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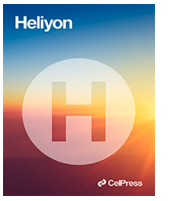

Research article

Efficacy of intestinal microorganisms on immunotherapy of non-small cell lung cancer

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ABSTRACT

While the 5-year survival rate of patients with advanced non-small cell lung cancer (NSCLC) has seen some improvement, the majority of NSCLC patients fail to respond to immunotherapy with immune checkpoint inhibitors (ICIs). It is critical to identify effective biomarkers that can enhance the efficacy of immunotherapy. The clinical data in the current study were collected from NSCLC patients treated with ICIs, and two groups were classified according to treatment effect: good group with consistent efficacy, poor group with only progressiveness. Differences in intestinal microbiota between the two groups were analyzed using 16s rRNA sequencing. Beta diversity analysis indicated differences between the two groups that were available for differentiation. Comparison of the number of common or unique operational taxonomic units (OTUs) among different groups suggested that there were 53 unique OTUs in the good group and 51 unique OTUs in the poor group. At the phylum level, there was a difference between the two groups for several bacterial groups with the highest abundance values, among which *Firmicutes, Actinobacteria* and *Fusobacteria* were more abundant in the good group. Members of the genera *Bifidobacterium* and *Lactobacillus* were abundant in the good group, while the abundance of *Bacteroides* was low. Biomarkers in the poor group included *Bacteroides, Bacteroidetes, Bacteroidia, Bacteroidales, Bacteroidaceae* and *Veillonellaceae*. The intestinal microbiota composition affected the immunotherapy process for NSCLC, which might offer more rational instructions for the clinical application of ICIs in NSCLC patients.

1. Introduction

On the basis of the latest national cancer statistics disclosed by the 2022 National Cancer Center, lung cancer remains the leading malignant tumor in terms of morbidity and mortality in China [1]. Lung cancer can be histologically categorized as either non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC) [2]. NSCLC accounts for the highest proportion and can be subdivided into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Unfortunately, patients with advanced NSCLC have a 5-year survival rate as low as 5 % [3].

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Immune checkpoint inhibitors (ICIs) have recently emerged as the most promising treatment option for advanced NSCLC [4]. In particular, the introduction of programmed death receptor 1 (PD-1) and programmed death receptor ligand 1 (PD-L1) inhibitors has brought about tremendous transformation to the treatment pattern for NSCLC, and consequently, the 5-year survival rate for advanced NSCLC patients has witnessed a notable increase ranging from 15.5 % to 23.2 % [5]. However, not all patients benefit from immunotherapy, and ICIs can give rise to a range of adverse events; therefore, the use of biomarkers can help improve the efficacy of treatment and reduce the adverse effects of ICIs.

The intestinal microbiota participates in the occurrence and development of malignant tumors in various ways, and the intestinal microbiota is of great significance in tumor immunotherapy [6–8]. Therefore, this study collected the clinical data of 50 NSCLC patients treated with ICIs in our hospital, statistically analyzed the efficacy, and grouped them according to efficacy to explore the feasibility of biomarkers, including intestinal microbes, as predictors of the efficacy of lung cancer immunotherapy.

2. Materials and methods

2.1. Clinical information

Informed consent was obtained from the patients with NSCLC who voluntarily agreed to participate in the study. Based on the effect of ICIs treatment and RECIST criteria 1.1 [9], these patients were divided into two groups: the good group was defined as a patient with complete (CR) or partial response(PR) or stable disease (SD) lasting at least 6 months, including 38 patients, and poor referred to patients whose therapeutic effect was ineffective or did not last for 6 months, including 12 patients (Table 1). Nine of these cases were selected for subsequent 16s of RNA sequencing.

2.2. Fecal sampling

The sampling process was strictly implemented in accordance with the guidelines for sterile procedures. To prevent contamination from urine or certain debris, the surface of fresh feces was gently wiped off using sterile cotton swabs, and one g of the inner layer of feces was removed by replacing the swab and placed in a sterile lyophilization tube.

2.3. Extracting and purifying DNA from intestinal microbes

The QlAamp DNA Stool Mini Kit (Qiagen, Germany) was used for DNA extraction; the concentration was detected using a QubitFluorometer kit (Life Technologies, USA). Sample integrity was determined using 1 % agarose gel electrophoresis. Following successful 1 % agarose gel electrophoresis, the samples were sent to the laboratory for 16S high-throughput sequencing analysis.

