RESEARCH ARTICLE

Targeting Renal Cell Carcinoma with Gambogic Acid in Combination with Sunitinib *in Vitro* and *in Vivo*

Xiao-Liang Jiang[1], Yao Zhang[1], Chun-Li Luo[2], Xiao-Hou Wu[1]*

Abstract

Purpose: To evaluated the effect of the gambogic acid (GA), one of the effective components of *Garcinia*, in combination with a new multi-targeted oral medication, sunitinib (SU) on renal cancer cell proliferation in vitro and on tumor growth in vivo. Methods: After treatment with GA or SU, either alone or in combination, MTT and FACS analysis were used to examine cell viability and cycle distribution of the renal carcinoma cell lines 786-0 and Caki-1. Western blotting was employed to examine the expression of proteins related to the cell cycle and vascular formation. Furthermore, a xenograft model was applied to study the antitumor efficacy of SU or GA alone or in combination, with immunohistochemistry to detect expression of proteins related to xenograft growth and angiogenesis. Western blotting was used to examine NF-κB signaling pathway elements in xenografts. Results: Treatment of 786-0 and Caki-1 cells with GA or SU resulted in decreased tumor cell proliferation, especially with joint use. Cells accumulated more strongly in the sub-G1 phase after joint treatment with GA and SU than treatment of GA and SU alone. Western blotting arrays showed 1 protein significantly upregulated, 2 proteins downregulated, and 2 proteins unchanged. Moreover, combined use of GA and SU inhibited the growth and angiogenesis of xenografts generated from Caki-1 significantly. Immunohistochemistry arrays showed downregulation of the expression of proteins promoting xenograft growth and angiogenesis, and Western blotting showed inhibition of the NF-κB signaling pathway after treatment by GA alone and in combination with SU in xenografts. Conclusions: Our results show that the joint use of GA and SU can provide greater antitumor efficacy compared to either drug alone and thus may offer a new treatment strategy for renal cell carcinoma.

Keywords: Renal cell carcinoma - 786-0 cells - caki-1 cells - gambogic acid - sunitinib

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Introduction

Renal cell carcinoma (RCC) is one of the most lethal urologic malignancies. It accounts for 2–3% of cancers worldwide (Jemal et al., 2009). Conventional therapies for RCC include chemotherapy, radiotherapy and immunotherapy, but unfortunately, all of these therapy above are successful only in small number of patients,and advanced and metastatic renal cell carcinomas are rather insensitive to chemotherapy and cytokine therapy (Gary et al., 2007; Merseburger et al., 2008). In recent years, with the depth of kidney cancer molecular mechanisms to understand, there have been new molecular targeted therapy drugs which have revolutionized the therapy of metastatic RCC. Among those agents, sunitinib(SU) is a novel small molecule targets of tyrosine kinase inhibitors for RCC to be approved by the FDA in December 2005,it can inhibit vascular endothelial growth factor (VEGF) receptor and platelet-derived growth factor receptor with both antiangiogenic and antitumoral activities. SU is now recommended for the first-line treatment of metastatic renal cell carcinoma. However, this medicine only prolong patients' life, drug resistance usually occurred in a period of time after treatment (Rini et al., 2009). Moreover, SU may lead to more potential adverse reactions due to its inhibition of multiple target molecular mechanisms compared with some single target drugs. So, for further improvement of prognosis, it seems that combination of chemotherapy drugs with different mechanisms are required, and combination therapies of SU with other targeted drugs ,such as bevacizumab, sorafenib, gefitinib have the potential to be more effective than single targeted therapies (Dudek et al., 2009; Feldman et al., 2009; Motzer et al., 2010)

Gambogic acid (GA) is the most abundant ingredient (Asano et al., 1996) from gamboge which comes from the trees of the genus Garcinia, GA could induce cancer cells apoptosis in cervical cancer (Zhang et al., 2010), breast cancer (Gu et al., 2009), gastric cancer (Wang et al., 2008). Furthermore, GA effectively inhibited tumor angiogenesis by significantly inhibiting effect of VEGF (Lu et al., 2007; Pandey et al., 2007). As to restrain tumor metastasis , GA also could suppresses the expression of matrix metalloproteinase (Qi et al., 2008) and inhibit

