

ORIGINAL ARTICLE

Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers

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Despite a long-suspected role in the development of human colorectal cancer (CRC), the composition of gut microbiota in CRC patients has not been adequately described. In this study, fecal bacterial diversity in CRC patients ($n=46$) and healthy volunteers ($n=56$) were profiled by 454 pyrosequencing of the V3 region of the 16S ribosomal RNA gene. Both principal component analysis and UniFrac analysis showed structural segregation between the two populations. Forty-eight operational taxonomic units (OTUs) were identified by redundancy analysis as key variables significantly associated with the structural difference. One OTU closely related to *Bacteroides fragilis* was enriched in the gut microbiota of CRC patients, whereas three OTUs related to *Bacteroides vulgatus* and *Bacteroides uniformis* were enriched in that of healthy volunteers. A total of 11 OTUs belonging to the genera *Enterococcus*, *Escherichia/Shigella*, *Klebsiella*, *Streptococcus* and *Peptostreptococcus* were significantly more abundant in the gut microbiota of CRC patients, and 5 OTUs belonging to the genus *Roseburia* and other butyrate-producing bacteria of the family *Lachnospiraceae* were less abundant. Real-time quantitative PCR further validated the significant reduction of butyrate-producing bacteria in the gut microbiota of CRC patients by measuring the copy numbers of butyryl-coenzyme A CoA transferase genes (Mann–Whitney test, $P<0.01$). Reduction of butyrate producers and increase of opportunistic pathogens may constitute a major structural imbalance of gut microbiota in CRC patients.

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Introduction

Colorectal cancer (CRC), also known as large bowel cancer, is one of the most common forms of cancer and the third most common cause of cancer mortality in the world (Jemal *et al.*, 2011). Although heredity is tightly associated with some forms of CRC (Lynch and de la Chapelle, 2003), diet is considered to be the most significant risk factor of this disease by many researchers. Incidence of up to 80% of CRC has been attributed to dietary factors in western populations by epidemiological surveys (Bingham, 2000). High rates of CRC are found in

populations consuming diets high in animal fat, red meat and processed meat, and low in unrefined grains, dietary fiber and vegetables (Chan and Giovannucci, 2010; Dahm *et al.*, 2010).

A highly diverse and dense microbiota exists in the lower part of the human gastrointestinal tract. Recent studies have shown that diet has a dominating role in shaping the structure of gut microbiota (De Filippo *et al.*, 2010; Walker *et al.*, 2010; Zhang *et al.*, 2010), which anaerobically ferments dietary components that are not completely digested and absorbed in the upper gastrointestinal tract (van Hylckama Vlieg *et al.*, 2011). Metabolites and antigens produced by gut microbiota may have significant roles in influencing CRC risk through their interactions with host metabolism and immunity (Davis and Milner, 2009; Saleh and Trinchieri, 2011).

The types of bacteria and their metabolites that may affect the progression of CRC have long been of

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interest. For example, acetaldehyde-producing bacteria, sulphate-reducing bacteria and 7α -dehydroxylating bacteria have been implicated as possible contributing factors to the risk of CRC, due to their metabolites possessing colonic inflammatory and tumor-inducing toxicities, including acetaldehyde (Homann *et al.*, 2000), hydrogen sulfide (Huycke and Gaskins, 2004) and secondary bile acids (Tong *et al.*, 2008; Bernstein *et al.*, 2009), so on. On the other hand, bacterial metabolites that may reduce CRC risk include butyrate and other short-chain fatty acids (Scharlau *et al.*, 2009), and conjugated linoleic acids (Palombo *et al.*, 2002), so on. Producers of these compounds, identified by isolation and *in vitro* characterization, have thus been regarded as beneficial bacteria for human colon health. These bacteria are widely distributed in different phylogenetic groups, including the butyrate producers in the order *Clostridiales* (Louis and Flint, 2009) and conjugated linoleic acids producers in the orders *Clostridiales*, *Lactobacillales*, *Bifidobacteriales*, and *Actinomycetales*, so on (Devillard *et al.*, 2007).

Researchers have also made efforts to search for gut bacterial types possibly related with CRC by comparing their abundance between CRC patients/high-risk populations and healthy populations. Moore and Moore (1995) found through cultivation of human fecal microbiota that *Bacteroides vulgatus*, *Eubacterium* spp., *Ruminococcus* spp., *Streptococcus hansenii*, *Bifidobacterium* spp. and *Faecalibacterium prausnitzii* were significantly higher in people with high CRC risk, while species *Lactobacillus* S06 and *Eubacterium aerofaciens* were significantly higher in people with low CRC risk. Using the same method, O'Keefe *et al.* (O'Keefe *et al.*, 2007) associated 7α -dehydroxylating bacteria to high CRC risk populations and *Lactobacillus plantarum* to low CRC risk populations. Using group-specific quantitative PCR (qPCR) assays, fecal amounts of *Desulfovibrio* sp. (Scanlan *et al.*, 2009) and *Enterococcus faecalis* (Balamurugan *et al.*, 2008) were found to be significantly higher in CRC patients than in healthy controls, while *Bacteroides/Prevotella* levels were significantly lower (Sobhani *et al.*, 2011). Differences were also observed in the overall structure of the gut microbiota between four healthy persons and four persons with colonic adenoma (Shen *et al.*, 2010), as well as between six healthy persons and six CRC patients (Sobhani *et al.*, 2011).

