学位論文の要旨

Elucidating colorectal cancer-associated bacteria through profiling of minimally perturbed tissue-associated microbiota

（組織関連微生物叢のプロファイリングによる大腸癌関連細菌の解明）

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Introduction: Colorectal cancer (CRC) is one of the most common cancers worldwide. In Japan, the number of annual deaths due to CRC has increased steadily and exceeded 50,000 in 2016. Colon cancer has a multifactorial etiology, and common risk factors include genetic mutations, unhealthy diet and lifestyle, and disruption of inflammatory processes. Furthermore, some bacteria have been implicated in colonic tumorigenesis, and the mechanisms underlying their oncogenic effects are starting to be understood. Current studies on tissue-associated microbiota in patients with CRC typically involve bowel preparation before sampling. However, bowel preparation can alter intestinal microbiota, and short-term changes in microbiome diversity and composition due to bowel preparation have been shown to introduce confounding effects in gastrointestinal microbiota studies (Harrell L et al., 2012). So, we evaluated the feasibility of identifying CRC-associated bacteria by profiling mucosal microbiota in tumors and adjacent noncancerous colonic tissues from patients who underwent colonic resection without preoperative bowel preparation.

Material and Methods: A cohort of patients diagnosed with CRC undergoing colonic resection at Yokohama City University Hospital was studied between October 2019 and March 2021. Tissue samples were collected from 11 patients undergoing surgery

according to Enhanced Recovery After Surgery protocols. Tissues were handled under sterile conditions, avoiding disruption of cancerous tissue and fecal material mixing. The samples were snap-frozen in liquid nitrogen and stored for analysis. Enrichment cultures were set up for DNA extraction, followed by amplicon sequencing of the V4 hypervariable region of the 16S rRNA gene and shotgun metagenomic sequencing. DNA extraction involved tissue pulverization, phenol-chloroform-based extraction, and purification (Tourlousse et al., 2021). Amplicon sequences were processed, and metagenomic data underwent preprocessing, human DNA removal, assembly, binning, and annotation. The ASV (amplicon sequence variants) and metagenome-assembled genomes (MAGs) were linked using MarkerMAG. Subsequently, data were analyzed for diversity, composition, and differential abundance using various bioinformatic tools in R. Differential abundance analysis was performed to identify ASVs showing significant differences between on-tumor and off-tumor samples. The study provides insights into the microbiota associated with CRC tissues and their genomic characterization using culture-based and molecular techniques, shedding light on potential microbial markers associated with CRC. The study protocol was approved by the Ethics Committee of Yokohama City University (B190600051, F220600030) and was registered in the University Hospital Medical Information Network (UMIN) under UMIN000038703, and in the Japan Registry of Clinical Trials (jRCT) under jRCT1030220239.

Results: This study analyzed colonic tissues from 11 patients undergoing colonic resection without preoperative bowel preparation. Tissue samples were collected from tumors and adjacent non-tumor regions at various distances from the tumor site,

generating a total of 98 samples. The V4 region of the 16S rRNA gene was sequenced, revealing microbiota compositions dominated by *Firmicutes*, *Bacteroidota*, *Proteobacteria*, and *Actinobacteriota*. Patient-wise grouping accounted for most microbiota profile variations ($R2 = 0.89$, $P < 0.001$). While alpha diversity varied between patients, the microbiota's on-/off-tumor location showed no significant association with diversity. Subject-matched on-/off-tumor microbiota analyses displayed variable dissimilarities across patients. Some patients exhibited distinctive on-tumor microbiota compared to off-tumor ones. Notably, genera like *Leptotrichia*, *Streptococcus*, and *Fusobacterium* were enriched in tumor samples. Localized enrichment of specific ASVs at the tumor site was observed, highlighting distinct microbial communities within tumors. Further analyses focused on ASVs enriched in on-tumor samples across all patients, revealing taxa like *Fusobacterium*, *Treponema*, and *Bacteroides* among the enriched ASVs. The study identified hitherto uncultured species and localized enrichment/depletion patterns near tumor sites, emphasizing the site-specific nature of certain microbial taxa. Shotgun metagenomics and binning of assembled contigs led to 115 medium-to-high-quality MAGs. Linking ASVs with MAGs identified genomes enriched at tumor sites, such as *Gemella morbillorum* and *Peptostreptococcus stomatis*, and "Fusobacterium_A ulcerans_A". Several MAGs represented taxa unique to uncultured microorganisms, indicating their potential role in colorectal cancer.

Discussion: The dissimilarity between on- and off-tumor microbiota varied widely among patients, potentially classifying patients based on distinct tumor-associated microbiota. Elevated abundance of specific bacteria that have been reported to be

associated with CRC in the past, such as *Fusobacterium*, *Peptostreptococcus stomatis*, and *Parvimonas micra*, was detected in tumor tissues, consistent with their associations in CRC pathogenesis. However, the exact role of these bacteria—whether as instigators of carcinogenesis or opportunistic occupants in the tumor microenvironment—remains unclear. Notably, the enrichment of these bacteria was localized to the tumor and diminished within a few centimeters from the tumor's edge.

Furthermore, *Fusobacterium* phylotypes other than *F. nucleatum*, particularly "Fusobacterium_A ulcerans_A," demonstrated significant enrichment at tumor sites, potentially encoding the FadA adhesin associated with CRC virulence (Rubinstein et al., 2013). Additionally, multiple species recovered from on-tumor tissue enrichment cultures were found in multiple patients, including *Erysipelatoclostridium ramosum*, *Clostridium_Q symbiosum*, and unclassified Collinsella MAGs, suggesting their possible roles in CRC.

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【論文目録】

Ⅰ　主論文

Elucidating colorectal cancer-associated bacteria through profiling of minimally perturbed tissue-associated microbiota

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Ⅱ　副論文

Ⅲ　参考文献