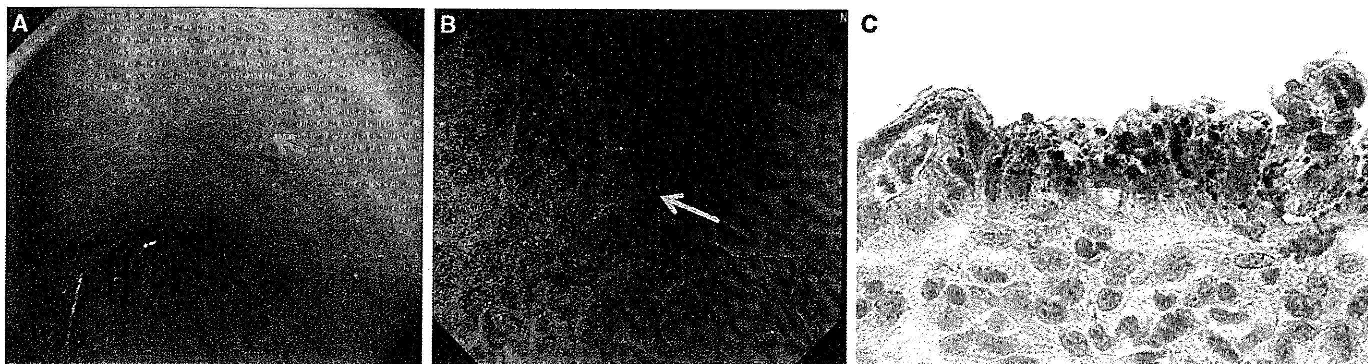

Fig. 3. (a) Standard endoscopic findings with white light of high-grade neoplasia of 0-IIc type. Slightly depressed reddened lesion (arrow) can be seen at the posterior wall of the gastric antrum. (b) Magnifying endoscopy (ME) with narrow-band imaging (NBI) findings. There is a clear demarcation line (arrow) between the background mucosa and the neoplastic lesion. Brownish subepithelial capillaries can be clearly visualized in the background mucosa. In contrast, as a fine speckled WOS is present in the neoplasia within the demarcation line, the subepithelial microvascular architecture cannot be visualized clearly. (c) Histopathological findings of the biopsied specimen from the neoplasia (oil red O staining, 200×). By oil red O staining, numerous round or oval amorphous LD are demonstrated within the neoplastic epithelium alone.

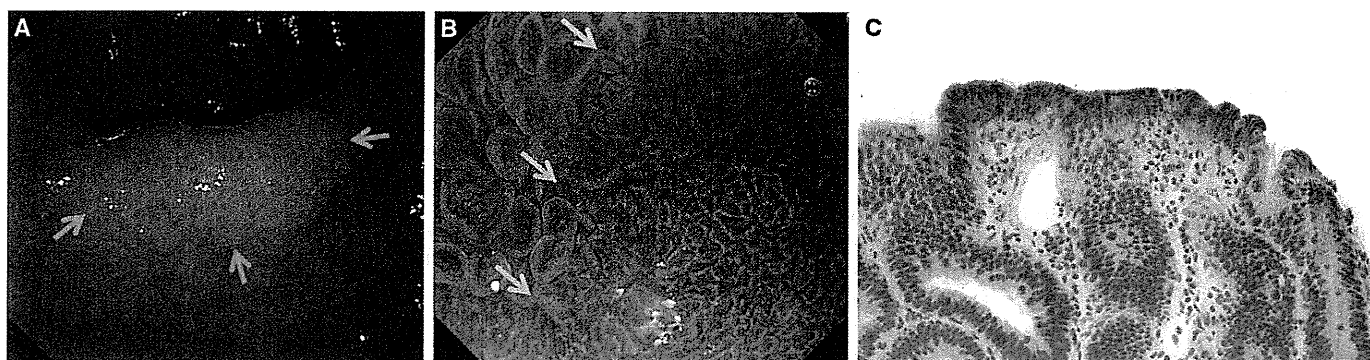

Fig. 4. (a) Standard endoscopic findings with white light of high-grade neoplasia of 0 IIa type. Slightly elevated pale lesion (arrows) is present at the lesser curvature of the gastric angulus. (b) Magnifying endoscopy (ME) with narrow-band imaging (NBI) findings. At the margin of the lesion, there is a clear demarcation line (arrows) between the background mucosa and the lesion. In the background mucosa, brownish subepithelial capillaries are clearly visualized by ME with NBI, but as the WOS in reticular morphology was present in the neoplasia within the demarcation line, the subepithelial microvascular pattern of the neoplasia is totally obscured. (c) Histopathological findings of the biopsied specimen from the neoplasia (oil red O staining, 50×). The accumulated LD can be detected only in the epithelium of the relatively elevated apical part between the crypts; they are absent in the cryptal part of the epithelium.

anatomical structure of the lamina propria in the stomach which is different from that of the small intestine. In the case of chylomicron retention disease, the accumulation of LD is limited to the epithelium. However, in our study, 61.5% of the lipid droplet-positive neoplasias showed both intraepithelial and subepithelial accumulation of LD. Therefore, it is speculated that the accumulated LD had been transported to the subepithelial part after forming chylomicron in the neoplastic cells with the intestinal phenotype. Nevertheless, as the mucosal lymphatics in the stomach are anatomically present only in the deepest level of the lamina propria,[29] LD cannot be easily transported into the lymphatics. Consequently, they may be retained within the superficial part of the mucosa for a longer period.

Although the presence of the WOS was dependent upon the presence of LD, we encountered two exceptions. In one case, WOS was detected by endoscopic observation using ME with NBI, but LD were not present on histological examination. To determine the reason, we reviewed the endoscopic findings using ME with NBI of that case, finding the density of the WOS was sparse and the distribution patchy. Accordingly, we speculated that a very thin section of the biopsy specimen failed to contain LD. The other case showed that although WOS was not identified using ME with NBI, LD were detected in the histological specimens. When we reviewed the histological findings carefully, we noticed that the size and the density of the LD were very small and remarkably low, respectively, compared with those in other LD-positive cases. Accordingly, we speculated that small low-density LD in the cells may not cause strong backward scattering of the light. However, we need to conduct further studies to clarify whether visualization of WOS depends upon the size and density of LD.

In conclusion, we have reported a novel and unique bio-optical finding, that is, the nature of WOS is the visualization

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Table 2. Histological distribution of LD ($n = 26$)

Distribution	No. lesions	
Intraepithelial	10	38.5%
Intraepithelial + subepithelial	16	61.5%
Subepithelial	0	0%

LD, lipid droplets.

Table 3. Phenotypic characterization according to the presence of the WOS

		WOS			
		Positive ($n = 26$)		Negative ($n = 23$)	
Phenotype	G	0	(0%)	11	(47.8%)
	GI	13	(50%)	4	(17.4%)
	I	13	(50%)	8	(34.8%)

G, gastric phenotype; GI, gastrointestinal phenotype; I, intestinal phenotype; WOS, white opaque substance.

of LD accumulated in the superficial part of epithelial neoplasias within the stomach.

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ORIGINAL ARTICLE

Reduced Diversity and Imbalance of Fecal Microbiota in Patients with Ulcerative Colitis

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Abstract

Background Clinical observations and experimental colitis models have indicated the importance of intestinal bacteria in the etiology of ulcerative colitis (UC), but a causative bacterial agent has not been identified.

Aim To determine how intestinal bacteria are associated with UC, fecal microbiota and other components were compared for UC patients and healthy adults.

Methods Fresh feces were collected from 48 UC patients. Fecal microbiota were analyzed by use of terminal-restriction fragment length polymorphism (T-RFLP), real-time PCR, and culture. The concentrations of organic acids, indole, and ammonia, and pH and moisture, which are indicators of the intestinal environment, were measured and compared with healthy control data.

Results T-RFLP data divided the UC patients into four clusters; one cluster was obtained for healthy subjects. The diversity of fecal microbiota was significantly lower in UC patients. There were significantly fewer *Bacteroides* and *Clostridium* subcluster XIVab, and the amount of *Enterococcus* was higher in UC patients than in healthy subjects. The fecal concentration of organic acids was significantly lower in UC patients who were in remission.

Conclusion UC patients have imbalances in the intestinal environment—less diversity of fecal microbiota, lower levels of major anaerobic bacteria (*Bacteroides* and *Clostridium* subcluster XIVab), and a lower concentration of organic acids.

Keywords Ulcerative colitis · Fecal microbiota · Diversity · *Bacteroides* · Organic acids

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Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease that has been increasing in Japan [1]. It often occurs in the prime of life (early 30 s) and is an intractable lifelong disease. Controlling the symptoms with drugs is a heavy burden for patients physically, mentally, and socially [1]. An understanding of the pathogenic mechanism of UC is needed to lengthen remission periods and improve quality of life. Although the exact etiology is unknown, multiple causes, for example immune system disorders [2, 3], intestinal bacteria [4–6], genetics [7, 8], and environmental factors, for example lifestyle and stress, have been implicated [9].

