Original Article
Distribution and gene mutation of enteric flora carrying β-glucuronidase among patients with colorectal cancer

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Abstract: Objective: To explore the difference of distribution in intestinal flora among colorectal cancer patients and healthy controls and investigate characteristics and changes of sequences in beta-glucuronidase (β-glucuronidase, β-G). Methods: Bacterial genomic DNA and *E. coli* DNA in feces were extracted from colorectal cancer patients and healthy controls respectively. Specific primers for β-G gene were designed and amplified by PCR as templates of fecal bacteria genomic DNA and *E. coli* DNA respectively. Results: Compared with normal control, the amount of *E. coli* in cancer group increased significantly, *Lactobacillus* and *Bifidobacterium* probiotics reduced significantly, and proportional quantity of anaerobic bacteria and aerobic bacteria reversed. The intestinal flora carry β-G in both groups, and homologies with uidA gene sequences encoding the β-G were 99% and 98% respectively. In colorectal cancer group the 1141th and 1148th A base were deleted. The 1149th A base mutated into T base, and the 1158th bit A base mutated into G base; however, in healthy control group the 1141th and 1148th position A base was deleted, and the 1149th A base mutated into T base. Conclusion: There are differences of intestinal flora distribution between cancer group and healthy control group. The gene mutation and deletion of intestinal flora of β-G gene appear at the same time at 1141th, 1148th and 1149th in both cancer group and healthy control group, and 1158th genetic mutation appears only in colon cancer group.

Keywords: Colon cancer, intestinal bacteria, *E. coli*, β-glucuronidase

Introduction

Colorectal cancer is a common malignant tumor and its morbidity is increasing [1, 2]. It has been reported that the pathogenesis and development of this disease were associated with intestinal flora [3, 4]. Human intestinal floras are important factors of intestinal environment. Host's heredity and external environment in which the host exists would affect the balance of human intestinal microecology. The host's healthy condition is also closely related to the balance of human intestinal microecology [5].

β-glucuronidase (β-G) is an acid hydrolase, its positive rate in *E. coli* is up to 97% and it has a high specificity [6]. β-G-mediated glucuronidation is the main pathway of detoxification in human body, while the activity of β-G carried by intestinal flora in colorectal cancer patients is obviously lower than that in health population [7, 8].

This paper aims to reflect changes of intestinal flora in colorectal cancer patients by analyzing the difference in intestinal microecology of stool samples between colorectal cancer patients and health population, to explore the intestinal flora in colorectal cancer patients, to investigate the relationship between the characteristics of β-G produced by the intestinal flora and colorectal cancer, and to compare sequence for β-G which is produced by *E. Coli* and can lead to formation of colorectal cancer between two groups, which may provide new ideas for the prevention and treatment of gastrointestinal cancer.

Materials and methods

Samples

Stool samples in colorectal cancer group were provided by Jilin City Cancer Hospital (30 samples). All the patients in colorectal cancer group did not receive antibiotics, hypertonic prepara-

Table 1. Culture conditions of enteric flora

Medium	Bacterium	Dilution	Culture environment	Culture time (h)
Sb medium	*Yeast*	10^{-4}, 10^{-5}, 10^{-6}	Aerobic	24
Staphylococcus aureus selective Agar 110	*Staphylococcus*	10^{-1}, 10^{-2}	Aerobic	24
EMB medium	*Escherichia coli*	10^{-3}, 10^{-4}	Aerobic	24
Bile esculin agar	*Enterococcus*	10^{-4}, 10^{-5}	Aerobic	48
BS medium	*Bifidobacterium*	10^{-4}, 10^{-5}, 10^{-6}	Anaerobic	24
LBS medium	*Lactobacillus*	10^{-4}, 10^{-5}, 10^{-6}	Anaerobic	48
PS medium	*Peptococcus*	10^{-5}, 10^{-6}	Anaerobic	24

Table 2. Comparison of enteric flora in both groups (Log10)

Enteric flora	Colorectal cancer group (n = 30)	Control group (n = 10)
Escherichia coli	7.64 ± 0.23	6.09 ± 0.10
Yeast	3.85 ± 0.91	3.48 ± 0.76
Staphylococcus	3.84 ± 1.38	3.77 ± 1.03
Enterococcus	6.42 ± 1.76	6.53 ± 0.92
Bifidobacterium	7.58 ± 0.93	9.19 ± 1.04
Lactobacillus	6.44 ± 1.13	7.79 ± 1.34

tions, viable microecological agents, stimulant laxatives, polyethylene glycol and other cathartic preparations one month before sampling. Stool samples in healthy control group were provided by the teachers and students from College of Medical Test in Beihua University (10 samples), and all the healthy controls had no history of gastrointestinal diseases or above-mentioned drug use before sampling.

