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Amentoflavone Suppresses Cell Growth and Invasion in Renal Carcinoma Cells by Activating PPARγ

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Abstract: This study intends to investigate the role of amentoflavone(AF) in human clear-cell renal cell carcinoma (ccRCC) and to elucidate underlying molecular mechanisms. Human RCC cell lines Caki-1 and 786-O were used in this study. Cell proliferation, apoptosis, cell cycle distribution and invasion assays were conducted to analyze the effect of AF against ccRCC *in vitro*. Xenograft model and pulmonary metastasis animal model were established to evaluate the vivo therapeutic efficacy and against pulmonary metastasis ability of AF, respectively. Our findings revealed that AF selectively suppressed tumor cell proliferation in a dose- and time-dependent manner. Treatment with AF significantly elevated the percent proportion of apoptotic cells and blocked cell cycle progression. Moreover, AF inhibited cell invasion in a dose-dependent fashion. Our findings revealed AF activated PPARγ, which accounts for its anti-tumor activities. To conclude, our findings suggest AF suppresses tumor growth and metastasis by activating PPARγ.

Keywords: PPARγ, ccRCC, amentoflavone.

1 Introduction

The most common histological subtype of RCC, the most lethal form of urologic cancer, is clear-cell renal cell carcinoma (ccRCC), which can be cured with partial or radical nephrectomy at early stage [Antonelli, Cozzoli, Zani, Zanotelli, and Nicolai (2007)]. Along with the application of target therapy in the treatment of ccRCC, the prognosis of patients with ccRCC has been markedly improved [Escudier, Eisen and Stadler et al. (2007); Hudes (2007); Motzer, Hutson and Tomczak et al. (2007); Motzer, Escudier, Oudard and Hutson et al. (2008)]. However, the clinical outcome remains unsatisfactory due to tumor recurrence and metastasis. Therefore, it is imperative in order to discover novel effective therapeutic agents against ccRCC.

Peroxisome proliferator-activated receptor gamma (PPARγ), belonging to the nuclear receptor superfamily of ligand activated transcriptional factors, has been found to be

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involved in the tumorigenesis and development of human malignancies [Fajas, Debril and Auwerx (2001)]. PPAR PPARγ is also highly expressed in a variety of human tumor tissues, including colorectal cancer [Choi, Kim and Kim et al. (2008)], non-small cell lung cancer [Han, Zheng and Roman (2007)] and gastric cancer [Fu, Sung and Wu et al. (2006)]. In ccRCC, PPAR-γ expression correlated with clinical parameters and serves as independent prognostic factors for prognosis [Zhu, Wei and Tian et al. (2015)]. *In vitro* studies also evidenced that activation of PPARγ blocks cell cycle progression and induces apoptosis in human ccRCC cell lines [Zhu, Wei and Tian et al. (2015)]. Furthermore, PPARγ agonist decreases the production of potent angiogenic factors such as VEGF and FGF [Yuan, Takahashi and Masumori et al. (2005)]. Taking together, these findings indicate that PPARγ is a promising target for ccRCC treatment.

Amentoflavone (AF), one of the flavonoid compounds extracted from Selaginella tamariscina Spring, has been found to have pharmacological activities against cancer [Banerjee, Van der Vliet and Ziboh (2002); Guruvayoorappan and Kuttan (2008); Lee, Lee and Oh et al. (2009)]. The pro-apoptotic effect of AF has been evidenced in melanoma, lung cancer, cervical cancer, breast cancer and colorectal cancer cells [Wu, Huang and Zhong et al. (2017); Jung, Lee and Lee et al. (2017); Yang, Xu and Peng et al. (2014); Pei, Liu and Hsu et al. (2012); Lee, Kim and Kang et al. (2011)]. Moreover, the anti-metastatic and anti-angiogenic effect of AF has also been reported in breast cancer [Chen, Chen and Liu (2015)]. However, whether it could exert anti-tumor activities against ccRCC and the relevant mechanisms remains elusive. In current study, the inhibitory effect of AF on ccRCC cell proliferation and invasion was investigated *in vitro* and *in vivo*. Moreover, our study was aimed to examine the role of PPARγ in the anti-tumor effect of AF against ccRCC.

