triplicate.

Colony formation. To determine the inhibition effect of SSd on colony formation in RCC cells, two different types of RCC cells-769-P and 786-O RCC cells were respectively seeded in 24-well plates (100 cells/well), and 10, 20 and 30 μM of SSd was added respectively into the wells for a 14-day culture. Then the cells were stained using crystal violet for colony counting.

DNA cell cycle. In the cell cycle test, the cells were treated respectively with 0 and 20μM of SSd for 48 h when a 60-80% confluence was obtained; the cells were washed twice with PBS before fixation with 70% ethanol at 4°C for 1 h; after another wash with PBS, 0.05 mg/ml RNase-contained propidium iodide (PI) solution was added to re-suspend the cells, which were then incubated for 30 min at room temperature avoiding light for DNA content analysis using flow cytometer.

Apoptosis. During the procedure of apoptosis quantitative determination, the 769-P and 786-O cells were processed respectively with 10 and 20 μM of SSd for 48 h. Annexin V-FITC Apoptosis Assay kit was employed for Annexin V and PI staining according to the operative instruction from the kit provider. And then flow cytometry was used for apoptotic cell analysis.

Western blot. When it came to western blot analysis, RIPA buffer was prepared using Tris-HCl (50 mM, pH 7.4), NaCl (150 mM), EDTA (1 mM), 1% NP-40, PMSF (1 mM), NaF (1 mM), Na_3VO_4 (1 mM), okadaic acid (1 mM), as well as 1 mg/ml pepstatin, aprotinin and leupeptin; the cells were lysed in RIPA buffer and Protease Inhibitor Cocktail (provided by Roche Inc.); 20 μg of protein was sampled, with 10-15% gel prepared for SDS-PAGE; the protein was transferred onto PVDF membranes (provided by Millipore) after electrophoresis procedure, followed by membrane blocking at room temperature for 1 h using 5% BSA diluted in PBS; then the membranes, after processed with diluted primary antibody solutions, were incubated overnight at 4°C; after another wash, anti-rabbit, anti-mouse or anti-goat IgG HRPs was used as the secondary antibody to treat the membranes before they were incubated for another hour; in the last step, ECL mixture (provided by Thermo Fisher Scientific) was employed for color developing for the blots[19].

Statistical analyses. In this study, SPSS 22.0 was used for all the statistical analyses. Quantitative data were presented as mean ± SE and one-way ANOVA was employed for inter-group comparisons. If statistical difference was shown, then Dunnett's t-test was established for further comparison between paired groups. Student's t-test was also used when only 2 groups were involved in statistical comparison. $P<0.05$ was considered to represent a significant statistical difference.

Results

Figure 1 shows the growth inhibitory effect of SSd (Figure 1A – chemical structure) on RCC cells by comparing the cell viability between groups treated with different doses of SSd as well as between different time points of the groups treated with the same dose of SSd. The results revealed that SSd presented a proliferation inhibitory activity in 769-P and 786-O cells. The viability rate of RCC cells was 67% (10 μM group), 34% (15 μM group) and 15% (20 μM group) respectively through a 48-hour SSd treatment (Figure 1B). Also, an declining viability rate was found at the time points of 24, 48 and 72 h in the groups treated with 15 μM of SSd (Figure 1C), indicating a dose- and time-dependent inhibitory effect on RCC cells.

For colony formation assay, 769-P and 786-O cells were respectively seeded into 24-well plates, 100 cells per well, and cultured for 14 days after SSd treatment (10, 20 and 30 μM, respectively). As shown in Figure 2, the colony counting was remarkably decreased with the increase of SSd dose, indicating that SSd can significantly inhibit colony formation in both of 769-P (Fig. 2B) and 786-O (Figure 2C) cells.

To understand how SSd influences cell cycle in RCC cells, flow cytometry was employed to determine the percentage of the cells at different phases after a 24-hour SSd treatment process (20 μM). The results of RCC cell cycle assay showed a significantly elevated percentage level of G0/G1 phase cells and an obviously declined level in S phase cells compared with

the control group (0 μM) (Figure 3), revealing that SSd has the ability to induce cell cycle arrest by inhibiting cell growth from G0/G1 phase.

