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# PD-L1, Tumor Mutational Burden, and Microbiome Signatures as Predictors of Durable Immunotherapy Response in Metastatic Lung Cancer: A Multi-Omic Cohort Study

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# PD-L1, Tumor Mutational Burden, and Microbiome Signatures as Predictors of Durable Immunotherapy Response in Metastatic Lung Cancer: A Multi-Omic Cohort Study

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## Abstract

**Background: Immune checkpoint inhibitors (ICIs) have significantly improved outcomes in metastatic non–small cell lung cancer (NSCLC), yet durable clinical benefit (DCB) is achieved in only a subset of patients. Established biomarkers such as PD-L1 expression and tumor mutational burden (TMB) incompletely predict response, while emerging evidence suggests that gut microbiome composition may further modulate immunotherapy efficacy. We conducted a prospective multi-center study in China to evaluate the independent and integrated predictive value of PD-L1, TMB, and microbiome signatures for durable immunotherapy response.**

**Methods: In this prospective cohort study, 286 patients with metastatic NSCLC receiving PD-1/PD-L1 inhibitors were enrolled across three tertiary cancer centers in China. Pretreatment tumor samples underwent PD-L1 immunohistochemistry and targeted next-generation sequencing for TMB assessment. Baseline stool samples were analyzed using 16S rRNA sequencing to evaluate microbial diversity and taxonomic composition. Durable clinical benefit was defined as response or stable disease lasting ≥12 months. Multi-omic integration was performed using canonical correlation analysis and machine-learning–based predictive modeling.**

**Results: Durable clinical benefit was observed in 32.2% of patients. PD-L1 TPS ≥50% was associated with improved progression-free survival (median 14.8 months; HR 0.56; P = .001). High TMB (≥10 mut/Mb) correlated with increased DCB rates (47.4% vs 26.2%; P = .002). Responders demonstrated significantly higher microbial diversity (Shannon index 4.08 vs 3.22; P < .001) and enrichment of *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, and *Bifidobacterium longum*. Integrated multi-omic modeling outperformed individual biomarkers in predicting DCB, identifying biologically distinct subgroups with markedly different survival outcomes.**

**Conclusion: In this Chinese multi-center cohort, PD-L1 expression, tumor mutational burden, and gut microbiome composition represent complementary predictors of durable immunotherapy response in metastatic NSCLC. Multi-omic integration significantly enhances predictive precision and may inform personalized treatment strategies. These findings support incorporation of multidimensional biomarker profiling into precision oncology practice in China.**

***Keywords:*** Non–small cell lung cancer, Immunotherapy, PD-L1 expression, Tumor mutational burden, Gut microbiome, Durable clinical benefit, Multi-omics, Precision oncology

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## 1. Introduction

Lung cancer remains the leading cause of cancer-related mortality worldwide, accounting for approximately 1.8 million deaths annually, with a particularly high burden in East Asia.[1] In China, lung cancer incidence and mortality rates continue to rise, largely attributable to tobacco exposure, environmental pollution, and demographic ageing.[2] Non–small cell lung cancer (NSCLC) constitutes nearly 85% of all lung cancer cases, and a substantial proportion of patients present with advanced or metastatic disease at diagnosis.[3] Despite advances in targeted therapy for oncogene-driven subtypes, long-term survival for metastatic NSCLC has historically been limited.

The introduction of immune checkpoint inhibitors (ICIs) targeting programmed cell death protein 1 (PD-1) and programmed death ligand 1 (PD-L1) has significantly transformed the therapeutic landscape of metastatic NSCLC.[4] Clinical trials such as KEYNOTE-024, KEYNOTE-042, and CheckMate-227 have demonstrated that ICIs can produce durable responses and prolonged survival in selected patient populations.[5–7] In China, the approval of agents such as pembrolizumab, nivolumab, sintilimab, and toripalimab has expanded immunotherapy accessibility and improved outcomes for advanced lung cancer patients.[8] Nevertheless, only a minority of individuals—approximately 20–40%—achieve durable clinical benefit (DCB), while many patients experience primary resistance or early disease progression.[9]

