



OPEN The gut microbiota as a potential biomarker in patients with EGFR-mutant lung cancer

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Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are highly effective against EGFR-mutant non-small cell lung cancer (NSCLC); however, identifying biomarkers that predict prognosis and adverse events is necessary. Although the gut microbiota is considered to be a biomarker for NSCLC without mutations, no studies have examined its potential as a biomarker for EGFR-mutant NSCLC. Here, we investigated the association between gut microbiota composition and diarrhea, a common side effect caused by EGFR-TKIs. In addition, we examined the association between the efficacy of EGFR-TKIs and the gut microbiota. A total of 21 NSCLC patients with EGFR mutations were enrolled. Fecal samples were collected prior to EGFR-TKI treatment and 16S rRNA metagenome sequencing was performed to evaluate the microbiota profile. In addition, α -diversity, β -diversity, and Linear discriminant analysis Effect Size (LEfSe) analyses were performed. The α -diversity of the gut microbiota was higher in patients with grade 0–1 diarrhea than in those with grade 2–3 diarrhea (Shannon, $p = 0.0367$). In terms of β -diversity, there was a significant difference in the best overall response between patients with a partial response (PR) to EGFR-TKIs and those with stable disease (SD)/progressive disease (PD) (weighted $p = 0.041$). Analysis of microbial composition revealed an increased abundance of *Ruminococcus* in the PR group. In patients taking EGFR-TKIs, a higher α -diversity may be associated with less severe diarrhea. In addition, a high abundance of *Ruminococcus* may be a potential biomarker for predicting favorable efficacy of EGFR-TKIs.

Keywords Lung cancer, EGFR, Gut microbiota, Best overall response, Diarrhea

Lung cancer is the leading cause of cancer-related mortality worldwide^{1,2}, with non-small cell lung cancer (NSCLC) being the major histological subtype. Approximately 50% of Asian NSCLC patients carry epidermal growth factor receptor (EGFR) mutations³, the most common of which are deletion of exon 19 and L858R substitutions in exon 21⁴. Since these mutations are associated with high sensitivity to EGFR-tyrosine kinase inhibitors (EGFR-TKIs), these drugs are the standard front-line treatment and have improved clinical outcomes for patients with advanced disease over and above those achieved by traditional cytotoxic therapies⁵. Although EGFR-TKIs are highly effective, biomarkers that predict efficacy and adverse events are needed in clinical practice.

Many have described an association between cancer and the gut microbiota. Recently, the relationship between the gut microbiota and the immune system has gained much attention⁶. Growing evidence supports the concept of the lung–gut axis, a bidirectional communication pathway between the intestinal and respiratory microbiota. Alterations in the gut microbiota can affect pulmonary immunity and inflammatory responses through microbial metabolites, such as short-chain fatty acids (SCFAs) and bile acids, and through systemic cytokine signaling. Conversely, lung inflammation or tumor-associated factors may disrupt intestinal homeostasis^{7–9}. These findings suggest that the gut microbiota may contribute not only to local intestinal physiology but also to the pathogenesis and treatment response of lung diseases, including lung cancer. Indeed, accumulating evidence indicates that modulation of the gut microbiota can influence immune responses and therapeutic efficacy in lung

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cancer. In the case of NSCLC patients lacking mutations, incorporation of immune checkpoint inhibitors (ICIs) into the treatment strategy has improved survival in some cases¹⁰. Thus, the gut microbiota is a useful biomarker for NSCLC without genetic mutations^{11–13}; however, no study has examined its potential as a biomarker in patients with EGFR mutations.

Here, we asked whether the gut microbiota is a potential biomarker for NSCLC with EGFR mutations. To do this, we evaluated the diversity and trends of the gut microbiota before treatment to determine its potential as a biomarker, as well as its association with adverse effects and responses to treatment.

Materials and methods

Study design

This prospective observational study was conducted at the Department of Respiratory Medicine, Hirosaki University Hospital, and approved by the Ethics Committee of Hirosaki University Graduate School of Medicine (approval number: 2020-085-1). All patients provided written informed consent before entry into the study. The clinical grade of diarrhea was evaluated in accordance with the common toxicity criteria for adverse events (CTCAE), version 5.0. All methods were performed in accordance with the relevant guidelines and regulations.