The effect of SSd on RCC cell apoptosis was measured by Annexin V positive cells and western blot analysis after 769-P and 786-O cells were respectively treated with 10 and 20 μM of SSd for 48 hours. According to the flow cytometry data (Figuer 4A), Annexin V positive cells increased after SSd treatment, and compared to the control group (0 μM), significant differences were found in SSd treatment groups, where the percentage of Annexin V positive cells was respectively 9.6% (10μM group) and 16.6% (20 μM group) in 769-P cells, and 21.2% (10μM group) and 49.8% (20μM group) in 786-O cells (Figure 4B). The western blot analysis demonstrated an increase in p53 after treatment with SSd (Figure 4C). These results indicate SSd has apoptosis inducing effect on RCC cells.

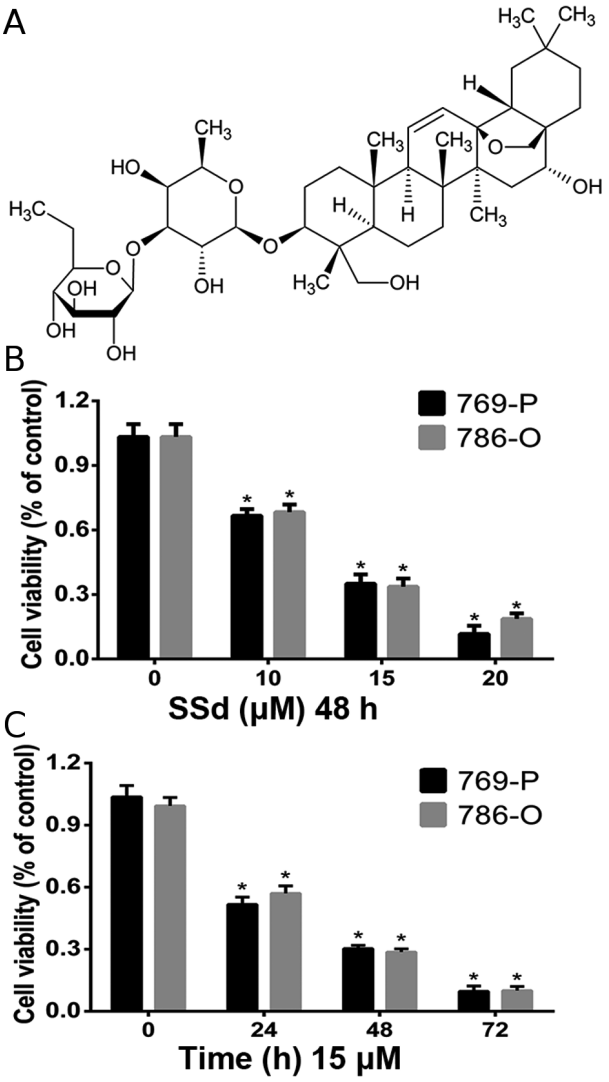

Figure 1. The growth inhibitory effect of SSd on RCC cells. (A) The chemical structure of SSd. (B) Cell viability was determined after RCC (769-P and786-O) cells were treated respectively with different doses of SSd (10, 15 and 20 μM) for 48 h. (C) Cell viability was determined at different time points (0, 24, 48, and 72 h) after RCC (769-P and 786-O) cells were treated with 15 μM of SSd. MTT assay was employed to determine the cell viability. *P<0.05 represents a statistically significant difference.

In this study, the mechanism of SSd on its cell proliferation inhibitory effect was also investigated. Further study on the expression of cell growth related genes suggested that SSd can significantly inhibit the activation of the EGFR pathway, as EGFR, phospho-EGFR, MEK, phospho-MEK, p38, phospho-p38 and its downstream c-Fos all showed a decrease after SSd treatment (Figure 5A, 5B). The expression of ERK, however, presented an increase tendency, which was considered to be related to its activity on (endoplasmic reticulum) stress induction.

As EGFR is known to regulate cell proliferation via the MAPK pathway [20], our exploration was mainly focused on verification of the hypothesis that EGFR/MAPK signaling pathway is the one through which SSd implements its regulatory function on RCC cell growth. EGFR inhibitor AG1478 [21] and p38 inhibitor SB203580 [22] were used as positive controls, and an inhibitory effect similar to these inhibitors was found in SSd, characterized by blocking EFG induced p-EGFR and activation of p38 (Figure 5C, 5D), which indicates that SSd can block the EGFR/p38 signaling pathway. Then MTT assay was employed to detect cell viability of 769-P and 786-O groups after treatment with SSd (2 h) and EGF (48 h), where EGFR inhibitor AG1478 or p38 inhibitor SB203580 was also used as positive control (Figure 5E, 5F). The results showed that SSd presented a significant inhibitory effect on cell proliferation simulated EGFR inhibitor or p38 inhibitor. So, a conclusion can be drawn based on the above findings that SSd inhibits cell growth via the EGFR/p38 MAPK signaling pathway.