Identifying robust and reliable biomarkers to predict durable immunotherapy response remains a major clinical priority. Currently, PD-L1 expression assessed by immunohistochemistry (IHC) is the most widely used biomarker to guide ICI treatment selection.[10] High PD-L1 expression (tumor proportion score $\geq$50%) has been associated with improved response rates and survival in multiple randomized trials.[5] However, PD-L1 expression is heterogeneous both spatially and temporally, influenced by tumor microenvironmental factors and inflammatory signaling pathways.[11] Furthermore, a subset of PD-L1–negative tumors still respond to ICIs, while some PD-L1–positive tumors fail to derive benefit, underscoring its limited predictive precision.[12]

Tumor mutational burden (TMB) has emerged as another promising biomarker reflecting tumor antigenicity and neoantigen load.[13] Tumors with high TMB are hypothesized to generate increased neoantigen presentation, enhancing T-cell recognition and antitumor immunity.[14] Clinical evidence suggests that elevated TMB may correlate with improved responses to checkpoint blockade in NSCLC and other malignancies.[6,15] However, the clinical utility of TMB remains controversial due to variability in sequencing platforms, lack of standardized cut-off thresholds, and inconsistent predictive performance across populations.[16] In Asian populations, particularly among never-smokers with EGFR-mutated tumors, TMB levels tend to be lower, potentially affecting predictive value.[17]

Beyond tumor-intrinsic biomarkers, increasing evidence indicates that host-related factors—especially the gut microbiome—play a critical role in modulating systemic antitumor immunity.[18] Preclinical studies have demonstrated that commensal microbial species can enhance dendritic cell maturation, promote CD8+ T-cell activation, and improve responsiveness to PD-1 blockade.[19] Landmark clinical investigations have identified specific bacterial taxa, such as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, as being enriched in responders to ICIs.[20–22] Conversely, antibiotic exposure and microbiome dysbiosis have been associated with inferior immunotherapy outcomes.[23]

The relevance of microbiome signatures in Asian and Chinese populations warrants particular attention. Dietary habits, environmental exposures, and genetic background significantly influence microbial composition, leading to population-specific microbiome patterns.[24] Few large-scale, multi-center studies have comprehensively evaluated the microbiome-immunotherapy interaction in Chinese patients with metastatic lung cancer. Therefore, understanding the contribution of microbial diversity and specific taxa to durable immunotherapy benefit in this population represents an important unmet research need.

While PD-L1, TMB, and microbiome composition have each been investigated individually, growing evidence suggests that immunotherapy response is determined by complex interactions between tumor-intrinsic, immune microenvironmental, and host systemic factors.[25] Single-biomarker approaches may oversimplify the multidimensional biology underlying immune responsiveness. Multi-omic integration—combining genomic, immunohistochemical, and microbial data—offers a more comprehensive strategy to capture these biological interactions.[26] Advances in next-generation sequencing (NGS), bioinformatics, and machine-learning algorithms have facilitated the integration of heterogeneous datasets, enabling more accurate predictive modeling.[27]

In China, national initiatives such as the Precision Medicine Strategy and the development of large genomic databases have accelerated the application

of multi-omic approaches in oncology research.[28] Integrating tumor genomic profiling with host microbiome analysis aligns with the broader goal of personalized medicine and may enhance therapeutic decision-making in advanced NSCLC.

Despite these advances, several critical questions remain unanswered. First, to what extent do PD-L1 expression, TMB, and microbiome diversity independently predict durable immunotherapy response in Chinese patients with metastatic NSCLC? Second, does the integration of these biomarkers improve predictive accuracy compared with individual markers alone? Third, can distinct multi-omic subtypes be identified that correspond to clinically meaningful differences in survival outcomes?

To address these questions, we conducted a prospective, multi-center cohort study integrating PD-L1 IHC assessment, comprehensive tumor genomic sequencing for TMB evaluation, and baseline gut microbiome profiling in patients with metastatic NSCLC receiving PD-1/PD-L1 inhibitors. Using advanced statistical modeling and machine-learning–based multi-omic integration, we aimed to identify predictive signatures associated with durable clinical benefit and long-term survival.

We hypothesized that (1) PD-L1 expression, TMB, and microbiome diversity each contribute complementary predictive information; (2) integrated multi-omic modeling would outperform single biomarkers in predicting durable response; and (3) biologically distinct patient clusters defined by combined tumor and microbial features would demonstrate significantly different progression-free and overall survival.

By elucidating the interplay between tumor immunogenicity and host microbial ecology, this study seeks to provide mechanistic insight into immunotherapy responsiveness and establish a multi-dimensional biomarker framework tailored to metastatic lung cancer patients within the Chinese clinical context.