Eligibility criteria

The inclusion criterion was advanced NSCLC scheduled for induction of the first-line treatment with EGFR-TKIs. Patients with concomitant intestinal diseases such as autoimmune or inflammatory bowel disease, infectious diseases requiring systemic treatment, psychosis or psychiatric symptoms that would make participation in the study difficult, poorly controlled diabetes mellitus (HbA1c $\geq 10\%$), pregnancy or lactation, or active symptomatic interstitial pneumonia or pulmonary fibrosis were excluded.

Sample collection

Fecal samples used for intestinal microflora analysis were collected using a stool collection kit (CYK20201025, Cykinso Inc., Tokyo, Japan). The storage container provided with the kit contained a preservation solution (Guanidine_solution 1), and the collected stool samples were placed in the container so that they were completely soaked in the preservation solution. Subjects were asked to collect stool specimens on the same day of their visit and bring them to the clinic. If it was difficult to collect the specimens on the same day, the subjects were asked to store specimens collected a few days before the visit in a cool and dark place. Specimens were stored at 4 °C until DNA extraction. Stool samples collected within a few days prior to the first day of EGFR-TKI treatment was used for analysis.

DNA extraction and gene amplification

DNA was extracted from fecal samples using a DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) and stored at -20 °C. Each library was prepared according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide using primer set 27Fmod/338R targeting the V1–V2 region of 16S rRNA genes. Next, 251-bp paired end sequencing of the amplicons was performed on a MiSeq system (Illumina, San Diego, CA) using a MiSeq Reagent v2 500 cycle kit.

Bioinformatics analysis

The paired end sequences obtained were merged, filtered, and denoised using DADA2. Taxonomic assignment was performed using the QIIME2 feature-classifier plugin and the Greengenes 13_8 database. The QIIME2 pipeline, version 2020.2, was used as the bioinformatics environment for processing of all relevant raw sequencing data.

Diversity analysis

Analysis of α - and β -diversity was performed for each individual sample. The Shannon index of α -diversity is a common metric that measures the α -diversity of species in a community mathematically. The Shannon index measures both species richness and evenness. β -diversity compares the evenness of the population of each species. Principal coordinate analysis (PCoA) assessment of β -diversity was used to identify differences between two groups (i.e., patients with a partial response (PR) vs. those with stable disease (SD)/progressive disease (PD)). β -diversity analysis was performed for weighted and unweighted UniFrac distances. Analysis of similarities (ANOSIM) is used widely as a multivariate method in microbiome studies. ANOSIM was conducted to observe the degree of separation between sample groups (PR vs. SD/PD). The null hypothesis that the average rank similarity between samples within a group is the same as the average rank similarity between samples belonging to different groups was tested by ANOSIM.

Linear discriminant analysis effect size (LEfSe) analysis

LEfSe determines the bacterial flora classification unit most likely to explain differences among groups. The best overall response, evaluated according to the response evaluation criteria in solid tumors (RECIST ver. 1.1), was determined for the two groups (PR vs. SD/PD), and LEfSe analysis was used to search for strains that differed significantly between the groups.

Statistical analysis

The statistical analysis was performed using JMP Pro 17.0 (SAS Institute Inc., Cary, NC). The difference in the Shannon index and relative abundance of *Ruminococcus* between the groups was evaluated using the Mann-Whitney U tests. P value < 0.05 were considered statistically significant in univariate analysis. The predictive cut-off value was obtained by constructing a receiver operating characteristic (ROC) curve. Progression free

survival and overall survival (OS) were evaluated using log rank tests, in which a *P* value <0.05 was considered significant.

Results
Patient characteristics

From July 2020 to July 2023, 21 treatment-naïve patients with EGFR-mutant NSCLC were judged eligible and enrolled in the study. The characteristics of the eligible patients are shown in the Table 1.

All patients included were Japanese and had adenocarcinoma histology. There were seven male and 14 female patients, with a median age of 70 years (range, 43–85 years). In total, 11 patients (52.4%) had never smoked. Most patients had a good Eastern Cooperative Oncology Group performance status (ECOG-PS) 0–1 (95.2%), and only one patient had ECOG-PS 3 (4.8%). Nine patients had the EGFR L858R mutation, and nine had deletions of exon 19 (19del). There were two uncommon mutations (L861Q and G719X), and one compound mutation (L858R + E709G). Seven patients were treated with osimertinib, 13 with afatinib, and one with elrotinib plus ramucirumab (RAM) (Fig. 1). No patient experienced a complete response. A total of 11 (52.4%) patients had a PR, nine (42.8%) had SD, and one (4.8%) had PD. Seven patients had diarrhea of grade 2 or higher, and 14 patients had no diarrhea or diarrhea of grade 1. None of the patients had been taking antibiotics prior to specimen collection.