2 Materials and methods

2.1 Cell culture

Human ccRCC cell lines 786-O and Caki-1 were purchased from ATCC (Shanghai, China). All cells were cultured in DMEM medium including 10% (v/v) heat-inactivated FEB, 2mM glutamine, 1% nonessential amino acids and 100 U/ml streptomycin and penicillin at 37°C in a humidified atmosphere containing 5% CO_2.

2.2 Cell viability detection

The proliferation of cells was assessed by CCK-8 (Beyotime, Shanghai, China). Briefly, culture plates were plated by 1×10^5 cells in total. Following incubation for indicated period of time, the number of viable cells was quantified by detecting absorbance at 450 nm (Tecan Group Ltd, Männedorf, Switzerland).

2.3 Cell apoptosis detection

Following treatment, PI-FITC Apoptosis kit (Beyotime, Shanghai, China) were used to stain ccRCC cells in accordance with the manufacturer's instructions. Then flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA) was employed to determine the apoptotic percentage of treated cells.

2.4 Cell cycle distribution

The cells were cultured in RPMI1640 medium containing 10% FBS for 48 hours before plated at a density of $1x10^5$/well in a 6-well culture plate. The cells were washed before fixed with ice-cold 70% ethanol at 4˚C for 2 hours. FACScan DNA analysis was performed following standard protocol.

2.5 Transwell invasion assay

Aliquot of cells were plated on 24-well Transwells over a bottom layer of Matrigel (Thermo Fisher Scientific, Waltham, MA, USA) for overnight in serum-free medium. Then 2×10^5 cells/ml cells in DMEM containing 1% FBS were trypsinized and re-suspended. The invasion assay was then performed following standard protocols and cells were pictured and counted from random selected five microscopic fields (×200). The results were presented by the ratio of invasive cells to control.

2.6 Quantitative real-time PCR (qRT-PCR)

The isolation of total RNA from cultured ccRCC cells was performed using TRIzol Reagent (Life Technology, Carlsbad, CA, USA). The isolated RNA was employed to cDNA synthesis by using the reverse transcription kit (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. The forward and reversed primer sequences were synthesized by Sangon (Shanghai, China) based on published sequence [Liu, Su and Qin et al. (2013)]. PCR reaction was performed using SYBR GREEN mastermix (Takara, Shanghai, China). GADPH was used as an endogenous control. The $2^{-\Delta\Delta Ct}$ method was used to calculate the expression of PPARγ relative to the endogenous control.

2.7 Western blot

Western blotting was performed following standard protocols. Proteins were detected with specific primary antibodies. Goat anti-rabbit IgG-HRP purchased from Beyotime (Shanghai, China) served as the second antibodies. β-actin, as internal control, was employed to normalized the relative levels of protein expression.

2.8 Luciferase assay for PPARγ activity

The luciferase reporter vector was constructed as previously described [Rocchi, Picard and Vamecq et al. (2001)]. Following treatment, cells were lyzed and the lysate was assayed for luciferase activity using Promega's Dual Luciferase (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol with empty vector as control. The luciferase activity was recorded as the folds of the activity of control cells.

2.9 In vivo tumor growth model

Male BALB/c nude mice (8-week old) were obtained from Animal Center of Southern Medical University. 786-O cells (10^7 cells) were injected into left flanks of mice model to establish tumor mass. When the tumor volume reached 100 mm^3, mice were randomly assigned into three groups (8 mice per group). Mice in vehicle group received

intraperitoneal (IP) injections of vehicle once daily. Mice in low dose group received IP injection of AF dissolved in vehicle at a dosage of 100 mg/kg/day. Mice in high dose group received IP injection of AF dissolved in vehicle at a dosage of 50 mg/kg/day. The body weight and tumor volume were monitored every three days.

