Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Nobiletin inhibits viability of human renal carcinoma cells via the JAK2/STAT3 and PI3K/Akt pathway

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Received May 25, 2020; **Accepted** June 30, 2020; **Published** July 31, 2020

Doi: http://dx.doi.org/10.14715/cmb/2020.66.5.33

Abstract: Nobiletin, a flavonoid found chiefly in oranges and lemons, has exhibited potential anti-proliferative and pro-apoptotic activities in various types of cancers. However, the inhibitory effect and mechanisms of nobiletin on renal cancer cells are unclear. CCK8 and plate clone formation assay were used to determine the effect of nobiletin on the proliferation of renal cancer cells, while scratch healing test was used to assay its effect on migration ability. The effect of nobiletin on the invasion of renal cancer cells was determined using Trans well chamber assay. Flow cytometry was used to determine the effect of nobiletin on apoptosis of renal cancer cells, while Western blotting assay was used to determine its effect on the expressions of JAK2/STAT3 and PI3K/Akt pathway proteins, and apoptosis-related proteins. Nobiletin inhibited the proliferation of renal carcinoma cells in a time- and dose-dependent manner. It inhibited the migration and invasion of renal cancer cells, and promoted their apoptosis. Western blot results showed that nobiletin inhibited the phosphorylations of JAK2, STAT3, PI3K, and Akt, and promoted the expressions of apoptosis-related proteins. Nobiletin inhibits the proliferation, invasion and migration of renal cell carcinoma by inhibiting JAK2/STAT3 and PI3K/Akt pathways, and promotes their apoptosis. These findings provide a new experimental basis for the application of nobiletin in the treatment of renal cancer.

Key words: Nobiletin; JAK2/STAT3 pathway; PI3K/Akt pathway; Renal carcinoma.

Introduction

Renal cell carcinoma (RCC), the most common types of renal carcinoma, occurs mainly in renal tubular epithelial cells, and accounts for more than 90% of all renal tumors (1-2). The development of kidney cancer is influenced by many risk factors such as obesity, hypertension, diet, smoking and lifestyle (3). Renal cell carcinoma (RCC) comprises clear cell carcinoma, papillary renal cell carcinoma, chromophobe cell carcinoma, collecting duct carcinoma, and an unclassified renal cell carcinoma. The most common among these is clear cell carcinoma which accounts for about 60 to 85% of RCC. At present, the main diagnostic methods for RCC are B-mode ultrasound and computed tomography imaging procedures. The major treatment for RCC is based on surgery. The pathogenesis of clear cell carcinoma is from Von Hippel-Lindau (VHL) gene mutations which lead to increased activity of hypoxia inducible factor 1 (HIF 1) and angiogenesis. Tyrosine kinase inhibitor (TKI) drugs are used for adjuvant therapy for targeting platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor, VEGFR). The high cost of targeted drug therapyandsurgical trauma has become a huge challenge for patients. Another approach is to consume anticancer foods, with the aim of preventing kidney cancer. These foods contain apigenin and garlicin. Similarly, flavonoids, found mainly in vegetables and citrus fruits, have anti-inflammatory, anti-angiogenic and pro-apoptotic effects (4-6).

Nobiletin is a flavonoid found chiefly in oranges and lemons. Studies have reported that nobiletin downregulated nitric oxide synthase and improved colitis induced by 2,4, 6-trinitrobenzene sulfonate (7). Nobiletin can prevent the synthesis of prostaglandin E1 in osteoporosis by inhibiting NF-κB (8). It also improved cognitive performance in animal models of Alzheimer's disease (9). In addition, a growing number of studies have shown that nobiletin has anti-tumor effects. It has been reported that nobiletin reduced the migration ability of HCC cells by inhibiting the expression of Akt and extracellular signal-regulated kinase (ERKs) (10). Nobiletin significantly inhibited the protein tyrosine kinase 2(PTK2)/SRC/STAT3 angiogenic signaling pathway, thereby arresting the proliferation of breast cancer cells (11). It also inhibited the proliferation of tumor cells by downregulating the expressions of HIF1 and Akt (12). In addition, combined treatment with nobiletin and chemotherapy has been shown to improve the efficacy of chemo therapy (13). These studies demonstrate that nobiletin exerts beneficial pharmacological effects in humans, and has a significant therapeutic effect on tumor proliferation and metastasis.