Discussion

Currently available medical interventions for RCC, one of the most common malignant tumors in the world, are still relatively low in satisfaction in terms of the efficacy and safety. Surgical intervention can do some help to early stage RCC only, while patients with advanced tumor are still in urgent need for more promising approaches. Although studies have proved that SSd can inhibit cell growth and induce apoptosis in malignancies such as liver cancer, prostate cancer and thyroid carcinoma [8, 23, 24], its effect in RCC remains unclear. Our study is the first to explore the role and mechanism of SSd in RCC treatment which has been proved in this research to be able to inhibit RCC cell growth in vitro.

It is well known that abnormal cell proliferation plays a major part in tumorigenesis. In this study, the inhibitory effect of SSd on cell proliferation was found in two RCC cell lines--769-P and 786-O by MTT assay. Consistent with the findings of R-Y LIU's studies, SSd, even at a relatively low dose

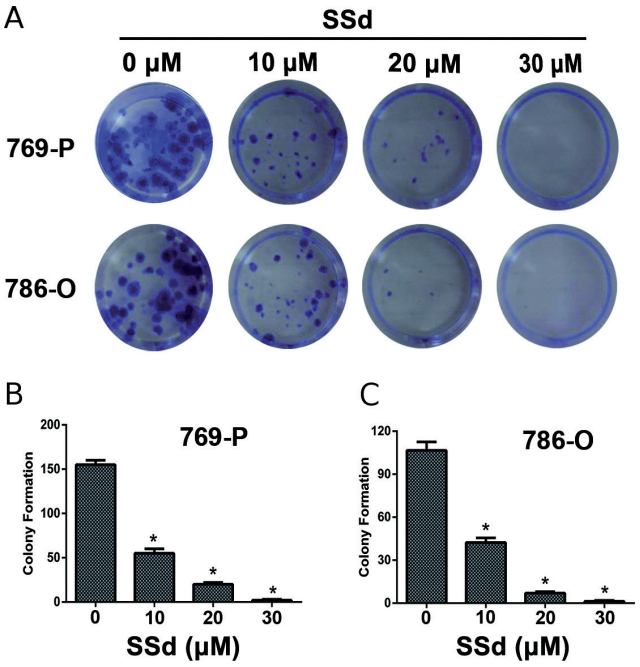

Figure 2. The inhibitory effect of SSd on colony formation of RCC cells. (A) Colony formation assay (24-well). 769-P and 786-O cells were respectively seeded into 24-well plates, 100 cells per well, and cultured after treatment respectively with different doses of SSd (10, 20 and 30 μM) for 14 days for crystal violet staining and colony number counting. (B and C) Colony quantification. *P<0.05 represents a statistically significant difference.

(20 μM), showed an effective inhibition on RCC cell growth in our study, suggesting a feature of low toxicity and good safety in the future clinical use, which is subject to further verification in subsequent clinical trials [9].

Cell cycle checkpoints are key factors to control cell cycle and influence the cell proliferation, and those at the G1-S transition and at the G2-M transition have great role in detecting and correcting DNA damage [25]. p53 serves as the most important tumor suppressor protein to regulate the cell cycle checkpoints, particularly those at the G1/S transition which can respond to DNA damage and induce G0/G1 arrest [26-28]. Apoptosis induction achieved through the death receptor pathway and the intrinsic or mitochondrial pathway is a vital mechanism in organ development by which the homeostasis is sustained [29]. As abnormal cell proliferation mostly accounts for the pathogenesis for tumor formation, interventions involving with inducing apoptosis and cell cycle arrest are considered to be potentially effective solutions in anti-tumor treatment. Ya-Ling Hsu revealed that the proliferation inhibitory effect and apoptosis promoting activity of SSd were obtained through inducing p53 and the Fas/FasL apoptotic system in human non-small cell lung cancer A549 cell lines. Cell apoptosis and cell cycle arrest induced by SSd in human liver cancer cells was also demonstrated in another study which associated the above effects with p53 stimulation as well as the further induction of Bax and p21 expression [9]. In our study, G0/G1 cell cycle arrest was successfully induced after SSd treatment, with an

