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Ginkgetin induces apoptosis in 786-O cell line *via* suppression of JAK2-STAT3 pathway

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ABSTRACT

Objective(s): Renal cell carcinoma (RCC) is insensitive to conventional chemotherapy. Ginkgetin effectively treats several carcinoma cells. However, little is known about effects of Ginkgetin on RCC. In the present study, using 786-O cells, we evaluate whether Ginkgetin exerts anticancer effects against RCC.

Materials and Methods: 786-O cells suspended in the medium containing Ginkgetin were cultured for 24 hr to 72 hr, and then MTT assay was used to study cytotoxic effect of Ginkgetin. Apoptosis in 786-O was measured by an FITC Annexin apoptosis detection kit. Protein expression was detected by Western blotting. 786-O cells with active Janus kinase 2 (JAK2)-Signal transducer and activator of transcription 3 (STAT3) were prepared by stimulant of interleukin-6 (IL-6), whereas 786-O cells with deactivated STAT3 were produced by small interfering RNA (siRNA) STAT3.

Results: Ginkgetin suppressed the growth of 786-O in dose and time-dependent manners with IC_{50} values of 7.23 μM. Ginkgetin induced apoptosis of 786-O cells and increased the levels of caspase-8, caspase-9, and caspase-3. Additionally, Ginkgetin treated 786-O cells showed decreased levels of JAK2 and phosphorylated-STAT3 whether or not IL-6 was pretreated. Interestingly, pretreatment of siRNA STAT3 exerted inhibitory effects on the growth of 786-O cells, and the observation could be further reinforced after the Ginkgetin treatment.

Conclusion: Our results indicate Ginkgetin possesses obvious inhibitory effects on the proliferation of 786-O, and this effect is probably due to its inhibition of JAK2/STAT3 pathway. Our findings imply Ginkgetin is a potential therapeutic medicine for RCC.

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Introduction

Renal cell carcinoma (RCC) is a common malignant disease of the human genitourinary system and comprises 90% of all kidney cancers (1). It is estimated the global age-standardized incidence rate was 4 per 100,000 population per year. Developed countries, such as Western Europe and North America, have a higher incidence of RCC, whereas in developing countries the rates are lower (2). At present, surgical intervention is the first option for treatment of RCC and causes limited benefits in patients mainly due to metastatic tumors, which accounts for up to the 25%–30% of the patients upon diagnosis of RCC (3). Additionally, RCC is highly resistant to traditional cytotoxic agents and radiotherapy (4). Therefore, overall survival of RCC patients is still very poor and a safe and effective anti-RCC drug is urgently needed.

Janus kinase 2 (JAK2)-Signal transducer and activator of transcription 3 (STAT3) signaling pathway plays an important role in the occurrence and development of tumor cells (5). STAT3 is an oncogene with several down-stream anti-apoptotic molecules, such as Bcl-2, Bcl-XL and others (5). When cells receive the pro-proliferative stimulation, STAT3 will be activated by JAK2, and the activated STAT3 modulates the cell survival and proliferation via its down-stream targets. In recent studies, abnormal JAK2-STAT3 signaling has been observed in several kinds of tumors, such as lung, gastric, and prostate cancers (6-8). Accordingly, JAK2-STAT3 could serve as a potential target for cancer therapy.

Ginkgetin is an active bioflavonoid constituent derived from Ginkgo biloba leaves and possess a variety of pharmacological properties, including anti-inflammatory, anti-virus, neuroprotective, and anti-tumor (9-12). It was first reported in 2000 that Ginkgetin induces apoptosis of ovarian adenocarcinoma cell line (OVCAR-3), cervical carcinoma (HeLa) and foreskin fibroblast (FS-5), with IC_{50} values of 3.0, 5.2, and 8.3 mg/ml, respectively. In 2013, Ginkgetin was further confirmed to inhibit prostate cancer cells and

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medulloblastoma proliferation in vitro (12-14). Conclusively, previous studies disclose Ginkgetin induced apoptosis in tumor cells is via activation of caspases, deactivation of survival genes, and inhibition of Wnt signaling pathway (12-14). To date, to the best of our knowledge, neither the precise effect of Ginkgetin against RCC nor the underlying molecular mechanisms have been well established. Therefore, in the present study, we aim to investigate whether Ginkgetin shows inhibitory effects on the growth of RCC cells, and then study whether Ginkgetin has inhibitory effects on the JAK2/STAT3 pathway in RCC cells.

