Kaempferol induces cell cycle arrest and apoptosis in renal cell carcinoma through EGFR/p38 signaling

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Abstract. Kaempferol has been shown to inhibit cell growth, induce apoptosis and cell cycle arrest in several tumors, but not in renal cell carcinoma (RCC). In the present study, we investigated the effects of kaempferol and the underlying mechanism(s) on the cell growth of RCC cells. MTT assay and colony formation assay were used to study cell growth, and flow cytometry was used to study apoptosis and cell cycles in different RCC cells treated with various doses of kaempferol. A significant inhibition on cell growth, induction of apoptosis and cell cycle arrest were observed in 786-O and 769-P cells after kaempferol treatment compared with the control group. Moreover, the results clearly showed that kaempferol causes a strong inhibition of the activation of the EGFR/p38 signaling pathways, upregulation of p21 expression and downregulation of cyclin B1 expression in human RCC cells, together with activation of PARP cleavages, induction of apoptotic death and inhibition of cell growth. Collectively, our results suggest that kaempferol may serve as a candidate for chemopreventive or chemotherapeutic agents for RCC.

Introduction

Renal cell carcinoma (RCC) is one of the most commonly diagnosed urological malignancies in China. Although surgery is the main therapy for RCC, some patients are already considered to have metastatic RCC (mRCC) at time of diagnosis (1). For those particular patients, the treatment relies mainly on systemic therapy including chemotherapy, radiation therapy and immune therapy, but their effects are limited (1,2). RCC expresses multidrug resistance transporters and is refractory to chemotherapy once it becomes metastatic (3). With this concern, novel agents are being developed to target mRCC; one approach to control RCC is growth inhibition wherein the disease is prevented, slowed by the administration of one or more non-toxic naturally occurring or synthetic agents (4).

Kaempferol, a flavonoid, is a yellow compound with a low molecular weight (MW:286.2 g/mol) (5). Kaempferol has been identified in many botanical families, and several epidemiological studies have evaluated the possible association between the consumption of foods containing kaempferol and a reduced risk of developing several disorders (6-8). For anticancer activity, kaempferol induced apoptosis in ovarian cancer (9), oral cavity cancer (10), osteosarcoma (11) and colon cancer (12). Kaempferol was also able to inhibit cell growth (13) and angiogenesis (14,15), which is necessary for solid tumor formation.

Epidermal growth factor receptor (EGFR) is one of the members of the ErbB receptor tyrosine kinase family and plays a critical role in a wide variety of cellular functions, including proliferation, differentiation and apoptosis (16). Several studies have shown that EGFR and other members of their family together with the growth factors that activate them are over-expressed in RCC tissue and cell lines (17,18). Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases. p38 is one of the MAPKs, and could regulate cell proliferation. EGFR could mediate p38 activation (19). In this study, we found kaempferol could functions mainly through the EGFR/p38 pathway to inhibit RCC cell growth.

Materials and methods

Cell culture. Human RCC cell lines (786-O and 769-P) were purchased from ATCC and were maintained in RPMI-1640 containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin. All cells were cultured at 37°C, in a humidified atmosphere containing 5% CO_2.

Reagents. Kaempferol was a gift from Dr Defeng Xu of Changzhou University; it was dissolved in DMSO at 50 mM and stored at -20°C. Antibodies against human Chk1, CDK2,

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p35, c-jun, cyclin B1 and GAPDH were purchased from Santa Cruz Biotechnology, Inc. Antibodies against p-EGFR, EGF, p-MEK, MEK and p-p38 were purchased from Cell Signaling Technology, Inc. AG1478 was from Calbiochem and SB203580 was purchased from Cell Signaling Technology, Inc. Annexin V-FITC Apoptosis Detection kit was from Nanjing Jiancheng Bioengineering Institute.

