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Research Article

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The effect of chemotherapeutic drugs on the intestinal flora of gastric cancer tumor-bearing mice

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Abstract – *Purpose*: To determine the effect of chemotherapeutic drugs on the intestinal flora of tumor-bearing mice using high-throughput sequencing technology. *Methods*: Twenty BALB/c nude mice were randomly divided into two experimental groups. Each experimental group was divided into a blank group and a drug group, each containing five mice. BGC823 cells were inoculated to all experimental groups. For the blank groups, physiological saline or glucose injection was used for intervention. For the drug groups, oxaliplatin or 5-fluorouracil was used for intervention. Changes in the intestinal flora of mice were detected via the high-throughput sequencing technology. *Results*: No significant difference in diversity between the blank and drug groups was found. A decrease in the expression of bacteroidetes was noted in the drug groups. *Conclusions*: The use of chemotherapeutic drugs did not significantly affect the intestinal flora diversity of tumor-bearing nude mice, but it did inhibit the expression of probiotics.

Keywords: Intestinal flora, Gastric cancer, Chemotherapeutic drug effect, High-throughput sequencing technology

Introduction

Gastric cancer is one of the most common malignant tumors in the world and the third most common cancer in China. It is estimated that China will have approximately 769,000 new cases and 490,000 deaths associated with the condition in 2015 [1]. Due to multiple drug resistance, the effective treatment rates of gastric cancer have not increased, despite the emergence of new chemotherapeutic drugs and regimens. With the development of second-generation sequencing technology, an increasing number of studies have shown that intestinal flora can inhibit the occurrence of tumor and participate in the regulation of tumor treatment. Studies have found that intestinal flora is significantly correlated with intestinal tumors but also plays a very important role in the occurrence and development of liver cancer, breast cancer, malignant melanoma, lymphoma, and other tumors. Therefore, the study of intestinal flora has become a hot topic in tumor research [2–4].

High-throughput sequencing technology can sequence hundreds of thousands to millions of DNA molecules in parallel. With the rapid development of the second-generation sequencing technology, in the study of intestinal flora, high-throughput sequencing technology can complete the collection and analysis of information rapidly, reflect the expression, and improve people's awareness of various effects of intestinal flora. This study aimed to investigate the effect of chemotherapeutic drugs on intestinal flora through the use of high-throughput sequencing technology detecting the feces collected from experimental mice.

Materials and methods

Reagents and antibodies

RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD, USA).

Fluorouracil (25 mg/mL) was purchased from Tianjin Jinyao Co., Ltd.

Oxaliplatin (50 mg) was purchased from Laboratoires Thissen Company.

DNA extraction kit for fecal samples (Beijing Tiangen Biochemical Technology Co., Ltd.); AxyPrepDNA gel recovery kit (AXYGEN).

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Cell line and culture

BGC823 cells were purchased from ATCC. The cell lines were cultured in RPMI-1640 medium containing 10% FBS and were incubated at 37 °C with 5% CO_2.

Animals and feeding

Twenty BALB/c-nude mice were provided by Nanjing Bairui Biotechnology Co., Ltd., license no.: SCXK (su) 2017-0007, animal certificate no. 201801754, male, weight: 16–18 g; mice were fed in separate cages. Mice were contained in an environment with a consistent temperature (25 ± 1 °C) and humidity (55 ± 10%).

Instruments and equipment

TransGen AP221-02: TransStart Fastpfu DNA Polymerase; the PCR instrument: ABI GeneAmp® 9700.

Experimental methods

Animal grouping, modeling, and administration

The 20 BALB/c-nude mice were randomly divided into two experimental groups following five days of adaptive feeding. Each experimental group was randomly divided into a blank and drug group, with five mice in each. All groups were inoculated with BGC823 cells under the right hypoderm of the back. Total cell suspension volume per mouse was 0.1 mL. Each mouse received a total of 2.0×10^6 cells per inoculation. After successful modeling, normal saline was given to the blank group, and 5-fluorouracil (25 mg/kg) was given to the drug group in experimental group 1; glucose injection was given to the blank group and, oxaliplatin (43 mg/kg) was given to the drug group in the experimental group 2; 0.1 mL per intraperitoneal injection, every other day for 14 consecutive days. See Table 1 for the specific grouping and medication administration procedures.

Table 1. Grouping and administration of mice.