2.4. High-throughput sequencing of 16S analysis of intestinal microbiota

The Illumina HiSeq sequencing platform was used for double-end sequencing, and the original data in fastq format were obtained by sequencing (the samples corresponded to a pair of sequences S_1. fastq and S_2.fastq). They were then paired and spliced into single sequences. A barcode was used to determine the sample corresponding to the sequence. QIIME software (Quantitative Insights into Microbial Ecology 2, q2cli version 2021.2.0) was used to filter the sequences for quality control, and chimeras were removed. Thus, a valid sequence was obtained.

2.5. Intestinal microbiota analysis method

Sequences with more than 97 % similarity were defined as an operational taxonomic unit (OTU). The alpha diversity of the microbial communities, including richness, chao1, ACE, Shannon, Simpson, and Invsimpson indices, were compared. Beta diversity was

Table 1
Patient clinical data.

Patient group (n = 50)		
Gender	Male	45
	Female	5
Age	<70	38
	≥70	12
Tissue types	Adenocarcinoma	30
	Squamous carcinoma	20
Staging	Stage III	5
	Stage IV	45
International cancer management	First line	32
	Second line	18
Therapeutic efficacy	Lasting efficacy	38
	Ineffective	12

analyzed using principal component analysis and the distance matrix heatmap bray_curtis. Linear discriminant analysis Effect Size (LEfSe) was applied to identify the best characteristic taxonomic level for each group. Groups with an LDA score greater than 4 and a value of *P* less than 0.05 were considered significant.

3. Results

3.1. Clinical information of the participants

Among the 50 NSCLC patients who received ICIs, the number of men was greater than that of women, and most of them were under 70 years of age. After immunotherapy and chemotherapy, 76 % of patients had a good effect and 24 % had a poor effect (Table 1).

3.2. Composition analysis of each classification level

By comparing the number of common or unique OTU among the different groups, it was found that there were 53 unique OTU in the good group, 51 unique OTU in the poor group, and 52 common OTU in the two groups (Fig. 1).

According to the species annotation results, a relative abundance bar chart was drawn for the high-abundance bacteria selected from the samples at each taxonomic level, so that the proportion of species composition could be determined. The dominant bacterial groups at the phylum level were *Firmicutes, Bacteroidetes, Actinobacteria,* and *Proteobacteria*. *Firmicutes, Actinobacteria*, and *Fusobacteria* were more abundant in the good group. At the class level, the bacterial groups with high abundance included *Clostridia, Bacteroidia,* and *Actinobacteria*, among which there were many *Bacteroidia* in the poor group. There were more bacterial groups at the order level, such as *Clostridiales, Bacteroidales,* and *Bifidobacteriales*, among which the abundance of *Bacteroidales* in the poor group was higher. At the family level, *Lachnospiraceae, Bacteroidaceae,* and *Bifidobacteriaceae* ranked highest in terms of abundance, among which *Bacteroidaceae* was more abundant in the poor group. The dominant bacterial groups at the genus level included *Blautia, Bifidobacterium,* and *Lactobacillus*, in which the abundance of *Bacteroides* in the good group was relatively low (Fig. 2A–B).

3.3. Alpha diversity analysis

In the study of community ecology, alpha was used to analyze the species diversity in each group. The dilution curves of intestinal flora richness and diversity plateaued or leveled off, indicating that the sequencing depth met the standard (Fig. 3A). The richness results, including α richness, chao1, ACE, Shannon, Simpson, and Simpson indices in the good and poor groups were not significantly different (Fig. 3B–G). These results indicated that there was no significant difference in species abundance and diversity between the good and poor groups.

3.4. Beta diversity analysis

The beta index was used to compare discrepancies between multiple groups of samples. Principal component analysis (PCA) indicated that the composition of gut microbiota was different between the good and poor groups (Fig. 4A). The distance matrix heatmap bray_curtis showed a sample discrepancy between the two groups (Fig. 4B). The similarity between samples in the same group was high, whereas that between samples in different groups was low.

