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Colorectal Cancer Grows and Proliferates Due to Crosstalk Between Intestinal Microbiota and Mucosal Epithelium

Tadashi Ohara, M.D., Ph.D.

Department of Gastroenterology, Kitashinagawa Third Hospital, Kohno Clinical Medicine Research Institute, Tokyo, Japan.

***Correspondence:**
Tadashi Ohara, M.D., Ph.D., Department of Gastroenterology, Kitashinagawa Third Hospital, Kohno Clinical Medicine Research Institute, 3-3- 7 Kitashinagawa, Shinagawa, Tokyo 140-0001, Japan, Tel: +81-3-3474- 1831; Fax: +81-5461-3740.

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ABSTRACT

Background/Aim: *The details of how intestinal microbiota are involved in the carcinogenesis, growth and proliferation of colorectal cancer have not been elucidated. This study investigated how intestinal microbiota were involved in colorectal carcinogenesis, growth and proliferation.*

Subjects and Methods: *Samples were feces and tumor epithelium of patients with colorectal cancer (n=18), and analyzed using a metagenomic analysis after collected genomic DNA from each sample.*

Results: *According to the functional analysis of common microorganisms in feces and tumor epithelium microflora of patients with colorectal cancer, there were no microorganisms which activate any carcinogenic pathways and cancer-related signals. There were observed microorganisms involved in tumor growth such as expression of transporters essential for amino acids uptake, DNA replication / repair, RNA degradation, transcription factors and microorganisms involved in tumor metabolism.*

Conclusions: *Although the colorectal carcinogenic mechanism was unknown, it has been suggested that colorectal cancer may grow and proliferate due to crosstalk between intestinal microbiota and tumor epithelium.*

Keywords
Colorectal cancer, Microbiota, Crosstalk between microorganisms and tumor epithelium.

Introduction
The combination of high-throughput DNA sequencing technology and advanced bioinformatics tools have led to rapid advances in the understanding of microbial ecology and the human microbiome. Short chain fatty acids (SCFAs) are produced by intestinal microbiota. We had reported that SCFAs such as butyric acid, isobutyric acid, and acetic acid have antitumor effects in colorectal cancer cultures [1]. Then, we had investigated the mechanism of SCFA's antitumor action from in silico analysis and reported that SCFAs suppresses molecules involved in tumor growth and tumor metabolism [2]. In silico analysis of the tumor pathology is a comprehensive and one-time computer analysis of biological functions and pathways based on experimental data (bioinformatics) using microarrays, metabolomics, proteomics and RNA-Seq. SCFAs suppress carcinogenesis by suppressing molecules in tumor growth and tumor metabolism.

The relationship between colorectal carcinogenesis and microbiota has not yet been elucidated in details. In order to investigate the relationship between colorectal carcinogenesis / growth / proliferation and intestinal microbiota, I analyzed the microbial flora in feces and tumor epithelium of patients with colorectal cancer in this time.

Subjects and Methods

Total eighteen patients with colorectal cancer were recruited for this study. Samples are feces and tumor epithelium of patients with colorectal cancer. All experiments were performed in accordance with the 1964 Declaration of Helsinki and its later amendments. Patients with colorectal cancer were informed of the experimental nature. Patients provided written, informed consent before participating. The study was approved by the Ethics Committee of Fukushima Daiichi-Hospital, Fukushima, Japan.

Fecal samples were collected from participants and genomic DNA was extracted and used for each analysis. The analysis of microbiota in colorectal tumor epithelium was performed in the same manner as feces. DNA was extracted from 200 μL of the suspension using an automatic nucleic acid extractor (Precision System Science, Chiba, Japan) after centrifugation at 5000 rpm for 1 min. MagDEA DNA 200 (GC) (Precision System Science) was used as the reagent for automatic nucleic acid extraction [3]. Each sample was stored at -4°C prior to DNA extraction.

Sequences of the 16S rRNA gene of gut microbiota were analyzed by next-generation sequencing using the MiSeq system (Illumina, San Diego, CA, USA), as previously described [4]. The V3 and V4 hypervariable regions of the 16S rRNA gene were amplified by polymerase chain reaction (PCR) from microbial genomic DNA using prokaryote universal primer sets [5]. The V3 and V4 regions of the 16S rRNA fragments were amplified in 25-μL PCR reactions containing ~30 ng DNA template, 12.5 μL 2× MightyAmp Buffer (v.2.0; Mg2+, dNTP plus; TaKaRa Bio, Inc., Shiga, Japan), 0.25 μM of each primer, and 0.625 U MightyAmp DNA polymerase (TaKaRa Bio, Inc.). The PCR conditions for DNA amplification were as follows: initial denaturation at 98˚C for 2 min, followed by 35 cycles of annealing beginning at 65˚C and ending at 55˚C for 15 s and extension at 68˚C for 30 s. The annealing temperature was lowered 1˚C every cycle until it reached 55˚C, which was maintained for the remaining cycles. PCR products were purified through a MultiScreen PCRu96 filter plate (Merck Millipore, Billerica, MA, USA). To prepare the amplicon pool, purified products were quantified by real-time quantitative PCR on a Rotor-Gene Q quantitative thermal cycler using MightyAmp reagents and SYBR Plus (TaKaRa Bio, Inc.). Barcoded amplicons were sequenced using paired ends and the MiSeq reagent kit (v.3.0; Illumina) on the MiSeq system (600 cycles, 2×284 bp/cycle; Illumina) [6].

