The therapeutic effect of phellopterin on colitis-associated cancer and its effects on TLR4/NF-κB pathway and macrophage M2 polarization

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ABSTRACT

This study was conducted to investigate the effect and mechanism of phellopterin on colitis-associated cancer (CAC). For this purpose, the CAC mouse model was established by the AOM/DSS method, and the therapeutic effects of phellopterin in different doses were compared. The levels of interleukin-6 (IL-6), IL-1β, IL-10, and tumor necrosis factor-α (TNF-α) in peripheral blood were detected by ELISA. The changes in T lymphocyte subsets and the expressions of CD163, CD206, Arg-1, and Ym-1 in colonic macrophages were detected. The expression of TLR4 and NF-κB p65 in the colon was tested by Western blot. Results showed that as against the Model group, the body weight and survival rate of mice treated with phellopterin were increased, the disease activity index, hematochezia rate, and tumor formation rate were decreased, the colon length was increased, and the number of tumors and spleen index were decreased (P < 0.05). As against the Model group, the proportion of CD4+ and CD8+ in the peripheral blood of phellopterin intervention mice increased, the content of IL-6, IL-1β, and TNF-α decreased, and the content of IL-10 increased. The expression of CD163, CD206, Arg-1, and Ym-1 in colonic macrophages was decreased. The protein expressions of TLR4 and NF-κB p65 in colonic tissue were decreased (P < 0.05). The effect of phellopterin intervention on CAC was dose-dependent. In conclusion, phellopterin can improve the symptoms and inflammatory response of CAC and inhibit the occurrence of colon cancer (CC) by inhibiting M2 polarization of macrophages and activation of the TLR4/NF-κB pathway.

Introduction

Colitis cancer (CC) is one of the top three malignant tumors worldwide. The occurrence and development of CC are affected by a variety of factors, mainly related to patients’ lifestyles, intestinal diseases, and family genetics (1). In addition, the abnormal expression of tumor-promoting/tumor suppressor genes is also an important factor inducing CC. Inflammatory bowel disease (IBD) is a recurrent chronic intestinal disease, which increases the risk of CC (2). With the repeated onset of inflammatory bowel disease, the course of the disease is prolonged, and CAC develops after prolonged treatment, which is the most serious complication of inflammatory bowel disease (3). Epidemiology shows that the risk of patients with inflammatory bowel disease developing into CC is about 1% after 8-10 years, and the risk increases to about 8% after 20 years, and about 20% after 30 years (4). The traditional treatment methods of CAC include surgery, radiotherapy, and chemotherapy, but the 5-year survival rate of patients is still only about 50% (5).

Traditional Chinese medicine (TCM) has the advantages of multiple targets, low recurrence rate, and few side effects. Recent studies have found that TCM can cooperate with chemotherapy and other treatment methods to improve the therapeutic effect (6). In addition, TCM can also become the main intervention method in the empty window period after surgery and radiotherapy and chemotherapy. Previous studies have confirmed that Shaoyao decoction in inflammatory bowel disease and CAC (7). A previous study found that the Decoction of Seven Ingredients Including White Peony Root could inhibit the classical inflammatory pathway TLR4/MyD88/NF-κB pathway. Thus, it can alleviate 2,4, 6-trinitrobenzene sulfonic acid-induced inflammatory response and relieve intestinal inflammatory symptoms (8). Moreover, it has been found that paony lactone, one of the effective components of paony, can regulate the expression of CDX2 through AMPK, thus alleviating the inflammatory response and apoptosis of enteritis (9). Common aucklandia root is an important component of the Decoction of Seven Ingredients Including White Peony Root, while phellopterin is one of the active ingredients of common aucklandia root. It confirmed that phellopterin has anti-tumor activity and can arrest the cell cycle to the G2/M phase (10). In addition, phellopterin can inhibit the secretion of TNF-α, transforming growth factor-β (TGF-β), and IL-4 (11). However, the role of phellopterin in colitis or CC has not

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Detection of peripheral blood T lymphocyte subsets

The mice serum was collected, and 20 μL of CD4+, CD8+, and other antibodies were added, respectively. Following shaking, the cells were kept in the dark and incubated for 20 min at ambient temperature. Subsequently, 2 mL of 1× hemolysis was added to each tube, mixed, incubated at ambient temperature in the dark for 10 min, centrifugation at 1,000 rpm for 5 min, and the supernatant was discarded. One mL of 0.01 M phosphate buffer was put into each tube, mixing, the mixture was centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. 0.3 mL of 0.01 M phosphate buffer was put into the flow tube, mixing, detection inside 1 h, analyzing the results by Flowjo software.