However, adequate knowledge has not yet been provided by previous studies due to the well-known limitations of culture-based methods and DNA fingerprinting techniques, or the small sample size. With the development of high-throughput DNA-sequencing technology and application of multivariate statistical tools, researchers are now able to do a 'microbiome-wide association study' to reveal microbial variations that are possibly linked to environmental changes such as the host health status, with higher resolution and fewer biases

(Andersson *et al.*, 2008; Dethlefsen *et al.*, 2008). In this study, we employed this type of strategy to reveal a significant segregation of the gut microbiota of CRC patients from healthy volunteers, and identified the bacterial phylotypes important for this segregation.

Materials and methods

Sampling

All the 46 CRC patients, aged 42–77 years, were from Shanghai Cancer Hospital, Shanghai Medical College, Fudan University (Shanghai, China). All patients were categorized according to histopathological features by TNM classification of malignant tumors after surgery. None of the patients were on any medication before sample collection. The 56 healthy volunteers, aged 40–54 years, were selected as controls during a routine physical examination and none of them had had gastrointestinal tract disorders or any antibiotics in the past 3 months before sample collection (Table 1). All participants were local residents of Shanghai city for more than 10 years. Informed consent was given by all individuals before participation. One fecal sample was collected from each control, and from each patient before surgery. Fecal samples were frozen immediately after sampling and stored at -80°C .

DNA extraction

Genomic DNA was extracted from each fecal sample by bead-beating extraction and phenol-chloroform purification, as described previously (Li *et al.*, 2008). The amount of DNA was determined by Fluorescent and Radioisotope Science Imaging Systems FLA-5100 (Fujifilm, Tokyo, Japan). Integrity and size of DNA were checked by 0.8% (w/v) agarose gel electrophoresis in 0.5 mg ml^{-1} ethidium bromide. All DNA samples were stored at -20°C until further processing.

Pyrosequencing

The V3 region of the 16S ribosomal RNA (rRNA) gene from each DNA sample was amplified using

Table 1 Clinical characteristics of all individuals

	Healthy volunteers	CRC patients
<i>n</i>	56	46
Age (median, range)	49, 40–54	60, 42–77
Male/female	27/29	24/22
BMI (median, range)	21.07, 20–22.68	ND
Stage I		8
Stage II		18
Stage III		17
Stage IV		3

Abbreviations: BMI, body mass index; CRC, colorectal cancer; ND, no data.

the bacterial universal forward primer 5'-NNNNNN NNCCTACGGGAGGCAGCAG-3' and the reverse primer 5'-NNNNNNNNATTACCGCGGTGCT-3'. The NNNNNNNNN was the sample-unique 8-base barcode for sorting of PCR amplicons into different samples, and the underlined text indicates universal bacterial primers for the V3 region of the 16S rRNA gene (Muyzer *et al.*, 1993). PCR amplification, pyrosequencing of the PCR amplicons, and quality control of raw data were performed, as described previously (Zhang *et al.*, 2010).

Bioinformatics and multivariate statistics

All reads were sorted into different samples according to barcodes. After removal of barcodes, the sequences were clustered using the program CD-HIT with 99.8% similarity (Li and Godzik, 2006). The most abundant sequence of each cluster was selected as a representative and aligned by NAST multi-aligner with template length ≥ 100 bases and percent identity $\geq 75\%$ (DeSantis *et al.*, 2006). RDP Classifier was used for taxonomical assignments of all sequences at 80% confidence level (Cole *et al.*, 2009). Relative abundances of different phyla and genera in each sample were calculated and compared between healthy volunteers and CRC patients using the Student's *t*-test (data of normalized distribution) or Mann–Whitney test (data of non-normalized distribution) via software SPSS 17.0 (SPSS Inc, Chicago, IL, USA). All aligned sequences were imported into ARB (Ludwig *et al.*, 2004) to construct a neighbor-joining tree. The tree, together with sequence abundance data, was then used for online Fast UniFrac analysis based on weighted metric (Hamady *et al.*, 2010). Operational taxonomic unit (OTU) was classified with DOTUR (Schloss and Handelsman, 2005). For each library, OTUs defined at 98% similarity level were used for coverage analysis using Good's coverage by $[1 - (n/N)] \times 100$ (Good, 1953), and richness and diversity estimations using Rarefaction analysis (aRarefact-Win software, S Holland, Stratigraphy Laboratory, University of Georgia, Athens, GA, USA, <http://www.uga.edu/strata/software>), Shannon diversity index (H') (R package 2.7.1), as well as Simpson's diversity index (1-D), and Buzas and Gibson's evenness index ($\exp(H')/S$) (PAST software, Ø Hammer, University of Oslo, Oslo, Norway, <http://www.nhm.uio.no/norlex/past/download.html>). The most abundant sequence of each OTU (98% similarity) was BLAST searched against the RDP database (Release 10) to determine the phylogeny of the OTU. Relative abundances of OTUs were used for principal component analysis, multivariate analysis of variance, and redundancy analysis via Matlab R2010a (The MathWorks, Natick, MA, USA) and Canoco for Windows 4.5 (Microcomputer Power, NY, USA).