Analysis of α-diversity of the intestinal gut microbiota

First, we evaluated the patients’ gut microbiota by assessing α-diversity indices. This was done using stool samples collected before introduction of EGFR-TKIs. The Shannon index for α-diversity was calculated to describe the diversity of each species. The data showed that the Shannon index was higher in patients with no diarrhea or grade 1 (G1) diarrhea than those with grade 2 (G2) or higher diarrhea (Shannon, *p* = 0.0367; Fig. 2), suggesting that a high diversity within the gut microbiota might be associated with no or mild diarrhea.

Association between the microbiota and EGFR-TKI efficacy

Next, we analyzed the β-diversity of the microbiota. β-diversity analysis was performed to compare “high efficacy” patients (the PR group) and “low efficacy” patients (the SD/PD group). ANOSIM revealed no significant differences in unweighted UniFrac distances between the PR and the SD/PD groups (PCoA, unweighted; *p* = 0.118; Fig. 3A). By contrast, the weighted UniFrac distances differed significantly between the two groups (weighted; *p* = 0.041; Fig. 3B). These results indicate that the composition of the gut microbiota of patients who respond well to EGFR-TKIs is different from that of those who do not.

LEfSe analysis conducted to investigate the strains responsible for this difference identified *g_Ruminococcus.s_* (*Ruminococcus*) in patients who achieved a PR. A list of strains with a log score of 3 or higher was plotted according to abundance of *Ruminococcus*, which was the focus of our study (Fig. 4). Univariate analysis revealed significant differences in the relative abundance of *Ruminococcus* between the PR and the SD/PD groups (*p* = 0.0018, Fig. 5). These results suggest that EGFR-TKIs may be more effective in those with a higher abundance of *Ruminococcus* in the gut microbiota.

Furthermore, we determined the percentage of the total microbiota should be made up of *Ruminococcus* to expect a PR (or better) effect. The predicted cut-off value for *Ruminococcus* was obtained by drawing a ROC

Clinical characteristics		Median (range)	<i>n</i> (%)
Patients			21
Age, years		70 (43–85)	
Sex	Male		7 (33.3)
	Female		14 (66.7)
Smoking status	Current		1 (4.8)
	Former		9 (42.8)
	Never		11 (52.4)
ECOG performance status	0–1		20 (95.2)
	≥ 2		1 (4.8)
Clinical stage	4		18 (85.7)
	Postoperative recurrence		3 (14.3)
EGFR mutations	L858R		9 (42.8)
	Exon19del		9 (42.8)
	Uncommon (L861Q and G719X)		2 (9.6)
	Compound (L858R + E709G)		1 (4.8)
Diarrhea			
	Grade < 2		14 (66.7)
	Grade ≥ 2		7 (33.3)

Table 1. Patient characteristics.

