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Inhibition effect of glycyrrhiza polysaccharide (GCP) on tumor growth through regulation of the gut microbiota composition

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ABSTRACT

Glycyrrhiza Uralensis Polysaccharide (GCP), as a macromolecular polysaccharide extracted from the Traditional Chinese Medicine (TCM) - Licorice has been proved to inhibit tumor growth in vitro and in vivo; however, the specific anti-tumor mechanism of GCP needs to be further investigated. In this study, we explore the anti-tumor mechanism of GCP from the angle of gut microbiota. Colon carcinoma cells (CT-26) were used to set up a tumor-bearing mouse model. After 14 days of GCP treatment, the weights of tumors were significantly reduced. In addition, HE staining of tissue sections reflected that GCP could effectively inhibit tumor metastasis. 16SrRNA high-throughput sequencing of fecal samples showed a significant change between the model group and GCP group in the composition of gut microbiota. Subsequently, gut microbiota depletion and fecal transplantation experiments further confirmed the relationship between the anti-tumor effects of GCP and gut microbiota. Following depletion of gut microbiota, GCP cannot inhibit tumor growth. Fecal transplantation experiments found that transplanting the feces of GCP-treated mice, to a certain extent, could inhibit tumor growth and metastasis. These results indicate that Glycyrrhiza Polysaccharides exert anti-tumor effects by affecting gut microbiota composition.

1. Introduction

Cancer is one of the major diseases threatening human health and its morbidity and mortality rate around the world are on the rise. In 2012, the incidence of malignant tumors was 199.4/10 million, the number of deaths caused by malignant tumors was 8.22 million, and the mortality of malignant tumors was 116.3/10 million.[1] The grim reality compels us to discover new ideas to combat tumor.

The human gut contains a large number of symbiotic bacteria, known as the largest symbionts,[2] which contains about 800 kinds of bacterial genus and more than 7000 strains, and its huge number (about 100 trillion, with the total weight of 1–2 kg) makes it an immune organism that cannot be ignored in the human body.[3] This community, by means of metabolizing indigestible carbohydrates, producing vitamins, preventing infection from pathogenic bacteria, anti-tumor and modulating host immune responses, demonstrates its indispensability in metabolism and protection of human health.[4,5] Existing studies have shown that the gut microbiota of patients with tumors is significantly different from that of normal people[6,7] and gut microbiota plays a key role in the treatment of tumor.[8]

Studies show that some TCM extracts play a therapeutic role by regulating the abundance of gut microbiota. For example, Ganoderma lucidum plays a role in weight loss by regulating the composition of gut microbiota.[9] Berberine could modulate the microbiota-gut-brain axis for high-fat diet-fed rats and thus improve their metabolic status,[10] and Isoliquiritigenin decreases the morbidity of colorectal cancer by modulating gut microbiota.[11]

Glycyrrhiza Uralensis Polysaccharide (GCP), the main efficacious ingredient extracted from Chinese licorice (Glycyrrhiza uralensis Fisch.), which is one of the basic herbs in TCM therapies, has shown its anti-cancer potential by restraining tumorigenesis and

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metastasis.[12,13] Previously, we investigated that GCP has an obvious anti-tumor effect in vitro.[14] Furthermore, other studies on GCP indicate that GCP could be utilized to regulate the immune system to achieve anti-tumor effects.[15,16] However, due to its high molecular weight, oral administration of GCP cannot be easily absorbed into the blood stream directly for functional activity. Until now, the way in which GCP exerts its capacity in vivo has remained unclear. Therefore, we assume that: gut microbiota may be the target for the anti-tumor effects of GCP.

In this study, we investigated the anti-tumor effects of GCP in vivo and their effects on tumor metastasis, and the effects of GCP on gut microbiota composition in tumor-bearing mice were investigated by high throughput sequencing of 16SrRNA. Two kinds of antibiotics were used to deplete the gut microbiota to verify whether the anti-tumor effect of GCP remained valid. Since most gut microbiota is difficult to culture, we selected a fecal transplanting experiment to further refine the effects of gut microbiota on the anti-tumor effects of GCP.

2. Materials and methods

2.1. Cell lines and cell culture

A colon carcinoma cell line (CT-26) was purchased from the Institute of Biochemistry and Cell Biology. The CT-26 cell line was routinely maintained in RPMI-1640 supplemented with 1% streptomycin/penicillin (P/S), L-glutamine and 10% Fetal Bovine Serum (FBS) at 37 °C and 5% CO_2. The reagents mentioned above were purchased from HyClone, Piscataway, New Jersey, USA. All cell lines were maintained in a humidified atmosphere. Standard protocols for cell culture were followed at the TUTCM laboratories.

2.2. Animals and groups

Six-to eight-week-old male BALB/c mice were purchased from BEIJING HUAFUKANG BIOSCIENCE CO. INC., housed in a pathogen-free facility in TIANJIN Hospital at the ITCWM Laboratory Animal Center. Effectively sterilized food and water were provided by the Laboratory Animal Center. All of the animal protocols were approved by the Local Ethics Committee for Animal Care and Use at TUTCM. In order to avoid the effects of coprophagy, mice from same group were housed in the same cage.

