Effects of Atractylon on Proliferation and Apoptosis of Intestinal Cancer Cells Through PI3K/AKT/mTOR Signaling Pathway

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ARTICLE INFO

Original paper

Article history:
Received: January 11, 2022
Accepted: April 01, 2022
Published: May 31, 2022

Keywords:
- atractylon, PI3K/AKT/mTOR signaling pathway, intestinal cancer cells, proliferation, apoptosis

ABSTRACT

The study aimed to explore the effects of atractylon on the proliferation and apoptosis of intestinal cancer cells through the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway. The intestinal cancer HT29 cell lines were cultured in vitro, and atractylon at different concentrations (15 and 30 mg/mL) was added. Then cell proliferative activity was detected via cell counting kit-8 (CCK8) assay, and the proportion of positive cells was determined using EdU staining. The content of interferon-γ (INF-γ), tumor necrosis factor-α (TNF-α) and matrix metalloproteinase-9 (MMP-9) was detected via enzyme-linked immunosorbent assay (ELISA), and the apoptosis of HT29 cells was detected through terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Moreover, reverse transcription-polymerase chain reaction (RT-PCR) was performed to determine the messenger ribonucleic acid (mRNA) levels of proliferation, apoptosis and PI3K/AKT/mTOR signaling pathway-related genes, and Western blotting was used to analyze the expression of the PI3K/AKT/mTOR signaling pathway. The cell growth status was poorer with a lower density in the 15 mg/mL atractylon group and basically normal morphological structure in the 30 mg/mL atractylon group. The number of cells significantly declined and the proliferative activity was also significantly weakened in the 30 mg/mL atractylon group. There were obviously more apoptotic cells in the 30 mg/mL atractylon group. Besides, INF-γ, TNF-α and MMP-9 were all evidently decreased in the 30 mg/mL atractylon group. Expressions of B-cell lymphoma-2 (Bcl-2), PI3K, AKT and mTOR obviously declined in the 30 mg/mL atractylon group, and they were raised in the NC group, while the expression of Caspase3 showed the opposite trends. Atractylon at an appropriate concentration can inhibit the proliferation and promote the apoptosis of intestinal cancer cells by suppressing the PI3K/AKT/mTOR signaling pathway, which can be used to treat colorectal cancer and other related diseases.

Introduction

Cancer is a kind of multifactorial disease, and cancer cells can up-regulate multiple defense mechanisms to evade drug treatment. Colon cancer is the second most common type of cancer in the world, as well as the fourth leading cause of cancer-related death (1, 2). The morbidity rate of colon cancer in developed countries is 10 times that in developing countries (3). In fact, the risk of colon cancer in Americans is about 20% in life, and the cases of colon cancer are increasing every year in China (4). Studies have demonstrated that the incidence of intestinal cancer is related to a variety of factors. In 2012, it was reported by the expert group of the International Agency for Research on Cancer that there is a correlation between body weight and cancer (5). According to epidemiological and cohort studies, obesity has a strong correlation with the risk of colorectal cancer (6). Despite great progress made in the treatment of colon cancer, its prognosis remains poor (7). Colon cancer is characterized by intra-abdominal metastasis and frequent postoperative recurrence, which are the main causes of poor prognosis (8). Therefore, exploring the molecular mechanism of colon cancer may offer valuable insights and help the development of new and effective therapeutic methods for colon cancer. In the present study, the basic mechanism of intestinal cancer was explored, hoping to help understand the pathogenesis of the disease. Atractylon is the main
The phoshatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway is involved in a variety of biological functions during the life process, and its functions have been gradually studied and perfected. It has been found in studies that the PI3K/AKT/mTOR signaling pathway can mediate cell proliferation and apoptosis (11, 12). Moreover, the PI3K/AKT/mTOR signaling pathway can exert a survival effect and inhibit abnormal cell death, which is mainly mediated through inhibiting such pro-apoptotic proteins as Caspase9 (13). Studies have demonstrated that the activation of the PI3K/AKT/mTOR pathway has important effects on the differentiation, proliferation and apoptosis of smooth muscle cells and vascular fibroblasts (14). In addition, it has been proved that PI3K and its downstream target AKT are also involved in the regulation of oxidative and inflammatory responses, and previous studies have shown that the PI3K/AKT/mTOR signaling pathway may serve as a feedback regulator of inflammatory oxidative stress (15). The over-activation of the PI3K/AKT signaling pathway participates in the development and metastasis of colon cancer, including cell proliferation, apoptosis and migration, which has been widely studied (16, 17). In addition, blocking the activity of PI3K/AKT in colon cancer cells exhibits a good anticancer effect (18). According to recent studies, the downstream factor of the PI3K/AKT pathway is mTOR, a potential therapeutic target for a variety of cancers, including non-small cell lung cancer and colorectal cancer (19). Moreover, the activated PI3K/AKT pathway has been proved to be associated with a poor prognosis of stage II colon cancer (20). The above results indicate that the PI3K/AKT pathway is of great importance for the occurrence and development of colon cancer, so it is an important target for the treatment of colon cancer. The mechanism of the PI3K/AKT/mTOR pathway in the pathogenesis of intestinal cancer has been partially studied, but its potential role in the proliferation and apoptosis of intestinal cancer cells remains unclear, and the downstream components of the PI3K/AKT/mTOR pathway are yet to be determined. Besides, there are few studies on the regulatory effect of atractylon on PI3K/AKT/mTOR and its influences on the proliferation and apoptosis of intestinal cancer cells.