Because inflammation is limited to the large intestine in UC, intestinal microbiota and its metabolites might be important in the etiology for UC. Interleukin (IL)-10 knockout (KO) mice and IL-2 KO mice spontaneously develop UC-like inflammation in a conventional environment, but not under germ-free conditions [2, 3, 10, 11], indicating the importance of intestinal bacteria in addition to dysfunction of the immune system. However, the involvement of intestinal bacteria in the development of UC has not been clarified. Some intestinal bacteria have been suggested as candidate agents. *Bacteroides vulgatus* has been found in biopsy samples of UC patients, and antibodies against this bacterium have been detected in the serum of UC patients [12]. More sulfate-reducing bacteria (SRB) reside in UC patients, producing cytotoxic hydrogen sulfide, which has been implicated in UC [13, 14]. Ohkusa et al. [15] detected *Fusobacterium varium* in the colonic mucosa of UC patients and reported that antibiotics against *F. varium* ameliorated disease symptoms. These bacteria also reside in healthy adults, however, and no bacterial species has been identified as UC-specific.

Most human microbiota belongs to four bacterial divisions: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* [16–18]. *Bacteroides*, *Eubacterium*, and *Ruminococcus* are the dominant genera in the adult intestine [18]. The effects of these dominant bacteria and their metabolites on the intestinal mucosa are not negligible. Intestinal microbiota metabolizes undigested carbohydrates into organic acids, for example acetic and butyric acids, in the lower intestinal tract. These acids reduce intestinal pH and suppress over-growth of pathogenic bacteria and the production of putrefactive components, for example indole and ammonia. Butyrate is an energy source for the intestinal mucosa and promotes growth and differentiation of colonocytes [19–23]. Intestinal organic acids, especially butyric acid, have immunomodulatory effects on colonic inflammation and a reinforcing effect on the intestinal defense barrier [19, 20, 24].

Microbiota colonization affects the development of the intestinal immune system, and proper immune function is regulated by the interaction between host and symbiotic bacteria [25, 26]. Normal intestinal microbiota is associated with both pro and anti-inflammation responses [25]. Fecal microbiota, innate and adaptive immune responses in the host, and the intestinal environment, including bacterial metabolites, are closely linked to gut health, and an imbalance in these factors has been implicated in intestinal disorders. Here, we investigated differences in fecal microbiota and organic acids between UC patients and healthy adults to clarify how intestinal microbiota is involved in the development of ulcerative colitis.

Materials and Methods

Reagents and Bacterial Strains

Blood liver agar (BL), Rogosa SL agar, Trypticase soy agar (TS), and desoxycholate hydrogen sulfide lactose agar (DHL) were obtained from Nissui Pharmaceuticals (Tokyo, Japan). *Bacteroides fragilis* JCM11019, *Bifidobacterium longum* JCM1217, *Blautia coccoides* JCM1395, *Clostridium perfringens* JCM1290, *Desulfovibrio alkalitolerans* JCM12612, *Enterococcus faecalis* JCM5803, *Fusobacterium varium* JCM3722, and *Methanobrevibactor arboriphilus* JCM13429 were obtained from the Japan Collection of Microorganisms, Riken Bioresource Center (Tsukuba, Japan). *Clostridium difficile* ATCC9689 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Chemicals, unless otherwise stated, were reagent-grade or higher and obtained from Sigma Chemical (St Louis, MO, USA) or Wako Pure Chemical Industries (Osaka, Japan).

Subjects and Study Design

Forty-eight UC outpatients at the Kenporen Osaka Central Hospital, Osaka, Japan, gave written consent to take part in this study. Eligible patients were aged 22–69 and had been diagnosed with UC by colonoscopy and histology (Table 1). Patients were categorized on the basis of their clinical activity index scores (CAI): remission, 0–4; mildly active, 5–7; moderately active, 8–11 [27]. Patients with fulminant UC, unable to take food orally, or treated with steroids or immunosuppressants were excluded from this study. None of the subjects was treated with antibiotics (e.g., metronidazole and ciprofloxacin) during this study. Most patients received oral or intrarectal treatment with 5-ASA compounds, for example Salazopyrin or Pentasa (Table S1). Nineteen patients were taking MiyaBM (viable *Clostridium butyricum* strain miyairi, 300 mg/6 tablets), and four patients were taking, daily, fermented milk supplemented with *Bifidobacterium breve* or a supplement containing *Bifidobacterium* (Table S1). All patients were

Table 1 Profile of subjects

	UC patients	Healthy adults
Number of subjects	48	36
Male/Female	26/22*	14/22
Age	22–69	22–67
Disease activity		
Remission (CAI 0–4)	35	
Active	13	
Mild (CAI 5–7)	5	
Moderate (CAI 8–11)	8*	
Medication		
Corticosteroids	1**	
5-ASA	40	
Probiotic (MiyaBM$^{(R)}$)	19	

CAI, clinical activity index; 5-ASA, 5-aminosalicylic acid

* One female patient with moderately active UC (UC68) was receiving diet-related folk remedies

** Only external application for hemorrhoids

omnivorous and were not on a restricted diet with one exception—one female patient (UC68) was excluded from the comparison of amount of bacteria and fecal concentrations of metabolites because she had received diet-related folk remedies (Table 1). The patients self-reported their bowel habits by using the Inflammatory Bowel Disease Questionnaire (IBDQ) with a visual analog scale (VAS). The average frequency of evacuation and time and date of the occurrence of diarrhea/loose stool was individually entered as the distance on the VAS (Table S2). The data from 36 healthy volunteers used as controls were obtained from Nemoto et al. [28]. Healthy volunteers were recruited at Tokushima University, Tokushima, Japan. All of the control subjects were omnivorous and were not taking antibiotics. All subjects were asked to provide a fresh stool sample. Patients and healthy volunteers collected samples at home and immediately placed them in an AnaeroPouch-Anaero (Mitsubishi Gas Chemical Company, Tokyo, Japan), where they were kept anaerobic and cold with refrigerants until received by the laboratory. The protocol was approved by the Ethics Committee of Kyoto Prefectural University and Osaka Chuo Hospital and the Ethics Committee of Tokushima University Hospital.

Fecal Microbiota Analysis: Culture

Bacteriological analysis was performed by use of the procedure of Mitsuoka et al. [29] with slight modification as described by Nemoto et al. [28]. Results were calculated as $\log_{10}$ of colony forming units (CFU) per gram (wet weight) of the initial material. Total numbers of fecal bacteria were counted after Gram staining.

Fecal Microbiota Analysis: Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

Fecal and bacterial DNA was extracted for T-RFLP analysis of fecal microbiota [28, 30, 31]. Briefly, 16S ribosomal DNA was amplified by use of universal PCR primers (Table 2) and digested with *Hha*I or *Msp*I (Takara). The

Table 2 Primers used in this study

Assay	Primer name	Sequence	Ref.
T-RFLP	27F-FAM	6FAM-AGAGTTTGATCCTGGCTCAG	[31]
	1492R	GGTTACCTTGTTACGACTT	
Bifidobacteria	Bif164F	CATCCGGCATTACCACCC	[32]
	Bif662R	CCACCGTTACACCGGGAA	
Sulfate-reducing bacteria	Des-f	CCGTAGATATCTGGAGGAACATCAG	[33]
	Des-r	ACATCTAGCATCCATCGTTTACAGC	
Enterococci	Enc-F	CCCTTATTGTTAGTTGCCATCATT	[34]
	Enc-R	ACTCGTTGTACTTCCCATTGT	
Clostridium subcluster I	CI-F1	TACCHRAGGAGGAAGCCAC	[35]
	CI-R2	GTTCTTCCTAATCTCTACGCAT	
Clostridium subcluster XI	CXI-F1	ACGCTACTTGAGGAGGA	
	CXI-R2	GAGCCGTAGCCTTTCACT	
Clostridium subcluster XIVab	CXIV-F1	GAWGAAGTATYTCGGTATGT	
	CXIV-R2	CTACGCWCCCTTTACAC	
Clostridium common probe	Clostridium probe	6FAM-GTGCCAGCAGCCGCGGTAATACG-TAMRA	
Bacteroides	AllBac296F	GAGAGGAAGGTCCCCAC	[36]
	AllBac412R	CGCTACTTGGCTGGTTCAG	
	AllBac375Probe	6FAM-CCATTGACCAATATTCCTCACTGCTGCCT-TAMRA	

length of the terminal-restriction fragments (T-RFs) was determined by use of Genetic analyzer and GeneScan Analysis Software. T-RF patterns were analyzed by use of BioNumerics ver.5.01 software (Applied Maths, Sint-Martens-Latem, Belgium) to construct dendrograms. Distances between samples were represented graphically by constructing a dendrogram based on Dice coefficients of the T-RFLP profiles. The Ward method was used to establish dendrogram type. Microbiota Profiler software (Infocom, Tokyo, Japan) was used to estimate the intestinal bacteria corresponding to T-RFs in UC patients and healthy subjects.

Diversity of Intestinal Microbiota

Diversity of intestinal microbiota was evaluated as the number of operational taxonomic units (OTUs) derived from T-RFLP analysis. Because 16S rDNA is highly and phylogenetically conserved, digested rDNA would yield genus and/or species-specific sizes of T-RFs because of different 16S ribosomal DNA sequences. OTUs that represent each length of T-RF might reflect single and/or phylogenetically-related bacteria. Therefore, each OTU can indicate one or more organisms, including uncultured, unnamed bacteria. Peaks over 5 % of maximum peak height of the sample were used as OTUs. Diversity was represented as the sum of OTUs derived from *Hha*I and *Msp*I digests.

