Comparison of human gut microbiota in control subjects and patients with colorectal carcinoma in adenoma: Terminal restriction fragment length polymorphism and next-generation sequencing analyses

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Abstract. Colorectal cancer (CRC) is the third leading cause of cancer-related deaths in Japan. The etiology of CRC has been linked to numerous factors including genetic mutation, diet, life style, inflammation, and recently, the gut microbiota. However, CRC-associated gut microbiota is still largely unexamined. This study used terminal restriction fragment length polymorphism (T-RFLP) and next-generation sequencing (NGS) to analyze and compare gut microbiota of Japanese control subjects and Japanese patients with carcinoma in adenoma. Stool samples were collected from 49 control subjects, 50 patients with colon adenoma, and 9 patients with colorectal cancer (3/9 with invasive cancer and 6/9 with carcinoma in adenoma) immediately before colonoscopy; DNA was extracted from each stool sample. Based on T-RFLP analysis, 12 subjects (six control and six carcinoma in adenoma subjects) were selected; their samples were used for NGS and species-level analysis. T-RFLP analysis showed no significant differences in bacterial population between control, adenoma and cancer groups. However, NGS revealed that i), control and carcinoma in adenoma subjects had different gut microbiota compositions, ii), one bacterial genus (*Slackia*) was significantly associated with the control group and four bacterial genera (*Actinomyces*, *Atopobium*, *Fusobacterium*, and *Haemophilus*) were significantly associated with the carcinoma-in-adenoma group, and iii), several bacterial species were significantly associated with each type (control: *Eubacterium coprostanoligens*; carcinoma in adenoma: *Actinomyces odontolyticus*, *Bacteroides fragiles*, *Clostridium nexile*, *Fusobacterium varium*, *Haemophilus parainfluenzae*, *Prevotella stercorea*, *Streptococcus gordonii*, and *Veillonella dispar*). Gut microbial properties differ between control subjects and carcinoma-in-adenoma patients in this Japanese population, suggesting that gut microbiota is related to CRC prevention and development.

Introduction

Colorectal cancer (CRC), the third leading cause of cancer-related deaths in Japan, has an etiology linked to numerous factors including genetic mutation, diet, life style, and inflammatory process. The human gut is continually colonized by complex microbial communities in which the combined number of cells (10^{11-13} cells/g in the colon) is greater than the total number of the host cells (1). Therefore, the human body harbors 10 times more exogenous cells than human cells. The human gut microbiota becomes relatively stable around 1 week after birth, begins to resemble that of an adult after weaning, and once established remains stable over lifetime (2). It is generally believed that each healthy individual has his or her own unique gut microbiota (3,4).

Numerous researchers have catalogued the gut microbiota of healthy humans and the gut microbiota associated with inflammatory bowel disease (IBD) (5-7) or obesity (8-10). In addition, a recent study suggests that the gut microbiota is associated with CRC development. Several plausible mechanisms in which the gut microbiota could interface with CRC have been proposed; for example, inflammation, DNA-damaging

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Abbreviations: T-RFLP, terminal restriction fragment length polymorphism; CRC, colorectal cancer; NGS, next-generation sequencing

Key words: colorectal cancer, microbiota, terminal restriction fragment length polymorphism, next-generation sequencing

effects, and non-DNA-damaging effects could each be mechanistically important (11).

There is a considerable amount of research confirming that the gut microbiota is a primary driver of inflammation in the colon and the inflammatory environment is related to CRC development (12). Microbial dysbiosis (i.e., disturbance of the normal microbial community) can increase the proportion of facultative anaerobic bacteria, which include potentially harmful inflammation-inducing microorganisms. Inflammation driven by such bacteria (e.g., *Bacteroides fragilis* and *Streptococcus bovis*) is thought to affect carcinogenesis because these bacteria can activate immune cells to release promitogenic and proangiogenic cytokines such as IL-6 and IL-17 (13-15). In fact, there is epidemiological data that suggest up to 15% of human cancer incidence is inflammation-associated (16,17).

DNA-damaging effects of microbiota in CRC are thought to be induced by microbes that produce numerous genotoxic substances and are thus linked to CRC development. For example, microbial-derived nitric oxide has the capacity to damage DNA (18,19). Reactive oxygen species (ROS) are also powerful instigators of mutation and could contribute to chromosomal instability and risk of CRC (20,21).

Carcinogenic effects of CRC-associated microbiota, effects that are unrelated to DNA damage, may be attributable to a number of bacterial metabolites. For example, hydrogen sulfide (H_2S) has been linked to CRC as a potential tumor-promoting agent. H_2S is produced by sulfate-reducing commensal bacteria as part of their normal metabolism (22). Although H_2S does not act as a direct DNA damaging agent, it modulates proliferation, apoptosis, and differentiation of colonic epithelial cells (23). Moreover, the gut microbiota metabolizes different dietary components to influence CRC development. For example, the gut microbiota metabolizes proteins from red meat to nitrosamine and heterocycle amines, and these metabolites are risk factors for CRC development (24,25). In contrast, a high intake of dietary fiber has been considered to be protective against CRC development (26,27); nevertheless, the beneficial effect of microbial fermentation of fiber and production of butyrate on CRC development is still an area of substantial controversy.

