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Effect of ginsenoside Rg3 on proliferation and apoptosis of 786-0 cells and AktmTORSTAT3 signaling in renal carcinoma

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Abstract

Surgery is currently the major approach treating kidney cancer, which is insensitive to chemo-/radio-therapy. However, due to the limitation of current diagnostic technique, certain number of patients has lost the opportunity of surgery. Therefore the searching for novel effective treatment approach is of critical importance. Ginsenoside Rg3 can inhibit proliferation and induce apoptosis of various tumor cells. It effect in kidney cancer, however, is still lacked. This study thus treated kidney cancer cell line 786-O with ginsenoside Rg3 to illustrate its effect on cell proliferation and apoptosis, and investigated possible mechanism. Gradient concentrations of ginsenoside Rg3 was used to treat 786-O cells, whose proliferation was measured by CCK8 assay. The apoptotic rate was quantified using flow cytometry. Western blotting was used to measure protein expression levels of p-Akt, p-mTOR and p-STAT3. Ginsenoside Rg3 significantly inhibited proliferation of kidney cancer cell 786-O in a dose- and time-dependent manner: higher concentration and longer treatment time caused more significant inhibition on cell proliferation. Similar patterns also occurred for the induction for cell apoptosis by ginsenoside Rg3. The expression levels of p-Akt, p-mTOR and p-STAT3, however, were decreased with higher dosages of ginsenoside Rg3, and were negatively correlated with apoptotic ratio. Ginsenoside Rg3 can exert anti-tumor role via inducing apoptosis and inhibiting proliferation of 786-O cells, possibly via the blocking of Akt/mTOR/STAT3 pathway.

Keywords: ginsenoside Rg; kidney cancer 786-O cell; cell apoptosis; cell proliferation.

Practical Application: Our study shows that Ginsenoside Rg3 can exert anti-tumor role via inducing apoptosis and inhibiting proliferation of 786-O cells. However, whether it possess the same effect in patients remains unclear and requires further investigations.

1 Introduction

Renal carcinoma is one common malignant tumor in urinary system, and is the second popular urinary cancer, occupying about 3% of total cancer cases in adults (Jemal et al., 2010). Due to the insensitive nature of kidney cancer against radio- or chemo-therapy, surgery occupies the most important part of treatment. However, due to the occurrence of distal metastasis of patients at the time of diagnosis, surgery may not achieve satisfactory effect on early stage renal carcinoma. Ever with complete removal during surgery, there were still 20% ~ 40% patients with post-operative reoccurrence or metastasis (Amato, 2005). Therefore the establishment of novel treatment method is of critical importance for improving survival rate of patients.

Ginsenoside Rg3 is one chemical compound extracted from Chinese herb ginseng, and has pluripotent functions including improving body immune function and facilitating local blood circulation. In addition, ginseng is consumed as a food and health supplement. Study has found the inhibition of tumor cell growth and induction of apoptosis by ginsenoside Rg3 (Luo et al., 2015; Zhang et al., 2015), in addition to the inhibition of cell invasion, migration, infiltration and tumor angiogenesis (Kim et al., 2012, 2014b; Lee et al., 2015; Liu et al., 2009). Moreover, ginsenoside also significantly improve the sensitivity of chemotherapy (Liu et al., 2009; Wang et al., 2015). All those previous studies have led to various studies about anti-tumor role of ginsenoside Rg3. Current studies, however, were limited in pulmonary carcinoma (Park et al., 2011; Wang et al., 2015), breast cancer (Kim et al., 2013, 2014a), pancreatic carcinoma (Guo et al., 2014; Jian et al., 2009) and gastric cancer (Kim et al., 2011). No study has been performed regarding the effect of ginsenoside Rg3 on renal cell carcinoma. This study thus selected 786-O cell line, which was treated with different concentrations of ginsenoside Rg3 to detect cell survival rate and apoptosis, in an attempt to illustrate the effect of ginsenoside Rg3 on human renal cell carcinoma cells.