3.5. Comparing microbiota across groups and selecting species markers

Biomarkers between groups were screened using LEfSe; that is, species with significant different between groups. The biomarkers in

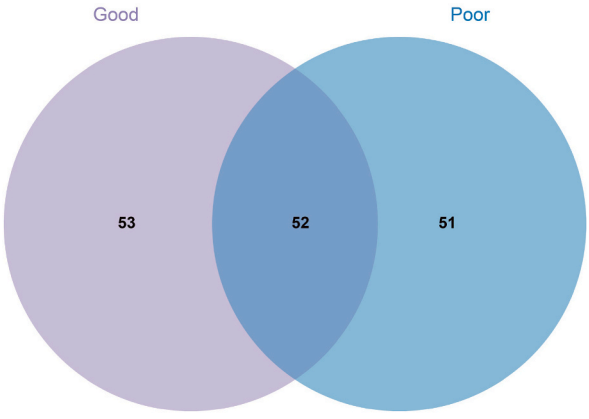

Fig. 1. OTU clustering and statistics.

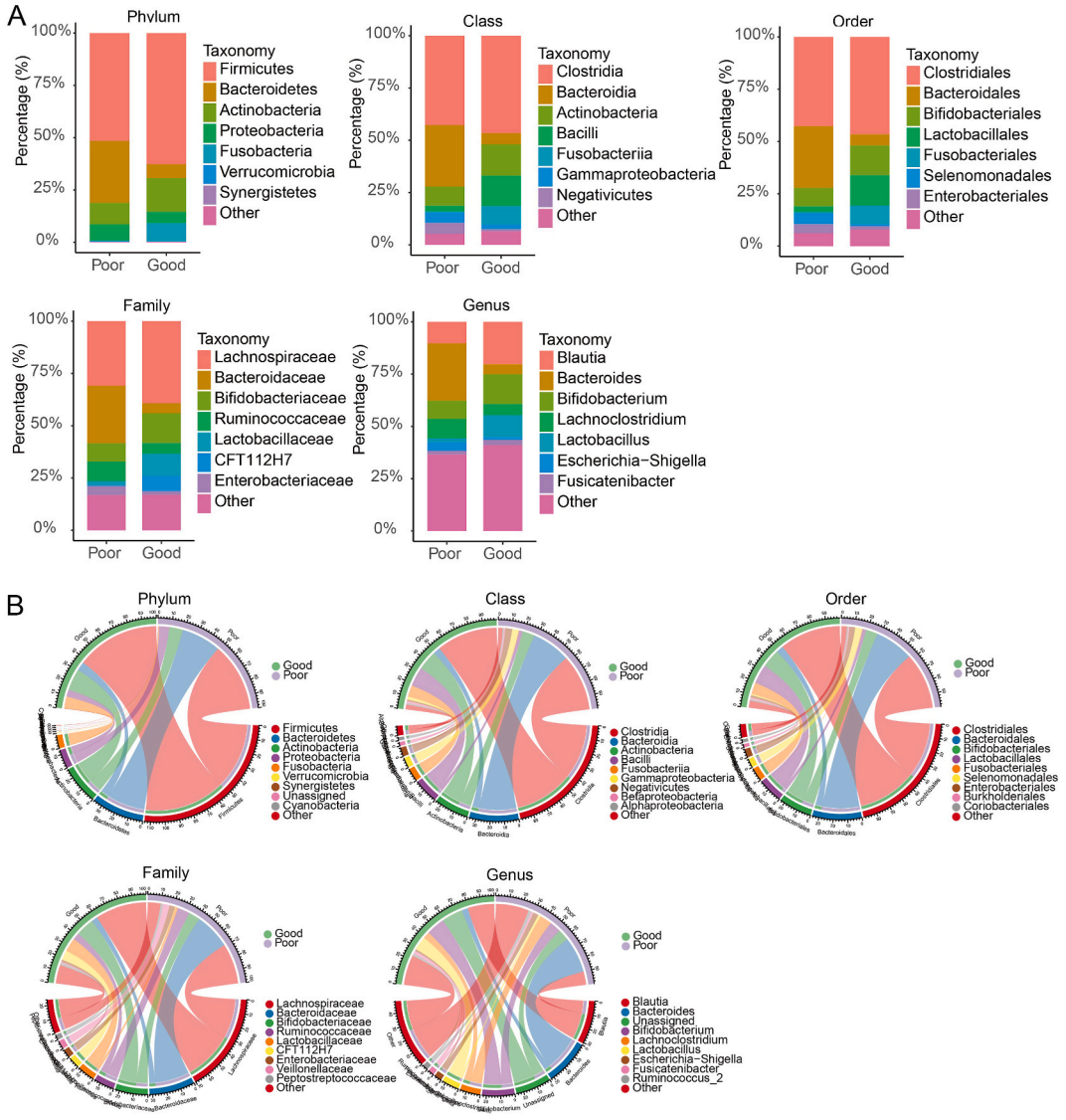

Fig. 2. Species abundance analysis at level of Phylum, Class, Order, Family and Genus. (A) Histogram of relative abundance. (B) Circus diagram of collinearity relationship between samples and species, the upper half represents different levels in different sample groups.

the poor group included *Bacteroides, Bacteroidetes, Bacteroidia, Bacteroidales, Bacteroidaceae* and *Veillonellaceae* (Fig. 5A–B).

3.6. Analysis of differences in the third-level classification of KEGG metabolic

Fig. 6 presents KEGG metabolic pathways with significant differences in the composition of the third level among the groups and their proportions in each group. Glycan biosynthesis and metabolism were more common in the poor group than in the good group. However, the Membrane Transport was higher in the good group.

3.7. Comparison of the abundance values of intestinal microorganisms associated with immunotherapy C-reaction protein (CRP) in different groups

Intestinal microorganisms associated with CRP and immunotherapy include *Thalassobaculum, Flavobacterium.3, Parvibaculum, Acidiferrobacter, Lachnoclostridium.25, Hydrogenophaga, and Lactococcus, Limnohabitans, Prosthecobacter* and *Lactococcus*. Comparing the abundance differences of these microorganisms between the poor and good groups, the results show that in the poor group, *Thalassobaculum*, *Flavobacterium.3*, *Parvibaculum*, *Acidiferrobacter*, *Lachnoclostridium.25*, *Hydrogenophaga*, *Lactococcus*, *Prosthecobacter* and *Lactococcus* were significantly higher than in the good group (Fig. 7A–G, 7I-J), while Limnohabitans was significantly lower than in