2.10 In vivo metastasis model

786-O cells (1×10^6 cells) were injected through the tail vein of mice to establish a pulmonary metastasis model. Treatment started following inoculation and the mice were sacrificed after 5 weeks' treatment. Hematoxylin-eosin staining was performed on lung tissue. Metastatic nodules in lungs were counted to assess the effect of AF on metastasis of ccRCC.

2.11 Statistical analysis

The data were expressed as the mean ± SD. One-way ANOVA followed by Dunnett's t-test were employed for statistical comparisons between cell lines. The analysis of experimental data was performed by GraphPad Prism. p value of less than 0.05 was considered as statistically significant.

3 Results

3.1 AF inhibits cell growth and induces apoptosis in ccRCC cells

To fully appreciate the anti-tumor effect of AF in ccRCC, the effect of AF on cell viability was first assessed. Two ccRCC cell lines, Caki-1 and 786-O, were incubated with AF for 24 hours and AF exhibited dose-dependent anti-growth activities in both cell lines, although Caki-1 showed higher sensitivity to AF treatment (Fig. 1A). When the treatment was prolonged to 48 hours, the anti-growth effect of AF was augmented in both cell lines, resulting in a further loss of cell viability in Caki-1 and 786-O cells, respectively. Next, whether AF could induce apoptosis in ccRCC cells was examined. Results from flow cytometry showed that AF treatment for 48 hours led to marked increase in apoptotic cell death (Fig. 1B). Moreover, the cleavage of capase-3 and PARP, which are markers for induction of apoptosis, was observed in Caki-1 and 786-O cells treated with AF (Fig. 1C).

