Original Article

Gut microbial composition changes in bladder cancer patients: a case-control study in Harbin, China

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Background and Objectives: This study aimed to explore the changes of gut bacteria in bladder cancer patients. **Methods and Study Design:** Newly diagnosed bladder cancer patients were recruited. All participants completed a questionnaire about personal behavior and diet. Pyrosequencing of the total genomic DNA extracted from human feces was carried out by Illumina HiSeq 2000. The copy number of target DNA for bacteria was determined by real-time quantitative PCR assay. Fecal short chain fatty acids contents were measured by gas chromatography (GC) analysis. The concentrations of lipopolysaccharide and D-lactic acid in serum were determined by enzyme-linked immunosorbent assay kits. **Results:** Fruit intake was significantly lower than in healthy controls. The numbers of *Clostridium cluster XI* and *Prevotella* in bladder cancer patients decreased. The numbers of domain bacteria and *Prevotella* were significantly and positively associated with fruit intake (r=0.002, $p<0.05$ for domain bacteria; r=0.004, $p<0.05$ for *Prevotella*). The concentration of butyric acid decreased significantly in bladder cancer patients, and the quantities of fecal butyric acid were significantly and positively associated with fruit intake (r=0.610, $p<0.01$). The concentrations of lipopolysaccharide and D-lactic acid, two sensitive markers of gut permeability, were greater in bladder cancer patients. **Conclusions:** Dysbiosis of gut microbiota, decreased butyric acid concentrations and impaired intestinal structural integrity were found in bladder cancer patients, which might be associated with inadequate fruit intake.

Key Words: bladder cancer, gut microbiota, short chain fatty acids, gut barrier, fruit intake

INTRODUCTION

Urothelial bladder cancer (BC) generally originates from urothelium. The overall incidence of bladder cancer was $5.71/10^5$ in 2014 in China, ranking the sixth in male cancers.[1] Transitional-cell carcinoma which accounts for over 90% of bladder cancer represents the most frequent histological type.[2] Approximately 75% of the newly diagnosed patients are non-muscle-invasive bladder cancer (Ta or T1 of Tumor, Node, Metastasis (TNM) stage), and 25% of the patients are muscle-invasive or metastatic diseases belonging to T2-T4 of TNM stage with bad prognosis.[3] Besides cigarette smoking and occupational exposure to carcinogen,[4] dietary factors, such as low consumption of fruit and vegetable as well as high consumption of processed meat are positively associated with BC risk,[2,5] whereas its underlying mechanisms remain unknown.

Recently, some studies explored the composition of urinary bacterial communities in urinary tract. A small pilot study revealed an enrichment of *Streptococcus* in urine samples from BC patients (n=8).[6] One possible mechanism is that microbiota produces proteases and then contacts with epithelium. These enzymes function as extracellular virulence factors in tissue degradation, evasion and destruction of host physical barriers. Bacterial evasion generates inflammation and oxygen radicals that drive cancer and cancer recurrence.[7]

Almost 99% of the microbial mass is located within the intestinal tract, and the microbiota and its host have co-evolved into a complex 'super-organism'.[8] Moreover, gut microbiota exerts not only local but also long-distance effects on the host. To communicate with distant organs, gut microbial signals should be firstly transmitted across the intestinal epithelium.[9] Failure of the intestinal barrier will cause the leakage of undesirable solutes, pathogenic microorganisms and toxins, which subsequently leads to

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inflammation and immune activation.[10] It has been suggested that targeting inflammatory cytokines may be an effective therapeutic strategy for bladder cancer since for their important role in cancer formation and progression.[11]

The human colonic microbiota produces an enormous quantity of molecules that impact on the gut homeostasis.[12] Short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate, are synthesized from dietary carbohydrates by bacterial fermentation in large intestine.[13] Most SCFAs are locally produced in large intestine by specific bacterial species. Butyrate is mainly produced by *Firmicutes*, whereas acetate is dominated by most anaerobes.[14] Approximately 80-90% of SCFAs are absorbed from the colonic lumen and partly utilized by colonic epithelial cells, and the rest are transferred to circulation or excreted in feces.[14] Therefore, protective effects by SCFAs are not only presented in intestinal mucosa but also extended to other compartments of the body. SCFAs might play an essential role in prevention pathological conditions such as inflammatory bowel disease and cancer for its anti-neoplastic, anti-inflammatory and immune modulatory properties.[15] Moreover, butyric acid showed the direct inhibitory effect on bladder cancer cell by inhibiting cell growth and inducing apoptosis *in vitro*.[16]

Although epidemiological evidence implied the intake of fruit and vegetable was negatively associated with bladder cancer, the underlying mechanism remains unclear. Here, we aim to profile the characteristics of gut microbiota in bladder cancer, which will be helpful to uncover the diet-associated mechanism of bladder cancer.