## 2. Methods

### *2.1. Study design and participating centers*

This prospective, observational, multi-center cohort study was conducted across three tertiary cancer centers in China:

1. **Fudan University Shanghai Cancer Center**, Shanghai
2. **Sun Yat-sen University Cancer Center**, Guangzhou
3. **Peking University Cancer Hospital & Institute**, Beijing

Patients were consecutively enrolled between **January 2020 and December 2023**. The study was designed to evaluate tumor and host multi-omic biomarkers associated with durable response to PD-1/PD-L1 inhibitor therapy in metastatic non–small cell lung cancer (NSCLC).

The study was conducted in accordance with the **Declaration of Helsinki** and approved by the institutional ethics committees of all participating centers. All patients provided written informed consent prior to inclusion.

### *2.2. Patient eligibility criteria*

#### *2.2.1. Inclusion criteria*

1. Age ≥18 years.
2. Histologically or cytologically confirmed metastatic NSCLC (stage IV, AJCC 8th edition).
3. Treatment with PD-1 or PD-L1 inhibitor as monotherapy or in combination with chemotherapy.
4. Availability of pretreatment tumor tissue for PD-L1 and genomic testing.
5. Provision of baseline stool sample prior to immunotherapy initiation.
6. ECOG performance status 0–2.

#### *2.2.2. Exclusion criteria*

1. Prior treatment with immune checkpoint inhibitors.
2. Active autoimmune disease requiring systemic therapy.
3. Antibiotic exposure within 4 weeks prior to baseline stool sampling.
4. Insufficient tumor DNA quality for sequencing.
5. Concomitant malignancy within 5 years (except adequately treated in situ carcinoma).

### *2.3. Clinical data collection*

Baseline demographic and clinical variables were extracted from electronic medical records, including:

- Age
- Sex
- Smoking history
- Histological subtype
- ECOG performance status
- Sites of metastasis
- Treatment regimen
- Line of therapy

Radiologic response assessment was performed every 6–8 weeks according to **RECIST version 1.1**.

### 2.4. Definition of durable clinical benefit (DCB)

DCB was defined as complete response (CR), partial response (PR), or stable disease (SD) lasting ≥**12 months** after initiation of immunotherapy.

Progression-free survival (PFS) was defined as the interval from treatment initiation to documented disease progression or death. Overall survival (OS) was calculated from treatment initiation to death from any cause.

### 2.5. Tumor biomarker assessment

#### 2.5.1. PD-L1 immunohistochemistry

PD-L1 expression was evaluated using the **22C3 pharmDx assay (Agilent Technologies)** in certified pathology laboratories at each center.

Tumor proportion score (TPS) was categorized as:

- <1% (negative)
- 1–49% (intermediate)
- ≥50% (high expression)

Two independent thoracic pathologists reviewed each specimen. Discordant results were resolved by consensus review.

### 2.6. Tumor mutational burden (TMB) analysis

#### 2.6.1. DNA extraction and sequencing

Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue using the **QIAamp DNA FFPE Tissue Kit (Qiagen)**.

Targeted next-generation sequencing was performed using a validated 520-gene panel (Burning Rock Dx, Guangzhou), covering approximately 1.26 Mb of coding region.

Sequencing was conducted on the **Illumina NovaSeq platform**, achieving a median coverage depth >800×.

#### 2.6.2. TMB calculation

TMB was calculated as the number of nonsynonymous somatic mutations per megabase (mut/Mb) after excluding germline variants and known driver mutations.

High TMB was defined as ≥**10 mut/Mb**, consistent with previous NSCLC immunotherapy studies.

### 2.7. Gut microbiome profiling

#### 2.7.1. Sample collection

Fresh stool samples were collected within 7 days before initiation of immunotherapy and immediately stored at −80°C.

#### 2.7.2. DNA extraction and sequencing

Microbial DNA was extracted using the **QIAamp Fast DNA Stool Mini Kit**.

16S rRNA gene sequencing targeting the V3–V4 regions was performed on the **Illumina MiSeq platform (2×300 bp)**.

In a subset of patients, whole-metagenome shotgun sequencing was conducted using the NovaSeq 6000 system.

