Original Article

Special tissue microbiota such as Cyanobacteria are associated with the immune microenvironment of lung adenocarcinoma

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Background: Lung cancer is the leading prevalent form of human cancer and has the highest mortality rate among all cancer types. The role and potential mechanism of the lung microbiome in lung cancer is still unknown. This study aims to investigate the microbiomes of lung cancer patients possessing different levels of infiltrated $CD8^+$ T cells and programmed cell death-1 (PD-1) receptors, and further assess the correlation between specific microbes and the immune environment of lung tumor.

Methods: We analyzed the microbiomes of lung cancer tissues from patients with different levels of infiltrated $CD8^+$ T cells and PD-1 expression using 16S rRNA gene sequencing. The relative abundance of dominant phyla and genera was compared, and the correlation between microbial composition and immune markers was explored.

Results: Our results showed that lung cancer tissues displayed similar microbiome profiles, including Proteobacteria, Bacteroidetes, and Actinobacteria as the dominant phyla; and *Chryseobacterium*, *Triticum aestivum* (bread wheat), and *Acinetobacter* as the dominant genera. We found that the relative abundance of *Chryseobacterium* was positively correlated with $CD8^+$ T cell infiltration and the level of PD-1 expression, while the relative abundance of *Acinetobacter* was negatively associated with the PD-1 level. In addition, higher beta diversity was identified in samples with low $CD8^+$ T cell infiltration, but no significant correlation between beta diversity and PD-1 expression was observed. Furthermore, the relative abundance of Cyanobacteria was significantly higher in both the CD8 high and PD-1 high groups.

Conclusions: Our study indicated that the lung microbiota played an indispensable role in the $CD8^+$ T cell-mediated tumor immune response. These findings shed light on valuable insights into the intricate interplay between the lung microbiome and the immune system in the progression of lung cancer, offing potential therapeutic strategies targeting the lung microbiome.

Keywords: Lung cancer; microbiota; immune microenvironment

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Introduction

Lung cancer is the most common human cancer and the leading cause of human cancer deaths worldwide (1). Although great advances in the treatment of lung cancer have been achieved in the past few decades, chemoresistance and severe life-threatening side effects due to conventional therapies result in the extremely low overall survival of lung cancer patients (2,3).

Polymorphic microbiomes are the new frontier in the field of oncology and also regarded as one of the hallmarks of human cancer (4). To date, only a few microbes, including *Helicobacter pylori*, hepatitis B virus, and human papillomavirus, have been intensively investigated in human tumorigenesis. In addition, specific microbes have been identified in the lung tumor environment (5-7) and demonstrated to be involved in regulating tumor behavior and the local immune microenvironment through multiple mechanisms, including modulating microbiome dysbiosis, genotoxicity, and virulence effects as well as metabolism, inflammation, and the immune response (8). Furthermore, lung infection is closely correlated with lung tumorigenesis (9-11), and the microbes within tumors have been reported to affect the development and progression of lung carcinoma in an inflammasome-dependent manner (8). However, the specific lung microbiota and their complicated interactions with lung cancer are still not fully understood.

Recently, immunotherapy has emerged as a promising treatment and cure for lung cancer patients. Several approaches of immunotherapy targeting lung cancer have been developed, such as immune checkpoint inhibitors, cancer vaccines, and adaptive T cell therapy. The blockade of immune checkpoint proteins, such as programmed cell death-1 (PD-1) and programmed death-ligand 1 (PD-L1), can activate T cell receptor-mediated cellular signaling, further promote tumor cell recognition, initiate the tumor immune response, and ultimately eradicate tumor cells (12). The delicate regulation of lung microbiota homeostasis also fundamentally determines the outcome of anti-PD-L1 immunotherapy (12). Moreover, the level of PD-L1 expression was shown to be positively correlated with the $CD8^+$ T cell tumor-infiltrating lymphocyte score and the prognosis of non-small cell lung cancer patients (13).

In this study, we identified several novel microbes that were involved in the regulation of the lung cancer microenvironment as well as assessing their correlation with $CD8^+$ T cell tumor-infiltration and the PD-1 expression level in human lung cancer patients. For the first time, our results provide a potential therapeutic strategy for lung cancer targeting specific lung microbes.

Highlight box

Key findings

- The relative abundance of specific lung microbiota, such as Cyanobacteria, was positively correlated with the expression levels of CD8 and programmed cell death-1 (PD-1).

What is known and what is new?

- The immune microenvironment of lung adenocarcinoma is associated with the presence of special tissue microbiota, such as Cyanobacteria. These microorganisms can modulate the immune response and promote tumor growth.
- The relative abundance of Cyanobacteria was significantly higher in both the $CD8^{high}$ and $PD\text{-}1^{high}$ groups.

What is the implication, and what should change now?

- The lung microbiome, such as Cyanobacteria, may be involved in shaping the tumor immune environment, which provides potential targets for the treatment of lung cancer.

Methods

Patient samples

Primary lung tumor tissues were harvested from 40 patients who underwent a lobectomy at Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, and did not receive antibiotic or systemic glucocorticoid treatment in the past 3 months. Patients with a history of chronic lung disease, active infection, neoadjuvant therapy, or active tuberculosis were excluded from this study. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The human sample collection and processing were approved by the Ethics Committee of Shenzhen Second People's Hospital (No. 20200601041-FS01), and informed consent was obtained from all individual participants.