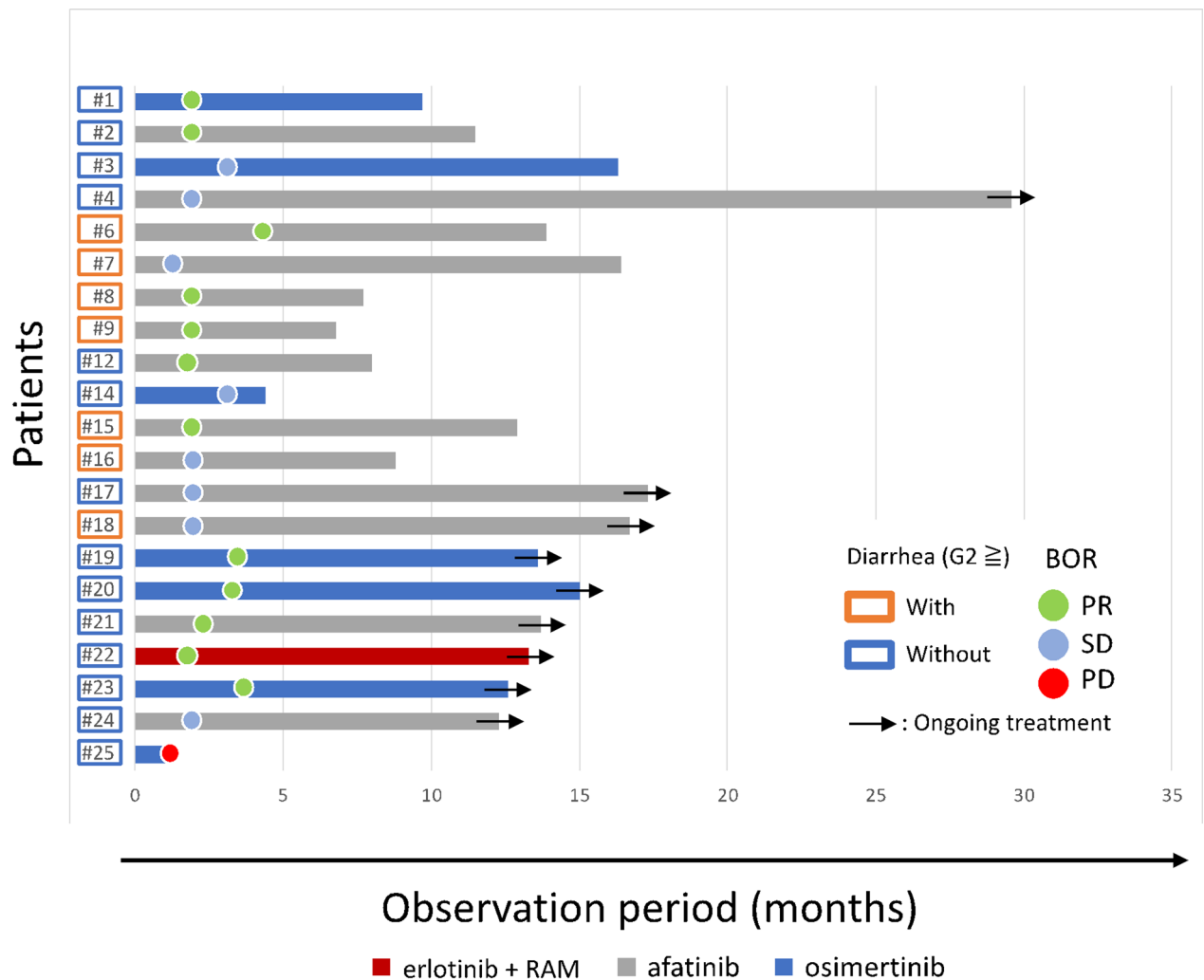


Fig. 1. Swimmer plot showing clinical course after initiation of EGFR-TKI treatment. Each bar represents one patient. This shows the clinical course of first-line treatment; patients who are still undergoing treatment are indicated by arrows.

curve with a cut-off value of 1.4% (Area under curve (AUC) 0.96, $p < 0.001$; the dotted line in Fig. 5). The results suggested that EGFR-TKIs treatment may be more effective when *Ruminococcus* makes up more than 1.4% of the total flora in stool samples before EGFR-TKI treatment.

Discussion

Here, we evaluated the gut microbiota profile, and the α - and β -diversity, in NSCLC patients with EGFR mutations. The data show that patients with higher α -diversity may have less severe diarrhea during/after EGFR-TKI treatment. The high abundance of *Ruminococcus* in those with good response suggests that this genus may be a potential biomarker for predicting favorable efficacy of EGFR-TKIs. Few reports have examined the association between EGFR-mutant NSCLC and the gut microbiota, and our study is the first to examine the gut microbiota of Japanese patients with advanced NSCLC carrying EGFR mutations.

Our findings are also related to the concept of the lung–gut axis, which refers to the bidirectional interaction between the intestinal and pulmonary microbiota^{7–9}. In this context, alterations in the gut microbiota may affect the tumor microenvironment, immune status, and therapeutic response in NSCLC. The observation that higher α -diversity was associated with less severe diarrhea and that *Ruminococcus* abundance correlated with better EGFR-TKI response suggests that intestinal microbial composition may modulate both gastrointestinal toxicity and systemic treatment efficacy through this axis.

