Respiratory microbiota diversity as a predictive biomarker for the efficacy of PD-1 blockades in patients with advanced non-small cell lung cancer: A retrospective exploratory study

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Abstract. Despite advancements in immunotherapy, particularly regarding programmed cell death protein 1 (PD-1)/programmed death-ligand 1 blockades, the clinical outcomes in non-small cell lung cancer (NSCLC) remain variable with limited predictive biomarkers currently available. The present study investigated respiratory microbiota diversity as a potential biomarker to predict the efficacy of PD-1 blockades in patients with advanced NSCLC. A retrospective analysis was conducted on 60 patients treated with PD-1 blockades from May 2019 to May 2023. Clinical data were collected and respiratory microbiota from deep induced sputum specimens were analyzed using 16S rRNA gene sequencing. An index of respiratory microbiota α diversity was applied and exploratory analysis was performed accordingly. The objective response rate (ORR) and disease control rate among the 60 patients receiving PD-1 blockades was 23.3% (95% CI, 13.4-36.0%) and 58.3% (95% CI, 44.9-70.9%), respectively. Analysis of prognostic data of patients with advanced NSCLC receiving PD-1 blockades monotherapy demonstrated a median progression-free survival of 3.4 months (95% CI, 2.54-4.26) and a median overall survival (OS) of 12.3 months (95% CI, 6.29-18.31). Patients were stratified into high and low α diversity groups based on the Shannon diversity index of respiratory microbiota. The ORR was increased in the high diversity group (26.7%) compared with that of the low diversity group (20.0%), although the difference was not statistically significant (P=0.542). Notably, the high diversity group demonstrated a longer median PFS (3.9 vs. 2.8 months; P=0.017) and median OS (16.8 vs. 6.8 months; P=0.016) compared with that of the low diversity group. These findings suggested that PD-1 blockades demonstrate promising therapeutic activity for patients with previously treated advanced NSCLC in clinical practice. Respiratory microbiota α diversity might serve as a potential biomarker to predict the efficacy of PD-1 blockades monotherapy in patients with advanced NSCLC in the future. Therefore, further prospective studies are warranted to validate these findings and to explore the underlying mechanisms by which respiratory microbiota might modulate the immune response to cancer therapy.

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Introduction

Lung cancer remains one of the leading causes of cancer-related mortality worldwide; ~2.2 million new cases of lung cancer are diagnosed each year, resulting in >1.8 million deaths annually (1). In China, lung cancer accounts for ~40% of the global burden with 0.81 million new cases and 0.71 million deaths reported annually (2). These statistics underscore the urgent need for more effective treatments to improve the prognosis of patients with lung cancer (3). Non-small cell lung cancer (NSCLC) constitutes ~85% of all lung cancer cases, with a large proportion of patients diagnosed at a locally advanced or metastatic stage, making them ineligible for surgical resection (4). The advanced stage at diagnosis notably limits the effectiveness of traditional treatments, such as surgery, chemotherapy and radiotherapy, which contributes to poor overall survival (OS) rates (5). Despite these challenges, recent years have witnessed remarkable advancements in the treatment of advanced NSCLC (6). The introduction of targeted therapies and immunotherapy agents has transformed the first-line treatment landscape, positioning advanced NSCLC as one of most successfully treated types of cancer with precision

medicine (7). Targeted drugs, particularly those addressing EGFR mutations and anaplastic lymphoma kinase rearrangements, have demonstrated high efficacy. For example, the median OS for EGFR-positive patients with advanced NSCLC is >36 months (8) and the 5-year survival rate for patients without driver mutations is >15% (9).

In addition to targeted therapies, immunotherapy has achieved notable breakthroughs in the treatment of advanced NSCLC, particularly in patients without driver gene mutations (10). Immune checkpoint inhibitors targeting programmed cell death protein 1 (PD-1), programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 have delivered notable survival benefits in this population (11). Consequently, PD-1/PD-L1 blockades have been established as the standard of care for second-line or later therapy in patients with advanced NSCLC (12). Moreover, recent clinical trials, including the Keynote and IMpower series, have demonstrated the efficacy of PD-1/PD-L1 blockades (pembrolizumab and atezolizumab) in combination with chemotherapy as the standard first-line treatment for advanced NSCLC (13,14). In China, domestically developed PD-1 blockades such as tislelizumab, sintilimab, camrelizumab and toripalimab have also been approved for the treatment of advanced NSCLC, further expanding the range of therapeutic options (15). These developments have made PD-1 blockades a predominant therapeutic approach for NSCLC in China, especially as access has improved following reductions in medical insurance costs (16).

Despite the progress achieved with PD-1/PD-L1 blockades, a significant clinical challenge remains their relatively low objective response rate (ORR), which is <20%. While preliminary biomarkers such as PD-L1 expression and tumor mutational burden (TMB) have shown potential for predicting immunotherapy efficacy, no other widely accepted biomarkers are currently available (17). This highlights the urgent need for additional predictive biomarkers to identify patients most likely to benefit from PD-1/PD-L1 blockades therapy in clinical practice (18). Given the variability in ORR with immunotherapy, identifying reliable biomarkers is key for guiding treatment decisions. Biomarkers such as PD-L1 expression, TMB and microsatellite instability have demonstrated potential in predicting responses to PD-1 blockades (19). However, these markers are not universally predictive and often require invasive tissue biopsies for assessment (20). Consequently, the development of non-invasive, easily accessible biomarkers is desirable to optimize clinical decision-making and improve patient outcomes.

The human microbiota is comprised of trillions of microorganisms residing in various body sites, and has emerged as a key player in modulating the immune system (21). In particular, the gut microbiota has been investigated for its role in systemic immune responses and its potential impact on the efficacy of cancer immunotherapy (22,23). Preclinical and clinical trials have demonstrated that specific gut microbiota compositions may potentiate the therapeutic activity of PD-1/PD-L1 blockades (23,24). The respiratory tract, including the lungs, also harbors a diverse microbiota that may contribute to local immune responses (25). Emerging evidence suggests that the respiratory microbiota may involve in the tumor microenvironment and the host's immune response to lung cancer (26). Moreover, the respiratory microbiota is intricately linked to respiratory physiology, immune function maturation and the maintenance of homeostasis (27). However, whether the respiratory microbiota serves a role in modulating the efficacy of PD-1 blockades in patients with advanced NSCLC remains unclear.