Serine/threonine protein kinase (Akt) can regulate cell proliferation and/or differentiation via activating relevant substrates. The mammalian target of rapamycin (mTOR) was a type of serine/threonine protein kinase with highly conserved sequence. It is directly regulated by Akt and can be phosphorylated by active Akt (Asnaghi et al., 2004). Signal transducers and activator of transcription 3 (STAT3) can be easily phosphorylated by activate mTOR kinase (Wang et al., 2008). Akt/mTRO/STAT3 pathway signal pathway is critical in cellular growth and metabolism (Wullschleger et al.,

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2006; Zhang et al., 2011). Study has found the close correlation between Akt/mTOR/STAT3 signal pathway and the occurrence and progression of renal cell carcinoma. For example, mTOR inhibitor can suppress the growth of kidney cancer 786-O cells and induce their apoptosis (Li et al., 2013). Therefore this study applied ginsenoside Rg3 to treat 786-O cells, whose expression of p-Akt, p-mTOR and p-STAT3 proteins was examined, in order to explore the possible mechanism of ginsenoside Rg3 on renal cancer.

2 Materials and methods

2.1 Reagents

Kidney cancer cell 786-O, fetal bovine serum (FBS) and RMPI1640 medium were purchased from Gibco (US). Ginsenoside Rg3 was purchased from Yatai Pharma. (China). CCK8 test kit was produced by Toyobo (Japan). Cell apoptosis kit was provided by Beyotime (China). Total protein extraction kit was produced by BestBio (China). Coomassie brilliant blue was purchased from Meiji Bio (China). SDS-PAGE, PBST buffer, electrophoresis approach and GIS-2020D gel imaging analysis system were all products of Sigma (US). Antibodies against p-Akt, p-mTOR, p-STAT3, and GAPDH were all provided by Abcam (US).

2.2 Kidney cancer 786-O cell culture

Renal cell 786-O was incubated in RMPI1640 medium containing 10% FBS with streptomycin and penicillin (100 U/mL each) in a humidified chamber with 5% CO_2 at 37 °C. Cells at log-phase were rinsed twice in PBS, and were digested in 0.25% trypsin for 1 min. Single-cell suspension was then prepared and was centrifuged at 2000 rpm for 5 min to discard the supernatant. Cells were then re-suspended in 1 mL medium for further study.

2.3 CCK-8 study

A total of 5 X 10^3 cells were seeded into 96-well plate. After 24-hour acclimation, ginsenoside Rg3 at gradient concentrations (0, 5, 15 and 45 μM) were added for treatment of 24, 48 or 72 hours. At each dosage and time point, there were 5 replicated studies (N = 5) in parallel with negative control wells. After treatment, the plate was washed in PBS twice. CCK8 solution was then added into each well (0.1 mL, 1:10 with medium) for 2.5-hour dark incubation at 37 °C. The absorbance value (A) at 450 nm was measured by a microplate reader for calculating cell viability ratio as: $(A_{\text{Treatment group}} - A_{\text{Blank control}})/(A_{\text{Control}} - A_{\text{Blank control}})$.

2.4 Flow cytometry

Annexin V/PI double fluorescent labelling was used to detect cell apoptosis. Cells at log-phase were prepared into cell suspension and were inoculated into 6-well plate (5 X 10^5 per well). After 24-hour acclimation, ginsenoside Rg3 at gradient concentrations (0, 5, 15 and 45 μM) were added for treatment of 48 hours. Cells were then digested in trypsin and rinsed twice in PBS. After centrifugation for 5 min at 1000 g, cells were re-suspended and counted for 1 X 10^6 cells to re-centrifuge at 1000 g for 5 min. After discarding the supernatant, 195 μL Annexin V-FITC biding buffer plus 5 μL Annexin V-FITC was added to re-suspend cells, followed by the addition of 10 μL PI dyes The mixture was incubated in dark at room temperature for 20 min and were incubated on ice. Flow cytometry was employed to detect cell apoptotic rate in triplicates.