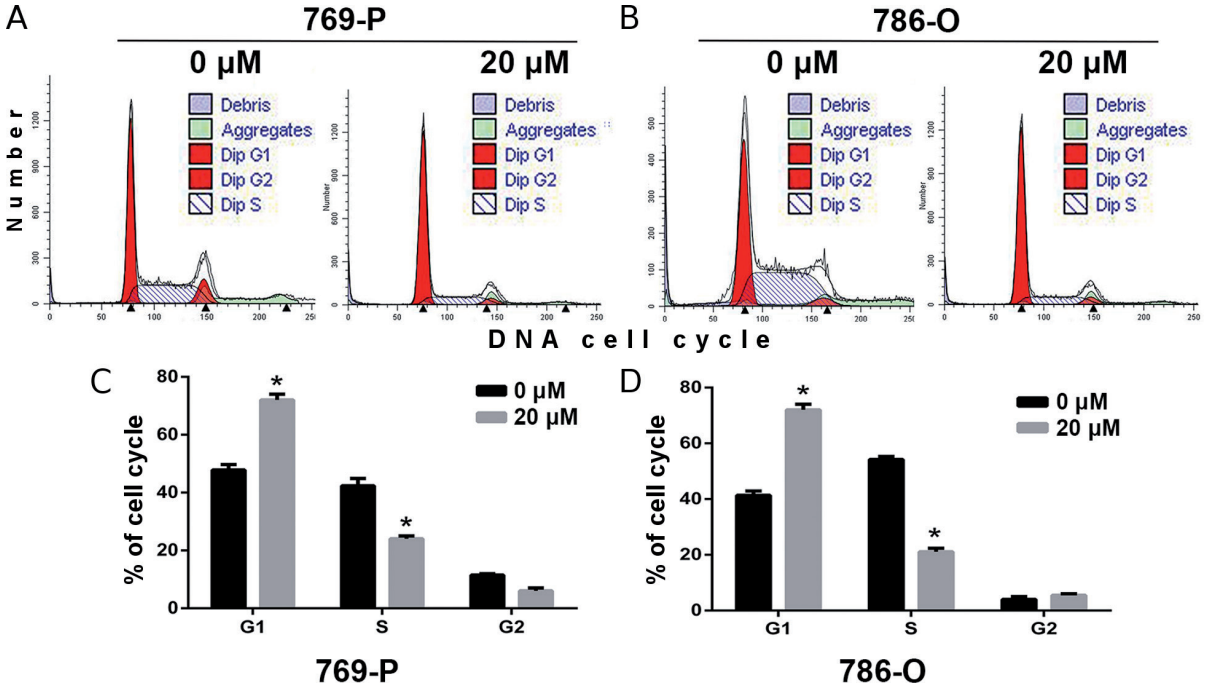

Figure 3. Activity of SSd on cell cycle arrest induction in RCC cell lines. (A) (B) Cell cycle analysis. 769-P and 786-O cells were treated respectively with indicated doses of SSd, fixed and stained with PI, and then analyzed by DNA contents using FACS. (C) (D) Comparison of cell quantification between different stages of the cell cycle. *P<0.05 represents a statistically significant difference.

increased expression of p53 protein, indicating that SSd can inhibit the proliferation of RCC cells.

The molecular mechanism of SSd-induced apoptosis and cell cycle arrest in RCC was also investigated in this study based on the previous finding that EGFR/p38 signaling pathways are those involving in cell growth, differentiation and migration, and are activated in RCC cells. In this study, a down-regulation suggesting apoptosis induction was detected in some cell growth related genes as well as phospho-p38 and its downstream c-Fos, with the exception of phospho-ERK which, however, showed an increase after SSd treatment, which might be explained by its activity on inducing ER stress [30]. The proteins upstream of p38 with cell growth regulation activity such as p-MEK and p-EGFR were also tested, leading to a conclusion that SSd may inhibit cell growth through the EGFR/p38 signaling pathway. The protein lever of EGFR, MEK, p38, as p-EGFR, p-MEK, p-p38, deceased with treatment of SSd, so we can guess that SSd not only decrease phosph level, but also degrade total protein level. EGFR inhibitor AG1478 and p38 inhibitor SB203580 were utilized in the study as positive controls to make a comparison between them and SSd on their cell proliferation inhibitory effects. The results of these tests showed a strong support to the hypothesis that the EGFR/p38 signaling pathway is closely associated with the cell growth inhibitory effect of SSd in RCC cell lines.