The present study aims to explore the effects of atractylon on the proliferation and apoptosis of intestinal cancer cells through PI3K/AKT/mTOR. In this study, the cells were cultured, the proliferation-and apoptosis-related molecules were detected, and the gene and protein assays were performed, hoping to confirm that atractyloininhibits proliferation and promotes apoptosis of intestinal cancer cells through PI3K/AKT/mTOR. To sum up, the experimental results enrich and perfect the theoretical basis of the effects of atractylon on the proliferation and apoptosis of intestinal cancer cells through PI3K/AKT/mTOR, and the PI3K/AKT/mTOR pathway may serve as a key target for the treatment of intestinal cancer.

Materials and methods

Commonly-used reagents and consumables

Intestinal cancer HT29 cells (ATCC), atractylon (Qilu Pharmaceutical), RIPA lysis buffer (Beyotime), loading buffer, protease inhibitor and bicinchoninic acid (BCA) protein concentration assay kit (Biosharp), and enzyme-linked immunosorbent assay (ELISA) kits of tumor necrosis factor-α (TNF-α) (Nanjing Jiancheng Bioengineering Institute), β-actin and secondary antibodies (Boster Biological Technology Co., Ltd.), primary antibodies (Santa), electrophoresis apparatus (Bio-Rad), microplate reader (Thermo), 2500 gel imager (Bio-Rad, USA), quantitative polymerase chain reaction (qPCR) instrument (7900 Fast, Applied Biosystems), TRIzol reagent, DEPC-treated water, SuperScript III RT kit and SYBR qPCR Mix (ABI).

Cell culture and grouping

Intestinal cancer HT29 cells purchased from ATCC were quickly taken out from the liquid nitrogen container, rapidly thawed in 60°C sterile water prepared in advance and centrifuged, and then the supernatant was discarded. The above operation was repeated several times. The cells were resuspended

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using the medium, inoculated into a 6-well plate at an appropriate density and incubated in the thermostatic incubator. The original medium was replaced every other day. The second-generation cells in good growth status were divided into the negative control (NC) group, 15 mg/mL atractylon group and 30 mg/mL atractylon group. The morphological changes in cells in each group were observed, and the cell samples were collected 24 h after stimulation.

The study was approved by the ethics committee of Yantai Hospital of Traditional Chinese Medicine.

**Cell counting kit-8 (CCK8) cell proliferation assay**

The cells in the logarithmic growth phase in each group were inoculated into a 96-well plate and cultured in the incubator with 5% CO2 at 37°C for 0, 24, 48 and 72 h. Then the medium was discarded, and 110 μL of developing solution was added into each well, followed by incubation in the incubator at 37°C for 1 h. Finally, the absorbance in each group was detected at 450 nm using an ultraviolet spectrophotometer, and plotted into the line chart to reflect the cell proliferative activity.

