Exploring the impact of non-small cell lung cancer tumor microbiome on the efficacy of chemotherapy and immune checkpoint inhibitors

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Abstract

Background: This study aimed to investigate the potential of intratumoral microbiota, gut microbiome, peripheral blood T-cell subsets, and inflammatory markers as predictive biomarkers for antitumor efficacy in patients with non-small cell lung cancer. Methods: This study observed patients with metastatic non-driver mutation non-small cell lung cancer who were initially diagnosed at the First Affiliated Hospital of Dalian Medical University’s Department of Oncology from August 2021 to July 2022 and completed at least four cycles of chemotherapy combined with immune checkpoint inhibitor treatment. Lung biopsy tissues, fecal, and peripheral blood samples were collected from these patients. Based on the efficacy of the combined chemotherapy and immunotherapy, patients were divided into an effective group and an ineffective group. The tumor microbiota, gut microbiome, peripheral blood T-cell subsets, and inflammatory markers were compared between the two groups. The lung and fecal microbiota were analyzed using 16S rRNA high-throughput sequencing. Flow cytometry was used to detect T-cell subsets, and enzyme-linked immunosorbent assay was employed to measure inflammatory factors. Results: A total of 21 patients were observed. There were significant differences in the tumor microbiota between the responsive and non-responsive groups, particularly the proportion of the genera Sphingomonas and Pseudomonas. The gut microbiome composition changed after treatment, but there were no differences between the two groups before treatment. There were no significant differences in T-cell subsets and inflammatory markers between the responsive and non-responsive groups. Conclusion: The composition of intratumoral microbiota in non-small cell lung cancer patients may serve as an indicator of response to chemotherapy combined with immunotherapy. The predictive value of gut microbiota, T-cell subsets, and inflammatory markers appears limited. Future research should further validate the predictive role of changes in gut microbiota on treatment outcomes.

Keywords: NSCLC; tumor microenvironment; gut microbiota; tumor-associated microbiota
Background

Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancers. Chemotherapy combined with immune checkpoint inhibitors is one of the main treatment methods for NSCLC without driver gene mutations. However, several challenges remain, such as how to avoid immune-related adverse reactions and the lack of predictive biomarkers for treatment efficacy. Therefore, exploring new predictive biomarkers is an essential task [1].

The gut microbiome’s impact on tumor development and the host’s immunity has received widespread attention in recent years [2]. The microbiome also contributes to the tumor microenvironment, and specific bacterial populations have been identified in cancers at mucosal sites [3]. The microbiome consists of fungi, bacteria, viruses, and archaea. Since bacteria can disrupt the cell cycle by producing specific toxins, affecting cell growth, and altering the expression of proteins that control DNA repair, cell division, and apoptosis, most studies on the microbiome’s impact on cancer have focused on bacterial communities [4–7]. Research has confirmed that the gut microbiome is associated with the incidence of lung diseases such as pneumonia and Chronic obstructive pulmonary disease [8]. The interaction between the gut microbiome and lung diseases is referred to as the “gut-lung axis” [9, 10]. Furthermore, the lung microbiota, especially within lung cancer tissues, is considered one of the driving factors in tumor development and plays a significant role in the inflammation-cancer transformation in lung tissue [3, 11, 12]. The mutual influence between the lung and gut microbiomes may affect therapeutic approaches targeting the tumor microenvironment, such as immune checkpoint inhibitors [13]. Studies have shown that changes in peripheral blood T cell subtypes and inflammatory factors can also reflect treatment efficacy [14]. An ideal predictive biomarker should possess excellent sensitivity and specificity, necessitating the screening of various clinical biomarkers to determine the optimal development pathway for predictive biomarkers.

This study is dedicated to the exploration of specific biomarkers within the tumor microenvironment, including the microbiota that infiltrates tumors, the gut microbiome, and the subsets of T cells and inflammatory factors in peripheral blood. The aim is to assess their potential as predictive indicators for the therapeutic efficacy of chemotherapy in combination with immune checkpoint inhibitors in patients with NSCLC who do not have driver gene mutations. The research is committed to laying the foundational groundwork for the optimal development of clinical efficacy indicators, thereby guiding the formulation and refinement of personalized treatment strategies.