Consistent with this concept, it is important to consider the overall taxonomic composition of the gut microbiota in EGFR-mutant NSCLC. In general, the gut microbiota of EGFR-mutant NSCLC patients is occupied by members of four bacterial phyla: *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria*⁷. Another study comparing the gut microbiota of lung cancer patients with that of healthy controls reported that *Actinobacteria* (Lung cancer (LC): 3%, Healthy control (HC): 1.56%) and *Fusobacteria* (LC: 1.14%, HC: 0.22%) were predominant in lung cancer patients¹⁴. *Fusobacteria* are linked to malignancy and are associated

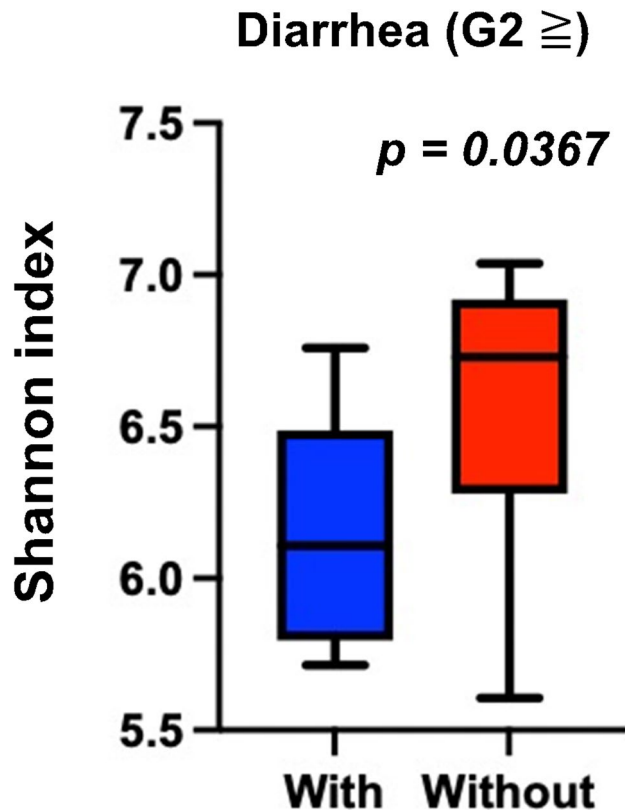


Fig. 2. Comparison of microflora diversity in individuals with and without grade 2 or higher diarrhea. Boxplot based on the Shannon index. *P* values were obtained using the Mann-Whitney U test.

with unfavorable prognosis and treatment failure¹⁵. Herein, we detected *Fusobacteria* in three patients in the SD/PD group (0.69–5.32% of the total), all of whom had a poor response. Although this number of patients is small, we think that *Fusobacteria* may be associated with a poor prognosis for NSCLC patients with EGFR mutations.

The gut microbiota is affected not only by drugs but also by other factors such as place of residence and diet, and the composition of the gut microbiota varies among ethnic groups¹⁶. Shoji and colleagues evaluated the gut microbiota in three groups of Japanese individuals: patients with lung cancer, patients with lung inflammation, and healthy controls¹⁷. Each group was represented by 20 phyla, with the main phyla being *Firmicutes*, *Actinobacteria*, and *Bacteroidota*. There were no significant differences between the three groups in the abundance of these phyla. Similarly, we found that *Firmicutes*, *Actinobacteria* and *Bacteroidota* accounted for more than 90% of the gut microbiota, a trend similar to that in previous reports. While the overall trends in the gut microbiota are consistent, *Akkermansia muciniphila* and *Bifidobacterium breve*, which are associated with effective immunotherapy in patients with NSCLC without mutations^{18,19}, were not identified in NSCLC patients with EGFR mutations. We speculate that this may be one reason why immunotherapy is less effective in these patients, although further investigation is necessary.

Our data demonstrated that α -diversity was higher in the group with no or less severe diarrhea. A previous report also showed a trend toward higher α -diversity in Japanese healthy individuals who did not have diarrhea²⁰. Taken together, the data suggest that the α -diversity of the gut microbiota is associated with no/less severe diarrhea, regardless of whether the subject has cancer. Also, our β -diversity and LEfSe analyses identified *Ruminococcus* in patients who were responsive to EGFR-TKI treatment. Previous studies show that ICI have better anti-tumor effects in patients with abundant *Ruminococcus*²¹, and that high levels of *Ruminococcus* correlate with durable clinical benefit²². It seems therefore that *Ruminococcus* abundance is associated with favorable outcomes after immunotherapy, making it a potential biomarker. In this study, we found a similar trend in EGFR-mutant NSCLC patients, suggesting that *Ruminococcus* abundance may also be a biomarker that can be used to estimate the efficacy of EGFR-TKI therapy.

