Analysis of the colonic mucosa associated microbiota (MAM) using brushing samples during colonoscopic procedures

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The mucosa-associated microbiota is an important component in human microbiota. The aim was to investigate mucosa-associated microbiota using brush samples during endoscopic procedures and compare with fecal microbiota. Seven patients who were planning to undergo a routine colonoscopy were recruited. Mucosal brushing samples were taken from 3 sites (terminal ileum, ascending and sigmoid colon), and a fecal sample was taken on the morning of colonoscopy. The samples were immediately placed in microcentrifuge tubes containing DNA stabilization reagent and analyzed using the next generation sequencer. The individual differences in microbiota were more evident than the differences of the sampling sites. Actinobacteria was more abundant and Bulleidia and Oriibacterium were significantly more abundant and the proportions of genes responsible for transcription factors and phosphotransferase system were higher in ileal mucous than those in the fecal samples. Brushing during colonoscopic procedure instead of using feces samples might be useful to analyze mucosa-associated microbiota.

Key Words: mucosa associated microbiota (MAM), colonic mucus, brushing samples, 16S rRNA sequencing, taxonomic composition

H uman gut microbiota is complex and diverse communities of commensal microorganisms.1,2 Intestinal commensal bacteria were divided into the two compartments, the transient luminal compartment and the mucosa-adherent compartment consisting of entrenched residents. Mucosa-adherent compartment is known as mucosa-associated microbiota (MAM).1,2 Many studies have focused on the fecal microbiota which are predominantly resident in the lumen. The fecal compartment is influenced by diet,3 while MAM is the more stable adherent compartment that adhere to mucosal surface of GI tract.4 There is increasing attention about the MAM of the GI tract in patients with a variety of diseases such as coeliac disease, irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), etc.5 Thus evaluating MAM in precise and accurate manners is recognized as a clinically important trial to characterize and understand the host-microbe interactions and their roles in the development of health promotion and diseases.

However, the standard research methodology for MAM has not been established. We have already reported that gastric mucus can be used for molecular testing to confirm the diagnosis of gastric MAM, Helicobacter pylori (H. pylori) infection and their clarithromycin susceptibility.6 In this study, we evaluated the intestinal MAM analysis by using brushing samples taken from ileum end, ascending and sigmoid colon during colonoscopic procedures and compared the microbiota between the brushing samples and feces.

Materials and Methods

Ethics. Ethical approval was obtained from Kawasaki Medical School Ethic and Medical Research Committee (no. 2094). Written informed consent was achieved from each research subject before enrollment. All patients were enrolled at the Division of Gastro-enterology of Kawasaki Medical School Hospital. The study was registered at the University Hospital Medical Information Network Center (UMIN000018601).

Patient recruitment and sample collection. We recruited consecutive 7 adults [6 women, mean age 54.1 years old (42–79)] who were referred for colonoscopy. Patients with antibiotic use within 3 months, colon cancer, history of coloectomy, active GI bleeding, hospital admission in the preceding six-month, were excluded. Brush samples were taken from 3 sites (terminal ileum, ascending and sigmoid colon) using endoscopic microbiology brush (COOK, Bloomington, IN) after usual preparation for colonoscopy using polyethylene glycol (PEG). Fecal samples were taken on the morning of colonoscopy.

DNA extraction, polymerase chain reaction (PCR) amplification, library preparation and 16S ribosomal RNA (16S rRNA) sequencing. DNA was extracted using lysis methods, and preparation of library of amplicons encoding 16S rRNA gene and sequencing were conducted as previously reported.8 The samples were profiled by high-throughput amplicon sequencing with dual-index barcoding using the Illumina Miseq platform (Illumina, San Diego, CA). The V3–V4 regions of the gene encoding 16S rDNA (460 bp) were tailed PCR amplified.9 PCR amplicons were purified using SPRI select beads (Beckman Coulter). DNA concentration of purified amplicons were measured using a Quanta Fluorometer and the QuantiFluor® dsDNA System (Promega, Madison, WI) and approximately equal amount of their DNA were pooled. The pooled sample was sequenced using Miseq Reagent Kit V3 (600 cycle) (Illumina, San Diego, CA) on...
the Misq system according to the manufacturer’s instructions. Sequence data were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (ver. 1.8.0).

