Altered Profile of Fecal Microbiota of Patients with Colon Diverticulitis

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Objective: During colonoscopies, clinicians often find feces accumulated in the colonic diverticula. We hypothesized that these feces and potential changes in fecal microbial communities contribute to colon diverticulitis. The aim of this study was to investigate potential changes in the fecal microbiota in symptomatic uncomplicated diverticular disease by terminal restriction fragment length polymorphism (T-RFLP) analyses of fecal microbiota from patients with colonic diverticula, and stool samples.

Materials: Fifteen patients with colon diverticulum and 28 healthy volunteers were enrolled. The fecal samples were classified as follows: Group I, feces from a healthy individual; Group II, feces in colonic diverticula obtained during colonoscopy; Group III, feces from the natural defecation of Group II patients.

Methods: Fecal microbiota profiles were evaluated by T-RFLP analysis.

Results: T-RF patterns of fecal microbiota were divided into three clusters. Most Group I samples were included in clusters A and B, whereas most Group II samples were included in cluster C. Operational taxonomic units of 657 bp ($p < 0.05$) and 955 bp ($p < 0.01$) differed in abundance between patients with colon diverticulum and healthy individuals.

Conclusions: T-RFLP analyses revealed that the fecal microbial communities in patients with diverticular disease differed from those of healthy individuals, particularly for the operational taxonomic units of 657 and 955 bp. Changes in the fecal microbiota, including *Lactobacillales* and *Clostridium* subcluster XIVa, may play a role in diverticular disease.

Key words: colon diverticulum, microbiota, T-RFLP, *Lactobacillales*, *Clostridium*

Introduction

Approximately 20% of patients with diverticulosis will experience inflammatory complications of the disease, ranging in severity from a single mild acute attack of diverticulitis to more severe attacks characterized by perforation and abscess formation¹⁾. Aging and reduced dietary fiber intake are the main pathological factors in this disease^{2) 3)}. The pathophysiology of diverticulitis involves an imbalance in colonic microbiota^{4) 5)}. During colonoscopy, feces are often found accumulated in the colonic diverticula. These feces differ in appearance

or solidity from those in the main intestinal tract. Thus, we hypothesized that the microbiota of feces in the diverticula may differ from that of normal feces and cause diverticulitis.

Terminal restriction fragment length polymorphism (T-RFLP) analysis enables the assessment of bacterial community structure and ecosystem diversity⁶⁾. Research findings obtained by T-RFLP are consistent with those obtained by next-generation sequencing (NGS), which is now widely used⁷⁾. In the current study, we investigated changes in the diversity of fecal microbial communities in the colon diverticula of patients with colon diverticulitis by

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T-RFLP.

Materials and Methods

1. Participants and method

This study was a pilot case-control study carried out between December 2013 and November 2015. Fifteen patients who were diagnosed as having colon diverticulitis by computed tomography within the past 6 months, and were found to have several right-sided colonic diverticula containing impacted feces by colonoscopy were included in this study. We performed colonoscopies to collect feces accumulated in the right-sided colonic diverticula with biopsy forceps, and all patients who underwent colonoscopy provided a natural defecation specimen for analysis on the same day. As a control, healthy volunteers also provided natural defecation samples. All samples were stored in 100 mM Tris-HCL (pH 9), 40 mM ethylenediaminetetraacetic acid, 4 M guanidine thiocyanate, and 0.001% bromothymol, immediately. Exclusion criteria included a history of or current colorectal cancer, a history of or current chemotherapy or radiation therapy, and current use of antibiotics, probiotics or immunosuppressants. Fecal microbiota profiles were evaluated by T-RFLP.

The fecal samples were classified as follows: Group I, stool samples from healthy individuals; Group II, samples of feces accumulated in the colonic diverticula; Group III, natural defecation samples from Group II patients. All procedures used in this study were approved by the ethical committee of Asakusa Hospital (Approval number 190007). Written informed consent was obtained from the patients for inclusion in this study and for the publication of this report and its accompanying images.