Primers

All the sequences of β-G gene (S69414.1) were searched from GeneBank, and the bioinformatics software Primer 5.0 was used to design the primer for PCR application. Sense: 5'-GGGCA-ACAA GCCGAAAGA-3' and anti-sense: 5'-GCG-TCGCAGAACATTACA-3' were synthetized by Shanghai General Biotech Co., Ltd.

Separation of normal intestinal flora from stool samples and colony counting

0.1 g naturally discharged and fresh stool was weighed and then placed in sterile 1 mL EP tubes. 0.9 mL saline was added. The mixture was placed on a vibrator and vibrated at 200 times/min for 30 min or until the stool was mixed uniformly with saline. Multiple proportions of dilutions were performed for the solu-

tion above, and 0.01 proportion dilution was taken in accordance with **Table 1**. The solution obtained was inoculated onto the following selective media (seven) with "L" rod, and then aerobic and anaerobic culture was performed for 24-48 h. Counting was performed using the formula (e.g., viable bacteria colony unit in 1 mL sample, (sample weight + dilution amount)/dilution ratio × sample weight × colony count (or ×10)) according to the plate count and dilution. The colony counting results were expressed as the logarithm of colony-forming unit per gram of stool (log10) [3].

Genomic DNA of intestinal flora was extracted in accordance with the extraction kit instruction for microbial genome in stool, and *E. coli* DNA was extracted by water-boiling method. The above-mentioned extracts were preserved at -20°C for usage.

