Intestinal microbiota predicts lung cancer patients at risk of immune-related diarrhea

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Aim: Previous studies showed that some patients after the treatment of anti-programmed cell death protein-1 (anti-PD-1) antibodies experienced immune-related diarrhea. In this study, we aim to explore the association between intestinal microbiota and immune-related diarrhea. **Methods:** We obtained the fecal samples of 26 advanced lung cancer patients before the first dose of anti-PD-1 antibodies. **Results:** Eight of 26 patients experienced diarrhea after the treatment of anti-PD-1 antibodies. At the phylum level, our study demonstrated that *Bacteroidetes* were higher in diarrhea-free (D-F) patients, while *Firmicutes* were lower. *Bacteroides* and *Parabacteroides* belonging to *Bacteroidetes* phylum and *Phascolarctobacterium* of *Firmicutes* phylum were more abundant in D-F patients. Whereas, *Veillonella* of *Proteobacteria* phylum was lower in D-F patients. **Conclusion:** Our study highlights that intestinal microbiota variation is correlated with the subsequent development of immune-related diarrhea.

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Keywords: 16S rRNA • anti-PD-1 antibodies • immune-related diarrhea • intestinal microbiota • PD-1

Background

Anti-programmed cell death protein-1 (PD-1) antibodies provide a novel effective option for lung cancer patients [1,2]. The Clinical trials CheckMate 017 and CheckMate 057 showed that nivolumab significantly improved 2-year overall survival rates in non-small-cell lung cancer (NSCLC) patients [3]. Clinical trial Keynote 010 suggested that pembrolizumab could prolong overall survival in NSCLC patients [4]. Moreover, an ongoing phase Ib clinical trial demonstrated that the objective response rate was 29% and the DCR was 33% in small cell lung cancer (SCLC) [5]. Thus, the anti-PD-1 antibodies nivolumab and pembrolizumab have been approved for treating advanced NSCLC and showed impressive efficacy in SCLC [6].

PD-1, a receptor expressed on the surface of numerous immune cells, effects programmed cell death via diminishing cell growth factor and survival signals [7]. Anti-PD-1 antibodies inhibit negative regulatory components of the immune response, boosting immune system by enhancing T cell action against the cancer cells [8]. Though anti-PD-1 antibodies improved objective response rate and prolonged overall survival in lung cancer patients, a number of patients treated with anti-PD-1 antibodies experienced immune-related adverse events (irAEs). Studies reported that the incidence of irAEs, including fatigue, rash/pruritus and mucositis, diarrhea and colitis, and pneumonitis, varies from 15 to 90%, and severe irAEs is estimated to range from 0.5–13% [9]. Furthermore, a previous study reported 6.0–16.0% patients treated with anti-PD-1 antibodies suffered immune-related diarrhea [10]. However, the mechanisms of immune-related diarrhea still need elucidation.

Intestinal microbiota contributes to immune system development and plays an important role in the regulation and maintenance of intestinal barrier function. The human intestines harbor more than 100 trillion bacteria that vary markedly in composition [11]. Researches indicated that commensal *Bifidobacterium* boost immunotherapy responses in mouse models of cancer and facilitate the efficacy of anti-PD-1 antibodies [12,13]. Moreover, intestinal microbiota could be used to predict the risk of immune-related colitis in patients treated with ipilimumab treatment, and bacterial metabolism pathways involved in polyamine transport and B vitamin biosynthesis protect patients from immune-related colitis [14]. Also, exacerbations of chronic gut and lung disease have been shown to share key conceptual features with the disorder and dysregulation of the microbial ecosystem [15]. However, the relationship

between intestinal microbiota and immune-related diarrhea is indeterminate. Also specific intestinal microbiota should be found out as biomarkers to predict immune-related diarrhea in patients after the treatment of anti-PD-1 antibodies. We try our best to find out the association between intestinal microbiota and immune-related diarrhea and furthermore we aim to identify specific intestinal microbiota as potential biomarkers for immune-related diarrhea in patients after the treatment of anti-PD-1 antibodies in our study. To the best of our knowledge, our study is the first to identify the potential microbiota-associated biomarkers that could predict the incidence of immune-related diarrhea after the treatment of anti-PD-1 antibodies.