In conclusion, the present study demonstrates for the first time that SSd has proliferation inhibitory effects in RCC cells. And its anti-proliferative activity is very likely to be mediated by apoptosis and cell cycle arrest induction via the

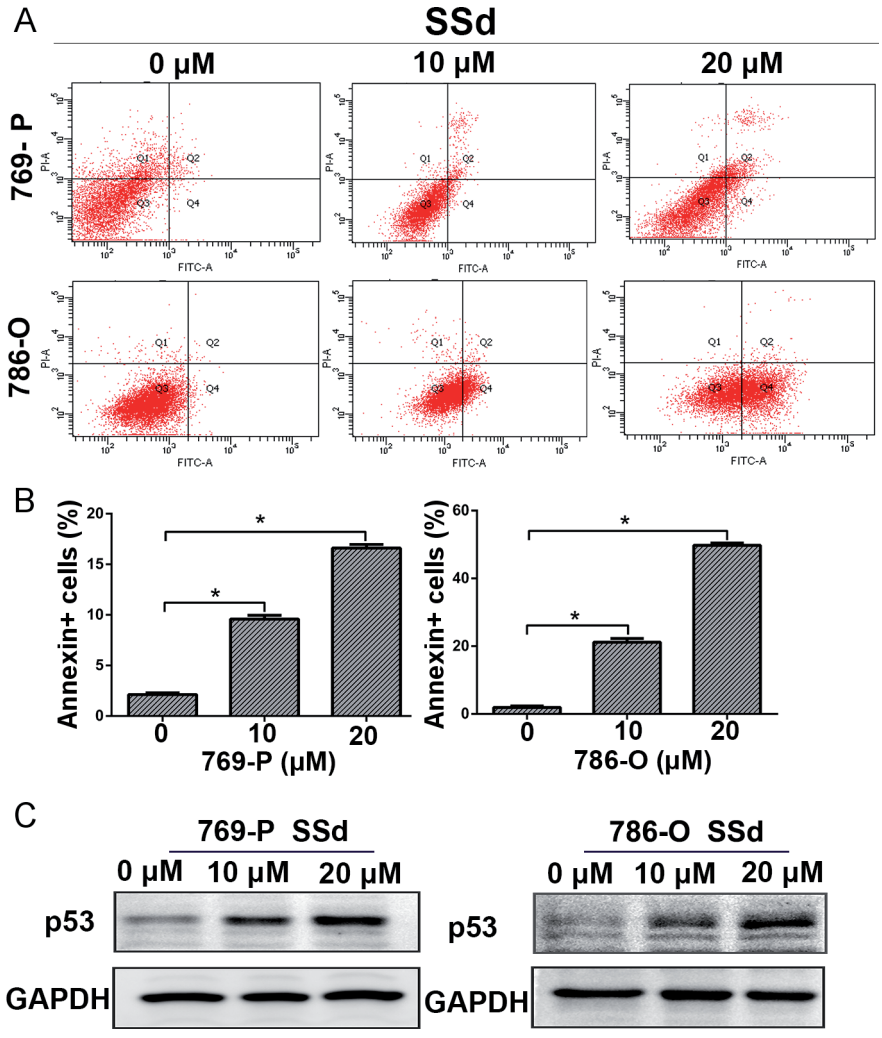

Figure 4. The effect of SSd on apoptosis induction in RCC cell lines. (A) 769-P and 786-O cells were treated respectively with 10 and 20 μM of SSd for 48 h, and then stained with Annexin V and PI for FACS analysis. (B) Comparison of Annexin V positive cell quantification between different dose groups. (C) p53 (1:1,000) analysis using western blotting assay after a similar SSd treatment procedure. *P<0.05 represents a statistically significant difference.

strong inhibitory activation on EGFR/p38 pathways as well as up-regulated expression of p53 protein [31]. The results of this study suggest that SSd could be considered as a promising candidate for further research and clinical use in RCC patients. Certainly, more pre-clinical studies are needed to verify the efficacy and safety of SSd treatment in RCC models, which could serve as the basis for the design of clinical trials in RCC patients.