**Bioinformatics analysis.** Processing of sequence data, including chimera check, operational taxonomic unit (OTU) definition and taxonomy assignment, was performed using QIIME ver. 1.8.0, USEARCH 6.1 and UCLUST 1.2.22. Open-reference OTU picking was performed at the 97% sequence similarity level against Greenegenes Database ver. 13.8.10-11

**α-Diversity and β-diversity.** The observed species, Chao1, and Shannon diversity indices were calculated by the phyloseq package of R software. α-Diversity was estimated using the UniFrac metric to calculate the distances between the samples using QIIME ver. 1.8.0. It was visualized by principal coordinate analysis (PCoA) and statistically analyzed using permutational multivariate analysis of variance (PERMANOVA) using R software. PERMANOVA was performed by vegan package.

**Functional differences of microbiome.** Potential differences in the microbiome at the functional level were evaluated by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software using 16S rRNA sequence profiles. Metagenome content was estimated based on reference bacterial genomes and the KEGG pathway database. The results were converted to relative abundance data for statistical analysis.

**Statistical analysis.** The relative abundance of bacterial genera and metagenome content predicted with PICRUSt, and diversity indices were compared between the sampling sites by paired one-way ANOVA in R software. *p*<0.05 was taken to indicate a significant difference and paired *t* test was also used to determine where the differences were.

**Results**

**The bacterial load of colonic mucus.** The α-diversity (observed species, Chao1, Shannon-Wiener Index) among brushing samples taken from different anatomical sites of lower GI tract and feces was compared. The observed species of brushing samples were lower compared to the feces samples and the statistical significant was observed among the four groups (Fig. 1, *p* = 0.047, paired ANOVA). However, there was no statistically significant difference between each group. Besides, there was no statistically significant difference in the other indexes of α-diversity of Chao1 and the Shannon index (Supplemental Fig. 1A and B*).

The overall structure of the gut microbiome among the different anatomical sites was evaluated using β-diversity indices calculated for UniFrac distance. PCoA revealed no microbial structural differences among the four different sites (PERMANOVA, *p* = 0.783 for weighted UniFrac and *p* = 0.237 for unweighted UniFrac). The samples from the same participant tended to locate closely together and were more similar regardless of anatomical location, indicating the existence of inter-individual similarity. However, the microbial structure of the feces samples tended to be different from those of brushing samples in PCoA plots for both weighted and unweighted UniFrac distance (Fig. 2A and B).

**The bacterial community profiles of colonic mucus.** In the bacterial community profile of phylum level, Actinobacteria phyla was tended to be more abundant in the brush samples than in the fecal samples. In contrast, Bacteroidetes was less abundant in the brush samples (Fig. 3). In the class (order) levels, Actinobacteria (Bifidobacteriales) and Bacilli (Lactobacillales) tended to be richer in brushing samples, while Bacteroidia (Bacteroides) was less rich (Supplemental Fig. 2A and B*).

**Comparative analyses of the taxonomic composition of the microbial community.** Comparative analysis of the taxonomic composition at the genus level between the brushing samples and fecal samples indicated a significant increase in the abundance of *Bulleidia* and *Oribacterium* in ileum brushing samples (Fig. 4A), *Veillonella*, *Bulleidia* and *Corynebacterium* in ascending colon (Fig. 4B), and *Lactobacillus* and *Corynebacterium* in sigmoid colon (Fig. 4C). In contrast, *Phascolarctobacterium*, *Oscillospira* and *Lachnospiraceae* were significantly abundant in the fecal samples.