Materials and Methods

Cell lines and culture

This study was approved by the ethics committee of Ningbo Urology and Nephrology Hospital, Zhejiang province, PR China. The human renal cell line, 786-O, was purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified eagle medium (DMEM, HyClone) in the presence of 10% fetal bovine serum (Gibco, USA) under the condition of 5% CO_2 at 37 ˚C. Ginkgetin (purity>95%, Nanjing PuYi biological technology Co, LTD, Nanjing, China) solution was disinfected by a 0.22 μm minipore membrane, and then stored at –20 ˚C for further use.

Cell viability assay

Survival evaluation of 786-O cells was performed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich , St. Louis, MO) assay(12-13). Briefly, 786-O cells were seeded in 96-well plates at a concentration of $1x10^4$ cells/well and received Ginkgetin treatment with different concentrations (2 μM–64 μM) or different incubation times (24 hr, 48 hr, and 72 hr). Four hours before the end of the study, 20 μl MTT solutions were added, followed by dissolution in dimethyl sulfoxide. Cell survival was quantified by measuring the OD value at 450 nm (Microplate Reader, Bio-Rad, CA, USA). Moreover, non-treated cell viability was set as 100%. Inhibition rate = (1-OD value of treatment group/OD value of control group) x100%.

Apoptosis assay

Pro-apoptotic activity was measured using an AnnexinV-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, San Jose, CA). 786-O cells were plated in 6-well plates ($5x10^4$ cells/well) and maintained for 48 hr in the presence or absence of Ginkgetin treatment. Following that, AnnexinV- FITC and propidium iodide (PI) were added to the culture medium according to instructions offered by the manufacturer. Finally, each sample was analyzed by a FACS Calibur cytometer (BD Bioscience, Franklin Lakes, NJ, USA). Three independent tests under the same conditions were performed.

Caspase activity assay

The activities of caspase-3, caspase-8 and caspase-9 were performed by a colorimetric protease assay kit according to instructions offered by the manufacturer (Keygen Biotech Co., Ltd., China). Briefly, 786-O cells were treated with Ginkgetin (4 μM, 8 μM, and 16 μM) for 48 hr, and then the cells were lysed by lysates, and the protein levels in lysates were measured by a bicinchoninic acid kit (Thermo Fisher Scientific, Waltham, MA, USA). Following that, the protein was incubated with caspase-3 (DEVD -pNA), caspase-8 (IETD-pNA) or caspase-9 (LEHD-pNA) colorimetric substrate at 37ºC for 4 hr. The cleavage of peptide substrate was monitored at 405 nm by a 96-well microplate reader (Bio-Rad, CA, USA).

Cell lysis and Western blotting

The cells were grown to 90% confluence and then treated with Ginkgetin (4 μM, 8 μM) for 48 hr. Following that, the cells were treated with detergent lysis buffer (50 mM Tris-HCl; 150 mM NaCl; 1% Triton X-100; 1 mM ethylenediaminetetraacetic acid;10 mM NaF; 1 mM Na_3VO_4), and the protein was extracted. For detecting the expression of JAK2, STAT3, and phosphorylated-STAT3 (p-STAT3), a Western blotting was used (5). Briefly, a total of 50 μg extracted protein was subjected to SDS-PAGE and transferred to the PVDF membrane. The target proteins were bio-labeled by primary antibodies against JAK2 (1:1000; Santa Cruz, CA, USA), STAT3 (1:2000; Santa Cruz, CA, USA), and p-STAT3 (1:2000; Santa Cruz, CA, USA) overnight, and then re-labeled by the peroxidase-conjugated secondary antibody for 1 hr at room temperature. Finally, protein bands were visualized by the enhanced chemiluminescence kit (Millipore). The protein levels were analyzed by image software and normalized to the β-actin levels.

Small interfering RNA and transient transfection

The small interfering RNA (siRNA) targeting human STAT3 and control siRNA were from Santa Cruz. 786-O cells were seeded in 6-well plates with the density of $1×10^6$/well. The cells were transfected with STAT3 using Lipofectamine 2000 (Invitrogen) according to the instructions. After transfection, the cells were maintained in plates for 24 hr and then received Ginkgetin treatment (8 μM) for another 48 hr. Finally, the cells were used for MTT and apoptosis assay.