PCR application

30 μl PCR reaction system was used and was prepared as follows: 3 μL 10× buffer solution, 1 μL each of 12.5 μmol/L upstream primer and downstream primer, 1 μL dNTPs (2.5 mmol/L), 2.4 μL $MgCl_2$, 2 μL Tag DNA polymerase (1 U/μL) and 2 μL DNA template were added, and the solution was diluted with sterile triple-distilled water to 30 μL. Standard strains were taken as the positive control, and sterile triple-distilled water was adopted as the negative control. Reaction conditions: pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, renaturation at 55°C for 45 s, extension at 72°C for 30 s, 30 cycles, and extension at 72°C for 30 s. 6 μL PCR products and 1 μL buffer solution above were mixed, and 2.0% agarose gel was used for electrophoresis. Finally, Gel Documentation and Analysis System was adopted to photograph [4].
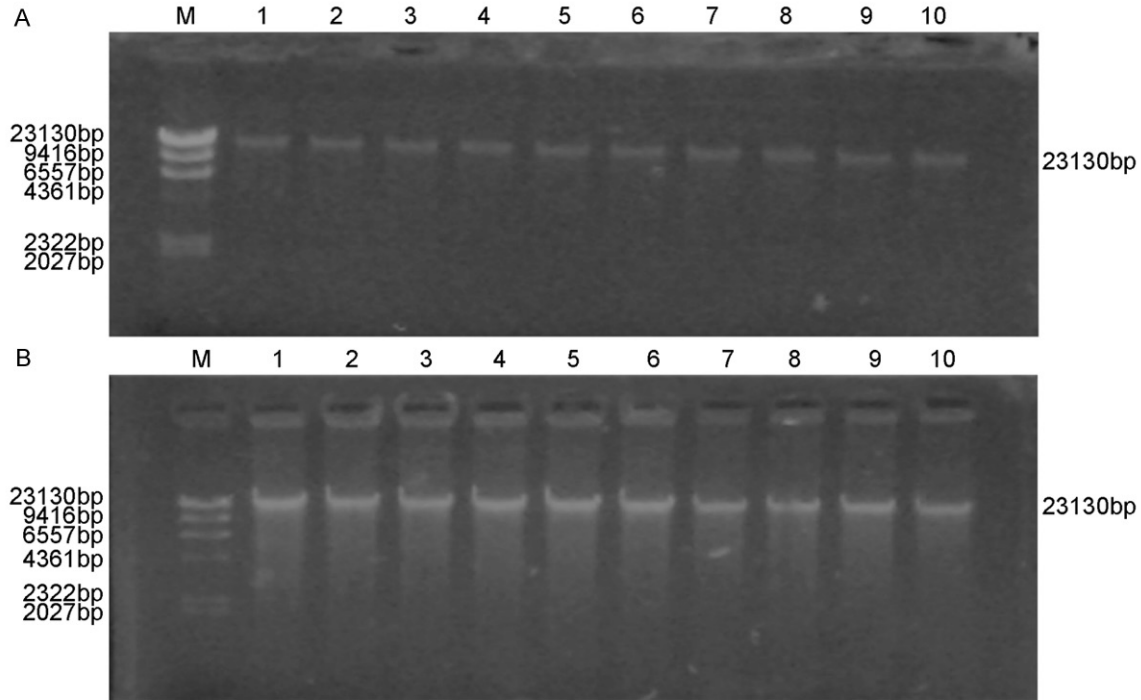
Figure 1. Agarose gel electrophoresis of total genome DNA of enteric flora in colorectal cancer group (A) and control group (B). M: marker; 1-10: total genome DNA of enteric flora in feces from ten random colorectal cancer group patients (A) or ten healthy control people (B).
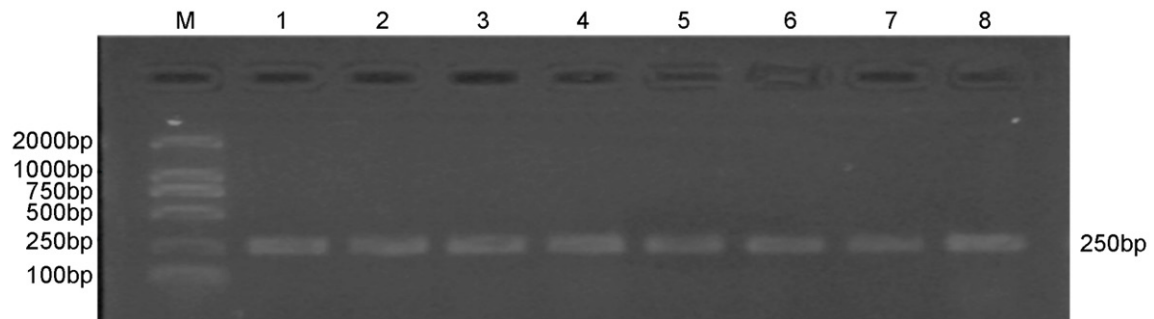

Figure 2. Agarose gel electrophoresis of PCR products in both groups. M: marker; 1-2: Escherichia coli DNA in control group; 3-4: total genome DNA of enteric flora in control group; 5-6: Escherichia coli DNA in colorectal cancer group; 7-8: total genome DNA of enteric flora in colorectal cancer group.

Purification of PCR products

Under a UV lamp, the target fragments of PCR products which were from the colorectal cancer and healthy control group and had received AGE (agarose gel electrophoresis) were excised using a sterile scalpel. DNA agarose gel extraction kit was used to purify and extract PCR products.

Sequencing

20 μl PCR products of the samples to be sequenced and 5 μl each corresponding geno-

type primer were submitted for test. The sequencing company was Sangon Biotech (Shanghai) Co., Ltd. The sequencing results would be compared by entering http://www.ncbi.nlm.nih.gov/BLAST and be compared to GENBANK database.