**PICRUSt predictions of the functional composition of the metagenome.** Potential differences in the function of the microbiome were evaluated using PICRUSt software. When comparing between feces and mucus samples, the proportions of the genes responsible for glycerolipid metabolism were significantly increased in mucus samples compared to feces samples (Fig. 5A–C). In addition, the proportion of genes responsible for transcription factors and phosphotransferase system and phosphotransferase system (PTS) were significantly increased in ileum compared to fecal samples.

**Discussion**

MAM would be an attractive target for analysis of microbiota and was mainly analyzed using biopsy. Our endoscopic brushing methodology was thought to be suitable to evaluate microbiota, because the bacterial load and the OTUs in the Next-Generation Sequencing analysis were not different between the brushing samples and feces samples. Most previous studies of MAM used samples collected by mucosal biopsy, however biopsy sampling was more invasive than brushing due to bleeding, especially in patients with a bleeding tendency. We previously assessed whether adherent gastric mucous to biopsy forceps instead of biopsy samples was suitable for the diagnosis of *H. pylori* infection. The sensitivity of our modified molecular detection using DNA extracted from gastric mucous, which was obtained by gently scraping gastric mucosa and present within the rapid urease test gel, was higher than that obtained by biopsy.17 Therefore, brushing may be more useful to detect few bacteria or sparsely localized bacteria covering extended mucosa. Moreover, brushing sample contains much less human DNA.13 The advantage of brushing is that it is noninvasive and makes it possible to avoid massive contamination of human cells in metagenomics analysis of the microbiome. Several papers investigating MAM in Japanese subjects have been previously published,13,14,15 however, there is no data comparing microbiome between brushing samples and feces taken from Japanese subjects.

In addition, the functional analyses of the microbiome using
PICRUSt software indicated some difference in the proportions of the genes between brushing and feces samples. The proportions of genes responsible for glycerolipid metabolism and PTS were increased in brushing samples compared to feces. PTS is a distinct system used by bacteria for glucose uptake where the source of energy is from phosphoenolpyruvate (PEP). Small bowel microbes are essential for host adaptation to dietary lipid changes by regulating gut epithelial processes involved in their digestion and absorption. Therefore, evaluating MAM instead of fecal bacteria seems to be important to investigate a variety of host functions involving intestinal function, micronutrient synthesis, and metabolism.

Kashiwagi et al. investigated the MAM profiles in healthy subjects and demonstrated that MAMs profiles were significantly different between the upper and lower gut. The phyla Deferribacteres, Lentisphaerae, and Archaea were detected only in the lower gut, not in the upper gut. Nishino et al. evaluated the colonic MAM profiles in patients with IBD among different anatomical sites (ileum, cecum and sigmoid colon) using methods similar to ours. In the controls, PCoA revealed no microbial structural differences between different anatomical sites indicating the existence of inter-individual similarity, which is similar to our data and the previous reports. However, our comparative analyses of the taxonomic composition of the microbial community revealed the abundance of Bulleidia and Oribacterium in ileum samples, Vellionella, Bulleidia and Corynebacterium in the ascending colon, Lactobacillus and Corynebacterium in the sigmoid colon. Clinical evidence about MAM in Japanese subjects is still lacking, and more investigations are required.