Cell viability. Cell viability was assessed using a tetrazolium-based assay (MTT assay). One thousand cells in 50 μl of media per well were plated in 96-well plates. Cells treated with or without different doses of kaempferol were incubated for various times, and then incubated with 0.5 mg/ml of MTT at 37°C for 1 h. Subsequently, the supernatant was dropped and dissolved with DMSO. Colorimetric analysis using a 96-well microplate reader was performed at the wavelength of 490 nm. The experiments were performed in triplicate.

Colony formation assay. RCC cells (786-O and 769-P) were respectively seeded in 24-well plates (100 cells/well) and cultured with different doses of kaempferol (50, 100 and 150 μM) for 14 days before staining. The colonies were stained with crystal violet and counted.

Quantitative detection of apoptosis. Cells (786-O and 769-P) were exposed to different doses of kaempferol (50, 100 and 150 μM) for 48 h. The cells were collected and subjected to Annexin V and propidium iodide (PI) staining using an Annexin V-FITC Apoptosis Detection kit, following the protocol provided by the manufacturer. Apoptotic cells were then analyzed by flow cytometry.

Cell cycle detection assay. After cells reached 60-80% confluence, they were treated with different doses of kaempferol (50, 100 and 150 μM). After 48 h, cells were washed twice with PBS and fixed with 70% ethanol for 1 h at 4°C, and then washed with PBS and resuspended with PI solution (0.05 mg/ml) containing RNase, and incubated at room temperature in the dark for 30 min. DNA content was then analyzed using the flow cytometer.

Western blot analysis. Cells were lysed in RIPA buffer (50 mM Tris-HCl/pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 1 mM okadaic acid, and 1 mg/ml aprotinin, leupeptin, and pepstatin) with Protease Inhibitor Cocktail (Roche Inc.). Individual samples (25 μg protein) were prepared for electrophoresis run on 12-15% SDS-PAGE gel and then transferred onto PVDF membranes (Millipore). After blocking the membranes with 5% BSA in PBS for 1 h at room temperature, the membranes were incubated with appropriate dilutions of specific primary antibodies overnight at 4°C. After washing, the blots were incubated with anti-rabbit, anti-mouse or anti-goat IgG HRPs for 1 h. The blots were developed in ECL mixture (Thermo Fisher Scientific Inc.).

Statistical analyses. All statistical analyses were performed using SPSS 16.0. Quantitative data are presented as mean ± SE and the differences among various treatment groups were compared by one-way ANOVA, followed by Dunnett's t-test for separate comparisons. When the comparison involved only
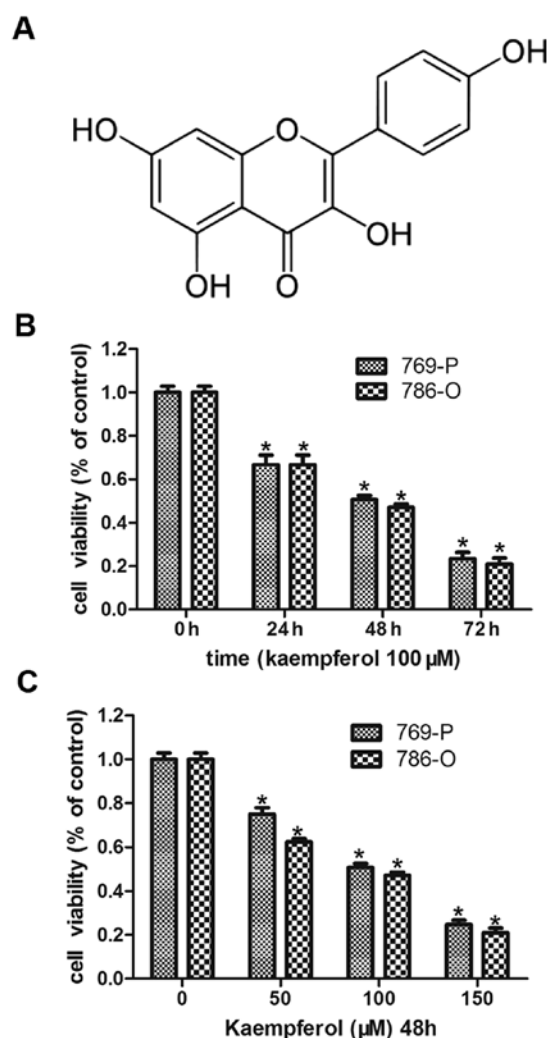