Methods

Study participants

Lung cancer patients treated with anti-PD-1 antibodies from January 2017 to June 2017 in Chinese PLA General Hospital were retrospectively analyzed. All subjects enrolled in this study signed an informed consent form before fecal sample collection. The inclusion criteria were as follows: advanced lung cancer patients were diagnosed by pathology and imaging tests, subjects have none history of inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) or bowel resection. Exclusion criteria were as follows: prior history of immune-therapy, suffering from diarrhea in the past 2 months, diarrhea status unknown, or administration of antibiotics in the preceding 2 weeks. Pembrolizumab was administered intravenously at a dose of 2 mg/kg body weight over 30 min every 3 weeks, and nivolumab at a dose of 3 mg/kg body weight every 2 weeks over 60 min. We divided the patients into progressed to diarrhea (PtD) group and diarrhea-free (D-F) group, based on whether they develop diarrhea or not after anti-PD-1 antibodies treatment. And we graded immune-related diarrhea according to the National Cancer Institute Common Toxicity Criteria (CTC, version 4.0). Clinical response was assessed via Response Evaluation Criteria In Solid Tumors, version 1.1 (RECIST v1.1) every two cycles until disease progression.

Sample collection

The fecal samples were obtained from patients before the first dose of anti-PD-1 antibodies. Samples were collected in a sterile container and stored at -80°C .

16S rRNA gene extraction & sequencing

16S rRNA sequence is used to profile fecal bacterial composition. Total genome DNA from samples was extracted using cetyltrimethylammonium ammonium bromide (CTAB)/sodium dodecyl sulfate (SDS) method. DNA concentration and purity were monitored by agarose gel electrophoresis. Then DNA was diluted to 1 ng/ μl using sterile water. The V3–V4 region of the 16S rRNA gene was amplified from the total DNA extracted. The length of the amplification was 478 bp. For every fecal sample, PCR amplification was performed using the specific primer with the barcode (forward primer: TCAGCCTACGGGNGGCWGCAG, reverse primer: GGACTACHVGGGTATCTAATCC) and Q5[®] Hot Start High-Fidelity 2X Master Mix (New England Biolabs). All PCR reactions were carried out in 50 μl reactions. It contains 2 μl template DNA, 25 μl Q5[®] Hot Start High-Fidelity 2X Master Mix (New England Biolabs), 2.5 μl forward primers, 2.5 μl reverse primers and 18 μl tri-distilled/deionized water. Thermal cycling was consisted of initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s, finally 72°C for 2 min and recovery to 4°C . The PCR products were mixed in equidensity ratios and then separated by electrophoresis on 2% agarose gel. The mixed PCR products were purified using a GeneJET Gel Extraction Kit (Thermo Scientific).

The DNA libraries were generated using a NEBNext Ultra DNA library prep kit for Illumina (New England Biolabs). After quantification via qPCR, the DNA library quality was assessed on Agilent Bioanalyzer 2100 system. At last, the libraries were sequenced on Illumina HiSeq2500 platform.

Sequencing analysis & statistics

As quality control, paired-end reads from the original DNA fragments were merged using FLASH [16], a very fast and accurate analysis tool, which was designed to extend the length of short reads by overlapping paired-end reads from fragment libraries that are sufficiently short. Then the merged sequences were used in analysis. The sequences containing less than 80% base scored over 30 (using the Phred quality score) were removed by QIIME1.8 [17]. Also the chimeras were eliminated in the analysis via USEARCHv6.1.

Sequence analysis was performed by QIIME software package [17]. We used uclust v 1.2.22 to cluster the sequences into operational taxonomic units (OTUs) based on 97% sequence similarity. Based on the greengene

database, we picked a representative sequences for each OTU and then used the RDP classifier to annotate taxonomic information for each representative sequence [18,19]. OTUs were grouped at two levels of classification (phylum and genus). To compute Alpha Diversity, we rarify the OTU table and calculate four metrics: Chao1 and abundance-based coverage estimator (ACE) were used to estimate the species abundance; Shannon index and Simpson index were used to estimate the species diversity. QIIME calculates both weighted and unweighted unifracs distance, which are phylogenetic measures of beta diversity. We used both of them for Principal Coordinate Analysis (PCoA). Principal coordinates Analysis (PCoA) plots were used to visualize the similarities or differences in intestinal microbiota composition between PtD group and D-F group [20].

