Tropical Journal of Pharmaceutical Research February 2020; 19 (2): 253-257
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i2.6

Original Research Article

Geraniin inhibits bladder cancer cell growth via regulation of PI3K/AKT signaling pathways

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Sent for review: 11 October 2019 *Revised accepted: 29 January 2020*

Abstract

Purpose: *The effect of geraniin on human bladder transitional carcinoma was not clear, this study was thus intended to reveal it and reveal the mechanism.*
Methods: *T24 cells were treated with 5, 10, and 20 μM of geraniin and the viability and apoptosis of T24 cells were determined using thiazolyl blue tetrazolium bromide (MTT) assay and flow cytometry. The protein expression levels of Cyclin D1, p21, BAL-2, BAX, cleaved caspase-3 and PI3K/AKT pathway were evaluated using western blot.*
Results: *Geraniin decreased T24 cell viability and induced T24 cell cycle arrest. The proportion of T24 cells in S phase was decreased by geraniin. Besides, geraniin promoted T24 cell apoptosis and regulated PI3K/AKT pathway.*
Conclusion: *Geraniin appears to regulate bladder cancer cell growth by decreasing the levels of PI3K and AKT phosphorylation. Thus, this agent may be useful in the management of bladder cancer*

Keywords: *Geraniin, T24 cells, Apoptosis, PI3K/AKT signaling*

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INTRODUCTION

Bladder cancer is a malignant tumor that affects urinary system, which is now second only to prostate cancer with a higher incidence in urogenital tumor system [1]. Patients are commonly treated with neoadjuvant chemotherapy and surgery. Though common chemotherapy regimens are reported, the effective proportion ranges only from 40 to 65 % [2], the development of new therapeutic drugs is thus necessary for increasing survival rate.

Botanically derived drugs, such as paclitaxel and camptothecin, are important for the treatment of malignant tumors due to their strong specific and pharmaceutical effects in cancer treatment. Besides, Phytohormones increase reactive oxygen species (ROS) levels in HL60 cells to stimulate caspase-3/7 activities and induce cell apoptosis. It has also been shown that scutellarin inhibits the dissociation and degradation of SKP2 by SKP1, which causes lung cancer cell line A549 to arrest in the G2/M phase [3]. In

particular, Geraniin is one of the typical natural scented tannin that has antioxidant, anti-viral, and anti-bacterial effects [4,5].

Related studies have demonstrated that geraniin could inhibit migration, invasion, and drug resistance in transforming growth factor-β1-treated A549 cells [6]. Geraniin induces breast cancer cell apoptosis by regulating the ROS-stimulated p38 mitogen-activated protein kinase pathway [7].In human colorectal cancer cells, by inducing chromosomal instability of mutations, geraniin could selectively arrest cell cycle [8]. In ovarian cancer cells, geraniin inhibits cell growth by inhibiting the nuclear factor-κB pathway and decreasing MCL-1 expression. In addition, geraniin reverses the resistance of human colon cancer cell line (HCT-8) to fluorouracil, cisplatin, and vincristine. The expression of the multidrug resistance gene *MDR1* may be inhibited by preventing nuclear translocation of the transcription factor YB1 [9-11]. Though Geraniin is an anti-tumor compound derived from traditional Chinese medicine, its anti-tumor effects and the underlying mechanism in bladder cancer is still unclear.

EXPERIMENTAL

Chemicals

Dulbecco's Modified Eagle Medium (DMEM), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS) phosphate-buffered saline (PBS) and TRIzol reagent were obtained from Invitrogen (Carlsbad, CA, USA). Geraniin was obtained from Sigma, St. Louis (MO, USA) with a purity of above 80 %. Annexin V-FITC/PI Apoptosis Detection kit was obtained from Beyotime (Shanghai, China). 3, 3'-diaminobenzidine (DAB) and bicinchoninic acid protein (BCA) assay were purchased from Phygene Lifescience (China).