2.5 Western blotting

Ginsenoside Rg3 at gradient concentrations (0, 5, 15 and 45 μM) was added for treatment of 48 hours. Cells were then homogenized in lysis buffer. Total protein was then extracted to obtain supernatant after centrifuge. The protein content was measured by coomassie brilliant blue. Denatured protein samples were loaded onto SDS-PAGE for separation, and were transferred to NC membrane. 5% defatted milk powder was used to block the membrane for 2 hours at room temperature. Primary antibody was added for 4 °C overnight incubation after PBST washing. On the second day, PBST-diluted secondary antibody containing 2.5% defatted milk powder for 60-min incubation. Chromogenic substrate was then added to the membrane, which was developed in dark followed by exposure. GIS-2020D gel image analysis system was employed to quantify optical density of protein bands for p-Akt, p-mTOR, p-STAT3 and β-actin. Using β-actin as the internal reference, relative expression level of p-Akt, p-mTOR and p-STAT3 was analyzed. The whole experiment was carried out for five repeats for each group (N = 5).

2.6 Statistical analysis

SPSS 19.0 software was used to process all collected data, of which those fitted normal distribution were presented as mean ± standard deviation (SD) and were compared by analysis of variance (ANOVA). LSD test was employed to compare means between two groups. Spearman rank-sum test was used for analyze correlation between non-parametric data. A statistical significance was defined when $p < 0.05$.

3 Results

3.1 Effect of ginsenoside Rg3 on 786-O cell proliferation

Using different concentrations of ginsenoside Rg3 on renal cell carcinoma 786-O for different time periods (24 hour, 48 hour and 72 hour). CCK assay was further replenished to detect the activity of 786-O cells. As shown in Table 1, ginsenoside Rg3 significantly inhibited the proliferation of cells in a dose-dependent manner and time-dependent manner, as higher concentration or longer treatment time resulted in more significant inhibitory effects. To sum, 45 μM ginsenoside Rg3 for 72 hour-treatment had the most significant inhibition.

Table 1. Ginsenoside Rg3 and 786-O cell proliferation.

Concentration	24 h	48 h	72 h
0 μM	1.00 ± 0.036	1.00 ± 0.035	1.00 ± 0.037
5 μM	0.94 ± 0.033*	0.89 ± 0.041*	0.82 ± 0.042*
15 μM	0.88 ± 0.037*	0.77 ± 0.039*	0.68 ± 0.044*
45 μM	0.76 ± 0.041*	0.69 ± 0.038*	0.58 ± 0.040*

*$p < 0.05$ compared to 0 μM group.