Statistical analysis

All values are expressed as means±standard deviations (SD). Dose–response curves were created using GraphPad Prism (version 5.0, GraphPad Software

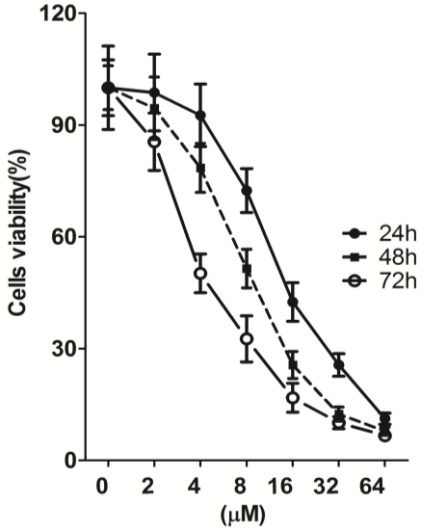

Figure 1. Inhibitory effects of Ginkgetin on the cell viability of 786-O cells. Cell viabilities were detected at different time points by MTT assay (n=5)

Inc, La Jolla, CA), and the half maximal inhibitory concentration (IC_{50}) values were calculated by the Probit procedure (SPSS software, version 15.0). Statistical analysis was performed using Student's t-test (SPSS software, Version 15.0) with $P<0.05$ considered significant.

Results

Ginkgetin inhibited 786-O proliferation

Figure 1 shows that Ginkgetin repressed proliferation of 786-O in dose- and time-dependent manners, and the IC_{50} of Ginkgetin against 786-O for 48 hr was 7.23 μM.

Ginkgetin induced apoptosis in 786-O

Figure 2. Indicates 786-O cells pretreated with Ginkgetin (4 μM, 8 μM, and 16 μM) showed higher levels of apoptosis than those of untreated cells (Figure 2B, $P<0.05$). These results suggest that Ginkgetin could enhance 786-O cell apoptosis.

Ginkgetin induced caspase cascade in 786-O

Figure 3. Indicated that Ginkgetin effectively promoted the activation of caspase-9, caspase-8, and caspase-3($P<0.01$). These results suggest activation

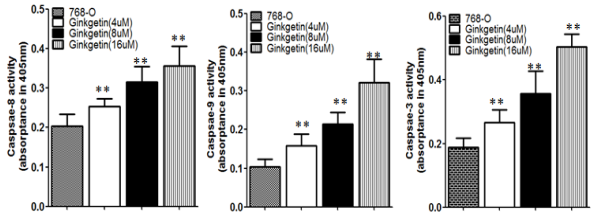

Figure 3. Ginkgetin activated caspase cascade in 786-O cells. Data represent mean±SD (n=5). $^{**}P<0.01$ as compared to 786-O cells without Ginkgetin treatment

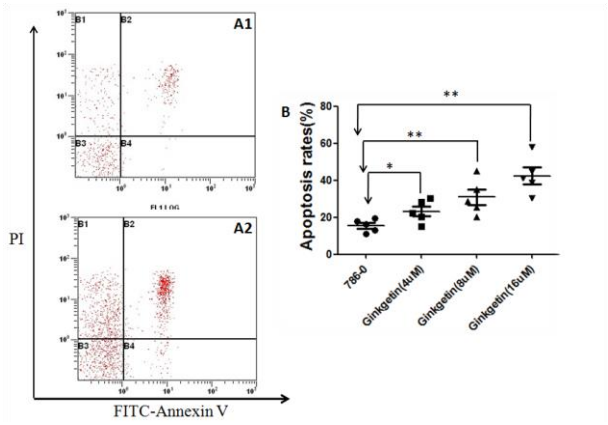

Figure 2. Ginkgetin induced apoptosis of 786-O *in vitro*. (A1): 786-O cells without Ginkgetin treatment; (A2): 786-O received Ginkgetin treatment (8 μM). (B): apoptosis rates of pretreated or untreated 786-O cells. Data represent mean±SD (n =3). $^{*}P<0.05$, $^{**}P<0.01$ as compared with 786-O cells without Ginkgetin treatment

of the caspase cascade was involved in Ginkgetin induced apoptosis.

Ginkgetin enhanced apoptosis of 786-O via blocking the JAK2/STAT3 pathway

Figure 4 shows that 786-O cells pretreated with IL-6 exhibited a notable increase in the expression of JAK2 and p-STAT3 compared with those without IL-6 treatment (Figure 4B-4C, $P<0.01$). However, Ginkgetin treatment obviously reduced the levels of JAK2 and p-STAT3 whether or not IL-6 was pretreated (Figure 4B-4C, $P<0.01$). As seen in Figure 5, pretreatment of siRNA STAT3 significantly reduced the STAT3 expression, inhibited the proliferation and promoted apoptosis in 786-O cells (Figure 5B-5D, $P<0.01$). Moreover, we observed Ginkgetin treatment reduced STAT3 expression, decreased viability and enhanced apoptosis in 786-O cells as compared to the cells treated with siRNA alone (Figure 5B-5D, $P<0.01$). These results suggest inhibition of JAK2/STAT3 pathway by Ginkgetin contributes to the apoptosis in 786-O cells.