Paired-end reads were concatenated using the FASTQ-join program with default options (Erik A. 2011). Only joined reads with quality value score >20 for >99% of the sequence were extracted using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Chimeric sequences were deleted using uSearch version 6.1 software [7].

Bacterial identification from analyses of the sequence reads was performed using Metagenome KIN software (v.2.2.1; World Fusion, Tokyo, Japan) and the TechnoSuruga Lab microbial identification database (DB-BA 10.0; TechnoSuruga Laboratory, Shizuoka, Japan) using a homology threshold of >97%.

To indicate specific or common taxonomies among the microbial-compositional structures of samples, Venn diagrams were constructed using the R Venn Diagram package (https://www.r-project.org/) based on the microbial-community results returned from DB-BA 10.0 analysis [8]. Heatmap clustering of the bacterial taxonomies identified in the fecal microbiota by the R amap and gplots packages was performed to visualize the abundance of taxonomies and similar microbial-compositional structures [9]. Data for each sample were aligned to a dendrogram constructed using the Ward algorithm based on a Euclidean distance matrix.

Operational taxonomic units (OTUs) were constructed using the raw data reads. To join two paired-end reads, we used FASTQ-join software with default options [10], and chimeric sequences were deleted with uSearch version 6.1 software [11]. OTUs at 97% sequence similarity were selected using the Greengenes database (http://greengenes.secondgenome.com/) andpick_open_reference_otus.py in the quantitative insight into microbial ecology (QIIME)1.8.0 pipeline [10]. QIIME is the genome structural analysis. Functional analysis of microbiota based on the Kyoto Encyclopedia of Genes and Genomes Orthology (KEGG) database was performed using PICRUSt version 1.1.1 [12]. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies.

Statistical analysis was performed with repeated measures analysis of variance for continuous variables and Pearson's chi-squared test for categorical variables. Differences in taxa abundance between groups were measured using an unpaired t test (assuming unequal variance), with significance determined at $p < 0.05$. All data were analyzed using SPSS (v.20.0; IBM Corp. Armonk, NY, USA).

Results

There were Heatmap clustering of taxonomy (species) identified in the fecal microbiota of patients with each part of colorectal cancer using 16s rRNA amplicon sequencing (Figure 1a). QIIME and PICRUSt of taxonomy (species) was each functional analysis tool in the fecal microbiota of patients with colorectal cancer. The colorectal cancer cases included ascending colon cancer, transverse colon cancer, descending colon cancer, sigmoid colon cancer and rectal cancer. In the analysis of these microbial flora, the structural analysis of fecal microorganisms by QIIME showed differences depending on each tumor site (Figure 1b), but the pathological function analysis of microorganisms by PICRUSt did not show any difference depending on the tumor site (Figure 1c).

Analysis by Venn diagram results of fecal microflora of three patients with colorectal cancer (rectal cancer, sigmoid colon cancer and descending colon cancer), and analysis results of

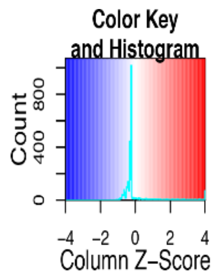

Figure 1a: Heatmap clustering of taxonomy (species) identified in the fecal microbiota of patients with each part of colorectal cancer using 16s rRNA amplicon sequencing.

The eighteen cases of colorectal cancer were ascending colon cancer (3 cases), transverse colon cancer (3 cases), descending colon cancer (4 cases), sigmoid colon cancer (4 cases), and rectal cancer (4 cases), all of which were advanced colorectal cancers.

The number on the left in Figure 1a is each colorectal cancer sample number.