The present retrospective exploratory study was conducted to analyze the respiratory microbiota profile of patients with advanced NSCLC treated with PD-1 blockades monotherapy. The primary objectives were to characterize the composition of the respiratory microbiota in these patients and to investigate its potential association with clinical outcomes of PD-1 blockades therapy.

Materials and methods

Study design and eligibility criteria. A substantial number of patients with advanced NSCLC are treated with PD-1 blockades monotherapy in clinical practice at the Tianjin Medical University Cancer Institute and Hospital (Hexi, China). Consequently, the present study was designed retrospectively to include patients who received PD-1 blockades monotherapy in Tianjin Medical University Cancer Institute and Hospital from May 2019 to May 2023. The inclusion criteria were: i) Histologically confirmed advanced NSCLC with pathological stage of IIIb or IV; ii) aged ≥18 years; iii) ECOG performance status score of 0-2; iv) treatment with PD-1 blockades monotherapy in clinical practice; and v) available measurable target lesions according to iRECIST criteria (28). Exclusion criteria included: i) A history of autoimmune diseases or prior treatment with steroids or other immunosuppressive therapies; ii) concurrent diagnosis of another tumor or a life-threatening disease; iii) lack of suitable specimens for respiratory microbiota analysis; and iv) EGFR-positive mutation status. Patients with EGFR-positive NSCLC were excluded from the present study due to their known lower response rates to PD-1 blockades therapy compared with that of EGFR wild-type patients (29). Excluding these patients reduced heterogeneity in treatment responses, allowing for a more focused analysis of the association between respiratory microbiota diversity and the efficacy of PD-1 blockades.

A total of 60 patients with advanced NSCLC who met the eligible criteria were included in the present study (Fig. 1). The primary endpoint was to elucidate the association between respiratory microbiota status and therapeutic outcomes of PD-1 blockades monotherapy in patients with advanced NSCLC. The present study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital (approval no. E20230112). Written informed consent was obtained from all patients included in the present study in accordance with the recommendations of the Declaration of Helsinki. Ethics approval was obtained in May 2023, and all data collection and analysis were performed retrospectively following approval. Only medical records of previously treated patients were screened and analyzed, ensuring compliance with ethical guidelines.

Therapeutic regimens and protocol for efficacy assessment. The PD-1 blockades used in the present study included tislelizumab (Beigene, Ltd.), sintilimab (Innovent Biologics, Inc.) and pembrolizumab (Merck Sharp & Dohme-Hoddesdon),

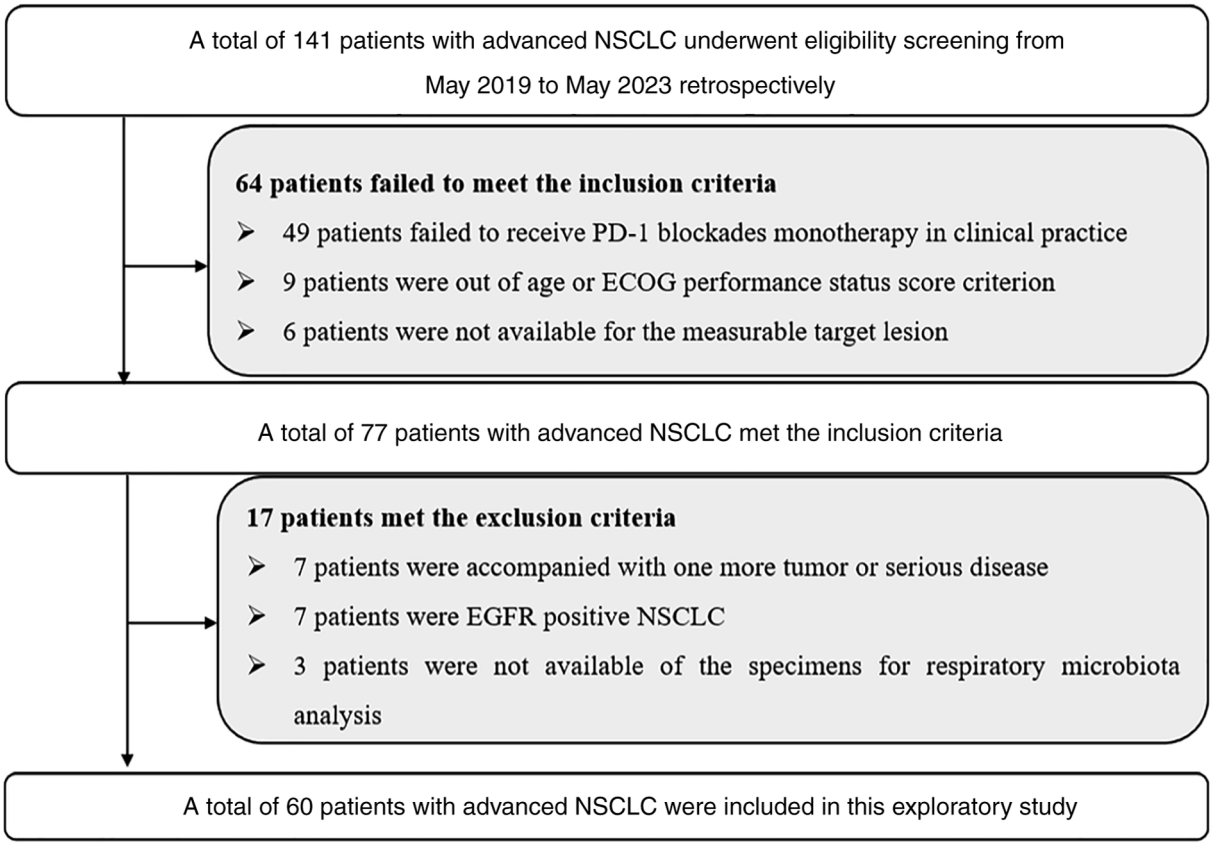

Figure 1. Study profile of the present retrospective analysis of 60 patients with advanced NSCLC who received PD-1 blockades monotherapy. NSCLC, non-small cell lung cancer; ECOG, eastern cooperative oncology group; PD-1, programmed cell-death protein 1.

which were chosen based on their availability and approval for use in clinical practice in China at the time of the present study. The specific dosage regimen for all three PD-1 blockades was intravenous administration with 200 mg on day 1 over 30 min, every 21 days as 1 therapeutic cycle. Administration of PD-1 blockades was discontinued upon disease progression or the occurrence of intolerable adverse reactions. The maximum treatment duration was set at 2 years. Efficacy of treatment was assessed using iRECIST criteria (28). Tumor response was evaluated through radiological CT or MRI scans at baseline and every 2 cycles when it was feasible. However, due to the retrospective nature of the present study, not all patients underwent radiological assessments every 2 cycles; some patients had efficacy evaluations based on clinical needs. The ORR and disease control rate (DCR) were assessed based on each patient's best response during treatment.