This study has several limitations that should be considered. First, our 16S V1–V2 sequencing approach did not provide accurate species-level resolution (e.g., *R. gnavus* vs. *R. bromii*). Previous studies suggest that members of the *Ruminococcus* genus play important functional roles in the gut, including participating in short-chain fatty acid production, bile acid metabolism, and maintenance of mucosal integrity^{23–25}. These functions may influence drug metabolism, mucosal protection, and ultimately the efficacy of EGFR-TKI therapy. Further studies using metagenomic shotgun sequencing are needed to clarify species-level contributions and their mechanistic impact.

In addition, use of the V1–V2 region may limit the comparability of our data with other studies that employed different target regions. This study did not include a control group. Instead, we referred to the report by Shoji

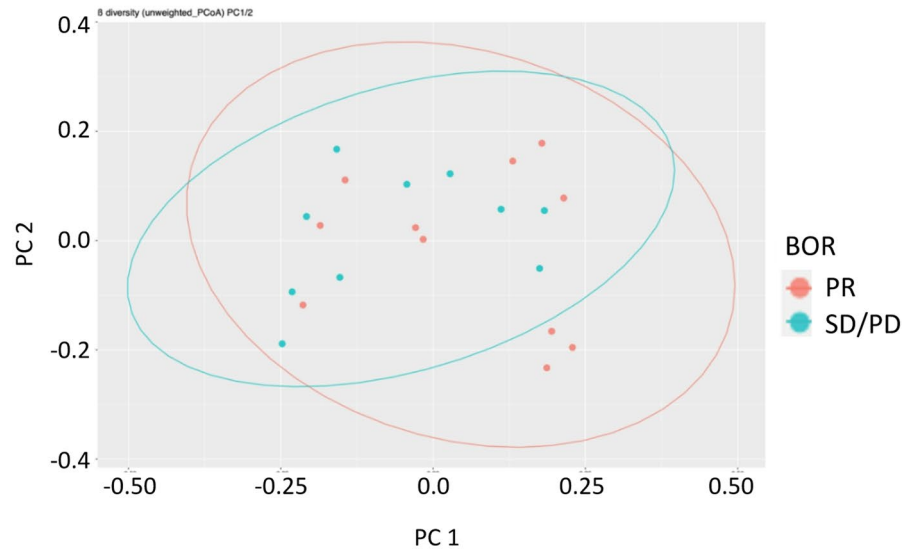
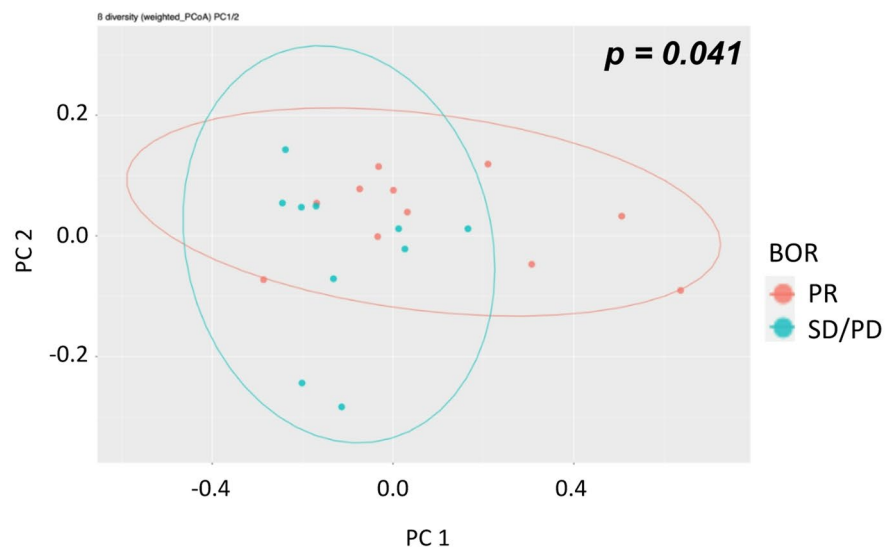
A: unweighted**B: weighted**

Fig. 3. Principal coordinates analysis of microbiota data based on genus-level unweighted (A) and weighted (B) UniFrac distances between patients in the PR and the SD/PD groups.

et al., which described characteristic oral and gut microbiota alterations in Japanese patients with lung cancer, as contextual information for the control cohort. Although their analysis targeted the V3–V4 region of the 16S rRNA gene, whereas our study targeted the V1–V2 region, making direct and accurate comparisons difficult, their findings provide useful context for interpreting our results. Inclusion of a control cohort, such as patients with wild-type EGFR, would allow us to better identify trends specific to EGFR-mutant NSCLC, and this is an important future research direction.