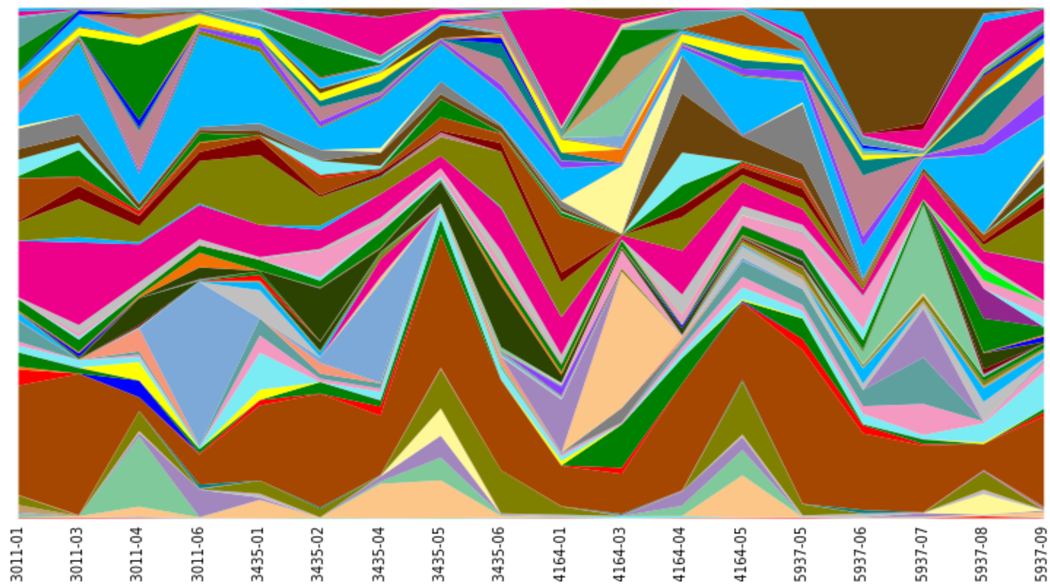

Figure 1b: QIIME of taxonomy (species) in fecal microbiota of colorectal cancer.

The number in the lower part of Figure 1b is sample number for each colorectal cancer.

The genomic structure of each microorganism was displayed in different color, individually.

Genomic structures of microorganisms in feces of patients with colorectal cancer were quite different.

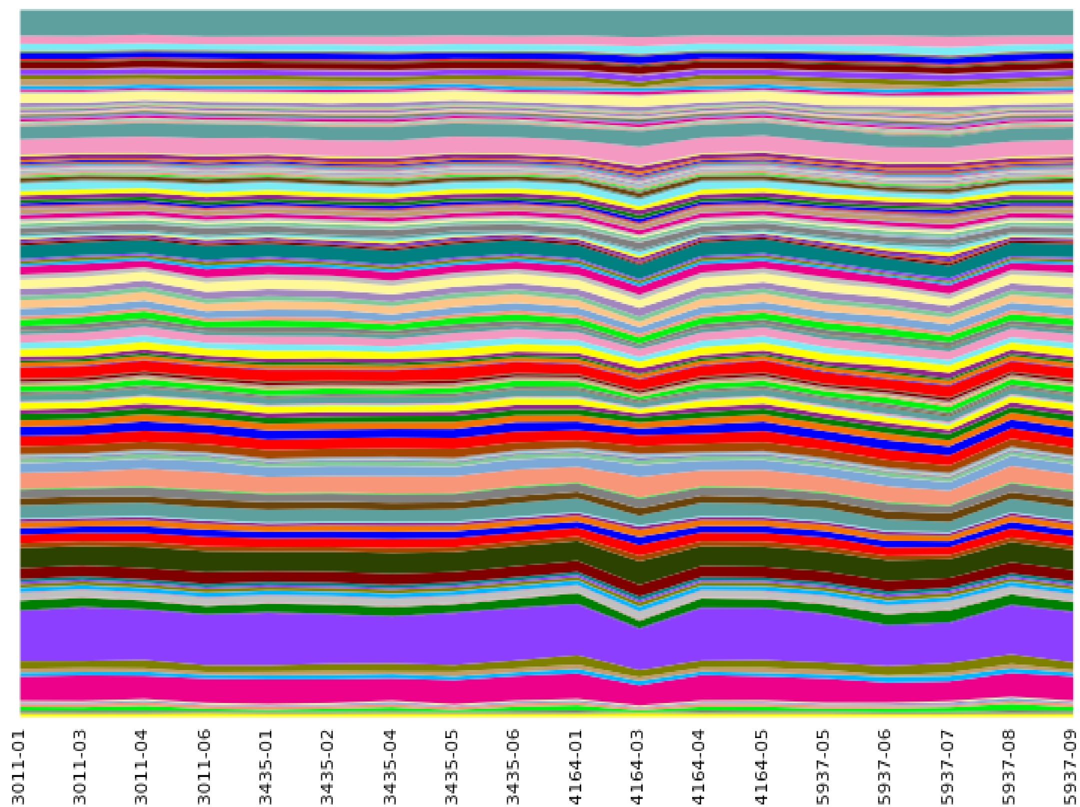

Figure 1c: PICRUSt of taxonomy (species) in fecal microbiota of colorectal cancer.

The number in the lower part of Figure 1c is sample number for each colorectal cancer.

The function of each microorganism was displayed in different color, individually.