Baseline and treatment characteristics were collected from the hospital's electronic medical record system. After disease progression following PD-1 blockades treatment, patients were followed up monthly via telephone to record subsequent treatments and survival status. Information on death and exact dates of death was obtained through inquiries with patients' relatives. The data cut-off date for the present study was 20th August 2024.

Collection of deep-induced sputum specimens and analysis of respiratory microbiota. The present study utilized suitable specimens that were routinely collected from patients for respiratory microbiota analysis. Respiratory microbiota specimens were collected from patients immediately before the initiation of PD-1 blockade therapy. Patients were instructed to cease antibiotic treatments 1 week prior to sample collection. Induced sputum samples were obtained through the following procedure: Inhalation of hypertonic saline (4.5% NaCl) via ultrasonic nebulization through the mouth while exhaling through the nose. The nebulization lasted for 10-15 min. After nebulization, the patient's back was gently tapped to promote sputum discharge. A total of 3-5 ml of pure sputum was induced and collected in a sterile disposable sputum collector. Total DNA was extracted from the sputum specimens using a bacterial total DNA magnetic bead extraction kit- QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), after extraction, the integrity and quality of DNA were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). and the specimens were stored at -80˚C for subsequent respiratory microbiota analysis. Total DNA specimens were analyzed using 16S rRNA gene sequencing. The 16S rRNA V3-V4 variable regions of the sputum DNA specimens were amplified using the following primers: Illumina_16S_341 forward, 5'-CCTAYGGGRBGC ASCAG-3' and Illumina_16S_806 reverse, 5'-GGACTACNN GGGTATCTAAT-3' (Illumina, Inc.). The final DNA library concentration was measured using a Qubit fluorometer, and each library was then diluted to 10 pM prior to sequencing to ensure optimal cluster generation. Sequencing libraries were prepared using the NEBNext® Ultra™ DNA Library Prep Kit

for Illumina® (cat. no. E7370; New England BioLabs, Inc.). Sequencing was performed on an Illumina high-throughput platform with a paired-end 300 bp configuration, providing high overlap between forward and reverse reads. The libraries were loaded onto the MiSeq (Illumina, Inc.) sequencing platform and run in 2x300 bp paired-end mode. This produced raw paired-end reads for each sample, with unique index barcodes allowing multiplexing of samples in a single run. PCR products were sequenced on the Illumina® platform (PE300; Illumina, Inc.) for analysis. Reads from each pair were then merged using FLASH software (v1.2.7), which overlapped the forward and reverse reads to reconstruct the full V3-V4 amplicon sequence. These merged reads (raw tags) underwent stringent quality filtering using QIIME (v1.7.0, https://qiime2.org/). These primers incorporated degenerate bases to enable broad coverage across diverse bacterial taxa. This design ensured inclusivity of various bacterial genomes present in respiratory microbiota samples, as validated by a previous study (30). The high-quality reads were then grouped into operational taxonomic units (OTUs) based on sequence similarity. OTU clustering was performed using Uparse software (v7.0.1001, http://drive5.com/uparse/)

16S rRNA gene sequencing targeting the V3-V4 regions was conducted using the Illumina MiSeq (Illumina, Inc.) platform with a target sequencing depth of at least 15,000 reads per sample. Quality filtering was applied to remove reads with a Phred score <20, and chimeric sequences were identified and excluded using the UCHIME algorithm. Reads were clustered into operational taxonomic units at 97% similarity using the QIIME pipeline, and rarefaction was performed at 15,000 reads per sample to standardize sequencing depth (31).

Based on the sequencing results of the 16S rRNA from respiratory microbiota, the 60 patients with advanced NSCLC were divided into high (H) α diversity group and low (L) α diversity group based on the median Shannon diversity index value of the cohort, which was calculated to be 5.09. The Shannon index, which combined richness and evenness of species within a sample, is a commonly used metric for microbiota diversity (32). The median cut-off was chosen rather than an arbitrary or literature-based threshold, because no universal clinical cut-off for the Shannon index in relation to PD-1 blockade efficacy was established currently (33). Additionally, to complement the findings from the Shannon diversity index, the Simpson diversity index was also used (34), and the 60 patients were also divided into H α diversity group and L α diversity group based on the median Simpson diversity index value (0.91). Among the 60 subjects, the Simpson diversity index was similar with and correlated with the Shannon diversity index in 55 patients (91.7%), indicating agreement between the two indices.

The H group was consisted of 30 patients, while the L group included 30 patients. Subsequent analyses were conducted to compare the therapeutic outcomes of PD-1 blockades monotherapy between the two groups.

Statistical analysis. The ORR in the present study was calculated as the proportion of patients who achieved complete response (CR) or partial response (PR) during treatment. The DCR was calculated as the proportion of patients who achieved CR, PR or stable disease (SD) during treatment. Data analysis was performed using SPSS (version 25.0; IBM Corp.). The association between respiratory microbiota groups and ORR or DCR was analyzed using the χ^2 test, with Fisher's exact test applied for sparse data (cut-off value of <5 samples). PFS and OS were analyzed using Kaplan-Meier curves plotted using the Stata software (v14.0; Stata Corp LP). The association between respiratory microbiota groups and PFS was compared using the log-rank test. PFS was defined as the time from the initiation of PD-1 blockade treatment to tumor progression or death, and OS was defined as the time from the initiation of PD-1 blockade treatment to death from any cause (35). Patients who had not experienced disease progression or death by the data cut-off were considered censored. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Baseline clinical characteristics, demographics and respiratory microbiota profiles of 60 patients with advanced NSCLC. Baseline clinical characteristics of the 60 patients with advanced NSCLC included in the present study were summarized in Table I. Patients enrolled were representative of common clinical presentations of advanced NSCLC with a median age of 64 years (range, 22-81 years). Adenocarcinoma and squamous cell carcinoma were diagnosed in 31 and 29 patients, respectively. All patients had previously treated advanced NSCLC, with prior first-line therapy reported in 13 patients and subsequent-line therapy in 47 patients. As described, three PD-1 blockades were administered: Tislelizumab, sintilimab and pembrolizumab, used in 28, 22 and 10 patients, respectively. The baseline clinical characteristics of patients in H and L α diversity groups were comparable, as no statistically significant differences were observed ($P>0.05$; Table I). Similarly, there was no statistically significant difference in the proportion of adenocarcinoma and squamous cell carcinoma cases between the H and L groups ($\chi^2=139$; $P=0.710$).