Detection of inflammatory factors in peripheral blood

The mouse serum was collected, and the standard preparation was carried out according to the instructions of the ELISA kit. After dilution, 50 μL of the standard was placed in the reaction well, and 50 μL of biotin-labeled antibody was added, shaken evenly, incubation for 1 h at 37℃. Following washing, the samples were dried, and 80 μL HRP-labeled affinity streptomyein was added. It was shaken evenly, and incubated at 37℃ in the dark for 30 min. Following washing, the mixture was dried, 50 μL each of working solutions A and B were added, shaken evenly, and incubated in the dark for 10 min at 37℃. 50 μL of termination solution was added, and the microplate reader was employed to measure the absorbance at 450 nm. The concentrations of IL-6, IL-1β, TNF-α, and IL-10 were calculated.

Quantitative reverse transcription PCR assay

Macrophages were isolated from the colon tissue of mice and inoculated in a DMEM medium having 10% FBS. The cells were placed in an incubator containing 5% CO₂ at 37℃ for 2 h and then fresh medium was replaced to incubate for another 48 h. The colonic macrophages in the logarithmic growth phase were isolated and identified by immunofluorescence single standard method. Tritol reagent was adopted to extract total RNA from cells, and the working solution was prepared based on guiding of the reverse transcription kit, and reverse transcription for cDNA was carried out. Then, a 20 μL reaction system was set up, and the expression of CD206, CD163, Arg1, ym-1, and GAPDH was tested. CD206, CD163, Arg1, ym-1, and GAPDH were calculated. CD206, CD163, Arg1, ym-1, and GAPDH were calculated. CD206, CD163, Arg1, ym-1, and GAPDH were calculated.

Western blot analysis

100 mg of mouse colon tissue was cut into pieces and fully ground with liquid nitrogen. The protein was extracted according to the RIPA method, and the protein concentration was detected by the BCA kit. The corresponding concentrations of separating glue and concentrating glue were prepared and subjected to SDS-PAGE gel electro-
phoresis. The membrane was transferred by wet method and blocked with 5% skim milk powder blocking solution for 2 h at ambient temperature. Then, first antibodies against TLR4 (1:2000), NF-κB p65 (1:2000), and GAPDH (1:2000) were added, and the cells were incubated overnight at 4°C. Then HRP-labeled IgG (1:10000) secondary antibody was added and incubated for 2 h at room temperature. ECL chemiluminescence staining was adopted for visualization, which was exposed and photographed under a gel imaging system. GAPDH as an internal control, the relative expression levels of TLR4 and NF-κB p65 proteins were tested.

Statistical analysis

SPSS 22.0 software was employed for statistical analysis. All results were expressed as mean ± standard deviation. One-way ANOVA was adopted for contrast among groups, and the LSD method was adopted for pairwise contrast among groups. The difference among groups was considered statistically significant when $P<0.05$.

Results

Effect of phellopterin on signs, disease activity, and survival rate of model mice

Weight changes and disease activity index of mice in different groups were observed as given in Figure 1A. The weight of mice in the Ctrl group remained stable, while the weight of mice in Model, Low-phellopterin, Middle-phellopterin, and High-phellopterin groups decreased gradually over time. The Model group exhibited the most significant weight loss, and on day 45, the body weight of mice in Model, Low-phellopterin, Middle-phellopterin, and High-phellopterin groups was lower as against Ctrl group; The body weight in Low-phellopterin, Middle-phellopterin, and High-phellopterin groups was lower as against Model group; The body weight in Middle-phellopterin and High-phellopterin groups was higher as against Low-phellopterin group, and the body weight in High-phellopterin group was higher as against Middle-phellopterin group; Similarly, the disease activity index of mice in Model, Low-phellopterin, Middle-phellopterin, and High-phellopterin groups increased gradually over time (Figure 1B). On day 45, the disease activity index of these groups was higher as against the Ctrl group; The disease activity index of the Low-phellopterin, Middle-phellopterin, and High-phellopterin groups was lower as against Model group; Additionally, the disease activity index in Middle-phellopterin and High-phellopterin groups was lower as against Low-phellopterin group, and the disease activity index in High-phellopterin group was lower as against Middle-phellopterin group. ($P<0.05$).