The data analyses were performed using R Statistical Language (v3.1.1) and GraphPad Prism (version 7.0a) software packages. Statistical analyses of clinical characteristics of the participants were performed using chi-square test. The data of Alpha diversity were expressed as the mean value $\pm$ standard deviation (SD) and compared using Student's t-test between PtD patients and D-F patients. Analysis of similarity (ANOSIM) was used to determine significant differences in microbial communities between the two groups. The test statistic R, ranges from -1 to 1: R = 1 implies differences between groups, while R = 0 implies that the groups are identical. The relative abundance of the intestinal microbiota was expressed as the median (P25, P75), and we used Mann–Whitney U test to compare the differences in intestinal microbiota composition. p-value < 0.05 was considered to be significant.

Results

Clinical characteristics of participants

26 lung cancer patients, including 18 male patients and eight female patients, treated with anti-PD-1 antibodies were enrolled in this study. The age of the patients ranged from 41 to 82 years old, and 18 patients were under 60 years old. Of the 26 patients, 22 were diagnosed with NSCLC and four were SCLC. 16 patients received the treatment of nivolumab and 10 patients were administered with pembrolizumab. Our results showed that eight patients experienced diarrhea after treatment were classified into PtD group, while the other 18 patients were in D-F group. No significant differences in gender, age, pathologic type and the kind of anti-PD-1 antibodies between the two groups were observed. The results of clinical response showed that one patient was partial response, two patients were stable disease and five patients were progressive disease in PtD group, while in D-F group, three patients gained partial response, 12 patients stable disease and three patients progressive disease. The objective response rates (ORRs) were 12.5 and 16.7% for PtD group and D-F group, respectively. The disease control rate (DCR) was 37.5% in PtD group and 83.3% in D-F group. Our results indicated that there were no significant differences in clinical response between patients in D-F group and PtD group was found in our study. In short, our results suggested that immune related diarrhea was not correlated with gender, age, pathologic type and the kind of anti-PD-1 antibodies. Detailed information is provided in [Table 1](#).

Clinical characteristics of the onset of diarrhea in PtD group

In our study, eight patients developed diarrhea after the initiation of anti-PD-1 antibodies. They experienced diarrhea vary from 1 to 10 weeks after using anti-PD-1 antibodies. The median onset time of patients experienced diarrhea was 4 weeks. Duration of diarrhea varied from 2 days to 14 days, and the median duration of diarrhea was 6.5 days. Bristol Stool Scale of patients suffering from diarrhea varying from type 5 to type 7. Three patients who suffered grade 1 diarrhea received the treatment of Loperamide, while the other 3 patients who suffered grade 2 diarrhea were treated with Loperamide and probiotic bacteria. Two patients experiencing grade 3 diarrhea were treated with prednisolone (80 mg/d p.o), which interrupted the use of anti-PD-1 antibodies. Satisfactorily, the relief of the symptom was obtained in all patients. And none of the patients was treated with antibiotics after initiation of PD-1 therapy and 2 weeks before PD-1 therapy. Detailed information is provided in [Table 2](#).

Alpha diversity & Beta diversity of intestinal microbiota before diarrhea

To verify the correlation between immune related diarrhea and intestinal microbiota, fecal samples were collected and microbiota composition was analyzed in both groups. A total of 3672 OTUs were identified in our study. A total of 3401 OTUs were identified in patients of D-F group, while 1965 OTUs in patients of PtD group. Our results showed that the patients of both groups shared 1334 identical OTUs, suggesting similar microbial populations harbored in both groups ([Figure 1](#)). To investigate the richness and evenness of microbiota in both groups, alpha diversity analysis was conducted. The ACE metrics was 819.96 ± 463.45 for the PtD group, and 910.12 ± 251.66 for the D-F group ([Figure 2a](#)). The Chao 1 metrics was 774.62 ± 436.46 and 902.55 ± 256.13

Table 1. Clinical characteristics of 26 lung cancer patients.