METHODS

Patients and stool sample collection

All participants were informed about the study purpose during the admission interview, and voluntarily signed consent forms prior to enrolling in the study. All procedures in this study were compliant with the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. This trial was registered at www.chictr.org.cn as ChiCTR-OOC-16007937. The privacy rights of human subjects were observed.

BC patients were recruited for this study at the First Affiliated Hospital of Harbin Medical University between October 1, 2014 and January 30, 2015. Healthy controls (HCs) were recruited from patients who visited the First Affiliated Hospital of Harbin Medical University for physical examination. All of the BC patients and HCs were local residents, and of Han ancestry. None of the patients were undergoing radiotherapy, chemotherapy or other medical interventions. The exclusion criteria were as follows: 1) a history of taking antibiotics, aspirin, other non-steroidal anti-inflammatory drugs or probiotics within the previous 6 months before enrollment; 2) a vegetarian diet; and 3) a history of any other cancer or inflammatory disease of the intestine.[17] According to the criteria, of the 74 subjects enrolled this study (40 for BC groups, 34 for HC group), a total of 26 newly diagnosed patients with histologically and 16 HCs were qualified.

Waxed tissue paper (Epitope Diagnostics, CA, USA) was used by participants, which was laid down on the water in the toilet bowl prior to defecation to avoid contamination. All of the fresh stool samples were collected into sterilized and portable plastic containers and then were placed on ice and transported to the laboratory immediately. Aliquots (200 mg/aliquot) were stored at -80°C until analysis.

Assessment of personal behavior and diet questionnaire

Data on the subjects' demographics, lifestyle factors and dietary information were collected from questionnaires by 2 qualified clinical doctors. All the participants completed a questionnaire that was designed to provide information on health-related issues, including dietary information, smoking status, drinking status, and medical history.[17] All of the participants were asked to recall the types and frequency of foods eaten in the past three months. The questionnaires included 69 kinds of food and drinks. According the criteria mentioned by Chen et al, foods were grouped into three categories: fruits, vegetables and grains.[17] Individual food intake was computed from the reported consumption frequency of each specified unit of food based on data from the Chinese Society of Nutrition regarding the nutrient content in the specified food types. According to the standard for evaluation developed by Chen et al., scores were assigned for smoking status, daily alcohol intake, food intake (vegetables, grains and fruits) and frequency of weekly vigorous exercise.[17]

DNA extraction and 16S rRNA gene sequencing analysis

Total genomic DNA was extracted from fecal samples (human and mice, separately) aseptically using the QIAamp DNA Stool Mini Kit (Qiagen, CA). Pyrosequencing of the total genomic DNA extracted from human feces was carried out by using an Illumina HiSeq 2000 by Sangon Biotech Co. Ltd. (Shanghai, China). The sequences were grouped into unique operational taxonomic units (OTUs) using 97% identity thresholds. The richness, Shannon index and Simpson rarefaction plot were calculated to compare the microbial diversity and richness among the HCs and four subgroups of BC patients (nTa=4, nT1=12, nT2=7, nT3=3). A Venn diagram with shared OTUs was shown to depict the similarities and differences among the five groups. UniFrac principal coordinate analysis (PCoA) analysis was applied to compare the bacterial communities of the different groups based on phylogenetic information. The heatmap figure was generated using the R-package gplots.[18]

RNA extraction, cDNA synthesis and real-time quantitative PCR assay

RNA was extracted with TRIzol Reagent (Life Technologies), and cDNA was synthesized from total RNA using the gDNA Removal and cDNA Synthesis Supermix Kit (TransGen Biotech, Beijing, China). The copy number of target DNA for bacteria was determined by comparison with serially diluted standards of plasmid DNA (10^2 to 10^7 copies) containing the respective amplicon for each set of primers and was run on the same plate under the same conditions.[19] Bacteria were quantified as log10 bacteria per gram of stool.[20] All primers used for real-time qPCR were listed in Table 1.

