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The influence of gut microbiota dysbiosis to the efficacy of 5-Fluorouracil treatment on colorectal cancer

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ABSTRACT

Colorectal cancer is one of the most frequently diagnosed cancers worldwide. Gut flora can modulate the host response to chemotherapeutic drugs. However, the understanding regarding the relationship between the gut microbiota and the antitumor efficacy of 5- Fluorouracil (5-FU) treatment is limited. Therefore, we compared the tumor size and profiled the gut microbiota of mice treated with 5-FU, combined with probiotics or ABX (an antibiotic cocktail of antibiotics) by using the Colorectal Cancer (CRC) mouse model and high-throughput sequencing. The results elucidated that ABX administration diminished the antitumor efficacy of 5-FU in mice and supplementation of probiotics upon 5-FU treatment could not significantly increase the efficacy of 5-FU treatment, despite improving mice body weight at day 33. There were significant differences in fecal bacteria community among the four groups (ANOSIM $p < 0.05$). ABX administration reduced microbiota biodiversity and altered microbiota community. The pathogenic bacteria included *Escherichia shigella* and *Enterobacter* significantly increased, while other commensal bacterial decreased *unidentified Firmicutes increased* and the opportunistic pathogens decreased after the administration of Probiotics. In addition, 5-FU treatment also changed the diversity and the community composition of the gut mirobiota. The relative abundance of genus *Lachnospiracea_NK4 A136, Bacteroides, Odoribacter, Mucispirillum,* and *Blautia* were significantly increased compared to the control group. Additionally, functional capacity analysis of gut microbiota using PICRUSt showed that genes involved in amino acid metabolism, replication and repair translation, nucleotide metabolism expressed much lower in FU.ABX group than the other groups. The current results suggest that ABX administration disrupted the gut microbiota in mice, which contributed to the reduction of antitumor efficacy of 5-FU.

1. Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality worldwide [1]. Surgical resection is curative for most of the cases at an early stage, but for advanced stages the systemic administration of cytotoxic chemotherapy is the most common therapeutic approach [2]. 5-fluorouracil (5-FU) is the most common and standardized chemotherapeutic agent used for CRC treatment, mostly based on the capecitabine's active metabolite [3]. 5-FU is a cytotoxic agent, which inhibits the growth of cancer cells by targeting thymidylate synthase enzyme, inducing RNA and DNA double strand breakage, provoking cell cycle arrest and apoptosis [4]. Despite making significant progress in the treatment of CRC during the last decade, only 10–15% of patients with advanced CRC could positively respond to the 5-FU treatment while the side effects to its cytotoxicity were serious [5]. Therefore, development of new strategies to overcome the adverse side effects and improve the response rates, are urgently needed.

Human Microbiome Project (HMPs) shows that the human intestine harbors more than 100 trillion microorganisms, about ten-times higher in numbers than the human cells and, expressing over 3.3 million bacterial genes, about 150 times larger than human genome [6,7]. These flora are considered as a "metabolic organ" in regard to their essential roles in maintaining human health and their involvement in various pathogenic disorders, such as colorectal cancer, inflammatory bowel disease (IBD), Crohn's disease, liver fibrosis, obesity, diabetes, rheumatoid arthritis(RA) and Asthma [8,9].

Recent reports evidently suggest that the gut microbiota may modulate the host response to chemotherapeutic drugs [10]. A study

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using a mouse model showed that the disruption of gut microbiota resulted in the lower cytokine production and tumor necrosis, leading to the impaired response of subcutaneous tumors to CpG-oligonucleotide immunotherapy and platinum chemotherapy [11]. The administration of cyclophosphamide (CTX) altered the composition of microbiota in the small intestine and induced the translocation of gram-positive bacteria provided the underlying immunological environment, which is important to maximize the therapeutic efficacy of CTX by invoking an anti-tumor adaptive immune response [12]. Furthermore, when the Taconic mice were orally supplemented with a cocktail of *Bifidobacterium spp*. in addition to anti-PD-L1, melanoma growth was almost completely ablated [13]. Recently, scientists have used the caenorhabditis elegans system to study the mechanism of gut microbiota response to the three chemotherapeutics agents, including 5-FU, 5-fluoro-2′-deoxyuridine (FUDR) and camptothecin (CPT), which reveals that bacteria modulate host chemotherapeutic drug response through active metabolic mechanisms [14]. However additional attempts are required to look into the gut microbiota response to the combination of different chemotherapeutic drugs and the underlying mechanisms involving host-flora interactions, in order to develop novel therapeutic strategies and provide precise treatments to cancer patients. Therefore, the aim of this study was to characterize the influence of gut microbiota on anti-tumor efficacy of 5-FU on colorectal carcinoma growth in mice.