Clinical features	PtD group (n = 8)	D-F group (n = 18)	p-value
Gender			0.667
– Man	5	13	
– Woman	3	5	
Age			0.197
– <60 years	4	14	
– ≥60 years	4	4	
Pathologic type			1.000
– NSCLC	7	15	
– SCLC	1	3	
Treatment			1.000
– Nivolumab	5	11	
– Pembrolizumab	3	7	
Clinical response			0.063
– Complete response (CR)	0	0	
– Partial response (PR)	1	3	
– Stable disease (SD)	2	12	
– Progressive disease (PD)	5	3	
– ORR	1(12.5%)	3(16.7%)	1
– DCR	3(37.5%)	15(83.3%)	0.060

CR: Complete response; DCR: Disease control rate; NSCLC: Non-small-cell lung cancer; OR: Overall response rate; PD: Progressive disease; PR: Partial response; SD: stable disease; SCLC: Small-cell lung cancer.

Table 2. Clinical characteristics of diarrhea of the eight patients.

Grade CTCAE	Bristol Stool Scale	Anti-PD-1 antibody	Occurrence in weeks after initiation of anti-PD-1 antibody	Duration of diarrhea (days)	Treatment of diarrhea	Outcome of diarrhea
1	Type 5	Nivolumab	2	5	Loperamide p.o	Resolved
2	Type 6	Pembrolizumab	3	8	Loperamide p.o.; probiotic bacteria p.o	Resolved
3	Type 7	Nivolumab	10	14	Pause of nivolumab, prednisolone 80 mg/d p.o	Resolved
1	Type 6	Nivolumab	1	3	Loperamide p.o	Resolved
2	Type 6	Nivolumab	5	6	Loperamide p.o.; probiotic bacteria p.o.	Resolved
2	Type 7	Nivolumab	7	7	Loperamide p.o.; probiotic bacteria p.o.	Resolved
3	Type 7	Pembrolizumab	8	11	pause of pembrolizumab, prednisolone 80 mg/d p.o	Resolved
1	Type 6	Pembrolizumab	2	2	Loperamide p.o	Resolved

Anti-PD-1: Anti-programmed cell death protein-1; CTCAE: Common Terminology Criteria for Adverse Events.

for PtD group and D-F group respectively (Figure 2B). The Shannon index value was 5.19 ± 1.50 for the PtD group and 5.53 ± 0.85 for the D-F group (Figure 2C). The Simpson index value was 0.91 ± 0.08 for the PtD group and 0.93 ± 0.07 for the D-F group (Figure 2D). These results indicates that species abundance and diversity were tended to be less in PtD group than in D-F group, although no statistic difference was found between both groups. Detailed information is provided in Table 3. Thereafter, Beta diversity analysis was performed to identify the difference between both groups. Our results demonstrated that the fecal microbiota in PtD group and D-F group were significantly different by weighted-UniFrac PCoA analysis (ANOSIM, weighted: $R = 0.573$, $p = 0.001$) (Figure 2E). These results showed that intestinal microbiota were different between patients with and without immune related diarrhea, suggesting intestinal microbiota is associated with immune-related diarrhea in patients receiving anti-PD-1 antibodies treatment.

Figure 1. A scaled Venn diagram. 1707 operational taxonomic units presented only in diarrhea-free patients; 631 operational taxonomic units presented only in progressed-to-diarrhea patients; and 1334 operational taxonomic units were shared in both groups. D-F: Diarrhea-free; PtD: Progressed to diarrhea.

Table 3. Alpha diversity of the two group.

Alpha diversity	PtD group	D-F group	p-value
ACE	819.96 ± 463.45	910.12 ± 251.66	0.618
Simpson	0.91 ± 0.08	0.93 ± 0.07	0.537
shannon	5.19 ± 1.50	5.53 ± 0.85	0.572
Chao1	774.62 ± 436.46	902.55 ± 256.13	0.459

D-F: Diarrhea-free; PtD: Progressed to diarrhea.