Table 1. Primers used for real-time qPCR

Target group	Primer	Sequence	Reference
Clostridium cluster I	F	5'-TACCHRAGGAGGAAGCCAC-3'	[20]
	R	5'-GTTCTTCCTAATCTCTACGCAT-3'	
Faecalibacteriumprausnitzii	F	5'-GATGGCCTCGCGTCCGATTAG-3'	[20]
	R	5'-CCGAAGACCTTCTTCCTCC-3'	
Clostridium cluster XI	F	5'-ACGCTACTTGAGGAGGA-3'	[20]
	R	5'-GAGCCGTAGCCTTTCACT-3'	
Lactobacillus group	F	5'-AGCAGTAGGGAATCTTCCA-3'	[20]
	R	5'-ATTYCACCGCTACACATG-3'	
Clostridium leptum subgroup	F	5'-TTACTGGGTGTAAAGGG-3'	[36]
	R	5'-TAGAGTGCTCTTGCGTA-3'	
Clostridium coccoides group	F	5'- AAATGACGGTACCTGACTAA-3'	[37]
	R	5'-CTTTGAGTTTCATTCTTGCGAA-3'	
Bacteroides-Prevotella group	F	5'-GAAGGTCCCCCACATTG-3'	[20]
	R	5'-CAATCGGAGTTCTTCGTG-3'	
Bacteroides fragilis group	F	5'-AYAGCCTTTCGAAAGRAAGAT-3'	[38]
	R	5'-CCAGTATCAACTGCAATTTTA-3'	
Bifidobacterium genus	F	5'-GGGTGGTAATGCCGGATG-3'	[37]
	R	5'-TAAGCCATGGACTTTCACACC-3'	
Atopobium cluster	F	5'-GGGTTGAGAGACCGACC-3'	[38]
	R	5'-CGGRGCTTCTTCTGCAGG-3'	
Enterobacteriaceae	F	5'-CATTGACGTTACCCGCAGAAGAAGC-3'	[20]
	R	5'-CTCTACGAGACTCAAGCTTGC-3'	
Domain Bacteria	F	5'-AGAGTTTGATCCTGGCTCAG-3'	[36]
	R	5'-GCTGCCTCCCGTAGGAGT-3'	
	R	5'-TGATCCACATCTGCTGGAAGGT-3'	

GC analysis of SCFA content in fecal samples

Fecal samples (2 g) were homogenized with 10 mL of deionized water for 10 min and centrifuged at 13200 g for 20 min at 4°C. The supernatant was immediately filtered through a 0.45 μm microfiber filter, and then 1 mL of supernatant was placed in a 1.5 mL GC vial with 100 μL of formic acid. Six SCFAs (≥99%, analytical standard, Sigma) (acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid and iso-valeric acid) were serially diluted to make standard curves separately. Then, the concentrations of six SCFAs were quantified by GC (Agilent 7890; Agilent Technologies, USA) equipped with a flame ionization detector (FID) according to the standards.[21] Total SCFAs concentrations were calculated as the sum of six SCFAs (acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid and iso-valeric acid).

Determination of lipopolysaccharide (LPS) and D-lactic acid levels in serum

For patients and healthy controls, venous blood was collected. All the serum was centrifuged after clotting at room temperature. Aliquots of each serum sample were stored at -80°C until analyzed. The concentrations of LPS and D-lactic acid were determined by enzyme-linked immunosorbent assay (ELISA) kits from Jiancheng Bioengineering Institute (Nanjing, P. R China) according to the manufacturer's instructions. The concentrations were spectrophotometrically quantified by measuring the absorbance at 450 nm. The results were expressed as μmol/mL for D-lactic acid and ng/mL for LPS.

Statistical analysis

The statistical analysis was performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Continuous data were reported as the mean ± SD. One-way analysis of variance (ANOVA) was used to compare the differences. Categorical variables were compared using chi-squared and Fisher's exact tests. Correlation was subjected to Pearson correlation analysis. A two-sided p value <0.05 was considered to indicate statistical significance.

RESULTS

Demographics and lifestyle-related factor analysis

Diet has an influence on the gut microbiota composition.[22] Moreover, diverse studies have demonstrated that cigarette smoking and dietary factors such as the decreased intake of fruit and vegetable were associated with an increased risk of BC.[23] Thus, we evaluated health-related issues including food intake (vegetables, grains and fruits), frequency of weekly vigorous exercise, smoking and drinking status for all individuals. No significant differences were observed in age, sex, body mass index or percent of overweight between two groups (Table 2). In addition, the ratios (scores from 0 to 4) of smoking, drinking alcohol and weekly vigorous exercise in the two groups showed no difference (Table 3). Data from food questionnaires indicated that fruit intake in the BC group was markedly reduced in contrast with that in the HC group (the ratio for fruit scores from 0 to 3, 14:8:4:0 for the BC group versus 2:9:5:0 for the HC group, p=0.026) (Table 3).

Changes in fecal microbial communities and microbial components in BC patients

Firstly, 83815 sequences were obtained for high throughput sequencing. Then, a total of 70702 optimized

Table 2. Clinicopathological parameters between patients and healthy controls

Parameters	HC (n=16)	BC (n=26)	*p* value
Ages (years)/ range[†]	58.5±10.6	62.2±9.80	0.162
Sex [% (n)]			0.095
Male	43.8 (7)	69.2(18)	
Female	56.3 (9)	30.8 (8)	
BMI (kg/m^2) [†]	23.8±3.07	24.3±3.30	0.606
Overweight [% (n)] [‡]	37.5 (6)	34.6 (9)	0.852
Tumor stage			
Ta	4		
T1	12		
T2	7		
T3	3		

HC: healthy controls; BC: bladder cancer patients.
[†]Data were expressed as the mean±SD.
[‡]Categorical variables were compared by a chi-square test or Fisher's exact test.