Real-time qPCR

Fecal DNA from each sample was subjected to real-time qPCR assays to determine the amounts of total

bacteria and *Bacteroides* spp. through detection of 16S rRNA genes, and the amount of butyrate-producing bacteria through detection of butyryl-coenzyme A (CoA) CoA transferase genes. PCR reactions were performed using iQ SYBR Green SuperMix (Bio-Rad, Richmond, CA, USA) on a DNA Engine Opticon 3 system (MJ Research, Waltham, MA, USA). Each 25- μ l reaction system contained 12.5 pmol of each primer for amplification of 16S rRNA genes of total bacteria, or 7 pmol of each primer for amplification of *Bacteroides* spp. 16S rRNA genes, or 25 pmol of each primer for amplification of butyryl-CoA CoA transferase genes. Primers and amplification conditions are listed in Supplementary Table S1. For each reaction, fluorescence detection was performed at the end of each cycle at 80 °C for 5 s. After all cycles, amplification specificity was monitored via dissociation curve analysis of PCR end products by increasing the temperature at a rate of 0.5 °C per 10 s from 60 to 95 °C with continuous fluorescence collection. Amplification efficiency was determined using Opticon Monitor Software (Version 3.1, MJ Research) from the slope of the line during the linear phase of the PCR reaction. Reactions with efficiency of 1.8–2.2 were selected for further analysis. A plasmid containing a *Bacteroides* spp. full-length 16S rRNA gene from a previous study (Li *et al.*, 2008) was prepared using the QIAGEN Plasmid Mini Kit (Qiagen, Hilden, Germany) and diluted from 1×10^3 to 1×10^9 (copies μ l $^{-1}$) to construct standard curves for the detection of total bacteria and *Bacteroides* spp. A plasmid containing a single butyryl-CoA CoA transferase gene amplicon was prepared and diluted from 1×10^3 to 1×10^9 (copies μ l $^{-1}$) to construct standard curves for the detection of butyrate-producing bacteria. Reactions for standard samples were performed in triplicate and others in duplicate. Copy number of the targeting gene in each reaction was determined by comparison to the standard curve via Opticon Monitor Software. The *Bacteroides* spp. specific 16S rRNA gene copy number or butyryl-CoA CoA transferase gene copy number in relation to total 16S rRNA gene copy number was calculated for each sample, and compared between healthy volunteers and CRC patients by Mann–Whitney test as above. The amounts of *Bacteroides* spp. measured by qPCR and pyrosequencing were compared using Spearman's rank correlation coefficient via SPSS 17.0 (SPSS Inc).

Accession number

The sequence information in this paper has been deposited in the GenBank Sequence Read Archive with accession number SRP005150.

Results

Richness and diversity analysis

In total, 278 877 usable raw reads were obtained from all 102 samples, with an average of 2734 ± 460

reads per sample. After CD-HIT clustering and NAST alignment, 21 043 unique representative sequences were generated. OTUs were delineated at 98% similarity level because higher thresholds generated a dramatic increase of OTU numbers, which might represent the microdiversity at subspecies level (Supplementary Figure S3) (Zhang *et al.*, 2009). The total number of OTUs at 98% similarity level was 4249 (Supplementary Table S2). The values of Good's coverage of all libraries were above 94%. While no rarefaction curve plateaued with the current sequencing, the Shannon diversity estimates of all samples already reached stable values at this sequencing depth, suggesting that although new phylotypes would be expected with additional sequencing, most diversity had already been captured (Supplementary Figure S1). There were no statistically significant differences with Shannon diversity index, Simpson's diversity index or Buzas or Gibson's evenness index between the gut microbiota of healthy volunteers and CRC patients (Student's *t*-test, $P > 0.05$; Supplementary Table S3).