The gut microbiota and its products are clearly linked to CRC. However, most research has focused on the association between the gut microbiota and advanced CRC, rather than early-stage cancer. Thus, it is not clear whether the gut microbiota plays a role at an early stage of colorectal carcinogenesis. In addition, most studies in this field have been carried out in Western countries, and it is unknown whether certain members of the gut microbiota particularly associated with CRC also exist in the Japanese population, whose dietary habits and lifestyles are different from those of Western populations. In this study, we used next-generation sequencing (NGS) subsequent to terminal restriction fragment length polymorphism (T-RFLP) analysis to investigate the human gut microbiota in a Japanese population. We specifically selected patients with carcinoma in adenoma for the NGS analysis to evaluate possible associations of gut microbiota with early-stage cancer. We identified several potential bacterial genera and species uniquely associated with control specimens or with carcinoma-in-adenoma specimens.

Materials and methods

Human subjects. Subjects who were under 65 years of age and had undergone colonoscopy at the Mie Prefectural General Medical Center, Yokkaichi, Japan, between 2012 and 2013 were enrolled in the study. To evaluate differences in gut microflora via T-RFLP analysis, the subjects were classified into three groups as follows: i), control subjects, who had normal colonoscopy; ii), adenoma patients, who were diagnosed with colon adenoma bases on the colonoscopy; and iii), cancer patients, who had recently been diagnosed with CRC. Exclusion criteria for all participants included current use of antibiotics, history of or current chronic bowel or liver disease, history of chemotherapy or radiation therapy, and regular use of immunomodulators (steroids, interferons, etc.) or probiotics. Assignment of the patients is shown in Fig. 1. All patients received an explanation of the procedures and possible risks associated with the study, and they gave their written informed consent to participate. This study was performed in accordance with the Declaration of Helsinki and was approved by our Institutional Ethics Committee (authorized no. 2011-5; Mie Prefectural General Medical Center, Yokkaichi, Japan). Stool samples were collected from each participant prior to polyethylene-glycol preparation of the bowel for colonoscopy; each sample was stored at 4˚C and submitted to Technosuruga Laboratory (Shizuoka, Japan) for the T-RFLP analysis, which is described below.

DNA extraction. Fecal samples (~4 mg each) were suspended in a solution containing 100 mM Tris-HCI, pH 9.0, 40 mM Tris-EDTA, pH 8.0, and 4 M guanidine thiocyanate. A 0.8-ml aliquot of each suspension was homogenized with zirconia beads in a 2.0-ml screw cap tube with a FastPrep 24 Instrument (MP Biomedicals, Santa Ana, CA, USA) run at 5 m/sec for 2 min and placed on ice for 5 min. Each sample was spun at 5,000 x g for 1 min; an automatic nucleic acid extractor (Precision System Science, Chiba, Japan) was then used to extract DNA from a 200-μl aliquot of each sample. MagDEA DNA 200 (GC; Precision System Science) was used as the reagent for automated nucleic acid extraction.

T-RFLP. The 16S rDNA was amplified from human fecal DNA using the fluorescent-labeled 516f primer (5'-TGCCAGC AGCCGCGGTA-3'; *Escherichia coli* positions 516-532) and 1510r primer (5'-GGTTACCTTGTTACGACTT-3'; *E. coli* positions 1,510-1,492). HotStarTaq DNA polymerase by Gene Amp PCR system 9600 (Applied Biosystems, Foster City, CA, USA) was used for each amplification reaction. The amplification program was as follows: preheating at 95˚C for 15 min and then 30 cycles of i), denaturation at 95˚C for 30 sec, ii), annealing at 50˚C for 30 sec, and iii), extension at 72˚C for 1 min, and finally, a terminal extension at 72˚C for 10 min. Amplified DNA was purified by a MultiScreen PCR96 Filter Plate (Millipore, Billerica, MA, USA) and verified by electrophoresis. The restriction enzymes were selected according to Nagashima *et al* (28,29). In brief, 16S-rDNA PCR products were purified and digested with 10 U of *Bsl*I (New England BioLabs, Ipswich, MA, USA) at 55˚C for 3 h. An ABI PRISM 3130xl genetic analyzer was used to analyze the resultant DNA fragments, namely fluorescent-labeled terminal restriction

fragments (T-RFs), and GeneMapper software (Applied Biosystems) was used to determine T-RF length and peak area for each sample. T-RFs were divided into 29 operational taxonomic units (OTUs). The OTUs were quantified as the percentage of individual OTU per total OTU area, which were expressed as the percentage of the area under the curve (% AUC). The reference database, Human Fecal Microbiota T-RFLP profiling (http://www.tecsrg-lab.jp/), was used to putatively identify the bacteria in each classification unit and the corresponding OTU.