Regarding the respiratory microbiota landscape (Fig. 2), the most abundant phyla identified at the phylum level among the 60 patients with advanced NSCLC were firmicutes (35.0%), proteobacteria (23.3%), bacteroidetes (21.7%), actinobacteria (11.7%) and other mycota (8.3%). At the genus level, the most abundant genera were *Streptococcus* (23.3%), *Prevotella* (16.7%), *Veillonella* (11.7%), *Acinetobacter* (10.0%) and other genera (38.3%). Based on the Shannon diversity index, patients were categorized into H group and L group, with 30 and 30 patients in each group, respectively.

Association between efficacy of PD-1 blockades monotherapy and respiratory microbiota α diversity status. Radiological assessment results for all 60 patients were collected and recorded. Treatment efficacy was evaluated based on the best response observed during therapy. Among the 60 patients, no CR or PR were observed in 14 patients, SD was found in 21 patients and progressive disease was documented in 25 patients, yielding an ORR of 23.3% (95% CI, 13.4-36.0%) and a DCR of 58.3% (95% CI, 44.9-70.9%).

Subsequent analysis of the association between respiratory microbiota α diversity status and therapeutic outcomes suggested that the ORR in H group and L group was 26.7 and 20.0%, respectively, which showed no statistical significance

Table I. Baseline characteristics of the 60 patients with advanced NSCLC according to respiratory microbiology H (n=30) and L (n=30) α diversity status.

Baseline characteristics	Overall, n (%)	L, n (%)	H, n (%)	χ^2	P-value
Age, years					
Median (range)	64 (22-81)			0.271	0.602
≥64	34 (56.7)	18 (60.0)	16 (53.3)		
<64	26 (43.3)	12 (40.0)	14 (46.7)		
ECOG performance status score				0.071	0.791
0-1	23 (38.3)	12 (40.0)	11 (36.7)		
2	37 (61.7)	18 (60.0)	19 (63.3)		
Sex				0.287	0.592
Male	38 (63.3)	20 (66.7)	18 (60.0)		
Female	22 (36.7)	10 (33.3)	12 (40.0)		
Pathological staging				0.741	0.389
IIIb	6 (10.0)	4 (13.3)	2 (6.7)		
IV	54 (90.0)	26 (86.7)	28 (93.3)		
Smoking status				0.884	0.347
Non-smoker	13 (21.7)	8 (26.7)	5 (16.7)		
Former/current smoker	47 (78.3)	22 (73.3)	25 (83.3)		
Histological category				0.067	0.796
Adenocarcinoma	31 (51.7)	16 (53.3)	15 (50.0)		
Squamous cell carcinoma	29 (48.3)	14 (46.7)	15 (50.0)		
Lines of previous treatment				0.884	0.347
First-line	13 (21.7)	5 (16.6)	8 (26.7)		
Subsequent line	47 (78.3)	25 (83.4)	22 (73.3)		
History of surgical resection				0.317	0.573
Yes	18 (30.0)	8 (26.7)	10 (33.3)		
No	42 (70.0)	22 (73.3)	20 (66.7)		
No. of metastatic lesions					
≤3	38 (63.3)	18 (60.0)	20 (66.7)	0.287	0.592
>3	22 (36.7)	12 (40.0)	10 (33.3)		
PD-1 blockades				0.543	0.762
Tislelizumab	28 (46.7)	13 (43.3)	15 (50.0)		
Sintilimab	22 (36.7)	11 (36.7)	11 (36.7)		
Pembrolizumab	10 (16.6)	6 (20.0)	4 (13.3)		

H, high; L, low; NSCLC, non-small cell lung cancer; ECOG, eastern cooperative oncology group; PD-1, programmed cell-death protein 1.

(χ^2=0.373; P=0.542) and the odds ratio (OR) was 1.46 (95% CI, 0.44-4.86). To further explore this, the microbiota profiles of patients in the L group who achieved PR, were analyzed. The results indicated no significant correlation between these cases and the microbiota characteristics typical of the H group (P>0.05). The PR values in the L group suggested that factors beyond respiratory microbiota diversity might contribute to PD-1 blockade efficacy. These factors might include genetic variations, baseline immune system status or other unmeasured clinical factors. Such findings emphasize the need for a multifactorial approach to identify robust biomarkers for predicting PD-1 blockade efficacy.

Furthermore, the DCRs in H group and L group were 70.0 and 46.7%, respectively, which demonstrated a notable difference (χ^2=3.360; P=0.067) and the OR was 2.67 (95% CI, 0.92-7.70). The waterfall plot illustrating the maximum reduction of target lesion during treatment in target lesions for patients in the H and L groups is presented in Fig. 3. Although target lesion shrinkage appeared more pronounced in the H group compared with that of the L group, the difference did not reach statistical significance (P=0.114).

Association between prognosis of PD-1 blockades monotherapy and respiratory microbiota α diversity status. The median follow-up duration of the present study was 10.3 months (range, 0.5-30.5 months) for all enrolled patients. The PFS and OS survival curves were presented in Fig. 4. The median PFS for the 60 patients with advanced NSCLC