Taxonomy-based comparisons of gut microbiota at the phylum and genus levels between healthy volunteers and CRC patients

The amounts of different phyla and genera were assessed by taxonomic assignment of all sequences using RDP Classifier. Among all bacterial groups revealed by the interpretable sequences, *Firmicutes* was the most predominant phylum, contributing 57.2% and 63.1% of the gut microbiota in healthy volunteers and CRC patients, respectively, followed by *Bacteroidetes*, which contributed 32.0% and 22.7%, respectively. *Proteobacteria*, *Actinobacteria* and *Fusobacteria* constituted the next most dominant phyla, contributing 2.81%, 2.22% and 2.20% of healthy volunteers, and 4.68%, 4.55% and 1.59%

of CRC patients, respectively (Supplementary Figure S2). Microbial compositions showed high inter-individual variability. For example, *Firmicutes* accounted for 16.6–94.5%, and *Bacteroidetes* 0.13–75.0% among all individuals (Supplementary Table S4). *Bacteroidetes* was statistically significantly more abundant in the gut microbiota of healthy volunteers than that of CRC patients, and *Proteobacteria* significantly less abundant in the gut microbiota of healthy volunteers ($P = 0.002$ and 0.045, respectively).

At the genus level, *Firmicutes* in the gut microbiota of CRC patients mainly consisted of *Faecalibacterium*, *Blautia*, *Enterococcus*, *Subdoligranulum*, *Dorea*, *Roseburia*, *Megamonas*, and *Streptococcus*, each constituting more than 1% of total bacteria in this population. Relatively abundant genera (>1%) in other phyla included *Bacteroides*, *Prevotella*, *Parabacteroides* and *Porphyromonas* of *Bacteroidetes*; *Bifidobacterium* and *Collinsella* of *Actinobacteria*; and *Escherichia/Shigella* of *Proteobacteria*. Statistically, the relative abundance of five genera: *Bacteroides*, *Roseburia*, *Alistipes*, *Eubacterium* and *Parasutterella* were found to be significantly higher in healthy volunteers than CRC patients, whereas another five genera: *Porphyromonas*, *Escherichia/Shigella*, *Enterococcus*, *Streptococcus* and *Peptostreptococcus* were significantly lower in healthy volunteers than CRC patients ($P < 0.05$; Table 2).

Structural comparison of gut microbiota between healthy volunteers and CRC patients with multivariate statistics

Multivariate statistical analyses were performed to compare the overall structure of gut microbiota of all samples. Principal component analysis based on the relative abundance of OTUs revealed a separation of the healthy and CRC individuals on the basis of the

Table 2 List of genera that were significantly different between H and C

Phylum	Genus	Relative contribution (%) ^a		Median, range (%)		P-value	Tendency in CRC patients compared with healthy volunteers ^b
		H	C	H	C		
<i>Bacteroidetes</i>	<i>Bacteroides</i>	21.49	14.04	20.9, 0.08–49.8	10.7, 0–59.9	0.005	↓
<i>Firmicutes</i>	<i>Roseburia</i>	3.59	1.56	2.39, 0–26.0	0.57, 0–9.53	0.003	↓
<i>Bacteroidetes</i>	<i>Alistipes</i>	1.11	0.67	0.50, 0–5.7	0.15, 0–11.0	0.039	↓
<i>Firmicutes</i>	<i>Eubacterium</i>	0.52	0.13	0, 0–4.68	0, 0–2.51	0.028	↓
<i>Proteobacteria</i>	<i>Parasutterella</i>	0.44	0.13	0, 0–4.10	0, 0–1.80	0.032	↓
<i>Bacteroidetes</i>	<i>Porphyromonas</i>	4.1e–3	1.74	0, 0–0.08	0, 0–47.4	0.020	↑
<i>Proteobacteria</i>	<i>Escherichia/Shigella</i>	0.87	2.45	0.07, 0–18.2	0.39, 0–15.1	2e–4	↑
<i>Firmicutes</i>	<i>Enterococcus</i>	0.09	2.40	0, 0–5.16	0.04, 0–59.8	5e–4	↑
<i>Firmicutes</i>	<i>Streptococcus</i>	0.66	1.19	0.27, 0–6.52	0.49, 0–8.58	0.018	↑
<i>Firmicutes</i>	<i>Peptostreptococcus</i>	1.3e–3	0.59	0, 0–0.04	0.04, 0–7.85	5.5e–6	↑

Abbreviations: C, CRC (colorectal cancer) patients; H, healthy volunteers.

^aRelative contribution of a genus in healthy volunteers or CRC patients was calculated as percentage of the sequences of this genus to all sequences in this population.

^b'↓' means decrease and '↑' means increase.