Statistical analysis

SPSS16.0 was used to analyze data. Two-sample t test for mean comparison in completely randomized design for all the results of the colorectal cancer and healthy control
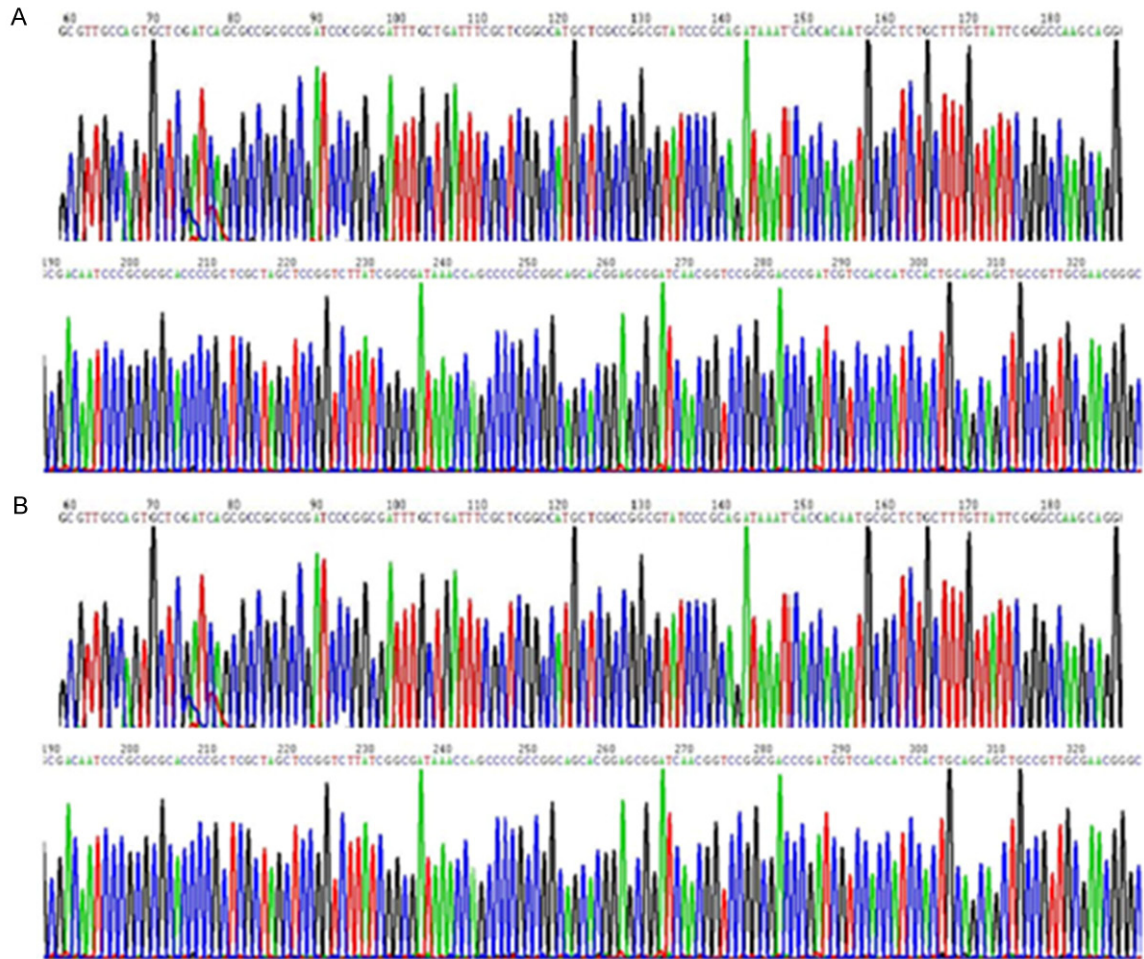
Figure 3. Sequence map of the obtained fragments from the PCR products of colorectal cancer group (A) and control group (B).

groups showed that the differences were all statistically significant ($P < 0.05$).

Results

Intestinal flora distribution in the stool of colorectal cancer and healthy control groups

30 colorectal cancer patients and 10 healthy persons were selected and included in the colorectal cancer and healthy control group, respectively. Anaerobic and aerobic cultures of intestinal bacteria were performed by using a variety of selective media. Results of two-sample *t* test for comparison intestinal flora of colorectal cancer and healthy control groups showed that the differences were all statistically significant (P < 0.05) (**Table 2**).

Agarose gel electrophoresis of genomic DNA of intestinal flora in stool

The genomic DNA was extracted from the samples of both groups and given agarose gel electrophoresis. Then a clear and bright band was observed at about 23130 bp, and the bands in both colorectal cancer and healthy control groups were the same in length (**Figure 1**).