Quantitative Analysis of Fecal Microbiota

As T-RFLP analysis is a semi-quantitative method, real time PCR was used to quantify fecal bacteria [28]. The primers and probes are shown in Table 2. SYBR Premix Ex Taq II (Takara) and Premix Ex Taq (Takara) were used with the ABI Prism 7000 and ABI Prism 7500 according to the manufacturer's instructions. The amplification program was obtained from the references in Table 2 [32–36]. The 16S ribosomal RNA gene of each bacterial strain listed in the section "Reagents and bacterial strains" was cloned into the pCR2-TOPO vector (Invitrogen, Tokyo, Japan) or the pGEM T-Easy vector (Promega, Tokyo, Japan) according to the manufacturer's procedure for use as a copy number standard. The results of real-time PCR for each sample were expressed as the copy number of bacterial 16S ribosomal DNA per gram feces (wet weight). The detection limits were: *Clostridium* I/XI/XIVab, 10^4 copies/10 ng; all other genera, 100 copies/10 ng fecal DNA. Gene recombination experiments for the preparation of copy number standards were approved by the University of Tokushima.

Fecal pH and Water Content

Stools (0.1–0.2 g) were homogenized with 1 ml water. Fecal pH was measured with a pH meter (Sartorius B021610 007). Water content was calculated as the weight difference before and after freeze-drying a portion of the fecal material (EYELA FDU-1000).

Fig. 1 Cluster analysis of fecal microbiota in healthy adults and patients with UC. T-RFLP patterns derived from *Hha*I and *Msp*I digests were analyzed by use of the Dice coefficient and the Ward algorithm. The dendrogram comprised four clusters for UC patients and one cluster for healthy adults. The clusters of UC were generated independently by disease types and state. *left* left-sided colitis, *RS* right-sided or segmental colitis

Measurement of Fecal Concentrations of Organic Acids, Indole, and Ammonia

Bacterial metabolites in the feces of UC patients were quantified as described elsewhere [28]. Portions (approximately 0.2 g) of homogenized stool were suspended in 1 ml water, centrifuged at 4 °C at 10 krpm for 10 min, and stored at −80 °C. Organic acids in the fecal extracts were extracted and measured by gas chromatography (C-7AG; Shimadzu, Kyoto, Japan) with external standards.

Fecal levels of indole were measured as described by Shioiri et al. [37]. Briefly, stools (approximately 0.1 g) were homogenized with nine volumes of 0.1 mM phosphate buffer (PB) and centrifuged. The supernatant was appropriately diluted with PB, and 0.2 ml diluted sample was mixed with 1 ml coloring solution (*P*-dimethylaminobenzaldehyde in a sulfuric acid–alcohol mixture) and kept at room temperature for 20 min. The absorbance at 570 nm was measured by use of a UV–visible spectrophotometer (Shimadzu UV-1200). As a control, 0–0.3 mM indole solution was prepared just before use. Ammonia concentration was measured by use of the Wako ammonia test.

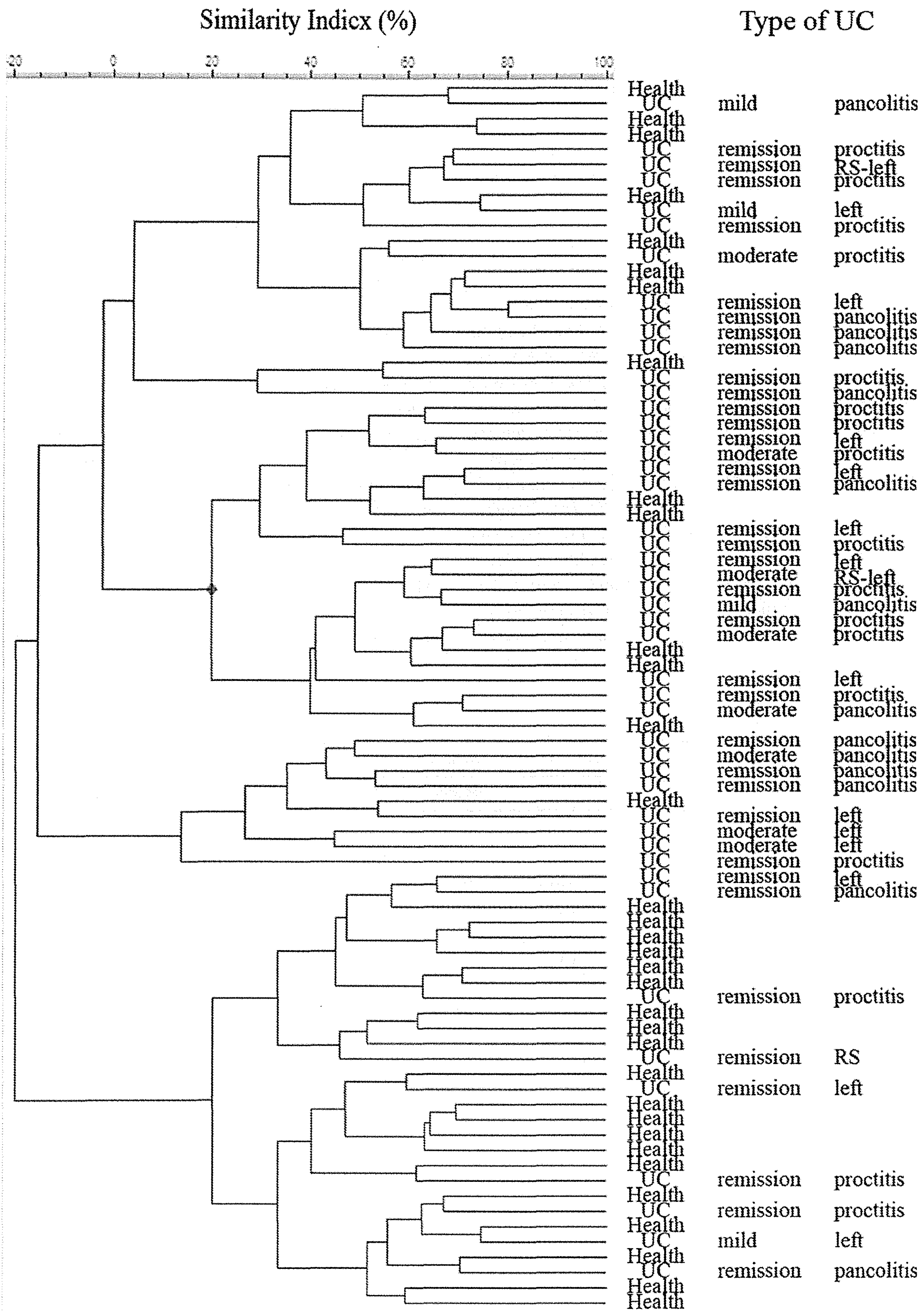
Statistical Analysis

The Mann–Whitney *U* test and the Spearman correlation coefficient by rank test were performed by using Microsoft Excel 2007 and Statcel2 add-in software (OMS publishing, Tokorozawa, Japan). A probability of less than 0.05 indicated statistical significance.

Results

T-RFLP Analysis and the Diversity of Fecal Microbiota

After T-RFLP analysis of 16S rDNA (using *Hha*I or *Msp*I digestion), a dendrogram was constructed by Dice correlation and use of the Ward algorithm. Four clusters of patients with UC and one of healthy subjects were roughly and weakly generated (Fig. 1). Clusters were not tied to inflammatory activity or location of UC. The number of


Similarity Indicx (%)
Type of UC
-20
0
20
40
60
80
100
Health
UC mild pancolitis
Health
Health
UC remission proctitis
UC remission RS-left
UC remission proctitis
Health
UC mild left
UC remission proctitis
Health
UC moderate proctitis
Health
Health
UC remission left
UC remission pancolitis
UC remission pancolitis
UC remission pancolitis
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OTUs from both the *Hha*I and *Msp*I digestions, which denote the diversity of the microbiota, was compared between the UC and healthy subjects. The diversity of fecal microbiota was lower in UC patients in remission (mean OTU = 41.0, interquartile range (IQR): 35.5–49.0, $P < 0.001$) and with active UC (mean OTU = 44.5, IQR: 38.0–46.5, $P = 0.029$) than in healthy subjects (mean OTU = 50.5, IQR: 44.3–60.5).

Analysis of Fecal Microbiota by Real-Time PCR

The copy number of 16S rDNA in sulfate-reducing bacteria (SRB, represented by *Desulfovibrio*), bifidobacteria, enterococci, *Bacteroides*, and clostridia in feces was investigated by use of real-time PCR (Table 3). The fecal moisture level did not differ significantly between UC and healthy subjects (Table 4). Therefore, wet feces were used as starting materials in subsequent experiments. The copy number of *Bacteroides* was lower in UC patients and was less than 10 % of that in healthy subjects. *Clostridium* subcluster I (represented by *C. perfringens*) and XI (*C. difficile*) were not significantly different between UC and healthy subjects. However, the *Clostridium* subcluster XIVab (represented by *B. coccoides*) was significantly smaller in UC patients, as was *Bacteroides*. The enterococci population was significantly larger in UC patients in remission than in healthy subjects. Fewer SRB were found in patients in remission than in controls, although some samples in both groups were below the detection limit. We also investigated methane-producing archea (methanogens) and *Fusobacterium*, but they were rarely detected in any of the subjects (data not shown).