Table 3. Personal behavior-related characteristics between patients and healthy controls

Parameters	HC (n=16)	BC (n=26)	*p* value
Smoking [%(n)][†,‡]			0.960
0	6.25 (1)	7.69 (2)	
1	18.8 (3)	26.9 (7)	
2	0.00 (0)	3.85 (1)	
3	6.25 (1)	3.85 (1)	
4	68.8(11)	57.7 (15)	
Weekly vigorous exercise [%(n)][†,‡]			0.163
1	93.8 (15)	76.9 (20)	
2	6.25 (1)	3.85 (1)	
3	0.00 (0)	19.2 (5)	
Alcohol [% (n)] [†,‡]			0.206
0	6.25 (1)	15.4 (4)	
1	43.8(7)	15.4 (4)	
2	0.00 (0)	3.85 (1)	
3	0.00 (0)	0.00 (0)	
Vegetables [% (n)] [†,‡]			0.381
0	0.00 (0)	0.00 (0)	
1	93.8 (15)	100 (26)	
2	6.25 (1)	0.00 (0)	
3	0.00 (0)	0.00 (0)	
Grains [% (n)] [†,‡]			0.106
0	0.00 (0)	0.00 (0)	
1	68.8 (11)	50.0 (13)	
2	25.0 (4)	50.0 (13)	
3	6.25 (1)	0.00 (0)	
Fruits [% (n)] [†,‡]			0.026
0	12.5 (2)	53.9 (14)	
1	56.3(9)	30.8 (8)	
2	31.3 (5)	15.4 (4)	
3	0.00 (0)	0.00 (0)	

HC: healthy controls; BC: bladder cancer patients.
[†]Categorical variables were compared by a chi-square test or Fisher's exact test.
[‡]Scores for personal behavior information were according to Chen et al.[17]

sequences were used for downstream analysis (on average, 14140±1855 reads per sample) and were clustered into 3233 OTUs. The results of richness diversities, Shannon index, and Simpson rarefaction plot indicated that bacterial diversities in the BC groups were reduced (data not shown).

On account of the decreased bacterial diversities in BC patients, the detailed distributions of fecal microbial communities at the phylum level were studied (Figure 1A). The main differences between the HC and BC groups were attributed to *Bacteroidetes* and *Firmicutes*, which accounted for up to 85% of the overall on average. The abundance of bacteria at the class level in all groups is presented in Figure 1B. The results of PCoA (Figure 1C) indicate that the fecal microbial communities of BC patients and HCs are separate as P1, P2 and P3 (43.3%, 33.5% and 17.4% of the explained variance, respectively).