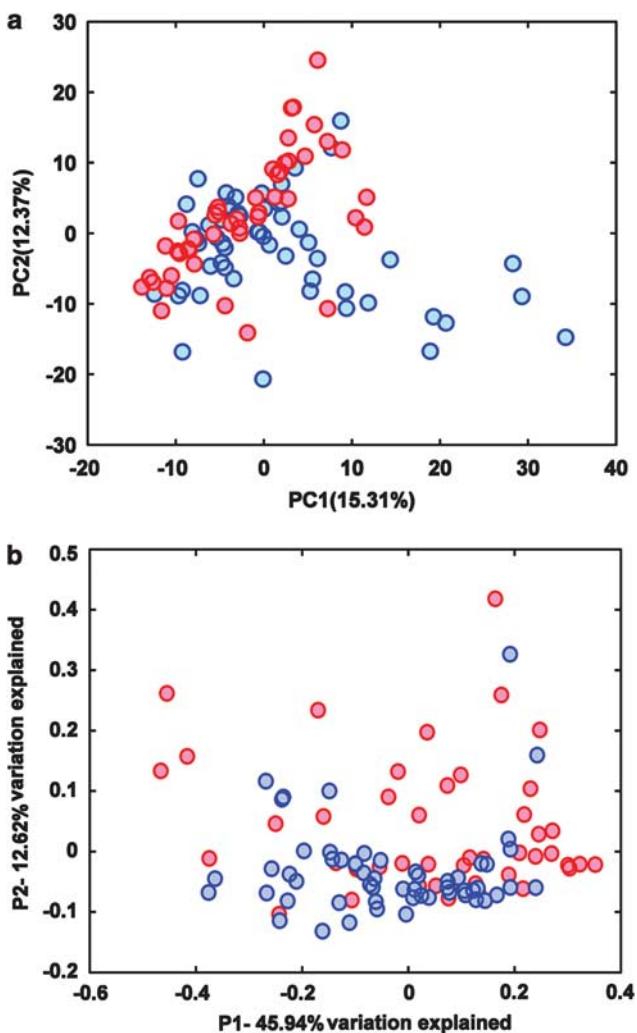


Figure 1 (a) Principal component analysis (PCA) scores plot based on the relative abundance of OTUs (98% similarity level). (b) PCoA (principal coordinate analysis) plot based on the weighted UniFrac metric. Blue circles represent healthy volunteers; red circles represent CRC patients.

first two principal component (PC) scores, which accounted for 15.31% and 12.37% of the total variations (Figure 1a). A multivariate analysis of variance test further indicated that the variation of all individuals exhibited by the first two PC axes was probably explained by disease status ($P=0.002$) rather than gender ($P=0.653$) or age (age groups of 40–49, 50–59, 60–69, 70–79 years, $P=0.165$). Principal coordinate analysis based on weighted UniFrac metric confirmed the above findings, showing a significant separation of healthy volunteers and CRC patients (Figure 1b). At other similarity level of OTUs (95%, 97%, 99% and 100%), consistent results of principal component analysis and multivariate analysis of variance were obtained (Supplementary Figure S4).

Redundancy analysis was performed using healthy or diseased as constrained explanatory variables and relative abundances of all OTUs as

response variables. Monte Carlo Permutation Test showed that the constrained ordination model by healthy or diseased was significant ($P=0.002$) and 5.3% of the variance in OTU abundance data can be explained by the canonical axis. We identified 48 OTUs as key variables, which were well fitted by the sample scores on the canonical axis; each had at least 5% of the variability in its values explained by this axis (Figure 2). On the redundancy analysis ordination plot, 19 OTUs were enriched in the gut microbiota of healthy volunteers, among which 10 OTUs belonged to the genera *Alistipes*, *Phascolarctobacterium*, *Oscillibacter*, or unclassified genera of the order *Clostridiales*; 4 OTUs belonged to the butyrate-producing genus *Roseburia*, and 1 OTU was closely related to the butyrate-producing bacterium A2-166 (100% similarity) of the family *Lachnospiraceae*. On the contrary, 29 OTUs were enriched in the gut microbiota of CRC patients, which mainly belonged to genera *Escherichia/Shigella*, *Klebsiella*, or *Citrobacter* of the family *Enterobacteriaceae*, and genera *Streptococcus*, *Enterococcus*, *Peptostreptococcus*, *Eggerthella*, *Fusobacterium*, *Gemella*, or some other bacteria of the order *Clostridiales*. Interestingly, two OTUs closely related to *B. vulgatus* (100% and 98.7% similarity, respectively) and one OTU related to *Bacteroides uniformis* (97.5% similarity) were enriched in the gut microbiota of healthy volunteers, while one OTU closely related to *Bacteroides fragilis* (98.1% similarity) was enriched in that of CRC patients. Two OTUs of the order *Bifidobacteriales* were also enriched in the gut microbiota of CRC patients (Figure 3). Mann–Whitney tests showed that the relative abundances of 46 OTUs out of the 48 were significantly different between healthy and CRC individuals ($P<0.05$, Supplementary Table S5).

Real-time qPCR

Group-specific qPCR was used to determine the relative amounts of *Bacteroides* spp. and butyrate-producing bacteria in all samples (Supplementary Table S6). *Bacteroides* spp. was found to be significantly more abundant in healthy volunteers than CRC patients ($P=0.005$). Quantification of *Bacteroides* spp. by qPCR and pyrosequencing showed a significantly high degree of consistency (Spearman correlation, $r=0.932$, $P<0.001$), indicating that differences at genus level between healthy volunteers and CRC patients found by pyrosequencing are reliable. The abundances of butyrate-producing bacteria, as represented by butyryl-CoA CoA transferase genes, were significantly higher in the gut microbiota of healthy volunteers than CRC patients ($P=1.5e-4$).