Agarose gel electrophoresis of PCR products from colorectal cancer and healthy control groups

The genomic DNA of intestinal flora extracted from the stool of both groups and *E. coli* DNA were amplified. The results of agarose gel electrophoresis method of genomic DNA of intesti-
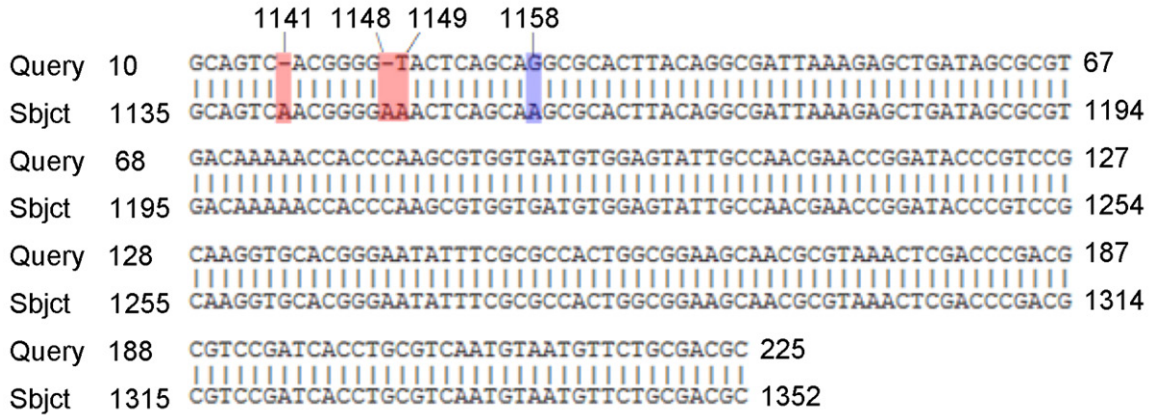
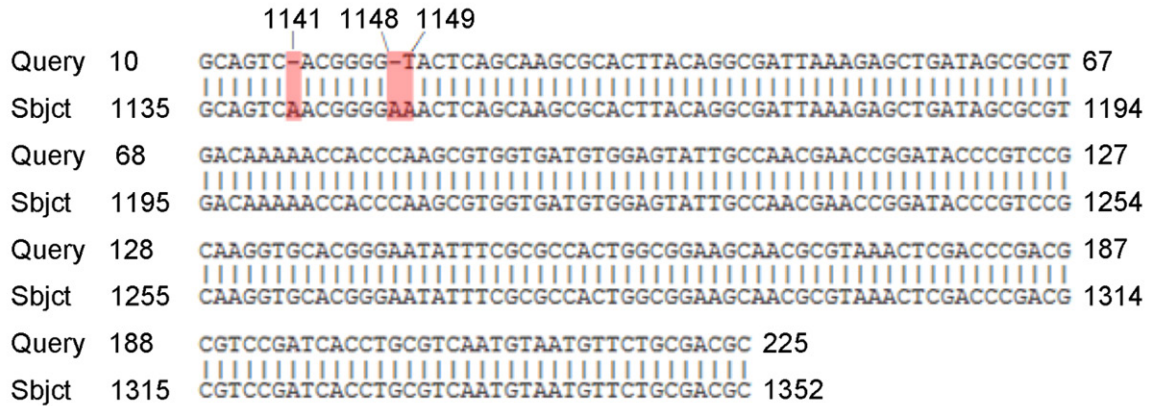Figure 4. BLAST analysis of GenBank in colorectal cancer group.

Figure 5. BLAST analysis of GenBank in control group.

nal flora from both groups as well as *E. coli* DNA showed a single bright band at 251 bp (**Figure 2**).

PCR product sequence map

The tested PCR products from both groups were sent to Sangon Biotech (Shanghai) Co., Ltd for sequencing. Sequence maps of the obtained fragments from the PCR products of colorectal cancer and healthy control groups are shown in **Figure 3**.

BLAST results of PCR products sequences

The BLAST analysis of GenBank showed Score =379 bits (205), Expect =2e-109, Identities =214/218 (98%) and Gaps =2/218 (0%) in uidA fragment sequences of *E. coli* isolated from the stool of colorectal cancer patients (**Figure 4**).

The BLAST analysis of GenBank showed Score =385 bits (208), Expect =4e-111, Identities =215/218 (99%) and Gaps =2/218 (0%) in uidA fragment sequences of *E. coli* isolated from the stool of healthy controls (**Figure 5**).