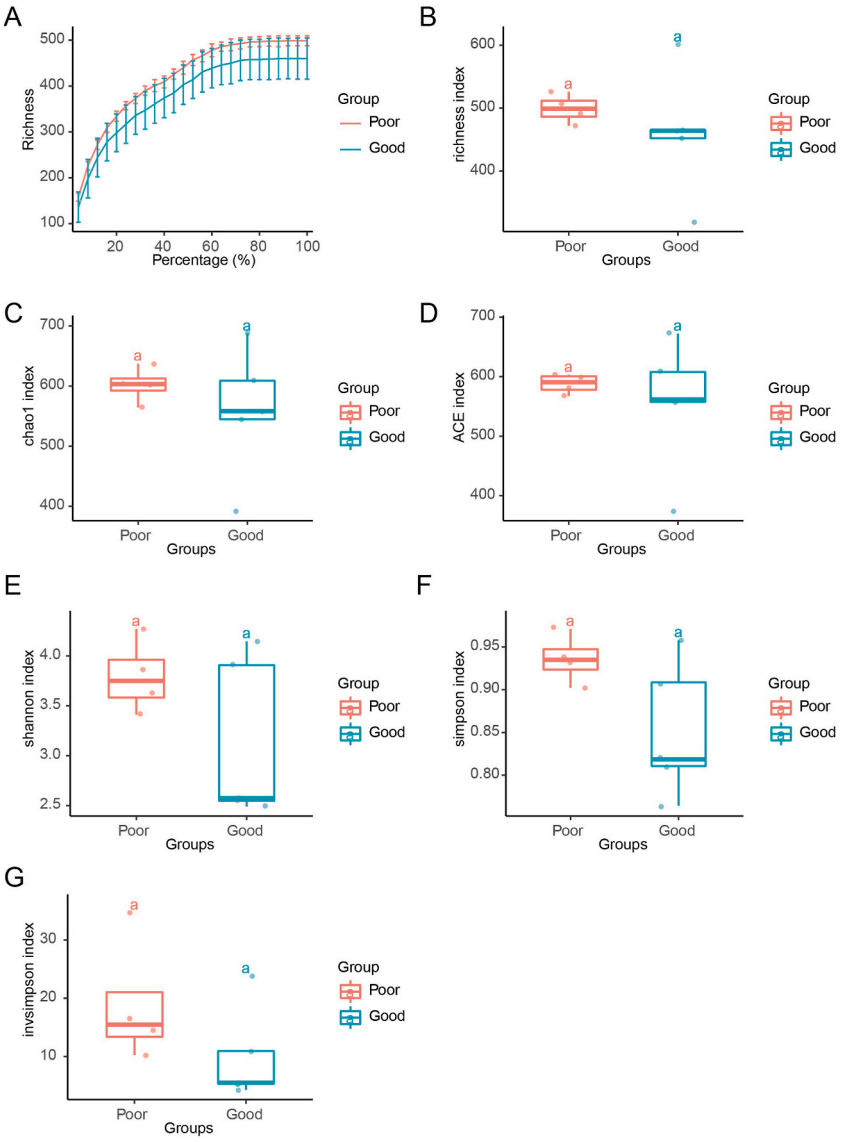

Fig. 3. Alpha diversity analysis. (A) Dilution curve. (B–G) Comparison of richness, chao1, ACE, shannon, simpson and invsimpson indexes between both groups.

the good group (Fig. 7H).

4. Discussion

Multiple clinical studies have confirmed that ICI markedly extend patients' progression-free survival (PFS) and overall survival (OS) at the stage of advanced NSCLC [10]. ICI inhibited interactions between the inhibitory receptors expressed on T cells and their related ligands to mediate immune cell activity and kill tumor cells [11]. It has been demonstrated that lung cancer is affected by microbial communities through their effects on immune responses, inflammation, metabolism, genotoxicity, and virulence [12]. In recent years, studies have found that regulating the intestinal microbiota can improve the efficacy of ICI and reduce adverse reactions. Intestinal microbiota diversity and some commensal bacteria are positively correlated with the efficacy of ICI [13].

A large number of microbial communities in the human body can help the body digest and absorb nutrients, participate in metabolic regulation processes, synthesize vitamins or regulate immune responses. Gut microbiota is essential for the onset and development of lung diseases since "gut-lung axis" was discovered [14,15]. Studies have found that patients with NSCLC and healthy people have different intestinal microbiota compositions, and some microbiota may be potential predictive markers of NSCLC [16]. Microbiota prediction models with specific intestinal microbiota markers can be used to predict early NSCLC [17]. Among treated patients with NSCLC, those with greater gut microbiota diversity had better treatment outcomes and considerably longer PFS [18]. In

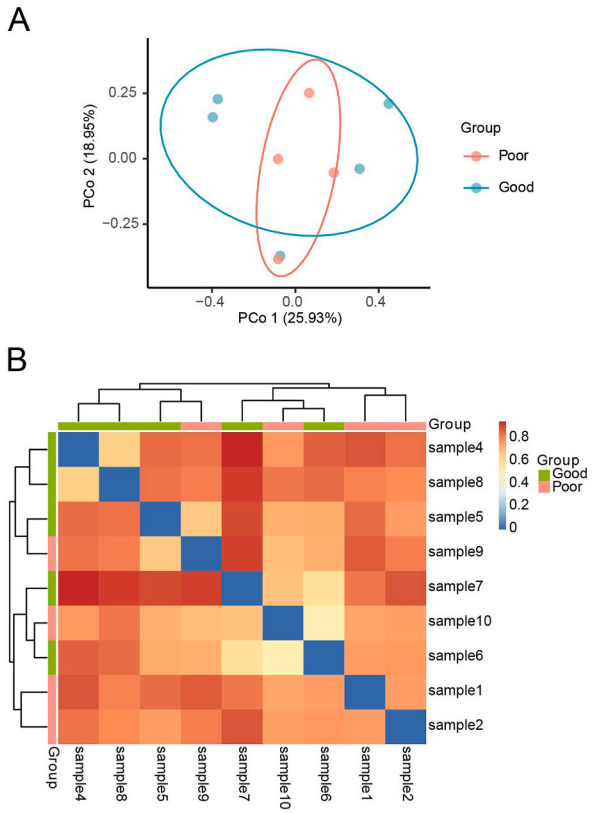

Fig. 4. Beta diversity analysis (A) PCA analysis. (B) Distance matrix heat map ray_curtis.

