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Relationship Between Adverse Events and Microbiomes in Advanced Hepatocellular Carcinoma Patients Treated With Sorafenib

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Abstract. *Background/Aim: Sorafenib results in several adverse events, the mechanism and predictors of which are unknown. Recently, it was reported that metabolism by microbiome changes the structure and effects of drugs. The blood levels of sorafenib may be affected by enterohepatic recycling of sorafenib due to microbial enzymes in the gut. We evaluated the relationship between adverse events caused by sorafenib treatment and microbiome in patients with advanced hepatocellular carcinoma. Materials and Methods: Twenty-five patients were classified into two groups based on the presence of hand-foot syndrome (HFS) or diarrhea within 12 weeks post-sorafenib treatment. Before sorafenib treatment, the fecal samples were analyzed targeting the V3-V4 region of 16s ribosomal RNA. Microbiome and predicted functional gene were compared between two groups. Results: The non-HFS group had a richer abundance of Veillonella, Bacillus, Enterobacter, Faecalibacterium, Lachnospira, Dialister, and Anaerostipes than the HFS group at genus level. Carotenoid biosynthesis and bacterial invasion of epithelial cells were enriched in the HFS group. The former three bacteria are classified as oral-origin bacteria, and the two predicted functions are associated with dysbiosis. The non-diarrhea group had a higher abundance of Butyricimonas and a lower abundance of Citrobacter, Peptostreptococcus, and Staphylococcaceae than the diarrhea group. Eight categories of predicted functional genes were detected with differences between the two groups. Conclusion: The non-HFS group had a higher relative abundance of oral-origin bacteria, which likely led to more robust dysbiosis in the gut. This dysbiosis may affect enterohepatic recycling. Additionally, the metabolism of these short-chain fatty acids in the gut may be different between the diarrhea and non-diarrhea groups.*

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Sorafenib is used worldwide as a standard treatment to improve prognosis in patients with hepatocellular carcinoma (HCC). It is a multitargeted tyrosine kinase inhibitor (TKI) that suppresses tumor proliferation and angiogenesis. TKIs can cause several common adverse events, including hand foot syndrome (HFS) and diarrhea. These adverse events not only necessitate a reduction in the dose of sorafenib, but also cause a decline in quality of life. In addition, it has also been reported that patients under sorafenib treatment who developed HFS because of it still have a longer survival time than those not under sorafenib treatment (1, 2). Therefore, it is important to elucidate the mechanisms of adverse events and establish a method for predicting them. The microbiome may be associated with drug effects and adverse events. For one, the microbiome may act protectively against gastrointestinal symptoms such as diarrhea (1). Microbiome diversity may also be linked to the effects of immune checkpoint inhibitors (2). Additionally, microbes can chemically modify many of these drugs (3). Microbiome research may be useful in clarifying the mechanism of adverse events and predicting such events.

In this regard, we hypothesized that some adverse events due to sorafenib are related to the patient's original microbiome and that the blood concentration of sorafenib is affected by the microbiome. It has previously been reported that blocking enterohepatic recycling by neomycin leads to a decrease in the blood concentration of sorafenib by 54% (4). Adverse events and blood levels are strongly correlated. Symptoms such as HFS improve when the dose is reduced. It has also been reported that some microbiomes and the intestinal environment may protect against diarrhea caused by chemotherapy. These mechanisms may be related to

sorafenib and the microbiome, but there is limited research on this topic. Thus, the present study aimed to determine potential relationships between the microbiome before sorafenib treatment and subsequent adverse events.

Materials and Methods

Study design. Microbiota from stool samples among patients with HCC were analyzed using a next-generation sequencer for this prospective study. Patients were classified into two groups based on the presence or absence of HFS or diarrhea within 12 weeks post-sorafenib treatment. Microbiota were compared using a linear discriminant analysis effect size algorithm (LEfSe), and gene function was predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). This study was approved by the Research Ethics Committee at Nagoya University Hospital on August 30, 2016 (Protocol number: 2015-0420). In accordance with the Declaration of Helsinki, written informed consent was obtained from all patients before registration. This study is registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN ID: 000020269). All the clinical and stool sample information were de-identified, and a database was constructed.

Patient selection. Between June 2016 and February 2018, 43 patients with advanced HCC started sorafenib treatment. Twenty-five of these patients agreed to participate in the study at the Nagoya University. HCCs were diagnosed clinically using contrast enhanced CT or MRI imaging based on guidelines established by the American Association for the Study of Liver Diseases (5). Four-phase (*i.e.*, unenhanced, late arterial, portal venous, and equilibrium phase) contrast enhanced CT examinations were obtained at baseline, at 2 and 6 weeks after sorafenib administration, and every 4-8 weeks thereafter (6). Antitumor response was evaluated according to the modified Response Evaluation Criteria in Solid Tumors (mRECIST) (7). The adverse events were assessed according to the Common Terminology Criteria for Adverse Events (CTC-AE) version 4.0.

All patients had been hospitalized at least 2 weeks after starting sorafenib administration. The starting dose (Nexavar; Bayer Yakuhin, Ltd., Osaka, Japan) was 800 mg/day, administered orally. However, out of concern for the possibility of having to discontinue sorafenib treatment at an early stage due to adverse events, the initial dose was set at 400 mg/day for patients who were 80 years or older, for those who had a body weight of 50 kg or less, for those with poor renal function, and for those who had a history of treatment for varices or ascites. In the case of drug-related adverse events, a dose reduction or temporary interruption was maintained until symptoms resolved to grade 1 or 2 according to guidelines provided by the manufacturer. Sorafenib therapy was continued until the occurrence of potentially fatal adverse events or until clinical tumor progression (8).