Although many studies have reported the anti-tumor effect of nobiletin, studies on the effect of nobiletin on RCC are few. In order to elucidate the potential mechanism involved in the anti-renal cancer effect of nobiletin, this study investigated the effect of nobiletin on the proliferation of renal cancer cells, and its effect on the JAK2/STAT3 and PI3K/Akt signaling pathways.

Materials and Methods

Cell culture and treatment

Kidney cancer cell line ACHN was purchased from Shanghai cell bank. The cells were cultured in a medium containing 100 U/mL penicillin, 100 g/mL streptomycin and 10% fetal bovine serum, and incubated at 37°C in an incubator containing 5%CO_2. Nobiletin was dissolved in DMSO to obtain a 50 mM stock solution which was diluted with medium to obtain working solution containing 0.5%DMSO. The control group was treated with an equivalent volume of DMSO in place of nobiletin.

CCK8 assay

Cells at logarithmic growth stage were inoculated into 96-well plates at a density of 3×10^3 cells per well, and then incubated in for 24 h. Then, different concentrations of nobiletin were added to the wells, followed by culturing for 48 h. Thereafter, the old medium was discarded, and CCK8 was added, according to the instructions on CCK8 kit. Absorbance was read at 450 nm (A450) in a microplate. Percentage cell survival was calculated using A450 in the experimental group and A450 in the control group. The experiment was repeated three times.

Plate clone formation experiment

Cells at logarithmic growth stage were inoculated into 96-well plates at a density of 400 cells per well, and then incubated for 24 h, as before. Then, different concentrations of nobiletin (80 and 120 μM) were added and cultured for 48 h. The medium containing nobiletin was discarded and replaced with a complete medium, the culturing was continued for 14 days. Thereafter, the cells were fixed with 4% paraformaldehyde for 40 min, and stained with 0.5% crystal violet at room temperature for 2 h.

Scratch healing experiment

Cells in logarithmic growth phase were inoculated into 6-well plate. A ruler and 200-μL pipette were used to scratch a line parallel to the bottom of the six-well plate. The old medium was discarded, and the cells were gently rinsed twice with PBS. Then, serum-free medium containing nobiletin was added to the experimental group, while only pure serum-free medium was added to the control group. Incubation was continued for 24 h. The scratches were observed and photographed under a microscope at 0 h and 24h.

Transwell chamber experiment

Matrigel was diluted in a pre-cooled medium, introduced into a chamber of Transwell plate and solidified by incubating it overnight in an incubator at 37°C. Then, 1×10^4 cells were inoculated in each cell. Serum-free medium containing nobiletin was added to the upper compartment of the experimental group. Serum-free medium was added to the upper compartment of the blank group, and medium containing 10% serum was added to the lower compartment of both groups. The cells were incubated in an incubator for 48 h. Cells below the upper compartment were fixed with 4% paraformaldehyde for 1 h and stained with 0.5% crystal violet for 2 h. Finally, the stained cells were counted under the microscope.

Flow cytometry

Cells at logarithmic growth stage were inoculated into 6-well plates at a density of 2×10^5 cells per well, and then incubated in the incubator for 24h. Then, different concentrations of nobiletin were added and cultured for 48h. The cells were then digested with trypsin and transferred to a centrifuge tube, where they were gently rinsed twice with PBS buffer to produce 1×10^6 / mL cell suspension. Then, 100μL of cell suspension was put into a 5-mL tissue culture tube, and 5μL of FITC-labeled membrane protein V and 5μLPI were added to the cell suspension. The mixture was gently rotated and incubated at room temperature in the dark for 15 min. Then, 400μL 1×buffer was added to each tube. Then, the tubes were placed on a flow cytometer for detection of apoptosis.

