The Comparison of Fecal Microbiota in Left-Side and Right-Side Human Colorectal Cancer

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Keywords
Colorectal cancer · Microbiome · \textit{Fusobacterium} · Tumor location

Abstract
Introduction: Microbiomes play a vital role in the development and progression of cancer. The clinical status, including prognosis, genetic mutations, and sensitivity to chemotherapy, differs depending on the location of colorectal cancer (CRC); however, the association between gut microbiota and the location of CRC is not entirely understood. This study was conducted to evaluate the differences in the gut microbiota in patients with CRC according to the location of the tumor. Methods: Fifty-six patients who underwent surgery for CRC between August 2018 and November 2019 were included in the study. Three patients who had received neoadjuvant therapy or antibiotic treatment within 1 month before surgery were excluded. The metagenomes of microbiota in preoperative feces were assessed using the V3–V4 region of 16S rRNA amplicon sequences. Results: The beta diversity of the Bray-Curtis distance was significantly higher in left-sided than in right-sided CRC. \textit{Fusobacterium} predominated in left-sided CRC according to the linear discriminant analysis effect size method. \textit{Blautia}, Erysipelotrichales, \textit{Holdemanaella}, \textit{Faecalibacterium}, \textit{Subdoligranulum}, and \textit{Dorea} constituted the dominant intestinal flora in right-sided CRC. Pathway analysis revealed that L-lysine fermentation and cob(II)yrinate a,c-diamide biosynthesis I were predominant in left-sided CRC. Discussion: This study demonstrated that fecal microbiota in left-sided CRC constitutionally and functionally differ from those in right-side CRC. These results will help to elucidate the biological differences according to tumor location and develop treatments for human CRC.

Introduction
Colorectal cancer (CRC) is the second most common cancer leading to death worldwide [1]. The overall survival of patients with unresectable CRC is currently approximately 30 months, despite advances in chemotherapy, including molecularly targeted therapies [2]. CRC is a genetically heterogeneous malignancy with diverse molecular profiles and exerts variable effects, depending on the location of the primary tumor. Right-sided CRC has
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Materials and Methods

Patients and Feces

We enrolled patients who underwent surgery for CRC with preoperative preservation of feces at the Shiga University of Medical Science Hospital, Otsu, Japan, between August 2018 and November 2019. Patients who had received preoperative chemotherapy or radiotherapy and antibiotics within 1 month before the date of surgery were excluded. The clinical data were retrospectively retrieved from the medical records. Fecal samples were collected before bowel preparation using metronidazole and magnesium citrate. For the primary tumor location, the splenic flexure was used as the boundary to classify right- and left-sided CRCs.

DNA Preparation and Amplicon Sequences

A 16s amplicon microbiome analysis was performed on DNA extracted from the fecal samples. The MPure Bacterial DNA extraction kit (M.P. Bio) was used to extract DNA from the feces. DNA libraries were prepared using a 2-step tail polymerase chain reaction (PCR) method. Briefly, the V3–V4 region of the bacterial 16S rRNA gene was amplified by PCR using a mixed primer set. The 16S rRNA gene was paired-end-sequenced using a MiSeq sequencer (Illumina, CA, USA) under 2 × 300 bp conditions, and then analyzed using the Qiime v2.0 package. Using the fastq_barcode_splitter in the FASTX Toolkit splitter, only sequences whose readout beginnings exactly matched those of the primers were extracted. Representative sequences from each operational taxonomic unit (OTU) were classified using the Silva database, and the abundance of OTUs per sample was obtained for subsequent analysis. DNA preparation and sequencing of 16s rRNA genes were outsourced to Seibutugiken. C.O. (Kanagawa, Japan).