Immunohistochemistry

The lung paraffin slides were dewaxed, rehydrated, and then blocked in 3% H_2O_2 to quench the endogenous peroxidase activity after heat-induced antigen retrieval. The slides were incubated with primary antibodies against PD-1 (IM362, LBP Medicine Science and Technology, Guangzhou, China) and CD8 (IR024, LBP Medicine Science and Technology) for 1 hour at room temperature. Nuclear counterstaining by hematoxylin was performed after incubation with secondary antibody followed by diaminobenzidine visualization. The images were captured and analyzed by a light microscope. Only positively stained T cells were counted for further evaluation.

Lung microbiome

The DNA library was established from the polymerase chain reaction-amplified and purified V3–V4 region of

Table 1 Clinical features of the study participants

Variable	$CD8^{low}$ group (N=22)	$CD8^{high}$ group (N=18)	PD-1^{low} group (N=21)	PD-1^{high} group (N=19)
Age (years)	61.32±9.1	59.44±8.9	62.43±8.8	58.32±9.0
Gender				
Male	2 (9.09)	10 (55.56)	5 (23.81)	7 (36.84)
Female	20 (90.91)	8 (44.44)	16 (76.19)	12 (63.16)
BMI (kg/m^2)	24.40±3.8	23±3.1	23.8±3.8	23.73±3.1
Tumor size (mm)	19.45±14.0	19.75±6.3	20±13.5	19.13±6.3
Lateral				
Left upper lobe	7 (31.82)	5 (27.78)	8 (38.10)	4 (21.05)
Left inferior lobe	3 (13.64)	4 (22.22)	4 (19.05)	3 (15.79)
Right superior lobe	8 (36.36)	5 (27.78)	5 (23.81)	8 (42.11)
Right middle lobe	2 (9.09)	1 (5.56)	1 (4.76)	2 (10.53)
Right inferior lobe	2 (9.09)	3 (16.67)	3 (14.29)	2 (10.53)

Data are presented as mean ± standard deviation or n (%). PD-1, programmed cell death-1.

16S rRNA by using Hieff NGS DNA Selection Beads (12601ES03) and then subjected to operational taxonomic unit clustering and taxonomic analysis by using USEARCH software (v8.0.1517). Alpha and beta diversity values were calculated by the Microbiome-Analyst platform after assessing the species richness (CHO1), Shannon index, Simpson index, and unweighted UniFrac or Bray Curtis distance, respectively. The difference of lung microbiome was further analyzed according to the linear discriminant analysis effect size.

Statistical analysis

Comparison of continuous and categorical variables was performed by using the Wilcoxon signed-rank test or the independent *t*-test and the chi-squared test or Fisher's exact test, respectively. The correlation of variables was determined by Spearman correlation. Data were shown as the mean ± standard deviation, where $P<0.05$ was considered statistically significant. The correlation network between different species was generated by using Gephi software.

Results

Participant profiling

Forty lung cancer patients receiving surgery were recruited (*Table 1*). The lung tumor samples were grouped into $CD8^{low}$ and $CD8^{high}$ groups or PD-1^{low} and PD-1^{high} groups, based on the median values of CD8 and PD-1 expression, respectively (*Figure 1*, *Table 2*).

Proteobacteria and Bacteroidetes were dominant microbiota of lung cancer patients

Species accumulation curves were applied to estimate the lung cancer microbiota. After comparison with the SILVA rRNA database, we found that the most abundant lung microbiota phyla belonged to Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Acidobacteriota, and Cyanobacteria (*Figure 2*). Further detailed analysis showed that *Chryseobacterium*, *Triticum aestivum* (bread wheat), *Acinetobacter*, *Oryza sativa* (indica group; long-grained rice), and *Sphingomonas* ranked as the top five most abundant genera in our study (*Figure 3*). Twenty-three core genera of the lung carcinoma microbiome were identified, including *Chryseobacterium*, *Rikenella*, *Delftia*, *Oryza sativa* (indica group; long-grained rice), and *Acinetobacter*.

Less beta diversity in $CD8^{high}$ microbiota

To further characterize the diversity and consistency of the microbiota, the alpha and beta diversity values were analyzed in different groups of lung cancer patients. Although the alpha diversity was not statistically different among groups, the beta diversity in the $CD8^{low}$ and $CD8^{high}$