Culture-Based Analysis of Fecal Microbiota

Total and viable cell numbers of fecal bacteria are shown in Table 3. The total number of fecal bacteria, which was counted after Gram-staining, was significantly smaller in patients with active UC than in healthy controls. The number in those in remission was also small, but not significant. Viable cell numbers of bifidobacteria, anaerobic G(−)R, anaerobic G(+)R, anaerobic cocci, and *Enterobacteriaceae* in feces were no different between UC and healthy subjects. Although the number of viable aerobic G(+)C was not significantly different between UC patients in remission and healthy subjects, it was significantly larger in active UC patients than in healthy controls.

Comparison of Fecal Organic Acids, Ammonia, Indole, Moisture, and pH

The concentration of organic acids in feces is shown in Table 4. The concentrations of acetic, propionic, butyric,

Table 3 Analysis of fecal bacteria

	Healthy subjects (n = 36)		Remission of UC (n = 35)			Active UC (n = 12)		
	Median	IQR	Median	IQR	P value	Median	IQR	P value
Total bacteria	11.3	11.2–11.6	11.3	11.2–11.4	0.064	11.2	11.1–11.3	0.018
Culture methods								
Bifidobacteria	9.7	9.4–10.0	9.8	9.5–10.0	0.808	9.9	9.6–10.0	0.885
Anaerobic G(−)R	10.2	9.8–10.4	9.9	9.6–10.2	0.078	9.8	9.6–10.1	0.135
Anaerobic G(+)R	9.8	9.5–10.0	9.8	9.3–10.1	0.836	9.8	9.3–10.1	0.900
Anaerobic cocci	9.2	8.6–9.3	9.2	8.8–9.7	0.613	8.8	8.6–8.8	0.613
Enterobacteriaceae	7.4	6.3–7.7	7.4	6.6–7.9	0.529	6.9	6.1–7.6	0.459
Aerobic G(+)C	6.9	6.6–7.5	7.5	6.5–8.5	0.191	7.8	7.6–8.1	0.039
Real time PCR								
Bacteroides	12.58	12.33–12.74	11.48	11.11–11.68	<0.001	11.26	11.12–11.35	<0.001
Bifidobacteria	12.29	12.04–12.62	12.46	12.00–12.65	0.737	12.00	11.42–12.67	0.210
Enterococci	7.96	7.44–9.09	9.17	8.26–9.94	0.008	8.46	7.44–9.64	0.351
Clostridium subcluster I	11.20	9.80–11.85	10.24	9.84–11.00	0.126	10.93	10.37–11.84	0.941
Clostridium subcluster XI	8.97	7.90–9.73	8.62	7.93–9.54	0.629	9.36	9.10–9.70	0.367
Clostridium subcluster XIVab	12.93	12.75–13.18	12.80	12.55–13.03	0.033	12.47	12.25–13.03	0.021
Sulfate-reducing bacteria	9.27	8.84–9.89	8.85	8.24–9.49	0.042	8.53	8.09–9.73	0.188

Total bacteria were counted under a microscope after Gram-staining. Values are median of $\log_{10}$CFU/g feces in culture method and median of $\log_{10}$ copies of 16S ribosomal gene/g feces in real time PCR. Values below the detection limit are not included. The Mann–Whitney U test was used

IQR, interquartile range; G(+)R, Gram-positive rod; G(−)R, Gram negative rod; G(+)C, Gram positive cocci

Values in italics have been changed because one UC patient had been excluded (UC68, diet-related folk remedies)

Table 4 Analysis of fecal components

	Healthy subjects (n = 36)		Remission of UC (n = 35)			Active UC (n = 12)		
	Median	IQR	Median	IQR	P value	Median	IQR	P value
Organic acids								
Total organic acid (μmol/g feces)	91.8	73.1–107.5	73.4	63.4–83.2	0.002	81.2	67.6–97.6	0.284
Acetic (μmol/g feces)	43.7	34.0–52.2	34.4	29.7–41.7	0.027	37.2	33.4–46.3	0.453
Propionic (μmol/g feces)	13.1	9.2–18.5	9.6	6.6–14.9	0.021	10.8	8.6–13.6	0.227
Butyric (μmol/g feces)	8.8	5.2–11.5	4.7	2.3–7.3	0.001	6.6	3.6–12.7	0.446
Lactic (μmol/g feces)	19.7	17.5–21.0	17.8	15.2–19.4	0.024	18.8	16.6–19.8	0.329
Others								
Ammonia (μg/g feces)	418.4	280.1–510.5	454.3	248.9–551.3	0.621	588.3	502.9–701.3	0.009
Indole (μmol/g feces)	0.78	0.58–1.22	0.63	0.48–1.24	0.476	0.39	0.29–0.45	0.007
Fecal pH	6.95	6.49–7.26	7.28	6.77–7.65	0.034	6.92	6.56–7.24	0.990
Moisture (%)	76.4	67.8–81.8	79.5	74.4–84.3	0.178	81.6	75.5–86.0	0.078

Total organic acid concentration was the sum of each measured concentration and includes isobutyric, isovaleric valeric, isocapuronic, capuronic, and succinic acids. Values are medians of μmol/g feces (wet weight) for organic acids and indole, μg/g feces (wet weight) for ammonia. IQR, interquartile range. Values below the detection limit are not included. The Mann–Whitney U test was used

Values in italics have been changed because one UC patient had been excluded (UC68, diet-related folk remedies)

and lactic acids were significantly lower in the fecal material of patients in remission than in healthy subjects, but were the same for active UC and healthy subjects. The total concentration of organic acids was also significantly lower in patients in remission than in healthy controls, but was not significantly different in samples from active UC patients. Other fecal components measured in this study are shown in Table 4. The moisture level was not significantly different between UC and healthy subjects. Fecal pH was significantly higher only in those in remission. The amount of ammonia was significantly larger, and that of indole was smaller, in those with active UC than in healthy subjects, but there was no significant difference between those in remission and the controls.

Effect of Medication, Probiotics, and Bowel Habits on Intestinal Bacteria Levels

Because 5-aminosalicylic acid (5-ASA) has antimicrobial activity [38] and the sulfapyridine moiety of Salazopyrin (a conjugate of 5-ASA and sulfapyridine) is an acknowledged antibiotic because of anti-folate activity, the amount of intestinal bacteria was plotted against different doses of 5-ASA compounds (Table S1). No significant correlation was found between the amount of intestinal bacteria and the dosing of 5-ASA compounds (Fig. S1). Intake of probiotics has been known to affect intestinal microbiota. Nineteen patients in this study took MiyaBM and four ingested *Bifidobacterium*-containing fermented milk or supplement, as described in Table S1. However, there was no correlation between MiyaBM intake and levels of the dominant anaerobic bacteria, and viable cell number of *Enterobacteriaceae* was negatively correlated with intake (Fig. S2).

UC patients reported their bowel habits in a questionnaire (IBDQ) (Table S2). Statistical analysis showed that the amount of *Clostridium* subcluster XIVab was negatively correlated with increased evacuation frequency and the number of times a day that the patient experienced diarrhea/loose stool. In addition, the amount of *Bacteroides* was significantly lower than that in healthy controls, irrespective of bowel habits (Fig.S3a,b).

Discussion

The healthy gut environment is complicated and controlled by the balance of the intestinal immune system, intestinal microbiota, and microbial metabolites produced by intestinal bacteria [23, 39]. Inflammatory bowel disease could be promoted by an imbalance of these elements in genetically susceptible persons. However, the bacteria responsible for UC have not been identified, and how the intestinal microbiota affects intestinal disorders is still unclear. Therefore, fecal microbiota and microbial metabolites, as indicators of the large intestinal environment, were compared between patients with UC and healthy adults to find a clue to the roles of intestinal bacteria in UC.

Dendrogram analysis of T-RFLP profiles divided fecal microbiota into one healthy cluster and four UC clusters (Fig. 1). The UC clusters were not dependent on disease activity and location of inflammation, indicating that fecal microbiota is basically different in UC patients. Most of the same species of bacteria appeared as T-RFs in both UC patients and healthy subjects, but the amounts of the dominant genera, *Bacteroides* and *Clostridium* subcluster XIVab, tended to be less in UC patients (data not shown).

Ando et al. [40] also analyzed fecal microbiota by T-RFLP and indicated that the diversity of fecal microbiota is altered in UC patients, and that unclassified bacteria, and known bacteria, contribute to the alterations. But, in our study, comparison of T-RFLP profiles revealed no increase of UC-specific bacteria, and the number of T-RFs was significantly less in UC patients. Less diversity has been reported in mucosa-associated microbiota in patients with Crohn's disease and in fecal microbiota in patients with UC [41, 42]. Reduced diversity of fecal microbiota might be a characteristic of inflammatory bowel disease (IBD). Martinetz et al. [43] reported a time-dependent decrease of diversity (i.e., unstable microbiota) in UC patients. In this study we analyzed fresh feces at one time point. We should analyze microbiota at different times or in different places to confirm the decrease in diversity.