Results

Analysis of the gut microbiota of 53 patients with CRC was included in the study (Table 1). Of the 53 patients with CRC, 24 were male and 29 were female; the mean age was 65.8 years in the right-sided CRC group and 70.1 years in the left-sided CRC group. Male sex was more common in the left-sided CRC group (p = 0.353). Patients with a past history of CRC were more common in the right-sided CRC group (p = 0.244). Tumor depth, differentiation, and distant metastasis were not significantly different between the two groups. However, lymph node metastasis was more common in the left-sided CRC group (p = 0.369), possibly due to the higher proportion of BRAF mutations and microsatellite instability. Alternatively, left-side CRC harbors more chromosomal instability and Her2 amplification [3]. In addition, chemotherapy sensitivity of these types of CRC differs according to tumor location [4]. The sidedness of CRC has been considered a factor for the biological differences between CRCs; however, the association of the gut microbiota and CRC location is largely unknown.

Gut microbiota are involved in food digestion, nutrition, and metabolism in the intestinal mucosa via short-chain fatty acids, and they are also involved in immune development and regulation [5]. Furthermore, they are a crucial component of the cancer microenvironment and play an essential role in cancer immunity [6]. Recently, advances in sequencing technology have made it possible to analyze several microbiomes, and the use of conserved 16s rRNA sequences of bacteria has enabled high-resolution bacterial sequences to be obtained without the need for culture [7]. Studies have specified the possibility of using the gut microbiota as a biomarker by detecting specific bacteria and using these for the early diagnosis of CRC [8]. However, no relationship between the microbiota and CRC tumor location is yet established. This study was conducted to evaluate the differences in the microbiota and tumor locations in patients with CRC.

| Clinicopathological characteristics of CRC patients | Right-sided (n = 16) | Left-sided (n = 37) | p value |
|---------------------------------------------|---------------------|--------------------|--------|
| Mean age, years                             | 65.8                | 70.1               | 0.244  |
| Male sex                                    | 6 (37.5)            | 19 (51.3)          | 0.353  |
| A past history of CRC                       |                      |                    |        |
| Yes                                         | 4 (25.0)            | 3 (8.1)            | 0.095  |
| Tumor depth                                 |                      |                    |        |
| m/sm                                        | 3 (18.7)            | 8 (21.6)           | 0.127  |
| mp                                          | 3 (18.7)            | 10 (27.0)          |        |
| ss/se/A                                     | 10 (62.5)           | 18 (48.6)          |        |
| Lymph node metastasis                       |                      |                    |        |
| Positive                                    | 4 (25.0)            | 13 (35.1)          | 0.468  |
| Differentiation                              |                      |                    |        |
| tub1/tub2                                   | 14 (87.5)           | 35 (94.5)          | 0.369  |
| muc/pap/por                                 | 2 (12.5)            | 2 (5.4)            |        |
| Distant metastasis                          |                      |                    |        |
| Yes                                         | 0                   | 5 (13.5)           | 0.122  |

Values express n (%), unless otherwise indicated.

Data Analysis

Read sequences obtained using fastq_barcode_splitter of the FASTX Toolkit v0.0.14 were extracted. After removing primer sequences, we derived a representative sequence and used the OTU table with a feature-classifier plug-in to compare the obtained representative sequences with Silva 138 (99% OTU full-length sequences) for phylogenetic estimation. Phylogenetic trees were then created using the alignment and phylogeny plug-ins.

Statistical Analysis

Statistical analysis was performed using Qiime2. The Kruskal-Wallis test was performed to examine the alpha diversity, and permutational multivariate analysis of variance (PERMANOVA) was performed to determine the alpha or beta diversity. A linear discriminant analysis (LDA) effect size algorithm (LEFSE) was used for detecting biomarkers [9]. Comparisons of bacterial species were performed by multivariate analysis using the analysis of the composition of microbiomes (ANCOM), followed by analysis with LEFSE. Pathway analysis was performed using PICRUSt2 in Qime2 with the Metabolic Pathway Database pathway, and then evaluated using LEFSE.