Figure 1. The effect of kaempferol on RCC cell growth. (A) The chemical structure of kaempferol. (B) RCC (786-O and 769-P) cells were treated with 100 μM kaempferol for different time periods (0, 24, 48, and 72 h). (C) RCC (786-O and 769-P) cells were treated with the indicated doses of kaempferol (50, 100 and 150 μM) for 48 h. Cell viabilities were detected by MTT assay. *P<0.05 was considered to indicate a statistically significant difference.

2 groups, Student's t-test was used. P<0.05 was considered to indicate statistically significant differences.

Results

Kaempferol inhibits cell growth and induces cell death in RCC cells. First, we demonstrated the effect of kaempferol (structure shown in Fig. 1A) on the growth of RCC cells (786-O and 769-P), as shown in Fig. 1. Kaempferol treatment inhibited the growth of 786-O and 769-P cells in both a dose- and a time-dependent manner. We fixed its concentration at 100 μM and treated RCC cells for 24, 48 and 72 h, resulting in ~78, 50 and 30% cell survival respectively (Fig. 1B), and then kaempferol treatment at 50, 100 and 150 μM doses resulted in the same tendency decreasing cell survival.

Kaempferol inhibits colony formation of RCC cells. We also tested the effect of kaempferol on colony formation, which is another type of proliferation assay. One hundred of each RCC cell type (786-O and 769-P) were seeded into 24-well plates,
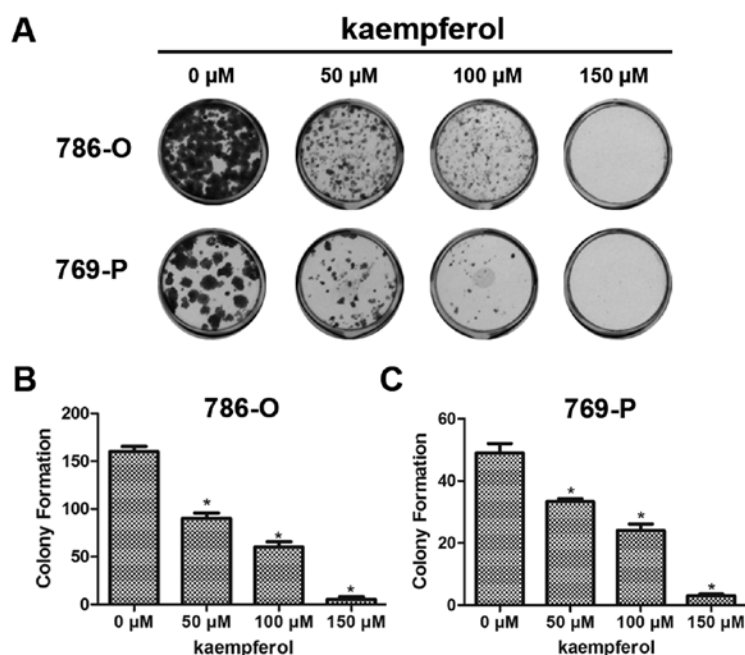
Figure 2. Kaempferol inhibits colony formation of RCC cells. (A) Colony formation assay (24-well). One hundred cells of 786-O or 769-P were seeded into 24-well plates and cultured with kaempferol at different doses (50, 100 and 150 μM) for 14 days. They were then stained with crystal violet and colony numbers were counted. (B and C) The quantification for colony formation. *P<0.05 was considered to indicate a statistically significant difference.
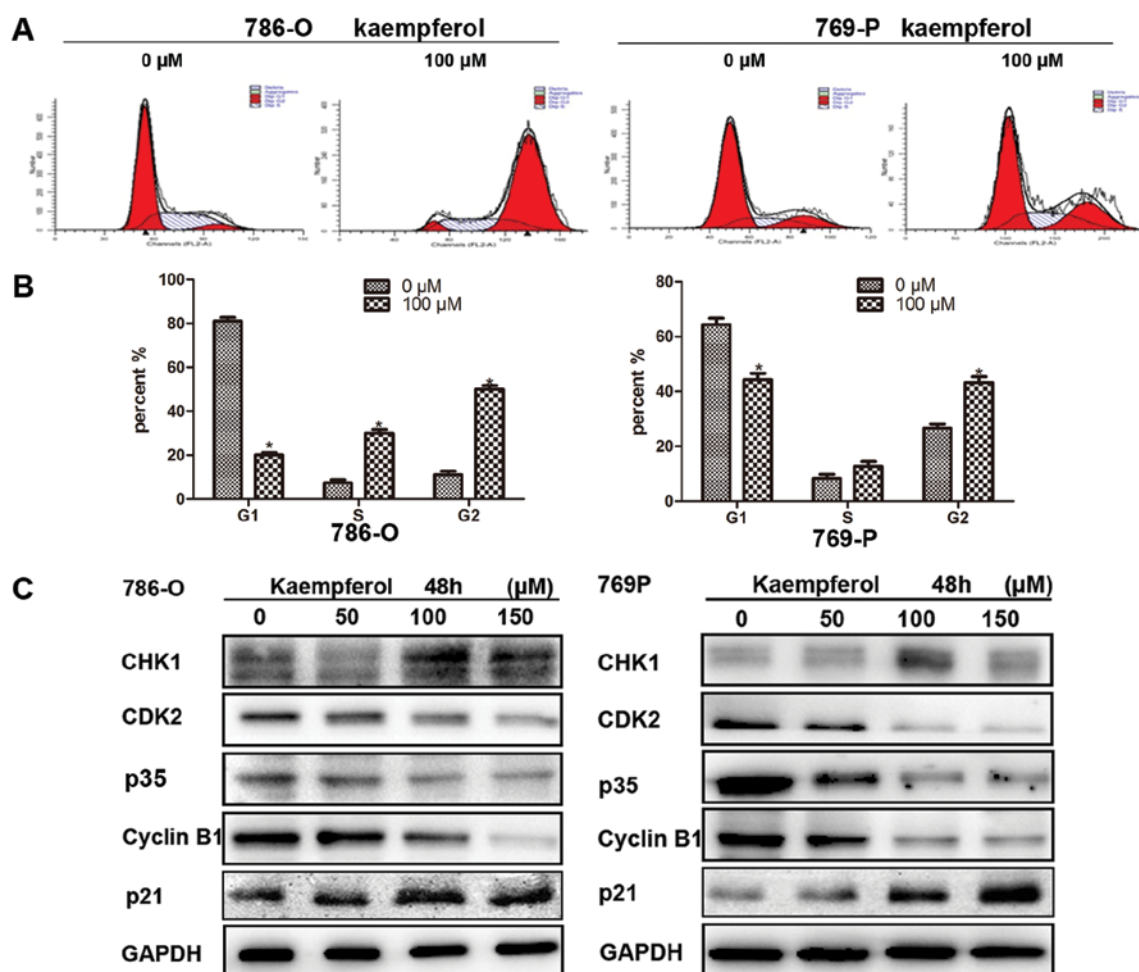