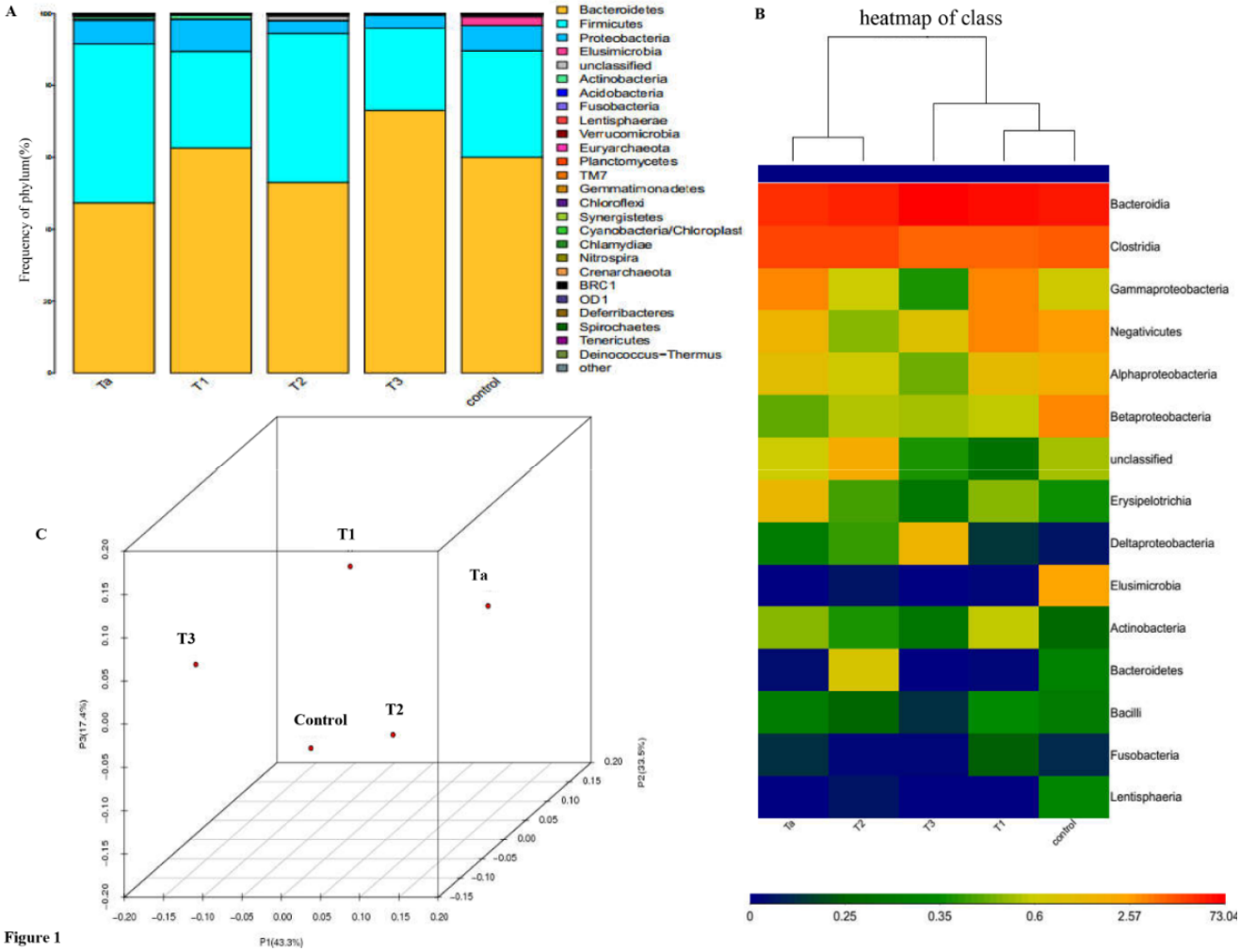

Figure 1

Figure 1. The composition of the fecal microbiota in the BC patients and healthy controls. (A) The compositional gut microbiota profiles are shown at the phylum level in the healthy control group and four subgroups of BC patients (each color represents one bacterial phylum). (B) The heatmap of specimens shows the relative abundance of main bacteria at the class level. (C) PCoA-estimated gut microbiota in the healthy control group and four subgroups of BC patients. HC: healthy controls; BC: bladder cancer patients

Table 4. Qualification of fecal bacteria from patients and healthy controls by real-time qPCR

Bacteria group	HC	BC	*p* value
Domain Bacteria	8.01±0.64	7.47±0.25	0.006
Phylum: *Firmicutes*			
Clostridium cluster I	4.46±0.77	3.96±1.46	0.191
Faecalibacterium prausnitzii	7.41±0.75	7.49±0.97	0.775
Clostridium cluster XI	4.97±0.67	4.40±0.85	0.031
Lactobacillus group	4.98±0.90	4.83±0.53	0.531
Clostridium leptum subgroup	7.41±1.17	6.82±1.10	0.122
Clostridium coccoides group	5.31±0.89	5.56±0.95	0.446
Phylum: *Bacteroidetes*			
Bacteroides-Prevotella group	7.54±1.21	6.52±0.90	0.004
Bacteroides fragilis group	5.94±0.81	6.06±1.17	0.732
Phylum: *Actinobacteria*			
Bifidobacterium genus	2.80±1.03	3.37±1.12	0.100
Atopobium cluster	5.88±0.71	5.47±0.77	0.128
Phylum: *Proteobacteria*			
Enterobacteriaceae	3.04±1.01	3.27±1.03	0.580

HC: healthy controls; BC: bladder cancer patients

Twelve dominant bacterial groups, mainly belonging to the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*, were analyzed by real-time qPCR (Table 4). The numbers of domain Bacteria, *Clostridium cluster XI* and *Prevotella* in BC patients were significantly lower than those in HC group ($p<0.05$). Other microorganism such as *Clostridium cluster I* and *Faecalibacterium*

prausnitzii were not significantly different between the two groups.

Correlation analysis was used in patients and healthy controls to analyze the relationship between fruit consumption and bacteria amounts; the numbers of domain bacteria and *Prevotella* were positively and significantly correlated with the fruit consumption (Figure 2A&B).

Decreased concentrations of SCFAs in BC patients

The microbiota is enriched in genes relevant for dietary nutrient absorption.[24] The quantities of SCFAs, the fermented metabolites of undigestible carbohydrates in the colon and cecum were measured. As shown in Figure 3A, the contents of butyric acid in the BC group (69.4±45.1 μmol/g) were lower than those in the HC group (125±39.7 μmol/g) (p<0.05). To explore whether a lower fruit intake is associated with a decreased concentration of butyrate, correlation analysis was performed for patients and healthy controls. According to the r value, butyric acid concentration is positively and significantly correlated with fruit consumption (Figure 3B).