Table 1. Clinicopathological characteristics of CRC patients

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of the patients was 67.0 years. Eleven patients had early-stage cancer and 42 had advanced cancer. Stage I was the most common stage. Seven patients had a history of treatment for CRC. The patients were divided into 2 groups according to the location of the primary tumor, with the splenic flexure as the boundary; 16 patients (30.1%) had a right-sided CRC and 37 (69.8%) had a left-sided CRC. The alpha diversity in the feces samples of the patients was examined using the OTU richness, Shannon index, and Pielou’s evenness, and we found no difference in the alpha diversity of the intestinal microbiota regarding the location of the primary CRC (Fig. 1a–c). Alternatively, the beta diversity of the Bray-Curtis distance was significantly higher for left-sided tumors (Fig. 1d, e).

We investigated the differences in the composition of the gut microbiota according to the location of the primary tumor (Fig. 2a). Firmicutes were the most dominant species in both left- and right-sided CRCs at the phylum level. Fusobacteriota was more abundant in left-sided CRCs at the phylum level whereas Micrococcaceae was significantly more abundant at the family level in right-sided CRCs (Table 2). LDA, combined with LEFSE, showed that Blautia, Erysipelotrichales, Holdemanela, Faecalibacterium, Subdoligranulum, and Dorea were predominant in right-sided CRCs. Interestingly, Fusobacterium was the abundant bacterial flora in left-sided CRCs (Fig. 2b, c).

To analyze the functional roles of the gut microbiota in CRC, we performed PICRUSt2 functional analysis of the gut microbiota according to the primary tumor site. We found that L-lysine fermentation and cob(II)yrinate a,c-diamide biosynthesis I were predominant in left-sided CRCs (Fig. 3).

**Discussion**

In this study, we investigated the differences in the gut microbiota according to primary tumor location in the feces of patients with CRC using next-generation se-
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The composition and function of the gut microbiota differed according to the sidedness of CRC, which is also a tumor characteristic associated with clinical prognosis [10].

*Fusobacterium* is an abundant component in patients with CRC compared with healthy controls, and serves as a biomarker [8]. *Fusobacterium* has been reported to be important in the induction of immune cells [11], along with other oral microbiomes and biofilms [12]. These biofilms induce inflammation in the colonic mucosa via interleukin-6 and are involved in colorectal carcinogenesis as an initiating factor [13]. Primary tumor progression influences the composition of the microbiota. However, no significant difference in tumor depth was ob-

![Fig. 2. The difference in the microbiome between left- and right-sided tumors in colorectal cancer. a The dominant phyla of microbiomes. b, c Histogram of the linear discriminant analysis (LDA) scores for differentially abundant genera calculated using the LDA effect size (LEFSE) according to the tumor location.](image)

| Table 2. Comparisons of families of bacterial genera were performed by multivariate analysis using ANCOM |
|---------------------------------------------------------------|
| **Percentile** | 0 | 25 | 50 | 75 | 100 | 0 | 25 | 50 | 75 | 100 |
| **Group** | L | L | L | L | L | R | R | R | R | R |
| **Fusobacteriota** | 1 | 1 | 7 | 101 | 587 | 1 | 1 | 1 | 1 | 24 | 6 |
| **Micrococcaceae** | 1 | 1 | 3 | 12 | 36 | 1 | 8.75 | 39 | 63 | 283 | 26 |
served between the 2 groups in this study, with 21.6% early cancers on the left side and 12.5% on the right side. These results suggest that *Fusobacterium* is dominant in left-sided CRCs, regardless of primary tumor progression.

We found that *Blautia*, Erysipelotrichales, *Holdemanelia*, *Facealibacterium*, *Subdoligranulum*, and *Doreaga* were predominant in right-sided CRCs, and *Fusobacterium* was the abundant bacterial flora in left-sided CRCs. Some studies have described the differences in microbiomes according to the location of primary CRC. Kohoutova et al. [14] reported on the microbiota from mucosal biopsy by colonoscopy after bowel preparation. They found a higher incidence of *Escherichia coli* phylogroup B2 in patients with right-sided CRC by culture and PCR. Gao et al. [15] analyzed the microbiota in 31 CRC mucosal tissues and compared the gut microbiota in the mucosa between proximal and distal CRCs. *Fusobacterium*, *Escherichia*, *Shigella*, and *Leptotrichia* in mucosal tissue were identified in relatively higher abundance in the proximal tumoral mucosa. The colon’s mucosal layer has a unique bacterial flora [16], and the microbiota in the feces differ from the mucosa-associated microbiota [17]. In addition, the administration of laxatives and antibiotics as well as surgical invasion can temporarily alter gut bacteria [18] (even though we detected *Fusobacterium* among the mucosa-associated bacteria in left-sided CRC).