**Detection of cytokines in each group**

After stimulation, the cells in good growth status in the three groups were selected from the incubator, and the medium was discarded. The cells were collected in the three groups, from which the proteins were extracted, and the protein concentration was calculated. Then the proteins were subjected to a water bath and centrifugation. Western blotting was performed: 10% separation gel and 5% spacer gel were prepared for protein loading and electrophoresis, and the protein was transferred onto a membrane using the semi-dry method, sealed, incubated with the primary antibody overnight and incubated again with the secondary antibody. The protein band was scanned and quantified using the Odyssey scanner, and the Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (F-R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’-GACATGCGCCCTGGAGAAAC-3’&lt;br&gt;5’-AGCCCGAGATGCCCCCTTATTG-3’</td>
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<tr>
<td>Bcl-2</td>
<td>5’-GGTGCTCTTGTAGATCTCTGG-3’&lt;br&gt;5’-CCATCGATCTTCAAGAATCTCT-3’</td>
</tr>
<tr>
<td>Caspase3</td>
<td>5’-TTCCGGTTAACCACAGTGTGAG-3’&lt;br&gt;5’-ATCGGCTCACCTTCTGCGCTC-3’</td>
</tr>
<tr>
<td>P3K</td>
<td>5’-TAGGTCTCGGTATCCCGAGAAAGGA-3’&lt;br&gt;5’-AGATCGGCAATCTTCCGC-3’</td>
</tr>
<tr>
<td>AKT</td>
<td>5’-GCTACGCCGGATCAGGAGCTAC-3’&lt;br&gt;5’-CTCACTCGCTCAACGAC-3’</td>
</tr>
<tr>
<td>mTOR</td>
<td>5’-CTCAGTGAAAGTGCCCAATCAGGTC-3’</td>
</tr>
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</table>

**Observation of cell proliferation in each group using EdU staining**

After drug intervention, the cells in each group were stained using the Click-iTEdU staining kit according to the instructions. Then the cells were photographed in 5 randomly-selected fields of view under a fluorescence microscope. Finally, EdU-positive cells were counted.

**TUNEL apoptosis assay**

The apoptosis of paraffin sections was detected using the in situ cell death detection kit (Roche, Germany), as follows: The paraffin sections were fixed, rinsed and infiltrated with 0.1% Triton X-100, followed by FITC end labeling of apoptotic DNA fragment using the TUNEL assay kit (Beyotime). The FITC-labeled TUNEL-positive cells were observed under the fluorescence microscope, and the TUNEL-positive cells were counted in 10 fields of view.

**Detection of gene expression using RT-PCR**

The RNA was extracted from cells collected and synthesized into DNA using the kits (TaKaRa) in accordance with the instructions. The primer amplification was performed for genes to be detected and internal reference gene using the system (20 μL): 2 μL of cDNA, 10 μL of qPCR mix, 2 μL of primers, and 6 μL of ddH2O, with 3 replicates in each reaction. Then the PCR amplification was performed: pre-denaturation at 95°C for 2 min, 94°C for 20 s, 60°C for 30 s, for a total of 40 cycles. The primer sequences of target genes and the internal reference GAPDH were designed according to those in the GenBank (Table 1). The expression levels of target genes were detected via qRT-PCR.

**Western blotting**

The cells in appropriate density and size were collected in the three groups, from which the proteins were extracted, and the protein concentration was calculated. Then the proteins were subjected to a water bath and centrifugation. Western blotting was performed: 10% separation gel and 5% spacer gel were prepared for protein loading and electrophoresis, and the protein was transferred onto a membrane using the semi-dry method, sealed, incubated with the primary antibody overnight and incubated again with the secondary antibody. The protein band was scanned and quantified using the Odyssey scanner, and the
level of protein to be detected was corrected using GAPDH. Finally, the protein expression was calculated through gray scanning.

Statistical analysis
All raw data obtained in the experiments were statistically analyzed using SPSS 20.0 software, the validity of data was retained, and multiple comparisons were performed. The experimental results were expressed as mean ± standard deviation (χ±SD). p<0.05 suggested that the difference was statistically significant. The bar graph was plotted using GraphPad Prism 7.0.

Results
Morphological observation of cells
At 24 h after treatment, the cell growth in clumps was rapid, and the cells were in an oval shape with an intercellular tight junction in the NC group (Figure 1A). In 15 mg/mL atractylon group and 30 mg/mL atractylon group, the cell growth morphology was uniform, the density was lower and the cell proliferation was slower (Figure 1B & 1C).

Results of CCK8 cell proliferation assay
The absorbance in each group at different time points was determined using the CCK8 proliferation assay. As shown in Figure 2, the proliferation ability of HT29 cells was significantly enhanced in NC group at 24, 48 and 72 h (p<0.05), *p<0.05

Detection results of cytokines in each group
For the early detection of the occurrence of intestinal cancer, the levels of inflammatory factors TNF-α, INF-γ and MMP-9 were detected. It was found that their levels were obviously higher in NC group than those in the other two groups, while they obviously declined in 30 mg/mL atractylon group (p<0.05) (Table 2), which suggests that 30 mg/mL atractylon can inhibit the production of inflammatory factors, further inhibiting the occurrence of intestinal cancer.