Western blotting assay

Cells in logarithmic growth phase were inoculated into 6-well plate. Different concentrations of nobiletin were added, followed by incubation for 24h. Thereafter, the cells were rinsed twice with PBS buffer and lysed on ice with RIPA lysate and 1%PMSF for 15 min. The cell lysates were centrifuged at a speed of 12,000 rpm at 4°C for 15 min, and the protein concentration of the supernatant was determined using the BCA protein assay kit. Equal amounts of protein (40μg) were subjected to 10-13% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was then sealed for 1 h and incubated with primary antibody (1:1000) at 4°C overnight. Following washing with TBST, the membrane was incubated with HRP-conjugated secondary antibody (1:5000) for 1 h at room temperature. The blots were detected using chemiluminescence. β-Actin was used as internal reference.

Statistical analysis

All data are expressed as mean ± standard deviation. Students *t*-test was used to analyze differences between two groups. Univariate ANOVA in SPSS 20.0 was used to analyze the differences amongst groups. Values of $p<0.05$ indicated statistically significant differences.

Results

Nobiletin inhibited the proliferation of renal cancer cells

Results from CCK8 showed that nobiletin inhibited cell proliferation in a dose-dependent manner (Fig. A). When the concentration of nobiletin was increased to 80μM, cell proliferation began to decline. At a concentration of 120μM, cell viability was further reduced. When the cells were treated with different concentrations of nobiletin for 12, 24 and 48 h, the inhibitory effect of nobiletin on cell proliferation was also time-dependent (Fig 1B). Thus, 80μM and 120μM were selected as the concentrations of nobiletinfor use in subsequent experiments. Colony-forming assay showed that the colony number in the control group was higher than that in the group treated with nobiletin (Fig. 1C).

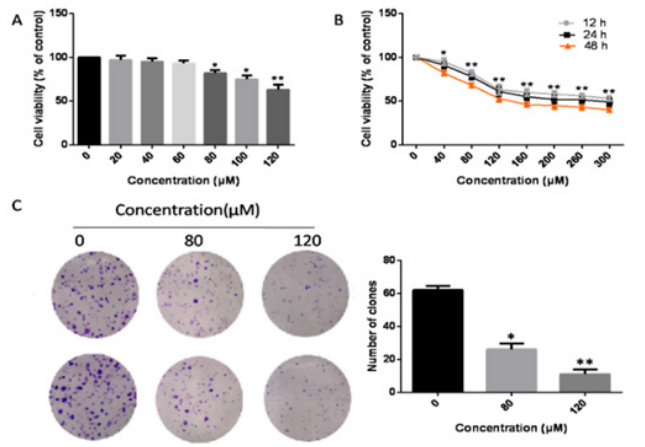

Figure 1. Effect of nobiletin on the proliferation of renal cancer cells. (A) The cells were treated with different concentrations of nobiletin, and cell viability was measured using CCK8.(B)The cells were treated with different concentrations of nobiletin for 12h,24h and 48h, and cell viability was measured using CCK8. (C) The cells were treated with nobiletin(80 and 120μM). The medium containing nobiletin was discarded and replaced with a complete medium, and culturing was continued for 14 days.*P<0.05, **p <0.01, compared with the control group.

