Panax notoginseng saponins prevent colitis-associated colorectal cancer development: the role of gut microbiota

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[ABSTRACT] Gut microbiota dysbiosis is a risk factor for colorectal cancer (CRC) in inflammatory bowel disease (IBD). In this study, the effects of Panax notoginseng saponins (PNS) on colitis-associated CRC progression were evaluated on an azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model. In vivo, PNS significantly relieved AOM/DSS-induced colon tumorigenesis and development by reducing the disease activity index (DAI) scores and colon tumor load. The 16S rRNA data of fecal samples showed that the microbiome community was obviously destructed, while PNS could recover the richness and diversity of gut microbiota. Especially, PNS could increase the abundance of Akkermansia spp. which was significantly decreased in model group and negatively correlated with the progression of CRC. Moreover, ginsenoside compound K (GC-K) was evaluated on the effects of human CRC cells, which was the main bio-transformed metabolite of PNS by gut microbiota. Our data showed that PNS played important role in the prevention of the progression of CRC, due to their regulation on the microbiome balance and microbial bio-converted product with anti-CRC activity.

[KEY WORDS] Panax notoginseng saponins; Gut microbiota; Colorectal cancer; Ginsenosides; 16S rRNA gene sequencing

[Introduction] Colorectal cancer (CRC) is a common malignancy worldwide, but its molecular mechanisms in tumorigenesis and progression are not fully understood yet. Nevertheless, the enhanced risk of colorectal carcinogenesis in ulcerative colitis (UC) is well-known, which is usually accompanied by chronic inflammation and gut microbiota dysbiosis\textsuperscript{[1,2]}. Mucosal inflammation caused by UC could generated reactive oxygen species to promote carcinogenesis. Except for genetically predisposed individuals, CRC is induced by environmental factors, such as bacteria, diet and so on\textsuperscript{[3,4]}. Therefore, the regulation of intestinal inflammation and gut microbiota were two key factors in the prevention and treatment of CRC.

Gut microbiota play a profound role on body immune homeostasis to prevent the tumorigenesis and development of CRC\textsuperscript{[3]}. Except for genetic factor, severe inflammatory-immunological factors are able to induce a tumorigenic milieu\textsuperscript{[3]}. For example, reduced mucus layer in inflammatory bowel disease (IBD) is susceptible to bacterial translocation to cause immune activation. In over-exuberant immune response, Th17-inducing bacteria enterotoxigenic Bacteroides fragilis could make Th17 cell infiltration into the colon, which mediates tumorigenesis of CRC\textsuperscript{[4]}. Panax notoginseng saponins (PNS) have been previously reported as anti-inflammatory and immunologically modulated botanical products\textsuperscript{[5,4]}. Moreover, PNS could de-
increase the expression of inducible nitric oxide synthase and cyclooxygenase-2 [9]. However, PNS are characterized as extremely low oral bioavailability, due to the weak absorption of parent compounds into circulatory system [10, 11]. In our previous study, PNS could interplay with gut microbiota in gastrointestinal tract, which could be bio-transformed to be novel bioactive compounds, such as the main metabolite ginsenoside compound K (GC-K) [12, 13]. The yielded metabolites may play an important role on anti-CRC effects. Simultaneously, the drug-microbial interactions could also reshape the environment of the mammalian intestine, while the most reported drugs are such as antibiotics, proton pump inhibitors and saponins [14, 15]. Therefore, the pertinent studies should not only focus on the bioactivities of PNS, but also refer to remodeling gut microbiota.

In this study, an azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced A/J mouse model, which suffered from acute and chronic intestinal inflammation to lead to death of epithelial cells to induce CRC [16], was constructed to evaluate the anti-neoplasm effect of PNS on preventing colorectal carcinogenesis. The disease activity index (DAI) scores, colon tumor load, colon length change and colon tissue histological assessment showed PNS could ameliorate colitis-associated CRC development. Moreover, the 16S rRNA sequencing analysis explored that the composition of the intestinal microbiota changed significantly and resulted in a partial recovery of AOM/DSS-associated dysbiosis with PNS treatment. Additionally, GC-K was also observed with significant proliferation inhibitory effects on HCT-116 and HT-29 cells. Therefore, due to the regulation on the microbiome imbalance and microbial bio-converted products with anti-CRC activity, PNS showed potential anti-CRC effects.