[1]*Department of Urology, The First Affiliated Hospital, Chongqing Medical University* [2]*Department of Laboratory Medicine, Chongqing Medical University, Yi Xue Yuan Road, Chongqing, China *For correspondence: 14246385@qq.com*

tumor cell adhesion by suppressing integrin signaling pathways (Li et al., 2011). Interestingly, GA inhibited proliferation in multidrug-resistant cancer cells (Wang et al., 2008) and increased the sensitivity of cancer cells to chemotherapeutic drugs, including docetaxel (Wang et al., 2008) and fluorouracil (Wang et al., 2009). Based on extensive anti-tumor effect of GA, it is currently being tested as an anti-cancer agent in a phase II clinical trial approved by the Chinese Food and Drug Administration. Although having received good clinical effect for patients with RCC, targeted therapies are not associated with long lasting responses, or bring some side effects after the use of high-dose. Consequently, it is the high time to develop new therapeutic strategies for the treatment of RCC. In this report, we have tried to analyze the effects of GA in combination with SU on renal cancer cell lines in vitro and on renal tumor xenografts in vivo.

Materials and Methods

Cell lines, antibodies and reagents

Two human RCC cell lines, Caki-1 and 786-O were used (both purchased from ShangHai cell bank, China). All these cells were cultured in DMEM medium supplemented with 10% fetal bovine serum(FBS)and incubated at 37°C in a humidified atmosphere containing 5%CO_2. GA, SU, 3-(4, 5)-dimethylthiahiazo(-z-y1)-3, 5-diphenytetrazoliumromide(MTT) and dimethyl sulfoxide(DMSO) were purchased from Sigma-Aldrich (Sigma, USA), GA and SU were dissolved in DMSO at a concentration of 25 mmol/L, respectively, aliquoted and stored at -20 °C. The mouse monoclonal antibody to p21, VEGF, Bcl-2, cyclin B1, Ki 67,β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA), and the rabbit polyclonal antibody to CD31 were from Dako North company (Carpinteria, CA). S-p kit was purchased from Beijing Zhongshan Golden Bridge Biotechnology Company. Matrigel were from Becton Dickinson company (Mountain View, USA).

Drug treatment

Cells were plated on day -1 and treated according to four protocols as follows: Untreated control, GA alone, SU alone, or GA in combination with SU. On day 0, new medium containing GA alone, SU alone, or GA in combination with SU (or DMSO) was added after the culture medium was removed. The assays were executed on day 2.

Cell viability assay

The influence of GA and SU on cell viability was analyzed by the MTT assay .Briefly, Caki-1 or 786-O cells were treated as described above. After the medium was removed and MTT solution were added, incubated for 1h,then MTT solution was replaced with 100 μl of DMSO. At last, the cells were then measured by a microplate reader (Bio-Rad) at a wavelength of 570 nm. Cell viability (%) was calculated by the following equation: [optical density (OD) of the treated wells]/(OD of the control wells) x 100%.

Cell cycle analysis

Caki-1 and 786-0 cells were treated with GA (0.5μM), SU (1μM), joint use of both, or DMSO as a control for 48 hours, then whole cells were harvested and fixed with 80% ethanol. All other experimental steps were conducted for FACS analysis as previously described (Abdelnour-Berchtold et al., 2010).

Western blotting analysis

After seeded in 96-well microtiter plates, cells were treated as described above. Preparation of cell lysates, protein fractionation, and transfer were as previously described (Iwata et al., 2011).After the separated proteins were transferred to a polyvinylidene difluoride membrane, 5% non-fat milk was used to block the membrane overnight, and then the membrane was incubated with a primary antibody followed by a secondary antibody. The following human reactive antibodies were used: anti-p21 (1/200), anti-β-actin (1/10000), anti-Bax (1/1000), anti-cyclin B1 (1/200), anti-Bcl-2 (1/1000) and anti-VEGF (1/1000). Proteins were visualized by using an Odyssey scanner instrument.

Treatment in mouse xenograft models

Animal experiments for this study were approved by the institutional animal care and use committee at Chongqing Medical University. 5-week-old male athymic BALB/c nu/nu mice (Experimental Animal Center of Chongqing Medical University, Chongqing, China) were maintained under pathogen-free conditions and provided with sterile food and water. Caki-1 cells ($2x10^6$) in 100 μl RPMI-1640 and 100 μl of matrigel were used to inject subcutaneously into each mouse. Once the transplanted tumors reached 25 mm3, mice were selected and randomly divided into four groups (8 mice per group) as follows: Untreated control, GA alone, SU alone, or a combination of GA and SU. Then all the mice were treated every day for 4 weeks, GA (5 mg/kg/day), SU (20 mg/kg/day), or in combination. GA was dissolved in 0.5% carboxymethyl cellulose Na (CMC-Na) and administered by gavage at dosages of 5 mg/kg. SU was dissolved in 20 mM citrate buffer at pH 3.5 then administered orally once daily at a dose of 20 mg/kg .The control group was given 0.5% CMC-Na containing no GA or given citrate buffer containing no SU. Caliper was used to measure tumor volumes. Tumor volume was calculated as [π/6 x large diameter x (small diameter)^2] .Body weight were measured once every week, On day 28, animals were sacrificed and tumors were carefully removed, weighed, and stored at -80°C.