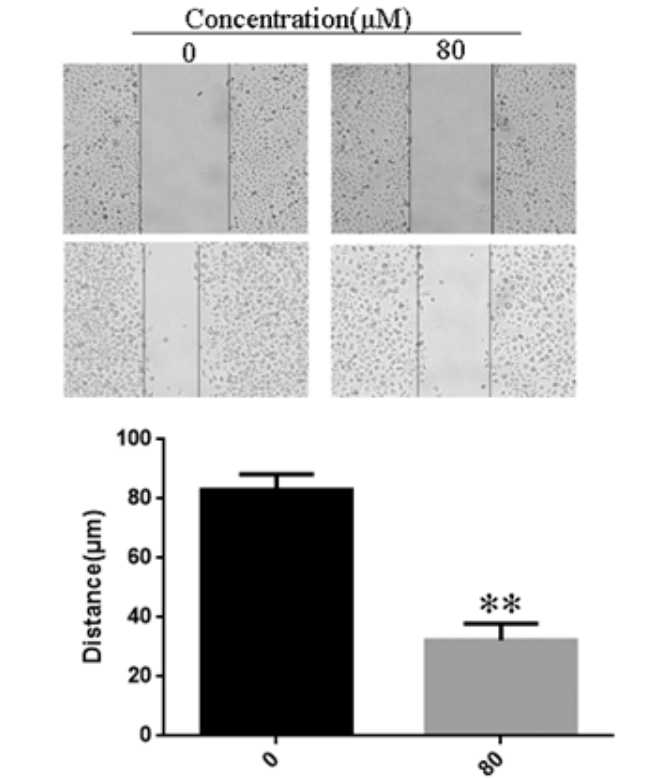

Figure 2. Nobiletin inhibited the migration of renal cancer cells. Data are mean ± SD, n=3. **P<0.01, compared with the control group.

Nobiletin inhibited migration of renal cancer cells

As shown in Fig. 2, nobiletinsignificantly reduced the migration capacity of renal cancer cells, when compared to the control group (p<0.01).

Nobiletin inhibited the invasion of renal cancer cells

Nobiletin significantly reduced the invasion ability of renal cancer cells, when compared with the control group (p <0.05). These results are shown in Fig 3.

Nobiletin enhanced apoptosis of renal cancer cells

The effect of nobiletin on apoptosis of renal carcinoma cells was detected using flow cytometry (Fig 4). Compared with the control group, apoptosis was significantly increased in the treatment group (p <0.05).

Nobiletin inhibited the phosphorylation of JAK2, STAT3, PI3K, and Akt in renal cancer cells

Western blot analysis was used to measure the expressions of proteins related to the JAK2/STAT3 and PI3K/Akt pathways, and the results showed that the phosphorylation levels of JAK2, STAT3, PI3K and Akt were significantly decreased after treatment with nobiletin (Fig. 5).

Nobiletin promoted the expressions of apoptotic proteins

Treatment of renal cancer cells with nobiletin led to marked increases in levels of pro-apoptotic proteins, cleaved caspase-3 and cleaved caspase-9, relative to the control group.

In contrast, the expression of the anti-apoptotic protein Bcl-2 decreased with increase in nobiletin concentration, while the expression of pro-apoptotic protein Bax increased with increase in nobiletin concentration (Fig. 6).

Discussion

At present, many studies have reported the pharmacological characteristics of nobiletin and its inhibitory effects on different types of tumors. Nobiletin is

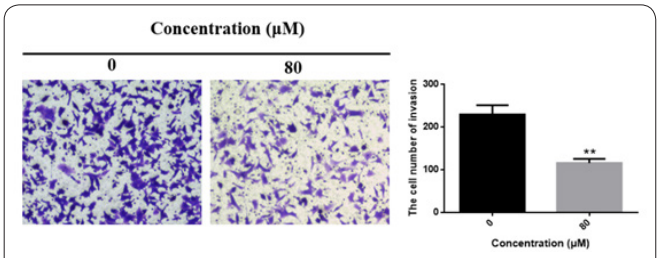

Figure 3. Effect of nobiletin on the invasion of renal cancer cells. (Data are mean ± SD, n=3).*P<0.05, compared with the control group.

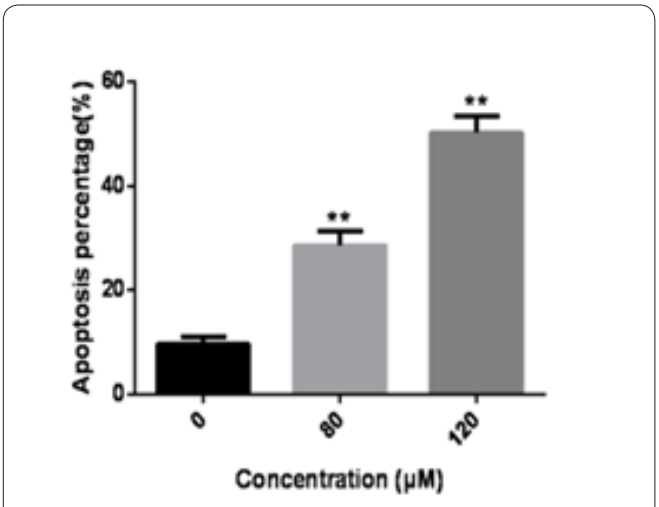

Figure 4. Nobiletin promotes the apoptosis of renal cancer cells. (Data are mean ±SD, n=3). Compared with the control group, **p<0.01.