Materials and methods

Experimental subjects

This study was approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University (PJ-KS-KY-2021-162). During the period from August 2021 to July 2022, patients with newly diagnosed metastatic NSCLC without driver mutations such as epidermal growth factor receptor, anaplastic lymphoma kinase, or receptor tyrosine kinase, who presented to the Department of Oncology at the First Affiliated Hospital of Dalian Medical University, were observed. According to the NCCN guidelines, these patients underwent a first-line treatment regimen that included at least four cycles of chemotherapy in combination with an immune checkpoint inhibitor.

Efficacy evaluation indicators

Efficacy was assessed based on RECIST (version 1.1), which categorizes the objective response into complete response, partial response, progressive disease, and stable disease at each evaluation. The R0 group was defined as those with complete response, partial response, and stable disease, while the R1 group included progressive disease.

Data collection

Collection of lung microbiota samples. Sampling method: lung tissue biopsies were obtained under CT guidance, and a 1-bar 18 g 15–20 mm pathological tissue was placed in the laboratory refrigerator at −80 °C within 30 min for preservation.

Collection of fecal samples. Subjects provided fecal samples twice, before treatment and after 2–4 treatment cycles, using a fecal kit (Jinan PLS Scientific bio-tech Co., Ltd., Jinan, China). Approximately 1–2 cm of the middle portion of the stool (approximately 2–3 g) was collected, and the specimens were transferred to a dedicated laboratory refrigerator at −80 °C within 30 min.

Collection of clinical data. Blood samples were collected from patients before treatment and after 2 and 4 treatment cycles for the detection of T cell subsets and interleukins.

Experimental methods

Detection of lung and intestinal microbiota. The lung and intestinal microbiota were analyzed using 16S rRNA sequencing technology: (1) Total DNA Extraction and Detection. (2) PCR amplification: primers corresponding to the 16S V3-V4 region were used: Forward primer 338F: ACTCCTACGGGAGGCAGCAG; reverse primer 806R: GGACTACHVGGGTWTCTAAT [15].

T cell subset detection. Flow cytometry was employed for the fluorescence labeling of T lymphocyte subsets to determine the percentage of each subset. The primary reagents used were from the MultiTEST IMK Kit (BD Biosciences, New Jersey, America), which included CD3-FITC/CD8-PE/CD45-PerCP/CD19-APC, and CD3-FITC/CD16 + 56-PE/CD45-PerCP/CD19-APC, along with FACSlysing solution.

Interleukin detection. Inflammatory factors, including IL-1, IL-6, IL-8, and IL-10, were detected using the enzyme-linked immunosorbent assay method.

Analytical method

Data analysis. Data pertaining to the pulmonary and intestinal microbiota were curated and processed using the UPARSE software. Sequence clustering of operational taxonomic units (OTUs) was performed at a 97% similarity threshold to mitigate the potential impact of sequencing depth on subsequent analyses of Alpha and Beta diversity. To standardize the dataset, all sample sequences were subsampled. Taxonomic classification of OTUs was accomplished by aligning against the Silva 16S rRNA gene database (v138) using the RDP classifier, with a confidence threshold set at 70%. The community composition of each sample was statistically assessed at different taxonomic levels. Functional predictions of 16S rRNA gene data were conducted using PICRUSt2 (version 2.2.0) [16].

Species abundance was computed by summing the abundance of aligned sequences with non-specific sequences in the database, yielding the overall abundance of a given species. Analysis of species diversity involving utilizing Alpha diversity indices, which serve to assess the diversity and richness of microbial communities within individual samples. The Chao1 index, focusing on the relative abundance of microbial communities, along with the Shannon and Simpson diversity indices, were employed to evaluate microbial ecological diversity within the community.