Discussion

Gut microbiota interact extensively with the host through metabolic exchange and co-metabolism of substrates to maintain the normal functions and

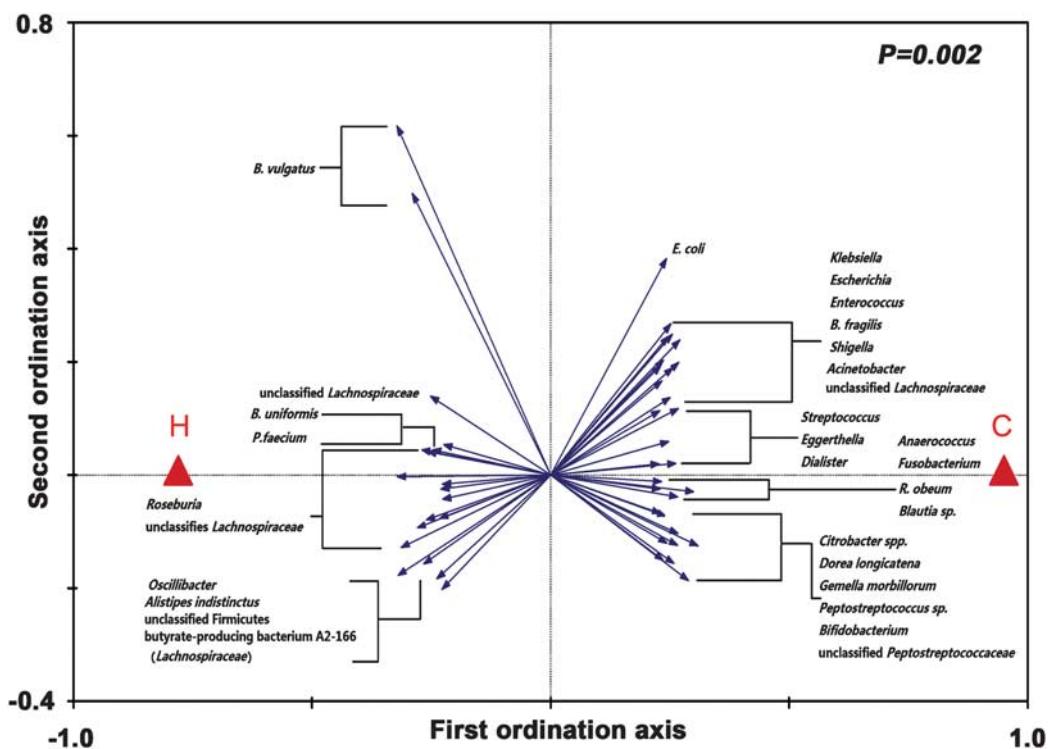


Figure 2 Biplot of the RDA (redundancy analysis) based on the relative abundance of OTUs (98% similarity level). Constrained explanatory variables are indicated by red triangles: H represents healthy and C represents diseased. OTUs that have at least 5% of the variability in their values explained by the canonical axis are indicated by blue arrows. Upper right shows *P*-value of Monte Carlo Permutation Test. B, *Bacteroides*; P, *Phascolarctobacterium*; E, *Escherichia*; R, *Ruminococcus*.