To evaluate differences in gut microbiota composition at the species level, samples from six control subjects and six patients with carcinoma in adenoma were selected for NGS; IBM SPSS software ver. 22 was used to match control and patient samples based on age, gender, and BMI. Subjects with carcinoma in adenoma were selected and subjects with advanced cancer were excluded to avoid the possibility of gut microbial environment alterations due to cancer progression.

Illumina library generation. NGS analysis of microbial community structure in each feces sample was performed with MiSeq (Illumina, San Diego, CA, USA), as previously described by Takahashi *et al* (30). Briefly, the V3-V4 region of 16S rDNA was amplified using 341F (5'-CCTACGGGAGG CAGCAG-3') (31) and 806R (5'-GGACTACHVGGGTWTCT AAT-3') (32) primers. In addition to the V3-V4-specific priming regions, these primers were complementary to standard Illumina forward and reverse primers. The reverse primer also contained a 6-bp indexing sequence (CAGATC, ACTTGA, GATCAG, TAGCTT, GGCTAC, CTTGTA, ATCACG, CGATGT, TTAGGC, TGACCA, ACAGTG and GCCAAT) to allow for multiplexing. The touchdown PCR method was used with a GeneAmp PCR system 9700 (Applied Biosystems) for thermal cycling. Each PCR reaction mixture (25 μl) contained 20 ng genomic DNA, 2X MightyAmp Buffer ver. 2 (Takara), 0.25 μM of each primer, and 1.25 units of MightyAmp DNA Polymerase (Takara). Each PCR amplification and preparation of amplicon pool were performed as described by Takahashi *et al* (30).

Illumina sequencing and quality filtering. As recommended by Illumina for the pooling of two libraries and described by Takahashi *et al* (30), each multiplexed library pool was spiked with 30% PhiX control to improve base calling during sequencing. Sequencing was conducted using a paired-end, 2x251-bp cycle run on an Illumina MiSeq sequencing system and MiSeq reagent Nano kit version 2 (500 cycle) chemistry. Paired-end sequencing with read lengths of ~251 bp was performed. After demultiplexing, a clear overlap in the paired-end reads was observed. This overlap allowed paired reads to be joined together with the fastq-join program (http://code.google.com/p/ea-utils/). Only reads that had quality value (QV) scores of ≥20 for >99% of the sequence were extracted for further analysis. All sequences with ambiguous base calls were discarded (30).

Bioinformatics analysis. Metagenome@Kin software (World Fusion Co., Ltd., Tokyo, Japan) was used to conduct homology searches of the TechnoSuruga Lab Microbial Identification Database DB-BA9.0 (TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan), which contains only bacteria with standing in the taxonomic nomenclature, with the 16S rDNA sequences.

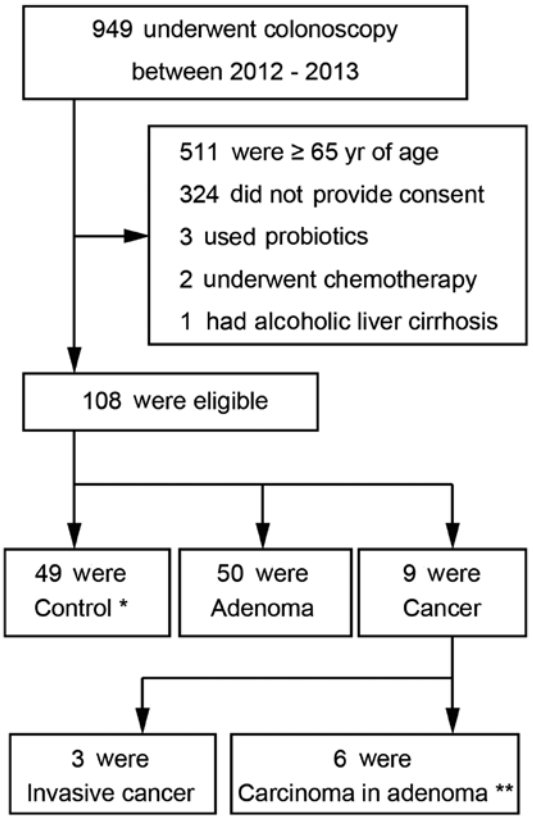

Figure 1. Flowchart showing the total number of participants enrolled and the final number of participants included in the study. Forty-nine control, 50 adenoma, and 9 cancer subjects (3/9 invasive cancer and 6/9 carcinoma in adenoma) were included in the study. *control and **used for NGS.

Statistical analysis. Data were analyzed using the Kruskal-Wallis test or the Mann-Whitney test (two-sided) for continuous variables and Fisher's exact test for categorical variables using IBM SPSS software ver. 22. P-values <0.05 were considered significant.