MTT

Human bladder transitional carcinoma cell line T24 in the logarithmic growth phase was digested, centrifuged, and the supernatant was discarded. Cell density was adjusted to 8×10^4 cells/mL with medium and inoculated with 100 µL per well. After 24 h of incubation, 5, 10, or 20 µM geraniin was added and then incubated and MTT was added. After removing the supernatant, DMSO was added and the absorbance was measured at 490 nm.

Flow cytometry

The harvested T24 cells were inoculated for 24 h, and then were treated with 5, 10, or 20 µM geraniin. 24h later, cells were collected and then washed twice with pre-cooled PBS. The staining reagents were added and incubated in the dark. Flow cytometry was performed by standard procedure.

To assess cell apoptosis, T24 cells that treated by geraniin were collected using centrifugation, washed by PBS, and suspended in binding buffer. Next, Annexin V-FITC and PI was added, mixed and measured.

Western blot

The T24 cells were treated with 5, 10, or 20 µM geraniin for 24 h, followed by lysed and centrifuged. Membranes were blocked and the Protein Gel Electrophoresis Chamber Systems (Cat. No. EI0001, Invitrogen) was performed at 100 V for about 90 min. Then, membranes were washed and incubated with the following antibodies overnight at 4 °C.

The primary antibodies (diluted 1:1,000) were as follows: cleaved caspase-3 (Asp 175, 9661#, Cell Signaling, USA), Cyclin D1 (Human Specific, MAB60001-SP, R&D System, USA), p21 (Human Specific, 9502#, R&D System, USA), BAX antibody (human, ab32503, Abcam, USA), BCL-2 (human, AF810-SP, R&D System, USA), Human PI3-Kinase Antibody (MAB2686-SP, R & D System, USA), PI3K (phospho Y464, ab138364, Abcam, USA), Human AKT (MAB2055-SP, R&D System, USA), and Human Phospho-AKT (MAB877-SP, R & D System, USA).

The secondary antibody was horseradish peroxidase-labeled goat anti-human immunoglobulin G (H + L) (diluted 1:500, A0201, Beyotime, China). The internal reference protein was β-actin (human, MAB8969-SP, R&D System, USA). TBST washes were then performed five times before chemiluminescence detection.

The gel was resuspended in 100 µL of loading dye and separated using 7.5 % polyacrylamide gel electrophoresis. The target protein in chemiluminescence was detected using ChemiDocTM XRS + gel imaging system.

Statistical analysis

Analysis was performed using SPSS (USA) 13.0 statistical software. Data were expressed as mean ± standard deviation. Data were analyzed using t-tests and one-way analysis of variance. Differences were considered significant at $p < 0.05$.

RESULTS

Geraniin decreased T24 cell viability

Figure 1 showed the viability of T24 cells after treatment with geraniin for 24-h. Compared with the control group, there was a significant decrease when treated with geraniin ($p < 0.05$). When the concentrations of geraniin were 5, 10, and 20 μM, the rates of cell viability were 80.7, 57.5, and 45.7 %, respectively. The viability of T24 cells was decreased dose-dependently, with a half-maximal inhibitory concentration (IC_{50}) value of 16.43 μM. These findings show that geraniin reduced T24 cell viability.

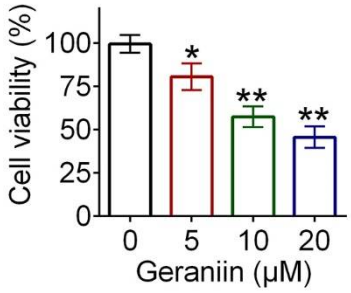

Figure 1: Effect of geraniin on the viability of T24 cells; *$p < 0.05$, ** $p < 0.01$ vs. control group, n = 3

Geraniin induced cell cycle arrest in T24 cells

Cyclin D1, a positive regulator of G1/S phase, forms a regulatory pathway with p21, could prevent cells from entering S phase and thus limit cell overgrowth. As shown in Figure 2A, when the concentration of geraniin was 5, 10, and 20 μM, T24 cells in G1 phase was 67.09, 76.69 and 88.74 % and T24 cells in the S phase was 28.36, 18.91, and 6.13 %, respectively. Figure 2B showed that the protein expression levels of Cyclin D1 and p21 was down-regulated ($p < 0.01$ vs. control), and up-regulated ($p < 0.01$ vs. control) by geraniin. These results showed that geraniin could induce T24 cell cycle arrest.