Statistical analysis. Alpha diversity indices such as Chao1 and Shannon were calculated using the Mothur software (http://www.mothur.org/wiki/Calculators), and the differences in alpha diversity between groups were analyzed using the Wilcoxon rank-sum test. Principal Coordinates Analysis based on Bray-Curtis distance was employed to assess the similarities in microbial community structures among samples, and the significance of the differences in microbial community structures between sample groups was analyzed using the non-parametric test PERMANOVA. The LEfSe analysis (Linear discriminant analysis Effect Size) (http://huttenhower.sph.harvard.edu/LEfSe/) (linear discriminant analysis > 2, P < 0.05) was utilized to identify bacterial taxa with significantly different abundances between groups, from phylum to genus level. Distance-based redundancy analysis was used to investigate the impact of clinical indicators on the structure of the
bacterial communities within the lung and gut. Linear regression analysis was performed to evaluate the influence of major clinical indicators identified in Distance-based Redundancy Analysis on the microbial alpha diversity indices. The Spearman correlation coefficient ($|r| > 0.6, P < 0.05$) was used to select species for correlation network analysis. As the clinical observation data were from small sample sizes, statistical analysis was performed using SPSS version 19.0. Quantitative data between two groups were analyzed using one-way ANOVA, while rate comparisons were carried out using rank-sum tests, with $P < 0.05$ considered statistically significant.

The flowchart of the experiment is shown below (Figure 1).

**Results**

This study enrolled 21 patients diagnosed with NSCLC and collected samples of their gut microbiota before and after treatment. The clinical efficacy assessment revealed that 11 patients exhibited partial effectiveness, while the treatment was ineffective in 10 patients. Data on T-cell subpopulations and inflammatory factors before and after treatment were systematically organized. The baseline clinical characteristics of the NSCLC patients included in the study are presented in Supplementary Table S1.

**Lung cancer-associated microbiota**

Supplementary Figures S1–S3 illustrate the diversity analysis of the lung cancer-associated microbiota, showing richness and even distribution based on indices such as Shannon, Simpson, and Chao1. At the phylum level (Figure 2), the predominant taxa, sorted by abundance, include Unclassified, Sphingomonadaceae, Pseudoalteromonadaceae, Moraxellaceae, Bacillaceae, Vibrionaceae, Pseudomonadaceae, Comamonadaceae, Burkholderiaceae, and Brevibacteriaceae.

![Figure 1](https://www.tmrjournals.com/cancer)

**Figure 1** The flowchart of the experiment

![Figure 2](https://www.tmrjournals.com/cancer)

**Figure 2** Illustrates the distribution of microbial communities at the taxonomic level of “class” across various samples in each lung cancer tissue

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At the genus level (Figure 3), revealing major taxa by abundance, excluding unclassified groups: Novosphingobium, Pseudoalteromonas, Acinetobacter, Pseudomonas, Anoxybacillus, Vibrio, Sphingomonas, Aquabacterium, and Brevibacterium.

At the genus level (Figure 4), Novosphingobium is significantly higher in the EG (effective groups) than in the IG (ineffective groups) ($P = 0.0020$). Ralstonia, though not highly abundant in the EG, is noticeably higher than in the IG ($P = 0.0364$). Pseudomonas is markedly more abundant in the IG ($P = 0.0053$), and Enterococcus, while not highly abundant, shows significant differences ($P = 0.0061$). Salinivibrio, Mycobacterium, and Arrobacter exhibit variations, albeit not prominently.