Results

Differences in bacterial community profiles between control, adenoma, and cancer subjects as determined by T-RFLP analysis. Demographic and clinical characteristics of the subject groups are shown in Table I. A total of 49 control subjects, 50 adenoma subjects, and 9 CRC subjects (3 with invasive cancer and 6 with carcinoma in adenoma) were enrolled in this study. Blood test results showed that total cholesterol and high-density-lipoprotein cholesterol levels were significantly lower in the cancer subjects. The average age and body mass index of the cancer subjects were each higher than those of the control and adenoma subjects. Differences in bacterial flora between the three groups are summarized in Table II. There were no significant differences in bacterial composition between each pair of groups.

Differences in bacterial communities between control and carcinoma in adenoma subjects by 16S rRNA sequencing.

Table I. Demographic and clinical characteristics of the study groups.

	Control (n=49)	Adenoma (n=50)	Cancer (n=9)[b]	P-value
Age (years)[a]	48.8±8.2	53.5±9.3	54.3±7.9	0.011
Gender, male; n (%)	21 (42.9)	28 (56)	4 (44.4)	0.399
BMI (kg/m^2)[a]	22.5±3.7	24.2±3.9	24.4±2.8	0.030
Constipation; yes, n (%)	11 (22.4)	8 (16.0)	5 (55.6)	0.042
Alcohol intake; yes, n (%)	23 (48.9)	26 (53.1)	3 (33.3)	0.646
Smoking; yes, n (%)	9 (18.8)	13 (26.5)	3 (33.3)	0.480
Laboratory data[a]				
HbA1c (JDS; %)	5.4±0.8	5.4±0.6	5.4±0.7	0.219
Total cholesterol (mg/dl)	206.2±34.7	195.2±32.1	174.6±39.5	0.021
Triglyceride (mg/dl)	113.9±85.5	128.7±80.0	135.0±104.0	0.229
HDL-cholesterol (mg/dl)	70.7±22.1	64.0±16.9	52.1±14.5	0.028

[a]Mean ± SD; [b]includes invasive cancer (n=3), carcinoma in adenoma (n=6); p-values are based on Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variable; BMI, body mass index; HDL, high-density lipoprotein.

Table II. Differences in bacterial flora based on T-RFLP analysis.

	Control	Adenoma	Cancer	P-value
Bifidobacterium	7.8±7.6	8.1±7.4	5.6±5.5	0.838
Lactobacillales	5.7±8.1	6.3±8.9	2.3±2.2	0.516
Bacteroides	40.1±12.9	37.5±15.0	39.1±7.0	0.835
Prevotella	2.5±6.8	2.7±7.2	0.6±1.1	0.637
Clostridium cluster IV	8.0±5.4	7.9±7.8	6.9±5.7	0.662
Clostridium subcluster XIVa	21.5±7.9	21.6±7.5	22.4±10.2	0.979
Clostridium cluster XI	2.0±4.0	1.4±3.0	2.9±2.5	0.144
Clostridium cluster XVIII	1.7±2.4	2.0±1.8	1.8±2.8	0.215

P-values are based on Kruskal-Wallis test; data are expressed as mean ± SD; TRFLP, terminal restriction fragment length polymorphism.

Our T-RFLP analysis showed no significant differences in bacterial population between the control, adenoma, and cancer groups. However, in order to determine the possible presence of bacteria correlated with health and cancer, we selected 12 subjects (six control and six carcinoma in adenoma) from the initial groups for NGS (Table III). Using our primer set and MiSeq platform combination, an average of 24,084 reads were obtained for each sequencing reaction. Fig. 2 shows the phylotype distribution for individual subjects in this study. The composition and relative abundance of the major bacterial phyla were similar, with *Bacteroidetes* and *Firmicutes* being the dominant phyla. However, after dividing the samples into two groups (control vs. carcinoma in adenoma) and performing statistical analyses, a significant increase in the proportion of *Fusobacteria* (control 0% vs. carcinoma in adenoma 4%) was observed in the carcinoma-in-adenoma group relative to the control group (Fig. 3). There were no between-group differences with regard to other bacteria.

Comparison of microbiomes at the genus level. Genus-level analyses identified one bacterial genus that was significantly associated with the control group (*Slackia*), and four bacterial genera were significantly associated with the carcinoma-in-adenoma group (*Actinomyces*, *Atopobium*, *Fusobacterium*, and *Heamophilus*) (Table IV).

Comparison of microbiomes at the species level. Species-level analyses identified one bacterial species (*Eubacterium coprostanoligens*) that was significantly associated with the control group and eight bacterial species (*Actinomyces odontolyticus*, *Bacteroides fragilis*, *Clostridium nexile*, *Fusobacterium varium*, *Heamophilus parainfluenzae*, *Prevotella stercorea*, *Streptococcus gordonii*, and *Veillonella dispar*) that were significantly associated with the carcinoma-in-adenoma group (Table V).