2. Material and methods

2.1. Materials and cell lines

5-FU was purchased from Sigma Aldrich (United States), vancomycin, imipenem, neomycin and metronidazole were purchased from Sangon Biotech (Shanghai, China). Live Combined *Bifidobacterium* and *Lactobacillus* tablets were purchased from Inner Mongolia Shuang Qi pharmaceutical industry. The mouse colon adenocarcinoma cell line CT26 was obtained from the Air Force Military Medical University of People's Liberation Army (PLA, China).

2.2. Tumor models and treatments

A total of 32, 6-8week-old female BALB/c mice, weighing 18.2 ± 3.6 g were purchased from the Experimental Animal Center of Xi'an Jiaotong University, and maintained under specific pathogen-free (SPF) conditions in our department's animal house facility with 12 h light/dark cycle. Mice were fed the rodent chow and water ad libitum. All animal experiments were approved by the Institutional Animal Use and Care Committee of Xi'an jiaotong University. CT 26 cells were cultured in RPMI1640 containing 10% fetal bovine serum (Gibco), 100 U /ml penicillin, and 100 μg/ ml streptomycin under a humidified atmosphere, which include 5% CO_2 at 37 °C. To develop a CRC tumor model, tumor cells were diluted in 2×10^6 cells/ml and injected subcutaneously into the right flanks of the BALB/c mice. Tumor growth was monitored every fourth day by vernier caliper. Seven days after the tumor inoculation, mice were randomly divided into four groups. Each group of mice received different treatments: 1) Control group: 0.9% saline. 2) FU group: 5-FU (20 mg/kg/) every three days. 3) FU.ABX group: 5-FU (20 mg/kg) combined with a cocktail of antibiotics (ABX: Vancomycin 0.5 g/L, Ampicillin 1 g/ L, Neomycin 1 g/L and Metronidazole 1 g/L); 4) FU.Probiotics: 5-FU (20 mg/kg) combined with probiotics every day. Tumor sizes were monitored every four days and volume was calculated using the following formula, from a previously established protocol [15]:

$$\text{Volume } (mm^3) = (\text{longest diameter}) \times (\text{shortest diameter})^2 / 2$$

Upon completion of the treatments, fecal samples were collected at the last day of the feeding period and stored at −80 °C.

2.3. Bacterial genomic DNA extraction

Total genomic DNA was extracted from 200 mg of fecal samples using a QIAamp DNA Stool Mini kit (Qiagen, Germany) according to the manufacturer's instructions. The concentration of the extracted DNA was measured by a NanoDrop2000 (Thermo Scientific, Waltham, MA, USA), and stored at −80 °C.

2.4. 16S rRNA gene high-throughput sequencing and data analysis

To develop the amplicon libraries, the V3-V4 region of the 16S rRNA gene was PCR-amplified with the primers 341 F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′), modified by adding Miseq barcodes for multiplexing. Pooled amplicons were paired-end sequenced (PE 2 × 250) by using an Illumina HiSeq 2500 platform according to the manufacture's protocol. Paired-end reads were merged and quality filtered using FLASH and QIIME [16]. Chimeras were detected and removed against the Gold reference database using the UCHIME algorithm [17]. Sequences with ≥97% similarity were assigned to the same OTUs. For each representative sequence, the GreenGenes Database was used based on RDP classifier algorithm to annotate. Alpha diversity analysis including Observed-species, Shannon, and Simpson index, Chao1, ACE and Good-coverage, were calculated with QIIME. Phylogenetic beta diversity distances, including unweighted and weighted

UniFrac distances were measured using QIIME. Principal-

component analysis (PCA) and NMDS (non-metric multi-dimensional scaling) coordination analysis were performed to visualize the similarities or dissimilarities of variables that best represented the pairwise distances between sample groups, which displayed by WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3).

2.5. Functional annotation and profiling

The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [18] together with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database as a reference was utilized to determine the OUT involved the relative abundance and enrichment of metabolic pathways [19]. Bootstrap Mann-Whitney *U* test with 1000 permutations was also used to identify gene pathways or OTUs with significantly different abundance between groups. The R packages were used for data analysis and plotting.

2.6. Statistical analysis

Mann–Whitney *U* test was used to test for significant differences in alpha diversity, and two-sided Student's *t*-test was used to test for significant differences in beta diversity between sample groups with multiple testing corrections applied according to the BenjaminiHochberg's false discovery rate (q value), based on global P values of the variables compared. A P value of < 0.05 with a q value of < 0.05 was considered indicative of significance. Statistical evaluations of the tumor size and body weight were performed using two-way analysis of variance (ANOVA) for multiple comparisons between each treatment group. All statistical analyses were carried out by GraphPad Prism (v 5.01) or R (v2.11.1) packages.

3. Results

3.1. Antitumor efficiency of 5-FU on mice with CRC among four groups

To determine the involvement of gut flora in 5-FU treatment, a CRC mouse model receiving 5-FU treatment was used, followed with the administration of ABX or probiotics (Fig. 1A). Compared with the control, 5-FU treatment effectively reduced the tumor volume in a 33-