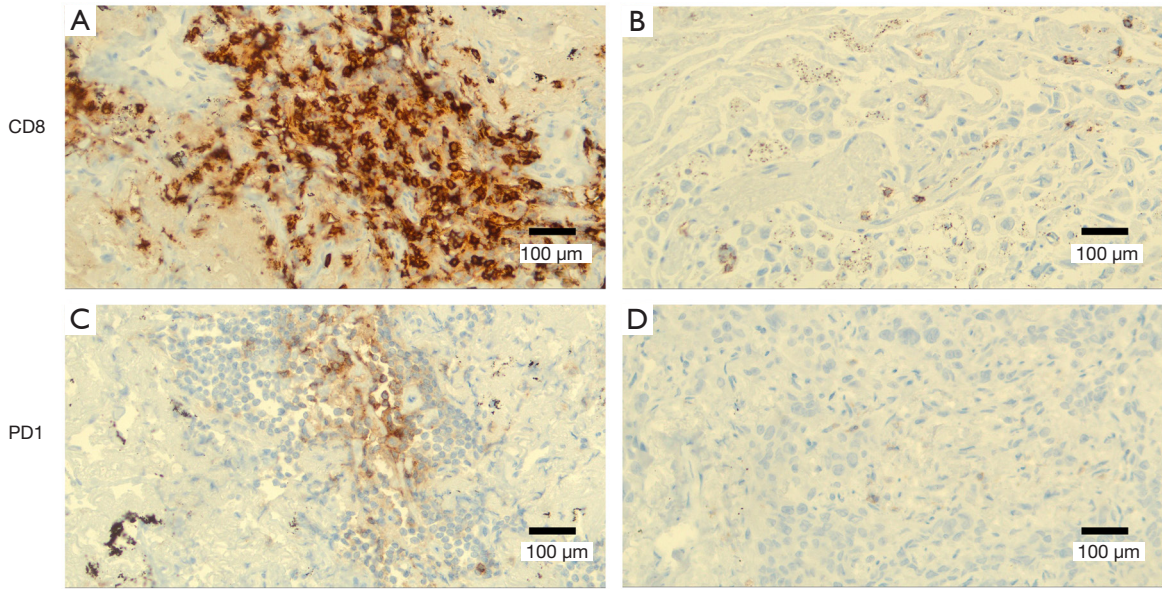

Figure 1 Immunohistochemical (by the EnVision method) staining of CD8 and PD-1 in tissues from enrolled participants. (A,B) Positive staining >80% and <3% is considered as $CD8^{high}$ and $CD8^{low}$, respectively; (C,D) positive staining >20% and <1% is considered as $PD\text{-}1^{high}$ and $PD\text{-}1^{low}$, respectively. PD-1, programmed cell death-1.

groups was statistically different according to the weighted UniFrac and Bray Curtis indexes between these two groups (P<0.05).

Cyanobacteria are involved in the infiltration of CD8⁺ T cells and PD-1⁺ T cells

The microbiota phyla with a relative abundance ≥5% were defined as the dominant phyla in our study. The top five and six dominant phyla of the lung microbiota were shown in *Figures 4*,*5*, based on the CD8 and PD-1 expression levels, respectively. The relative abundance of Cyanobacteria, WS2, *Nitrospinae*, and TA06 was significantly greater in the $CD8^{high}$ group than $CD8^{low}$ group (*Figure 4*). In addition, the relative abundance of Cyanobacteria, Synergistetes, and *Nitrospinae* was significantly greater in the $PD\text{-}1^{high}$ group than $PD\text{-}1^{low}$ group; and the relative abundance of Acidobacteriota, FBP, and TA06 was significantly less in the $PD\text{-}1^{high}$ group than $PD\text{-}1^{low}$ group (*Figure 5*).

We further defined the microbiota genera with a relative abundance of ≥1% as the dominant genera in our study. The top 12 and 10 dominant genera of the lung microbiota were identified in the $CD8^{high}$ and $CD8^{low}$ groups, and the levels of *Triticum aestivum* (bread wheat), *Oryza sativa* (indica group; long-grained rice), and *Acinetobacter* were significantly different between the $CD8^{high}$ and $CD8^{low}$ groups (*Figure 6*). Furthermore, the top 10 and 11 dominant genera of the lung microbiota were detected in the $PD\text{-}1^{high}$ and $PD\text{-}1^{low}$ groups; however, the genera between these two groups were not significantly different.

Specific lung microbiota are positively correlated with the infiltration of CD8⁺ T cells and PD-1⁺ T cells

The Spearman correlation was used to assess the association between the relative abundance of microbiota and the CD8 or PD-1 expression level. The relative abundance of Cyanobacteria, Fibrobacteres, Synergistetes, and Kiritimatiellaeota was positively correlated with CD8 expression; while Synergistetes was positively associated with the PD-1 level (*Figure 7*).

Discussion

The tumor microenvironment is a dynamic ecosystem within tumors that involves in complicated communication between cancer cells and adjacent noncancerous cells; it plays a key role in tumorigenesis and cancer progression through promoting tumor growth and metastasis (12,14). The lung is the organ responsible for respiration and

Table 2 Quantification of the immunohistochemical staining results

Sample ID	$CD8^+$ percentage, %	$PD\text{-}1^+$ percentage, %	Group I	Group II
SY01	60	20	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
SY02	40	5	$CD8^{high}$ group	$PD\text{-}1^{low}$ group
SY03	70	3	$CD8^{high}$ group	$PD\text{-}1^{low}$ group
CK01	30	<1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
SY04	50	<1	$CD8^{high}$ group	$PD\text{-}1^{low}$ group
SY05	40	15	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
CK02	10	<1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
SY06	80	10	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
SY07	60	10	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
SY08	40	20	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
SY09	50	20	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
SY10	80	10	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
CK03	30	20	$CD8^{low}$ group	$PD\text{-}1^{high}$ group
CK04	5	<1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
SY11	70	10	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
CK05	20	<1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
CK06	20	20	$CD8^{low}$ group	$PD\text{-}1^{high}$ group
CK07	5	<1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
SY12	40	15	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
SY13	40	5	$CD8^{high}$ group	$PD\text{-}1^{low}$ group
CK08	10	5	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
CK09	10	3	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
CK10	20	10	$CD8^{low}$ group	$PD\text{-}1^{high}$ group
CK11	30	5	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
SY14	50	10	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
SY15	80	10	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
CK12	30	1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
CK13	5	<1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
CK14	10	<1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
CK15	10	<1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
CK16	10	<1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
CK17	20	10	$CD8^{low}$ group	$PD\text{-}1^{high}$ group
CK18	10	<1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
CK19	30	5	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
SY16	40	20	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
CK20	3	<1	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
SY17	50	10	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
SY18	70	20	$CD8^{high}$ group	$PD\text{-}1^{high}$ group
CK21	5	5	$CD8^{low}$ group	$PD\text{-}1^{low}$ group
CK22	30	20	$CD8^{low}$ group	$PD\text{-}1^{high}$ group

PD-1, programmed cell death-1.