Sample collection and DNA isolation. Stool samples were collected in the hospital before sorafenib treatment. Samples were immediately stored at –80˚C. DNA was isolated using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) and stored at –80˚C until further analysis. Isolated DNA were amplified using universal primers (forward: 5'-**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGC**CTACGGGNGGCWGCAG-3' and reverse: 5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAATCC-3') to target the V3-4 regions of bacterial 16S rRNA, along with the Kapa HiFi Hotstart Ready Mix (KAPA Biosystems, Boston, MA, USA). The bold portions of the primers denote the Illumina overhang adapter. PCR conditions were 95˚C for 3 min, 25 cycles of 95˚C for 30 s, 55˚C for 30 s, 72˚C for 30 s, and 72˚C for 5 min. PCR amplicons were purified using AMPure XP magnetic purification beads (Beckman Coulter, Brea, CA, USA). Individual samples were barcoded through a second PCR cycle with the following conditions: 95˚C for 3 min, 8 cycles of 95˚C for 30 s, 55˚C for 30 s, 72˚C for 30 s, and 72˚C for 5 min. PCR products were pooled to construct the sequencing library, which was sequenced using an Illumina MiSeq sequencer to generate pair-end reads using the MiSeq Reagent Kit v3 with 2×300 reads and 600 cycles (Illumina, San Diego, CA, USA).

16S rRNA gene sequencing. We analyzed rRNA gene sequencing data obtained from MiSeq separately using the full Greengenes database, version 13.8 (9). USEACH 6.1 software (10) and QIIME 1.9.1 software (http://qiime.org) were used to make the barcode and trim the primers and barcodes to remove chimeric sequences. For chimeric sequences, we used the FASTA file of Greengene (version 13.8) as references. We used Greengene (version 13.8) for open reference picking to compare the microbiota and to analyze with LEfSe. We used Greengene (version 13.5) for close reference picking for PICRUSt. Operational taxonomic unit (OTU) formation was performed using optimized QIIME reference picking (11) at 97% similarity. Alpha diversity and hierarchical clustering and heatmap visualization were analyzed with MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/) with a default setting. Alpha diversity was evaluated with a Shannon index at the genus level. A clustering algorithm was calculated with an average distance using a Pearson's correlation coefficient between the two groups at the genus level; the result was figured with fewer than 1,500 features.

Statistical and microbial analysis. The two groups were compared using the Chi-square tests or Fisher's exact tests for categorical variables and Student's *t*-tests or Mann-Whitney *U*-tests for continuous variables, as appropriate. Data were analyzed using IBM SPSS Statistics version 24. The microbiome was compared between two groups using LEfse povided by the Galaxy module (http://huttenhower.sph.harvard.edu/galaxy/). This program was used online with default settings. Microbiota were compared from the phylum to genus level (12).

PICRUSt was used to test whether changes in gut microbial taxa would alter gut microbiota function. It could predict the functional gene content of bacterial communities. Predicted functional genes were categorized into Kyoto Encyclopedia of Genes and Genome (KEGG) orthology (http://www.kegg.jp/). The KEGG is a database detailing molecular interactions, reactions, and relationship networks for seven categories. KEGG functional classes at level 3 were compared by functional gene category using a statistical analysis of taxonomic and functional profiles (13).

Results

Study samples. Patient characteristics are shown in Table I. The average age was 72.9±8.18 years, and the average body mass index was 22.9±3.4. The average follow-up period was 556±612.9 days. Serum albumin was 3.61±0.45 g/dl, total

Table I. *Patient background*.

No.	Age	Gender	BMI	Etiology	CPS	BCLC Stage	HFS	Diarrhea	mRECIST	DM	PPI
1	79	M	28.29	NBNC	6	C	No	No	PD	No	PPI
2	76	M	18.62	HCV	5	B	HFS	No	SD	No	PPI
3	76	F	19.27	NBNC	5	B	HFS	No	SD	DM	No
4	68	M	19.43	HBV	5	C	No	No	SD	No	No
5	62	F	19.55	NBNC	5	B	HFS	Diarrhea	PR	No	PPI
6	71	M	25.56	NBNC	5	B	HFS	No	SD	DM	PPI
7	82	M	24.79	NBNC	6	B	No	Diarrhea	SD	DM	No
8	67	M	23.01	NBNC	6	B	HFS	Unknown	Unknown	No	No
9	64	M	26.25	NBNC	5	C	No	No	SD	No	No
10	81	M	20.59	NBNC	5	C	No	No	SD	No	No
11	71	M	18.74	HBV	5	C	HFS	No	SD	No	No
12	85	M	21.55	HCV	5	B	HFS	No	PR	No	PPI
13	68	M	25.33	NBNC	6	C	No	Diarrhea	PR	DM	PPI
14	73	F	26.3	HCV	5	C	No	No	Unknown	No	No
15	68	M	19.25	NBNC	5	C	HFS	Diarrhea	PR	DM	PPI
16	73	M	24.77	NBNC	5	C	HFS	No	SD	No	PPI
17	52	M	22.56	HBV	5	C	HFS	No	SD	DM	No
18	77	M	17.9	HCV	5	C	No	No	PD	No	No
19	74	M	25.89	NBNC	6	C	HFS	Diarrhea	PR	DM	PPI
20	73	M	26.11	HCV	5	C	HFS	Diarrhea	PR	No	No
21	74	M	21.2	HCV	7	C	No	No	Unknown	No	No
22	85	F	27.67	HCV	6	C	HFS	Diarrhea	SD	No	No
23	86	M	17.7	HCV	5	B	HFS	No	SD	No	PPI
24	61	M	27.67	NBNC	5	C	HFS	No	SD	DM	No
25	78	M	22.61	NBNC	5	C	HFS	No	SD	No	No