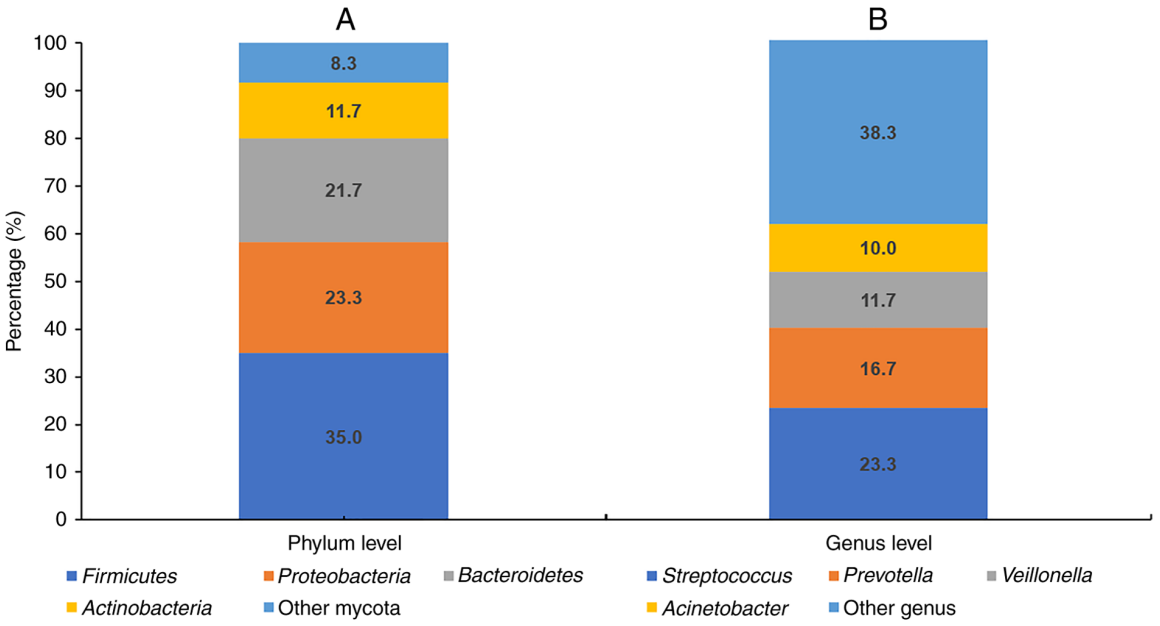

Figure 2. Respiratory microbiota composition in 60 patients with advanced non-small cell lung cancer receiving PD-1 blockade monotherapy. (A) Microbiota composition at the phylum level. (B) Microbiota composition at the genus level. PD-1, programmed cell-death protein 1.

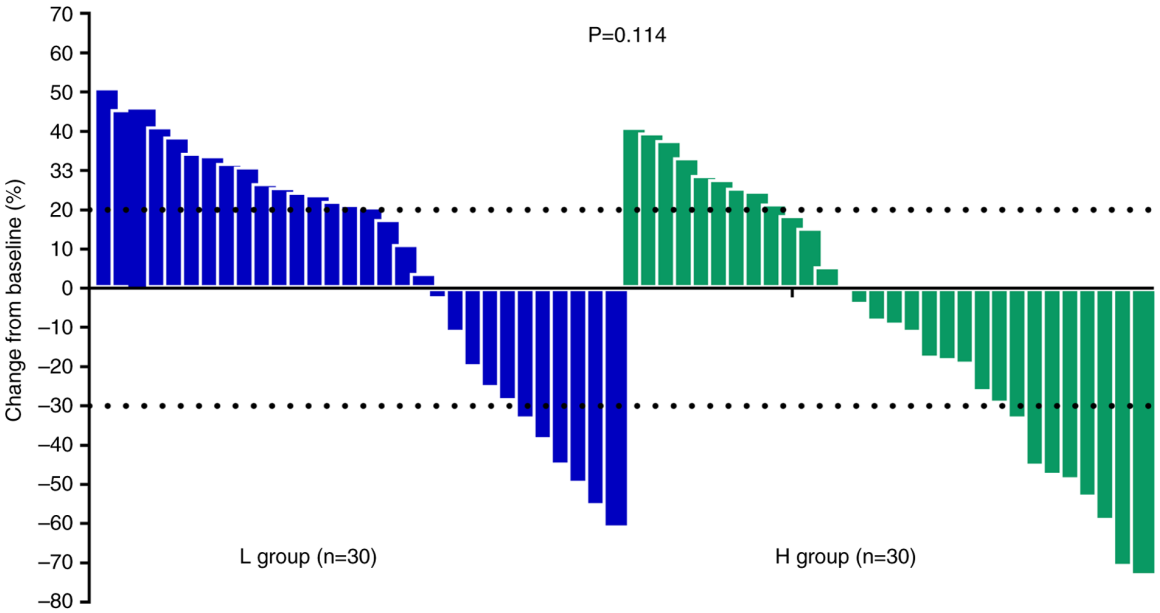

Figure 3. Best percentage change in target lesion (maximum reduction of target lesion during treatment) among 60 patients with advanced non-small cell lung cancer according to respiratory microbiology α diversity status. Dashed lines represent response evaluation criteria in solid tumors (RECIST)-defined thresholds (54): +20% (upper line) for progressive disease and -30% (lower line) for partial response. Each bar represented a single patient. Blue column, L group; green column, H group. H, high α diversity; L, low α diversity; PD-1, programmed cell-death protein 1.

receiving PD-1 blockades monotherapy was 3.4 months (95% CI, 2.54-4.26) with a 6-month PFS rate of 27.53% (95% CI, 16.57-39.63%) and a 12-month PFS rate of 14.72% (95% CI, 6.23-26.66%). Additionally, the median OS for the 60 patients with advanced NSCLC was 12.3 months (95% CI, 6.29-18.31) with a 12-month OS rate of 53.06% (95% CI, 39.69-64.76%) and a 24-month OS rate of 24.95% (95% CI, 12.19-40.02%).

The association between respiratory microbiota α diversity status and PFS was subsequently analyzed (Fig. 5). The median PFS was 3.9 months (95% CI, 2.06-5.74) for the H α diversity group and 2.8 months (95% CI, 1.85-3.77) for the L group. The 6-month PFS rates were 38.14% for the H group and 16.25% for the L group, while the 12-month PFS rates were 26.70 and 4.06%, respectively. The differences demonstrated statistical significance (χ^2=5.670; P=0.017). The association between respiratory microbiota α diversity status and OS was also analyzed (Fig. 6), the median OS was 16.8 months (95% CI, 9.45-24.15) for the H group and 6.8 months (95% CI, 0.00-14.63) for the L group. The 12-month OS rates were 63.33% for the H group and 42.76% for the L group, while the 24-month OS rates were 43.29