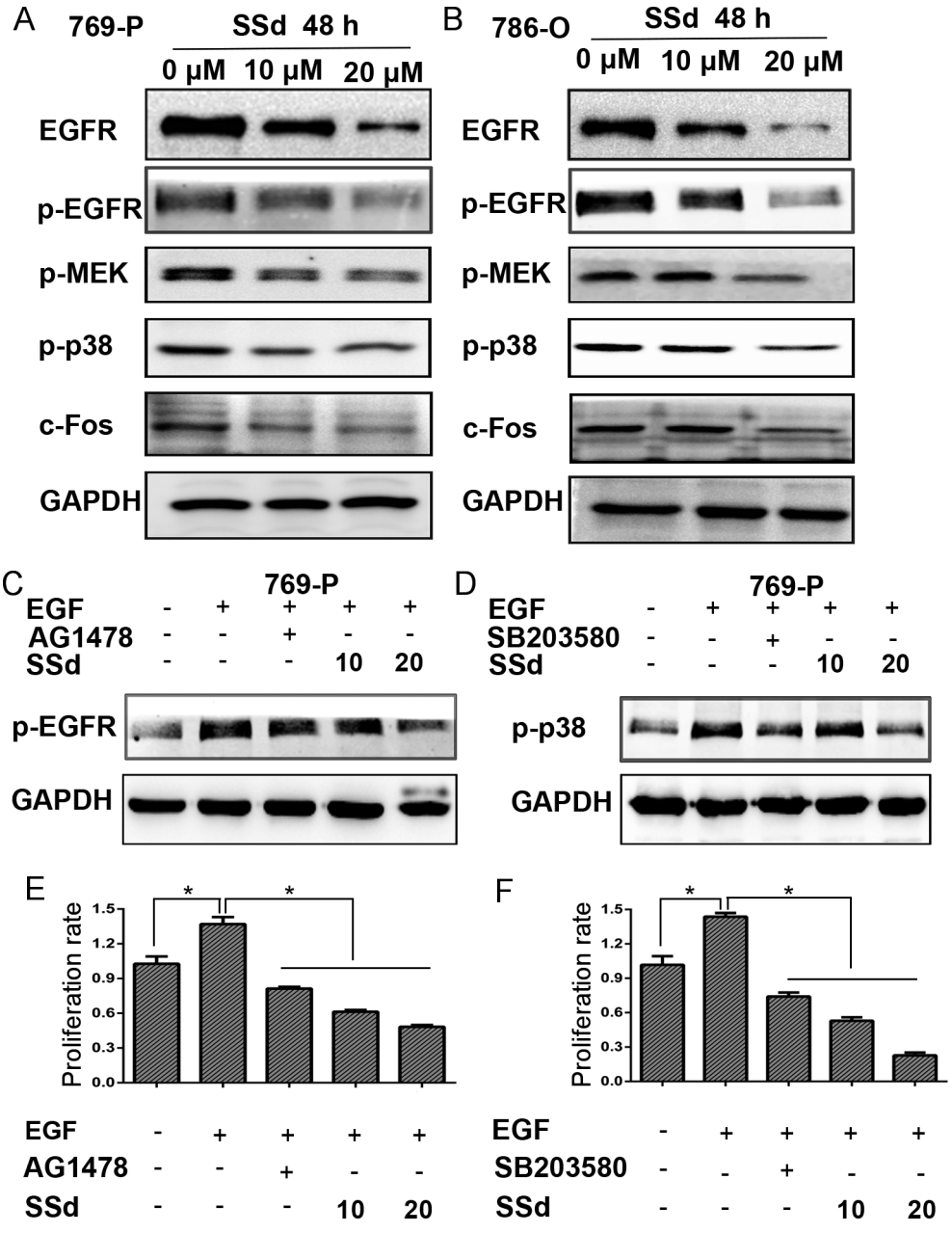

Figure 5. SSd performs its cell growth inhibitory function via the EGFR/p38 MAPK signaling pathway. (A and B) SSd-treated 769-P and 786-O cells were analyzed to determine EGFR, p-EGFR, MEK, p-MEK, p38, p-p38, and c-Fos by western blotting, with β-Actin, GAPDH used as a loading control. (C and D) 769-P cells were treated respectively with different doses of SSd for 48 h, with AG1478 (EGFR inhibitor) or SB203580 (p38 inhibitor) as the positive control; before cell collection, the cells were treated with EGF for 30 min to activate p-EGFR or phospho-p38, which was determined using western blotting. (E and F) Cells were seeded in 24-well plates, and then treated with SSd (2 h) and EGF (48 h) for cell proliferation rate determination by MTT assay, with AG1478 or SB203580 used as the positive control. *P<0.05 represents a statistically significant difference.

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