Most notably, the proportions of *Actinomyces odontolyticus*, *Bacteriodes fragilis*, and *Heamophilus parainfluenzae* were significantly higher in feces from carcinoma-in-adenoma subjects than in those from control subjects; in fact, these bacteria were barely detectable in feces from control subjects (Fig. 4A-C). In contrast, the proportions of *Eubacterium coprostanoligens* were significantly higher in feces from control subjects than in those from carcinoma-in-adenoma subjects; this bacteria was barely detectable in feces of carcinoma-in-adenoma subjects (Fig. 4D).

Although the genus *Slackia* was significantly associated with control subjects (Fig. 5A), there were no statistically significant between-group differences in the relative proportion of each individual *Slackia* species. *Slackia* species as a whole, however, were more abundant in feces from control subjects compared with that from carcinoma-in-adenoma subjects (Fig. 5B).

Table III. Characteristics of the study subjects participating in next-generation sequencing analysis.

Participant ID	Health status	Gender	Age (years)	BMI	Tumor size (mm)	Tumor location
N1	Healthy	M	46	25.1		
N2	Healthy	M	39	19.6		
N3	Healthy	F	55	24.7		
N4	Healthy	F	49	20.3		
N5	Healthy	M	56	25.63		
N6	Healthy	M	57	23.15		
C1	Cancer	M	48	24.5	10	Sigmoid
C2	Cancer	F	64	26.9	20	Sigmoid
C3	Cancer	M	40	20.5	13	Sigmoid
C4	Cancer	M	62	24.9	20	Sigmoid
C5	Cancer	M	61	24.3	15	Sigmoid
C6	Cancer	F	49	19.46	10	Descending

BMI, body mass index; F, female; M, male.

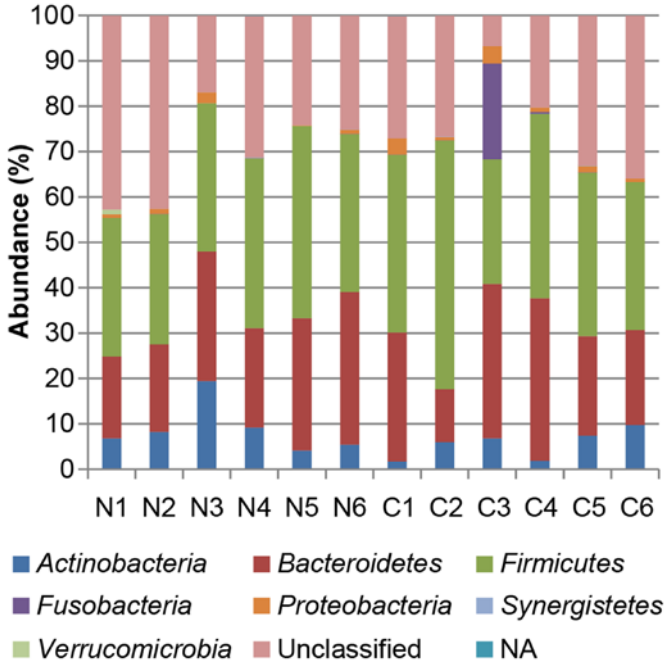

Figure 2. Phylum-level classification of bacteria identified in individual stool samples. N-numbered samples were obtained from normal control subjects, whereas C-numbered samples were obtained from carcinoma-in-adenoma subjects. Each bar represents the percent contribution of phylum-level profiles grouped by N-C status or for each individual. The phyla represented by each color code are shown below the figure. NA, not assigned.

Discussion

Using NGS, we found that the gut microbiota differs between control and carcinoma-in-adenoma subjects; however, our initial T-RFLP analysis did not reveal any statistically significant differences in relative proportions of bacterial flora between control, adenoma, and carcinoma-in-adenoma subjects. We identified several potential gut microbial members significantly associated with the control and carcinoma-in-adenoma groups.

Table IV. Bacterial genera with significantly different group-specific representation.

	Ave. N (%)	Ave. C (%)	P-value[a]
Actinomyces	0.022	0.116	0.037
Atopobium	ND	0.005	0.022
Fusobacterium	0.004	3.84	0.004
Heamophilus	0.002	0.027	0.020
Slackia	0.162	0.009	0.049

[a]P-values are based on Mann-Whitney U test; N, normal control subjects; C, carcinoma-in-adenoma patients; ND, not determined.

Phylum-level analyses revealed that the relative proportion of *Fusobacterium* was significantly higher in carcinoma-in-adenoma subjects than in control subjects. *Fusobacterium* has been studied recently because of its correlations with CRC (33,34). There are two studies (35,36) that investigated the mechanisms by which *Fusobacterium nucleatum* in the gut could be associated with CRC. The first study was conducted by Kostic *et al*; it suggested that *F. nucleatum* induced a nuclear factor-κB (NF-κB)-driven proinflammatory response to promote CRC (35). The second study was by Rubinstein *et al*; it provided mechanistic insights, most notably that the actions of *Fusobacterium* spp. were presumably mediated via binding of FadA, a virulence factor expressed on the bacterial cell surface, to receptors on host epithelial cells; this FadA-receptor binding seemed to modify barrier function, increase inflammation through the modulation of the tumor microenvironment, and activate pro-oncogenic signals to promote CRC (36).