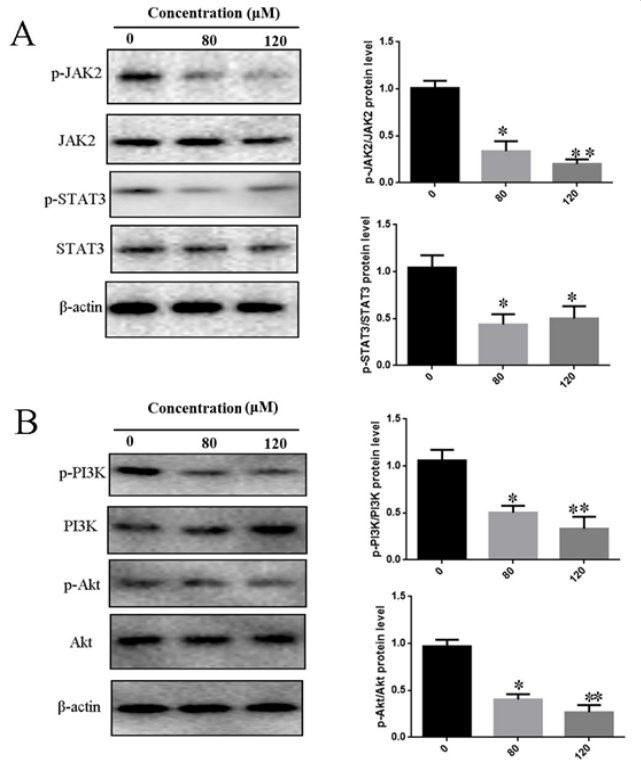

Figure 5. Effect of nobiletin on the expressions of JAK2, p-JAK2, STAT3, p-STAT3, PI3K, p-PI3K, Akt and p-Akt. The cells were treated with nobiletin NTP. After 24 h incubation, total protein was extracted and subjected to SDS-PAGE. The expressions of Bax, Bcl-2, cleaved Caspase-3 and cleaved Caspase-3 proteins were determined using Western blot analysis, and quantified using ImageJ program. β-Actin was used as an internal control. **P*<0.05, compared with the control group; **p*<0.05, ***p*<0.01, AKT compared with the control group.

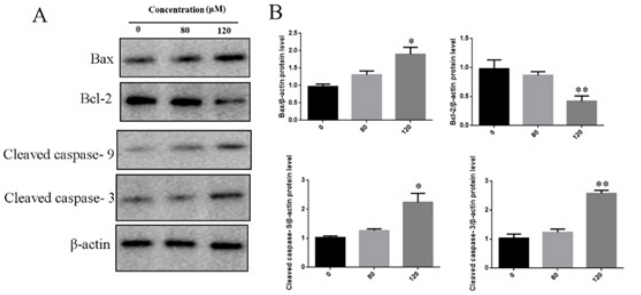

Figure 6. Effect of nobiletin on the expressions of Bax, Bcl-2, cleaved Caspase-3 and cleaved Caspase-9. The cells were treated with nobiletin NTP. After 24 h incubation, total protein was extracted and subjected to SDS-PAGE. The expressions of Bax, Bcl-2, cleaved Caspase-3 and cleaved Caspase-9proteins were determined using Western blot analysis, and quantified using ImageJ program. β-Actin was used as an internal control. **P*<0.05, compared with the control group.