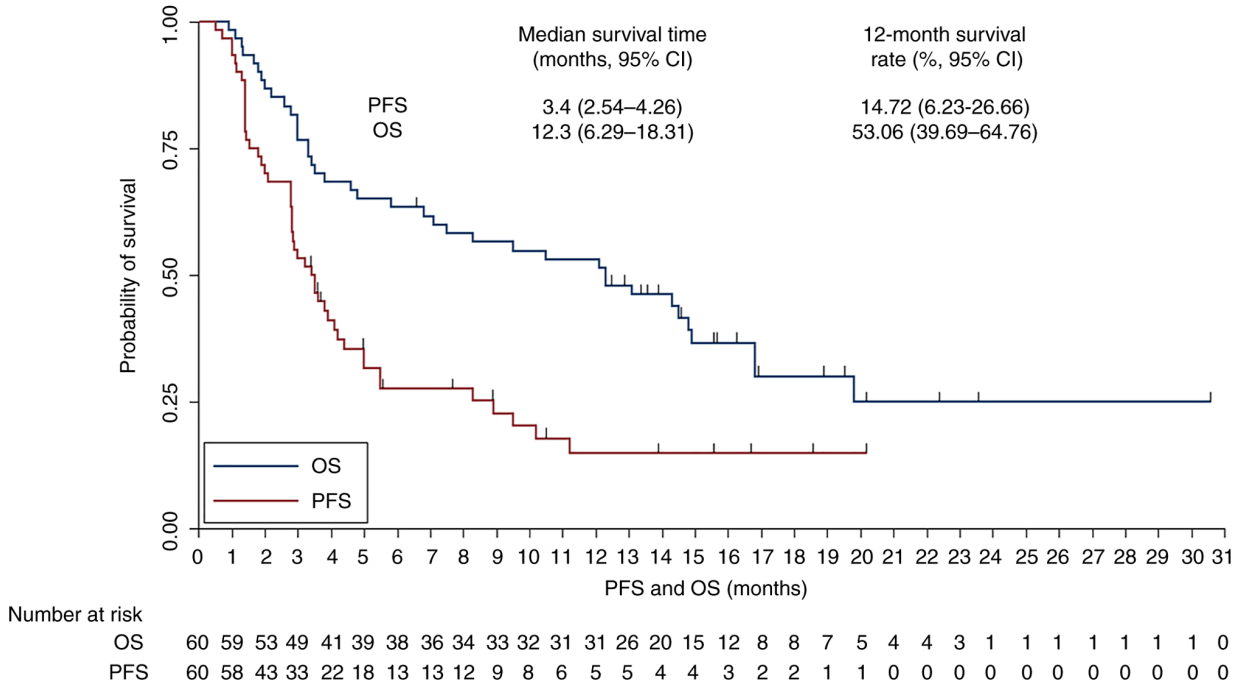

Figure 4. PFS and OS curves for 60 patients with advanced non-small cell lung cancer who received PD-1 blockades monotherapy. OS, overall survival; PFS, progression-free survival; PD-1, programmed cell-death protein 1.

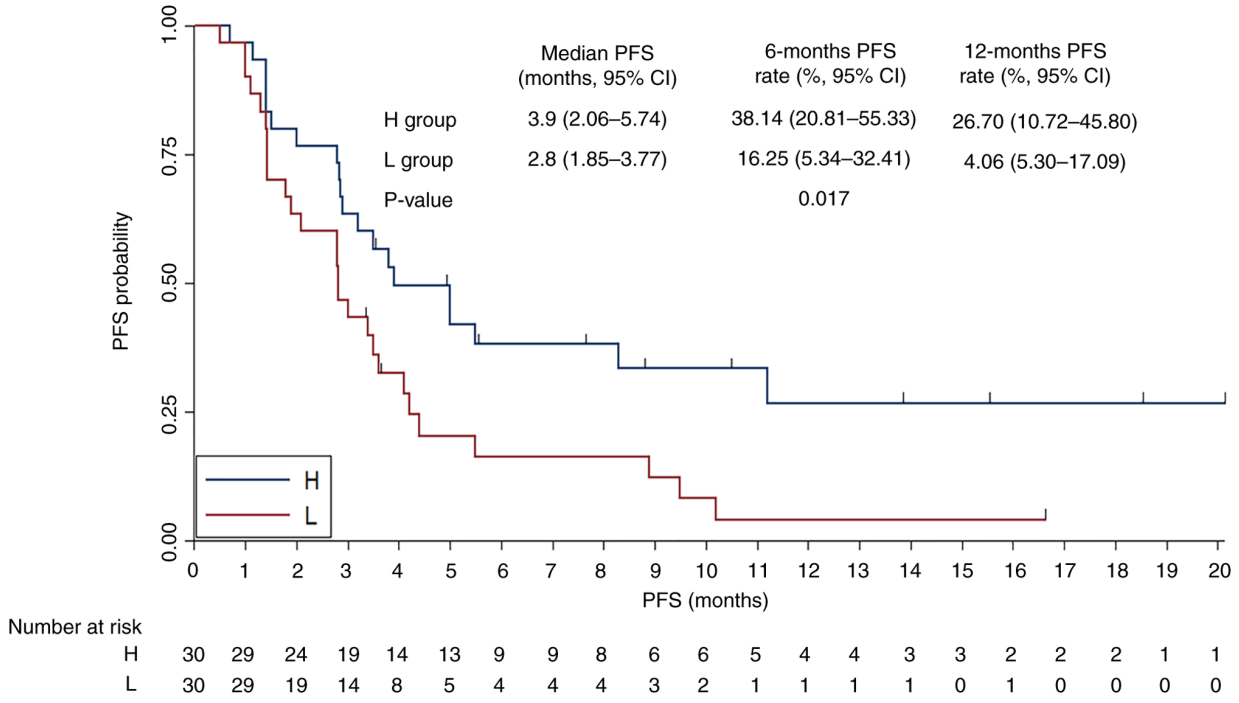

Figure 5. PFS for 60 patients with advanced non-small cell lung cancer according to H and L respiratory microbiology α diversity status. PFS, progression-free survival; H, high α diversity; L, low α diversity.

and 11.85%, respectively and the difference was statistically significant (χ^2=5.809; P=0.016).