BMI: Body mass index; CPS: Child Turcotte Pugh score; BCLC: Barcelona Clinic Liver Cancer staging system; HFS: hand-foot syndrome; mRECIST: modified response evaluation criteria in solid tumors at 6 weeks from sorafenib treatment; DM: diabetes mellitus; PPI: proton pump inhibitor; HCV: hepatitis C; HBV: hepatitis B; NBNC: non-B non-C hepatitis.

bilirubin was 1.08±0.53 mg/dl, and platelet count was $127±65×10^3$ cells/μl. Eight patients were treated for diabetes mellitus, and 10 patients used proton pomp inhibitors. There were no significant differences between the HFS and non-HFS groups or between the diarrhea and non-diarrhea groups on any of the aforementioned characteristics.

A total of 64.0% (16/25) of patients had HFS, and 29.1% (7/24) had diarrhea. In terms of additional adverse events, appetite loss was observed in 54.2% (13/24), hypertension in 37.5% (9/24), and fever in 28.6% (6/21) of the sample cohort. One patient was transferred to another hospital; thus, details of adverse events other than HFS could not be collected. We divided the patients into two groups based on the presence of HFS (HFS group or non-HFS group) or diarrhea (diarrhea or non-diarrhea group), which were diagnosed within 12 weeks from start of sorafenib treatment.

Profiling microbiota and comparison of alfa diversity. Hierarchical clustering and heatmaps of microbiota are shown in Figure 1. The samples are sorted by HFS group in Figure 1a and diarrhea group in Figure 1b. As can be seen in the figures, distinct phylogenetic trees were revealed. Related bacteria may differ depending on the type of adverse event. Conversely, the bacteria present in the lower row of the campylobacter shown at the bottom of the figure are common to both HFS and diarrhea events. These bacteria may be common to these two events and/or may not necessarily impact each event. The alpha diversity from the Shannon index did not differ significantly between the HFS and non-HFS group or between the diarrhea and non-diarrhea group. The average Shannon index was 2.223±0.359 in the HFS group and 2.217±0.425 in the non-HFS group (Figure 2a, p=0.452). Figure 2b shows the Shannon index as 2.176 ± 0.295 in the diarrhea group and 2.24±0.421 in the non-diarrhea group (p=0.664).

Comparison of relative abundance between the HFS and non-HFS group with LEfSe. Eight genus types and one family differed significantly between the HFS and non-HFS group (Figure 3a). The non-HFS group had a richer relative abundance of seven genus types (*Veillonella*, *Faecalibacterium*, *Lachnospira*, *Dialister*, *Bacillus*, *Entero-bacter*, and *Anaerostipes*), as well as the family *Bacillaceae*. Each relative abundance percentage was as follows: (HFS *vs*. non-HFS) *Veillonella* (0.842±1.213% *vs*. 4.837±6.354%), *Faecalibacterium* (2.257±2.592% *vs*. 6.319±2.882%), *Lachnospira* (0.382±0.706%