Figure 3 Depicts the distribution of microbial communities at the taxonomic level of “genus” across various samples in each lung cancer tissue

Figure 4 Illustrates the inter-group differences in microbial communities at the taxonomic level of “genus” in lung tumor-associated microbiota
In our investigation of the gut microbiota in lung cancer patients, we observed distinct taxonomic compositions at various hierarchical levels. At the phylum level, predominant taxa included Firmicutes, Proteobacteria, and Bacteroidetes. Within the class level, the major groups identified were Clostridia, Gammaproteobacteria, and Bacteroidia. Order-level analysis revealed significant abundances of Clostridiales, Enterobacteriales, and Bacteroidales. The top three families, ranked by abundance, were Ruminococcaceae, Lachnospiraceae, and Enterobacteriaceae. Furthermore, the most abundant genera comprised Faecalibacterium, Bacteroides, Blautia, Escherichia, and Prevotella. The study systematically conducted a relative abundance analysis of the gut microbiota in lung cancer patients at various taxonomic levels, including phylum, class, order, family, and genus. Species within each sample were methodically ranked based on the abundance of microbial communities. Subsequently, statistical software was employed to analyze and graphically represent these abundance rankings through column charts depicting relative abundances. These charts vividly depict the relative abundance of species across various taxonomic levels, offering a visual insight into the taxonomic makeup linked to lung cancer.

Gut microbiota
Relative abundance analysis at various taxonomic levels (phylum, class, order, family, and genus) was conducted on the gut microbiota of lung cancer patients. The resulting bar graphs, displaying the relative abundance of species ranked by high-to-low abundance, are shown in Figure 5 at the class level, where the top 10 classes include Lachnospiraceae, Ruminococcaceae, Streptococcaceae, Coriobacteriaceae, Bacteroidaceae, Peptostreptococcaceae, Enterobacteriaceae, Lactobacillaceae, Erysipelotrichisaceae, and Bifidobacteriaceae.

At the genus level (Figure 6), presents the top 10 genera by abundance: Blautia, Streptococcus, Faecalibacterium, Collinsella, Bacteroides, Dorea, Eubacterium hallii group, Romboutsia, Lactobacillus, and Subdoligranulum.

Before treatment, no significant differences were observed at the genus level between the R0 and R1 groups (refer to Supplementary Figure S4). A comparative analysis of the gut microbiota of lung cancer patients who completed two treatment cycles was conducted using LEfSe analysis (Figure 7). This analysis identified OTUs that

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**Figure 5** Displays the abundance of intestinal microbial communities at the taxonomic level of “family”

**Figure 6** Illustrates the abundance of intestinal microbial communities at the taxonomic level of “genus”
exhibited the most prominent distribution disparities, indicating statistically significant differences within the gut microbiota. In the bar chart, colors represent different groups, and the length of the bars signifies the LDA score, indicating the impact of species displaying significant intergroup differences. Within the R0 group, 10 species exhibited marked disparities, ranked based on their degree of variation, including Lachnospiraceae, NK4A214-group, Mogibacterium, TM7x, Saccharimonadaceae, Catenibacillus, Lachnospiraceae-FC9020-group, and Butyrivibrio. Notably, within the R0 group, Lachnospiraceae held a prominent position within the order Lachnospirales. Conversely, within the R1 group, Negativicutes and Veillonellales were identified as predominant species.

**Discussion**

In recent years, the microbiota has ascended as a pivotal focus of investigation. Studies have elucidated that an array of microbial species are capable of modulating or accelerating the progression of cancer [17, 18]. A growing corpus of research, delineating the microbial architecture within the pulmonary regions of individuals with NSCLC vis-à-vis healthy subjects, has substantiated significant variances [19]. There exists a discernible association between particular microbial communities and NSCLC. These microorganisms and their metabolic outputs, through a myriad of mechanisms such as metabolic processes, inflammatory responses, and immune interactions, alter the host's internal microenvironment to favor their proliferation. However, these changes concurrently influence the genesis, evolution, and therapeutic responses of tumors [3, 8, 20].

In the lungs of healthy individuals, the microbial load is markedly low, with a dynamic state of equilibrium maintained by continual microbial ingress and egress. The microbial flora of the upper respiratory tract primarily migrates into the pulmonary system during respiration, with host defense mechanisms concurrently purging these microbes. Alterations in the lung microenvironment of cancer patients precipitate diverse bacterial colonization, and the metabolic activities of these bacteria further remodel the lung cancer microenvironment [11].