Excessive LPS and D-lactic acid in BC patients

SCFAs, especially butyrate, act as a key energy source for intestinal epithelial cells (IECs) and impact on mucosal integrity by stimulating the growth of IECs.[25] LPS and D-lactic acid, known as two sensitive markers of gut permeability, can penetrate across the intestinal epithelium when the intestinal barrier is impaired.[26,27] As shown in Figure 4, the concentrations of LPS and D-lactic acid in the BC group were both significantly higher in contrast with those in the HC group (7.89±2.85 ng/mL versus 3.19±1.00 ng/mL for LPS, 49.3±47.0 μmol/mL versus 14.5±4.67 μmol/mL for D-lactic acid, both p<0.05).

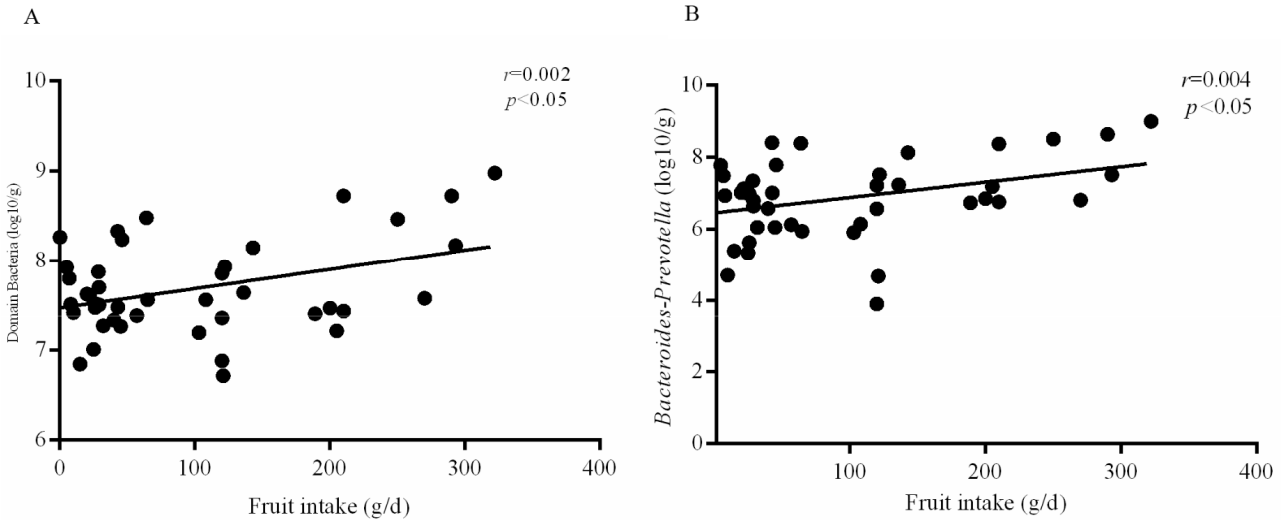

Figure 2. Relationship of bacteria amounts (log_{10}/g) and fruit intake (g/d) in the BC patients and healthy individuals. Pearson's correlation analysis was performed. (A) For the relationship between domain bacteria and fruit intake, r=0.002, and p<0.05. (B) For the relationship between Prevotella and fruit intake, r=0.004, and p<0.05. HC: healthy controls; BC: bladder cancer patients