Group	Intraperitoneal injection and treatment
Experiment 1 blank group	Normal saline
Experiment 2 blank group	Glucose injection
Experiment 1 drug group	5-fluorouracil 25 mg/kg
Experiment 2 drug group	Oxaliplatin 43 mg/kg

Sample collection

Mice were put to death 24 h after the last gavage, fixed on an anatomical plate, routinely sterilized, and dissected. The contents of the cecum within 10 cm at the end of the blind part were collected aseptically and placed in a dry, sterilized test tube.

DNA extraction and high-throughput sequencing of intestinal flora

The collected mouse fecal samples were used to extract the total DNA of the microorganism in the samples using the fecal DNA extraction kit. Extraction steps were performed according to the kit instructions. One percent agarose gel electrophoresis was used to detect the extracted genomic DNA. The extracted DNA was stored at −20 °C and saved for later use. PCR amplification and Illumina high-throughput sequencing of 16S rRNA gene V4 in the samples were performed by Nanjing Bairui Biotechnology Co., Ltd. Specific primers with barcodes were synthesized for region V4 of the 16S rRNA gene. PCR amplification was performed using TransGen ap221-02: TransStartFastpfu DNA polymerase reaction system. Each sample was tested in triplicate. PCR products from the same sample were mixed and tested by electrophoresis using a 2% agarose gel. Referring to the preliminary quantitative results of electrophoresis, the PCR products were detected and quantified using the QuantiFluorTM-ST blue fluorescence quantification system, followed by mixing in a corresponding ratio. Paired end (PE) reads obtained by sequencing were spliced according to their overlap relation, and meanwhile, the sequence quality was qualitatively controlled and filtered. After identifying the samples, Mothur software was used to conduct operational taxonomic unit (OTU) clustering for the sequences, which was used for similarity analysis between samples, and the sparse curve of samples was drawn to calculate the alpha diversity of samples. Alpha diversity analyzes species diversity in a single sample, including abundance-based coverage estimators (ACE) value, Chaol value, Shannon index, etc. Chaol and ACE value predict the species of microbes in the samples according to the measured number of OTU. Shannon index is a diversity index, the higher the Shannon index, the richer the species in this sample.

Statistical analysis of data

SPSS 23.0 statistical software was used to analyze experimental data, and one-way ANOVA was used for significance analysis. Significance level = 0.05, and $P < 0.05$ was considered statistically significant. Using the two-tailed Student's t-test statistical method examined whether there was a significant difference in Beta diversity between the two groups.

Results and analysis

Species abundance and diversity of intestinal flora in mice

Using UCLUST [5] in QIIME [6] (version 1.8.0) software to cluster Tags and obtain OTU at 97% similarity level and using Mothur (version v.1.30) software to evaluate the Alpha diversity index of samples. To compare the diversity index between samples, the number of sequences contained in samples was standardized during analysis. The alpha diversity index statistics of each sample at 97% similarity level are shown in Table 2. As per Table 2, the experimental groups' highest and lowest OTU numbers were (387.00 ± 18.38) and (346.50 ± 14.85), respectively. This shows that

Table 2. Operational taxonomic unit number and alpha diversity of intestinal flora of mice in each group.

Group	OTU	ACE	Chaol	Shannon
Experiment 1 blank group	355.40 ± 20.33	392.71 ± 25.21	401.95 ± 22.52	4.01 ± 0.16
Experiment 1 drug group	361.00 ± 17.94	392.26 ± 19.78	399.10 ± 19.31	4.10 ± 0.17
Experiment 2 blank group	387.00 ± 18.38	412.01 ± 17.79	416.25 ± 19.45	4.44 ± 0.26
Experiment 2 drug group	346.50 ± 14.85	375.52 ± 14.40	375.88 ± 9.11	3.99 ± 0.31