extracted mainly from citrus plants, and its anti-tumor effects include inhibition of proliferation and cell cycle, and promotion of apoptosis (14). Furthermore, it targets metastasis of tumor cells. Lee *et al*. (15) reported that nobiletin reduced the expressions of matrix metallopeptidase 2(MMP2) and MMP9 in gastric cancer cells through the PTK2/PIK3CA pathway, thereby reducing the invasion and migration ability of gastric cancer cells. The antitumor effect of nobiletin has two key aspects: inhibition of proliferation and promotion of apoptosis. Studies have suggested that nobiletin reduced the expression of VEGF by inhibiting the activation of Akt, thereby reducing angiogenesis and inhibiting the proliferation of tumors (16). Tang *et al*. (17) used flow cytometry to determine the pro-apoptotic effect of nobiletin on tumors. It has been reported that nobiletin inhibited proliferation, migration and invasion of gastric and prostate cancer cells (18-19). However, there have been very few reports on the inhibitory effect of nobiletin on the proliferation of renal cancer cells. In this study, it was found that the ACHN cells were inhibited in a time-dependent and dose-dependent manner.

Compared with normal cells, tumor cells have stronger proliferation capacity which involves a series of signaling pathways. To investigate the underlying mechanism, the JAK2/STAT3 and PI3K/Akt pathways were studied in this work. The JAK2/STAT3 pathway is widely involved in the signaling of various important growth factors *in vivo*. STAT3 interacts with relevant transcription factors and associates JAK2/STAT3 with other signaling pathways to form a network and promote tumor growth cooperatively (20). The inhibition of Akt may further induce cell growth arrest or apoptosis (21). Another focus of anti-tumor therapy is to control angiogenesis in which JAK2 promotes neovascularization by up-regulating VEGF expression. In this study, it was found that the phosphorylation levels of JAK2, STAT3, PI3K and Akt decreased with increase in nobiletin concentration, indicating that nobiletin significantly inhibited the activation of JAK2, STAT3, PI3K and Akt. Our results were consistent with previous report that Nobiletin can inhibits glioma cell growth through suppressing MAPK and Akt pathways [22].It is possible that different type cancer cells may have some different response mechanism.

In addition to inhibiting the proliferation of tumor cells, this study also demonstrated that nobiletin inhibited the migration and invasion of renal cancer cells. Earlier studies have shown that inhibition of Akt expression suppressed cell migration (23-24).The results of this study indicate that nobiletin inhibited the activation of Akt, which may be one of the mechanisms by which nobiletin inhibited the invasion and migration of renal cancer cells.

Apoptosis, known as type I programmed cell death, can be divided into two pathways: endogenous and exogenous pathways. The former activates caspase after the release of cytochrome c into the cytoplasm, while the latter is activated by the Fas death receptor (25). It is known that Bcl-2 and Bax control mitochondrial integrity in endogenous apoptosis (26-27). In the mitochondrial apoptosis pathway, Bcl-2 has an anti-apoptotic effect, while Bax promotes cell apoptosis after mitochondrial injury. Hence, the induction of apoptosis depends on the balance between these two proteins. Nobiletin is also a key factor in anti-tumor activity since it promotes the apoptosis of tumor cells. In this study, it was shown that nobiletin reduced the expression of Bcl-2in renal cancer cells, while the expression level of Baxis up-regulated. The present study also found that nobiletin increased the expression levels of cleaved caspase-3 and cleaved caspase-9 in a dose-dependent manner. These results indicate that nobiletin exerts a significant pro-apoptotic effect.

In summary, the results obtained in this study suggest that nobiletin inhibits the proliferation, invasion and migration of renal cancer cells, and promotes their apoptosis by inhibiting JAK2/STAT3 and PI3K/Akt pathways. These findings provide new prospects for the application of nobiletin and new targeted therapies for the treatment of renal cancer.

Acknowledgments
None.

Conflicts of interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Wenquan Zhou; Zhenyu Xu, Ding Wu, Dian Fu, Chaopeng Tang, Jingping Ge, Zhengyu Zhang andWenquan Zhou collected and analysed the data; Zhenyu Xu wrote the text and all authors have read and approved the text prior to publication.

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