Consistent with the results of T-RFLP analysis, real-time PCR revealed significantly fewer *Bacteroides* and *Clostridium* subcluster XIVab in UC patients than in healthy adults. Colitis therapy with immunosuppressants and anti-inflammatory drugs, for example the 5-ASA compounds [38, 44], intake of probiotics, dietary habits, and bowel habits can affect intestinal microbiota. We investigated whether decreases of dominant anaerobes, for example *Bacteroides* and *Clostridium* subcluster XIV, in UC patients depends on these factors. Significantly lower levels of *Bacteroides* and *Clostridium* XIVab were not correlated with the 5-ASA compounds administered in this study (Fig. S1). Finegold et al. [45] showed that a mixture of typical antifolate sulfa drugs, was very poorly active against anaerobic intestinal bacteria. Therefore, a decrease of *Bacteroides* and *Clostridium* XIVab in UC patients is not because of the colitis medications, although an effect cannot be completely excluded. The intake of probiotics containing *Clostridium butyricum* also did not affect levels of these dominant anaerobes in UC patients (Fig. S2). However, increasing frequency of evacuation and diarrhea/loose stool were negatively correlated with the amount of *Clostridium* subcluster XIVab, and *Bacteroides* was significantly lower than in the healthy controls, irrespective of bowel habits and other factors under analysis. Among minor aerobic bacteria, both culture and real-time PCR showed an increase in enterococci in UC patients. Although enterococci are normal residents of the human intestine, monoassociation of *Enterococcus faecalis* reportedly enhances gene expression of proinflammatory chemokines in intestinal epithelial cells [46], and leads to the development of colorectal inflammation in IL-10 KO mice to the same extent as in conventionalized IL-10 KO mice [47]. These reports suggest that an increase of enterococci has a promoting effect on UC.

Because probiotic bifidobacteria reportedly ameliorate UC [48–50], we expected lower levels of bifidobacteria might correlate with the occurrence or relapse of colitis. Instead, the level of bifidobacteria in UC was the same as that in healthy adults. Although sulfate-reducing bacteria (SRB) has been correlated with the occurrence of UC [6], amount of SRB was smaller in UC patients than in healthy adults. Although 5-ASA can inhibit sulfate-reducing bacteria-mediated production of hydrogen sulfide in vitro, the counts and carriage rates of SRB were not significantly different from those in controls [51]. In this study, neither treatment with 5-ASA compounds nor bowel habits alone significantly affected SRB, but they may possibly exert an effect in combination.

Fecal concentration of organic acids in UC patients in remission was significantly lower than those in healthy subjects. It was slightly higher in patients with active UC than in remission patients and significantly correlated with an increase in evacuation frequency (Fig. S5). These effects on organic acid levels occur:

1. because of inflamed mucosa that cannot efficiently utilize organic acids produced by intestinal bacteria; and
2. because of the rapid movement of intestinal contents, for example during diarrhea.

A lower concentration of organic acids was consistent with the decrease of *Bacteroides*, one of the most dominant species in the human gut, which produces abundant organic acids by acidic fermentation [18, 52]. *Bacteroides* spp. efficiently ferment carbohydrates to acetate, propionate, and succinate. A bowel habit-dependent decrease of *Clostridium* subcluster XIVab, which includes butyrate-producing bacteria, might also be implicated in the lower concentration of organic acids in UC patients, but this might be hidden by the effect of increasing evacuation/diarrhea. Besides butyric acid, these organic acids also contribute to intestinal homeostasis by supplying anti-inflammatory effects and energy for colonocytes [52, 53]. As acetate and lactate are intermediates in carbohydrate fermentation by intestinal microbiota [52, 54], lower concentrations of these in UC patients indicate depressed metabolic activity of microbiota. Reduced levels of organic acids in IBD patients have also been reported by Takaishi et al. [55] and Marchei et al. [56]. Although we did not quantify *Fecalibacterium prausnitzii* or *Clostridium* subcluster IV, these bacteria have been repeatedly detected in healthy subjects and their reduction has been characterized in Crohn's disease patients [41, 57]. Therefore, it is possible that a decrease of these commensal bacteria contributes to the lower concentration of organic acids in the feces of UC patients.

The concentration of putrefactive metabolites is significantly different between active UC and healthy controls (Table 4). Although a higher intake of protein increases the

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Scientific Research

Effect of Genetic Polymorphism of *CYP2A6* on Individual Susceptibility to Colorectal Tumors in Japanese Smokers

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ABSTRACT

Tobacco smoking is a risk factor for colorectal cancer and adenomas. To clarify the effect of genetic factors on the risk for tobacco-related colorectal tumors in a Japanese population, we performed a case-control study on 300 patients with two or more tumors and 181 healthy controls; all were genotyped for *CYP2A6*4*, *CYP2A6*7* and *CYP2A6*9*. Cigarette smoking increased colorectal tumor risk (trend-test $P < 0.0000005$). Current smokers plus ex-smokers (ever-smokers) with the *CYP2A6*4/*4* genotype (whole gene deletion) showed the lowest risk among smokers [odds ratio (OR), 0.17; 95% confidence interval (CI), 0.05 - 0.62 compared to ever-smokers with the wild-type *CYP2A6*1/*1*]. When the participants were classified into four phenotype groups based on estimated CYP2A6 activity [*i.e.*, normal (**1/*1*), intermediate (heterozygotes for the **1* and a variant allele), slow (heterozygotes and homozygotes for variant alleles except for **4/*4*) and poor (**4/*4*)], the ORs (95% CIs) in ever-smokers of the normal, intermediate, slow and poor groups were 6.75 (2.73 - 16.76), 4.59 (2.10 - 10.06), 3.89 (1.69 - 8.95) and 1.17 (0.31 - 4.40), respectively, compared with never-smokers with normal CYP2A6 activity. The susceptibility to colorectal tumors was dependent on the predicted phenotype among ever-smokers (trend-test $P = 0.015$), but not among never-smokers (trend-test $P = 0.47$). Stratifying the subjects with respect to cumulative tobacco exposure and estimated CYP2A6 activity, we found the highest risk of colorectal tumors in subjects with higher CYP2A6 activity and higher cumulative tobacco exposure (trend-test $P = 0.000023$); the lowest risk was found in subjects with the lowest estimated CYP2A6 activity independent of tobacco exposure (trend-test $P = 1.00$). These results suggest that the gene-environment interaction (*i.e.*, the CYP2A6-smoking interaction) strongly affects the individual susceptibility to tobacco-related colorectal tumors.

Keywords: P4502A6; Tobacco Smoking; Colorectal Cancer; Colorectal Adenomas; Case-Control Study

1. Introduction

Colorectal cancer is now one of the most frequent causes of cancer mortality worldwide [1]. The etiology of colorectal cancer is complex and multifactorial, involving both genetic and environmental factors. Numerous case-control and cohort studies have been conducted to investigate the relationship between colorectal cancer and lifestyle factors, including the consumption of red or well-done meat [2], fat [3], and alcohol [4] and cigarette smoking [5]. Cigarette smoking has consistently been identified as a potential risk factor for colorectal adenomas [5], which are recognized as precursor lesions for most cases of colorectal cancer [6]. The association between colorectal cancer and smoking has recently been reported in many studies [5,7,8].

Tobacco smoke contains more than 60 carcinogens, including *N*-nitrosamines, polycyclic aromatic hydrocarbons (PAHs) and aromatic amines [9]. To exert their carcinogenicity, these carcinogens require metabolic activation, and this activation is mediated mainly by phase I drug-metabolizing enzymes. Among phase I enzymes, the cytochrome P450 (P450 or CYP) family plays a crucial role in this respect. CYP2A6, one of the major members of the P450 family in humans, is involved in the metabolic activation of carcinogens, particularly tobacco-related *N*-nitrosamines [10-12]. CYP2A6 also catalyzes the elimination of nicotine (this elimination is reported to be a major factor in the maintenance of smoking behavior and tobacco dependence [13]) by metabolizing nicotine to cotinine [14], and cotinine to trans-3'-hydroxy-cotinine [15]. Genetic polymorphisms of *CYP2A6* that influence the activity of CYP2A6 have been reported to affect

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tobacco-related lung cancer risk and smoking behavior [16].

To date, many variants of *CYP2A6* have been identified (URL: http://www.imm.ki.se/CYPalleles/cyp2a6.htm). Among these, the *CYP2A6*4* allele, a whole-gene-deletion polymorphism [17-19], is thought to be the most important allele in Asians because it has an allele frequency of up to 20%; however, its frequency is low in Caucasians [19,20]. Subjects homozygous for *CYP2A6*4* have been reported to show very low capacity to form cotinine from nicotine [21]. The *CYP2A6*7* allele causes an amino acid substitution (Ile471Thr) that leads to reduced enzyme activity [20]. The *CYP2A6*9* allele, which contains a −48T to G nucleotide substitution in the TATA box of the 5'-flanking region, has been reported to reduce the expression levels of CYP2A6 mRNA and protein in human livers [22]. Both the *CYP2A6*7* and *CYP2A6*9* alleles are also more common in Asians than in Caucasians [23].