IHC analysis of xenograft tumors

Transplanted tumor pathological tissue specimens were fixed in 4% formaldehyde solution, embedded in paraffin, and consecutively made 4μm thick slices, then proceed to the immunohistochemical staining. All staining steps were performed according to kit instructions. The primary antibodies and incubation conditions were mouse mono¬clonal anti-Ki 67, 1:500 dilution, in ready-to-use form at room temperature for 30 min and rabbit polyclonal

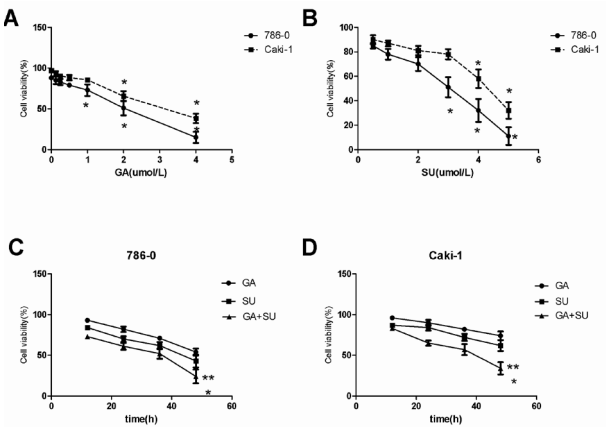

Figure 1. GA and SU Inhibit the Growth of Renal Cancer Cell Lines. (A, B) Cells were treated with GA and SU at the indicated concentrations for 48 h. (C, D) 786-0 and caki-1 cells were treated with GA (0.5uM), SU (2uM) or SU (2uM) in combination with GA (0.5uM) from 0 to 48h. Viability of cells was determined by MTT assay. Data are presented as the mean±SD of each group of cells from three independent experiments. *P<0.05 vs control group; **P<0.05 vs SU and GA alone treatment group

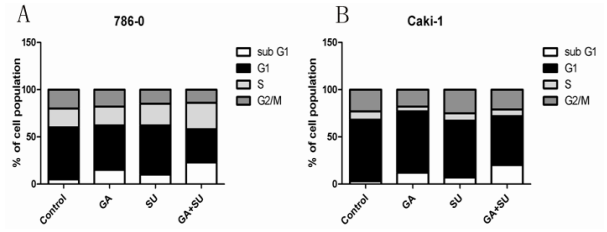

Figure 2. Effect of GA Alone or Combined with SU on Renal Cancer Cell Cycle. (A) 786-0 or (B) Caki-1 cells were treated for 48 hours with GA (0.5μM) or SU (2μM) either alone or in combination, or DMSO (Control). Cells were harvested and processed for cell cycle analysis. One of three similar experiments is shown

anti- CD31, 1:500 dilution, 4°C overnight. the microvessel density (MVD) score was described by others (Miyake et al., 2012).

Western bloting analysis of xenograft tumors

After xenografts were lysed in ice-cold lysis buffer, all other experimental steps were proceeded as described above. Anti-NF-κB p65 (1/1000) were used from Abcam (Abcam, USA). β-actin was used as the internal standard.

Statistical analysis

The chart drawing and statistical analysis rely PRISM software version 5.00 (GraphPad Software, San Diego, CA, USA) to complete, data are expressed as mean ± S.D., and the one-way ANOVA and Mann-Whitney U test was used in statistical analysis for comparison. P<0.05 was considered to be statisti¬cally significant.