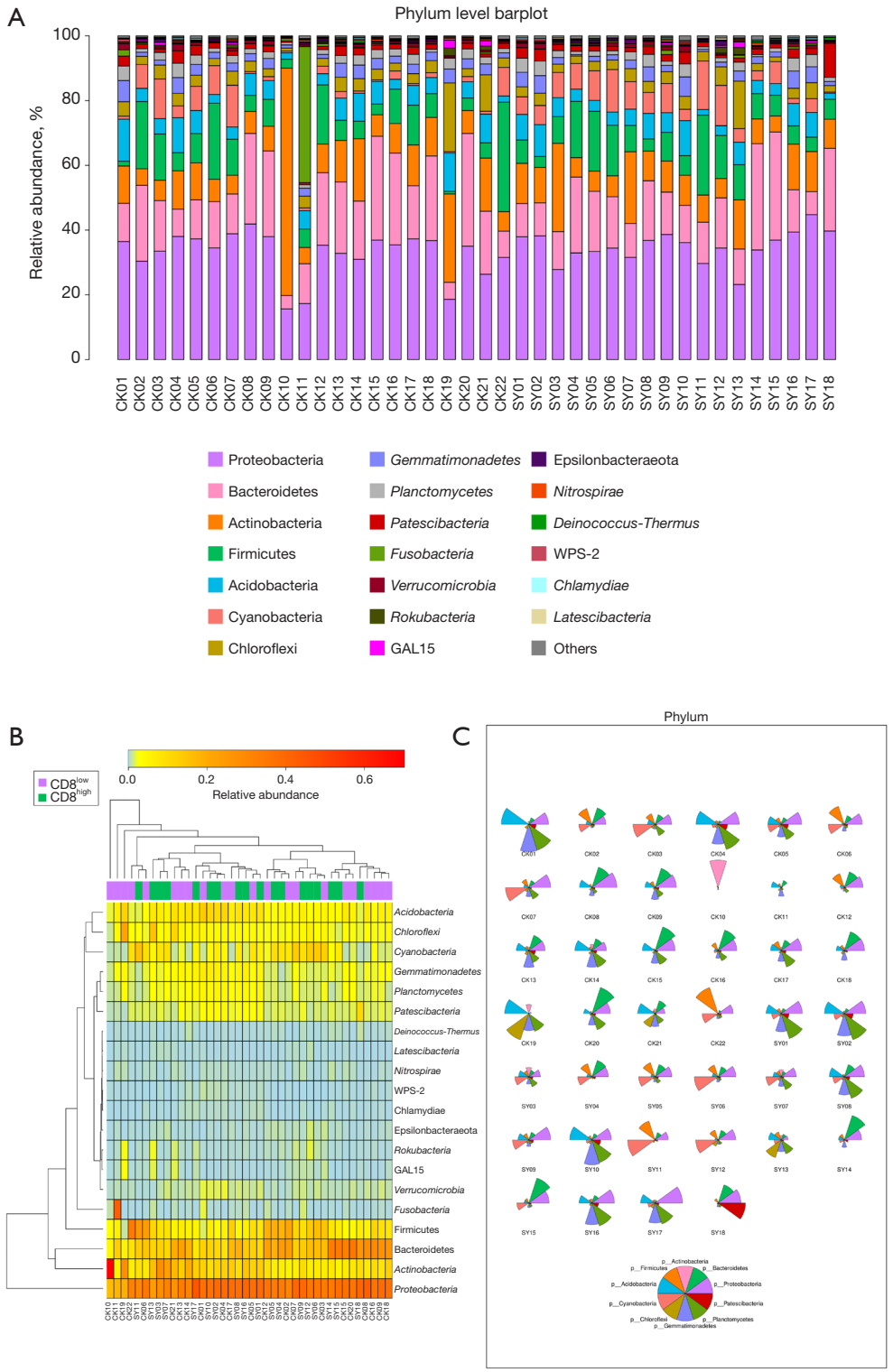

Figure 2 Microbiome phylum profiles of lung cancer patients. (A) Bar plot of the top 20 phyla according to the 16S rRNA level; (B) heatmap of the top 20 phyla based on the relative abundance; (C) star plot of the top 10 phyla based on the relative abundance.