Functions of microorganisms which observed in feces of patients with colorectal cancer were almost same.

microorganisms' structure and pathophysiological function by QIIME and PICRUSt tools were shown follows. Figure 2a is Heatmap clustering of taxonomy(species) identified in the fecal microbiota of patients with each part of colorectal cancer. Figure 2b is QIIME of taxonomy (species) in fecal microbiota of patients with each part of colorectal cancer. Figure 2c is PICRUSt of taxonomy (species) in fecal microbiota of patients with each part of colorectal cancer. Figure 2d is the functional analysis of fecal microorganisms (species) of patients with colorectal cancer by PICRUSt was confirmed the following functions; transporters, ABC transporters, phosphotransferase system, chaperon system, DNA repair / recombination / replication, transcription factor, ribosome, cysteine, methionine, phenylalanine, tyrosine, valine, leucine, isoleucine, alanine, aspartic acid, glutamine metabolism, tryptophan affects biosynthesis / metabolism, amino acid-related enzymes, TCA circuit, fructose / mannose metabolism, glycolysis / glycation, purine / pyrimidine metabolism, pentos phosphate pathway, energy metabolism, nitrogen metabolism, methane metabolism, and one carbon pool by folic acid. Sixty-seven microbial species were identified by Venn diagram as common microorganisms in feces of patients with colorectal cancer (Figure 2d, Table 1).

In the same analysis of the microbiota of the tumor epithelium of patients with colorectal cancer as described above, the Heatmap clustering of taxonomy (species) was different from that of feces (Figure 3a). Similarly, QIIME analysis also showed results that were not uniform in terms of molecular structure, similar to feces (Figure 3b). In addition, analysis of PICRUSt of the microflora of the tumor epithelium confirmed that it has the same function

Figure 2a: Heatmap clustering of taxonomy (species) identified in fecal microbiota_of patients with each part of colorectal cancer using 16s rRNA amplicon sequencing.

These are fecal microbial examination results of studies on rectal cancer(X01), sigmoid colon cancer(X02) and descending colon cancer(X03).

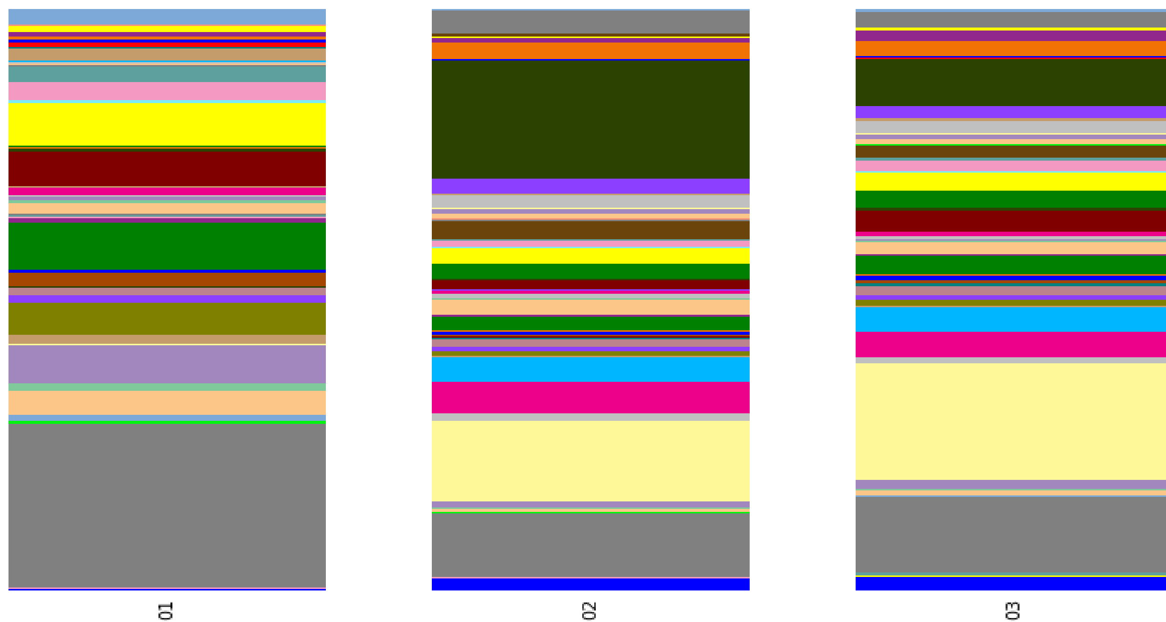

Figure 2b: QIIME of taxonomy (species) in fecal microbiota of patients with each part of colorectal cancer.

Genomic structures of microorganisms of feces in rectal cancer (01), sigmoid colon cancer (02), and descending colon cancer (03) showed different, respectively. In each colorectal cancer, the genomic structure of the microorganism was apparently different.