Figure 3. Kaempferol induces RCC cell cycle arrest. (A) Cell cycle analyses. After treating with the indicated doses of kaempferol, cells were collected fixed, and stained with PI, and then DNA contents were analyzed by FACS. (B) The quantification of cell percentage for each cell stage. (C) Cell lysates from 786-O and 769-P were analyzed to detect CHK1, CDK2, p35, cyclin B1 and p21. GAPDH was used as loading control. *P<0.05 was considered to indicate a statistically significant difference.
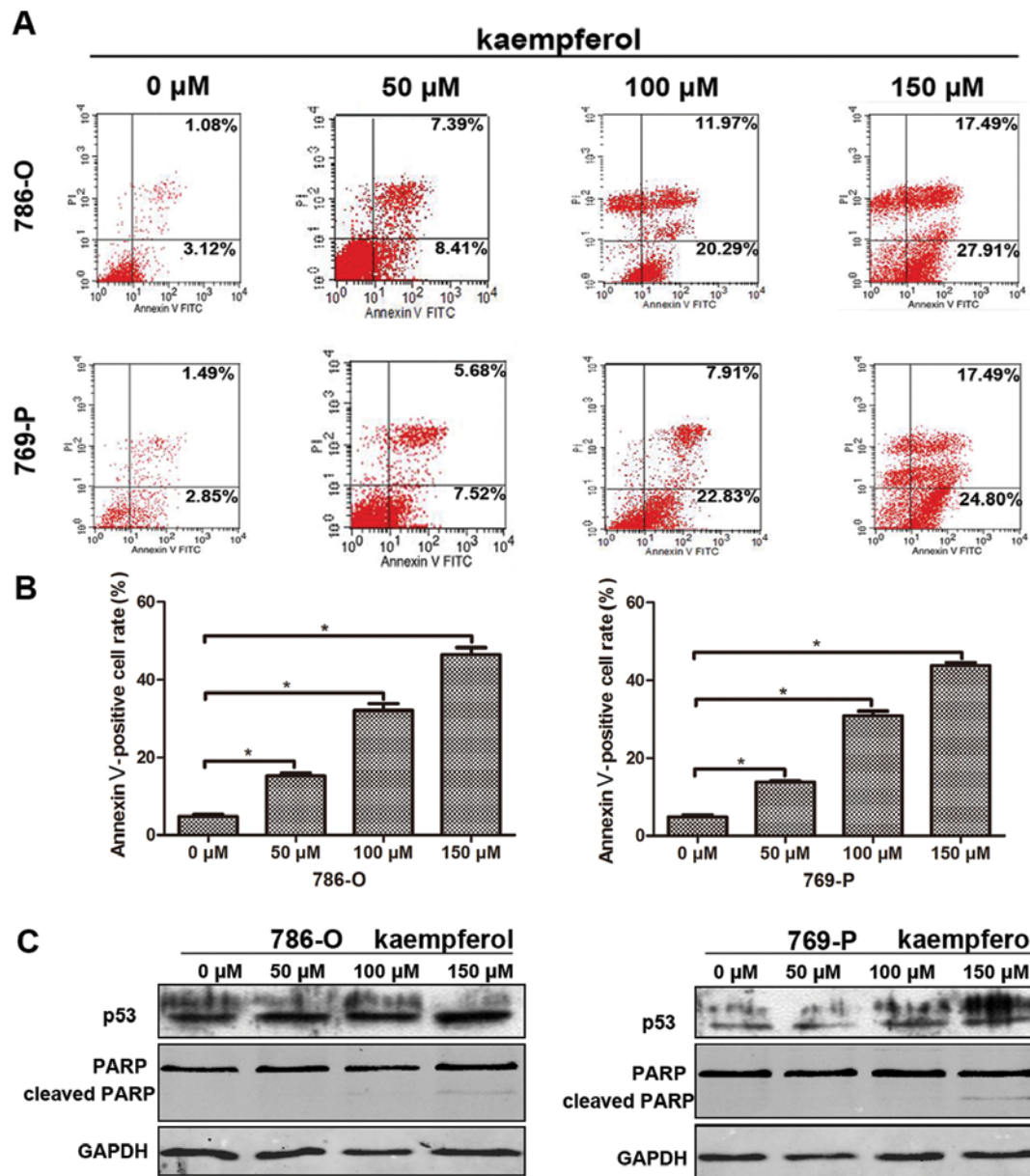
Figure 4. Kaempferol induces RCC cell apoptosis. (A) After treating with the indicated doses of kaempferol (50, 100 and 150 μM) for 48 h, 786-O and 769-P cells were collected and stained with Annexin V and PI, and then analyzed by FACS. (B) The quantification for Annexin V positive cells. (C) After similar treatment, total lysates were analyzed to detect p53 (1:1,000) and cleaved PARP (1:1,000) by western blotting. *P<0.05 was considered to indicate a statistically significant difference.