Discussions

The incidence of colorectal cancer is gradually increasing with diet structure changes in China [9]. It has been proved that the changes in the intestinal environment were caused by poor eating habits, and the incidence of colorectal cancer was also closely related to the changes in the intestinal environment [10, 11]. An important factor in affecting the intestinal environment is intestinal flora. The bacteria in stool include aerobe, anaerobe, facultative anaerobe, fungi, a small amount of *yeast, proteusbacillus vulgaris, Aerobacter aerogenes, Gemmatimonadaceae* and others, among which anaer-

obe accounts for 99%. Most of bacteria in human body have a beneficial role to health while they also cause some opposite effects. Generally, they mainly paly the beneficial role in human health under normal circumstances; however, they would cause a pathogenic effect when the human body environment changes, because balanced steady-state system may be destroyed to lead to the micro dysbiosis [12, 13].

In this study, qualitative and quantitative analyses were performed for the intestinal flora from both groups. Two-sample *t* test for mean comparison of all the results between both groups showed that the differences were all statistically significant. Compared with the control group, the quantity of *E. coli* in the colorectal cancer group increased significantly; moreover, *Lactobacillus*, *Bifidobacterium* and other probiotics reduced significantly; and proportional quantity of anaerobic bacteria and aerobic bacteria reversed. The possible reasons are as follows: the intestinal lesions in colorectal cancer patients would cause a decrease in peristalsis and absorption function so that the intestinal environment changes and result in an increase of aerobe quantity. The significant increase in the quantity of *E. coli* of the colorectal cancer group and the obvious decrease in the quantity of *Bifidobacterium* were related to decrease of the intestinal immune function [14, 15]. The proportion of *lactobacillus* that can produce lactic acid in healthy control group was higher than that in colorectal cancer group. Thereby, lower risk of colorectal cancer in healthy population may be associated with inhibition effect of colorectal cancer from some beneficial bacteria in human intestinal tract. When the intestinal microecology is in a steady state, the short-chain fatty acids produced by the fermentation of intestinal bacteria in cecum and right colon can reduce pH value of stool to inhibit oncogenic cell growth, thus promoting apoptosis of cancer cells [6, 16].

β-G from intestinal flora can convert methylazoxymethanol glucoside in it to cancerogenic substance. If methylazoxymethanol glucoside is added into general diet, colorectal cancer may occur. However, it would not have oncogenic potential if it is taken by germ-free mice [6, 8, 17]. It is indicated that β-G from stool can catalyze procarcinogen to convert to cancerogenic substance, and β-G-mediated glucuronidation

in human intestinal flora is the main pathway in detoxification of human body. The activity of β-G in intestinal flora in colorectal cancer patients was obviously lower than that in health population. Thereby, the detoxification in colorectal cancer patients was reduced to cause colorectal cancer. At present, no study has proved whether there was any difference in β-G gene sequences of intestinal flora between colorectal cancer patients and healthy population.

In this experiment, genomic DNA in intestinal flora from both groups and *E. coli* DNA were amplified by PCR technique, and PCR products were sequenced. β-G was carried by intestinal flora in both groups. The sequences in both groups were compared to uidA sequence encoding β-G in GenBank (Accession No. S69414.1), and the results showed that the homology was 99% and 98%, respectively. In colorectal cancer group, the base A at the 1141st and 1148th site were deleted, the base A at the 1149th site mutated into T, and the base A at the 1158th site mutated into G; however, in healthy control group, the base A at the 1141st and 1148th site were deleted, and the base A at the 1149th site mutated into T. It was found that colorectal cancer patients had the same base deletion and mutation with healthy controls at 1141st, 1148th and 1149th site, but it was observed that the base A mutated into G at the 1158th site only in colorectal cancer group. Therefore, differences in uidA sequences between β-G carried by human intestinal flora encoded in GenBank may cause decrease of β-G activity due to evolution and mutation of *E. coli* in human intestine. Methylazoxymethanol is formed by hydrolysis of β-G-dimethylhydrazine carried by intestinal flora, and methylazoxymethanol would continue to de degraded into methyldiazonium ion so as to decrease detoxification. Thereby, carcinogenic substances are easily produced to greatly increase the risk of gastrointestinal cancer. The 1158th site mutation would cause colorectal cancer. The aforementioned conclusion still needs to be verified by further experiments. In the present experiment, β-G of intestinal flora is not quantified, and expression of mRNA is not studied. Therefore, further verification is still needed, which will be of great importance in studies of intestinal microecology among colorectal cancer patients.

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