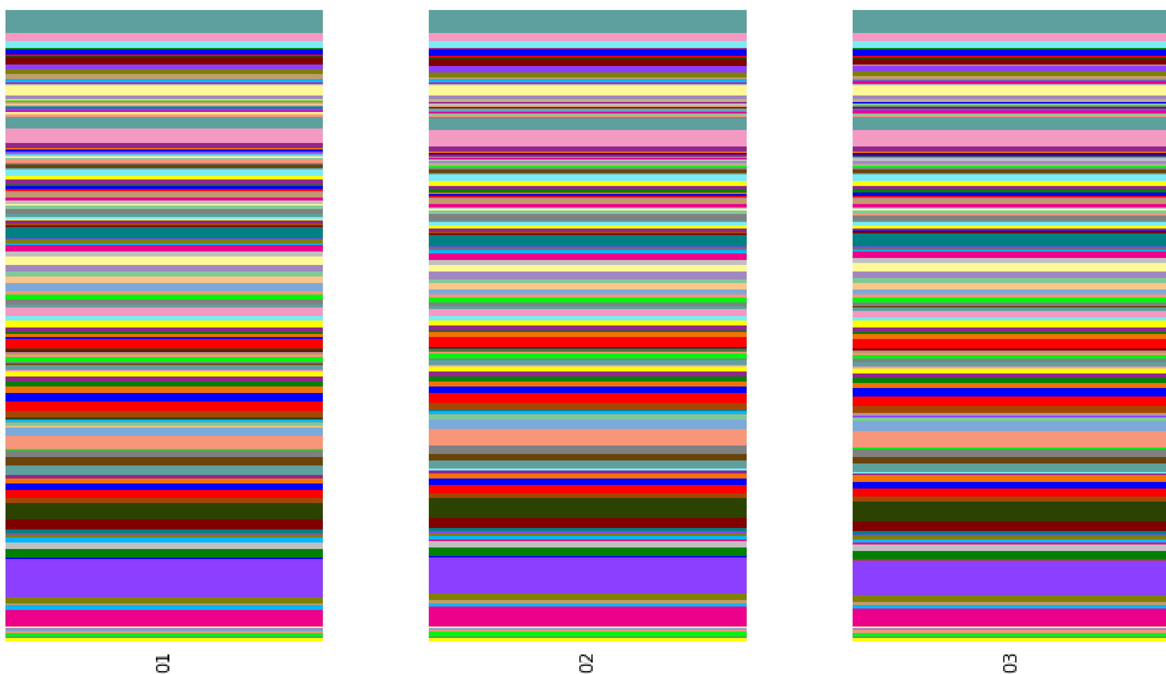

Figure 2c: PICRUSt of taxonomy (species) in fecal microbiota of patients with each part of colorectal cancer.

Functions of microorganisms of feces in rectal cancer (01), sigmoid colon cancer (02), and descending colon cancer (03) is shown, respectively.

Functions of fecal microorganisms in each colorectal cancer were same and uniformity.

The function of each microorganism is shown in a different color. It was confirmed that microorganisms found in each colorectal cancer have same common functions.

Common functions of microorganisms in feces of patients with colorectal cancers were transporter, ABC transporter, phosphotransferase system, chaperon system, DNA repair / recombination / replication, transcription factor, ribosome, cysteine, methionine, phenylalanine, tyrosine, valine, leucine, Isoleucine, alanine, aspartic acid, glutamine metabolism, tryptophan biosynthesis / metabolism, amino acid-related enzymes, TCA circuit, fructose / mannose metabolism, glycolysis / gluconeogenesis, purine / pyrimidine metabolism, Pentos phosphate pathway, energy metabolism, nitrogen metabolism, methane Microorganisms that affect metabolism and 1 carbon pool due to folic acid, individually.

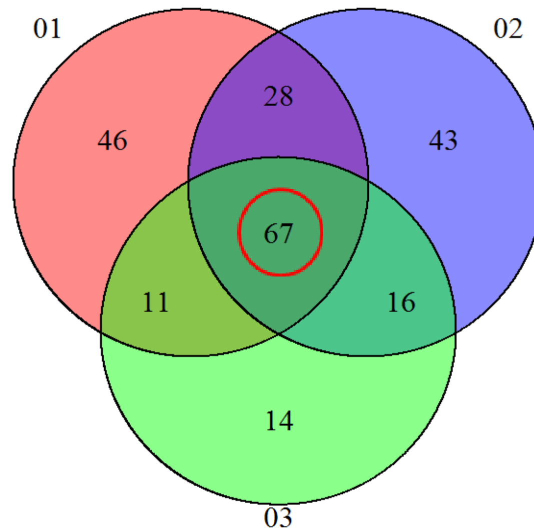

Figure 2d: Venn diagram showing taxonomy (species) of fecal microbial compositional structures in patients with colorectal cancer.

The Venn diagram shows the number of common microbial taxonomies according to overlapping regions. Sample identifiers: 01 as rectal cancer, 02 as sigmoid colon cancer and 03 as descending colon cancer, respectively.

Sixty-seven common microorganisms circled in red were confirmed as the result.