Results

Cytotoxicity of GA and SU on 786-0 and Caki-1 cells in vitro

MTT assay was used to test the impact of drugs on cell viability under a series of concentrations of GA and SU (Figure1A, B). The results showed that when the GA

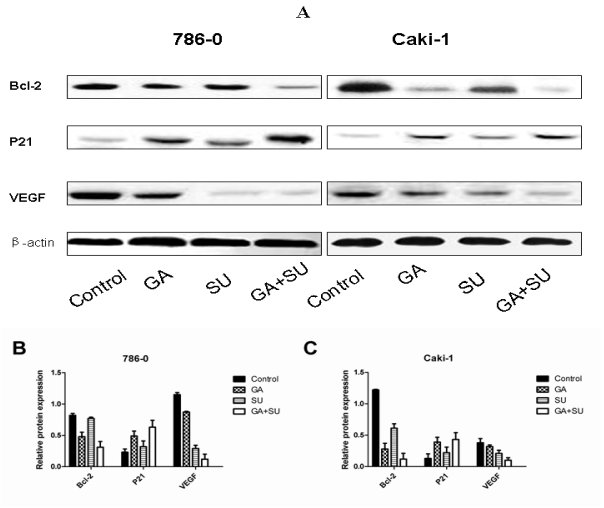

Figure 3. Effect of GA and SU on Protein Expression in RCC Cell Lines in Vitro. Cells were treated as described in the legend to Figure 2. Whole cell lysates were analyzed by Western blotting using the indicated antibodies. (A) Effect of GA and SU on the expression of cell cycle-related and angiogenesis-related proteins. The data shown are representative of three independent experiments. (B, C) The intensity of the bands was normalized to the internal standard β-actin and presented as a ratio to control. Each value represents the mean ± SD (n=3)

concentrations of more than 2uM and 1uM, respectively, caki-1 and 786-0 cell viability can be significantly inhibited. While cell viability significantly change caused by the SU concentration of the Caki-1 and 786-0 were 4uM and 3uM, respectively. So, a concentration of 0.5 uM GA and 2uM SU was selected for the subsequent experiments.

Effect of GA alone or joint use of SU on renal cancer cell proliferation

To characterize the effects of GA alone or joint use of SU on the proliferation of caki-1 and 786-0 cells, viable cells were treated with drug at the indicated concentrations for 0-48h (Figure1C, D). Appraisal of proliferation by MTT assay showed that GA in combination with SU inhibited caki-1 and 786-0 cell proliferation in a time-dependent manner. There was significantly decrease for the combined treatment group in the cell proliferation compared with SU alone and GA alone treatment group at 48 h for both caki-1 and 786-0 cells.

Effect of GA alone or in combination with SU on renal cancer cell cycle

After caki-1 and 786-0 cells were treated by GA alone ,SU alone or in combination with GA for 48h, we then analyzed the response of the cell cycle to drug using propidium iodide staining and FACS. We found that joint treatment caused a more prominent sub-G1 population when compared to monotherapy by cell cycle analysis (Figure 2).Our results show that the pro-apoptotic effect of SU combined with GA is better to single SU and GA treatment.

Effect of GA and SU on expression of protein in RCC cell lines in vitro

To further investigate the mechanism of more prominent sub-G1 population after treated by SU combined

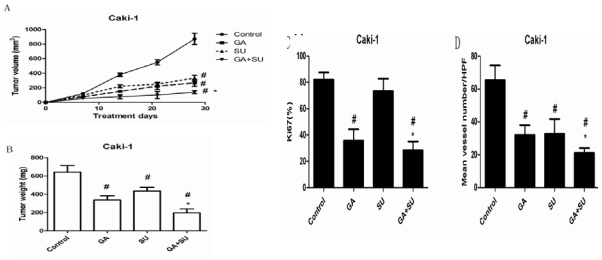

Figure 4. Effect of GA Alone or Joint Use with SU on the Caki-1 Xenografts. (A) When the Caki-1 xenografts reached 25 mm^3, mice were selected and then randomized into four groups and treatments were started with GA (5 mg/kg/day), SU (20 mg/kg/day), a combination of both (GA+SU) or vehicle (Control), Eight s mice were included in each group. (B)After 28 days of treatment, tumor xenografts were harvested and tumor weight was measured. (C) The proliferative activity was evaluated by Ki-67 immunohistochemical staining. The percentage of positive tumor cells was then calculated as Ki-67 labeling index using 4 fields. (D) The angiogenesis activity was evaluated by CD31 immunohistochemical staining. The data are presented as the mean ± SD. $^{\#}P < 0.05$ vs control, $^{*}P < 0.05$ vs SU and GA alone treatment group, respectively

with GA, Western blotting analysis was performed. In the 786-0 and Caki-1 cells, the SU induced up-regulation of P21 was reinforced by co-treatment with GA (Figure 3B, C). In addition, Bcl-2 expression was inhibited significantly in the co-treated cells than SU-treated and GA-treated cells (Figure 3B, C). However, significant changes in cyclinB1 and Bax expression were not found in the two kinds of cells (data was not shown). As the SU primarily anti-tumor mechanism was antiangiogenic, we also investigated the expression of VEGF protein, which was the important factors of angiogenesis. As shown in Fig3B,C, the expression of VEGF was decreased by GA and SU alone treatment, and simultaneously, the SU-induced down-regulation of VEGF was strengthened by co-treatment with GA.