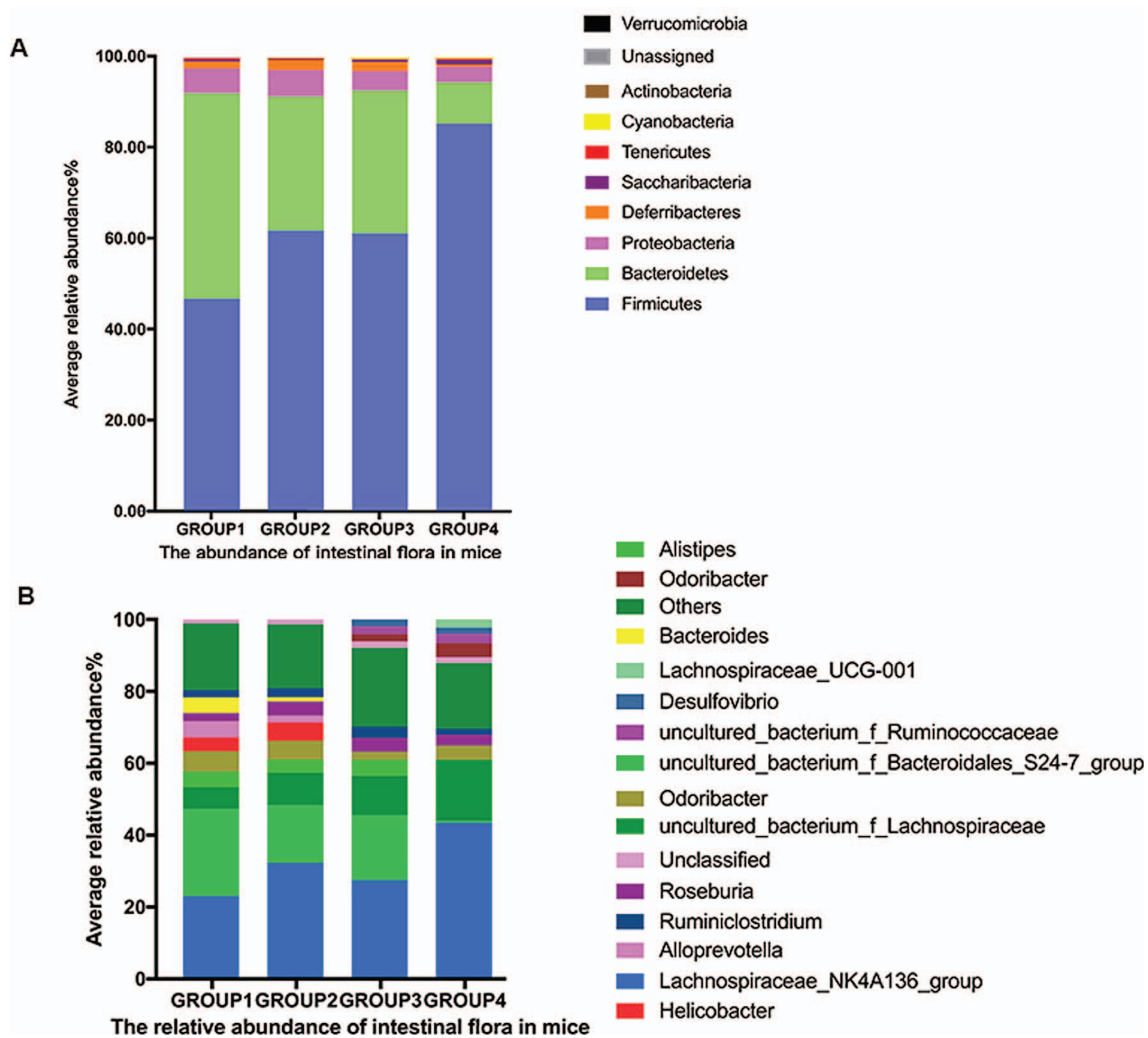

Figure 1. Analysis of intestinal flora structure. (A) The abundance of intestinal flora in mice; (B) the relative abundance of intestinal flora in mice; (GROUP 1: Blank group of experimental group 1; GROUP 2: Drug group of experimental group 1; GROUP 3: Blank group of experimental group 2; GROUP 4: Drug group of experimental group 2).

each experimental groups of mice have a high abundance of intestinal contents. When comparing the drug group and the blank group, $P > 0.05$, suggesting no statistical difference in the microbial abundance between them.

Although the difference between microbial abundance is not big, concrete numerical comparison can still be found. The diversity of intestinal flora in mice in the 5-fluorouracil group is increased, and it is decreased in the oxaliplatin group. The alpha diversity analysis showed that the diversity index is generally higher in the blank group than in the drug group, but still the diversity index $P > 0.05$ reveals the intestinal flora diversity was not significantly changed in tumor transplanted nude mice after given 5-fluorouracil or oxaliplatin.