Geraniin promoted T24 cell apoptosis

When geraniin concentrations were 5, 10, and 20 μM, the apoptotic proportion were 18.5%, 30.2 %, and 36.5 % ($p < 0.05$, $p < 0.01$ and $p < 0.01$ vs. control, respectively). Moreover, in T24 cells that treated with 5, 10, and 20 μM of geraniin, the early apoptotic proportions were 10.3, 20.9, and 21.3 %, while the late apoptotic proportions were 7.46, 9.28, and 16.0 %, respectively (Figure 3A). Totally, geraniin promoted T24 cell apoptosis.

The results in Figure 3B showed that, after T24 cells were treated with geraniin, the protein expression levels of BAX and cleaved caspase-3were up-regulated, and the anti-apoptotic protein BCL-2 was down-regulated (all $p < 0.01$ *vs.* control).

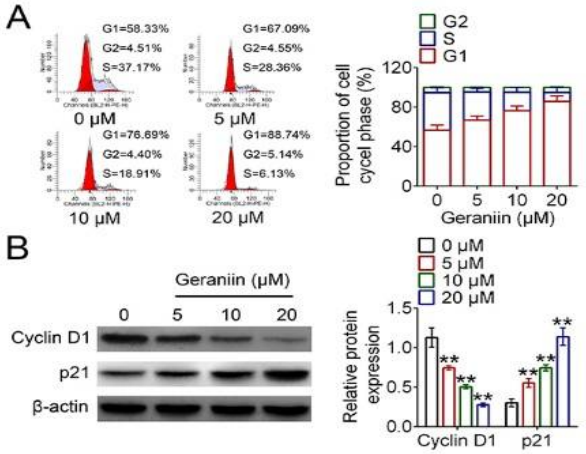

Figure 2: Effect of geraniin on T24 cell cycle arrest. (A) Cell cycle analysis was performed by flow cytometry. (B) Cyclin D1 and p21 protein expression levels were detected by western blot; *$p < 0.05$, ** $p < 0.01$ vs. control group, n = 3

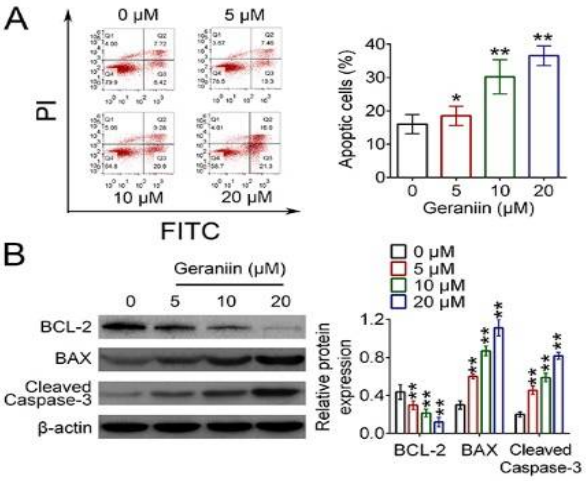

Figure 3: Effect of geraniin on the expression of apoptosis related proteins. (A) Cell apoptosis was detected by flow cytometry. (B) The protein expression levels were measured by using western blot; *$p < 0.05$, ** $p < 0.01$ vs. control group, n = 3

Geraniin regulated PI3K/AKT pathway

Western blotting was used for detecting the protein expressions of apoptotic pathway. The results were shown in Figure 4. The protein expression levels of p-PI3K and p-AKT were down-regulated (all $p < 0.01$ vs. control group), butPI3K and AKT were not significantly affected. The ratios of p-PI3K/PI3K and p-AKT/AKT were

decreased when the concentration of geraniin was increased ($p < 0.01$ vs. control group).