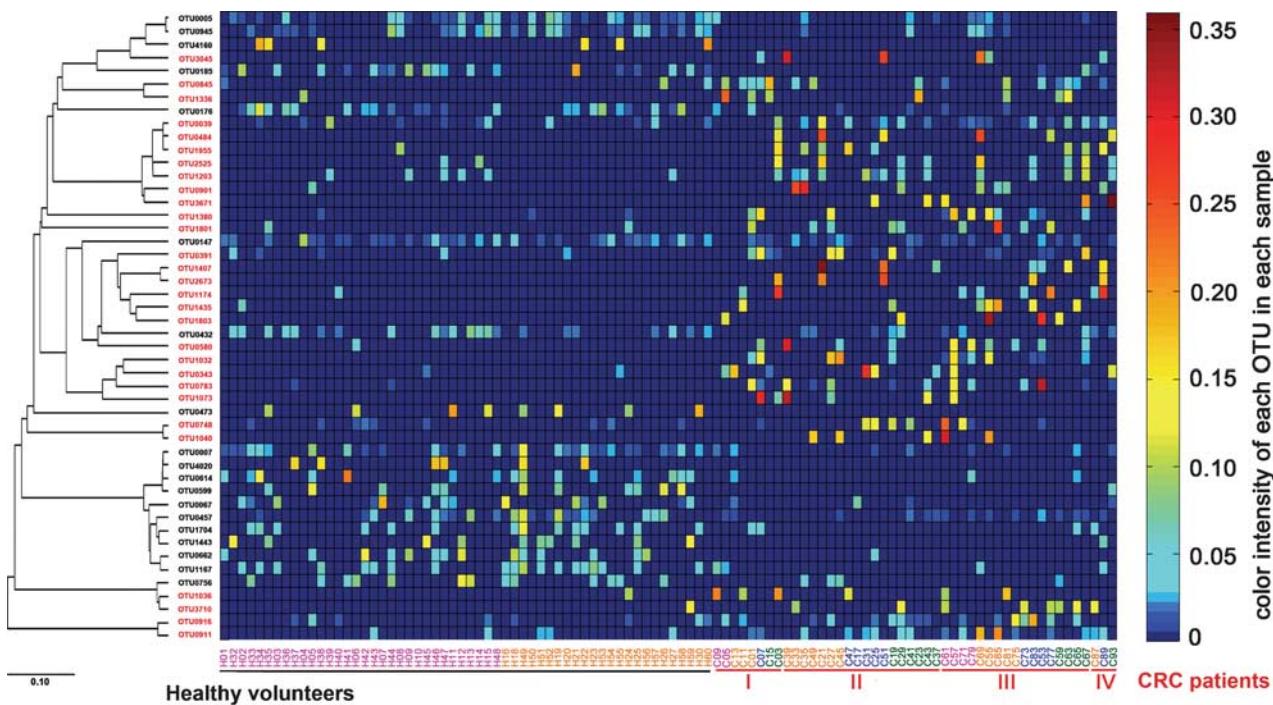


Figure 3 Relative abundances of the 48 OTUs identified as key variables for the differentiation of microbiota of healthy volunteers and CRC patients. OTUs shown in black are the ones enriched in healthy volunteers and those in red are the ones enriched in CRC patients. Phylogenetic tree of the OTUs on the left is constructed with neighbor-joining method in ARB. For each OTU, the color intensity in each sample is normalized to represent its relative ratio in all samples. Individuals at the bottom are arranged according to disease status (healthy; stage I, II, III and IV of CRC) and age inside each stage (from youngest to oldest, color code: pink for age 40–49 years; orange for 50–59 years; blue for age 60–69 years; green for age 70–79 years).

health status of the intestinal tract and the whole body (Nicholson *et al.*, 2005). In this study, we revealed differing gut microbiota structure patterns in CRC patients compared with healthy persons. Significance of the associated structural shift of gut microbiota in the progression of human diseases has been highlighted by several recent publications. In our earlier study, we observed significant differentiation of gut microbiota between healthy rats and carcinogen-treated rats that developed colonic precancerous lesions, but were still apparently healthy (Wei *et al.*, 2010). Alterations of gut microbiota have also been suggested to occur in Crohn's disease patients, ulcerative colitis patients (Frank *et al.*, 2007; Willing *et al.*, 2009, 2010), children with celiac disease (Nadal *et al.*, 2007), and infants suffering from allergic inflammation (Kalliomaki and Isolauri, 2003).

An important part of the structural imbalance is a significant depletion of butyrate-producing bacteria in the gut microbiota of CRC patients. Adequate amounts of butyrate has been implicated in the protection against colitis and CRC by reducing oxidative damage to DNA, inducing apoptosis in DNA-damaged cells, inhibiting tumor cell growth, and decreasing the activity of co-carcinogenic enzymes (Pryde *et al.*, 2002; Rose *et al.*, 2007). The *in vivo* supply of butyrate largely depends on the butyrate-producing bacteria, which are mainly classified into two groups: the *Clostridium leptum* cluster (or clostridial cluster IV, represented by *F. prausnitzii*) and the *Clostridium coccoides* cluster (or clostridial cluster XIVa, represented by *Eubacterium rectale/Roseburia* spp.) of the phylum *Firmicutes* (Louis and Flint, 2009). Here we showed reduced amounts of *C. coccoides* (*Roseburia* spp. and *Eubacterium* spp.) in the gut microbiota of CRC patients by pyrosequencing analysis. We further confirmed the depletion of butyrate producers in CRC patients by measuring the levels of partial butyryl-CoA CoA transferase gene, which is involved in the butyrate formation pathway in the majority of human colonic butyrate producers (Louis and Flint, 2007).

Balamurugan *et al.* (Balamurugan *et al.*, 2008) found by qPCR assays that *F. prausnitzii* decreased approximately fourfold in 20 CRC patients compared with 17 healthy volunteers. In our study, *Faecalibacterium* constituted more than 11% of microbiota of either healthy volunteers or CRC patients, with no difference in between. No OTU related to *F. prausnitzii* was found to contribute to the structural differentiation between CRC patients and healthy controls. Instead, our results show that a decrease in *Roseburia* spp. is more closely associated with the occurrence of CRC. It has been suggested by two dietary intervention studies that, population densities of *Roseburia/E. rectale* spp. have a strong correlation with fecal butyrate concentrations in response to altered carbohydrate supply while *F. prausnitzii* does not (Duncan

et al., 2007; Ramirez-Farias *et al.*, 2009). This indicates the importance of *Roseburia* spp. and *E. rectale* in the production of butyrate *in vivo*. A culture-based study has also suggested that *Roseburia* spp. are the most important active conjugated linoleic acids producers in the human gut (Devillard *et al.*, 2007). Our findings here have suggested that *Roseburia* spp. may have important roles in protecting hosts from CRC.