Combination effect of SU with GA in 786-0 xenograft tumors

Since our aim was to establish a new therapeutic strategy for advanced metastatic clear cell RCC which is not so sensitive to SU, the caki-1cell line, whose IC50 (50% inhibitory concentration) for the treatment during 48 hours with SU was significantly higher than 786-0 cells(Figure 1B),was selected for the in vivo study. To further assess anti-tumor effects in vivo under the joint applications of SU with GA, nude mice bearing caki-1 tumor xenografts were treated with SU, GA or joint use of both drugs for 28 days, respectively. The doses of SU and GA was decided by our previously study which has been found to inhibit the growth of renal cancer xenografts, data was not shown. The tumor size (Figure 4A) and weight (Figure 4B) for xenografts were significantly smaller after treated by SU or GA than untreated (control)xenografts. Furthermore, the growth of combined GA and SU treated xenografts was significantly inhibited when compared to monotherapy. At the same time, there was no significant body weight decrease was observed during 28 days of drug treatment process, which proved the treatments were tolerated without obvious toxicity. In conclusion, these results show that the antitumor efficacy of SU combined with GA is greater than SU and GA used alone.

Effect of SU alone or joint use with GA on tumor growth and angiogenesis

To investigate the tumor growth and angiogenesis mechanism, immunostainings of Ki-67 and CD31 were used respectively. The Ki 67 labeling index data revealed that tumors from mice treated with GA without SU had decreased levels of proliferation as compared to the control mice. While combination treatment with SU and GA led to a significant decrease in the Ki 67 labeling index compared to SU monotherapy (Figure 4C). MVD analysis revealed that mice treated by SU and GA treatment alone group of mice compared to control group demonstrated decreased MVD, respectively, while there was a significant decrease in the MVD for combination treatment group with SU and GA than SU monotherapy (Figure 4D).

Effect of GA and SU on protein expression related to NF-κB signaling pathway in Caki-1 xenografts

Finally, we focused on GA-induced inhibition of tumor growth and angiogenesis and investigated the change of p65 protein expression related to NF-κB signaling pathway. The expression of nuclear p65 protein was nearly three fifths lower in the cells treated with GA than in the control cells. Moreover, level of nuclear p65 protein decreased significantly in the joint drug treatment group compared to single-drug treatment group (Date was not shown).

Discussion

In the past 10 years, targeted therapy utilizing tyrosine kinase inhibitors has proven efficacious and is widely used in advanced RCC. SU, as a novel small molecule targets of tyrosine kinase inhibitor for RCC, has been confirmed objective response rate of up to 40% for the treatment of advanced renal cancer (Motzer et al., 2006a; Motzer et al., 2006b) and recommended as first-line treatment option by a number of guidelines for diagnosis and treatment of renal cell carcinoma. However, not all patients received SU treatment receive complete responses and most of the patients progress during therapy at last (Rini et al., 2009) ,and at the same time, compared with other single target drugs, SU may lead to more potential adverse reaction due to its inhibition of multiple target molecular mechanism. Therefore, new therapy strategies are urgently needed to get complete responses and improve patient outcomes. It seems that combination therapies of SU with other targeted drugs, such as bevacizumab , sorafenib , gefitinib (Dudek et al., 2009; Feldman et al., 2009; Motzer et al., 2010), could be more effective than single targeted therapies ,whereas there is no study has evaluated the combination with GA.. In the first set of experiments, we found that there was only a little decrease of cell viability in the cells treated with SU or GA alone, however, cell viability was significantly reduced by co-treatment with GA and SU at 48h.From these observations, the combination treat with GA and SU seems to be more effective against Caki-1 and 786-0 cells proliferation in vitro than treat with SU and

GA alone, respectively.