Discussion

The present study retrospectively analyzed the efficacy of PD-1 blockades monotherapy in 60 patients with advanced NSCLC. The preliminary findings suggested that PD-1 blockades might provide survival benefits for patients with advanced NSCLC in clinical practice. Furthermore, the association analysis between respiratory microbiota status and therapeutic outcomes demonstrated that higher α diversity of respiratory microbiota was associated with increased PFS and OS. These findings indicated that respiratory microbiota diversity could

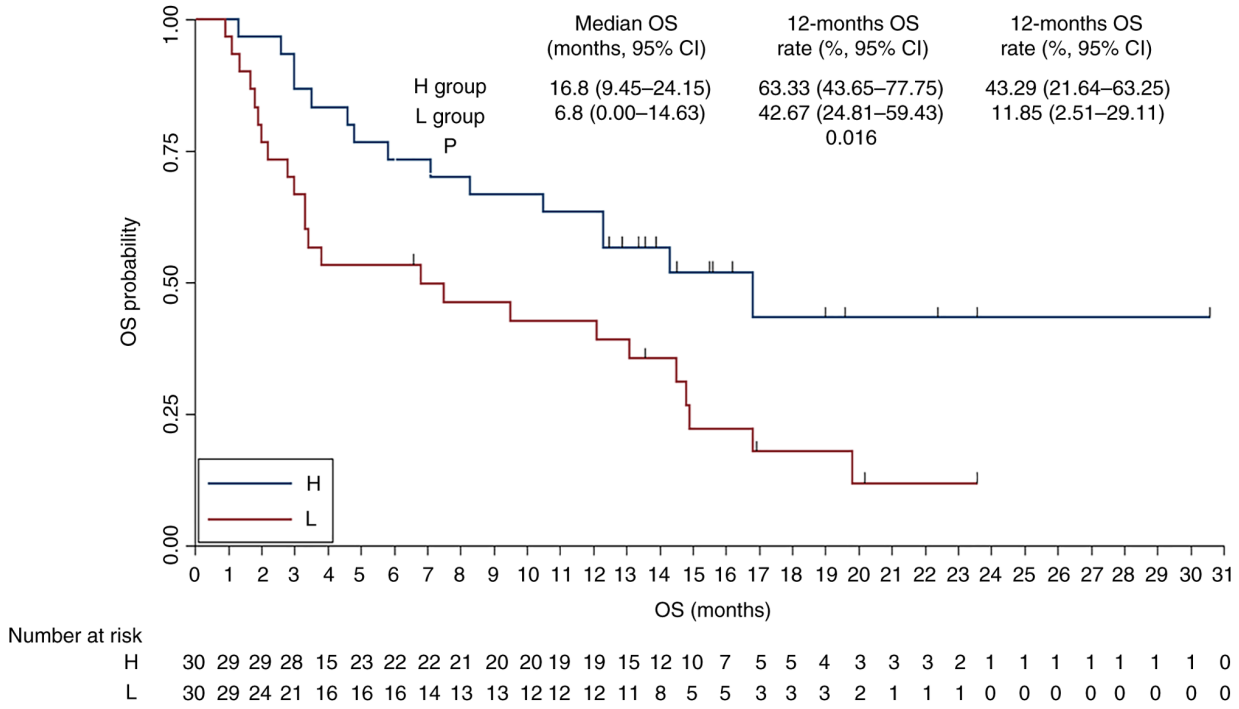

Figure 6. OS for 60 patients with advanced non-small cell lung cancer according to H and L respiratory microbiology α diversity status. PFS, progression-free survival; H, high α diversity; L, low α diversity.

be used as a potential biomarker to predict the efficacy of PD-1 blockades. Nevertheless, these results warrant further confirmation with prospective clinical trials in the future.

As a highly heterogeneous respiratory malignancy, lung cancer demonstrates a rising incidence in the Chinese population and remains the leading cause of cancer-related deaths in China currently (36). Despite breakthroughs in therapy of advanced NSCLC with targeted drugs and PD-1/PD-L1 blockades offering substantial survival benefits in first-line treatment (37), PD-1 blockades have only been available in China since 2018. Consequently, a considerable number of patients who had previously undergone chemotherapy and experienced disease progression have been able to receive PD-1 monotherapy, achieving notable survival benefits in clinical practice (38). In the present study, only 22% of patients had previously received first-line treatment, while 78% of the 60 patients with advanced NSCLC included were treated with PD-1 blockades therapy as third-line or later treatment. Given the demonstrated efficacy of PD-1 blockades in both lung adenocarcinoma and squamous cell carcinoma in prior clinical trials (39,40), ~52% of the patients in the present study were diagnosed with either lung adenocarcinoma or squamous cell carcinoma, consistent with findings from previous research (41). Furthermore, the inclusion of both imported and domestic PD-1 blockades such as tislelizumab, sintilimab and pembrolizumab, reflects the current clinical therapeutic trend, which highlights the widespread use of various PD-1 blockades in the treatment of advanced NSCLC in China, aligning with findings from a previous exploratory analysis of PD-1 blockades in NSCLC (42).

Although designed as a retrospective analysis, the present study evaluated the therapeutic outcomes and prognosis of PD-1 blockades monotherapy in 60 patients with advanced NSCLC in clinical practice. Therapeutic outcomes suggested that in a real-world setting, the ORR of PD-1 blockades monotherapy in patients with advanced NSCLC was 23.3%, with a DCR of 58.3% and a median PFS of 3.4 months. These outcomes were where increased in comparison to those reported for second-line monotherapy with docetaxel in advanced NSCLC, which demonstrated an ORR of ~10%, a DCR of 55% and a median PFS of 3 months (43). However, it seemed that the efficacy and PFS results in the present study were slightly decreased to those reported for pembrolizumab as the second-line treatment in advanced NSCLC in the Keynote-010 trial, which achieved an ORR of ~20% and a median PFS of 4 months (44). It could be considered that the discrepancy might be due to two main factors; first, the Keynote-010 trial recruited only patients with PD-L1 positive expression (>1%), while the present retrospective study did not perform PD-L1 expression testing on enrolled patients potentially including some patients with PD-L1-negative expression, which might result in worse therapeutic outcomes. Second, as a retrospective study, patient management and medication adherence in this analysis were relatively inferior compared with the rigorously controlled conditions of phase III clinical trials. A previous study also concluded that therapeutic outcomes from retrospective study tended to be slightly worse than those from clinical trials (45). Due to the relatively long follow-up period, OS analysis was also carried out in the present study. The results highlighted that the median OS for the 60 patients with advanced NSCLC was 12.3 months with a 12-month OS rate of 53.06% (95% CI, 39.69-64.76%), which was slightly longer compared with those previously reported for docetaxel monotherapy and increased in comparison with the OS outcomes reported for pembrolizumab monotherapy in patients with advanced NSCLC (46). This improvement may be attributed

to the introduction of various new targeted therapies and immunotherapy agents since 2018, particularly the approval of anlotinib as a third-line therapy for advanced NSCLC, which has provided significant survival benefits (47). As a result, patients in the present study who experienced disease progression after PD-1 blockades treatment were also able to receive subsequent treatments such as anlotinib or PD-L1 blockades, which may have contributed to survival benefits.