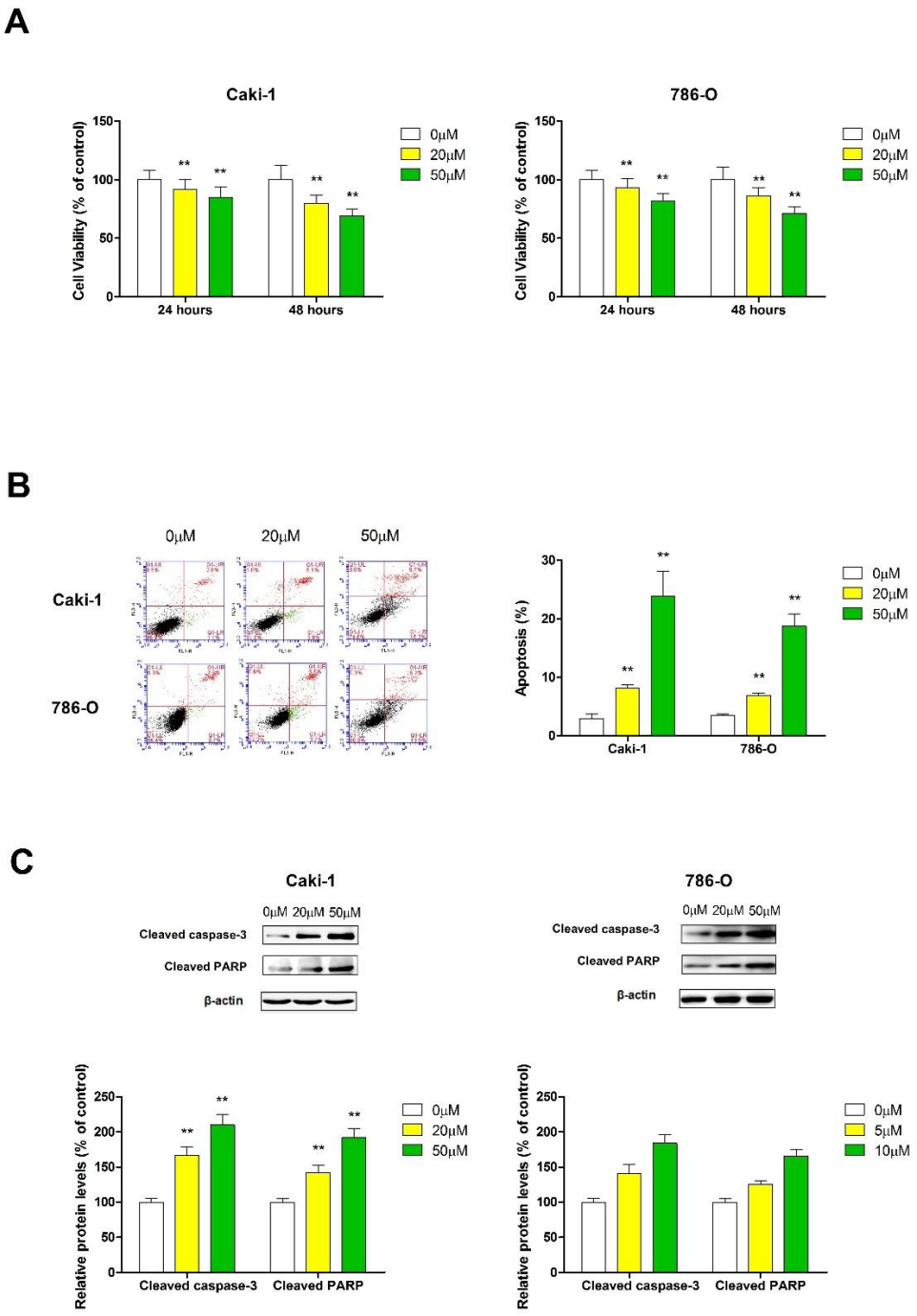

Figure 1: AF inhibits cell growth and induces apoptosis in ccRCC cells. A. AF suppresses cell proliferation in a dose- and time-dependent fashion; B. AF exerts pro-apoptotic effect in ccRCC cells; C. Treatment with AF activates caspase-3 and PARP. ***p*<0.01 *vs*. control.

3.2 AF blocks cell cycle progression

Next, we examined the contribution of cell cycle regulation in the anti-tumor activity of AF. As shown in Fig. 2A, treatment with AF resulted in significantly increase in cell population at G1/G0 phase with a decrease in both S and G2/M phases. The expression of cyclin A (a regulatory molecule for DNA replication in S phase) and cyclin D1 (a molecule promoting the passage of G1/S checkpoint and entry of S phase) were significantly lowered by AF (Fig. 2B). Taken together, these results suggested that AF induces cell cycle arrest in at G0/G1 phase in ccRCC cells through modulating the regulatory molecules cyclin A and cyclin D1.