Materials and Methods

Chemicals and reagents

Azoxymethane (AOM, Sigma, USA) and dextran sulfate sodium (DSS, molecular weight, 36-50 kDa, MP Biomedicals, USA) were used. McCoy’s 5A medium and RPMI-1640 medium were purchased from Biological Industries (BI, Israel). Fetal bovine serum (FBS) was obtained from Omega Bio-tek (Norcross, GA, USA). Mixture PCR product purification kit was purchased from Qiagen (Hilden, Germany). Sequencing library generation kit was purchased from Illumina (San Diego, USA).

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PNS preparation and HPLC-DAD-Q-TOF-MS/MS analysis

The air-dried root of P. notoginseng was purchased from Wenshan city (Yunnan, China) and was extracted by heat-refluxing with 70% ethanol (1 : 10) for 3 cycles (2 h each time). Then, all the filtrates were concentrated under vacuum at 50 °C and dried by vacuum-drier at 60 °C to obtain the PNS. HPLC analysis was conducted on an Agilent 1290 Series system connected with a diode-array-detector and a 6530 accurate-mass Q-TOF LC-MS instrument (Agilent, CA, USA) to analyze the contents of PNS. The detailed information about P. notoginseng and PNS extract were described in our previous publication [13].

Animals and treatment protocols

The experimental protocols were approved by the Central South University Animal Management Committee and conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals. Male A/J mice (6–8 week, 20 ± 2 g) were obtained from SIPPR-BK Laboratory Animal Co., Ltd. (Shanghai, China). All mice were caged in a room with a 12/12 h light/dark cycle, the conditioned temperature (22 ± 2 °C) and relative humidity (60% ± 5%). After acclimated to these conditions for at least 7 days, mice were randomly divided into 4 groups (n = 10): the control group, the AOM/DSS model group, the low-dose (30 mg kg⁻¹·d⁻¹) group and the high-dose (90 mg kg⁻¹·d⁻¹) group. The control group was intraperitoneally injected with saline, while the other groups were administrated with intraperitoneal injection of AOM (7.5 mg kg⁻¹). One week later, drinking water was fed free with 1% DSS for 7 consecutive days, followed by normal drinking water. The low-dose and high-dose groups received PNS at 220 or 660 ppm in standard AIN-93M chow (Trophic Animal Feed High-Tech Co., Ltd., China). The mice were sacrificed on week 13, and tissue samples were collected for further pathological observations. The stool samples were collected during week points 1, 3, 5 and 8 for 16S rRNA sequencing (shown in Fig. 1).

Disease activity index and histologic assessment

The DAI was calculated to evaluate the severity of AOM/DSS–induced colitis in the acute phase as described previously, including weight loss, stool consistency change and rectal bleeding [9]. The animals were scored for the DAI at the same time from day 1 to 14. The distal colorectal segments were immersed in 4% paraformaldehyde and stained with hematoxylin and eosin (H&E) to perform histopathology assay.

![Fig. 1 The animal experimental protocol](image-url)
**DNA extraction and PCR amplification**

Microbial genomic DNA was extracted and stored in −20 °C until further processing from each sample using the E. Z. N. A. Stool DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). The concentration of DNA was spectrophotometrically determined by NanoDrop instrument (Shimadzu), and the purity of DNA was monitored on 1% agarose gels. Subsequently, DNA was diluted to 1 ng μL⁻¹ using sterile water. The variable region V4 of the bacterial 16S rRNA gene from each sample was amplified using the bacterial universal primer 515F 5′-barcode- GTGCCAGCMGCCGCGGTAA-3′ and 806R 5′-barcode GGACTACHVGGGTWTCTAAT-3′, while the barcode was a six-base unique sequence to each sample. All PCR reactions were carried out in 30 μL of mixtures with 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μmol·L⁻¹ of forward-reverse primers and approximate 10 ng of template DNA. PCR thermal cycling conditions were set as initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s and elongation at 72 °C for 30 s, with a final extension at 72 °C for 5 min.