Differences in the intestinal microbiota composition between PtD group & D-F group

To identify the difference of intestinal microbiota in both groups, intestinal microbiota composition were analyzed. Eight phyla (Figure 3A) and 36 genera (Figure 4A) were identified based on the OTUs observed. Our results showed that the *Firmicutes* phylum and the *Bacteroidetes* phylum account for over 80% of whole microbiota in both groups. Whereas, *Firmicutes* phylum was the most abundant (52.21%) in patients of PtD group, the *p*-value was 0.0054, while *Bacteroidetes* phylum (57.39%) was in D-F group, the *p* value was less than 0.001. Moreover, comparing with patients in PtD group, the phylum of *Bacteroidetes* were significantly higher and the *Firmicutes* phylum were obviously lower in D-F group (Figure 3B & C). Subsequently, we compared the relative abundance of the members of intestinal microbiota at the genus level. We found that *Bacteroides* and *Parabacteroides*, both of which belong to the *Bacteroidetes* phylum, were significantly abundant in D-F group (Figure 4B & C). However, *Phascolarctobacterium* from *Firmicutes* phylum was higher in D-F group (*p* = 0.0090, Figure 4D). Our results also showed that *Veillonella* from *Proteobacteria* phylum was less abundant in D-F group than that in PtD group (Figure 4E). Detailed information was showed in Table 4. Our findings suggested that *Bacteroides* and *Parabacteroides* from *Bacteroidetes* phylum, *Phascolarctobacterium* from *Firmicutes* phylum and *Veillonella* from *Proteobacteria* phylum probably increased the risk of immune related diarrhea in patients receiving anti-PD-1 antibody treatment.

Discussion

Blockade of PD-1 by nivolumab or pembrolizumab leads to irAEs in a number of patients [10]. Previous studies reported that intestinal microbes play a great role in irAEs of anti-PD-1 antibodies in mouse models of cancer [13]. However, the correlation between intestinal microbiota and immune-related diarrhea in human is still elusive.

In this study, we compared the intestinal microbiota between patients in PtD group and D-F group. In line with previous research on patients, our results revealed no significant difference in the richness and diversity of intestinal microbiota between PtD patients and D-F patients [14]. Moreover, consistent with previous studies, our results demonstrated that the majority of all microbial populations identified in our participants belonged to one

Figure 2. Analysis of Alpha diversity and Beta diversity in progressed to diarrhea group (red) and diarrhea-free group (blue). (A) The estimator of community richness abundance-based coverage estimator between PtD and D-F groups. (B) The estimator of community richness Chao1 between PtD group and D-F group. (C) The estimator of community diversity Shannon index between PtD group and D-F group. (D) The estimator of community diversity Simpson between PtD and D-F group. (E). Weighted-UniFrac Principal Coordinate Analysis (PCoA) of samples between PtD group and D-F group (ANOSIM, weighted: $R = 0.573$; $p = 0.001$). D-F: Diarrhea-free; PtD: Progressed to diarrhea.

of the two phyla *Bacteroidetes*, *Firmicutes* [21,22]. These findings suggested that immune-related diarrhea in patients receiving anti-PD-1 antibodies may not correlate with richness or diversity of intestinal microbiota at baseline.

Then, we analyzed intestinal microbiota composition between both groups. Comparing with patients in D-F group, we found that the relative abundance of the two dominant phyla (*Bacteroidetes* and *Firmicutes*) were significantly different in patients in PtD group. Furthermore, we found that the members belonging to *Bacteroidetes* phylum were higher in D-F group than in PtD group, while the *Firmicutes* phylum were lower. The members belonging to *Bacteroidetes* phylum, which have great influence on limiting inflammation, are common in human GI tract [21,23]. They stimulate Th1 and Th17 responses in gut and have the potential for inducing regulatory cells (Tregs), which could result in resistant to many immune-related diseases such as IBD and rheumatoid arthritis in mouse models [24–26]. *Firmicutes* is another abundant group of the intestinal microbiota. It has been reported that the

Figure 3. Intestinal microbiota composition and differences between the two groups at phylum level. Operational taxonomic units with relative abundance less than 1% were assigned to be others. **(A)** The composition of intestinal microbiota at phylum level, each bar represents one patient. **(B)** Relative abundance of the phylum *Bacteroidetes* in PtD and D-F patients. **(C)** Relative abundance of the phylum *Firmicutes* in PtD, and D-F patients. D-F: Diarrhea-free; PtD: Progressed to diarrhea.

Table 4. Differences in the fecal microbiota at the phylum level and the genus level between the two groups.