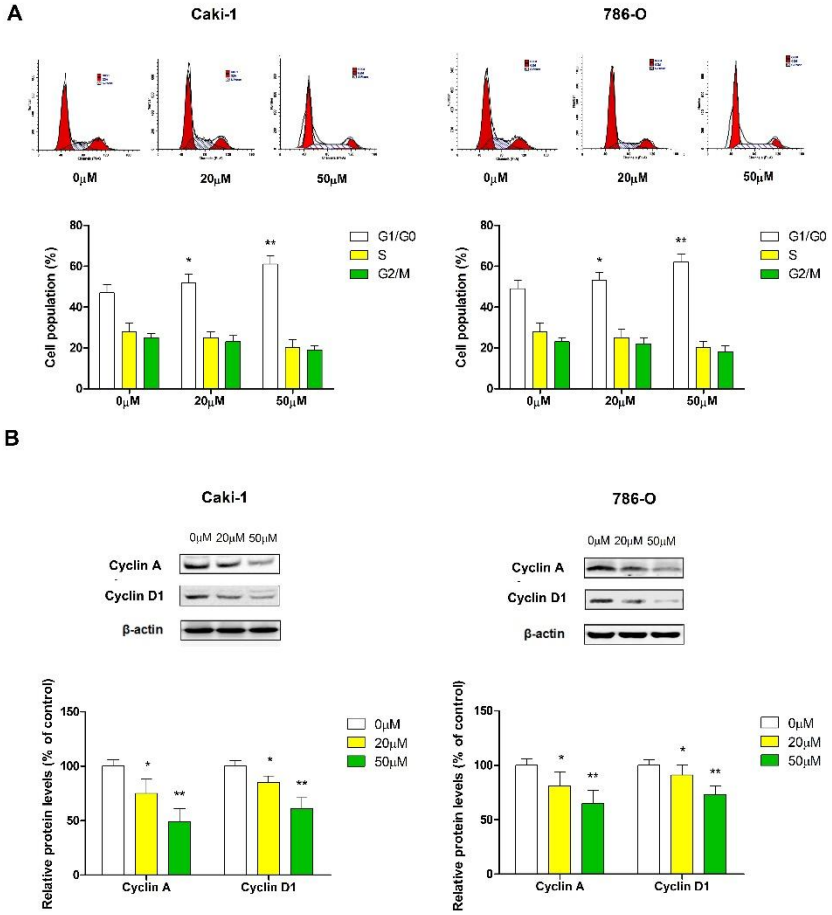

Figure 2: AF blocks cell cycle progression in ccRCC cells. A. AF induces cell cycle arrest at G1/G0 phase; B. AF modulates the expression of molecules involved in cell cycle regulation. * p<0.05 *vs*. control, ** p<0.01 *vs*. control.

3.3 AF decreases the cell invasion in ccRCC

The effect of AF on cell motility of ccRCC was also assessed. Our results from Transwell invasion assay also showed that ccRCC cells treated with AF presented with significantly compromised invasiveness compared with cells treated with vehicle (Fig. 4A). Altogether, our results showed that AF promoted cell death and suppressed cell invasiveness. Moreover, the effect of AF on the molecular markers of cell invasion, MMP-2 and MMP-9, was also examined. As shown in Fig. 3B, AF repressed the expression of MMP-2 and MMP-9 in both Caki-1 and 786-O cells in a dose-dependent fashion.

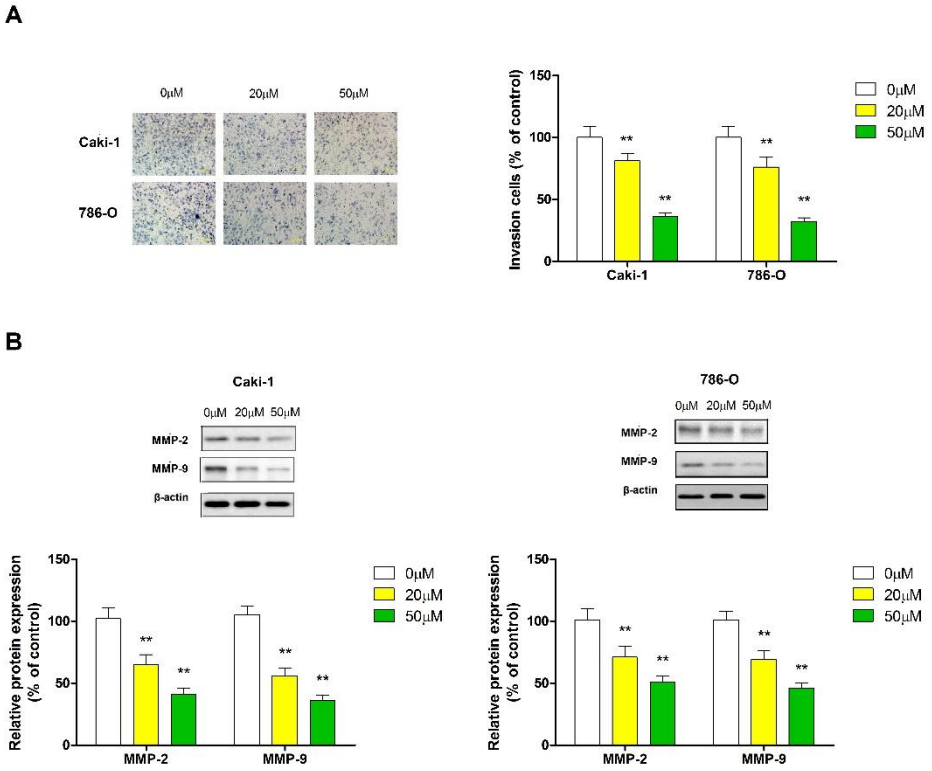

Figure 3: AF suppresses cell invasion of ccRCC cells. A. AF suppresses cell invasion. B. AF downregulates the expression of MMP-2 and MMP-9 in ccRCC cells. ** $p<0.01$ *vs*. control.