We then examined whether the combined effect of GA and SU was different compared with effect of SU alone in cell cycle. Using cell cycle assays, we showed that the cell population in the subG1 phase was increased by drug treatment in all cell lines. In particular, the ratio of subG1 was increased more potently by co-treatment than by treatment with GA and SU alone. We then examined the mechanisms of this combination effect by measuring the protein expression levels in cells. As a proto-oncogene, Bcl-2 gene has the role of inhibition of apoptosis, it can also inhibit the cell death caused by a variety of cytotoxic factors and overexpression of Bcl-2 can enhance the resistance of the cells to the most cytotoxin. Downregulation of Bcl-2 expression has been shown to improve chemosensitivity in clinical studies with various carcinomas. Inhibition of Bcl-2 by using antisense oligonucleotides also can enhance the efficacy of chemotherapy in renal cell carcinoma (Kausch et al., 2005). In pheochromocytoma tumor cells, SU could induce apoptosis by inhibit signaling pathways such as Bcl-2 (Saito et al., 2012).While on the other hand, treatment of cells with GA could also inhibit the expression of gene products related to antiapoptosis such as Bcl-2 (Pandey et al., 2007). In this study, we found that Bcl-2 expression was lower in the co-treatment group than in the control or single drug treatment group, that maybe due to the downregulation of the expression of Bcl-2 at the protein levels by joint use of GA and SU.

P21 gene was a member of cyclin dependence kinase inhibitor, it was not only able to induce the G1 phase of the cell cycle arrest but also capable of inducing G2 phase stagnation (Medema et al., 1998; Ando et al., 2001) and Cyclin B1 is key regulator of the transition from the G2 to the M phase. GA could induces G2/M arrest in human gastric carcinoma (Yu et al., 2007) and GA also could induce p21 expression independent of p53 (Rong et al., 2009). In our study, we found p21 expression was moderately increased and there was not apparent change in the co-treatment group with GA and SU for cyclin B1, compared to the control or SU and GA treatment alone groups. In addition, protein level of pro-apoptotic Bax remained unchanged. Therefore, we speculated that G1 arrest could due to the raise of the p21. Simultaneously we also conjectured that apoptosis could be potentiated by the co-treatment mainly because of the downregulation of anti-apoptotic Bcl-2, while there was no change for pro-apoptotic Bax.

VEGF is generally regarded as the most effective pro-angiogenic growth factor, it can not only stimulate endothelial cell proliferation, migration but aiso form new vessels. Overexpression of VEGF has been observed in many human carcinomas, and GA significantly inhibited VEGF mRNA and protein levels in tumor cells (Lu et al., 2007; Pandey et al., 2007). In our study, the protein expression of VEGF in the cell lines was decreased significantly in the co-treatment group with GA and SU than control or monotherapy group. Therefore, we hypothesized that the combination therapy can further inhibit tumor angiogenesis.

We then examined whether the combined effect of GA and SU was generally observed in xenograft tumor, we observed that volume and weight were significantly reduced by co-treatment with GA and SU, compared to the control or monotherapy group, and there was not apparent loss of body weight in the mice treated with the drugs. From these observations, it seems that combination therapy with GA and SU will be secure and effective against Caki-1 cell proliferation in vivo. To further investigate the mechanisms of this combination effect, we measured protein expression levels of ki-67 and CD31 in isolated xenografts by using immunohistochemical method. Ki-67 protein is generally considered to be the cell proliferation activity biomarkers and research tools, reduced ki-67 protein expression means the decline in the ability of cell proliferation in general. Assessment of the Ki 67 labeling index and MVD confirmed that the combination therapy inhibited tumor growth and angiogenesisas compared to monotherapy. Previous studies have shown that GA could induce cell apoptosis and inhibit proliferation via several mechanisms, such as inhibiting the NF-κB signaling pathway (Lu et al., 2012), while inhibition of NF-kappaB activity could reduce chemoresistance to 5-fluorouracil in human stomach carcinoma (Uetsuka et al., 2003), to further study the mechanism of anti-tumor of GA in combination with SU, we then investigated whether nuclear p65, whose protein level represents the degree of activation of the NF-kB signaling pathway, was changed after treated by GA or joint use with SU by utilizing western blotting. we further confirmed that GA can indeed decrease NF-κB transcriptional activity and GA in combination with SU had more significant inhibition effect for NF-kb signaling pathway.

However, there are some obvious limitations for our study, only two kinds of renal carcinoma cells were used and in vivo data were generated only in Caki 1 xenograft tumor, so other RCC cell lines are needed to use for further evaluate the clinical potential of joint use of GA and SU.

In conclusion, we found that GA combined with SU in the treatment of human kidney cancer cells play a synergistic anti-tumor effect. Our findings also provide some inspiration for future joint clinical application of GA and SU. However, whether the clinical application can achieve a satisfactory clinical outcome, it requires further validation.

Acknowledgements

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