2. Extraction of DNA

Using the fluorescently labeled 516 forward primer (5'-TGCCAGCAGCCGCGGTA-3') and 1492 reverse primer (5'-GGTTACCTTGTTACGACTT-3'), the 16S rRNA genes were amplified from fecal DNA. The 5' end of the forward primers was labeled with 6-carboxyfluorescein (6-FAM), which was synthesized using a MultiScreen PCR₉₆ filter plate (Millipore, Tokyo, Japan). The PCR amplification of DNA samples was performed according to the protocol described by Nagashima *et al*⁷⁾. The

purified polymerase chain reaction products (3 µl) were digested with 10 U of *Bs*II (New England Biolabs Inc., Ipswich, MA, USA) at 55°C for 3 h. The T-RF lengths were determined using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Tokyo, Japan) in GeneScan mode.

3. T-RFLP analysis

T-RF patterns were classified into 29 operational taxonomic units (OTUs) in accordance with the Nagashima method^{7) 8)}. Individual OTUs were quantified as a percentage of the total OTU area, expressed as a percentage of the area under the curve (%AUC), which indicates the predominance of the different microorganisms that comprise each peak⁶⁾. *Bs*II-digested T-RFLP profiles with predicted bacterial species were evaluated (Techno-Suruga Laboratory Co., Ltd., Shizuoka, Japan). Cluster analyses were performed, and a dendrogram of the T-RFLP profiles was generated by applying Ward's method. In this study, we focused on OUT of 657 bp including *Lactobacillales*, which is strongly associated with irritable bowel syndrome (IBS), and OUT of 956 bp reported to decrease in inflammatory bowel disease cases⁹⁾.

4. Statistical analysis

The T-RF peak patterns were compared between patients with colon diverticulitis and healthy individuals using the Wilcoxon rank sum test. The canonical judgment function coefficient was obtained from discriminant analysis of the three groups. Each OTU was compared among the three groups using one-way analysis of variance (ANOVA). Differences with a p-value of less than 0.05 were regarded as significant. The data were analyzed using SPSS (IBM SPSS Statistics 20.0, Tokyo, Japan).

Results

Twelve men and three women, mean age 70 years [range: 60–77 years], were examined. Of these patients, six (40%) also had diverticula in the left-sided colon. Multiple diverticula were defined as three or more diverticula in the right-sided colon. To prevent constipation, most patients had been taking oral magnesium oxide. None of the patients had a history of colorectal cancer or severe cardiovascular disease. Feces from 28 healthy

Figure-1 Analysis of fecal bacterial communities from all participants

The dendrogram shows the similarities of intestinal microbiota terminal restriction fragment length polyphism (T-RFLP) patterns from 43 fecal samples. The data from T-RFLP analysis used the restriction enzyme *Bs**II*. Solid triangles (▲) represent colonic diverticulosis, and open circles (○) represent healthy samples. Eleven of 28 healthy individuals (39%) were included in cluster A.

volunteers, 23 men and five women, mean age of 66.5 years [range: 54–81 years], without constipation, colon diverticula by colonoscopy, or serious disease were collected as control samples. None of the control participants had been taking any intestinal regulators or received any oral antibiotics within the past one month. Although a few control participants had diabetes mellitus or hypertension,

their conditions were being controlled with medication.

The dendrogram shows a comparison between fecal microbial communities in Group I and Group II. The data are from cluster analyses of fecal microbial communities from *Bs**II*-digested T-RF patterns (Figure-1). T-RF patterns were assigned to three major clusters (clusters A, B and C) on the

Table-1 Terminal restriction fragment length polymorphism profiling of fecal samples from healthy individuals and those with diverticular disease