**MiSeq sequencing of 16S rRNA gene amplicons**

Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit with additional index codes. The library quality was assessed on the Qubit 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina HiSeq 2500 to generate 250 bp paired-end reads.

**Bioinformatics Analysis**

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence, and then merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/). Comparing with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) according to UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html), the chimera sequences were removed from the tags to generate the Effective Tags. Sequences analysis was performed on Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/), while sequence with ≥ 97% similarity was assigned to the same operational taxonomic units (OTUs). Representative sequence for each OTU was screened and annotated taxonomic information based on the Green-Gene Database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi).

In order to analyze alpha diversity, Chao1 and ACE were calculated to estimate the species abundance, while Observed Species were analyzed to evaluate the amount of unique OTUs found in each sample. Simpson indexes were used to determine the evenness of each sample.

Alluvial diagrams were visualized by the ggplot2 package of R software to show changes in the microbiota profile. The linear discriminative analysis (LDA) effect size (LEfSe) analysis was performed on LEfSe software to assess the effective size (> 4) of each differential abundant taxon or OTU, and the cladogram was displayed according to effective size.

**Cell proliferation analysis**

Human CRC cell lines (HCT-116 and HT-29 cells) were obtained from Cell Center of Central South University. HCT-116 and HT-29 cells were cultured in McCoy’s 5A medium containing 10% FBS. The two cell lines were maintained in an incubator at 37 °C with a humidified atmosphere of 5% CO₂ and sub-cultured every two or three days.

Cell proliferation was measured using MTS assay kit. Cells (HT-29, 3 × 10⁴ cells/well; HCT-116, 5 × 10⁴ cells/well) were seeded into a 96-well plate at logarithmic phase, and then treated with 10, 20, 40, 60 and 80 μmol·L⁻¹ of GC-K. 0.5% dimethyl sulfoxide (DMSO) and vials without cells were used as negative and vehicle control, respectively. After 24 and 48 h incubation, the medium were discarded and followed by adding MTS (20 μL of MTS/100 μL of medium). Optical density (OD) was measured at 490 nm to calculate the cell proliferation rate (DMSO vehicles set at 100%).

**Statistical Analysis**

The one-way ANOVA with LSD post-hoc test was used to analyze data. Pearson correlation analysis was performed on SPSS software (Version 23). Significant differences were set as *P < 0.05 and **P < 0.01.

**Results**

**PNS suppressed AOM/DSS-induced acute colitis and colorectal carcinogenesis**

After AOM/DSS treatments, mice in low/high dose groups showed significant activity reduction, debilitation, loss of appetite and weight, diarrhea and rectal bleeding with high DAI scores. After the cessation of DSS on day 8, the above symptoms were gradually relieved in low/high dose groups. Meanwhile, the disease severity reached its highest level on Day 10 in model group. It suggested that PNS significantly promoted recovery from the colitis due to the reduction of the DAI score in a dose-depended manner (Fig. 2A).

Colon length was measured to represent the severity of inflammation. Compared with the control group, the colorectal segment of the model group was significantly shortened, while the intestinal segment of PNS administration group had no significant difference (Figs. 2B and 2C). The colorectal segment was longitudinally oriented to measure the malignant tumor load. Tumorigenesis was obviously increased in the model group. However, in the PNS treatment group, the tumor load was significantly decreased (Figs. 2D and 2E).

H&E staining histological assay was employed for the colon damage evaluation in model group, which were characterized by the enlarged epithelial cell nuclei, deep stained nuclei and obviously disordered structure. The glandular hyperplasia structure was irregular and difficult to identify. Goblet cells were reduced, while crypts were arranged disorderly. Mixed inflammatory cells infiltrated the submucosa to form ulcers. Moreover, the number of goblet cells and
crypts were increased. Relieved dysplasia and infiltrating inflammatory cells also showed a significant reduction in inflammatory injury (Fig. 2F).