and cultured with kaempferol at 50, 100 and 150 μM doses for 14 days. As shown in Fig. 2, it is evident that kaempferol significantly inhibited colony formation of both 786-O (Fig. 2B) and 769-P (Fig. 2C) cells.

Kaempferol induces cell cycle arrest in RCC cells. We further detected the effect of kaempferol on cell cycle arrest by flow cytometry. After kaempferol treatment for 24 h at 100 μM, most cells arrested mainly at phase G2-M stage 52.36% in 786-O cell and 43.45% in 769-P cells (Fig. 3A and B). Consistently, we observed that several cell cycle related gene expressions were altered, for example, Chk1 and p21waf1/Cip1 increased, while CDK2, p35 and cyclin B1 decreased in 786-O and 769-P cells after treatment for 48 h with different doses

of kaempferol (Fig. 3C). The results of Fig. 3A-C show that kaempferol induced cell cycle arrest.

Kaempferol induces RCC cell apoptosis. We also investigated the effects of kaempferol on apoptosis in 786-O and 769-P cells after they were treated with kaempferol for 48 h at 50, 100 and 150 μM. The flow cytometry data (Fig. 4A) showed Annexin V positive cells increased after treatment with kaempferol and there are significant differences compared with the control group, with Annexin V positive cells increased to 15.8, 32.26 and 45.4% in 786-O cells, and 13.2, 30.74 and 42.29% in 769-P cells (Fig. 4B). Next, we used western blotting to detect apoptosis related gene expression, and we found p53 and cleaved PARP increased after treatment with kaempferol (Fig. 4C).