Figure 1. *Continued*

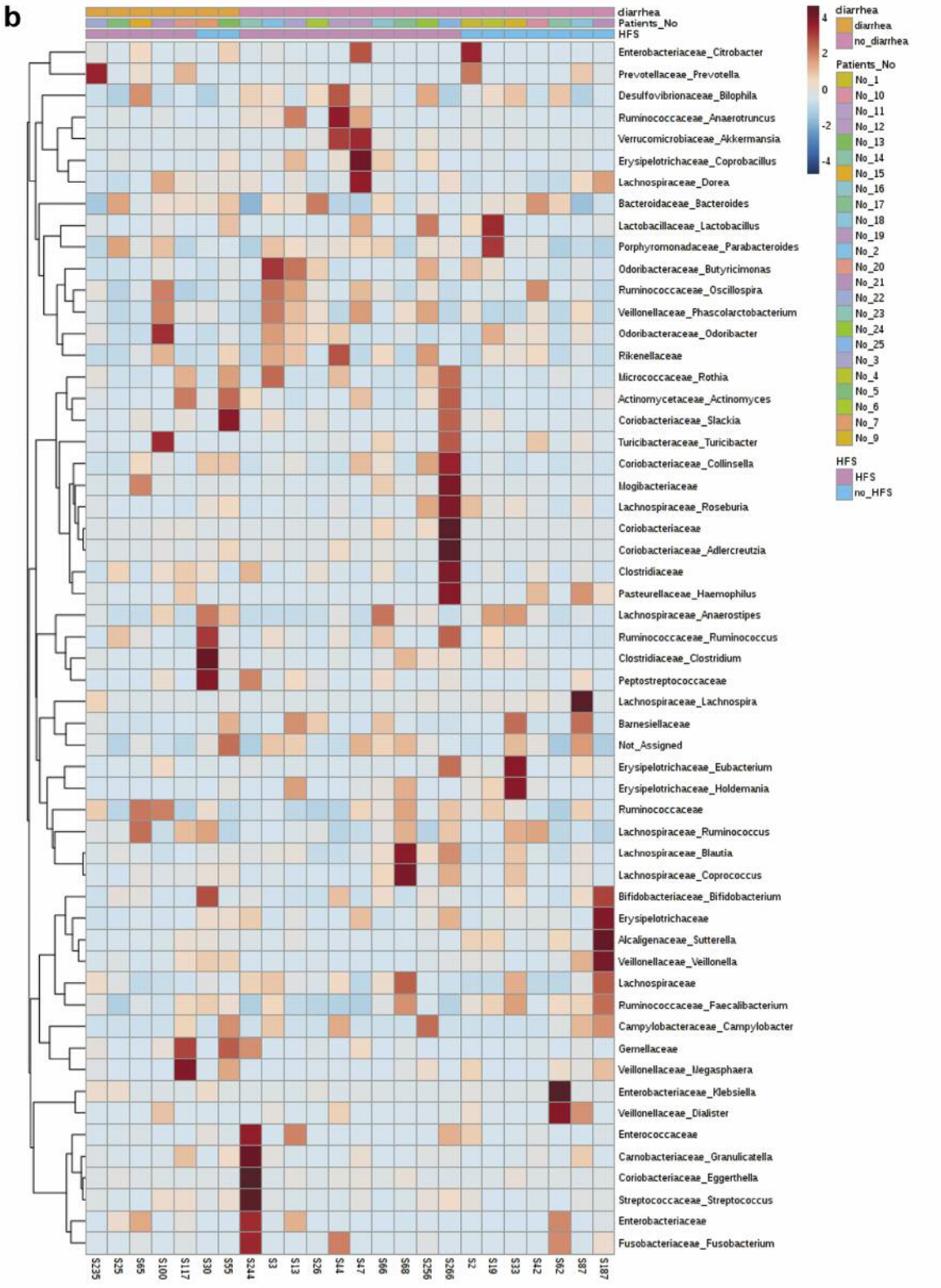

Figure 1. *Hierarchical clustering and heatmap. The three colored bars on the top of the figure show the grouping category or patient number. The samples are sorted by HFS/non-HFS group (a) or diarrhea/non-diarrhea group (b). The left 16 patients in pink on the third bar belong to the HFS group, and the right 9 patients belong to the non-HFS group. The left 7 patients in orange on the first bar belong to the diarrhea group, and the right 17 patients belong to the non-diarrhea group. The phylogenetic tree shown on the left side differs between the two figures. Clustering between the two figures differed, suggesting that related bacteria may differ depending on the type of adverse event.*

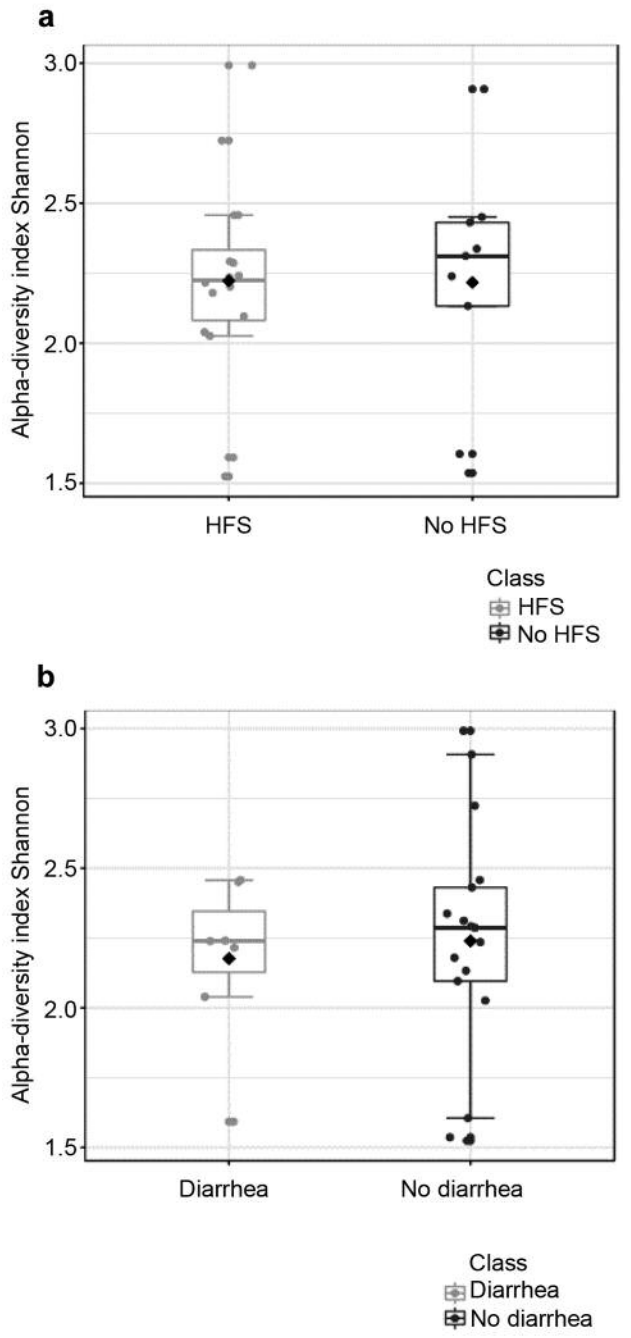

Figure 2. *Comparison of alpha diversity. There were no significant differences between the two groups. a) HFS and non-HFS group, b) diarrhea and non-diarrhea group.*

vs. 2.227±4.611%), *Dialister* (0.081±0.141% *vs.* 0.511±0.532%), *Bacillus* (0.016±0.066% *vs.* 0.02±0.055%), *Enterobacter* (0.002±0.007% *vs.* 0.028±0.059%), and *Anaerostipes* (0.069±0.124% *vs.* 0.201±0.175%). The HFS group had a richer relative abundance of one genus, "g_," which belonged to the *Desulfovibrionaceae* family; however, this was not identified with the OTUs for the bacterial name at the genus level.