**Alpha diversities and taxonomic differences of gut microbiota**

In this study, Chao1 (richness estimation index) and Simpson index (diversity index) were used to analyze bacterial alpha diversity (Figs. 3A and 3B). Chao1 in the model group continuously decreased (week 0 vs week 8, $P < 0.05$), indicating gradually dropped intestinal bacterial species. However, in PNS administration groups, the microbial spe-
Fig. 3  Gut microbota profiles at different week check-points. Chao 1 index (A); Simpson index (B); An alluvial diagram based on the relative abundance of the top five gut microbials at phylum level (C); The relative abundance of Akkermansia spp., week 0 vs week 8, *P < 0.05 (D); The relative abundance of Alistipes spp., week 0 vs week 8, *P < 0.05 and **P < 0.01 (E)
cies richness was partially recovered. The Simpson index in model group increased uniformly at week 3, 5 and 8, by comparing with week 0. To alpha diversity indexes, reduced rare species and proportion of dominant bacteria indicated that the normal gut microbiota profiles were sharply destroyed by AOM/DSS. After ceasing DSS administration, the gut microbiome community began to against dysbiosis. In this process, the enriched species were easier to re-collocate in intestinal tract. However, the rare species had poor recovery ability, which led to the decreased microbial richness and diversity. In contrast, PNS treatment could partially prevent the deterioration of diversity to promote homeostasis.

Taxonomical assignation was performed at phylum level. The differences of relative abundance of the top five gut microbials at phylum with day check-points were shown in Fig. 3C. In model group, the proportion of Firmicutes was continuously increased, but Verrucomicrobia was decreased (week 0 vs. week 8, \( P < 0.05 \)). The PNS administration group had significantly increased levels of Firmicutes and Verrucomicrobia at week 5. Proteobacteria was significantly dropped off in the low-dose group (week 3 vs week 8, \( P < 0.05 \)). However, a relatively stable composition was observed in the high-dose group. Therefore, the gut microbiome community could be partially restored by PNS treatment.

In genus level, compared with week 0, the relative abundance of \( \text{Akkermansia} \) spp. was dropped off gradually at model group, but slightly restored at week 5 in low-dose group (Fig. 3D). In addition, the proportion of \( \text{Alistipes} \) spp. was observed to be continuously raised after AOM/DSS treatment (Fig. 3E). Therefore, the increased \( \text{Alistipes} \) spp. was a possible carcinogenic risk factor for AOM/DSS-induced A/J mice.

**Biomarker discovery and correlation analysis of gut microbiota**

Using LEfSe biomarker discovery tool, the gut microbiota of mice in each group were analyzed to identify the specific phylotypes with OTU levels associated with CRC. In genus level, the biomarkers for the model group were \( \text{Parabacteroides} \) spp. and \( \text{Rikenellaceae RC9 gut group} \) spp., while the biomarkers for low-dose group were \( \text{Akkermansia} \) spp. Those biomarkers presented high LDA scores (LDA > 4) and were enriched in Firmicutes, Bacteroidetes and Verrucomicrobia phylum (Figs. 4A and 4B).

The association was found according to Pearson’s correlation. Fecal \( \text{Akkermansia} \) spp. relative abundance in mice were inversely associated with AOM/DSS induced tumor size(Fig. 4C, coefficient = −0.7669, \( P < 0.05 \)). Therefore, PNS might enhance anti-CRC effects through adjusting the proportion of \( \text{Akkermansia} \) spp.