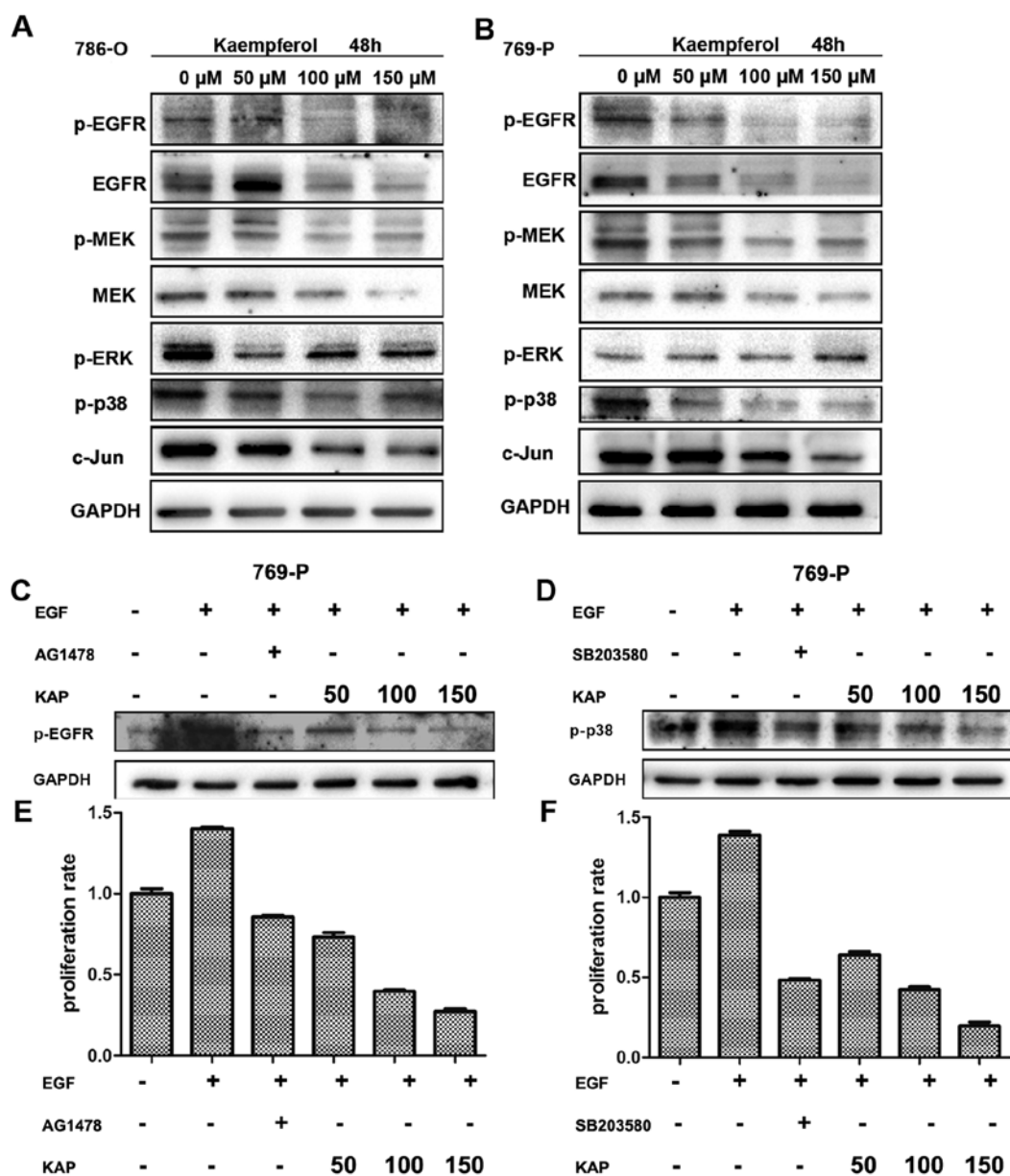
Figure 5. Kaempferol goes through the EGFR/p38 MAPK signaling pathway to inhibit cell growth. (A and B) 786-O or 769-P cells lysates were analyzed to detect p-EGFR, EGFR, p-MEK, MEK, p-p38, p-ERK and c-jun by western blotting. GAPDH was used as a loading control. (C and D) 786-O cells were treated with AG1478 (EGFR inhibitor) or SB203580 (p38 inhibitor) and different doses of kaempferol for 48 h; before cell collection, cells were treated with EGF for 30 min to activate p-EGFR or phospho-p38, and then p-EGFR or phospho-p38 expression was detected by western blotting. (E and F) Cells were seeded in 24-well plates, treated with AG1478 or SB203580 and kaempferol for 2 h. EGF was added for 48 h, and then MTT assay was applied to detect cell proliferation.

Kaempferol inhibits cell growth through the EGFR/p38 MAPK signaling pathway. Since we showed that kaempferol inhibits cell proliferation, the underlying mechanisms were then further investigated. We screened the expression of some growth related genes, and binding kaempferol significantly inhibited the activation of the EGFR pathway, as shown in Fig. 5A and B, EGFR and phospho-EGFR, MEK and phospho-MEK, phospho-p38 and its downstream c-jun, decreased after treatment with kaempferol. However, we also found that the expression of ERK increased, which may be related to stress, since we know kaempferol induces (endoplasmic reticulum) stress.