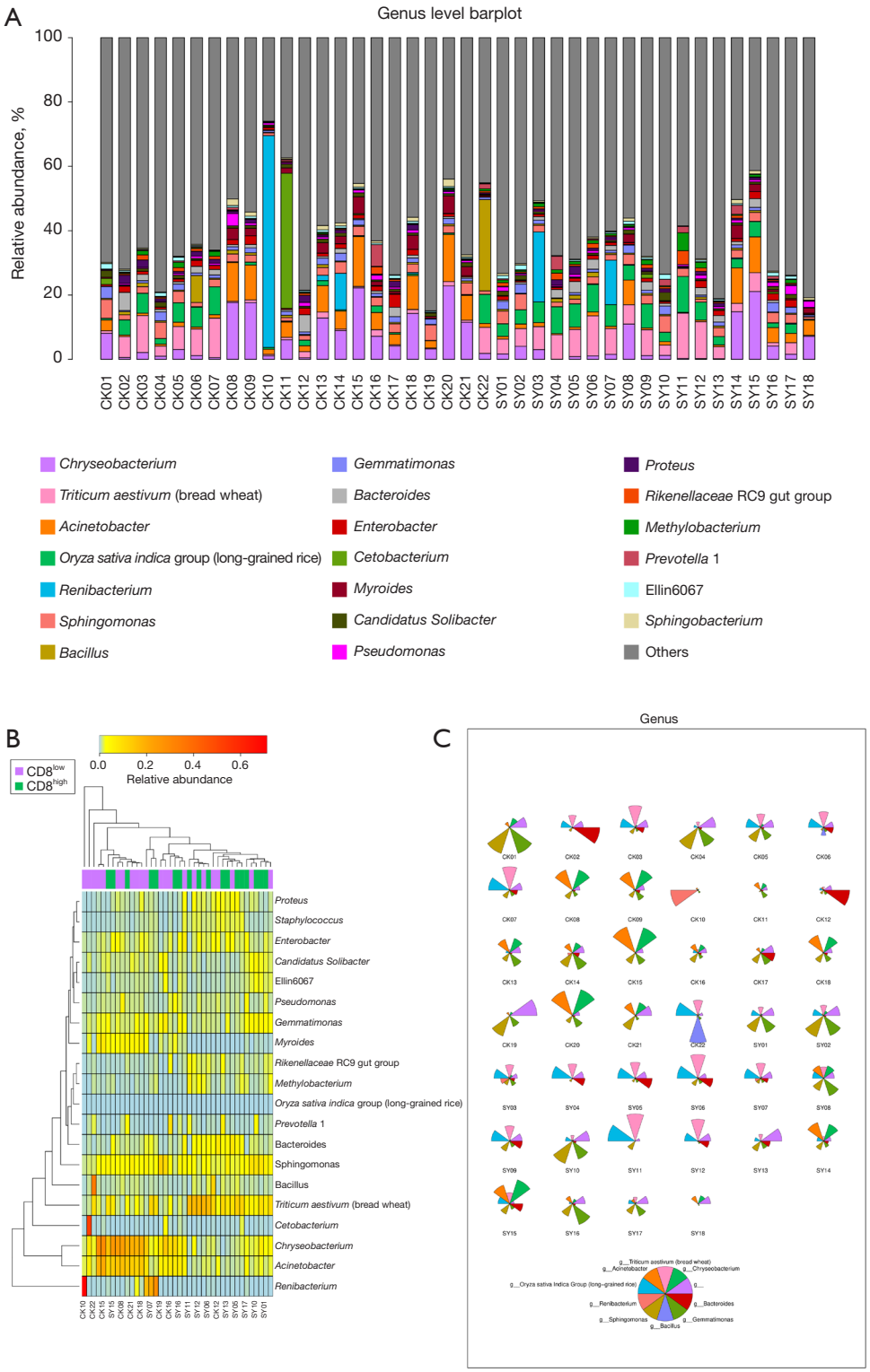

Figure 3 Microbiome genus profiles of lung cancer patients. (A) Bar plot of the top 20 genera based on the relative abundance; (B) heatmap of the top 20 genera based on the relative abundance; (C) star plot of the top 10 genera based on the relative abundance.