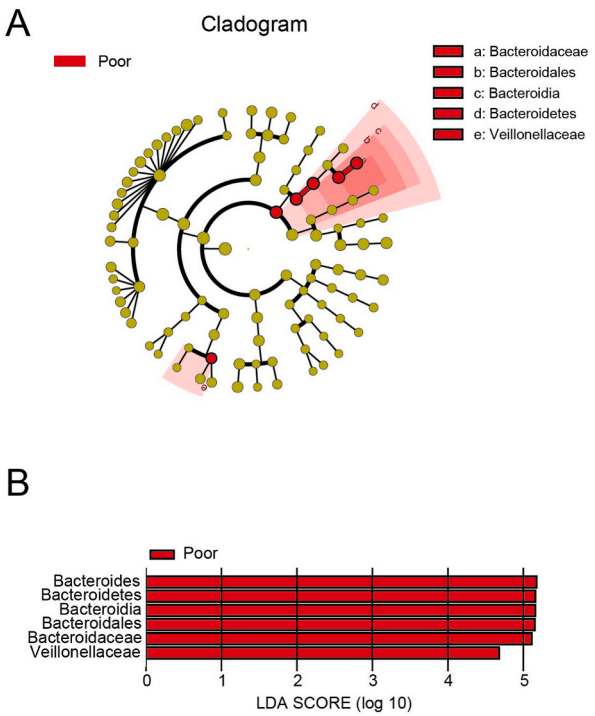

Fig. 5. LEfSe analysis of intestinal flora in poor groups. (A) Evolutionary branching diagram. (B) LDA effect size.

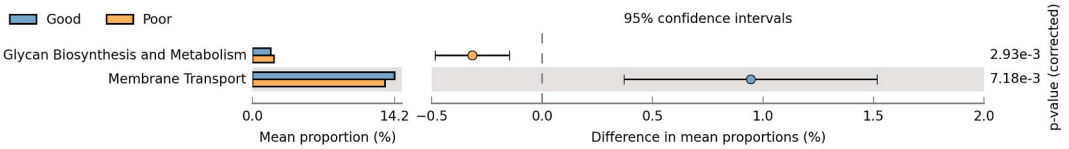

Fig. 6. Differential analysis of KEGG metabolic pathways in the third layer classification. Note: Different colors in the figure represent different groups. The proportion, confidence interval and P-value of differences are shown on the right, and the abundance level is shown at the bottom.