Comparison of relative abundance between the diarrhea and non-diarrhea group with LEfSe. Figure 3b outlines the bacterial differences between patients with or without diarrhea. At the genus level, the non-diarrhea group had a higher relative abundance of genus *Butyricimonas* (0.056±0.079% *vs.* 0.275±0.377%). The diarrhea group had a higher relative abundance of genus *Citrobacter* (0.074±0.078% *vs.* 0.035±0.098%), *Peptostreptococcus* (0.006±0.01% *vs.* 0.001±0.004%), and *Staphylococcus* (0.006±0.009% *vs.* 0.001±0.002%). The order *Erysipelotrichales* in the non-diarrhea group and family *Staphylococcaceae* and "g_," which belongs to the *Pseudomonadaceae* family, in the diarrhea group was also relatively more abundant than in the non-diarrhea group.

Differences in microbial function in the gut by metagenome predictions using PICRUSt. Microbiota play several roles in the gut, ranging from immune induction to metabolism. Figure 4 shows significant differences in microbial functioning based on microbiota predicted by PICRUSt. PICRUSt is a bioinformatics software package designed to predict metagenome functional content from 16S rRNA surveys. In other words, PICRUSt can predict what role each bacterium can play. Significant differences between the HFS and non-HFS group are shown in Figure 4a. The bar graph indicates the absolute abundance of functional genes in each group. Factors other than "carotenoid biosynthesis" and "bacterial invasion of epithelial cells" do not reveal significant results, as the absolute abundance is nearly zero. Microbiota in the HFS group had more functional genes for carotenoid biosynthesis and bacterial invasion of epithelial cells than the non-HFS group.

Figure 4b shows 10 categories of functional genes whereby significant differences emerged between the diarrhea and non-diarrhea group. The bacteria in the diarrhea group had more functional genes reflected in glycerophospholipid metabolism, atrazine degradation, and amoebiasis compared to the non-diarrhea group, while the bacteria in the non-diarrhea group had more various functional genes, reflected in nicotinate and nicotinamide metabolism, type II diabetes mellitus, vitamin B6 metabolism, carbohydrate digestion and absorption, and ribosome biogenesis in eukaryotes, compared to the diarrhea group. "Replication, recombination and repair proteins" and "amino acid metabolism" also differed significantly but were unclassified for detailed functionality. Differences in microbial functioning between the diarrhea and non-diarrhea group included more categories than differences observed between the HFS and non-HFS group.

