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High-throughput 16S rRNA gene sequencing reveals alterations of mouse intestinal microbiota after radiotherapy

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ABSTRACT

The mammalian gastrointestinal tract harbors a highly complex microbial community that comprises hundreds of different types of bacterial cells. The gastrointestinal microbiota plays an important role in the function of the host intestine. Most cancer patients undergoing pelvic irradiation experience side effects such as diarrhea; however, little is currently known about the effects of irradiation on the microorganisms colonizing the mucosal surfaces of the gastrointestinal tract. The aim of this study was to investigate the effects of gamma irradiation on the compositions of the large and small intestinal microbiotas. The gut microbiotas in control mice and mice receiving irradiation treatment were characterized by high-throughput sequencing of the bacterial 16S rRNA gene. Irradiation treatment induced significant alterations in the bacterial compositions of the large and small intestines at the genus level. Unexpectedly, irradiation treatment increased the number of operational taxonomic units in the small intestine but not the large intestine. In particular, irradiation treatment increased the level of the genera *Alistipes* in the large intestine and increased the level of the genus *Corynebacterium* in the small intestine. By contrast, compared with that in the corresponding control group, the level of the genera *Prevotella* was lower in the irradiated large intestine, and the level of the genera *Alistipes* was lower in the irradiated small intestine. Overall, the data presented here reveal the potential microbiological effects of pelvic irradiation on the gastrointestinal tracts of cancer patients.

1. Introduction

Recent studies have shown that the human body is inhabited by at least ten times more microorganisms than the number of somatic and germ-line cells it contains [1]. The host gut and its microbiota have co-evolved intricate relationships, and the mammalian gastrointestinal tract is colonized by 10–100 trillion microorganisms that are essential to host cell maintenance in health and disease; therefore, the human gut microbiota has attracted increasing interest from medical researchers. Comprehensive 16S rRNA gene-based analyses of fecal microbial communities have demonstrated that the gut microbiota is highly diverse between individuals; however, *Bacteroidetes* and *Firmicutes* are the predominant phyla in most individuals [2,3]. The majority of studies examining the beneficial and/or pathogenic influences of microbes on intestinal diseases have focused on irritable bowel or inflammatory bowel diseases [4,5]; however, recent evidence has linked the composition of the gut microbiota to gastrointestinal responses to irradiation [6–9].

Radiotherapy is a common treatment for cancers, especially gynecological and colorectal cancers. Approximately 70% of all cancer patients receive radiotherapy treatment [6,10,11] and these patients typically present clinical symptoms of gastrointestinal irradiation injury [8,12,13]. Pelvic radiotherapy of gynecological cancer patients causes changes in the microbial composition of the gut; Nam et al. [14] demonstrated that radiation therapy changes the intestinal levels of the *Firmicutes* and *Fusobacterium* phyla significantly. Furthermore, Crawford and Gordon [7] used germ-free mice models to demonstrate microbial regulation of intestinal radio-sensitivity. These two studies focused on the large intestine microbiota and cell function, respectively; however, several

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studies [6,8,15,16] have suggested that injury to the small intestine in cancer patients receiving pelvic irradiation leads to adverse side effects such as diarrhea. Nevertheless, analyses of the small intestine microbiota are lacking because sampling of this region is challenging. Therefore, the objective of this study was to investigate and compare the microbial compositions of fecal samples from irradiated mouse large and small intestines using Illumina MiSeq high-throughput sequencing.

2. Materials and methods

2.1. Animal preparation

Male 8–10-week-old C57BL/6 mice were purchased from Orient Bio (Seongnam, Republic of Korea) and housed with access to irradiated food and water. All mouse experiments were performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of Jeju National University. The mice were irradiated with a single 8 Gy dose using a Cobalt 60 source irradiator [17]. The small and large intestinal contents were collected three days after irradiation. During the study, the mice were fed a commercial AIN-76A Purified Rodent Diet (Dyets Co. Ltd, Bethlehem, PA) *ad libitum*; however, the food consumption was limited to 2–3 g/day.