The sigmoid colon could be the most suitable site of MAM analysis using brushing samples because of bacterial load richness, different bacterial profiles from feces, and convenient location for colonoscopy. The mucous layer in distal colon is known to be thicker than in the proximal colon, therefore sigmoid mucous layer is thought to be more stably reserved than ascending mucous. Although our results indicated little variability across the longitudinal axis of the colon and ileum end, similarly with the previous report, the difference in the bacterial community profile of brushing samples from fecal samples was detected. Dominant Actinobacteria and Proteobacteria and less Bacteroidetes, were recognized in ileum end and ascending colon compared to sigmoid colon. Moreover, significantly abundant bacteria in ileum and ascending colon brushing samples were Bulleidia and Oribacterium and both are obligate anerobe and isolated from the oral cavity of patients with periodontitis. Both bacteria in the duodenal mucosa were reported to be associated with obese. Some MAM might be more specifically and stably detected in the brushing samples taken from ileal mucosa. Interestingly, the functional analyses revealed higher proportion of genes responsible for transcription factors in ileum samples. MAM analysis using surgical samples of ileocecal resection is important to confirm the specific function of the ileal MAM. Ileum is the important site of gut immunity, because of the presence of many T helper (Th) cells including Th17 cells and innate lymphoid cells (ILC). The differentiation and functions of Th cells depend on the induction of lineage-specific transcription factors, including the so-called master regulators: T-bet for Th1, GATA3 for Th2, and RORγt for Th17. ILC subsets also depend on...
Fig. 4. Comparative analyses of the taxonomic composition of the microbial community at the genus level. Representative genera with significant differences between groups are presented with the displayed histograms and p value determinations. Comparison of ileum mucus (A), ascending colon mucus (B), and sigmoid colon mucus (C) with feces.
Fig. 5. PICRUSt predictions of the functional composition of metagenome using 16S rRNA gene data and a database of reference genomes. The KEGG database functional categories are shown with the displayed histograms and p value determinations. Comparison of ileum mucus (A), ascending colon mucus (B) and sigmoid colon mucus (C) with feces.
their development and functions by transcription factors, T-bet, GATA3, and RORγt.²⁵ Gury-BenAri et al.²⁵ characterized the spectrum of transcriptional identities of small intestinal ILCs and described signals integrated by ILCs from the microbial microenvironment to generate phenotypic and functional plasticity. Therefore, evaluating ileal MAM might be important to elucidate the pathological role of gut microbiota in the various diseases. We need to further studies investigating the relationship between ileum MAM and transcription factors of gut immune systems in the future.

One of limitations of the study is the small samples size. In addition, we used PEG preparation for colonoscopy which may affect microbiota compositions by removing luminal resident bacteria. Rectal swabs without bowel preparation appeared to be a convenient sampling method.²⁶ However, it was reported that the bacterial community in rectal swabs without preparation was highly similar to that in feces samples.²⁷ The microbiota data in our brushing may represent more intensely mucosa attached bacteria. The further studies in a larger number of patients with various diseases comparing the healthy subjects are required to investigate the specificity for the relationship of target diseases with bacteria such as obesity with Akkermansia muciphila and colorectal cancer with Fusobacterium.

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Abbreviations
IBD inflammatory bowel disease
IBS irritable bowel syndrome
ILC innate lymphoid cell
MAM mucosa associated microbiota
OTU operational taxonomic unit
PCoA principal coordinate analysis
PCR polymerase chain reaction
PEG polyethylene glycol
PEP phosphoenolpyruvate
PTS phosphotransferase system
IQIME Quantitative Insights into Microbial Ecology
16S rRNA 16S ribosomal RNA
Th T helper

Conflicts of Interest
The authors have no conflict of interest to declare in this study. Co-authors, YK, SH, KO and MT belong to the Miyarisan Pharmaceutical Co., Ltd.