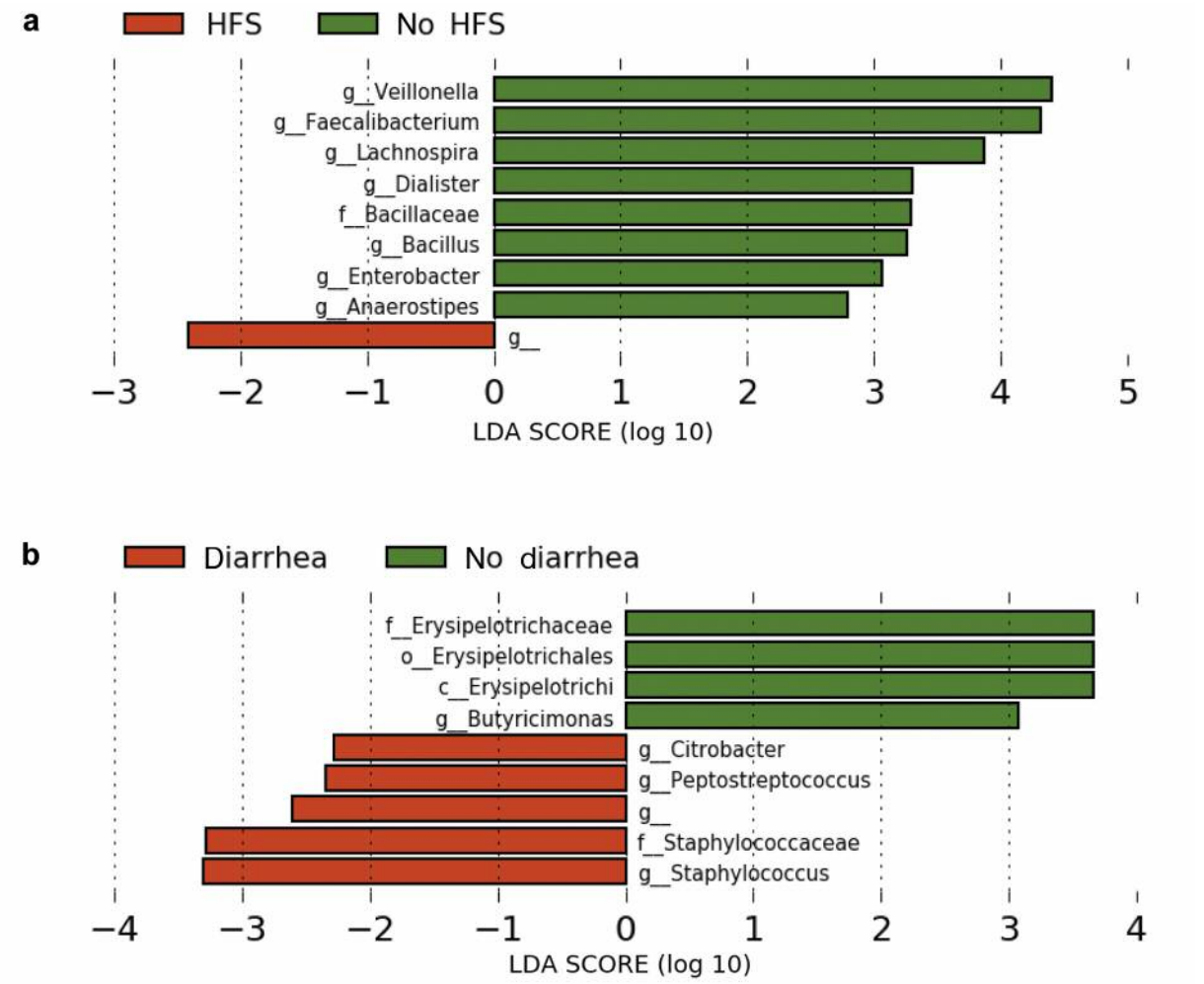

Figure 3. *Comparison of relative abundance between the groups with LEfSe. a) The detected bacteria show significant differences between the HFS and non-HFS group. Seven types of bacteria (green bar) indicate higher relative abundances in the non-HFS group than in the HFS group at the genus level. Only "g_" is the OTU formation clustered by QIIME at 97% similarity that was not defined by bacterial name based on the Greengene database (version 13.8) at the genus level. Veillonella, Bacillus, and Enterobacter were recognized as bacteria of oral origin. b) The detected bacteria show significant differences between the diarrhea and non-diarrhea group. The non-diarrhea group had a higher relative abundance of Butyricimonas and a lower relative abundance of Citrobacter, Peptostreptococcus, and Staphylococcus. Order Erysipelotrichales in the non-diarrhea group and family Staphylococcaceae in the diarrhea group were detected as more abundant.*

Discussion

Recently, the mechanism, prognosis, treatment with pro/prebiotics, etc. of cancer and microbiome have been reported (14). In addition the relationship between bacterial gut metabolism and drug effects or toxicity has gained worldwide attention. The microbiome has various genes for metabolism, including roles designed to help absorb nutrients from food. The microbiome metabolizes dietary fiber down into short chain fatty acids that are absorbed in the gut (15). Similarly, drugs are affected by microbiome metabolism. For instance, chemotherapy drugs can be influenced by the microbiome in terms of inflammation and immunity (16). Additionally, Zimmermann *et al*. reported that the abundance of genes within identified drug-metabolizing proteins significantly correlated with a bacterial community's capacity to metabolize the respective drug (3). In short, many drugs are chemically modified by microbes.

In clinical practice, Jin *et al*. reported a strong correlation between gut microbiome diversity and responses to anti-PD-1 immunotherapy among patients with advanced non-small cell lung cancer (2). Among patients with melanoma, mechanisms for modulating responses within the microbiome *via* improved effector T cell function in the host