Another feature of the structural imbalance of gut microbiota in CRC patients is the significant increase in a variety of opportunistic pathogens. Some researchers have proposed hypotheses for the oncogenic potential of bacteria during the progression to CRC, while the mechanisms were suggested to include induction of inflammation or production of mutagenic toxins (Collins *et al.*, 2010). Inflammation is commonly associated with CRC. Colitis-associated cancer, a subtype of CRC, develops directly from inflammatory bowel disease. Other sporadic CRCs, which are not associated with clinically detectable inflammatory bowel disease, also display robust inflammatory infiltration and increased expression of proinflammatory cytokines (Terzic *et al.*, 2010). Inflammation associated with CRC has been suggested to be related to bacterial infection. Through isolation and cultivation from biopsy samples, Martin *et al.* (Martin *et al.*, 2004) have found increased proportion of hemagglutinin expressing *Escherichia coli* in Crohn's disease and colon cancer patients compared with healthy controls. It has also been suggested that enteropathogenic *E. coli* possesses the ability to downregulate DNA mismatch repair proteins and, therefore, promotes colonic tumorigenesis (Maddock *et al.*, 2009). *Streptococcus bovis* promotes the progression of preneoplastic lesions by increasing cell proliferation and interleukin-8 production in a rat model (Ellmerich *et al.*, 2000), and its wall-extracted antigens promote carcinogenesis in Caco-2 (human colorectal adenocarcinoma epithelium) cells by over-expression of cyclo-oxygenase-2 (Biacr *et al.*, 2004). *E. faecalis* produces extracellular superoxide that damages colonic epithelial cell DNA both *in vitro* and *in vivo* (Huycke and Moore, 2002; Huycke *et al.*, 2002), and its population was found to be significantly higher in CRC patients compared with healthy volunteers by qPCR assays (Balamurugan *et al.*, 2008). Both these studies and our results suggest that *Enterococcus*, *Streptococcus*, and *Escherichia/Shigella* may contribute to the differentiation of the gut microbiota of CRC patients from healthy controls.

Previous studies have suggested that a significant reduction of the phylum *Bacteroidetes* occurs in Crohn's disease/ulcerative colitis patients compared with controls (Ott *et al.*, 2004; Frank *et al.*, 2007). However, in our study, we have found different changes among genera of this phylum that, *Bacteroides* decreased while *Porphyromonas* increased significantly in the gut microbiota of CRC patients compared with controls. Furthermore, key OTUs

in the genus *Bacteroides* also showed different enrichments between healthy volunteers and CRC patients. Previous studies have suggested that different *Bacteroides* strains may influence the host health in different ways. A *B. vulgatus* strain was shown to protect against *E. coli*-induced colitis in interleukin-2-deficient mice (Waidmann *et al.*, 2003), while interleukin-10 knockout ($\text{Il10}^{-/-}$) mice monoassociated with pig isolates of *B. vulgatus* have significantly reduced colitis-associated colorectal tumor multiplicity compared with common $\text{Il10}^{-/-}$ mice (Uronis *et al.*, 2009). In contrast, the common colonic commensal, enterotoxigenic *B. fragilis* was reported to trigger cellular proliferation, colonic colitis and strongly induce colonic tumors in multiple intestinal neoplasia (Min) mice (Wu *et al.*, 2009). These studies are in agreement with our finding that the OTU related to *B. fragilis* was more abundant in CRC patients and the two related to *B. vulgatus* were more abundant in healthy persons. It is also reported that non-toxigenic human commensal bacterium *B. fragilis*, which expresses a single microbial molecule polysaccharide A, has the potential to protect animals from experimental colitis induced by *Helicobacter hepaticus* infection (Mazmanian *et al.*, 2008). The possible strain-specific bioactivities of *Bacteroides* group members in human gut suggest that more work at the gene or strain level is still needed to identify the effects of gut microbes relevant to the pathogenesis of CRC.

With this design of cross-sectional cohort study, it is not possible to untangle the causal relationship between gut microbiota and CRC. As samples were collected from patients already diagnosed with CRC, the changes of gut microbiota identified in this work might be just a consequence of CRC. However, these imbalanced changes might contribute to the further progression of CRC through the possible mechanisms discussed above. One way to help clarify the possible roles of gut microbiota in CRC could be correcting this structural imbalance by using designed nutritional interventions or gut microbiota transplantation from healthy donors (Borody *et al.*, 2003).

In summary, by comparing the gut microbial composition of CRC patients and healthy volunteers, we have identified a structural imbalance of the gut microbiota, represented by the reduction of butyrate producers and the increase of opportunistic pathogens, which may be a significant feature of human CRC. The structural imbalance of gut microbiota revealed in this work may provide insights for in-depth analysis of host-microbe interactions and determination of their roles in cancer development.

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