Our recent studies have clarified that the mutant alleles described above reduce the susceptibility to squamous cell carcinoma and small-cell carcinoma, which are known to be types of lung cancer frequently induced by exposure to tobacco smoke [16,24,25]. The reduced risk in those with the *CYP2A6*4* allele was also seen in the reduced occurrence of oral cancer in betel/quid chewers with the same allele [26]. However, there is little information on the effects of *CYP2A6* polymorphisms on the risks for other smoking-related tumors, including colorectal cancer and adenomas.

In the present study, we found that *CYP2A6* polymorphisms, *CYP2A6*4*, *CYP2A6*7* and *CYP2A6*9*, reduced the tumor risk in combination with exposure to tobacco. We herein provide evidence that CYP2A6 activity is one of the principal factors that determine individual susceptibility to smoking-related colorectal cancer and adenomas.

2. Materials and Methods

2.1. Study Subjects and Data Collection

This study was approved by the ethics committees of the Osaka Medical Center for Cancer and Cardiovascular Diseases, the Osaka Central Hospital and Hokkaido University. All study participants were Japanese and were recruited from June 1993 to September 1997 at the Osaka Medical Center for Cancer and Cardiovascular Diseases. Cases were defined as patients with two or more colorectal tumors removed endoscopically within 3 months before recruitment. The colorectal tumors in this study included both adenomas (mild atypia, moderate atypia or severe atypia) and adenocarcinomas but not advanced or invasive cancer. Patients with one or more adenocarcinomas were assigned to the adenocarcinoma group, and those with adenomas only to the adenoma group. Individuals with a history of intestinal or gastric resection (other than appendectomy), familial adenomatous polyposis, ulcerative colitis, other malignant tumors and severe illness were excluded from the case group. Control subjects were healthy volunteers who required hospitalization for a health checkup. The ages of case and control subjects were defined at the time of the first hospital visit. Signed consent forms and completed questionnaires were collected from all case and control subjects before the collection of blood. The questionnaire covered smoking status (never-, ex- or current smoker), the total duration of smoking (excluding intermediate periods without smoking) and the average number of cigarettes smoked daily. The number of pack-years was calculated as a measure of cumulative cigarette smoking. One pack-year was defined as smoking 20 cigarettes daily for 1 year. Ex-smokers were defined as subjects with a minimum smoking history of 0.5 pack/day for at least 1 year. Current and ex-smokers were often combined into a group referred to as "ever-smokers."

2.2. Genotyping of CYP2A6

The *CYP2A6*4* allele genotyped in this study was the *CYP2A6*4A* variant, which is identical to *CYP2A6*4C* [20]. The genotyping of *CYP2A6*4* was based on polymerase chain reaction (PCR)/restriction fragment length polymorphism and was performed by a previously described method [20] with minor modifications. Briefly, a novel forward primer named 2A6-B6 (5'-CCT CAT CAC ACA CAA CTT CCT C-3') and a reverse primer named 2A6-UTRAS1 (5'-TGT AAA ATG GGC ATG AAC GCC C-3') [20] were used to amplify the common regions of *CYP2A6*1* and *CYP2A6*4*. The reaction mixture contained 1 × LA PCR Buffer II, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM 2A6-B6, 0.2 μM 2A6-UTRAS1, 0.5 U of LA Taq DNA polymerase (TaKaRa BIO, Shiga, Japan), and approximately 50 ng of genomic DNA in a final volume of 25 μL. PCR was carried out under the following conditions: initial denaturation at 94˚C for 3 min; followed by 30 cycles of denaturation at 94˚C for 30 s, annealing at 64˚C for 30 s and extension at 72˚C for 1.5 min; and subsequently a final extension at 72˚C for 3 min. The PCR products consisted of 1358-bp fragments from the *CYP2A6*1* allele and 1356-bp fragments from the *CYP2A6*4* allele; these fragments were digested with Eco81 I. Fragments of 824 bp and 728 bp were derived from the *CYP2A6*1* and *CYP2A6*4* alleles, respectively. The *CYP2A6*7* allele was genotyped using a two-step allele-specific-PCR method [20] in which the PCR products described above were used as templates.

For *CYP2A6*9* genotyping, a new method based on allele-specific-PCR was developed for this study. In the first PCR, a 440-bp fragment from the 5'-flanking region of exon 1 of *CYP2A6* was amplified using the primers

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2A6 up-0.1 kb and 2A6 ex1R, as described previously [22]. In the second PCR, 2A6 TATA (5'-TCC CTC TTT TTC AGG CAG TA<u>T</u>-3') and 2A6 TAGA (5'-TCC CTC TTT TTC AGG CAG TA<u>G</u>-3') were employed as forward primers. The reaction mixture (25 μL) contained 1 μL of the first PCR products; 1 × PCR Buffer II, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM 2A6 TATA or 0.2 μM 2A6 TAGA, 0.2 μM 2A6 ex1R; and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). Amplification was performed by initial denaturation at 94˚C for 1 min; followed by 20 cycles of denaturation at 94˚C for 30 s, annealing at 64˚C for 30 s and extension at 72˚C for 45 s; and subsequently a final extension at 72˚C for 1 min. An aliquot (5 μL) of second PCR products (387 bp) was analyzed by electrophoresis with a 1% agarose gel. Alleles that were not classified as *CYP2A6*4*, *CYP2A6*7* or *CYP2A6*9* were assigned to the wild-type *CYP2A6*1* allele.

In the analysis of putative CYP2A6 phenotypes, subjects were tentatively assigned to four groups based on their *CYP2A6* genotypes according to the definitions of Fujieda *et al.* [16]: the putative "normal" phenotype group, *CYP2A6*1/*1*; the "intermediate" group, those heterozygous for the *CYP2A6*1* allele (*CYP2A6*1/*4*, **1/*7* and **1/*9*); the "slow" group, those heterozygous or homozygous for variant alleles except for those homozygous for the *CYP2A6*4* allele (*CYP2A6*4/*7*, **4/*9*, **7/*7*, **7/*9* and **9/*9*); and the "poor" group, *CYP2A6*4/*4*.

2.3. Data Analysis

Differences in the distribution of relevant characteristics between cases and controls were evaluated using *t* tests and χ^2 tests. Compliance with Hardy-Weinberg equilibrium among controls was examined using the χ^2 test. Associations between susceptibility to colorectal tumors and smoking-related indicators or the *CYP2A6* genotypes/phenotypes were assessed using odds ratios (ORs). ORs and 95% confidence intervals (CIs) were calculated from logistic regression models to adjust for age and sex. When an association between smoking and colorectal tumor risk was detected, the never-smoker group was defined as the reference. The tertile values of continuous smoking variables (smoking duration, daily cigarette consumption and pack-year smoked) among ever-smokers in the controls were used as cutoff points. To investigate the impact of *CYP2A6* genotypes and the putative CYP2A6 phenotypes on colorectal tumor risk, the ORs among the never- and ever-smokers were estimated by comparing with the reference group of the never-smokers with the *CYP2A6*1/*1* genotype and the putative normal phenotype, respectively. Subsequently, among ever-smokers, the effects of the *CYP2A6* genotypes and putative CYP2A6 phenotypes on the risk were assessed, defining ever-smokers with the *CYP2A6*1/*1* genotype and the normal phenotype, respectively, as the reference groups. More detailed investigations were conducted in association with the combination of putative CYP2A6 phenotypes and cigarette smoking. The median values of smoking duration, daily cigarette consumption and pack-years smoked were used as the cutoff points.

To investigate whether the estimated risks were dependent on increased smoking variables, trend test was performed by assigning ordinal scores as continuous variables in the logistic regression models. When the median or tertile values of the smoking indicators among the control group were used as the cutoff points, *P* values for the trend were estimated with the ordinal scores 1 - 3 assigned to the three levels of smoking exposure (1 for never-smokers, 2 for light smokers and 3 for heavy smokers). When the trends of associations between putative CYP2A6 phenotypes and the risks were tested, ordinal scores of 1 - 4 were assigned to the normal, intermediate, slow and poor groups, respectively. All tests of statistical significance were two-sided. A *P* value of 0.05 was considered the threshold of significance. All statistical analyses were carried out by using the statistical software SAS, version 5.0 (SAS Institute, Inc., Cary, NC).

3. Results

The characteristics and smoking-related indicators of the colorectal tumor patients and healthy controls are summarized in **Table 1**. The mean ages of the case and control groups were significantly different ($P = 0.0048$). There more men than women in both case and control groups; however, the gender distributions were not significantly different between the two groups ($\chi^2 = 0.59$, $P = 0.44$). More ever-smokers (*i.e.*, ex-smokers and current smokers combined) were present in case group than in the control group (72.3% versus 51.9%; $\chi^2 = 20.6$, $P = 0.0000058$). Ever-smokers in the case group had significantly longer durations of smoking ($P = 0.0013$) and higher values of pack-years smoked ($P = 0.000080$) than ever-smokers in the control group. No significant difference was found in the daily cigarette consumption between cases and controls ($P = 0.080$).

Table 2 shows the association between the various smoking-related indicators (smoking status, smoking years, daily cigarette consumption and pack-years smoked) and the risk for colorectal tumors. Ever having smoked cigarettes was associated with a significantly increased risk for colorectal tumors (OR, 2.95; 95% CI, 1.85 - 4.70). In particular, current smokers were at the highest risk (OR, 3.16; 95% CI, 1.90 - 5.26). The association with smoking was also examined in terms of smoking years, daily cigarette consumption and pack-years. The highest exposure levels were associated with

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Table 1. Characteristics and smoking-related indicators of colorectal tumor patients and healthy controls enrolled in the present study.