As is well known, EGFR goes through the MAPK pathway to influence cell proliferation (20). To delineate the growth

inhibition of RCC cells upon kaempferol treatment was mainly through the EGFR/MAPK signaling pathway, we used EGFR inhibitor AG1478 (21) and p38 inhibitor SB203580 (22) to study their effects on cell growth. First, as shown in Fig. 5C and D, kaempferol blocked EGF induced p-EGFR and activation of p38, similar to their inhibitors. This indicated that kaempferol blocked the EGFR/p38 signaling pathway.

We used the MTT assay to detect cell viability of 786-O and 769-P after treatment with EGR as well as EGFR inhibitor AG1478, or p38 inhibitor SB203580 and kaempferol for 48 h, as shown in Fig. 5E and F. Kaempferol significantly inhibited cell growth simulated EGFR inhibitor or p38 inhibitor. Collectively, we suggest kaempferol inhibited cell growth via the EGFR/p38 signaling pathway.

Discussion

Kaempferol has been reported to inhibit the cell growth of several types of cancer and to induce apoptosis. However, in RCC, the effect of kaempferol remains unclear. This study is the first to demonstrate that kaempferol inhibits RCC cell line growth *in vitro*.

As our results show, kaempferol inhibited the cell growth of two different RCC cell lines, 786-O and 769-P. In previous studies, kaempferol was shown to inhibit prostate cancer cell (13), hepatocyte (23) and lung cancer cell (24) growth. This suggests kaempferol has a wide anti-proliferation capacity. In addition, our data showed 50 μM kaempferol has an effect on cell growth, which is μM grade and a relatively low concentration, suggesting it has a low toxicity and is relatively safe; however, *in vivo* studies to test and verify this are required.

Cell cycle progression is tightly controlled by a subfamily of cyclin-dependent kinases (CDKs), the activity of which is regulated by several activators (cyclins) and cyclin-dependent kinase inhibitors (CDKIs) (25). In several other types of cancer, kaempferol was demonstrated to cause G2/M phase arrest (26-29), but in our results we also observed G1/S phase arrest in RCC cell lines, which is associated with p21waf1/cip1 upregulation in RCC cells. Kaempferol could induce DNA damage, increase ATM, and lead to CHK1/2 activation, which has several effector substrates, including cyclin B1.

An aberrant activation of several growth signaling pathways and evasion of apoptosis have been recognized as hallmarks of cancer cell survival and growth including RCC (30,31) cells and the inhibition of growth mediated by blocking the EGFR/p38 pathway. We screened some growth related genes and found phospho-p38 and its downstream c-jun was downregulated, but, notably, phospho-ERK increased after treatment with kaempferol in our study, which may mediate cell apoptosis (24) or may be related to cell stress related with ER stress (32). Huang *et al* have already found that kaempferol could induce ER stress in osteosarcoma cells (11), and ERK acts as a stress consequence. Next, we detected some candidates upstream of p38, such as c-Met (20), and EGFR (33), which could lead to DNA synthesis and cell proliferation. Therefore, we suggest that kaempferol may function through the EGFR/p38 signaling pathway to inhibit cell growth. We utilized EGFR inhibitor AG1478 and p38 inhibitor SB203580 to confirm whether kaempferol has the same effects with inhibitors. Our results support the hypothesis that the EGFR/p38 signaling pathway is involved in growth inhibition by kaempferol in RCC.

In conclusion, the present study showed that kaempferol causes a strong inhibition of the activation of EGFR/p38 signaling pathways, upregulation of p21 expression, and down-regulation of cyclin B1 expression in human RCC cells together with activation of PARP cleavages, induction of apoptotic death, and inhibition of cell growth. Further studies are required to establish the efficacy of kaempferol in pre-clinical RCC models, which may be useful in supporting a rationale for a clinical trial in RCC patients.

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