The diversity of the gut microbiota is reduced in patients with CRC compared with that in healthy individuals [19]. Some studies have reported increased diversity in patients with CRC compared with in healthy individuals [20, 21]. In this study, the alpha diversity was not altered based on the tumor location but the beta diversity was increased in left-sided CRC, suggesting that the intercommunity diversity varies according to the location of the primary CRC.

Metabolites from the gut microbiota have a biological function linked to several diseases, including inflammatory bowel disease [22]. The clinical association of the metabolites with cancer incidence or progression is largely unknown. In this study, Micrococcaceae was significantly more abundant in right-sided CRC; it is involved in L-lysine synthesis. Microbiomes in right-sided CRC synthesize L-lysine, and the pathway of L-lysine fermentation to acetate and butanoate was also involved in left-sided CRC. Butyrate is a short-chain fatty acid essential for colonic epithelium homeostasis [23]. *Fusobacterium nucleatum* and *Intestinimonas* AF211, which belong to clostridial cluster IV, possess enzymes that ferment lysine under anaerobic conditions [24].

As part of the tumor microenvironment, the gut microbiota can influence the incidence and progression of CRC by direct stimulation from bacterial bodies and metabolites, such as the butyrate paradox [19, 23]. In this study, we found that bacterial species and metabolites differ according to CRC location. The results suggest the

**Fig. 3.** Histogram of the linear discriminant analysis (LDA) scores for differentially abundant pathways analyzed using the LDA effect size method according to tumor location.

| Pathways       | Descriptions                                                                 |
|----------------|------------------------------------------------------------------------------|
| PWY0_1297      | Superpathway of purine deoxyribonucleosides degradation                      |
| PWY0_1298      | Superpathway of pyrimidine deoxyribonucleosides degradation                  |
| PWY_2941       | L-lysine biosynthesis II                                                     |
| PWY_7315       | dTDP-N-acetylthomosamine biosynthesis                                        |
| PWY_3801       | Sucrose degradation II (sucrose synthase)                                    |
| P163_PWY       | L-lysine fermentation to acetate and butanoate                               |
| PWY_7377       | cob(II)lyrinate a,c-diamide biosynthesis I (early cobalt insertion)         |
involvement of characteristic gut microbiota and metabolites as factors that cause the clinical course and treatment to differ according to the CRC location. Therefore, focusing on specific gut microbiota could predict the efficacy of molecular-targeted and chemotherapeutic drugs and could lead to the development of new prognostic and therapeutic methods.

This study has some limitations. First, it was a single-center study with patients from a relatively uniform ethnic background in Japan. Because of the diversity of the gut microbiota and how they are strongly influenced by age, race, food, and lifestyle [25], an international multicenter validation is needed to confirm our findings. Second, because this study excluded healthy individuals, projecting the results as a potential biomarker for CRC screening is difficult. Third, we did not examine the long-term prognosis of the patients, including recurrence and sensitivity to anticancer drugs. The association between the gut microbiome and cancer prognosis should be evaluated.

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Statement of Ethics

The study complied with the ethics standards of the Declaration of Helsinki and was approved by the Ethics Committee of Shiga University of Medical Science (ref. No. R2018-037). Written informed consent was obtained from each patient.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

T.M. and M.T. described and designed this project. T. M. performed the next-generation sequencing analysis. H.M., D.Y., Z.H., H.M., T.U., M.K., S.K., H.I., and T.S. collected the clinical samples and data and supported the analysis. M.O., A.A., and M.T. revised this article. All authors read and approved the final version of the manuscript.
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