Genus-level and species-level analyses showed that the genus *Slackia* and the species *Eubacterium coprostanoligens* were present in significantly higher proportions in control subjects compared with carcinoma-in-adenoma subjects. *Slackia* is one of the few characterized equol-forming gut

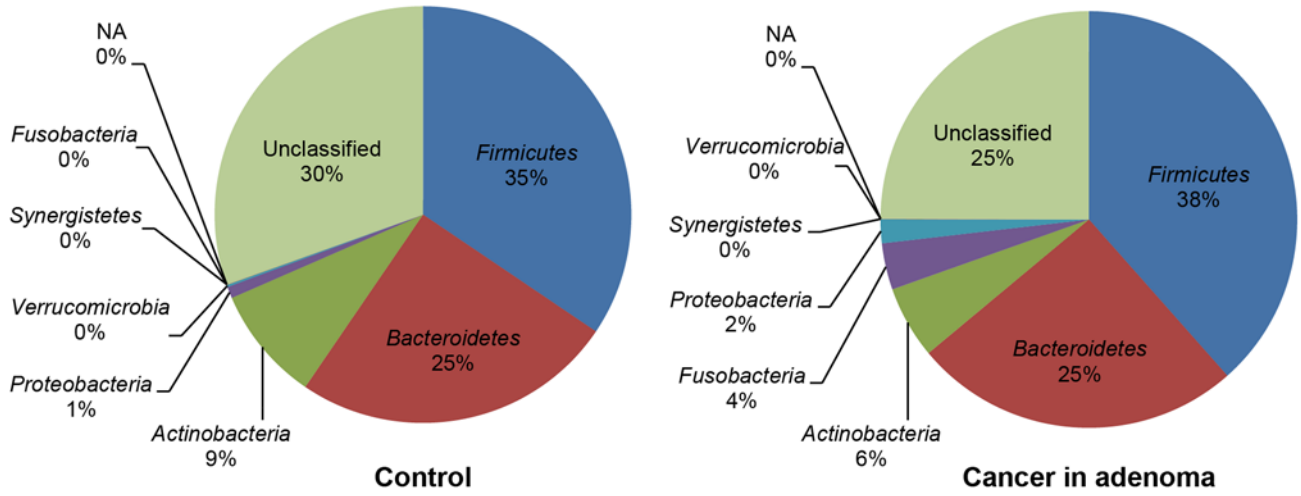

Figure 3. Average phylum distribution of gut microbiomes of control and carcinoma in adenoma subjects. NA, not assigned. *P<0.05.

Table V. Bacterial species with significantly different group-specific representation.

	Ave. N (%)	Ave. C (%)	P-value[a]
Actinomyces odontolyticus	ND	0.036	0.007
Bacteroides fragilis	0.015	0.658	0.0046
Clostridium nexile	0.067	0.661	0.036
Eubacterium coprostanoligens	0.650	ND	0.022
Fusobacterium varium	ND	0.268	0.022
Heamophilus parainfluenzae	0.001	0.022	0.020
Prevotella stercorea	ND	1.186	0.022
Streptococcus gordonii	0.002	0.031	0.014
Veillonella dispar	0.004	0.198	0.042

[a]P-values are based on Mann-Whitney U test; N, normal control subject; C, carcinoma-in-adenoma patient; ND, not determined.

bacteria isolated from humans (37). Equol is produced from daidzein (a soy isoflavone) by intestinal bacteria in some, but not all, adults (38). An individual's capacity for equol production depends on the representation of equol-forming bacteria in the individual's intestine (39). Equol is produced in only 20-30% of adults who consume soy diets containing isoflavones in Western countries; in contrast, it is produced in no less than 50-60% of adults in Asian countries, and these adults more commonly consume soy diets (40-42). Soy isoflavones are often referred to as phytoestrogens; they have an estrogen-like chemical structure and can bind to estrogen receptors (43). Equol is the active form of soy isoflavones in the human intestine, and equol shows a stronger estrogen-like activity than daizein because it affects hormone-dependent diseases (44). Equol is anticipated to have a protective effect on prostate cancer development and to reduce the risk of mammary tumors (45,46). We expect that the equol-forming bacteria *Slackia* have a preventive effect against CRC, as well.