	Cases (n = 300)	Controls (n = 181)	*P* value
Age, years			0.0048
Mean ± SD	56.1 ± 6.3	58.4 ± 10.1	
Median	57	58	
Range	41 - 67	35 - 82	
Gender, n (%)			0.44
Men	244 (81.3)	142 (78.5)	
Women	56 (18.7)	39 (21.5)	
Smoking status, n (%)			0.0000058
Never-smokers	83 (27.7)	87 (48.1)	
Ever-smokers	217 (72.3)	94 (51.9)	
Ex-smokers	85 (28.3)	42 (23.2)	
Current smokers	132 (44.0)	52 (28.7)	
Pack-years among ever-smokers			0.000080
Mean ± SD	35.1 ± 18.7	28.8 ± 17.8	
Median	33.8	25.4	
Smoking duration among ever-smokers, years			0.0013
Mean ± SD	27.6 ± 9.4	23.8 ± 9.7	
Median	30	20	
Cigarettes/day among ever-smokers			0.080
Mean ± SD	25.1 ± 11.5	23.1 ± 10.1	
Median	20	20	

Table 2. Association between cigarette smoking and risk of colorectal tumors.

	Cases (n = 300) n (%)	Controls (n = 181) n (%)	Adjusted odds ratio (95% CI)*	*P* value*
Smoking status				
Never-smokers	83 (27.7)	87 (48.1)	1.00 (reference)	–
Ever-smokers	217 (72.3)	94 (51.9)	2.95 (1.85 - 4.70)	
Ex-smokers	85 (28.3)	42 (23.2)	2.65 (1.52 - 4.59)	0.00055
Current smokers	132 (44.0)	52 (28.7)	3.16 (1.90 - 5.26)	0.0000091
Smoking duration, years†				
<20	29 (9.7)	26 (14.4)	1.53 (0.78 - 2.97)	0.21
20 to <30	74 (24.7)	35 (19.3)	2.41 (1.36 - 4.26)	0.0028
≥30	114 (38.0)	33 (18.2)	4.74 (2.70 - 8.32)	0.000000056
		Trend test‡	2.20 (1.69 - 2.85)	0.0000000030
Cigarettes/day†				
<20	44 (14.7)	27 (14.9)	2.08 (1.12 - 3.86)	0.021
20	72 (24.0)	37 (20.4)	2.53 (1.44 - 4.43)	0.0012
>20	101 (33.7)	30 (16.6)	4.42 (2.48 - 7.87)	0.00000046
		Trend test‡	2.11 (1.58 - 2.82)	0.00000047
Pack–years†				
<20	46 (15.3)	28 (15.5)	2.14 (1.15 - 3.99)	0.016
20 to <34	63 (21.0)	38 (21.0)	2.04 (1.17 - 3.58)	0.013
≥34	108 (36.0)	28 (15.5)	5.25 (2.93 - 9.41)	0.000000025
		Trend test‡	2.08 (1.61 - 2.70)	0.000000029

*Adjusted by logistic regression for age and gender; †Tertile values of ever-smokers among controls were used as cutoff points; ‡Based on a trend variable assigned values 1 - 4, including never-smoker (value 1).

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the highest risk, as shown by the groups smoking for ≥30 years (OR, 4.74; 95% CI, 2.70 - 8.32), smoking >20 cigarettes/day (OR, 4.42; 95% CI, 2.48 - 7.87) and having smoked ≥34 pack-years (OR, 5.25; 95% CI, 2.93 - 9.41). Elevated risks for colorectal tumors were significantly associated with increased smoking years, daily cigarette consumption and pack-years (trend-test P = 0.00047, 0.015 and 0.0026, respectively), even when the tests for trend excluded never-smokers.

To confirm the effects of *CYP2A6* genotypes on the risk for colorectal tumors in ever- and never-smokers, all case and control subjects were genotyped for *CYP2A6*1*, **4*, **7* and **9* (**Table 3**). The distribution of *CYP2A6* genotypes among controls were in Hardy-Weinberg equilibrium in ever-smokers (χ^2 = 2.81, P = 0.97) and never-smokers (χ^2 = 5.74, P = 0.77). This fact indicates that control subjects were sufficiently random and representative in both groups. Among never-smokers, no positive association was seen between *CYP2A6* genotype and the risk for colorectal tumors. Compared to never-smokers with *CYP2A6*1/*1* as a reference group, estimated risks among ever-smokers were significantly increased in *CYP2A6*1/*1*, **1/*4*, **1/*7*, **1/*9*, **4/*7*, **4/*9* and **7/*9* genotypes (e.g., *CYP2A6*1/*1*: OR, 6.85; 95% CI, 2.76 - 17.01). To investigate the impact of *CYP2A6* genetic polymorphisms among smokers only, ever-smokers with the *CYP2A6*1/*1* genotype were defined as the reference group. This analysis revealed that ever-smokers with the *CYP2A6*4/*4* genotype had a significantly reduced risk (OR, 0.17; 95% CI, 0.05 - 0.62). The ORs among ever-smokers with other genotypes tended to be lower than 1, but these ORs were not significant.

To further investigate the effects of *CYP2A6* genetic polymorphisms on the tumor risk in smokers, we classified all study subjects into four groups (normal, intermediate, slow and poor metabolizers) according to putative CYP2A6 phenotypes predicted from the *CYP2A6* genotypes, as previously defined by Fujieda *et al.* [16]. Among never-smokers, the putative CYP2A6 phenotypes did not affect the risk for colorectal tumors (trend-test P = 0.47, see **Table 4**). However, compared with CYP2A6-normal never-smokers, the ORs in predicted normal, intermediate and slow phenotype groups among ever-smokers were significantly increased to 6.75 (95% CI, 2.73 - 16.76), 4.59 (95% CI, 2.10 - 10.06) and 3.89 (95% CI, 1.69 - 8.95), respectively. However, compared with CYP2A6-normal ever-smokers, the estimated risk in CYP2A6-poor ever-smokers was significantly low (OR, 0.17; 95% CI, 0.05 - 0.62). These results clearly indicate that the risk of colorectal tumors in smokers is decreased by polymorphisms that reduce the activity of CYP2A6 (trend-test P = 0.015).

Table 4 shows the effects of putative CYP2A6 phenotypes on the risk of colorectal tumors with stratification by smoking-related indicators. The ORs among ex-smokers and current smokers relative to CYP2A6-normal never-smokers were significantly high, except for these in CYP2A6-poor groups. With respect to pack-years smoked, the highest OR was seen in groups with the putative normal phenotype with >25 pack-years smoked (OR, 10.03; 95% CI, 3.55 - 28.34) (see **Table 4**). In subjects with at least some CYP2A6 activity (*i.e.*, all except

Table 3. Effect of *CYP2A6* genotypes on risk of colorectal tumors in never- and ever-smokers.

	CYP2A6 genotypes									
	**1/*1*	**1/*4*	**1/*7*	**1/*9*	**4/*4*	**4/*7*	**4/*9*	**7/*7*	**7/*9*	**9/*9*
Never-smokers										
Cases/controls*	16/24	13/13	10/6	17/13	5/5	7/7	9/5	2/5	1/4	3/5
Adjusted odds ratio (95% CI)†	1.00 (reference)	1.50 (0.54 - 4.16)	2.60 (0.77 - 8.77)	2.15 (0.80 - 5.77)	1.84 (0.44 - 7.68)	1.63 (0.46 - 5.72)	2.87 (0.79 - 10.38)	0.78 (0.13 - 4.72)	0.40 (0.04 - 4.00)	0.93 (0.19 - 4.58)
P value†	–	0.44	0.12	0.13	0.38	0.45	0.11	0.80	0.44	0.93
Ever-smokers										
Cases/controls*	52/15	36/16	24/13	44/15	5/8	12/6	15/6	7/2	13/6	9/7
Adjusted odds ratio (95% CI)†	6.85 (2.76 - 17.01)	4.59 (1.83 - 11.52)	3.56 (1.34 - 9.47)	5.65 (2.26 - 14.15)	1.19 (0.32 - 4.46)	3.81 (1.13 - 12.84)	4.80 (1.48 - 15.59)	5.40 (0.97 - 30.17)	4.56 (1.36 - 15.25)	2.36 (0.70 - 7.94)
P value†	0.000034	0.0012	0.011	0.00021	0.80	0.031	0.0090	0.055	0.014	0.16
Adjusted odds ratio (95% CI)‡	1.00 (reference)	0.67 (0.29 - 1.54)	0.52 (0.21 - 1.27)	0.83 (0.36 - 1.89)	0.17 (0.05 - 0.62)	0.56 (0.18 - 1.75)	0.70 (0.23 - 2.14)	0.79 (0.15 - 4.27)	0.67 (0.21 - 2.07)	0.35 (0.11 - 1.09)
P value‡	–	0.34	0.15	0.65	0.0071	0.32	0.54	0.81	0.48	0.072

*Never-smokers were made up of 83 cases and 87 controls, and ever-smokers were 217 cases and 94 controls; †Adjusted ORs for age and gender compared to never-smokers with *CYP2A6*1 *1* genotype as the reference group; ‡Adjusted ORs for age and gender compared to ever-smokers with *CYP2A6*1 *1* genotype as the reference group.