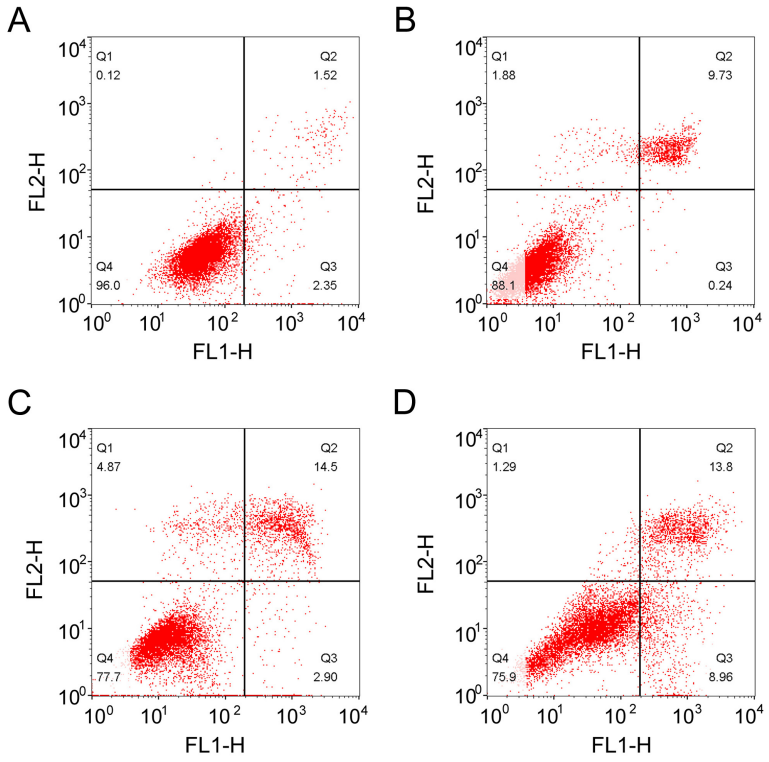

Figure 1. Effect of ginsenoside Rg3 on apoptosis of 786-O cells. A, 0 μM; B, 5 μM; C, 15 μM; D, 45 μM.

Table 2. Protein expression of p-Akt, p-mTOR and p-STAT3 after ginsenoside Rg3 treatment.

Protein	0 μM	5 μM	15 μM	45 μM	P value
p-Akt	1.35 ± 0.10	1.08 ± 0.09*	0.89 ± 0.08*	0.63 ± 0.09*	<.0.001
p-mTOR	1.21 ± 0.07	0.95 ± 0.08*	0.78 ± 0.06*	058 ± 0.07*	< 0.001
p-STAT3	1.02 ± 0.08	0.91 ± 0.07	0.75 ± 0.09*	0.53 ± 0.06*	< 0.001

*p < 0.05 compared to 0 μM group.

3.2 Effects of ginsenoside Rg3 on cell apoptosis

Using different concentrations (0, 5, 15 and 45 μM) of ginsenoside Rg3 on renal cell carcinoma 786-O for 48 hours, flow cytometry was employed to quantify apoptosis of 786-O cells. As shown in Figure 1, in which upright quadrant showed late-stage apoptosis cells while downright quadrant represented early-stage apoptotic cells, the apoptotic percentage of control group was (5.29 ± 1.18) %. Such ratio, however, was elevated to (9.14 ± 1.35)% when applying 5 μM ginsenoside Rg3, and was further elevated to (15.26 ± 2.03)% and (23.18 ± 1.46)% for 15 μM and 45 μM group, respectively. There was a significant difference of apoptotic percentage at all concentrations of ginsenoside Rg3 compared to control group ($p < 0.05$). With elevated dosage of drugs, cell apoptotic rate was also increased, displaying a dose-dependent manner.

3.3 Ginsenoside Rg3 regulates p-Akt, p-mTOR and p-STAT3 protein expression

Using different concentrations (0, 5, 15 and 45 μM) of ginsenoside Rg3 on renal cell carcinoma 786-O for 48 hours, Western blotting was employed to detect protein level of p-Akt, p-mTOR and p-STAT3. As shown in Table 2 and Figure 2, with elevated concentration of ginsenoside Rg3, expression level of p-Akt, p-mTOR and p-STAT3 proteins was gradually decreased.

3.4 Correlation between protein level and cell apoptosis

Using different concentrations (0, 5, 15 and 45 μM) of ginsenoside Rg3 on renal cell carcinoma 786-O for 48 hours, we detected both cell apoptotic ratio and protein levels of p-Akt, p-mTOR and p-STAT3 in cells. Spearman correlation analysis was employed to show significantly negative relationship between cell apoptotic ratio and protein levels of p-Akt ($r = -0.72$, $p < 0.01$), p-mTOR and ($r = -0.81$, $p < 0.01$) p-STAT3 ($r = -0.75$, $p < 0.01$).