Actinomyces odontolyticus	Faecalibacteriumprausnitzii
Agathobacter rectalis	Flavonifractor plautii
Akkermansia muciniphila	Flintibacter butyricus
Alistipes onderdonkii	Fusicatenibacter saccharivorans
Alistipesputredinis	Fusobacterium mortiferum
Bacteroides caccae	Fusobacteriumsimiae
Bacteroides dorei	Haemophilus parainfluenzae
Bacteroideseggerthii	Intestinibacter bartlettii
Bacteroides faecichinchillae	Lachnospira pectinoschiza
Bacteroidesfaecis	Lactobacillus salivarius
Bacteroides fragilis	Not determined
Bacteroides massiliensis	Olsenella profusa
Bacteroidesovatus	Parabacteroides distasonis
Bacteroides plebeius	Parabacteroidesmerdae
Bacteroides stercoris	Peptostreptococcus stomatis
Bacteroides uniformis	Phascolarctobacterium faecium
Bacteroidesvulgatus	Phascolarctobacterium succinatutens
Bacteroides xylanisolvens	Prevotella salivae
Barnesiella intestinihominis	Rejected hit
Bifidobacterium longum	Romboutsia ilealis
Blautiacaecimuris	Roseburiafaecis
Blautia faecis	Roseburia intestinalis
Blautia luti	Roseburiainulinivorans
Blautia wexlerae	Ruminococcusbromii
Clostridium bolteae	Ruminococcus gnavus
Collinsella aerofaciens	Ruminococcus torques
Corynebacterium simulans	Streptococcus anginosus
Enterococcus faecalis	Streptococcussalivarius
Enterococcus lactis	Streptococcus sanguinis
Enterococcus raffinosus	Subdoligranulum variabile
Enterococcus rivorum	Sutterella stercoricanis
Enterococcus thailandicus	Veillonelladispar
Escherichia coli/Shigella	
Eubacterium eligens	
Eubacteriumhallii	

Table 1: Identified common microorganisms of feces in patients with colorectal cancer.

There were 67 common microorganisms which identified by Venn diagram.

Figure 3a: Heatmap clustering of taxonomy (species) identified in tumor epithelial microbiota of patients with each part of colorectal cancer using 16s rRNA amplicon sequencing.

These are results of microbial examination in tumor epitheliums studies on rectal cancer (X01), sigmoid colon cancer (X02) and descending colon cancer (X03).

Figure 3b: QIIME of taxonomy (species) in tumor epithelial microbiota of patients with each part of colorectal cancer.

Genomic structures of microorganisms of tumor epithelium in rectal cancer (01), sigmoid colon cancer (02) and descending colon cancer (03) showed apparently different similar to fecal examination, respectively.

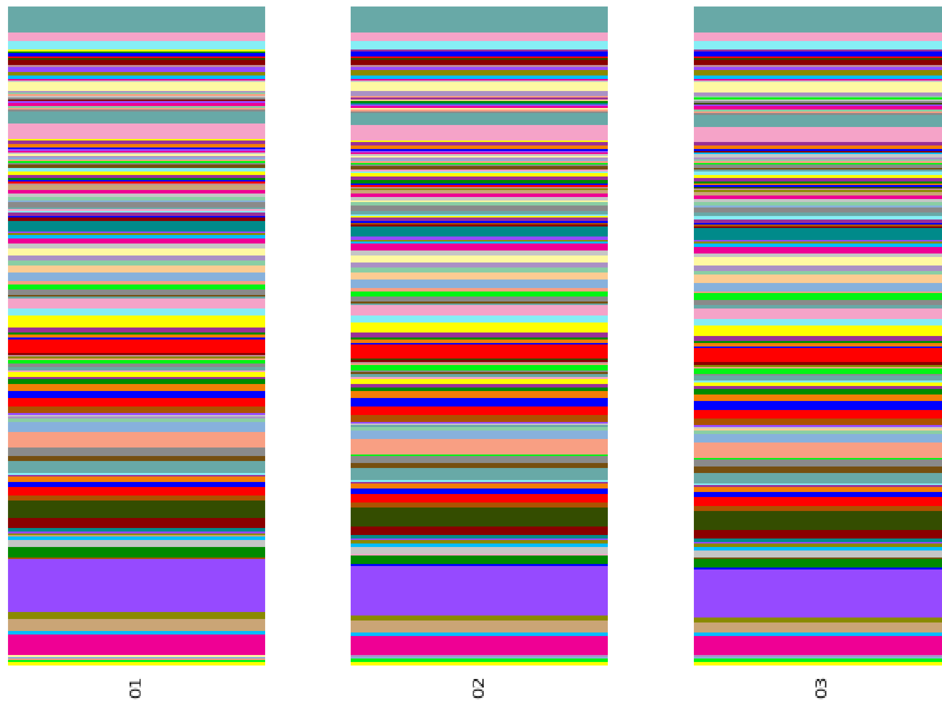

Figure 3c: PICRUSt of taxonomy (species) in tumor epithelial microbiota of patients with each part of colorectal cancer.

Functions of microorganisms of tumor epithelium in rectal cancer (01), sigmoid colon cancer (02), and descending colon cancer (03) is shown, respectively.

Functions of microorganisms of tumor epithelium in each colorectal cancer were same and uniformity.

The function of each microorganism is shown in a different color. It was confirmed that microorganisms found in each colorectal cancer have same common functions.

Common functions of microorganisms in tumor epithelium of patients with colorectal cancers were transporters, ABC transporters, amino acid-related enzymes, leucine, valine, isoleucine, methionine, phenylalanine, tryptophan biosynthesis / metabolism, glycolysis / gluconeogenesis, TCA cycle, Pentos phosphate pathway, pyruvate metabolism, purine / pyrimidine metabolism, DNA repair / replication, protein export and RNA degradation / transcription, individually.