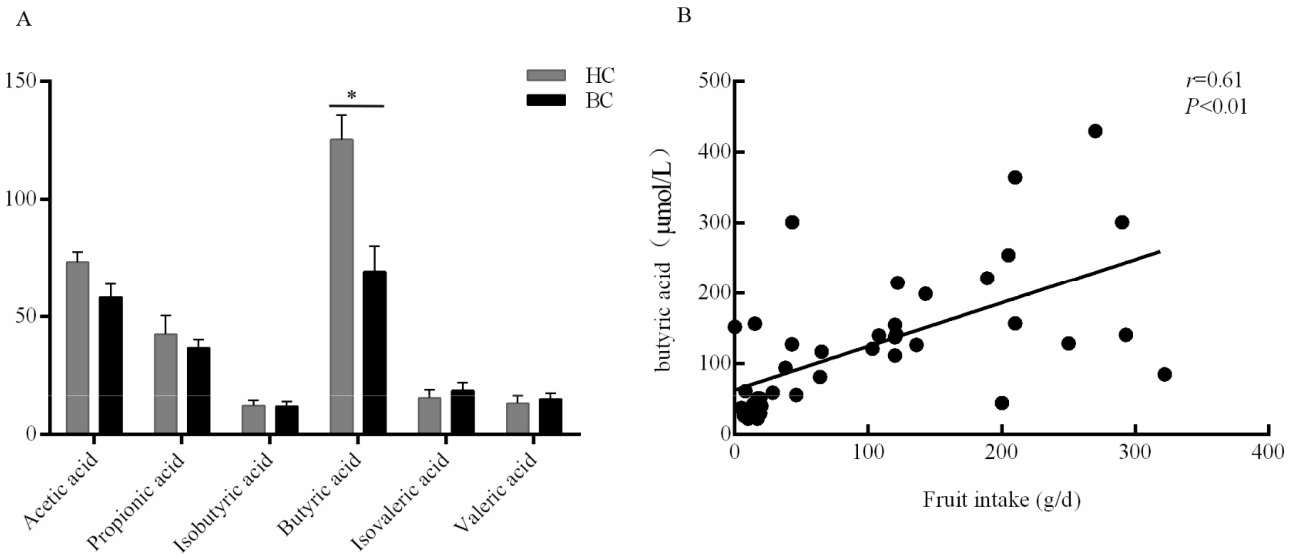

Figure 3. SCFAs concentrations in BC patients. (A) GC analysis of SCFAs concentrations in fecal samples from BC patients and HCs. (B) Relationship of butyric acid (μmol/g) and fruit intake (g/d) in the BC patients and healthy individuals. Pearson's correlation analysis was r=0.610, with p<0.01. HC: healthy controls; BC: bladder cancer patients

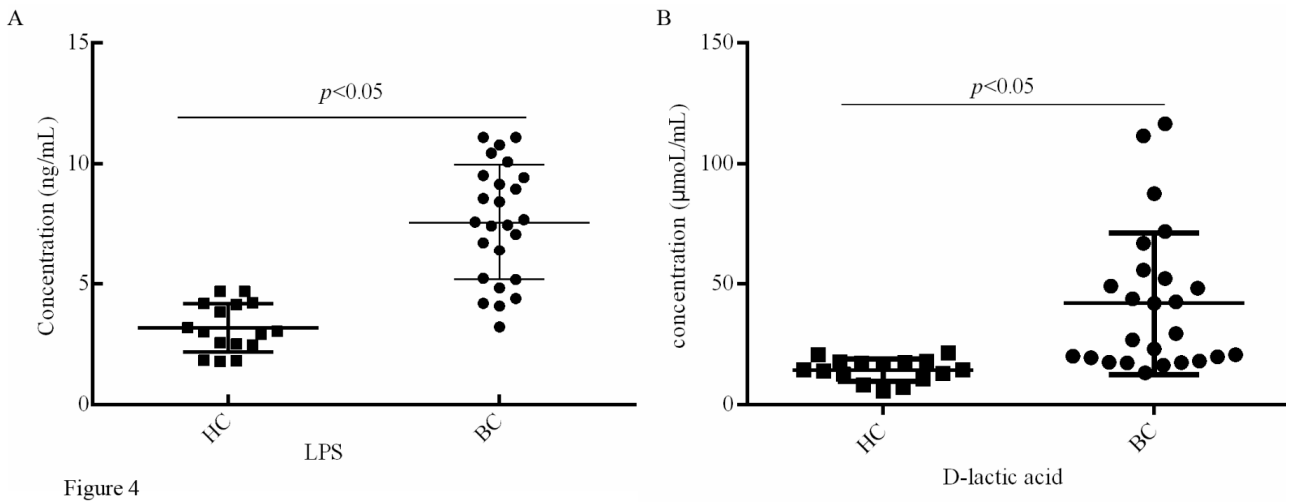

Figure 4. Concentrations of LPS and D-lactic acid in the BC patients and healthy controls. HC: healthy controls; BC: bladder cancer patients

DISCUSSION

BC is a most costly cancer to treat, and there is a pressing need for new and potential therapeutic targets and pathways. Our previous work has demonstrated that abnormal fecal microbiota composition and impaired gut epithelial barrier are to be found in N-butyl-N-(4-hydroxybutyl)-nitrosamine induced bladder cancer in mice.[28] Here we have explored the gut microbiota composition in BC patients as a possible therapeutic consideration .

In the present study, the numbers *of Clostridium cluster XI* and *Prevotella* were both significantly lower in BC patients than those in the HC group. *Clostridium cluster XI* is known to be a SCFA-producing microorganism.[29] *Prevotella* is positively associated with dietary fiber consumption and induces the fermentation of complex polysaccharides.[30] A high level of *Prevotella* is found in healthy people, but not patients with colorectal or breast cancer, consistent with our findings.[31] Meanwhile SCFAs (especially butyric acid), were decreased in patients. Butyrate is essential for growth and proliferation of IECs, and maintains homeostasis of the intestinal mucosal barrier.[25] Butyrate, also known as a histone deacetylase inhibitor, significantly inhibits cell growth and inducts apoptosis in bladder cancer cell lines *in vitro.*[25] Exposure to butyrate can sensitize bladder cancer cells to chemotherapeutic agents *in vitro.*[32] The decreased levels of butyrate in BC patients might delay the proliferation and development of IECs and also decrease its direct anticancer effect on bladder cancer.