Second, with respect to diarrhea severity, the observed difference in the Shannon index between the G0–G1 and G2–G3 groups was borderline significant ($p = 0.0367$). This was a two-group comparison and thus multiple testing correction was not applied; however, this borderline statistical significance is a limitation of the present study. Further validation in larger cohorts and complementary analyses, including β -diversity comparisons, are necessary to confirm this association between α -diversity and diarrhea severity.

Third, different types of EGFR-TKIs (osimertinib, afatinib, and erlotinib + RAM) were administered in this cohort, and the type of EGFR-TKI could potentially influence the gut microbiota composition. However, the total number of samples was limited ($n = 21$) and therefore further subgroup analyses according to EGFR-TKI type were not statistically feasible. We acknowledge this is an important limitation and plan to increase the

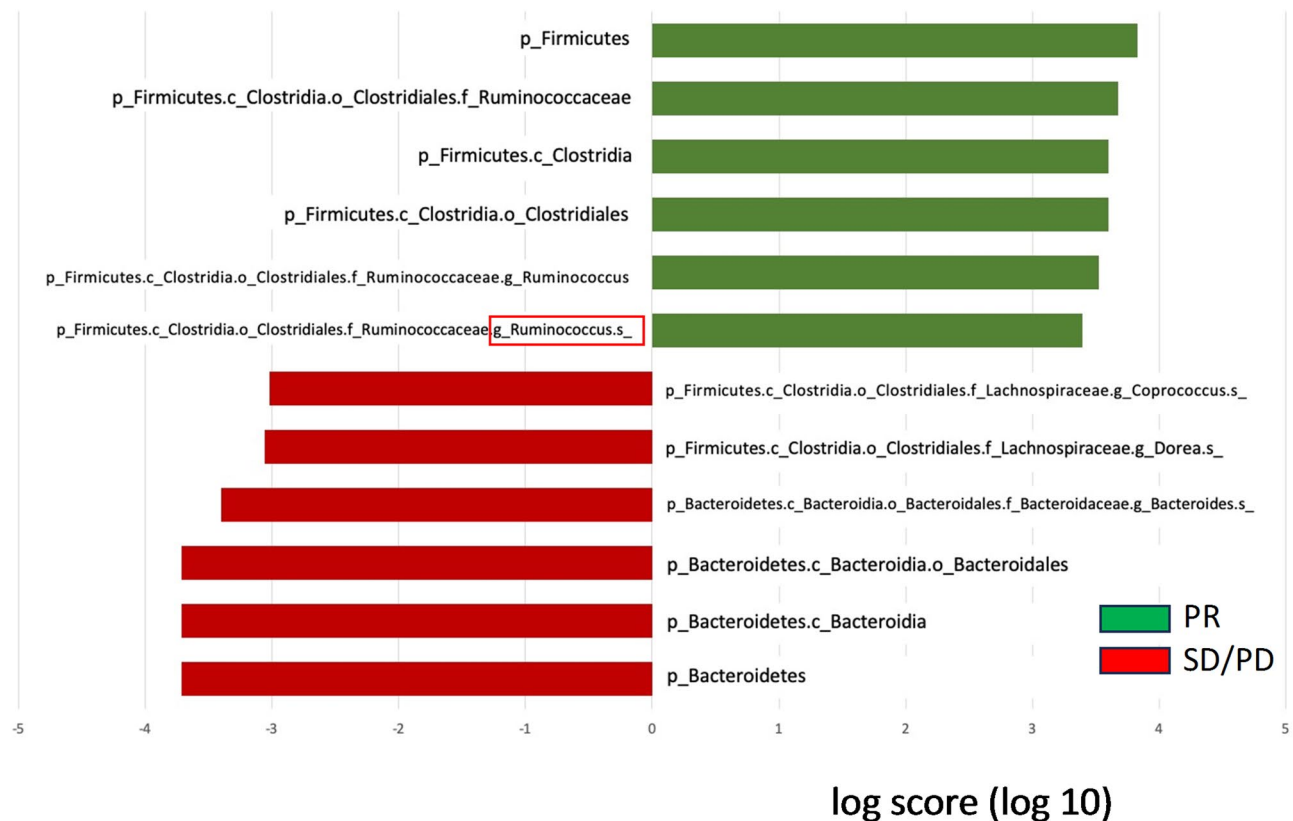


Fig. 4. Results of LEfSe analysis of differentially abundant taxa in the gut microbiota; based on best overall response (the PR vs. SD/PD groups).