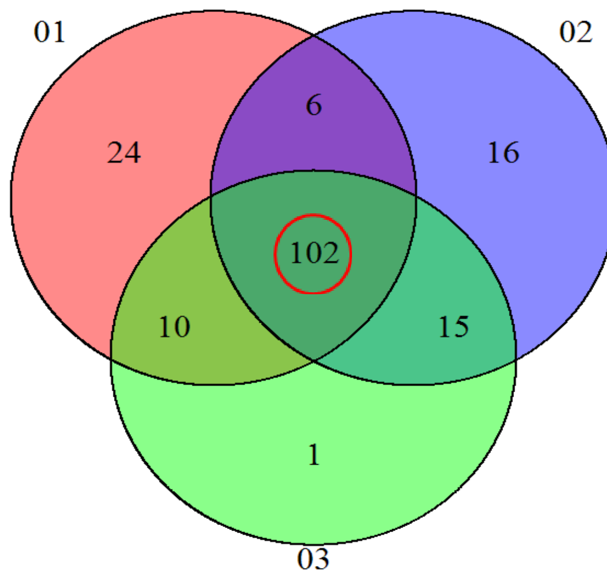

Figure 3d: Venn diagram showing taxonomy (species) of microbial compositional structures of tumor epithelium in patients with colorectal cancer.

The Venn diagram shows the number of common microbial taxonomies according to overlapping regions. Sample identifiers: 01 as rectal cancer, 02 as sigmoid colon cancer and 03 as descending colon cancer, respectively.

One hundred-two common microorganisms circled in red were confirmed as the result.

Microorganism
Actinomyces cardiffensis
Acutalibacter muris
Akkermansia muciniphila
Alistipes finegoldii
Alistipes indistinctus
Alistipes onderdonkii
Alistipes putredinis
Alistipes shahii
Anaerococcus vaginalis
Anaerostipes caccae
Anaerotruncus colihominis
Bacteroides caccae
Bacteroides cellulosilyticus
Bacteroides coprocola
Bacteroides faecichinchillae
Bacteroides massiliensis
Bacteroides nordii
Bacteroides ovatus
Bacteroides plebeius
Bacteroides stercoris
Bacteroides thetaiotaomicron
Bacteroides uniformis
Bacteroides vulgatus
Barnesiella intestinihominis
Bilophila wadsworthia
Blautia faecis
Blautia hansenii
Blautia hydrogenotrophica
Blautia wexlerae
Butyricicoccus desmolans
Butyricicoccus pullicaecorum
Butyricimonas virosa
Cloacibacillus evryensis
Clostridium aldenense
Clostridium bolteae
Clostridium citroniae
Clostridium glycyrrhizinilyticum
Clostridium hylemonae
Clostridium innocuum
Clostridium lavalense
Clostridium nexile
Clostridium ramosum
Clostridium scindens
Clostridium symbiosum
Clostridium xylanolyticum
Desulfovibrio intestinalis
Dialister invisus
Dialister pneumosintes
Dorea formicigenerans
Eggerthella lenta
Eisenbergiella tayi
Enterococcus faecalis
Escherichia coli/Shigella
Eubacterium callanderi
Eubacterium fissicatena
Eubacterium infirmum
Eubacterium nodatum
Flavonifractor plautii
Flintibacter butyricus
Fretibacterium fastidiosum
Frisingicoccus caecimuris
Fusobacterium nucleatum
Fusobacterium simiae
Fusobacterium varium
Hespellia porcina
Holdemania filiformis
Hungatella hathewayi
Intestinimonas butyriciproducens
Klebsiella variicola
Longicatena caecimuris
Not determined
Odoribacter splanchnicus
Oscillibacter ruminantium
Parabacteroides distasonis
Parabacteroides goldsteinii
Parabacteroides merdae
Parvimonas micra
Peptostreptococcus stomatis
Phascolarctobacterium succinatutens
Porphyromonas gingivalis
Porphyromonas uenonis
Prevotella nigrescens
Prevotella oralis
Prevotella oris
Pseudomonas aeruginosa
Pyramidobacter piscolens
Rejected hit
Roseburia hominis
Roseburia inulinivorans
Ruminococcus faecis
Ruminococcus gauvreauii
Ruminococcus gnavus
Ruminococcus torques
Ruthenibacterium lactatiformans
Slackia exigua
Solobacterium moorei
Streptococcus anginosus
Streptococcus salivarius
Streptococcus sinensis
Sutterella wadsworthensis
Tannerella forsythia
Veillonella atypica

Table 2: Identified common microorganisms of tumor epithelium in patients with colorectal cancer.
There were 102 common microorganisms which identified by Venn diagram.

uniformly as feces. Its specific functions were transporters, ABC transporters, amino acid-related enzyme, leucine, valine, isoleucine, methionine, phenylalanine, tryptophan biosynthesis / metabolism, glycolysis / gluconeogenesis, TCA cycle, pentose phosphate pathway, pyruvate. It affected metabolism, purine / pyrimidine metabolism, DNA repair / replication, protein export, RNA degradation and transcription factors (Figure 3c). One hundred-two microbial species were identified by Venn diagram as common microorganisms in tumor epithelium of patients with colorectal cancer (Figure 3d, Table 2).