Analysis of intestinal flora structure

Illumina sequencing was performed on the sequence of 16S rRNA gene v3–v4 in all samples. The results from each experimental group are shown in Figure 1: In two experimental groups, Firmicutes and Bacteroidetes were mainly detected, and the rest were Proteobacteria, Deferribacteres,

Saccharibacteria, Tenericutes, Cyanobacteria, and Actinobacteria. In experimental group 1, the relative proportion of Firmicutes was 47.06%, and Bacteroidetes was 45.08% in the blank group. While in the 5-fluorouracil group, the relative proportion of Firmicutes was 61.99%, and Bacteroidetes was 29.47%. At the genus level, ranked in order of abundance: Lachnospiraceae NK4A136 group, uncultured bacteriumum f Bacteroidales S24-7 group, uncultured bacteriumum f Lachnospiraceae, Odoribacter, Helico-bacter, Alloprurevotella, Roseburia, Bacteroides, Rumminiclostridium. In experimental group 2, the relative proportion of Firmicutes was 61.40%, and Bacteroidetes was 31.38% in the blank group. While in the oxaliplatin group, the relative proportion of Firmicutes was 85.54%, and Bacteroidetes was 9.01%. At the genus level, in order of abundance: Lachnospiraceae NK4A136 group, uncultured bacterium f Lachnospiraceae, uncultured bacterium f Bacteroidales S247 group, Alistipes, Roseburia, Odoribacter, uncultured bacterium f Ruminococcaceae, Rumminiclostridium, Lachnospiraceae UCG-001.

Discussion

Human gastrointestinal microflora is a complex micro-ecosystem, and the balance of gastrointestinal microecology is closely related to human health. Malignant tumors are formed by the abnormal proliferation and differentiation of local tissue cells under various tumorigenic factors in the body. In recent years, with the development and application of high-throughput sequencing technology, the study of gastrointestinal flora in tumor research is becoming increasingly extensive, providing new ideas for the diagnosis and treatment of tumors.

Intestinal flora is closely related to the occurrence and development of tumors. Under physiological conditions, the intestinal flora can repair itself. However, once the composition of intestinal flora changed beyond its ability to rebalance itself. This imbalance of bacterial flora, and the interaction of hematopoietic cells, which regulate the inflammatory response, are causes of tumor onset. It has been found that certain bacteria can induce the occurrence of tumors, such as *Streptococcus gallolyticus*, *Enterococcus faecalis*, enterotoxigenic bacteria, etc. [7]. In this experiment, we found that the feces of mice in the two experimental groups had high bacterial diversity, but the diversity index showed no significant difference between the blank and drug groups. This indicates that the change in fecal bacterial diversity caused by tumors in mice could not be affected by chemotherapeutic drugs.

Chemotherapeutic drugs can affect the treatment effect of intestinal flora in tumors. Probiotics are the most important physiological bacteria in the intestinal tract of humans and various other mammals and are also the main components in intestinal mucosa flora. Bifidobacterium and other probiotics can antagonize the growth of multiple tumors, but also prevent the occurrence and development of multiple tumors [8]. In the current study on bacterial group imbalance, Håkansson et al. [9] induced digestive tract mucositis in rats with dextran sulfate sodium (DSS) and quantitatively detected the dominant intestinal flora. It was found that the number of bifidobacterium, lactobacillus, and rumen coccus genera decreased. Stringer et al. [10, 11] induced intestinal mucositis of DA mice with 5-fluorouracil and irinotecan, respectively. After drug administration, the total number of intestinal bacteria decreased, and the number of bifidobacteria and lactic acid bacteria also decreased significantly. Meanwhile, after studying fecal samples from 16 participants with various types of malignant tumors and who received different chemotherapy regimens, they found the number of bifidobacteria and lactobacillus in the intestinal flora of the patients was reduced compared with that of healthy people. Similar to that, our study also found that the number of bacteroidetes was lower in mice treated with 5-fluorouracil or oxaliplatin than in the blank group using high-throughput sequencing in fecal samples. Through quantitative analysis of feces of patients before and after chemotherapy, Xu et al. [12] found that intestinal flora of patients with gastric cancer was unbalanced, and the application of chemotherapeutic drugs could inhibit the growth of probiotics, further aggravates the imbalance of intestinal flora, which would, in turn, increase adverse reactions to chemotherapy, and influence the treatment of tumors. The results of our study also showed that the use of chemotherapeutic drugs inhibited the expression of probiotics.

Conclusion

This study revealed that both 5-fluorouracil therapy and oxaliplatin therapy might not significantly affect the intestinal flora diversity of tumor-bearing nude mice, but they could inhibit the expression of probiotics. Previous studies and literature suggest that this may lead to bacterial imbalance, affect drug efficacy and increase adverse reactions to treatment.

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Declaration of conflict of interest

The authors declare no potential conflicts of interest.

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