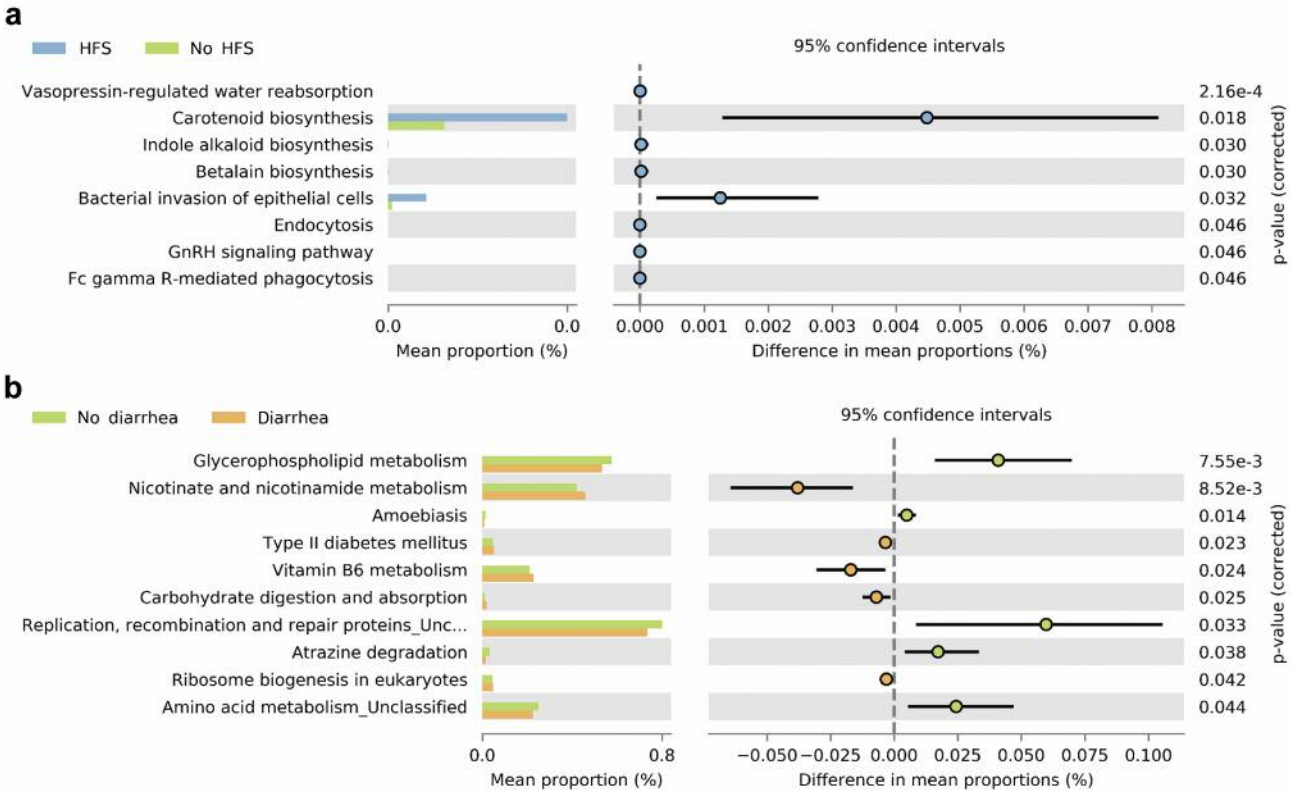

Figure 4. *Comparison of bacterial function predicted by PICRUSt. a) The functional categories of bacteria revealing significant differences between the HFS and non-HFS group. PICRUSt predicted and compared the genes for metabolism and other functions. Eight KEGG pathways were detected, but the categories other than "carotenoid biosynthesis" and "bacterial invasion of epithelial cells" were near zero. Carotenoid biosynthesis and bacterial invasion of epithelial cells may be related to dysbiosis. b) The functional categories of bacteria revealing significant differences between the diarrhea and non-diarrhea group. Ten categories of functional genes differed significantly between the diarrhea and non-diarrhea group. "Replication, recombination and repair proteins" and "amino acid metabolism" also differed significantly but were unclassified within the detail functionality. The differences in microbial functions between the diarrhea and non-diarrhea group included more categories than between the HFS and non-HFS group. Divergent gut metabolism may indicate the presence of different intestinal environments. PICRUSt: Phylogenetic investigation of communities by reconstruction of unobserved states; HFS: hand foot syndrome; KEGG: Kyoto encyclopedia of genes and genomes.*

and tumor microenvironment also had a significant impact (17). In terms of adverse events, Fei *et al*. reported a relationship between lower diversity in the microbiome and chemotherapy-induced diarrhea due to capecitabine combined with oxaliplatin in patients with colorectal cancer (1). However, the relationship between sorafenib and the microbiome had not yet been reported.

Sorafenib is a multitargeted, orally active small-molecule TKI that inhibits Raf kinase and the vascular endothelial growth factor receptor (VEGFR) intracellular kinase pathway (18). Sorafenib is widely used for patients with advanced HCC that cannot be treated with other therapies. Sorafenib has a statistically significant survival benefit over supportive care alone among patients with advanced HCC, as observed in a SHARP trial (19). In this SHARP trial, only grade 3 or 4 adverse events occurred significantly more often in the treated group, which included diarrhea (8% *vs*. 2%) and hand-foot skin reactions (8% *vs*. less than 1%). Any diarrhea (39% *vs*. 11%) and hand-foot skin reaction (21% *vs*. 3%) grade also occurred at a higher frequency in the sorafenib group than in the placebo group. Recently, the relevance of HFS in relation to sorafenib for HCC, along with good prognoses, have been reported (20). A meta-analysis comprising a total of 6,011 patients across 24 clinical trials for evaluating adverse events reported a 35.4% (95% CI=0.29-0.43) incidence of rash/desquamation and a 39.0% (95% CI= 0.32-0.47) incidence of HFS (21).

TKI targeting VEGFR has prominent dermatological adverse events, and diarrhea is also recognized as a common symptom. There are several reports of predictors affecting the effectiveness of sorafenib. Yin Hsun Feng *et al*. reported that GRP78 can be a predictive biomarker in HCC patients treated with sorafenib (22). However, predictors of adverse

events and the mechanism(s) are not adequaltely reported. In our previous study (8), we reported the prevalence of major adverse events: HFS (40.2%), fever (33.3%), hypertension (32.4%), appetite loss (15.7%), rash (24.5%), and diarrhea (5.9%). We hypothesized that some of these adverse events would be related to the microbiome. As a preliminary experiment, we examined microbiome differences depending on the presence of adverse events. We, thus, reported on the relationship between the microbiome and HFS or diarrhea. The reasons for the exclusion of the other four adverse events mentioned above are as follows. No significant differences in patients' microbiomes were observed in relation to hypertension and appetite loss. Furthermore, there was a debate regarding fever; it was decided to exclude it because fevers are affected by factors that also impact the microbiome, including infections. In addition, rashes were also excluded because symptoms often overlap with those related to HFS. Thus, we analyzed microbiomes by focusing on HFS and diarrhea.

HFS usually includes the development of localized and tender lesions, which appear as blisters or hyperkeratosis in areas of trauma or friction. Findings from histological examinations included epidermal parakeratosis and dyskeratosis with bandlike areas of necrotic keratinocytes. The dermis contains a dense, superficial perivascular lymphocytic infiltrate and some degree of non-leukocytoclastic vasculitis (23). As one candidate mechanism, researchers hypothesize that alterations to specific small vessels are commonly traumatized by frequent impact or pressure on the skin requiring continuous endothelial repair involving the VEGF receptor (24).