**GC-K inhibited human colon cancer cells growth**

After GC-K treatment, the proliferation of HCT-116 and HT-29 cells was both suppressed dose- and time-dependently in each case (Fig. 5). At the dosage of 10 \( \mu \text{mol·L}^{-1} \), HT-29 and HCT-116 cells proliferation was inhibited by 77.3% \( \pm 3.5\% \) (\( P < 0.01 \) vs control) and 90.5% \( \pm 2.6\% \) (\( P < 0.05 \) vs control) for 48 h exposure of GC-K. In addition, the proliferation of HT-29 and HCT-116 cells for 24 h were significantly less than that for 48 h. Remarkable correlations were observed between cell viability and GC-K concentration (24 h: \( r = -0.7892 \) in HCT-116 cells, \( r = -0.9661 \) in HT-29 cells, \( P < 0.01 \); 48 h: \( r = -0.9193 \) in HCT-116 cells, \( P < 0.01 \); \( r = -0.9746 \) in HT-29 cells, \( P < 0.01 \)). The data implied that the HCT-116 and HT-29 cells were significantly sensitive to GC-K which led to significant proliferation suppression of CRC cells.

**Discussion**

PNS could be metabolized by gut microbiota. Indeed, several metabolites have been reported as bioactive substances. It’s reported that ginsenosides have anti-inflammatory and antitumor activities. The involved molecular mechanisms include inhibiting the expression of IL-1β, IL-6, IL-8, TNF-α, IFN-γ through the NF-κB signal transduction pathway, sensitizing CRC cells to docetaxel and fluorouracil, and inducing tumor cell apoptosis via the AMPK signaling pathway. The beneficial biotransformation by gut microbiota in human intestine plays an inevitable role to achieve pharmaceutical action of \( \text{P. notoginseng} \), similar with other traditional Chinese medicines, such as American ginseng. However, the subsequent modification of gut microbiota profiles are still ambiguous. In this paper, PNS could prevent colitis-associated CRC development. Then, 16S rRNA gene sequencing technology was employed for analyzing the gut microbiome composition.

PNS could significantly reduce colonic inflammation in the acute phase with a dose-dependent manner according to DAI and histology evaluation. The colorectal carcinogenesis and shortening of colon length were significantly smaller than those of the model group, indicating that PNS had anti-CRC effects in vivo. According to 16S rRNA sequencing analysis, we observed that the gut microbiome community underwent obviously differences after AOM/DSS and PNS administration, e.g., \( \text{Alistipes} \) spp., \( \text{Akkermansia} \) spp. and \( \text{Alis-}

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Additionally, patients with a positive response to the immune checkpoint inhibitor PD-1 antibody have an much higher level of *A. muciniphila* [24].

In our study, increasing abundance of *Akkermansia* spp. was obviously observed after PNS administration, which was inversely associated with tumor volume. In addition, according to alpha diversity analysis, gut microbial diversity and richness had also been restored in low/high dose group. Therefore, PNS may play an anti-inflammatory and anti-CRC effect by regulating the proportion of *Akkermansia* spp. and promoting the recovery of disordered gut microbiome community. Moreover, we have also shown that the main gut microbial metabolite GC-K had potential anticancer effects on CRC *in vitro*. Other gut microbial metabolites, such as ginsenoside Rh2, protopanaxadiol and protopanaxatriol, need further study to investigate their bioactivities on anti-CRC effects. Moreover, anti-CRC effects of gut microbiota, such as enhancing host immunity response and modulating the tumor microenvironment, should also be inevitably further investigated.

**Conclusion**

PNS may prevent colitis-associated CRC development
mediated by gut microbiota. The abundance of Akkermansia spp., which could be restored by PNS, was negatively correlated with the development of CRC. Moreover, the main biotransformed product (GC-K) could inhibit the proliferation of human CRC cells. However, the anti-CRC effects and mechanisms of gut microbiota modified by PNS need further study.

**Abbreviation**

AOM, azoxymethane; CRC, colorectal cancer; DAI, disease activity index scores; DMSO, dimethyl sulfoxide; DSS, dextran sulfate sodium; FBS, fetal bovine serum; GC-K, ginsenoside compound K; H&E, hematoxylin and eosin; IBD, inflammatory bowel disease; LDA, linear discriminative analysis; LEfSe, the LDA effect size; OD, Optical density; OTUs, operational taxonomic units; PNS, Panax notoginseng saponins; SCFAs, short chain fatty acids; UC, ulcerative colitis.

**References**