Figure 2A and Figure 2B demonstrate that the hematochezia rate and tumor formation rate were 0.0% in the Ctrl group and 100.0% in the Model group. In the Low-phellopterin, Middle-phellopterin, and High-phellopterin groups, the hematochezia rates were 80.0%, 70.0%, and 55.0%, respectively, while the tumor formation rates were 75.0%, 65.0%, and 40.0%, respectively. In Figure 2C, except for the Ctrl group, all four groups of mice experienced fatalities (samples were collected immediately after death and stored at -80°C). At 15 weeks, the survival rates were 50.0% in the Model group, 60.0% in the Low-phellopterin group, 70.0% in the Middle-phellopterin group, and 85.0% in the High-phellopterin group. The survival rate in the Model, Low-phellopterin, Middle-phellopterin, and High-phellopterin groups was lower as against the Ctrl group; The hematochezia rate and tumor formation rate of the Low-phellopterin, Middle-phellopterin, and High-phellopterin groups were lower as against Model group, while the three groups had a higher survival rate. The Middle-phellopterin and High-phellopterin had a lower hematochezia rate and tumor formation rate, as well as a higher survival rate as against the Low-phellopterin. The High-phellopterin presented lower hematochezia and tumor formation rates and a higher survival rate relative to the Middle-phellopterin ($P<0.05$).

Effects of phellopterin on the organs of the model mice

Figure 3 illustrates that, in comparison to the Ctrl group, the colon length decreased and the spleen index increased in the Model, Low-phellopterin, Middle-phellopterin, and High-phellopterin groups; In contrast, the Low-phellopterin, Middle-phellopterin, and High-phellopterin groups had an increased colon length and decreased number of tumors and spleen index relative to the Model group; Fur-
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Moreover, the Middle-phellopterin and High-phellopterin groups had an increased colon length and decreased number of tumors and spleen index relative to the Low-phellopterin group; The High-phellopterin group demonstrated an increased colon length and decreased number of tumors and spleen index relative to the Middle-phellopterin group ($P<0.05$).

**Effect of phellopterin on the level of T lymphocyte subsets in model mice**

Based on Figure 4, the Model, Low-phellopterin, Middle-phellopterin, and High-phellopterin groups all exhibited a significant decrease in peripheral blood CD4+ and CD8+ levels. However, CD4+/CD8+ was increased in these groups; In comparison to the Model group, the Low-phellopterin, Middle-phellopterin, and High-phellopterin groups suggested an increase in peripheral blood CD4+ and CD8+ levels, while CD4+/CD8+ was decreased; Additionally, in contrast to the Low-phellopterin group, the Middle-phellopterin and High-phellopterin groups suggested an increase in peripheral blood CD4+ and CD8+ levels, and an obvious decrease in CD4+/CD8+; As against the Middle-phellopterin, the High-phellopterin exhibited an increase in peripheral blood CD4+ and CD8+ levels, and a clear decrease in CD4+/CD8+ ($P<0.05$).

**Effect of phellopterin on inflammatory response in model mice**

In Figure 5, it can be observed that the IL-6, IL-1β, and TNF-α were increased, while the IL-10 was lower in the peripheral blood of the Model, Low-phellopterin, Middle-phellopterin, and High-phellopterin groups; In contrast, as against the Model group, the phellopterin groups indicated decreased IL-6, IL-1β, and TNF-α, and increased IL-10; The Middle-phellopterin and High-phellopterin groups had lower IL-6, IL-1β, and TNF-α, and higher IL-10 as against the Low-phellopterin ($P<0.05$).