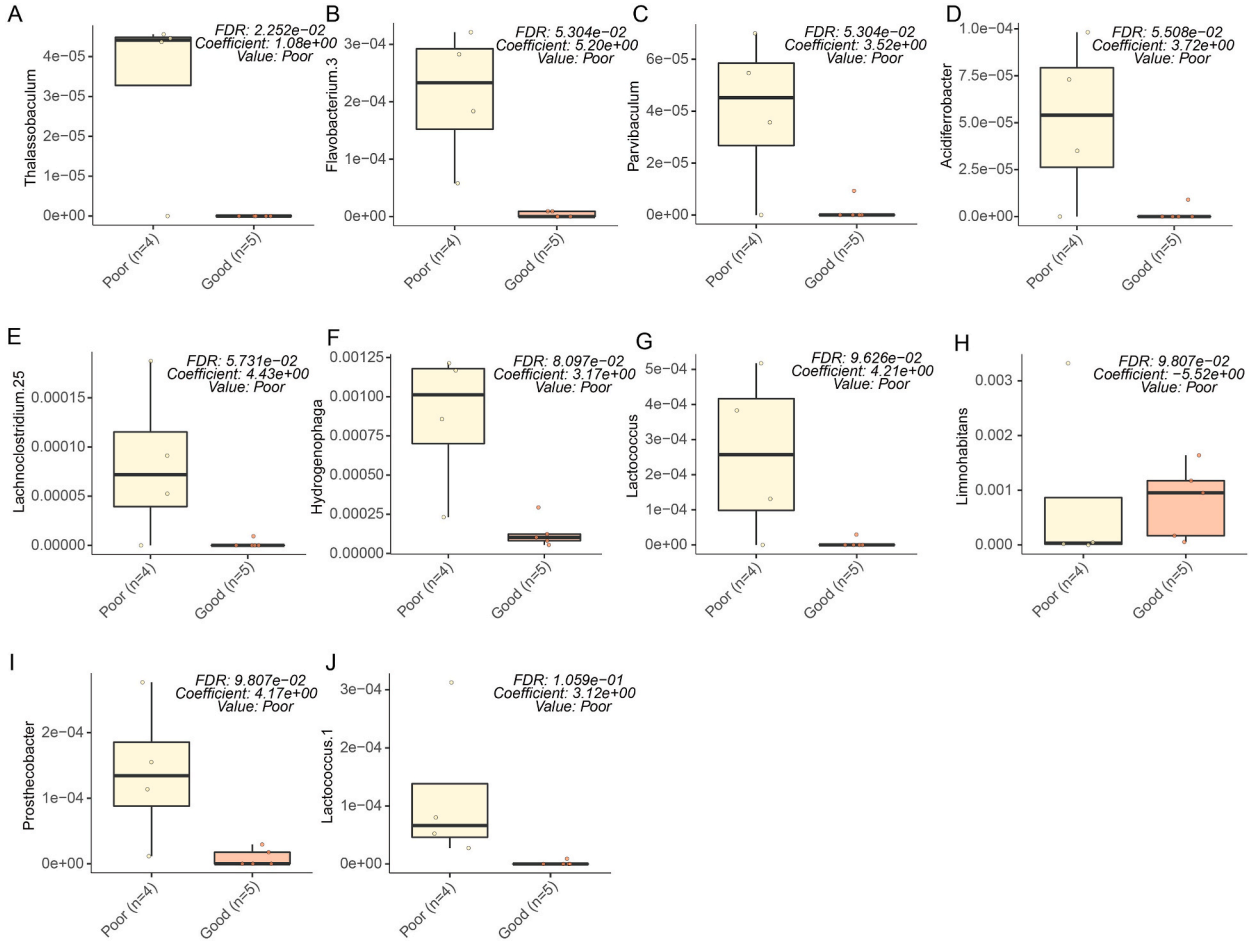

Fig. 7. Comparison of abundance values of intestinal microorganisms associated with immunotherapy markers CRP in different groups. (A) Thalassobaculum. (B) Flavobacterium.3. (C) Parvibaculum.(D) Acidiferrobacter. (E) Lachnoclostridium.25. (F) Hydrogenophaga. (G) Lactococcus. (H) Limnohabitans. (I) Prosthecobacter. (J) Lactococcus.1.

tumor tissues, intestinal microbiota increases the clinical effectiveness of ICIs by increasing the recruitment of CCR9+, CXCR3+, and $CD4^+$ T lymphocytes [19]. It has been found that *Akkermansia muciniphila* is associated with a positive response to ICI treatment, and supplementation with the herb will increase the response [20]. This study divided NSCLC patients into good and poor groups based on ICI efficacy, and used 16s rRNA sequencing to identify biomarkers.

16s rRNA sequencing analysis of intestinal microbiota differences between the good and poor groups indicated no difference in intestinal microbiota alpha diversity between both groups, but there was a significant difference in microbial composition. These results indicate differences in the microbiota between the good and poor groups. In order to further identify potential biomarkers, we compared the number of common or unique OTUs among different groups, which suggested that there were 53 unique OTUs in the good group and 51 unique OTUs in the poor group. At the phylum level, there was a difference between the two groups for several bacterial groups with the highest abundance values, among which *Firmicutes, Actinobacteria,* and *Fusobacteria* were more abundant in the good group. At the genus level, *Bifidobacterium* and *Lactobacillus* were abundant in the good group, whereas *Bacteroides* were scarce. However, it is not yet clear whether these microbial differences are the result of different treatment outcomes or the cause. Further in-

depth research is needed. Firmicutes is a group of bacteria that widely inhabit the human gastrointestinal tract, and their abundance and diversity are relatively high. Fecal samples composed of bacterial communities of *Proteus, Firmicutes, Bacteroides,* and *actinomycetes* have been reported to improve the efficacy of anti-PD-1 immunotherapy [20]. Therefore, we speculate that this may be the reason for the higher abundance of *Firmicutes* in the good group.