Table 2. Detection results of cytokines

<table>
<thead>
<tr>
<th>Group</th>
<th>TNF-α (fmol/mL)</th>
<th>INF-γ (μg/L)</th>
<th>MMP-9 (ng/mL)</th>
</tr>
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<tbody>
<tr>
<td>NC</td>
<td>88.05±2.35</td>
<td>30.21±0.85</td>
<td>300.4±0.8</td>
</tr>
<tr>
<td>15 mg/mL atractylon</td>
<td>60.52±3.54</td>
<td>15.32±1.20</td>
<td>200.1±2.1</td>
</tr>
<tr>
<td>30 mg/mL atractylon</td>
<td>40.34±2.14</td>
<td>5.02±1.34</td>
<td>98.2±2.5</td>
</tr>
</tbody>
</table>

Cell proliferation in each group observed through EdU staining
To further detect the effect of atractylon on the proliferation ability of intestinal cancer cells, EdU staining was performed to evaluate the cell proliferation ability in the NC group and 30 mg/mL atractylon group. The results showed that the number of EdU-positive cells was evidently smaller and the cell proliferation was less in 30 mg/mL atractylon group than those in the NC group (p<0.05) (Figure 3).
Figure 3. Results of EdU staining in each group. The number of EdU-positive cells is evidently smaller and the cell proliferation is less in 30 mg/mL atractylon group than those in NC group ($p<0.05$), showing statistically significant differences. A. 30 mg/mL atractylon group. B: NC group.

Results of TUNEL apoptosis assay

TUNEL staining was used to detect the apoptosis level in each group. As shown in Figure 4, there were fewer TUNEL-positive cells and they almost could not be observed in NC group. The number of TUNEL-positive cells in 15 mg/mL atractylon group and 30 mg/mL atractylon group was remarkably larger than that in the NC group, and it was the largest in 30 mg/mL atractylon group ($p<0.05$), indicating that atractylon can promote the apoptosis of HT29 cells.

Figure 4. Apoptosis level in each group detected using TUNEL staining. There are fewer TUNEL-positive cells and they almost cannot be observed in NC group. The number of TUNEL-positive cells in 15 mg/mL atractylon group and 30 mg/mL atractylon group is remarkably larger than that in NC group, and it is the largest in 30 mg/mL atractylon group ($p<0.05$). A: NC group.. B: 15 mg/mL atractylon group. C: 30 mg/mL atractylon group.

Results of RT-PCR

The results of RT-PCR manifested that the gene expressions of Bcl-2, PI3K, AKT and mTOR evidently declined in 15 mg/mL atractylon group and 30 mg/mL atractylon group, and they were increased in NC group, while the gene expression of Caspase3 displayed the opposite trends ($p<0.05$) (Figure 5), which demonstrates that atractylon inhibits cell proliferation and promotes apoptosis, further suppressing the occurrence of intestinal cancer.

Figure 5. Gene detection results. The gene expressions of Bcl-2, PI3K, AKT and mTOR evidently decline in 15 mg/mL atractylon group and 30 mg/mL atractylon group, and they are increased in NC group, while the gene expression of Caspase3 displays the opposite trends ($p<0.05$). * $p<0.05$ vs. NC group, £ $p<0.05$ vs. 15 mg/mL atractylon group

Results of Western blotting

The results of Western blotting manifested that the protein expressions of Bcl-2, PI3K, AKT and mTOR evidently declined in 15 mg/mL atractylon group and 30 mg/mL atractylon group ($p<0.05$), while they showed the opposite trends in NC group (Figure 6). The above findings demonstrate that atractylon inhibits cell proliferation and promotes apoptosis through suppressing the PI3K/AKT/mTOR signaling pathway, further inhibiting the occurrence of intestinal cancer.

Figure 6. Protein expression. The protein expressions of Bcl-2, PI3K, AKT and mTOR evidently decline in 15 mg/mL atractylon group and 30 mg/mL atractylon group ($p<0.05$), while they show the opposite trends in NC group ($p<0.05$). * $p<0.05$ vs. NC group, £ $p<0.05$ vs. 15 mg/mL atractylon group. A: Western blot result. B: Quantification analysis of Western blot.
Discussion