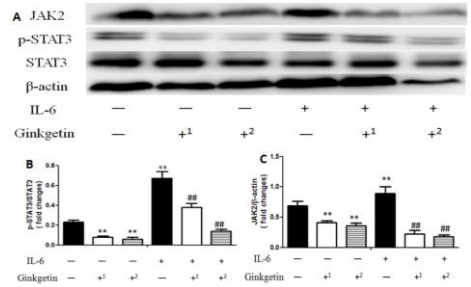

Figure 4. Ginkgetin down-regulated Janus kinase 2/signal transducer activator of transcription 3 pathway in 786-O cells. (A): representative protein bands of JAK2, p-STAT3, and STAT3; (B): p-STAT3 /STAT3 ratios; (C): JAK2/β-actin ratios. Data were presented as mean±SD (n=5). $^{**}P<0.01$ as vs 786-O cells (IL-6-/Ginkgetin-); $^{\#\#}P<0.01$ as vs 786-O cells (IL-6+/Ginkgetin-); IL-6 (15 ng/ml, 48 hr) were shown as "+"; Ginkgetin(4 μM, 8 μM) were shown as "+1" and "+2", respectively. Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3)

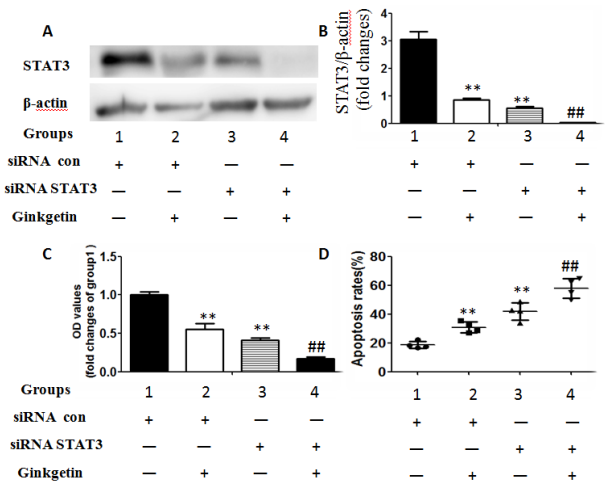

Figure 5. Ginkgetin suppressed the growth of signal transducer activator of transcription 3 small interfering RNA transfected 786-O cells. (A): representative protein bands of STAT3; (B): STAT3/β-actin ratios; STAT3 siRNA significantly enhanced the cytotoxicity of Ginkgetin(8 μM, 48 hr) in 786-O cells, as indicated by the cell viability (C) and apoptotic rates (D). Data was presented as mean±SD (n =5). $^{**}P<0.01$ as compared to group 1; $^{\#\#}P<0.01$ as compared to group 3. Signal transducer and activator of transcription 3(STAT3), small interfering RNA (siRNA)

Discussion

In previous studies, Ginkgetin was reported to inhibit the proliferation of ovarian adenocarcinoma cells (OVCAR-3, IC_{50}:3.0 mg/ml), cervical carcinoma cells (HeLa, IC_{50}:5.2 mg/ml), and foreskin fibroblast (FS-5, IC_{50}:8.3 mg/ml) (12-13). In the present study, the anti-tumor activity of Ginkgetin was investigated using 786-O RCC cells. It was first shown that Ginkgetin significantly inhibited the growth of 786-O cells *in vitro* with IC_{50} 7.23 μM, and this result is similar to previous investigations (12-13). Although the precise mechanism of growth inhibitory effect is unclear, a lot of papers have declared that apoptosis is involved. In the present study, it was observed that Ginkgetin (4–16 μM) significantly induced apoptosis in 786-O cells within 48 hr of treatment. Interestingly, the other important finding of this work is that Ginkgetin prefers to induce apoptosis in the late-stage rather than the early stage, and therefore, further study should be performed to reveal it. Moreover, it is well acknowledged that caspase-9 is a key protein involved in intrinsic apoptosis pathway, while caspase-8 plays a central role in the extrinsic pathway (15). Pro-apoptotic activity of caspase-9 and caspase-8 are both dependent on the activation of their down-stream signal molecule, caspase-3. To study the mechanism that mediates Ginkgetin induced apoptosis, activities of caspase-8, caspase-9, and caspase-3 were assayed in Ginkgetin-treated cells. Interestingly, we observed that Ginkgetin treatment resulted in increased levels of caspase-8, caspase-9, and caspase-3, which imply Ginkgetin triggers extrinsic and endogenous apoptotic pathways simultaneously.