2.2. Sample collection, DNA extraction, and sequencing

Immediately after the mice were euthanized, the contents of the small and large intestines, excluding the cecum, were obtained by manual extrusion. All samples were placed immediately into sterile plastic tubes using alcohol-sterilized spatulas. In total, five mice were sampled (two from the control group and three from the irradiation group), resulting in a total of ten samples from the large and small intestines. All of the samples were snap-frozen in liquid nitrogen and stored at −80 °C until analysis. DNA was extracted from the samples using the UltraClean® Fecal DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA), according to the manufacturer's instructions. Total DNA was quantified using a NanoDrop® ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). For paired-end sequencing, the bacterial 16S rRNA genes were amplified from the fecal samples using a barcode-tagged primer set designed for use with the MiSeq platform (Illumina, San Diego, CA). The sequences of the primers targeting the V4 hyper-variable region of the bacterial 16S rRNA genes [18,19] were as follows: 515F, 5′- GTGCCAGCMGCCGCGGTAA-3′; and 806R, 5′-GGACTACHVGGGTWTCTAAT-3′. Polymerase chain reaction (PCR) was performed using a published protocol [20,21] with slight modifications. Briefly, the 20 μl of PCR mixtures contained 10 μl of 2 × PCR Master Mix Solution (Solgent, Daejeon, Republic of Korea), 1 μM primers (final concentration), and approximately 10 ng of template DNA. The following thermal cycling conditions were used: initial denaturation at 95 °C for 5 min; followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and then a final extension at 72 °C for 5 min. The amplified products were purified using the LaboPass Gel and PCR Clean-up Kit (Cosmogenetech, Seoul, Republic of Korea). DNA was quantified using a spectrophotometer and mixed in equivalent proportions. Sequencing was performed by Macrogen (Seoul, (Republic of Korea)) using the Illumina MiSeq system (Illumina), according to the manufacturer's instructions.

2.3. Bioinformatics data analysis

The modified pipeline described on the mothur website (http://www.mothur.org/wiki/MiSeq_SOP) was used for the bioinformatics analysis. The bacterial sequence reads were compared to a reference database of known 16S rRNA genes obtained from the Ribosomal Database Project (RDP). The bacterial sequences were assigned taxonomically based on the RDP classifiers [22]. Rarefaction curves, Shannon indices, Good's coverages, and Chao1 nonparametric richness estimators were determined using the mothur package [23]. To increase the analysis quality, reads that included chimeric sequences that were unassigned and/or related to non-bacterial species, such as chloroplasts and mitochondria, were disregarded. Before performing diversity calculations, the library sizes were normalized to that of the smallest library. The calculations were repeated 100 times using random sub-samples of sequences. A 3% dissimilarity level between sequences was used to calculate the diversity estimators. The microbial community structures in different samples were compared using the Fast-UniFrac tool [24], based on the phylogenetic relationships between representative reads (operational taxonomic units or OTUs) from different samples. Trees were constructed using the FastTree program [25]. The Fast-UniFrac sample clustering results were used to compare the hierarchical relationships of the samples [24]. A Mann–Whitney U-test [26] was performed to compare the diversity indices between two groups. Unless otherwise stated, the proportion of total reads representing each taxonomic group was calculated.

3. Results

3.1. Clustering of the mouse gut microbiotas

Table 1 summarizes the sequencing reads, diversity indices, and sample coverages of the mouse intestinal samples included in the study. The control group comprised mice 1 and 2, and the irradiation group comprised mice 7, 8, and 9. After quality control processing and removal of chimeric reads, a total of 51,505 sequences (from 60,394 raw reads) were used for abundance and diversity analyses, as well as taxonomic comparisons. Based on unweighted pair group method with arithmetic mean clustering (using the mothur program) and a principal coordinates analysis using the UniFrac tool, the bacterial communities of the small intestinal samples were divided into two clear groups (Fig. 1); the first group comprised control samples 1 and 2, and the second group comprised irradiated samples 7, 8, and 9. By contrast, a clear division of these groups was not observed for the large intestinal microbiota.

Next, the diversity indices were compared using the qualified sequence reads. The detailed diversity estimates of the large and small intestinal microbiota are shown in Table 1. In the control group, the large intestinal microbiota was significantly different to that of the small intestine (ANOVA, $P = 0.004$). Although the irradiated large and small intestinal samples clustered separately, the difference between the microbiotas in these organs was not significant (ANOVA, $P = 0.292$). The small intestinal microbiota differed significantly between the control and irradiated groups (ANOVA, $P = 0.003$), whereas the large intestinal microbiota did not (ANOVA, $P = 0.586$). Based on the observed number of OTUs, the number of OTUs estimated using the Chao method, and the Shannon indices, the diversity of the small intestinal microbiota in the irradiated group was higher than that in the control group (Table 1 and Fig. 2); in particular, the OTUs were significantly different ($P < 0.05$) between these two groups (Fig. 2b). By contrast, the diversity indices of the large intestinal microbiota were comparable for the control and irradiation groups (Fig. 3).

Rarefaction curves of the OTUs in the large and small intestinal microbiotas did not appear to approach a horizontal asymptote, indicating that the sequencing effort did not saturate diversity