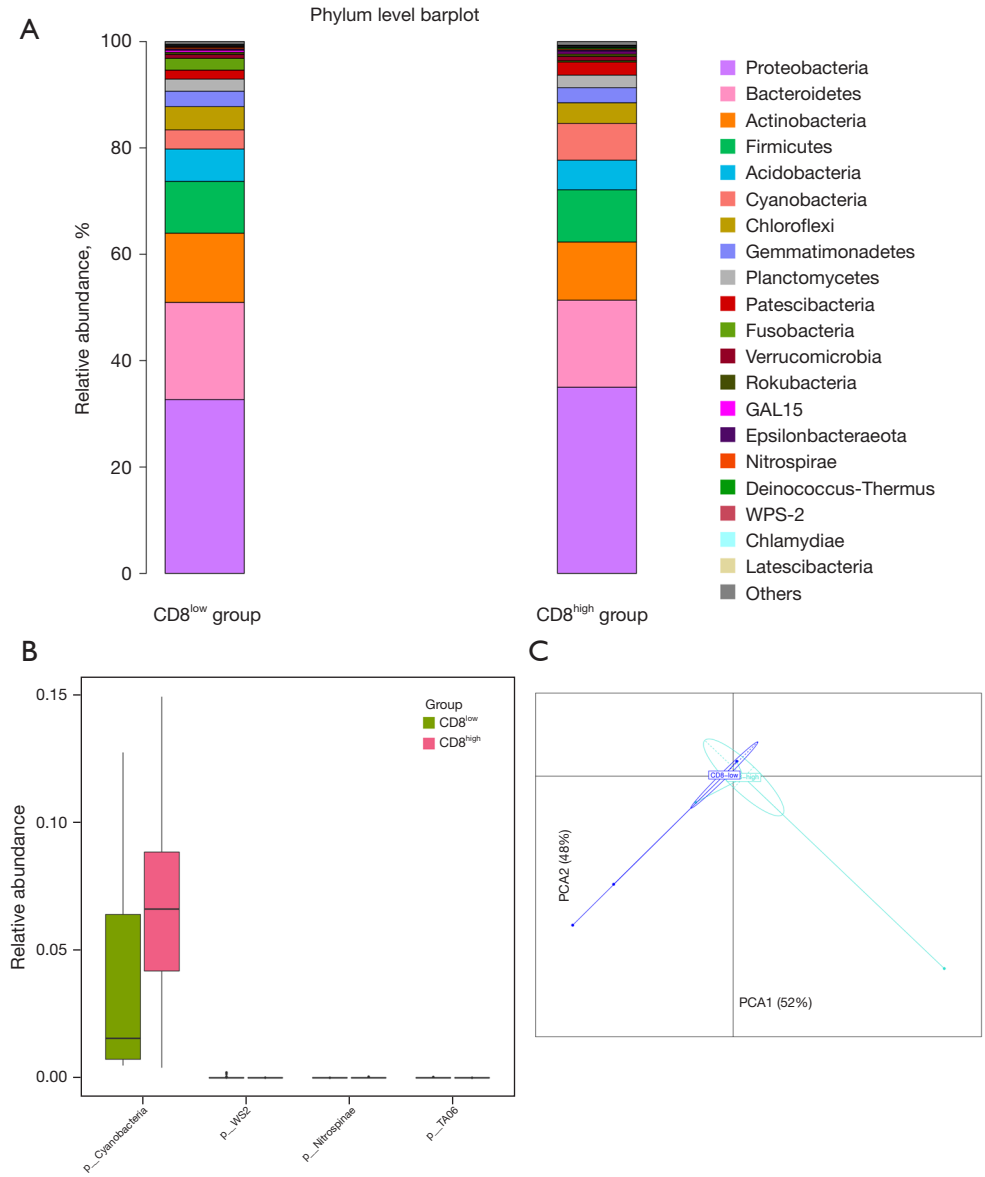

Figure 4 Correlations between the microbiome phyla and the CD8 expression levels. (A) No difference of the microbiome phylum relative abundance between the $CD8^{low}$ and $CD8^{high}$ groups was observed. (B) Box plot of the relative abundance of different phyla. The relative abundance of Cyanobacteria, WS2, *Nitrospinae*, and TA06 was significantly greater in the $CD8^{high}$ group compared to the $CD8^{low}$ group. Cyanobacteria harbored a greater relative abundance than the other phyla. (C) PCA showed the different relative abundances of phyla between the $CD8^{low}$ and $CD8^{high}$ groups. PCA, principal component analysis.

harbors diverse local microbial communities, which can affect lung tumorigenesis through regulating microbiome dysbiosis, chronic inflammation, and toxic metabolite synthesis (5-7). In this study, we found specific dominant microbes in lung cancer patients, including Proteobacteria, Bacteroidetes, and Actinobacteria; our results are supported by a previous study by Apopa *et al.*, who identified similar dominant microbes in different lung cancer patients, including Proteobacteria and Bacteroidetes (15). Proteobacteria have also been recognized as the dominant microbes in nonmalignant lung tissues (5). These findings suggest that the similar lung microbiota profiles are shared