sample size in future studies to enable stratified analyses according to EGFR-TKI type. Finally, we were unable to obtain OS data for all patients because the study period was short (3 years).

In conclusion, we report novel findings regarding the gut microbiota of EGFR-mutant NSCLC patients. Higher α -diversity before the start of treatment may predict no/less severe diarrhea as a side effect. In addition, *Ruminococcus* is a potential biomarker for predicting a better response. Because stool examination is noninvasive, it is beneficial for patients with EGFR-mutant NSCLC.

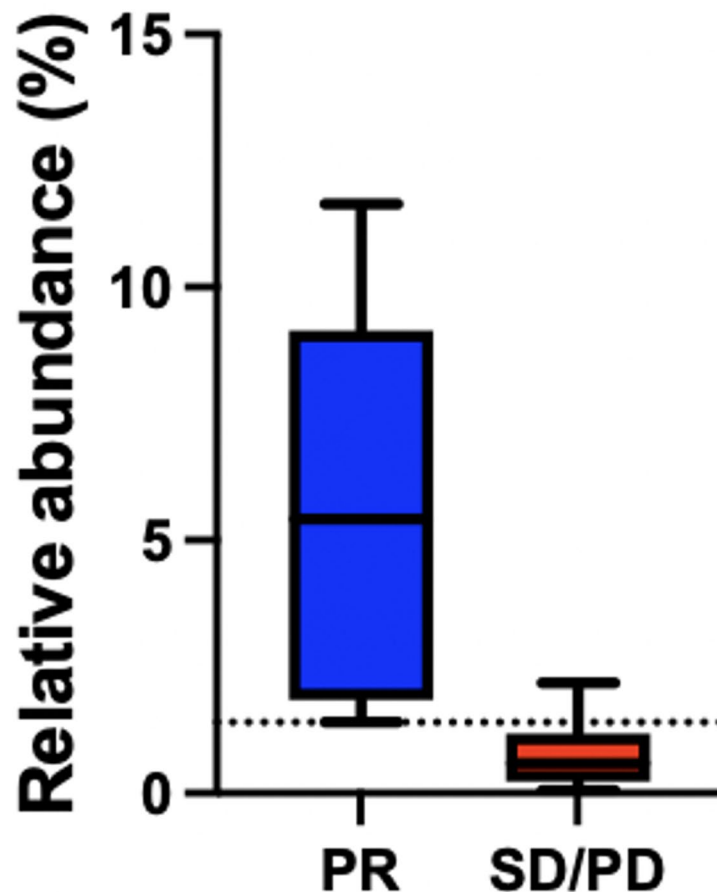


Fig. 5. Univariate analysis of the best overall response (PR vs. SD/PD groups) according to abundance of *Ruminococcus*. *P* values were calculated using the Mann-Whitney U test. The dotted line shows 1.4%, which is the cut-off value of *Ruminococcus*.

Data availability

NGS data were deposited at DDBJ Sequence Read Archive (DRA) with an accession number PRJDB35436 (<https://www.ncbi.nlm.nih.gov/sra/?term=PRJDB35436>).

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Author contributions

C.Tabe, S.Tasaka, and H.Fujii conceived the study. D.Motooka., T.Fujita, S.Tasaka, and H.Fujii supervised the study. C.Tabe and D.Motooka. performed experiments and wrote the manuscript. D.Motooka., T.Makiguchi., T.Fujita S.Tasaka, and H.Fujii revised the manuscript. T.Makiguchi., K.Taima, H.Tanaka, M.Itoga, Y.Ishioka, T.Akita, M.Ishidoya, K.Chubachi., T.Fukushima, Y.Tanaka, H. Odagiri., Y.Kameyama, and Y.Kobori supported the clinical experiments. All authors read and approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine (Approved No.: 2020-085-1).

Additional information

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