3.4 AF elevates PPARγ expression in ccRCC cells

Next, whether AF could regulate PPARγ was investigated. As shown in Fig. 4A and 4B, AF treatment resulted in a dose-dependent increase in the expression of PPARγ at both mRNA and protein levels. Moreover, AF significantly increased the transcriptional activity of PPARγ in both Caki-1 and 786-O cells (Fig. 4C).

3.5 Activation of PPARγ by AF mediates the anti-cancer effect of AF

Based on our beforementioned results, we postulated that AF exerted anti-tumor effect by modulating PPARγ. To further explore the role of PPARγ in the anti-tumor effect of AF, a PPARγ antagonist, GW9662, was used. Cell proliferation assay showed that the ant-growth effect of AF was significantly compromised by PPARγ antagonist (Fig. 5A). Correspondingly, flow cytometric analysis showed that AF-induced apoptosis was significantly attenuated by PPARγ antagonist (Fig. 5B). AF-induced blockade of cell cycle progression was also significantly reversed by PPARγ antagonist (Fig. 5C). Moreover, suppression of cell invasion by AF was also abrogated by PPARγ antagonist. Taken together, our findings highlighted that activation of PPARγ mediated the anti-tumor activity of AF.

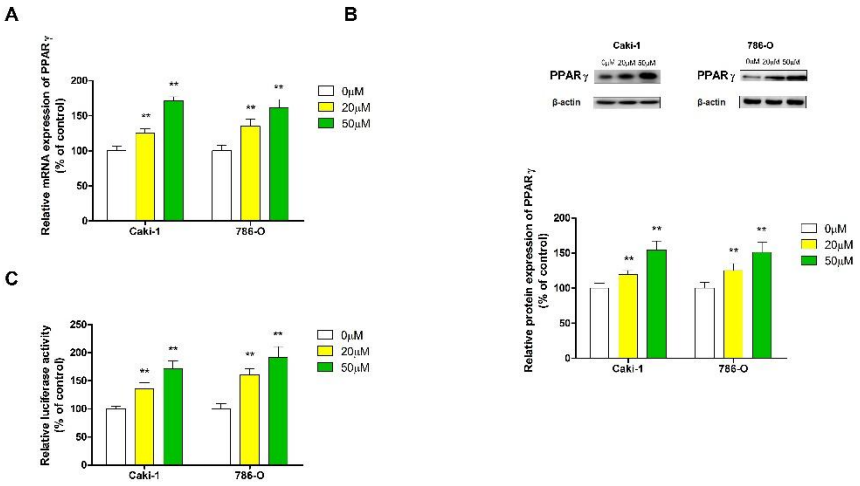

Figure 4: AF upregulates the expression and activity of PPARγ. A. AF increases the mRNA expression of PPARγ in ccRCC cells; B. AF increases the protein expression of PPARγ; C. AF increases the transcriptional activity of PPARγ. ** $p<0.01$ *vs*. control.