Furthermore, the present study also investigated the association between respiratory microbiota and therapeutic outcomes. The respiratory microbiota analysis suggested that the most common phylum level among patients with advanced NSCLC were Firmicutes (35.0%), Proteobacteria (23.3%), Bacteroidetes (21.7%) and Actinobacteria (11.7%). And the most common genera in genus level were *Streptococcus* (23.3%), *Prevotella* (16.7%), *Veillonella* (11.7%) and *Acinetobacter* (10.0%). These microbiota findings were consistent with previous research by Apopa *et al* (48), who analyzed lung biopsy specimens from 29 patients with lung cancer using 16S rRNA sequencing and reported that Bacteroidetes and Proteobacteria were predominant phyla in patients with lung cancer. Besides, another study highlighted that the significant differences existed in lung microbiota between patients with lung cancer and healthy individuals, noting that tumor tissue microbiota exhibited lower α diversity compared with healthy lung tissue (49). These findings underscored a potential role of respiratory microbiota in the occurrence and development of lung cancer. Moreover, correlation between respiratory microbiota α diversity and prognosis observed in the present study suggested that higher α diversity of respiratory microbiota was associated with superior therapeutic outcomes among patients receiving PD-1 blockades therapy (median PFS, 3.8 vs. 2.8 months; median OS, 16.8 vs. 6.8 months). To the best of our knowledge, the present results indicated for the first time that α diversity of respiratory microbiota might be used as a potential biomarker to predict the therapeutic outcomes of PD-1 blockades clinically. The higher ORR and DCR observed in the high α diversity group might be explained by the role of microbiota diversity in modulating immune responses. A diverse microbiota composition was thought to support a more balanced and robust immune microenvironment, which might enhance the efficacy of PD-1 blockades by promoting regulatory T-cell function, pro-inflammatory cytokine production and effective antigen presentation (50). A previous study also reported similar findings, where microbiota diversity was associated with improved responses to immunotherapy, likely due to its influence on systemic immune priming (51). Jin *et al* (52) explored the relationship between gut microbiota diversity and therapeutic outcomes of PD-1 blockades in patients with advanced NSCLC who included 37 patients with advanced NSCLC and found that higher gut microbiota diversity was significantly associated with higher ORR and longer PFS. Additionally, an exploratory study Jang *et al* (53) included a total of 84 patients with advanced NSCLC to investigate the relationship of lung microbiome with immunotherapy response in lung cancer. It was found that abundances of *Neisseria* and *Veillonella* differed significantly in relation to PD-L1 expression levels and immunotherapy response, suggesting that human microbiome might serve a certain role in the immune system *in vivo*. Collectively, these results were consistent with the present findings, highlighting that the respiratory microbiota might involve in immune responses and mediate differences in the therapeutic outcomes of PD-1 blockades in clinical practice. However, the specific mechanisms by which respiratory microbiota contributed to the efficacy of PD-1 blockades remained unclear and warranted further investigation. Understanding these mechanisms might provide valuable insights into optimizing immunotherapy strategy and improving patient outcomes. While the present study focused on the association between respiratory microbiota diversity and clinical efficacy outcomes of PD-1 blockades therapy, the potential influence of microbiota on immune-related adverse events remained an area for future exploration. It has been suggested that that certain microbial profiles might affect immune modulation and inflammatory responses (33). Future studies should examine the relationship between respiratory microbiota diversity and adverse events of immunotherapy, which might offer predictive insights for immune-related adverse events risk management in clinical practice.

The present study had several limitations that should be acknowledged. Firstly, designed as a real-world study, the sample size was relatively small and the conclusion remains to be confirmed in larger patient cohorts. Additionally, the retrospective nature of this analysis meant that patient management was less rigorous compared with that of well-controlled clinical trials, potentially introducing biases. Besides, as the present study collected microbiota specimens only at baseline, it was not possible to track potential changes in bacterial flora during PD-1 blockades therapy. Future prospective trials should consider longitudinal sampling of microbiota to assess dynamic changes during PD-1 blockades therapy and their association with treatment outcomes. In addition, targeting specific bacterial taxa, such as *Akkermansia muciniphila* and *bifidobacterium* species, might provide valuable insights, as these had been previously linked to enhanced immunotherapy responses. Finally, incorporating immune-related biomarkers, such as cytokine levels, regulatory T-cell counts and PD-L1 expression, could offer a more comprehensive understanding of the interactions between microbiota and the immune system in the context of PD-1 blockades. Furthermore, the present study was limited to a diversity analysis due to data constraints, and b diversity analyses could not be performed. Future studies should include b diversity metrics, such as Bray-Curtis or UniFrac distances, to assess differences in microbial composition between patients and explore their potential association with treatment outcomes of PD-1 blockades. Despite these limitations, the present study provided preliminary insights into the therapeutic outcomes of PD-1 blockades monotherapy in patients with advanced NSCLC and found a significant association between respiratory microbiota α diversity and therapeutic outcomes. These findings may have potential clinical implications in the selection of PD-1 blockade therapy for advanced NSCLC in clinical practice.

The present findings suggested that microbiota diversity testing might have potential as a predictive biomarker for PD-1 blockades efficacy. If further validated, this testing could be integrated into clinical practice to guide treatment selection and stratify patients in immunotherapy trials in the future. Additionally, microbiome-targeted interventions to increase

microbial diversity could be explored as a novel approach to enhance treatment outcomes for patients with low diversity in clinical practice.

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Availability of data and materials

The data generated in the present study may be found in the figshare repository (accession no. 10.6084/m9.figshare.28102844.v1) at the following URL: https://doi.org/10.6084/m9.figshare.28102844.v1.

Authors' contributions

LZ, PW and WDZ designed the study, performed the data of this study and wrote this manuscript. MJL, XPL, BY and TX collected the data, conducted the experiment and participated in the patients' follow-up. PW and WDZ guided the design and supervised the present study, LZ and WDZ confirm the authenticity of all the raw data. All agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work were appropriately investigated and resolved. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital (approval no. E20230112; Hexi, China). Written informed consent was obtained by each enrolled patient according to the recommendations of the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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