Eubacterium, a beneficial genus of fecal bacteria, includes many species that produce butyrate (47,48). Butyrate is regarded as the most important nutrient for epithelial cells of the colon, and it plays an essential role in the energy metabolism and normal development of these cells (49). Several studies have shown that butyrate is a beneficial inhibitor of colon carcinoma cell proliferation because it induces apoptosis in human colon carcinoma cells (50-52). *Eubacterium coprostanoligens* is a cholesterol-reducing bacterium (53). Cholesterol-reducing bacteria convert cholesterol to coprostanol, which is not absorbed by the human gastrointestinal system, thereby leading to reduced cholesterol levels. There is strong epidemiological evidence that links high fat consumption to increased risk of CRC (54,55). We thus expect *Eubacterium coprostanoligens* to be another prospective inhibitor of CRC.

Genus-level analyses showed that four genera (*Actinomyces*, *Atopobium*, *Fusobacterium*, and *Haemophilus*) were present in significantly higher proportions in carcinoma-in-adenoma subjects than in control subjects. Species-level analyses showed that eight species (*Actinomyces odontolyticus*, *Bacteroides fragilis*, *Clostridium nexile*, *Fusobacterium varium*, *Haemophilus parainfluenzae*, *Prevotella stercorea*, *Streptococcus gordonii*, and *Veillonella dispar*) were present in significantly higher proportions in carcinoma-in-adenoma subjects than in control subjects. Here we focused on only three of these species (*Actinomyces odontolyticus*, *Bacteroides fragilis*, *Haemophilus parainfluenzae* sp); each was highly represented in carcinoma-in-adenoma subjects, but barely detected in control subjects.

Actinomyces odontolyticus are often present in the oral cavity and gastrointestinal tract of healthy humans. Some *Actinomyces* spp. are known to be opportunist pathogens associated with several colon-related diseases such as CRC and Crohn's disease. *Bacteroides fragilis* is a Gram-negative obligate anaerobe persistently preset in the colon of nearly all humans. It accounts for only 0.5% of the human gut microbiota; nevertheless, it has enterotoxigenicity and is considered to be pathogen important to CRC. Chronic inflammation may lead to the hypermethylation of DNA and drive cells to malignancy (56). Persistent enterotoxigenic *Bacteroides*

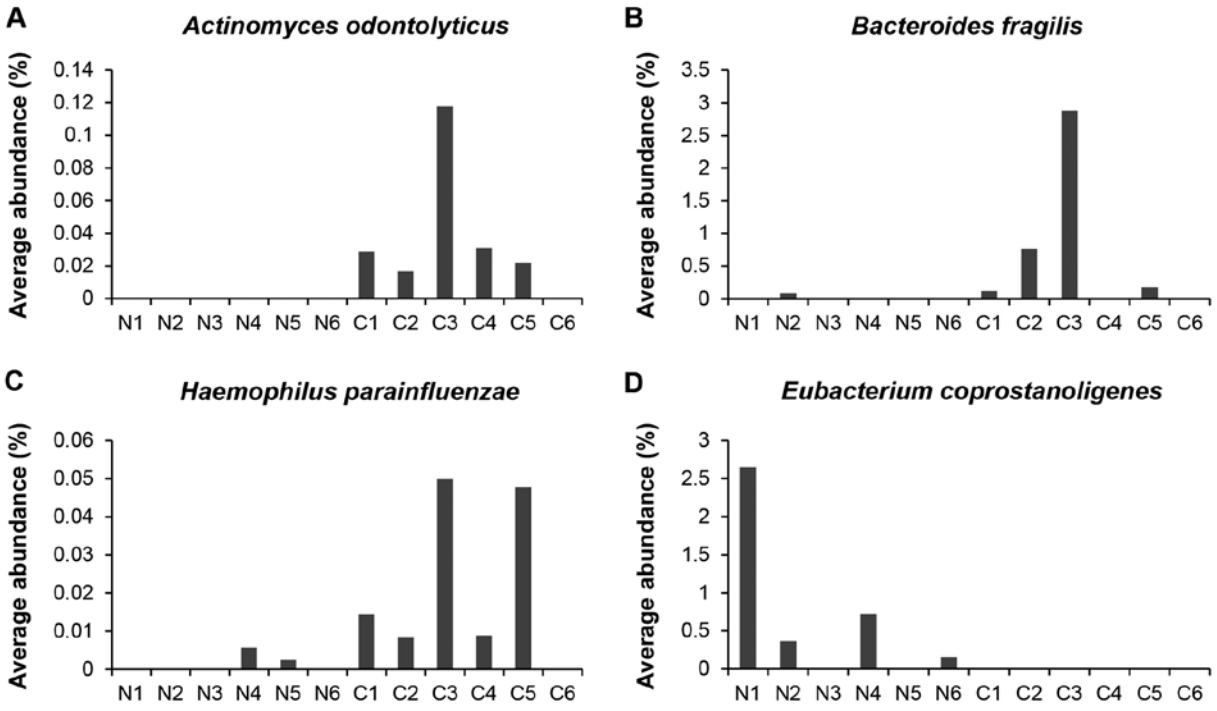

Figure 4. (A) Average abundance of *Actinomyces odontolyticus* in individual stool samples. N-numbered samples were obtained from normal control subjects and C-numbered samples were obtained from carcinoma-in-adenoma subjects. (B) Average abundance of *Bacteroides fragilis* in individual stool samples. (C) Average abundance of *Haemophilus parainfluenzae* in individual stool samples. (D) Average abundance of *Eubacterium coprostanoligenes* in individual stool samples.