STAT3 is a transcription factor in the cytoplasm. STAT3 is activated by JAK2 through phosphorylation of its tyrosine residues. The binding of cytokines to the JAK2 bonded cytokine receptors induces the activation of JAK2. Activation of STAT3 (5-8) triggers anti-apoptotic proteins to modulate cell survival and proliferation. Accumulating evidence has underscored the critical role of JAK2/STAT3 in modulating the viability and growth of cancer cells. It has been reported that tissue biopsies from patients with colon carcinoma reveal enhanced STAT3 signaling (16). Recently a paper reported about 40% mutants in STAT3 were observed in patients with large granular lymphocytic leukemia (17). On the other hand, in studies of carcinoma cells, blocking expression of STAT3 by siRNA usually causes the enhanced apoptosis of cancer cells and decreases their growth (18). Of note, treatment of JAK2 with siRNA markedly reverses paclitaxel resistance in human ovarian cancer cells, and pretreatment with siRNAs targeting JAK2 prevents gastric epithelial cells from increasing proliferation and migration (19-20). In the studies of RCC, several studies found inhibition of JAK2/STAT3 pathway decreases RCC growth and promotes its apoptosis (21, 22). Accordingly, these results demonstrate normalization of the JAK2/STAT3 pathway in RCC is a useful therapeutic strategy. However, a large number of STAT3 inhibitors have been reported, and there is a paucity of selective small molecule STAT3 targeting agents under clinical development.

In this paper, we speculate Ginkgetin exerts growth suppressive effects in 786-O *via* modulating the JAK2/ STAT3 pathway. It should be noted that Ginkgetin selectively suppressed the growth of prostate cancer cells, lung cancer cells and osteosarcoma cells with activated STAT3 rather than the cells without STAT3 activation, such as PC-3 and LNCap cells (23-25). The earlier investigations observe Ginkgetin inhibits expression and function of STAT3 *via* interfering with phosphorylation at tyrosine 705 and reducing its nuclear localization. Ginkgetin was also shown to inhibit the binding of STAT3 to the DNA and has no effects on STAT1 or STAT5 phosphorylation (23). However, in our study, it was observed that Ginkgetin treatment not only decreased expression of p-STAT3 and STAT3 but also reduced the level of JAK2. Accordingly, based on our findings and the previously published reports, it can be concluded that Ginkgetin has inhibitory effects on JAK2/STAT3 pathway *via* several targets rather than STAT3 alone. Moreover, it can be suggested that Ginkgetin may be a natural STAT3 inhibitor and designing STAT3 inhibitors based on the chemical structure of Ginkgetin may be a useful strategy. It is noteworthy that Interleukin-6 (IL-6) is an activator

of the JAK2/STAT3 pathway that can induce oncogenesis (26), and therefore, we used IL-6 as a positive stimulant. In an earlier study, IL-6 induced p-STAT3 activation in DU-145 and LNCap cells, and Ginkgetin therapy resulted in 31.6% reduction in the level of p-STAT3 (23-25). However, IL-6 seems to have no effects on the expression of STAT3. Our results showed similar results as evidenced by 47.1% decrease in the expression of p-STAT3 through Ginkgetin treatment. These observations indicate that Ginkgetin could repress the IL-6 mediated activation of JAK2/STAT3. Moreover, just as described previously, JAK2 could activate the other down-stream molecules that induce RCC proliferation, such as PI3K (27-28). To better understand the role of STAT3 in Ginkgetin-induced apoptosis of 786-O, siRNA targeting STAT3 was used to knockdown STAT3 expression in 786-O cells and then measured proliferation and apoptosis of RCC. Interestingly, we found 786-O cells transfected with STAT3 siRNA displayed observably reduced levels of growth and enhanced apoptosis rates. Ginkgetin treatment further reduced STAT3 expression and inhibited cell viability in 786-O cells. These findings indicate depletion of STAT3 has synergistic anti-tumor effects with Ginkgetin, and Ginkgetin might possess other pro-apoptotic mechanisms that differ from targeting STAT3.

Conclusion

Ginkgetin markedly exhibits a pro-apoptotic effect on 786-O cells. Additionally, we find the reduced JAK2/STAT3 pathway contributes to the Ginkgetin induced apoptosis in 786-O cells. These results indicate Ginkgetin is a promising therapeutic drug for RCC patients.

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Conflict of interest

The authors declare that they have no conflict of interest

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