The CT-26 cells suspension was washed with sterile PBS, and cell density was adjusted to 1×10^7 mL. A tumor-bearing mouse model was established by injecting 0.1 mL of CT-26 cell suspension under the left armpit of each mouse. When the average volume of tumor reached 100 mm^3, the tumor-bearing mice were randomly divided into model group (Group-M) and GCP group (Group-G). Normal animals were used as a control (Group-N). Intra-gastric sterile saline solution (1 m l/kg) was administered for 14 days in the model group; intra-gastric GCP (extracted by the TUTCM herbal pharmaceutical company, Tianjin, China., 500 mg/kg in 0.2 ml sterile saline solution) was administrated for 14 days in GCP group. Body weight was recorded each day. Tumor size was measured every day with a digital caliper. The following formula was used to calculate tumor volumes[17]: $V = L \times W^2 \times 1/2$, where V = volume, L = length, and W = width. On the 15th day, the mice were weighed and sacrificed, and tumors, spleen and lungs were stripped, weighed and stored in 10% neutral buffered formalin tissue-fixed solution for the subsequent hematoxylin-eosin staining procedure. The formula for calculating the tumor inhibition rate is: (model group tumor weight - GCP group tumor weight)/model group tumor weight × 100%. Spleen index formula is spleen weight (mg)/body weight(g).

Specific pathogen free (SPF) mice were gavaged with the Sterile Saline Solution twice a day for 30 days. Then, CT-26 cells were inoculated as above. SPF group mice would be randomized into SPF model group and SPF GCP group (n = 5). In the SPF model group, intra-gastric sterile saline solution was administered for 14 days; in the SPF GCP group, intra-gastric GCP was administered for 14 days.

For the commensal microbes depleted experiment, two kinds of broad-spectrum antibiotics, Cephradine (belonging to the cephalosporins class) and Gentamicin (belonging to the aminoglycosides class), were used to deplete gut microbiota.[18] These antibiotics were dissolved in Sterile Saline Solution and the final concentration for Cephradine and Gentamicin was 67 mg/kg and 2 mg/kg, respectively. Mice were gavaged with the above antibiotics twice daily for 30 days. The bacterial DNA was extracted to assess efficiency of depletion. After charged with tumor, antibiotic-treated (ABX) mice would be randomized into an ABX model group and ABX GCP group (n = 5). In the ABX model group, intra-gastric sterile saline solution was administered for 14 days; in the ABX GCP group, intra-gastric GCP was administered for 14 days.

For the fecal microbiota transplantation (FMT) experiment, stools from model group mice (model donor mice) and GCP group mice (GCP donor mice) were collected daily under a laminar flow hood in sterile conditions. Stools from the same group were pooled together and 100 mg stool from each group was resuspended in 1 mL of sterile saline. Before centrifugation at 800 g for 3 min, the solution was mixed for 10 s with a benchtop vortex (Vortex-Genie 2, Scientific Industries, USA; speed 9). The supernatant was collected and used as transplant material. Fresh transplant material must be prepared 10 min before oral gavage to recipient animals on the transplantation day to avoid any change of bacterial composition. Tumor-bearing mice (recipient animals) were set up as above and divided into a FMT model group and FMT GCP group (n = 5). For the FMT model group mice, 0.1 mL fresh transplant material from model donor mice was given by oral gavage each day for 14 days; for the FMT GCP group mice, 0.1 mL fresh transplant material from GCP donor mice was given by oral gavage each day for 14 days.[19]

2.3. Fecal DNA extraction and 16SrRNA gene sequencing

About 200 mg of fresh stool samples were collected from each mouse before being sacrificed, and then a TIANamp Stool DNA Kit (BEIJING TIANGEN BIOTECH CO., LTD) was utilized to extract bacterial DNA from fecal samples. DNA was quantified by the Nanodrop 2000. Then DNA from each sample was taken as a template to amplify the V3 and V4 hypervariable regions of ribosomal 16SrRNA genes. In short, the purified 1 μg of genomic DNA was fragmented into an average size of 300–400 bp and ligated with adapters. Extracted DNA was diluted to a concentration of 1 ng/μl and stored at −20 °C until further processing. The diluted DNA was used as a template for PCR amplification of bacterial 16SrRNA genes with the barcoded primers and HiFi Hot Start Ready Mix (KAPA). For bacterial diversity analysis, V3–V4 variable regions of 16SrRNA genes were amplified with universal primers 343F and 798R. Amplicon quality was visualized using gel electrophoresis, purified with AMPure XP beads (Agencourt), and amplified for another round of PCR. After being purified with the AMPure XP beads again, the final amplicon was quantified using Qubit dsDNA assay kit. Equal amounts of purified amplicon were pooled for subsequent sequencing.

2.4. Bioinformatic analysis

Raw sequencing data were in FASTQ format. Trimmomatic software[20] was used for the preprocessing of paired-end reads to

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