Taxon	The median (P25, P75)		p-value
	PtD group (n = 8)	D-F group (n = 18)	
Phylum			
<i>Bacteroidetes</i>	0.3157 (0.2989, 0.3403)	0.5739 (0.4742, 0.6304)	<0.001
<i>Firmicutes</i>	0.5221 (0.4575, 0.6289)	0.3226 (0.2684, 0.4362)	0.0054
Genus			
<i>Bacteroides</i>	0.2189 (0.0432, 0.2882)	0.3795 (0.2583, 0.4672)	0.0398
<i>Parabacteroides</i>	0.0080 (0.0006, 0.0189)	0.0400 (0.0080, 0.0791)	0.0145
<i>Phascolarbacterium</i>	0.0013 (0.0004, 0.0127)	0.0289 (0.0056, 0.0690)	0.0090
<i>Veillonella</i>	0.0079 (0.0030, 0.0747)	0.0006 (0.0004, 0.0069)	0.0348
D-F: Diarrhea-free; PtD: Progressed to diarrhea.			

D-F: Diarrhea-free; PtD: Progressed to diarrhea.

Figure 4. Intestinal microbiota composition and differences between the two groups at genus level. (A) The composition of intestinal microbiota at genus level, each bar represents one patient. **(B)** Relative abundance of the genus *Bacteroides* in PtD and D-F patients. **(C)** Relative abundance of the genus *Parabacteroides* in PtD and D-F patients. **(D)** Relative abundance of the genus *Phascolarctobacterium* in PtD and D-F patients. **(E)** Relative abundance of the genus *Veillonella* in PtD and D-F patients. D-F: Diarrhea-free; PtD: Progressed to diarrhea.

Firmicutes phylum is correlated with faster colonic transit [27]. Increased relative abundance of *Firmicutes* may lead to an increase in gut epithelial permeability and low grade inflammation, which was considered as possible disease mechanisms of IBS [28,29]. Thus, we speculated that *Bacteroidetes* phylum and *Firmicutes* phylum were associated with diarrhea in lung cancer patients treated with anti-PD-1 antibodies. Alteration in composition of the colonized bacteria can result in GI disorders including diarrhea and colitis [30–32]. Recent evidences suggested that the relative abundance of *Firmicutes* phylum is increased and the *Bacteroidetes* phylum is decreased in cancer patients experiencing diarrhea after radiotherapy [33]. These findings also partially support our speculation. To more comprehensively analyze the difference of intestinal microbiota, intestinal microbiota composition were analyzed at the genus level. We found two members of *Bacteroidetes* phylum were significantly higher in D-F group, including *Bacteroides* and *Parabacteroides*, suggesting an role of both microbiota in resistance to immune-related diarrhea in patients of D-F group. Moreover, our results demonstrated that *Phascolarctobacterium* of *Firmicutes* phylum is higher in patients of

D-F group. *Phascolarctobacterium* could produce short-chain fatty acids and adjust the metabolism of the host [34]. Studies also reported that significant decrease in genus *Phascolarctobacterium* correlated with presence of colonic inflammation [32]. Surprisingly, we observed an increased relative abundance of *Vellionella* in patients of PtD group, which was similar with previous researches on fecal samples of irritable bowel patients [35,36]. Taken together, these findings imply that *Bacteroides*, *Parabacteroides*, *Phascolarctobacterium* and *Vellionella* could be potential biomarkers for predicting immune-related diarrhea in patients treated with anti-PD-1 antibodies.

We speculated that the differences probably due to the different immune status and dietary behavior between the two group. Previous studies reported that adaptive immunity could shape the oral and colonic microbiome, which partly confirmed our speculation [37]. Also, it has been reported that intestinal microbiota is modulated by metabolic derangements, such as nutrition overload and obesity [38]. Furthermore, it has been found that a long-term diet with high protein can lead to higher *Bacteroides* [39]. Thus, we inferred that consuming diet of rich protein would increase the abundance of *Bacteroides* and avoid the occurrence of immune-related diarrhea. We will analyse the dietary patterns of the patients in the further study. Moreover, our results suggested the addition of probiotics before the treatment of anti-PD-1 antibodies might reduce the risk of immune-related diarrhea.