**Effect of phellopterin on M2-type polarization of colonic macrophages in model mice**

Figure 6 shows that the expression levels of CD163, CD206, Arg-1, and Ym-1 in colonic macrophages were increased in the Model, Low-phellopterin, Middle-phellopterin, and High-phellopterin groups; However, the expression levels of these markers were decreased in the Low-phellopterin, Middle-phellopterin, and High-phellopterin groups ($P<0.05$).
As opposed to the Model group, those were decreased in the Middle-phellopterin and High-phellopterin groups contrasting with the Low-phellopterin; Those had a clear decrease in the High-phellopterin group as against the Middle-phellopterin ($P<0.05$).

**Effect of phellopterin on TLR4/NF-κB pathway in the colon of model mice**

The results in Figure 7 indicate that the protein expression levels of TLR4 and NF-κB p65 were increased in the colon of the Model, Low-phellopterin, Middle-phellopterin, and High-phellopterin groups. Those were decreased in the colon of the phellopterin groups as opposed to the Model group. Those were decreased in the Middle-phellopterin and High-phellopterin groups as opposed to the Low-phellopterin. Those were decreased in the High-phellopterin group as opposed to the Middle-phellopterin ($P<0.05$).

**Discussion**

There is a close relationship between inflammatory bowel disease and inflammation-related CC. The risk of progression to inflammation-related CC gradually increases in patients with IBD 8-10 after diagnosis, and with the prolongation of the duration of inflammation, the extent of colonic damage in patients expands and the degree of inflammation deepens (12,13). This article used the AOM/DSS method to establish a mouse model of CC associated with inflammation. As the increase of modeling time, the body weight of the model mice gradually decreased, and the hematochezia rate and mortality gradually increased, the colon length decreased, and the spleen index increased. The spleen is an important peripheral immune organ in the body, which is rich in lymphocytes and macrophages and participates in humoral immunity (14). In this article, a mouse model of inflammation-related CC was successfully prepared using the AOM/DSS method, which laid a foundation for subsequent research. According to TCM, the weakness of the spleen and stomach caused by diet fatigue, stagnation of liver qi, and damage to the spleen and stomach, will cause the injury of large intestine mucosa and induce the occurrence of inflammation-related CC (15). The method of invigorating qi and invigorating the spleen is commonly used in the treatment of CC. Decoction of Seven Ingredients Including White Peony Root is a TCM compound, that has been used to treat ulcerative colitis in clinical practice (16). As the main component of Decoction of Seven Ingredients Including White Peony Root, common aucklandia root has attracted much attention in anti-tumor, anti-inflammatory, action on the digestive system, and anti-bacterium (17). In addition, phellopterin was found to be one of the effective components of common aucklandia root by TCMSMP database screening (OB $\geq$ 30, DL $\geq$ 0.18). Phellopterin has good anti-tumor activity (18). Therefore, this article analyzes the effect and mechanism of phellopterin in the treatment of inflammation-related CC.

The immune status of the body can regulate the occurrence and development of tumors. After the occurrence of tumors, the body can generate specific immune responses to tumor antigens, and cellular and humoral immunity coordinate with each other to regulate the tumor process (19). T lymphocytes mediate cellular immunity in the process of tumor immunity. T lymphocytes can be divided into CD4+ and CD8+ according to the molecular phenotype of surface cluster differentiation antigens. CD4+ T lymphocytes belong to helper T lymphocytes, which can be stimulated by tumors to produce cytokines. While directly acting on tumors, they can also induce anti-tumor inflammatory responses (20). After being stimulated by tumor antigens, CD8+ T lymphocytes can differentiate and directly kill tumor cells (21). This article found that peripheral blood CD4+ and CD8+ levels were decreased in mice with inflammation-related CC models, whereas phellopterin intervention increased peripheral blood CD4+ and CD8+ levels. At present, it is generally believed that the pathogenesis of inflammation-related CC follows the “inflammation-dysplasia-canceration” pathway, in which inflammatory factors, immune microenvironment, and TLR4/NF-κB related pathway play an important role in the pathogenesis of inflammation-related CC (22). IL-6 is a kind of pro-inflammatory factor, which is involved in mediating cellular immune response and is closely related to tumor size and metastasis (23). TNF-α, mainly produced in the initiation of inflammation, is a pro-inflammatory factor (24). IL-1β is a typical multifunctional cytokine and a kind of pro-inflammatory cytokine, which is mainly produced by lymphocytes, macrophages, and monocytes (25). IL-10 can negatively regulate the secretion of IL-6 and other factors, and inhibit inflammatory response (26). This article examined the changes in the levels of peripheral blood inflammatory factors IL-6, IL-1β, IL-10, and TNF-α, and revealed that the IL-6, IL-1β, and TNF-α in the peripheral blood of the model mice were raised, and the IL-10 was decreased. Phellopterin could inhibit the expression of pro-inflammatory factors (IL-6, IL-1β, TNF-α) and increase the content of anti-inflammatory factors (IL-10). Phellopterin can reduce the levels of pro-inflammatory factors, increase the levels of anti-inflammatory factors, inhibit the inflammatory response, and prevent the occurrence of inflammation-related CC.