Conversely, the mechanism(s) underlying diarrhea due to sorafenib have not been elucidated. Only one report suggested that diarrhea is related to diminished pancreatic exocrine function, but this report evaluated a mere 20 patients (25). Furthermore, diarrhea is a very common adverse event with various TKI treatments. Thus, sorafenib could easily induce diarrhea by affecting the intestinal epithelium. Skin hydration with topical urea-based creams is important in preventing HFS. Recently Naganuma A et al reported in a prospective open-label trial that prophylactic β-hydroxy-β-methylbutyrate, L-arginine and L-glutamine supplementation prevented sorafenib-induced HFS (26). This also reduced the incidence of diarrhea. These dietary amino acids have been linked to the metabolism of microbiome in the gut (27) and the Gut-Microbiome-Immune Axis (28).

The average plasma exposure of sorafenib is influenced by the microbiome. The Food and Drug Administration of the United States of America recommends that co-administration of sorafenib with oral neomycin should be carefully considered. Neomycin will cause dysbiosis because neomycin destroys a wide range of microbiomes, including gram positive and negative bacteria. Neomycin decreases the area under plasma concentration versus the sorafenib time curve by 54%, probably due to eradication of gastrointestinal bacteria, preventing enterohepatic recycling of sorafenib (4). It has been reported that microbial enzymes to sorafenib is related to enterohepatic recycling. Vasilyeva A *et al*. have reported that β-glucuronidases produced by intestinal microbiota increased blood levels of sorafenib because sorafenib-glucuronide excreted into the gut lumen can be cleaved by microbial its enzymes, which is then reabsorbed, supporting its persistence in the systemic circulation (29). Ervin *et al*. reported that microbial β-glucuronidase enzymes caused reactivation of the inactive regorafenib-glucuronide. This involved a subset of the four bacteria with the highest proportion of Ruminococcus gnavus (30). In our study, it is difficult to accurately assess species-level microbiomes because our data are based on V3-V4 region of 16s ribosomal RNA, so we cannot assess whether these bacteria are reduced. However, dysbiosis in patients with cirrhosis increase oral origin bacteria and autochthonous taxa such as Ruminococaceae relatively decreases (31, 32). In our study, the non-HFS group had a higher relative abundance of oral-origin bacteria (*Veillonella*, *Bacillus*, and *Enterobacter*). There was no significant difference in the difference of Ruminococaceae, but the median of relative abundunce was higher in the HFS group than in the non-HFS group (HFS: 1.49% vs non-HFS 1.31%). The non-HFS group may reduce some of enzyme due to simillar dysbiosis to cirrhosis patients. It is presumed that several enzymes are involved in enterohepatic circulation. We hypothesize that dysbiosis related to some unknown microbial enzymes also alters these enzymes to reduce blood levels of sorafenib. In clinical practice, HFS usually improves after dose reduction. Taken together, it may be difficult for patients with dysbiosis to have an increased concentration of sorafenib. Results of our PICRUSt procedure indicated that patients had dysbiosis because "carotenoid biosynthesis" (33) and "bacterial invasion of epithelial cells" (34) are both factors associated with dysbiosis. Kanbayashi *et al*. (35) reported that avoidance of proton pump inhibitors were one of the predictive factors for the development of HFSR. Proton pump inhibitor can also make the highr relative abundance of oral-origin bacteria and dysbiosis. It suspected that the same mechanism similar to our hypothesis were occurred.

The non-diarrhea group had a higher relative abundance of *Butyricimonas*, which is a butyric acid producing bacteria, than the diarrhea group. Butyric acid producing bacteria protect against mucosal inflammation by increasing the number of regulatory T cells and down-regulating inflammatory cytokines and stimulating IL-10 (36). Butyrate has a central role in suppressing inflammatory and allergic responses. *Citrobacter* and *Staphylococcus*, which were detected in the diarrhea group, are bacteria that can cause opportunistic infections and produce acetic acid.

Peptostreptococci metabolizes butyric acid. The metabolism of these short-chain fatty acids in the gut may be different between the diarrhea and non-diarrhea groups. They are also categorized as oral-origin bacteria. Normally, intestinal immunity prevents these bacteria from growing in the intestine. It is likely that the patients had an intestinal environment that allowed various bacteria to easily grow. PICRUSt results also showed that various metabolic and other functions differed between the two groups. These affected the intestinal environment and immunity. Furthermore, the present results support the possibility that the intestinal environment differed between the two groups. In other words, patients may have had an intestinal environment that was prone to diarrhea prior to sorafenib treatment.

Certain study limitations should be noted. Although the present data provide a first step, the sample size was very small. Thus, more patients need to be recruited for further consideration. We also think that the Shannon indices did not reveal significant results due to sample size issues. Finally, while we mentioned dysbiosis and concentrations of sorafenib in the discussion, we did not measure relative concentrations in the blood or liver. Future research should focus on the blood concentration of sorafenib and dysbiosis.

Conclusion

This is the first study to evaluate the relationship between the microbiome and adverse events due to sorafenib treatment. The non-HFS group had a greater relative abundance of oral-origin bacteria, which likely led to more robust dysbiosis in the gut. This dysbiosis may affect enterohepatic recycling of sorafenib. Additionally, the non-diarrhea group may have a protective intestinal environment to guard against diarrhea within the microbiome.

Conflicts of Interest

None to be declared.

Authors' Contributions

KY, TK and TH conceived the project and designed experiments. TI, YI, MN and RM collected and processed the samples in the field with the help of MI and MF. KY performed the data analyses and wrote the manuscript. TH, KT, IM and MF edited the manuscript. All Authors read and approved the final manuscript.

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