References
1. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 2006; 124: 837–848.
2. Li G, Yang M, Zhou K, et al. Diversity of duodenal and rectal microbiota in biopsy tissues and luminal contents in healthy volunteers. J Microbiol Biotechnol 2015; 25: 1136–1145.
3. Sundin J, Rangel I, Fuentes S, et al. Altered faecal and mucosal microbial composition in post-infectious irritable bowel syndrome patients correlates with mucosal lymphocyte phenotypes and psychological distress. Aliment Pharmacol Ther 2015; 41: 342–351.
4. Oswald IP. Role of intestinal epithelial cells in the innate immune defence of the pig intestine. Vet Res 2006; 37: 359–368.
5. Sonnenburg JL, Angenent LT, Gordon JI. Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? Nat Immunol 2004; 5: 569–573.
6. Dlugosz A, Winckler B, Lundin E, et al. No difference in small bowel microbiota between patients with irritable bowel syndrome and healthy controls. Sci Rep 2015; 5: 8508.
7. Matsumoto H, Shiotani A, Nishibayashi H, et al. Molecular detection of H. pylori using a different gastric mucous to biopsy forceps. Helicobacter 2016; 21: 548–553.
8. Hayashi A, Mikami Y, Miyamoto K, et al. Intestinal dysbiosis and biotin deprivation induce alopecia through overgrowth of Lactobacillus murinus in mice. Cell Rep 2017; 20: 1513–1524.
9. Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S rRNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 2013; 41: e1.
10. Navas-Molina JA, Peralta-Sánchez JM, Gonzalez A, et al. Advancing our understanding of the human microbiome using QIME. Methods Enzymol 2013; 531: 371–444.
11. Nishino K, Nishida A, Inoue R, et al. Analysis of endoscopic brush samples identified mucosa-associated dysbiosis in inflammatory bowel disease. J Gastroenterol 2018; 53: 95–106.
12. Langille MG, Zaneveld J, Caporaso JG, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 2013; 31: 814–821.
13. Lavelle A, Lennon G, Docherty N, et al. Depth-dependent differences in community structure of the human colonic microbiota in health. PLoS One 2013; 8: e78835.
14. Kobayashi T, Andoh A. Numerical analyses of intestinal microbiota by data mining. J Clin Biochem Nutr 2018; 62: 124–131.
15. Takagi T, Naito Y, Inoue R, et al. The influence of long-term use of proton pump inhibitors on the gut microbiota: an age-sex-matched case-control study. J Clin Biochem Nutr 2018; 62: 100–105.
16. Takagi T, Naito Y, Inoue R, et al. Correction to: Differences in gut microbiota associated with age, sex, and stool consistency in healthy Japanese subjects. J Gastroenterol 2019; 54: 96–98.
17. Kashiwagi S, Naito Y, Inoue R, et al. Mucosa-associated microbiota in the gastrointestinal tract of healthy Japanese subjects. Digestion 2019. DOI: 10.1159/000496102.
18. Martinez-Guryn K, Nebert T, Frazier K, et al. Small intestine microbiota regulate host digestive and absorptive adaptive responses to dietary lipids. Cell Host Microbe 2018; 23: 458–469.e5.
19. Atuma C, Strugala V, Allen A, Holm L. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am J Physiol Gastrointest Liver Physiol 2001; 280: G922–G929.
20. Matsuoka K, Ota H, Akamatsu T, Sugiyama A, Katsuyama T. Histochemistry of the surface mucus gel layer of the human colon. Gut 1997; 40: 782–789.
21. Eckburg PB, Bik EM, Bernstein CN, et al. Diversity of the human intestinal microbial flora. Science 2005; 308: 1635–1638.
22. Angelakis E, Armougou F, Carrière F, et al. A metagenomic investigation of the duodenal microbiota reveals links with obesity. PLoS One 2015; 10: e0137784.
23. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. Science 2015; 350: 981–985.
24. Robinette ML, Fuchs A, Cortez VS, et al.; Immunological Genome Consortium. Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. Nat Immunol 2015; 16: 306–317.
25. Gury-BenAri M, Thaiss CA, Serafini N, et al. The spectrum and regulatory landscape of ingestive innate lymphoid cells are shaped by the microbiome. Cell 2016; 166: 1231–1246.e13.
26. Budding AE, Grassman ME, Eck A, et al. Rectal swabs for analysis of the intestinal microbiota. PLoS One 2014; 9: e101344.
27. Bassis CM, Moore NM, Lolans K, et al. Comparison of stool versus rectal swab samples and storage conditions on bacterial community profiles. BMC Microbiol 2017; 17: 78.