Discussion

Many unsolved problems remain regarding the relationship between colorectal carcinogenesis / growth / proliferation and intestinal microorganisms. The question of whether there are specific microorganisms involved in colorectal cancer, and how intestinal microbes affect the carcinogenic and growth / proliferative mechanisms is the problem which must be solved.

Functions of microorganisms in feces and tumor epithelium were followings : transporters, ABC transporters, phosphotransferase system, chaperon system, DNA repair / recombination / replication, transcription factor, ribosome, cysteine, methionine, phenylalanine, tyrosine, valine, leucine, isoleucine, alanine, aspartic acid, glutamine metabolism, tryptophan affects biosynthesis / metabolism, amino acid-related enzymes, TCA circuit, fructose / mannose metabolism, glycolysis / glycation, purine / pyrimidine metabolism, pentos phosphate pathway, energy metabolism,

nitrogen metabolism, methane metabolism, and one carbon pool by folic acid, leucine, valine, isoleucine, methionine, phenylalanine, tryptophan biosynthesis / metabolism, gluconeogenesis, pyruvate. purine / pyrimidine metabolism, DNA repair / replication, protein export, RNA degradation and transcription factors.

Transporters and ABC transporters are essential for the uptake of molecules such as amino acids as a crosstalk between intestinal microbiota and intestinal epithelium. At above-mentioned results, it confirmed that the function of microorganisms in feces and tumor epithelium affects the tumor growth and tumor metabolism. Intestinal microorganisms take in substances necessary for living organisms.

According to the functional analysis of microorganisms in feces and tumor epithelium of patients with colorectal cancer, there were no microorganisms which activate any carcinogenic pathways as the cancer-related signals, Wnt signal, VEGF signal, TGFβ signal, mTOR signaling pathway or MAPK signaling pathway. It was the surprising and unexpected results that microorganisms of patients with colorectal cancer did not affect cancer-related pathways, but rather molecules involved in cancer growth and metabolism. These results are considered to be important discoveries which will be a breakthrough for future colorectal cancer and intestinal microbiological research.

Colorectal cancer research has been conducted so far because microorganisms such as Fusobacterium nucleatum (F. nucleatum) are considered to be involved in carcinogenesis [13, 14, 15, 16, 17, 18, 19]. However, F. nucleatum was found in feces but not in tumor epithelium in this study. It has been reported that F. nucleatum is also found in normal mucosal epithelium and not in large amounts in tumor epithelium. Regarding the relationship between F. nucleatum and colorectal cancer, F. nucleatum is involved in the progression of colorectal cancer, but there are negative reports on colorectal carcinogenesis, and the details of colon carcinogenesis have not been clarified [20].

On the other hand, we had reported that the antitumor effect of SCFAs produced by intestinal microbiota is due to the suppression of tumor growth and metabolic system molecules [2]. At the metabolome analysis of patients with colorectal cancer, it had reported that a large amount of s-acetylmethionine and methionine metabolites were present in the tumor tissue [21]. This report suggests that colorectal cancer may growth on the methionine cycle. It has been reported that methionine is taken up by tissues via LT1 which is an amino acid transporter expressed on tissue cell membranes [22,23].

Results of this study also confirmed the presence of microorganisms involved in the expression of transporters which take up amino acids such as methionine in intestinal microbiota of patients with colorectal cancer.

It was confirmed that the fecal and tumor epithelial microorganisms of patients with colorectal cancer have functions related to the uptake of amino acids such as transporters essential for tumor growth and proliferation, and also confirmed that the above-mentioned microorganisms have functions of DNA repair / replication, protein export, RNA degradation and transcription factors essential for tumor growth / proliferation / metabolism. In other words, it was conformed that colorectal tumor growth and proliferation due to crosstalk between intestinal microorganisms and mucosal epithelium in this analysis. Although it is necessary to carry out same studies by increasing the number of cases, these are new findings. However, this study did not provide data suggesting a direct involvement of intestinal microorganisms in colorectal carcinogenesis.

These results should be the foundation of new therapeutic strategies for colorectal cancer. If drugs of capable of simultaneously inhibiting the methionine metabolic cycle and specifically inhibiting the pathogenic microorganisms of colorectal cancer can be developed, it will be epoch-making therapeutic drugs for colorectal cancer. Elucidation of the mechanism of colorectal carcinogenesis is a mandatory task which must be done.

Conclusions

I have examined the relationship between colon carcinogenesis / growth / proliferation and intestinal microbiota. From the results of these examination, it was suggested that intestinal microorganisms involved to tumor growth and proliferation among the systems in colorectal carcinogenesis /growth / proliferation. However, this study did not confirm the involvement of intestinal microbiota in colorectal carcinogenesis.

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