We have shown that the concentrations of LPS and D-lactic acid, were both increased in patients, indicating a defect in gut intestinal integrity. Due to poor intestinal integrity, LPS entering the blood will stimulate the related immune cells or inflammatory cells to secrete inflammatory cytokines, which in turns aggravates or even initiates carcinogenesis. Our previous studies indicate that the concentration of IL-6 in serum is elevated in mice with N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced bladder cancer accompanied by breakage of intestinal epithelium and the presence of inflammatory lesions in colon and cecum.[28]

The present report shows that fruit consumption is positively associated with the numbers of domain bacteria and *Prevotella*, and amount of butyrate. Thus, inadequate fruit consumption might partly alter gut microbiota and SCFAs in BC patients. Dietary factors and dietary patterns play a critical role in modulation of gut microbiota.[22] BC patients consumed less than 20 g fruit per day, which is well below recommendations (200 g/day).[33] Possibly and consequently, the richness and composition of the intestinal flora, and also SCFAs were altered. The average fruit intake among Chinese is lower than the recommended intakes.[34] The Chinese Nutrition Society in 2012 showed the average intake of dietary fiber to be around 13 g, below the recommended 25 g. Inadequate dietary fiber, probably as a food marker of a wider range of food factors, is associated with lower overall mortality and cancer prevalence.[35] Gut microbiota may serve as a bridge between dietary factors and cancer. Future research can pursue this possibility in more detail.

In summary, we have identified a gut microbiota-associated mechanism for bladder cancer (Figure 5). However, our study is of a relatively small number of cases in all stages reflecting the low incidence of BC so that larger sample sizes will be required for the required confidence in these findings.

AUTHOR DISCLOSURES

The authors declare no conflict of interest.

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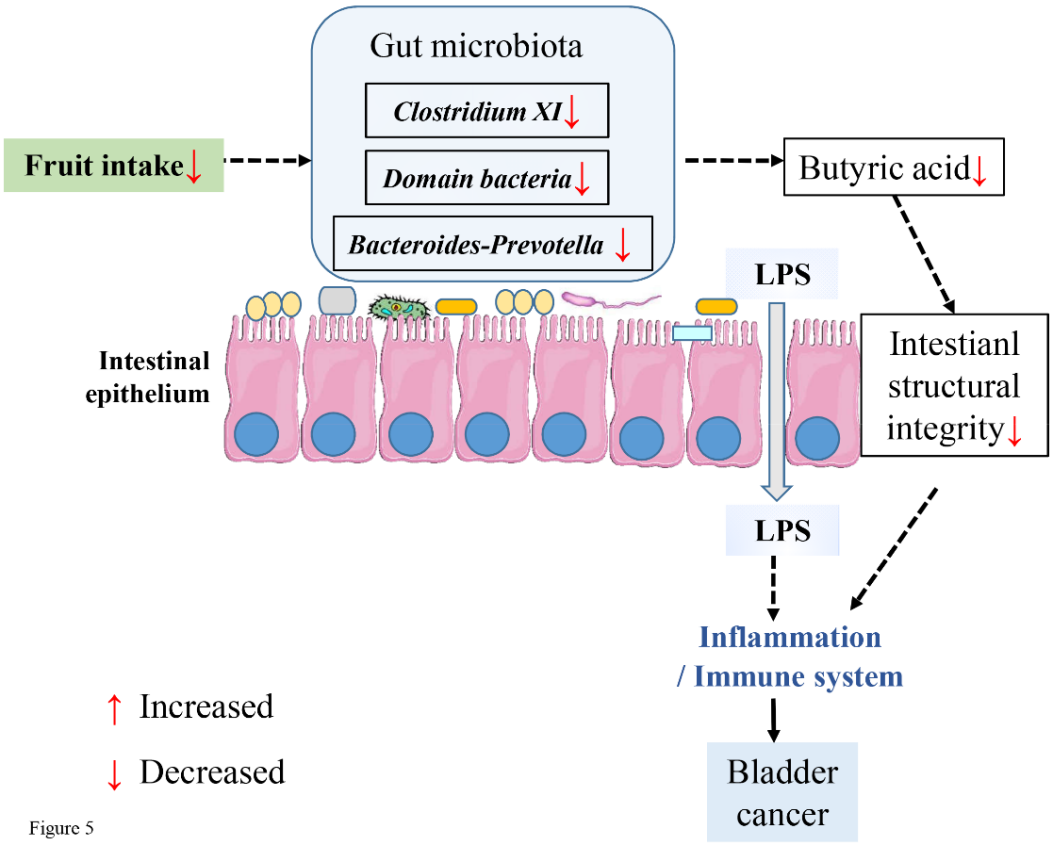

Figure 5

Figure 5. A conceptual diagram by which gut microbiota might increase the risk of urothelial bladder cancer.

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