OTU	Genus	Group I ^{a)} (n = 28)	Group II ^{b)} (n = 15)
106	<i>Clostridium</i> subcluster XIVa	2.53 ± 0.92	0.37 ± 0.19*
317	<i>Prevotella</i>	6.83 ± 2.49	0.42 ± 0.42*
369	<i>Clostridium</i> cluster IV	0.41 ± 0.41	3.15 ± 1.22*
657	<i>Lactobacillales</i>	5.01 ± 1.58	12.56 ± 2.65*
749	<i>Clostridium</i> cluster IV	5.76 ± 0.95	1.00 ± 0.52**
919	<i>Clostridium</i> cluster XI <i>Clostridium</i> subcluster XIVa	1.52 ± 0.26	2.72 ± 0.40*
940	<i>Clostridium</i> subcluster XIVa <i>Enterobacteriales</i>	5.27 ± 0.61	13.99 ± 3.08*
955	<i>Clostridium</i> subcluster XIVa	5.06 ± 0.51	2.31 ± 0.76**

Data are mean ± standard deviation of the percentage of the total area under the curve (%AUC). OTU, operational taxonomic unit. ^{a)} Group I: stool samples from healthy individuals. ^{b)} Group II: fecal samples collected from the diverticula of patients with colonic diverticulosis.

*p < 0.05, **p < 0.01

Figure-2 Box plot comparing the diversity of terminal restriction fragment length polymorphism profiles in fecal microbiota between the three groups. The canonical judgment function coefficient was obtained from discriminant analysis.

basis of their similarities. Of the 28 healthy individuals, 11 were in cluster A, 12 were in cluster B, and five were in cluster C. For the 15 patients with diverticular disease, T-RF patterns from 14 of 15 samples from Group II were in cluster B, and the

remaining one in cluster A. The %AUC values of representative OTUs after *Bs*II digestion are shown in Table-1. The OTUs (369, 657, 919, 940 bp) were increased in Group II. In particular, OTUs of 657 bp (*Lactobacillales*) and 940 bp (*Clostridium* subcluster XIVa, *Enterobacteriales*) were significantly increased in Group II. In contrast, OTUs (106, 317, 749, and 955 bp) predicting the *Clostridium* genus were decreased in Group II compared with Group I, with a significant decrease in OTUs of 749 bp (*Clostridium* cluster IV) and 955 bp (*Clostridium* subcluster XIVa). The canonical judgment function coefficient was obtained from discriminant analysis of Groups I, II and III. The results showed that the scores of Groups II and III were significantly different from those of Group I using one-way ANOVA (both p < 0.01). There were no significant differences between Groups II and III (Figure-2).

OTUs were then compared among the three groups using one-way ANOVA. An OTU of 657 bp (*Lactobacillales*) was increased in Groups II and III compared with Group I (p < 0.05). Additionally an OTU of 955 bp (*Clostridium* subcluster XIVa) was decreased in Groups II and III compared with Group I (p < 0.01, Figure-3).

Figure-3 Analysis of operational taxonomic units (OTU) in stool samples from the three groups

Discussion

The prevalence of colonic diverticulitis has increased worldwide. In Japan, diverticula were located predominantly in the right side of the colon in 50.0% of cases, bilaterally in 29.3% of cases, and in the left side in 20.7% of cases¹⁰⁾.

The prevalence of colonic diverticulosis increases with age. In this pilot study, we focused on the right diverticulum, which is the most common location of diverticulosis in the Japanese population. In our follow-up studies, we will examine the left diverticulum.

The pathophysiology of colon diverticulitis is complex and may be related to genetic factors, abnormal colonic motility, abnormality in the autonomic nervous system, macroscopic morphological changes in the large intestine, visceral hypersensitivity, and an imbalance in colonic microflora²⁾. To our knowledge, this study is the first to show an altered profile of intestinal microbiota in feces accumulated in the diverticula. Information on microbial diversity in patients with diverticular disease is relatively scarce. Here, we utilized a T-RFLP approach to compare microbial diversity in gastrointestinal populations from patients with diverticular disease and healthy individuals.