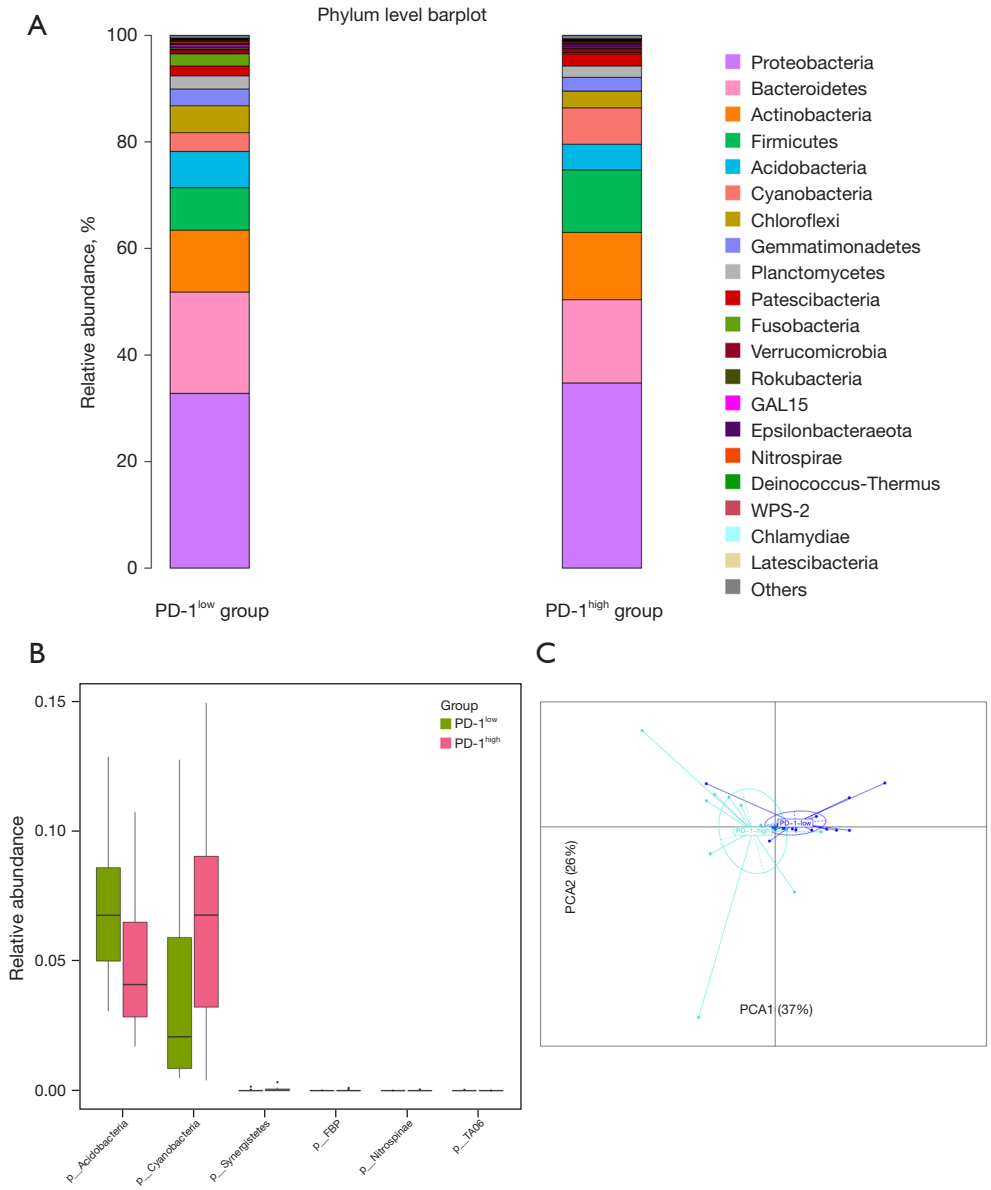

Figure 5 Correlations between the microbiome phyla and the PD-1 expression levels. (A) No difference of the relative abundance of microbiome phyla between the PD-1^{low} and PD-1^{high} groups was observed. (B) Box plot of the relative abundance of different phyla. The relative abundance of Cyanobacteria, etc. was enriched in different groups. Cyanobacteria and Acidobacteriota showed a greater relative abundance compared to other phyla. (C) PCA showed the difference of the relative abundance of phyla between the PD-1^{low} and PD-1^{high} groups. PCA, principal component analysis; PD-1, programmed cell death-1.

among different lung carcinoma patients.

We further investigated the relationship between lung microbiota and the T cell-mediated tumor immune response. Our results showed that the relative abundance of Cyanobacteria was positively correlated with the infiltration of CD8$^+$ and PD-1$^+$ T cells. PD-1 is a cell surface marker that weakens T cell activity to avoid self-tissue damage during the immune response; it is permanently activated in cancer, autoimmune disease, and chronic infections (16). In addition, the expression levels of interleukin-2, interferon gamma, and tumor necrosis factor-alpha are obviously decreased in weakening T cells inside tumors, followed by