According to some studies, secondary metabolites of intestinal actinomycetes have inhibitory effects on malignant tumor cells [21]. The study indicated that *Lactobacillus, Clostridium,* and *Intercampococcus* were the intestinal microbiota positively correlated with ICI efficacy in Japanese NSCLC patients, whereas *Choleliophila, Sarteria,* and *Parabacteroides* were negatively correlated [22]. Many inflammatory bowel diseases, ulcerative colitis, Crohn's disease, and colonic cystitis can be improved by *bifidobacterium* members for the three disorders [16]. Moreover, *bifidobacterium* is capable of inhibiting spoilage bacteria and decomposing carcinogens, thereby counteracting the effects of cancer and reducing inflammation. Supplementing germ-free or antibiotic-treated mice with deletions of *bifidobacterium* increased the degree of tumor growth inhibition by PD-1 inhibitors [23]. This is consistent with the finding that the abundance of *Actinobacteria*, *Lactobacillus*, and *Bifidobacterium* is higher in the good group compared to the poor group. Lavoie and Garrett [24] found that elevated levels of *Bacteroidetes* and *Proteobacteria*, along with reduced levels of *Firmicutes*, have been linked to CRC. The bacterium *Bacteroides fragilis*, a member of the *Bacteroidetes*, has been consistently linked to colorectal neoplasms and implicated in promoting CRC [25]. The LEfSe analysis in this study indicated that the abundance of *Bacteroidetes* was significantly higher in the poor group than in the good group, which may be a potential biomarker.

The membrane transport pathway is crucial for cell vitality and growth, and therefore essential for the survival of bacteria in the intestinal ecosystem [26]. Odamaki et al. [27] demonstrated that the function of transport proteins is related to the nutritional changes in the composition of the intestinal microbiota. In CRC, one of the main mechanisms of chemoresistance is the low expression of important membrane proteins, such as solute carrier (SLC) transport proteins, leading to reduced drug transport into tumor cells [28]. In this study, the membrane transport pathway in the good group was significantly higher than in the poor group, which may be one of the reasons for the better treatment outcomes in the good group. Fang et al.'s [29] research indicates that in CRC, there is an increase in abundance of the glycan biosynthesis and metabolism pathway. Studies have suggested that the glycan biosynthesis pathway is enriched with miRNA targets related to colorectal cancer-associated bacteria, and miRNA can attract specific microorganisms to the tumor microenvironment by regulating polysaccharide biosynthesis, thereby promoting tumor development [30]. This aligns with the conclusion in this study that the glycan biosynthesis and metabolism pathway in the poor group is significantly higher than in the good group.

The study enrolled a limited number of participants, and the human body was heterogeneous; therefore, it is necessary to further explore the characteristics of intestinal microbial composition in lung cancer patients treated with ICI to provide a basis for lung cancer immunotherapy.

5. Conclusion

This study evaluated ICI treatment on the intestinal microbiome and revealed differences in the gut microbiota between the good and poor groups. At the genus level, the abundance of *Bifidobacterium* and *Lactobacillus* in the good group was high, while the abundance of *Bacteroides* was low. The biomarker screened in the poor group was *Bacteroides*, we could regard it as an indicator of the therapeutic effect. The dynamic characteristics of the intestinal microbiome were analyzed according to the treatment effect of ICI, which can provide a better basis for immunotherapy in NSCLC patients.

Ethics approval and consent to participate

This study followed the CIOMS (Council for International Organizations of Medical Sciences) International Ethical Guidelines for Biomedical Research Involving Human Subjects and the WMA (World Medical Association) Declaration of Helsinki. The procedures followed in this study were approved by the Ethics Committee of Chongqing Hospital of Traditional Chinese Medicine (Approval No. 2022-KY-KS-XH). All participants provided informed consent to participate in the study.

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Data availability statement

The data used to support the findings of this study have been included in this article. The data relevant to my research were not deposited in public repositories.

CRediT authorship contribution statement

Hua Xu: Writing – original draft, Conceptualization. **Yongchun Deng:** Writing – original draft, Conceptualization. **Qing Zhu:** Visualization, Data curation. **Feng Li:** Visualization, Data curation. **Na Liu:** Writing – review & editing, Formal analysis. **Jun Cheng:** Project administration, Funding acquisition. **Min Qiu:** Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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