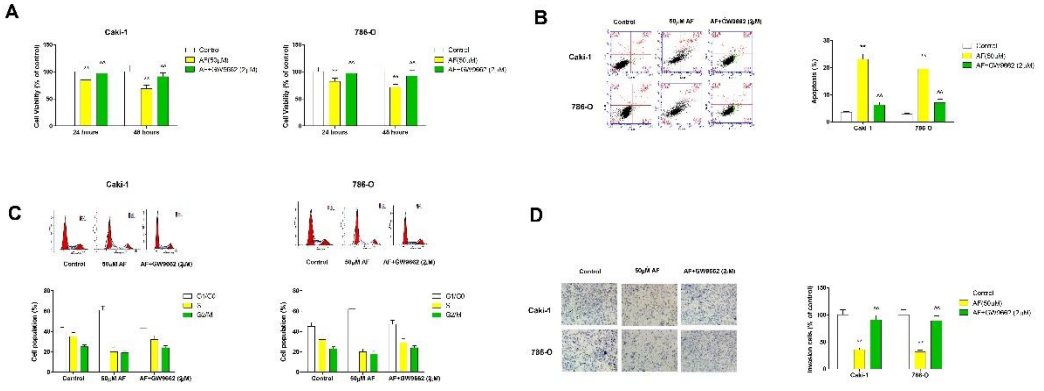

Figure 5: PPARγ antagonist (GW9662) attenuates the anti-cancer activity of AF in ccRCC cells. A. GW9662 significantly attenuates the anti-growth effect of AF; B. GW9662 overexpression significantly abrogates AF -induced apoptosis; C. GW9662

significantly abolished AF-induced cell cycle arrest; D. GW9662 significantly abolished AF-induced suppression on cell invasion. ** $p<0.01$ *vs*. control.

3.6 AF suppresses ccRCC tumor growth and pulmonary metastasis

The efficacy of AF against ccRCC *in vivo* was evaluated in a xenograft animal model. As shown in Fig. 6A, the tumor growth was significantly delayed by AF treatment, as indicated by the tumor volume (Fig. 6A). Pulmonary metastasis was evaluated by the number of metastatic foci in lung tissue. As shown in Fig. 6B, AF treatment effectively suppressed the metastasis of ccRCC in animal model. Altogether, the *in vivo* results indicated that AF could exert anti-tumor effect *in vivo*.

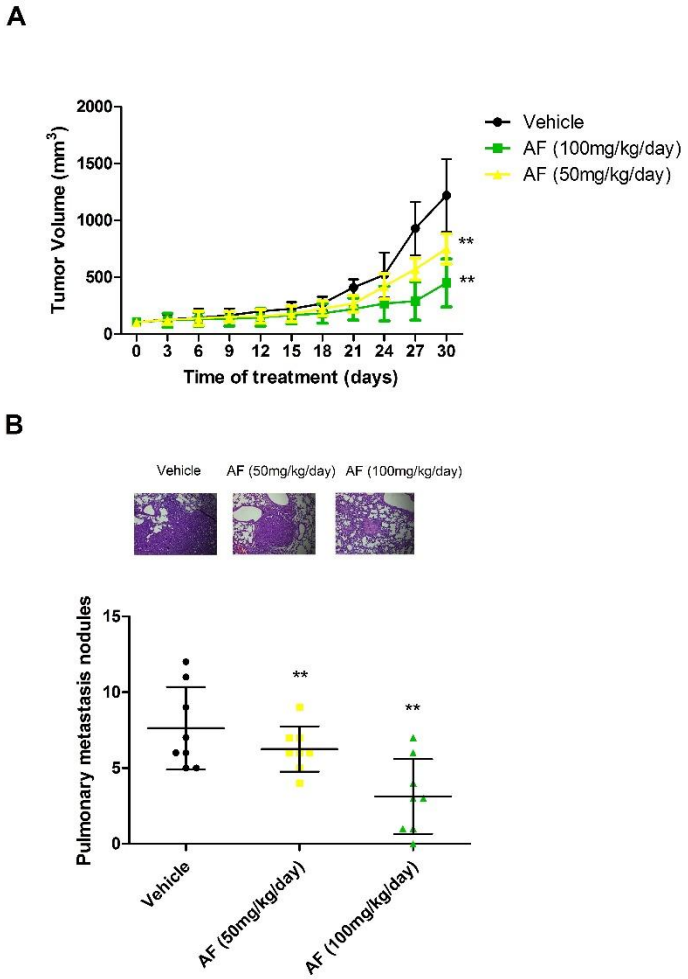

Figure 6: Effect of AF on tumor growth and pulmonary metastasis *in vivo*. A. Effect of AF on tumor volume. B. Effect of AF on number of pulmonary metastasis nodules. ** $p<0.01$ *vs*. vehicle.