In our experiments, we utilized 16S rRNA gene sequencing to analyze the microbiota within lung cancer, a technique that enables the detection of microbial diversity at various taxonomic levels including phylum, class, order, family, and genus. We observed that the overall microbial diversity was relatively rich and evenly distributed, with varying levels of abundance expressed at different taxonomic strata. From the perspective of screening for predictive biomarkers of therapeutic efficacy, we could directly analyze the data at the family and genus levels. In this study, at the genus level and
arranged by microbial abundance, the predominant genera identified were Sphingomonas, Pseudomonas, Acinetobacter, Stenotrophomonas, Bacillus, Vibrio, Sphingobacterium, Aquabacterium, and Bacillus cereus. There were significant differences in the microbial communities between the treatment-responsive and non-responsive groups. Sphingomonas was notably higher in the responsive group compared to the non-responsive group; Rothia, although not significantly abundant, displayed a significant difference; Pseudomonas was considerably more abundant in the non-responsive group, and Enterococcus, while not highly abundant in the non-responsive group, differed significantly compared to the responsive group. For dominant genera such as Sphingomonas and Rothia, studies have suggested that they may help regulate inflammatory responses and promote epithelial cell repair. However, further research is required to determine their impact on tumors, particularly lung tumors. Enterococcus, a normal member of the gut microbiota, could be present in the lungs due to natural breathing movements that introduce environmental microbes into the lung, or it may be influenced by the “gut-lung axis”, with its metabolic byproducts creating a conducive microenvironment.

Within a healthy human body, the diversity of the gut microbiota helps maintain normal digestive and absorptive functions, as well as a dynamic equilibrium of the gut microbiome [21]. Although the gut microbiota is diverse, certain microbial groups constitute a core

Figure 8 Displays the line chart comparing the effectiveness of T-cell subgroups. Subfigures a-f represent the proportions of CD3+ T cells among lymphocytes, CD3+CD4+ T cells among lymphocytes, CD3+CD8+ T cells among lymphocytes, CD3-NK+ cells among lymphocytes, Th/Ts ratio, and R2 representing lymphocyte proportions among nuclear cells, respectively. EG, effective group; IG, ineffective group.
Advances in the lung function is one of the hallmarks of healthy individuals. This also corroborates the alteration in the gut microbiome structure of NSCLC patients, with a higher proportion of Akkermansia, Bacteroides, and Lactobacillus. While the gut microbiome structure of NSCLC patients is proven to be distinct from that under normal conditions, due to the limited sample size in the experiment, the exact proportions of the existing core microbiome could not be determined. The comparison was made against the overall microbiome to draw conclusions.

Interestingly, our findings revealed that after chemotherapy combined with immunotherapy in lung cancer patients, different treatment response groups exhibited distinct trends in alterations of the gut microbiome. In the effective group, 10 species showed significant differences, among which the order Spirochaetales and the family Spirochaetaceae were significantly elevated. In the ineffective group, the class Clostridia and the order Veillonellales were notably increased. Most members within the phylum Firmicutes, such as lactobacilli and Ruminococcus, are beneficial bacteria, although some harmful bacteria like Staphylococcus also belong to this phylum. Veillonella is a genus of Gram-negative anaerobic cocci. Studies have suggested that Veillonella might serve as a specific biomarker for the remission or stabilization of advanced gastric cancer in patients treated with monoclonal antibodies. Furthermore, its abundance and genetic diversity in the gut microbiota, along with that of Streptococcus, may be primarily due to changes in nutrient utilization rates induced by alterations in food intake, leading to high population dynamics. These dynamic populations could profoundly affect the interactions between the local host and microbes, thereby modulating the physiological and immune functions of the gut. Our preliminary work has shown that chemotherapy can induce changes in the gut microbiome leading to the atrophy of intestinal villi, and that supplementing with probiotics can reverse these changes, suggesting that the gut microbiome could predict treatment toxicities [24]. Our goal is to identify highly sensitive predictive markers of therapeutic efficacy. Although this study’s sample size limits generalization compared with other studies, gut microbiome markers seem to show certain deficiencies when compared with tumor microbiome markers in this regard.