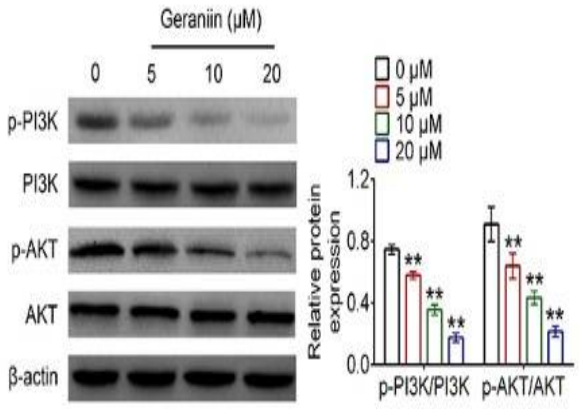

Figure 4: Effect of geraniin on PI3K/AKT pathway. Western blot was used for detecting the protein expression levels; ** $p < 0.01$ vs. control group, n = 3

DISCUSSION

Bladder transitional cell carcinoma is the most common type seen (about 90 %) in bladder cancer patients [12,13]. Thus, T24 cells were used in this study. Geraniin a yellow crystalline polyphenolic compound, has been reported to induce apoptosis of T24 cells which might has potential for possible cancer treatment [14].

Apoptosis is gene-regulated cell death, and it is extremely important for maintaining internal environmental stability and normal physiological activities. Tumors achieve unlimited proliferation through escape of programmed death [15]. This study demonstrated that geraniin has an anti-tumor effect by inhibiting the growth of T24 cells. In detail, geraniin inhibited the viability, caused the cessation of S phase in the cell cycle, downregulated the receptor proteins cyclin D1 and p21, and induced cell apoptosis in T47 cells. Bcl-2 family regulates mitochondria permeability via a mechanism in which cells receive a signal for apoptosis, and Bax is oligomerized and transferred from the cytoplasm to the outer membrane of the mitochondria [16]. Bax then interacts with the anion channels on the membrane, releasing apoptotic factors from the mitochondria into the cytoplasmic matrix. Bcl-2 forms a dimer with pro-apoptotic Bax. If the relative amount of Bax is higher than that of BCL-2, the number of BAX homodimers increases, leading to the opening of the mitochondrial permeability transition pore, and activates caspase-3 downstream. Caspase-3 is important for the apoptotic process [17]. Here, cleaved caspase-3 was up-regulated, indicating that geraniin induced apoptosis of T24 cells, which may also require the participation of caspase family proteins.

The PI3K/AKT pathway is one of the pathways that regulate cell function [18]. Phosphatidylinositol 3-kinase (PI3K) is the initial part of this pathway [19,20]. The second messenger produced by PI3K can be used to transmit information to the cells, ultimately affecting cell proliferation, differentiation, survival, and migration. The PI3K/AKT signaling pathway is critical for the development and progress of tumors [21].

s a protein kinase, AKT is activated by various growth and survival factors [22]. It is a key molecule in the PI3K-induced wortmannin sensitive signaling pathway and in regulating cell survival and apoptosis. Activation of AKT is accomplished by phosphorylation of Thr308 site of the activation loop and the Ser473 site. Activation of AKT requires simultaneous phosphorylation at both sites and thus promotes cell proliferation by inhibiting apoptosis. Based on the results in our study, it can be concluded that geraniin down-regulates PI3K/AKT signaling pathway to regulate cell growth by inhibiting viability and promoting apoptosis in T24 cells. However, the specific regulatory molecular mechanism of geraniin-induced apoptosis needs to be further studied.

CONCLUSION

Geraniin exerts anti-tumor effect in T24 cells by inhibiting cell viability and inducing cell apoptosis. Its anti-tumor activity is closely related to the apoptotic mechanism, PI3K/AKT pathway, which provides a pharmacological basis for the development of natural compounds for cancer treatment.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. Junwei Xu designed all the experiments and revised the paper. Ning Qin and Yebin Yao performed the experiments, Tao Chen and Wenbo Jiang wrote the paper.

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