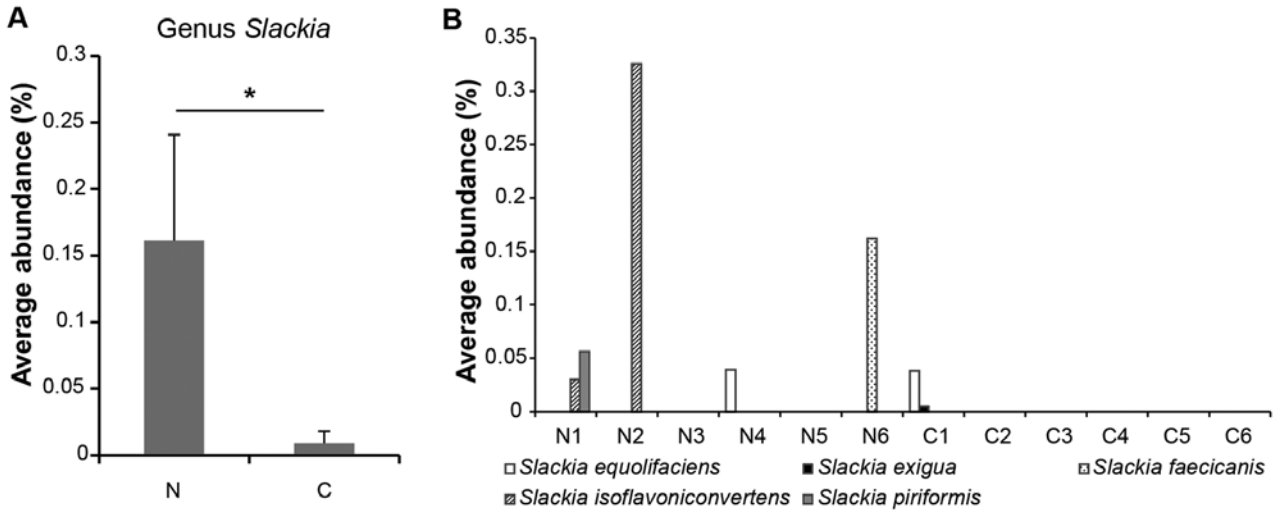

Figure 5. (A) Average abundance of the genus *Slackia* in individual stool samples. N-numbered samples were obtained from normal control subjects and C-numbered samples were obtained from carcinoma-in-adenoma subjects. *P<0.05. (B) Average abundance of *Slackia* species in individual stool samples.

fragilis (ETBF) infection may increase the risk of colon carcinogenesis (57). *Haemophilus parainfluenzae* is a commensal species, which belongs to the phylum *Proteobacteria*. It is an opportunistic pathogen that may induce invasive infections such as pneumonia and endocarditis.

The control subjects in our study harbored beneficial bacterial species, whereas the carcinoma-in-adenoma subjects harbored harmful bacteria species that could act as opportunist pathogens and/or inflammation drivers. In the case of progressive CRC, the colonic environment can be modified by multiple factors such as epithelial cell apoptosis and cancer cachexia; therefore, it is often complicated whether the current microbial environment is a sequel of CRC or the gut microbiota is a driver of CRC development by way of inflammatory responses. However, the fact that the gut microbial profiles differed significantly even between control subjects and carcinoma-in-adenoma (i.e., relatively-early-stage cancer) subjects on the genus and species levels suggested that the microbial environment including the gut microbiota was an important etiologic factor for

CRC. In order to determine the exact triggers of CRC, we should also carefully observe non-CRC patients harboring inflammation-driving microbes in a future long-term study of CRC progression.

We acknowledge this research was limited to the characterization of microbiota and that stool metabolites were not analyzed. We believe further related research that includes analysis of stool metabolites will definitely improve our understanding of the mechanisms that lead to CRC.

In conclusion, the results of the present study in a Japanese population showed that gut microbiota differed between control and carcinoma-in-adenoma subjects. In particular, the results suggested that the gut microbiota served as a driver of carcinogenesis because changes in the composition of the gut microbiota were observed even in carcinoma in adenoma, which is an early-stage cancer. However, further study will be necessary to clarify the precise mechanisms by which the gut microbiota drives carcinogenesis and to identify the cancer-associated microbial members. An improved understanding of mechanisms that cause gut microbiota metabolites to interface with carcinogenesis should lead to improved diagnostic, preventative, and therapeutic approaches; for example, probiotics may become useful as more natural and less disruptive treatments for the prevention of CRC and/or other GI-related disorders.

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