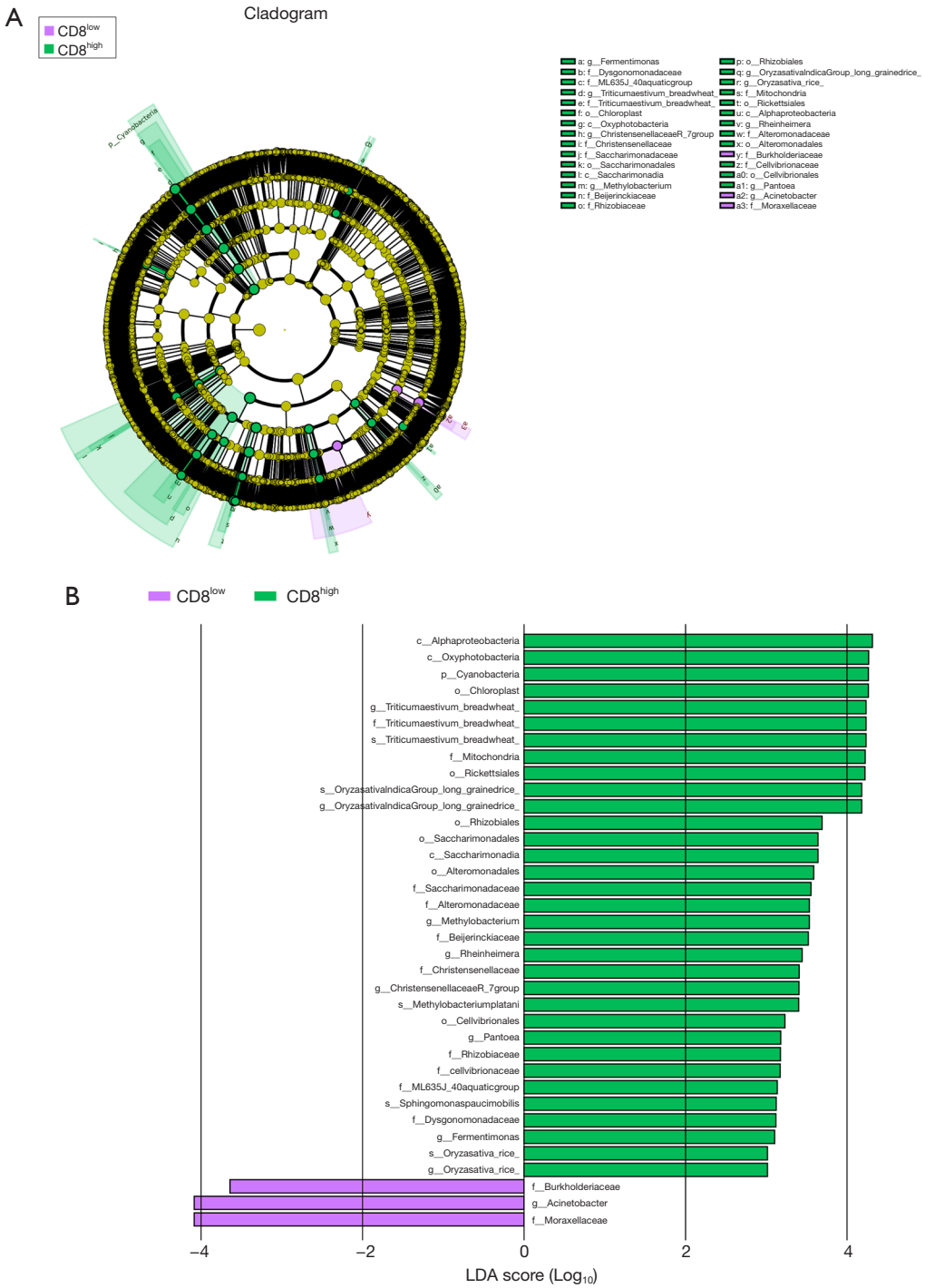

Figure 6 Linear discriminant analysis effect size analysis showed the difference in the microbiota genera between the $CD8^{low}$ and $CD8^{high}$ groups. Species evolution tree (A) and LDA between the $CD8^{low}$ and $CD8^{high}$ groups (B). P<0.05, LDA score >3. LDA, linear discriminant analysis.

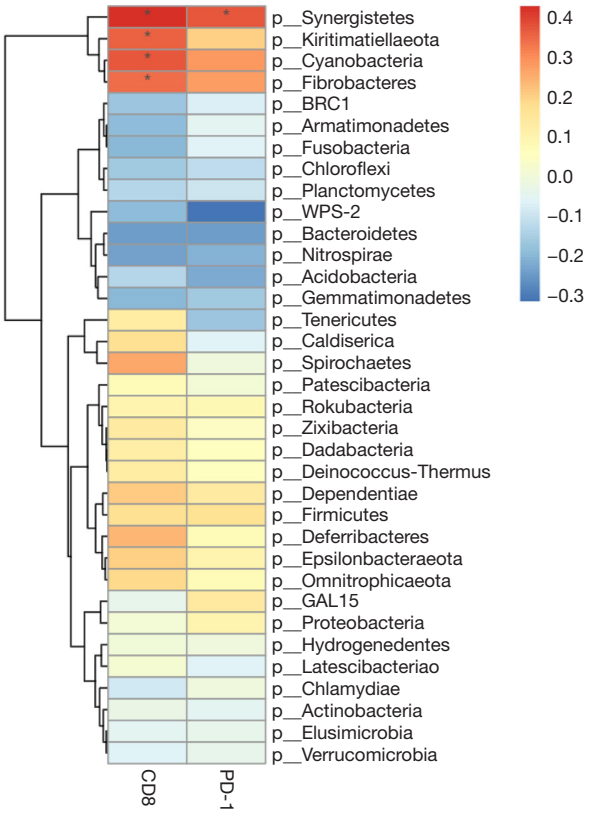

Figure 7 Spearman correlation showed the correlation between specific microbes and the CD8 or PD-1 expression level. *0.3< correlation coefficient <0.8, P<0.01. PD-1, programmed cell death-1.

cell cycle arrest.

Our results also demonstrated an association between Cyanobacteria and the antitumor immune response. Cyanobacteria inside tumors can secrete microcystin, which is correlated with decreasing CD36 and increasing PARP1 levels in non-small cell lung cancer tissues (15). CD36 is a transmembrane fatty acid transporter, and its inactivation significantly impairs tumor growth via reducing regulatory T cell infiltration and promoting $CD8^+$ T cell proliferation (17). $CD8^+$ T cells are effective immune cells that target cancer, and tumor cell infiltration is a biomarker of cancer prognosis (13). Although activated $CD8^+$ T cells have been reported in multiple human cancers, they cannot eliminate all tumor cells due to failure of the T cell-mediated immune response within the tumor microenvironment, including the loss of tumor antigen, the decrease of major histocompatibility complex-I, the upregulation of CTLA-4, the activation of the PD-1/PD-L1 pathway, and the secretion of inhibitory cytokines and chemokines. Moreover, the lower T cell activity induced by PD-L1/PD-1 signaling plays a vital role in the immunosurveillance escape of cancer cells. Our study showed that the abundance of specific microbiota was positively correlated with the infiltration of $CD8^+$ or $PD\text{-}1^+$ T cells, but no association between CD8 and PD-1 was observed. Interestingly, both the CD8 and PD-1 levels were correlated with the relative abundance of Cyanobacteria, suggesting the potential promoting effects of Cyanobacteria in the outcomes of PD-1/PD-L1 immunotherapy.

Conclusions

In conclusion, we demonstrated the influence of the lung microbiota on the development of lung cancer. Therefore, targeting certain microbiota may become a novel immunotherapeutic strategy for the treatment of lung cancer.

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Footnote

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-107/dss

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-107/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-107/coif). All authors report this study was funded by Shenzhen Science and Technology R&D Funds-Basic Research (Key Project) (No. JCYJ20200109120208018) and 2024 Hospital-Level Clinical Research Key Project of Shenzhen Second People's Hospital (No. 20243357014). The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Shenzhen Second People's Hospital (registration number: 20200601041-FS01), and informed consent was obtained from all individual participants.

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