The core of cellular immunotherapy lies in T cells, which occupy an essential place in the treatment and research of lung cancer. Tumor cells can present antigens to CD8+ T cells and activate them via major histocompatibility complex class I molecules, but in our experimental results, although there were changes in T cell subsets, these were insufficient to validate this concept. This may be related to the flow cytometry detection technique used, which did not allow for a more refined classification. Our research group is currently conducting studies on the monitoring of peripheral blood immune function in lung cancer patients, including the activation status of T cells, Ts cells, and the proportions of Th1/2/17 cells.

Research reports indicate that elevated levels of IL-8 and IL-10 in peripheral blood suggest an enhanced tumor immune escape, while an increase in IL-6 may indicate a higher possibility of adverse reactions to immunotherapy [25-28]. Peripheral inflammatory factors reflect the body's complex immune status. Studies have shown that the genus Bacteroides can inhibit the production of the pro-inflammatory cytokine IL-8 and can also activate T cells that produce IL-10 to counteract inflammation. However, this point was not reflected in the data from our experiment. Nevertheless, by observing the line graphs, it is noticeable that there is a downward trend in IL-8 levels at the early stages of treatment, with a significant decline. In contrast, IL-10 levels did not show a significant fluctuation. This may also be related to the presence of this bacterial group. It is considered that the small sample size or the short data collection period might be factors; further enlargement of the sample is needed to verify its potential.

Researchers have reported that the tumor microbiome in lung cancer development is an important factor in the effectiveness of immunotherapy. Therefore, understanding the interaction between the tumor microbiome and the immune system has important implications for the treatment of lung cancer and further research in this field.
cancer primarily consists of oropharyngeal bacteria and respiratory tract commensals based on an analysis of bronchoscopic lavage fluid from lung cancer patients [29]. However, this sampling method may not fully reflect the developmental patterns of lung cancer. Clinical pathology observations have confirmed that lung cancer often originates in the distal respiratory tract, with peripheral lung cancer being more prevalent. As lung cancer progresses, airway obstruction occurs, leading to isolation of the tumor microenvironment from the normal airway and suggesting that intrapulmonary bacteria may predominantly originate from the bloodstream, particularly in advanced lung cancer cases.

To address this, we have opted to perform microbial analysis by directly sampling tumor tissues via lung puncture biopsies in order to more accurately depict the composition and functionality of intratumoral bacteria within the tumor microenvironment. However, this sampling approach presents significant technical challenges in clinical practice and is constrained by medical ethics, making large-scale multicenter clinical studies exceedingly difficult and limiting the collection of patient cases. Nonetheless, the aim of our study is to explore novel clinical efficacy predictors through data analysis of a limited number of clinical samples, providing insights for future research directions.

Following an analysis of four potential predictive indicators, we have chosen to further investigate intratumoral bacteria. To gain a comprehensive understanding of the types and functions of intratumoral bacteria, we plan to employ metagenomic analysis of the microbial community. We eagerly anticipate the publication of our research results and aim to contribute new insights to the field of lung cancer treatment.

Conclusion and outlook
Our research suggests that differences in the composition of the microbiome within lung cancer may lead to variations in treatment efficacy. The dominance of the genus Sphingomonas indicates effective treatment, while the dominance of the genus Pseudomonas often suggests ineffective treatment. Moving forward, we will employ more precise diagnostic methods to identify specific bacteria within lung cancer that affect treatment outcomes, investigate their mechanisms of influence, and develop predictive assays for treatment efficacy. The trends in changes of the gut microbiome following lung cancer treatment may play a role in prognostic assessment, thus necessitating further in-depth studies. Although our study did not identify a predictive role for peripheral blood T cells and inflammatory factors in tumor treatment, it did reveal differential trends. In our next steps, we will utilize more sophisticated flow cytometry techniques to explore the activation of Th cells and Ts cells, and their predictive role in the efficacy of lung cancer treatment.

References