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Table 4. Effects of putative *CYP2A6* phenotypes on risk of colorectal tumors in never-smokers and ever-smokers.

	Putative CYP2A6 phenotypes*				
	Normal	Intermediate	Slow	Poor	Trend test†
Never-smokers					
Cases/controls	16/24	40/32	22/26	5/5	
Adjusted odds ratio (95% CI)‡	1.00 (reference)	1.98 (0.88 - 4.43)	1.39 (0.58 - 3.32)	1.83 (0.44 - 7.66)	1.14 (0.79 - 1.65)
P value‡	–	0.099	0.46	0.41	0.47
Ever-smokers					
Cases/controls	52/15	104/44	56/27	5/8	
Adjusted odds ratio (95% CI)‡	6.75 (2.73 - 16.76)	4.59 (2.10 - 10.06)	3.89 (1.69 - 8.95)	1.17 (0.31 - 4.40)	
P value‡	0.000036	0.00014	0.0014	0.81	
Adjusted odds ratio (95% CI)§	1.00 (reference)	0.68 (0.35 - 1.34)	0.58 (0.27 - 1.21)	0.17 (0.05 - 0.62)	0.68 (0.50 - 0.93)
P value§	–	0.26	0.25	0.0070	0.015
≤25					
Cases/controls	13/7	30/25	22/11	1/4	
Adjusted odds ratio (95% CI)‡	3.39 (1.06 - 10.82)	2.29 (0.95 - 5.51)	3.78 (1.39 - 10.32)	0.42 (0.04 - 4.26)	0.86 (0.53 - 1.40)
P value‡	0.039	0.064	0.0094	0.46	0.54
>25					
Cases/controls	39/8	74/19	34/16	4/4	
Adjusted odds ratio (95% CI)‡	10.03 (3.55 - 28.34)	7.84 (3.29 - 18.68)	4.06 (1.62 - 10.20)	2.06 (0.43 - 9.78)	0.59 (0.30 - 0.90)
P value‡	0.000020	0.0000033	0.0029	0.36	0.014
Trend test *P* value¶	0.000023	0.00021	0.086	1.00	

*Putative CYP2A6 phenotypes: normal, *CYP2A6*1/*1*; intermediate, *CYP2A6*1/*4*, *1/*7 and *1/*9; slow, *CYP2A6*4/*7*, *4/*9, *7/*7, *7/*9 and *9/*9; poor, *CYP2A6*4/*4*; †Based on a trend variable assigned values 1 - 4 for putative normal, intermediate, slow and poor phenotypes, respectively; ‡Adjusted ORs for age and gender compared to never-smokers with putative normal phenotype as a reference group; §Adjusted ORs for age and gender compared to ever-smokers with putative normal phenotype as a reference group; ¶Based on a trend variable assigned values 1 - 3 for never-smoker, ever-smoker with ≤25 and >25, respectively.

those with *CYP2A6*4/*4*), the estimated risks for colorectal tumors were elevated with increased pack-years smoked (putative normal phenotype group, trend-test $P = 0.000023$; intermediate, $P = 0.00021$; and slow, $P = 0.086$). The ORs in subjects assigned to the putative normal or intermediate group were significantly dose dependent with regard to daily cigarette consumption and smoking duration (data not shown). In the CYP2A6-poor group, there was no evidence that the estimated risk was dependent on smoking-related indicators (pack-years, trend-test $P = 1.00$). Individuals who had smoked for >25 pack-years was strongly affected by the *CYP2A6* genotype (trend-test $P = 0.014$). In contrast, no significant association between the susceptibility to colorectal tumors and putative CYP2A6 phenotypes was observed in light smokers or never-smokers (trend-test $P = 0.54$ or $P = 0.47$, respectively). Thus, the combination of *CYP2A6* genetic polymorphism and cigarette smoking was clearly associated with the risk for colorectal tumors.

4. Discussion

Enzymes such as *N*-acetyltransferase [27-29], CYP1A1 [30,31], microsomal epoxide hydrolase [29,31-33], sulfotransferase [29] and glutathione *S*-transferase [28-30] are known to activate tobacco-related carcinogens (mainly PAHs and aromatic amines). Thus, a close association between genetic polymorphisms of these enzymes and susceptibility to colorectal cancer or adenomas is to be expected. However, few studies have provided definitive evidence that colorectal tumor risk is influenced by genetic polymorphisms of these enzymes, despite positive associations between the risk and smoking. The results of this current study are the first to show a clear association between *CYP2A6* genotypes and the risk of colorectal cancer and adenomas. Supporting this association, we previously reported that *CYP2A6* polymorphism is one of the principal determinants of the risks of tobacco-related lung cancer and betel/quid-related oral cancer [16,24-26].

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Nowell *et al.* [34] reported a relationship between colorectal cancer risk and the urinary metabolite ratio of caffeine, which probably reflects the phenotype of CYP-2A6, although genetic variants were not analyzed. They demonstrated that subjects with high or medium putative CYP2A6 activity had an increased risk for colorectal cancer (high: OR, 2.9; 95% CI, 1.6 - 5.0 and medium: OR, 2.0; 95% CI, 1.0 - 3.7) compared with subjects with low activity. Their data strongly supported our concept that CYP2A6 could be one of the most important factors contributing to colorectal cancer risk. In contrast, Sachse *et al.* [35] reported that, in a Caucasian population, no significant association was seen between *CYP2A6* inactive alleles, particularly *CYP2A6*2*, and colorectal cancer risk (OR, 0.51; 95% CI, 0.28 - 1.06). This discrepancy between our results and theirs may be explained by several possibilities. First, ethnic differences exist in the frequencies of *CYP2A6* variants; the frequency of inactive alleles is lower in Caucasian than in Japanese populations [23]. The frequencies of the *CYP2A6*4*, *CYP2A6-*7* and *CYP2A6*9* alleles among controls in this study were 19.8%, 16.9% and 20.7%, respectively. These allele frequencies were similar to those previously observed in a large-scale Japanese population (χ^2 = 0.93, P = 0.82) [16] (in that study *CYP2A6*10* and *CYP2A6*11* alleles were classified as *CYP2A6*7* and *CYP2A6*1*, respectively) indicating that no selection bias was evident in our study population. In Caucasians, the frequencies of the *CYP2A6*4*, *CYP2A6*7* and *CYP2A6*9* alleles are reported to be 0.5%, 0.0% and 5.2%, respectively [23]. As a result, the statistical power is likely insufficient in the report by Sachse *et al.* [35]. Second, they analyzed the samples without distinguishing smokers from non-smokers [35]. We previously proposed that the positive relationship between cancer risk and CYP2A6 polymorphism can be detected only in smokers [16,20,25,26]. In accordance with our earlier studies, we found that CY-P2A6 polymorphisms altered the susceptibility to colorectal tumors in smokers but not in non-smokers (**Table 3**). Finally, most of the case subjects in the present study had colorectal adenomas, and most adenocarcinomas in the present study were cancer in adenomas; those with advanced or invasive cancer were excluded. The adenoma-carcinoma sequence is now widely accepted as a central pathway of carcinogenesis. Adenomas are recognized as the precursor lesions for the majority of cases of colorectal cancer [6]. The main environmental risk factors for colorectal adenomas are meat and fat consumption [36] and cigarette smoking [5], which are similar to those for colorectal cancer. However, it has been reported that the risk for colorectal adenomas was more strongly increased by cigarette smoking than was the risk for colorectal cancer. The effects of *CYP2A6* genetic polymorphism on the susceptibility to colorectal adenomas are thought to be clearer than those on the susceptibility to colorectal cancer.

We propose possible mechanisms for the correlation between *CYP2A6* genotypes and the susceptibility to smoking-related colorectal cancer. CYP2A6 is reportedly responsible for the metabolic activation of tobacco-related *N*-nitrosamines, including *N*-nitrosodiethylamine, *N*-nitroso-norntine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, *N*-nitroso-piperidine and *N*-nitrosopyrrolidine [11,12]. DNA adducts with these *N*-nitrosamines have been detected in human colonic tissues [37]. In addition, when human colon microsomes were incubated with known carcinogen methyl-*n*-pentylnitrosamine, the addition of coumarin, a typical substrate of CYP2A6, inhibited methyl-*n*-pentylnitrosamine metabolite formation [38]. These results suggest that CYP2A6 is expressed in human colon. We recently clarified that CYP2A6 mRNA and protein were over-expressed in colorectal adenocarcinoma. Kumarakulasingham *et al.* [39] examined CYP2A6 protein expression in normal colon and colorectal cancer tissues. Considering these lines of evidence together with our results, it seems reasonable to assume that CYP2A6 expressed in colon tissues is a factor critical for colorectal tumorigenesis.

In conclusion, the findings of this current study indicate that *CYP2A6* genetic polymorphisms alter the susceptibility to colorectal tumors in Japanese smokers, particularly in individuals highly exposed to tobacco smoke.

5. Abbreviations

PAHs, polycyclic aromatic hydrocarbons; CYP, cytochrome P450; OR, odds ratio; CI, confidence interval.

6. Competing Interests

There are non-financial competing interests in this study.

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