Tumor-associated macrophages are a prominent type of immune cell infiltrating the tumor microenvironment of colorectal cancer and have an obvious role in its progression (27). Intestinal macrophages are key players in maintaining immune homeostasis and regulating inflammation in the intestine. In response to various environmental stimuli, they can adopt a pro-inflammatory M1 phenotype or an anti-inflammatory M2 phenotype (28,29). Normally,
intestinal macrophages protect the gut from inflammatory damage. However, an imbalance in the polarization of intestinal M1/M2 macrophages, resulting from genetic and environmental factors, can disrupt the regulation of intestinal inflammation and turn a physiological inflammatory response into pathological intestinal damage (30). Studies have confirmed that inhibiting the M2 polarization of macrophages can effectively prevent the occurrence of colorectal cancer (31). CD163 and CD206 are markers of M2-type macrophages that promote tumor progression (32). In addition, when macrophages are polarized to the M2 phenotype, the expression of Arg-1 and Ym-1 is also up-regulated (33). It found that the expression of CD163, CD206, Arg-1, and Ym-1 in colonic macrophages of model mice was up-regulated, and phellopterin could inhibit the expression of CD163, CD206, Arg-1, and Ym-1 in model mice. Phellopterin could inhibit M2 polarization of macrophages, slow down the process of inflammatory reaction, and play a role in anti-inflammatory cytokines leading to inflammatory injury.

TLR is an important pattern recognition receptor in innate immunity, which is mainly expressed in immune cells. TLR can specifically recognize pathogenic molecular patterns, induce T cells to differentiate into immune-competent cells with different functions, secrete cytokines with different functions, and initiate the innate immune response of the intestinal mucosa (34). Previous studies have confirmed that the expression of TLR4 is up-regulated in inflammatory bowel disease and CC tissues, which is involved in the formation of colitis-related tumors (35). Studies have shown that the activation of the LPS/TLR4/NF-κB signaling pathway releases many inflammatory factors and inflammatory mediators, which can promote the occurrence and metastasis of colorectal cancer (36). TLR4 and NF-κB p65 protein expression was up-regulated in the colonic tissues of model mice. NF-κB activation can lead to autonomous genetic changes in tumor cells, regulate the secretion of inflammatory factors, and recycle the growth and invasion of tumor cells (37). In addition, NF-κB activation can lead to the expansion and deepening of inflammatory response, leading to tumor deterioration. Continuous activation of NF-κB can also promote cell proliferation, and improve the survival rate, growth rate, and inflammation-cancer transition of cancerous cells (38). Subsequently, these results suggest that phellopterin may play an important role in inhibiting inflammation-cancer transformation.

Phellopterin can inhibit the activation of the TLR4/NF-κB signaling pathway and M2 polarization of macrophages, reduce the inflammatory response, and prevent the occurrence of inflammation-related CC. In this article, only an animal model of inflammation-related CC was prepared to analyze the therapeutic mechanism of phellopterin. It is also necessary to prepare an in vitro model to analyze the mechanism of phellopterin blocking the inflammation-cancer transformation chain. In conclusion, it can provide reference materials for the development of new therapeutic drugs for inflammation-related CC, as well as the prevention and treatment of inflammation-related CC.

References

14. Lewis SM, Williams A, Eisenbarth SC. Structure and function