4 Discussions

Compared with conventional chemotherapeutics, natural compounds can exert potent anti-tumor effect and cause much less adverse effects. Therefore, a number of bioactive chemical structures from nature source have been studies for their anti-tumor activity. Epidemiological evidence has shown that consumption of flavonoids, a large group of polyphenolic compounds that are present in foods and beverages of plant origin, is inversely correlated with the incidence of cancers [Bosetti, Rossi and McLaughlin et al. (2007)]. AF is one of the active ingredients of Selaginella tamariscina Spring, and has been reported to have anti-neoplastic properties against a variety of human malignancies [Banerjee, Van der Vliet and Ziboh (2002); Guruvayoorappan and Kuttan (2008); Lee, Lee and Oh et al. (2009)], which include apoptosis induction and cell cycle arrest [Wu, Huang and Zhong et al. (2017); Jung, Lee and Lee et al. (2017); Yang, Xu and Peng et al. (2014); Pei, Liu and Hsu et al. (2012); Lee, Kim and Kang et al. (2011)], suppression, metastasis and angiogenesis [Chen, Chen and Liu (2015)]. Moreover, a number of molecular targets and mechanisms have been proposed to be responsible for the anti-tumor effects of AF. For instance, AF treatment was associated with the downregulation of COX-2/iNOS expression in human lung adenocarcinoma cells [Banerjee, Van der Vliet and Ziboh (2002)]. In breast cancer cells, the anti-proliferative and pro-apoptotic effects of AF were attributed to its inhibition of fatty acid synthase [Lee, Lee and Oh et al. (2009)]. The anti-angiogenic and anti-metastatic effects in breast cancer was found to be correlated to the suppression of NF-κB activation [Chen, Chen and Liu (2015)]. In this study, our findings revealed that AF exerted anti-cancer activities *in vitro* and *in vivo* through upregulating PPARγ. To our knowledge, this is the first experimental evidence showing that AF could exert anti-tumor effect in ccRCC.

PPARs belong to the nuclear receptor gene superfamily, containing three subtypes: α, β (or δ) and γ. Among them, PPARγ is the most frequently investigated and is closely associated with tumors. Previous studies have demonstrated that upon activation with specific ligands, PPARγ can inhibit the growth of human tumor cells *in vitro* and *in vivo* [Chen, Wang and Fu et al. (2009)]. PPARγ and its ligands have thus become one of the main focuses of basic tumor research. In the context of RCC, PPAR-γ expression correlated with clinical parameters and serves as independent prognostic factors for prognosis [Zhu, Wei and Tian et al. (2015)]. Moreover, a few of agents have been found to function as antineoplstic agents in ccRCC by activating PPARγ. Research has found that two PPARγ ligands, pioglitazone and 15-deoxy-Delta12,14-prostaglandin J2, were able to suppress cell proliferation and induce apoptotic cell death *in vitro* in ccRCC cells [Yuan, Takahashi and Masumori et al. (2005)]. Furthermore, PPARγ ligands were found to repress the secretion of both vascular endothelial growth factor and basic fibroblast growth factor [Yuan, Takahashi and Masumori et al. (2005)]. Another study by Guan's group showed that pioglitazone and troglitazone blocked cell cycle progression in addition to induction in ccRCC cells [Yang, Zhang and Xin et al. (2005)]. A preclinical study also evidenced that activation of PPARγ by a chemically designed ligand could induce cell cycle arrest and apoptotic cell death *in vitro* in ccRCC cells and *in vivo* in orthotopic model of RCC, further supporting that PPARγ is a promising therapeutic target for ccRCC [York, Abdelrahim and Chintharlapalli et al. (2007)]. In our study, we also

found that AF could increase the expression level of PPARγ. Moreover, PPARγ antagonist, GW9662, could significantly abolish the anti-cancer activities of AF, indicating that PPARγ activation mediated the antineoplastic activities of AF.

5 Conclusion

In conclusion, the current study showed that AF exhibited anti-cancer effect *in vitro* and *in vivo* in ccRCC. Furthermore, the anti-cancer effect of AF is mediated by its upregulation of PPARγ. Our results revealed that AF has the potential to be employed in the treatment of ccRCC in clinical settings.

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