Intestinal cancer is a common type of cancer in the world, and its occurrence is related to many factors, according to studies. In recent years, despite the great progress made in the treatment of intestinal cancer, its prognosis is still very poor (21). The PI3K/AKT signaling pathway is related to the proliferation, apoptosis and migration of colon cancer cells, which has been widely studied. Blocking the activity of PI3K/AKT in colon cancer cells exhibits a good anticancer effect (22). Therefore, exploring the pathogenesis of colon cancer may provide important references for the development of new and effective therapeutic methods for colon cancer. The present study aims to explore the effects of atractylonon on the proliferation and apoptosis of intestinal cancer cells through PI3K/AKT/mTOR. Therefore, the cells were cultured, the proliferation- and apoptosis-related molecules were detected, and the gene and protein assays were performed, hoping to confirm that atractyloninhibits proliferation and promotes apoptosis of intestinal cancer cells through PI3K/AKT/mTOR. It was found that the cell growth in clumps was rapid, and the cells were in an oval shape with an intercellular tight junction in the NC group. In the 15 mg/mL atractylon group and 30 mg/mL atractylon group, the cell growth morphology was uniform, the density was lower and the cell proliferation was slower. The absorbance in each group at different time points was determined using the CCK8 proliferation assay. The results revealed that the proliferation ability of HT29 cells was significantly stronger in NC group than that in the other two groups at 24, 48 and 72 h, while it was the weakest in the 30 mg/mL atractylon group. To further detect the effect of atractylon on the proliferation ability of intestinal cancer cells, EdUstaining was performed to evaluate the cell proliferation ability in the NC group and 30 mg/mL atractylon group. The results showed that the number of EdU-positive cells was evidently smaller and the cell proliferation was less in 30 mg/mL atractylon group than those in the NC group, consistent with previous studies (23).

Studies have demonstrated that inflammatory factors play important roles in the occurrence and development of intestinal cancer, especially TNF-α, INF-γ, etc., and the excessive production of them will cause irreversible damage to cells (24). In this study, for the early detection of the occurrence of intestinal cancer, the levels of inflammatory factors TNF-α, INF-γ and MMP-9 were detected. It was found that their levels were obviously higher in the NC group than those in the other two groups, while they obviously declined in the 30 mg/mL atractylon group, which suggests that 30 mg/mL atractyloninhibit the production of inflammatory factors, further inhibiting the occurrence of intestinal cancer. Apoptosis is regulated by apoptosis genes, which can remove harmful substances in cells. Moreover, as the defender of the body, it supplies energy to the production and metabolism of subcellular structure and maintains cell stability (25). In this study, TUNEL staining was used to detect the apoptosis level in each group. The results manifested that there were fewer TUNEL-positive cells and they almost could not be observed in the NC group. The number of TUNEL-positive cells in 15 mg/mL atractylon group and 30 mg/mL atractylon group was remarkably larger than that in the NC group, and it was the largest in 30 mg/mL atractylon group, indicating that atractyloninhibit the apoptosis of HT29 cells. The activation of PI3K triggers a series of intracellular events, leading to the activation of AKT and mTOR and inducing the expressions of multiple target genes that regulate cell proliferation and differentiation (26, 27). In this study, the results of RT-PCR manifested that the gene expressions of Bcl-2, PI3K, AKT and mTOR evidently declined in 15 mg/mL atractylon group and 30 mg/mL atractylon group, and they were increased in NC group, while the gene expression of Caspase3 displayed the opposite trends, which demonstrates that atractyloninhibits cell proliferation and promotes apoptosis, further suppressing the occurrence of intestinal cancer. Besides, the results of Western blotting manifested that the protein expressions of Bcl-2, PI3K, AKT and mTOR evidently declined in 15 mg/mL atractylon group and 30 mg/mL atractylon group, while they showed the opposite trends in the NC group. The above findings demonstrate that atractyloninhibits cell proliferation and promotes apoptosis by suppressing the PI3K/AKT/mTOR signaling pathway, further inhibiting the occurrence of intestinal cancer. To sum up, the effect of atractylon was confirmed through the above experiments, but there are some deficiencies in this study. In the future, the deeper regulatory
mechanism can be further explored using more molecular experiments.

In conclusion, it was found that atractylon may regulate the proliferation and apoptosis of intestinal cancer cells by inhibiting the PI3K/AKT/mTOR signaling pathway, and it can be used as a therapeutic drug for patients with intestinal cancer. The therapeutic effect and prognosis of patients can be evaluated using the PI3K/AKT/mTOR signaling pathway. In the subsequent study, animal experiments can be introduced to further verify and explore the mechanism of atractylon in regulating the proliferation and apoptosis of intestinal cancer cells through the PI3K/AKT/mTOR signaling pathway from multiple levels in vivo and in vitro.

Acknowledgements
Not applicable.

Funding

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
JJM wrote the manuscript. JJM and QZ were responsible for cell culture and CCK8 assay. JPM and LD perform PCR and ELISA. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the ethics committee of Yantai Hospital of Traditional Chinese Medicine.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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