However, there are a number of limitations in the present study. Since only 26 patients were enrolled in our study, data of more participants are needed. Also, this study was conducted retrospectively, prospective analysis of large number of subjects should be performed to confirm the findings of our results. In addition, we only collected stool samples on the basis of administration, We will carry out further study to collect stool samples on the basis of dose and duration of administration of the drug. Finally, the molecular mechanisms of the intestinal microbiota regulating immune related diarrhea in patients treated with anti-PD-1 antibodies are also needed in the future.

Conclusion

In conclusion, our study suggested that intestinal microbiota was associated with immune-related diarrhea in patients treated with anti-PD-1 antibodies, and might be potential biomarkers helping to predict diarrhea in patients treated with anti-PD-1 antibodies.

Future perspective

Our study aims to identify the association between intestinal microbiota and immune-related diarrhea and to identify specific intestinal microbiota as potential biomarkers for immune-related diarrhea in patients after the treatment of anti-PD-1 antibodies. Our analysis demonstrated that Bacteroidetes were higher in D-F patients, while Firmicutes were lower, at the phylum level. Two families of the Bacteroidetes phylum (*Bacteroides* and *Parabacteroides*) and a family of the Firmicutes phylum (*Phascolarctobacterium*) were more abundant in D-F patients. Whereas, *Veillonella* of *Proteobacteria* phylum was lower in D-F patients. So we inferred that consuming diet of rich protein would increase the abundance of *Bacteroides* and avoid the occurrence of immune-related diarrhea. Moreover, our results suggested the addition of probiotics before the treatment of anti-PD-1 antibodies might reduce the risk of immune-related diarrhea. The main target was to find methods to avoid the onset of immune-related diarrhea in the next 5 to 10 years.

Ethical conduct of research

All subjects enrolled in this study signed an informed consent form before fecal sample collection. Fecal samples and clinical characteristics used in this study were approved by the ethics committee of Chinese PLA General Hospital. The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Availability of data & materials

All data collected or analyzed during this study are included in this article and its additional files.

Author contributions

Y Hu conceived this study, T Liu and Q Xiong carried out experiments and analyzed the data. T Liu wrote the manuscript. Q Xiong and L Li revised the manuscript. Y Hu supervised the manuscript. T Liu and Q Xiong are equal contributors. All authors had read and approved the submitted manuscript.

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Executive summary**Background**

- Anti-programmed cell death protein-1 (PD-1) antibodies bring a new option for lung cancer patients. However, previous studies showed that a number of patients suffered from immune-related diarrhea after the treatment of anti-PD-1 antibodies.
- Intestinal microbiota plays a vital role in gastrointestinal dysregulation including diarrhea. Nevertheless, the association between intestinal microbiota and immune-related diarrhea is still indeterminate.
- Our study aims to identify the association between intestinal microbiota and immune-related diarrhea. Furthermore, we try to find out specific intestinal microbiota as potential biomarkers for immune-related diarrhea in patients after the treatment of anti-PD-1 antibodies.

Methods

- The fecal samples of 26 advanced lung cancer patients were collected before the first dose of anti-PD-1 antibodies.
- We retrospectively analyzed the onset of diarrhea and fecal bacterial composition. After the treatment of anti-PD-1 antibodies, eight of 26 patients experienced diarrhea.

Results

- Diarrhea-free (D-F) patients and patients with diarrhea had similar microbial richness and diversity. However, the bacterial composition of the microbiota in patients with diarrhea were remarkably different from patients without diarrhea.
- Further analysis demonstrated that *Bacteroidetes* were significantly richer in diarrhea free patients than those experienced diarrhea, while *Firmicutes* were poorer, at the phylum level.
- Moreover, *Bacteroides* and *Parabacteroides* belonging to the *Bacteroidetes* phylum and *Phascolarctobacterium* belonging to the *Firmicutes* phylum were more abundant in D-F patients. Whereas, a family of *Proteobacteria* phylum (*Veillonella*) was lower in D-F patients than those experienced diarrhea.

Conclusion

- Our study identified that intestinal microbiota variation is related to the onset of immune-related diarrhea. Identifying these biomarkers is useful for us to predict and prevent immune-related diarrhea earlier.

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