3.5 Discussion

As one compound extracted from Chinese herbs, ginsenoside Rg3 has drawn lots of research interests due to its potential anti-tumor effect, which has been demonstrated in surveys and studies both *in vitro* and *in vivo*. For example, non-small cell lung cancer patients had longer lifespan with the application of ginsenoside

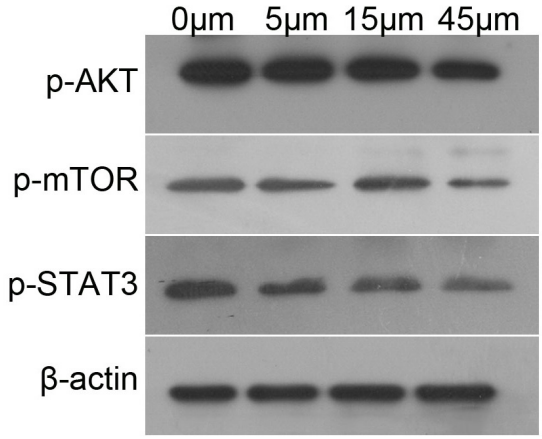

Figure 2. Effect of ginsenoside Rg3 on p-Akt, p-mTOR and p-STAT3 protein expression in 786-O cells.

Rg3 after surgery (Lu et al., 2008). No direct evidence, however, has reported regarding the effect of ginsenoside Rg3 on renal cell carcinoma. This study thus focused on the anti-tumor effect of ginsenoside Rg3 on kidney cancer, in order to provide novel approach for cancer treatment.

Our results found that, with elongated ginsenoside Rg3 treatment time and higher concentration, the survival ratio of renal cell carcinoma gradually decreased overtime. 45 μM ginsenoside Rg3 for 72 hour-treatment resulted in the most significant-42%-proliferation inhibition. Therefore ginsenoside Rg3 had potent inhibition on proliferation of 786-O cells in a time- and dose-dependent manner, consistent with previous studies showing the inhibition of the proliferation of colon adenocarcinoma cell by Cheddar cheese (Rafiq et al., 2020) or colon carcinogenesis by synbiotic sheep milk ice cream (Balthazar et al., 2021), indicating their anti-cancer properties. Moreover, after 48-hour treatment using ginsenoside Rg3, the apoptotic percentage of cells was further elevated, suggesting the induction of cell apoptosis by ginsenoside Rg3 for inhibiting 786-O cell proliferation and anti-tumor effects. Previous study has suggested the induction of ginsenoside Rg3 in tumor cell apoptosis including human glioblastoma U87MG (Choi et al., 2013), colon cancer HT-29 cells (Yuan et al., 2010). This study demonstrated that ginsenoside Rg3 may also induce cell apoptosis of 786-O cells and inhibit cell proliferation, although further studies are still needed before engaging in clinical trials.

Akt/mTOR/STAT3 signaling pathway is closely correlated with cell growth and metabolism (Dziennis & Alkayed, 2008). For example, glycyrrhizic acid can inhibit over-proliferation of leukemia tumor cells via blocking Akt/mTOR/STAT3 signaling pathway (He et al., 2015). Inhibitor of Akt/mTOR/STAT3 signaling pathway might also induce apoptosis of renal carcinoma cells and inhibit cell growth (Li et al., 2013). This study further tested protein levels of p-Akt, p-mTOR and p-STAT3. Results showed gradually decreased p-Akt, p-mTRO and p-STAT3 with higher dosages of drugs, and were negative correlated with cell apoptotic level, thus suggesting the possible involvement of the inhibition of Akt/mTOR/STAT3 signaling pathway in apoptosis as induced by ginsenoside. Similar results obtained consistent results, as ginsenoside Rg23 can induce the apoptosis of ovarian cancer HO-8910 cells (Wang et al., 2014). Moreover, ginsenoside Rg23 also may induce apoptosis of leukemia U937 cells and HL-60 cells via depressing Akt, p-Akt, mTOR and p-mTOR proteins (Qiu et al., 2014).

4 Conclusion

In summary, this study explored the effect of ginsenoside Rg3 on renal cell carcinoma. Preliminary data showed that ginsenoside Rg3 might induce the apoptosis of renal cell carcinoma 786-O cells and thus inhibition of cell proliferation. The mechanism of inhibiting Akt/mTOR/STAT3 signaling pathway for cell apoptosis by ginsenoside Rg3, however, involves complicated processes and required further study to illustrate the precise mechanism.

Conflict of interest

None.

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