T-RFLP analysis facilitates the comparison of complex microbiota diversity, because the fluorescently labeled T-RFs are able to be visualized^{11) 12)}. Notably, no studies have shown whether the fecal

microbiota communities accumulated in the diverticula differ from those of stool specimens in the same patients. If the fecal matter accumulated in the diverticula of a patient with diverticulitis and the microbiota of the patient's natural defecation were the same, it could be assumed that microbiota analysis would be easier. Our findings suggest that there were no differences in these communities. Moreover, our cluster analysis dendrogram indicates that the fecal microbial communities of patients with diverticular disease differed from those of healthy volunteers. These differences involved a significant decrease in the *Clostridium* genus, including *Clostridium* cluster IV and sub-cluster XIVa, and a significant increase in *Lactobacillales* and *Enterobacteriales* in patients with diverticular disease. Decreased *Clostridium spp.* is a common feature in many studies of inflammatory bowel disease (IBD)^{13) 14)}. Linnings *et al.* demonstrated that patients with diverticular disease had higher amounts of *Enterobacteriaceae* in the colon mucosa than patients without diverticula¹⁵⁾. However, it is still unclear whether diverticulitis occurs due to changes in the gut microbiota. Spiller and Sloan hypothesized that the presence of diverticula generates a unique environment that contributes to important changes in the diversity of fecal microorganisms with potential effects on the intestinal immune system¹⁶⁾. The differences between the feces of patients with diverticular disease and those of healthy individuals suggest that dysbiosis contributes to diverticular disease.

In this study, we focused on two OTUs (657 and 955 bp). In patients with diverticular disease, we found that an OTU of 657 bp (*Lactobacillales*) was increased, whereas that of 955 bp (*Clostridium* subcluster XIVa) was decreased. The OTU of 657 bp included *Streptococcus spp.* (*S. parasanguinis*, *S. salivarius*, *S. thermophilus*, *S. vestibularis*, *S. pasteurii*), and *Lactobacillus spp.* (*L. salivarius*, *L. cellobiosus*). *Lactobacillus* is a beneficial microbial genus, acting to prevent intestinal infection and activate the immune system. Thus, it seems counter-intuitive that *Lactobacillus* was increased in patients with diverticular disease. On the other hand, it has been reported that *Lactobacillus* is increased in patients with IBS characterized by abnormal bowel movements⁹⁾. *Streptococcus*, however, may increase inflammation in the human gut. Little is known about *Clostridium* subcluster XIVa, with the exception that it contains many species that produce butyric acid. Butyrate has many beneficial effects in the host such as trophic and anti-inflammatory effects on epithelial cells¹⁷⁾. Thibault *et al.* showed that the butyrate transporter is down-regulated in the colonic mucosa of patients with IBD¹⁸⁾. In addition, butyrate-producing bacteria are decreased in the gut microorganisms of the intestinal mucosa and feces of patients with IBD¹⁹⁾. Furusawa *et al.* demonstrated that butyrate induces the differentiation of colonic regulatory T cells in mice²⁰⁾. *Clostridia* can induce colonic regulatory T cells, which are involved in the suppression of inflammatory and allergic responses²¹⁾. These results suggest that an insufficiency of butyrate may be associated with the pathogenesis of IBD. Similarly, decreases in *Clostridium* subcluster XIVa found in patients with diverticular disease may result in a reduction in butyric acid in the bowel, which may contribute to diverticular disease development.

This study had some limitations. Firstly, we did not control for sex, medication, or diet; instead, patients with stomachache were subjected to colonoscopy, which may have led to selection bias. In our follow-up studies, we will control for subject characteristics such as sex, medication, and localization of diverticulitis in the colon. Secondly, the number of patients was small as this was only a pilot study. Additional studies are needed to confirm our findings. Thirdly, although T-RFLP analysis pro-

vides reproducible comparisons of microbial communities⁶⁾, it does not provide high-resolution data, as does NGS. Finally, this study was performed at a single institution. Recent studies have demonstrated that regional characteristics affect the fecal microbiota; thus, our observations may be limited to the region in which our hospital is located^{22) 23)}.

In conclusion, T-RFLP analyses revealed that the fecal microbial communities in patients with diverticular disease differed from those of healthy individuals, particularly for the operational taxonomic units of 657 and 955 bp. Changes in the fecal microbiota, including *Lactobacillales* and *Clostridium* subcluster XIVa, may play a role in diverticular disease.

Conflict of interest

The authors declare no conflicts of interest in association with the present study.

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