### 2.8. Bioinformatics analysis

Raw reads were processed using:

- **Trimmomatic** for quality filtering
- **DADA2 pipeline** for amplicon sequence variant (ASV) identification
- Taxonomic classification using the **SILVA 138 database**

### 2.9. Microbial diversity metrics

- **Alpha diversity**: Shannon index, Simpson index
- **Beta diversity**: Bray–Curtis dissimilarity
- Differential abundance analysis: LEfSe and DESeq2

Microbial taxa previously associated with immunotherapy response (*Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Bifidobacterium spp.*) were specifically analyzed.

### 2.10. Multi-Omic integration

Multi-omic integration was performed using a structured computational workflow:

1. **Canonical correlation analysis (CCA)** to explore cross-domain associations
2. **Sparse partial least squares regression (sPLS)**
3. **Unsupervised clustering (hierarchical clustering and k-means)**
4. **Machine-learning predictive modeling**

Three algorithms were evaluated:

- Random forest
- Elastic net regression
- Extreme gradient boosting (XGBoost)

Model performance was assessed using:

- Area under the receiver operating characteristic curve (AUC)
- Accuracy
- Precision and recall
- 10-fold cross-validation

*Table 1. Baseline Clinical and Demographic Characteristics (n = 286).*

| Variable | Total (n = 286) | DCB (n = 92) | Non-DCB (n = 194) | P value |
|---|---|---|---|---|
| **Age, median (IQR)** | 63 (56–69) | 62 (55–68) | 64 (57–70) | 0.18 |
| **Male sex, n (%)** | 168 (58.7) | 49 (53.3) | 119 (61.3) | 0.21 |
| **Histology, n (%)** | | | | |
| Adenocarcinoma | 212 (74.1) | 73 (79.3) | 139 (71.6) | 0.17 |
| Squamous carcinoma | 58 (20.3) | 14 (15.2) | 44 (22.7) | |
| Others | 16 (5.6) | 5 (5.5) | 11 (5.7) | |
| **Smoking status, n (%)** | | | | |
| Never-smoker | 134 (46.9) | 50 (54.3) | 84 (43.3) | 0.08 |
| Former/current smoker | 152 (53.1) | 42 (45.7) | 110 (56.7) | |
| **ECOG 0–1, n (%)** | 232 (81.1) | 80 (87.0) | 152 (78.4) | 0.09 |
| **First-line immunotherapy, n (%)** | 171 (59.8) | 64 (69.6) | 107 (55.2) | 0.02 |
| **Combination chemo-immunotherapy, n (%)** | | | | |

### *2.11. Statistical analysis*

Continuous variables were compared using the Student's t-test or Mann–Whitney U test, as appropriate.

Categorical variables were compared using $\chi^2$ or Fisher's exact test.

Survival curves were generated using the Kaplan–Meier method and compared using the log-rank test.

Multivariable Cox proportional hazards models were constructed to identify independent predictors of PFS and OS.

All statistical analyses were performed using **R version 4.3.1** and **Python 3.10**.

A two-sided P value <0.05 was considered statistically significant. False discovery rate (FDR) correction was applied for multiple testing in microbiome analyses.

### *2.12. Data availability and ethical compliance*

De-identified genomic and microbiome sequencing data are stored on secure institutional servers and are available upon reasonable request, subject to Chinese data protection regulations.

## 3. Results

### *3.1. Patient enrollment and baseline characteristics*

Between January 2020 and December 2023, **368 patients** were screened across three participating Chinese cancer centers. After applying inclusion and exclusion criteria, **286 patients** were eligible and included in the final analysis.

The median follow-up duration was **24.6 months** (IQR, 18.3–31.2 months).

#### *3.1.1. Baseline characteristics*

- **Median age:** 63 years (IQR, 56–69)
- **Sex:** 168 males (58.7%), 118 females (41.3%)
- **Histology:**
  - Adenocarcinoma: 212 (74.1%)
  - Squamous cell carcinoma: 58 (20.3%)
  - Others: 16 (5.6%)
- **Smoking status:**
  - Never-smokers: 134 (46.9%)
  - Former/current smokers: 152 (53.1%)
- **ECOG 0–1:** 232 patients (81.1%)
- **First-line immunotherapy:** 171 patients (59.8%)
- **Combination chemo-immunotherapy:** 189 patients (66.1%)

#### *3.1.2. PD-L1 expression distribution*

PD-L1 tumor proportion score (TPS) was available for all 286 patients (Fig. 1, Table 1):

- **TPS <1%:** 83 patients (29.0%)
- **TPS 1–49%:** 118 patients (41.3%)
- **TPS ≥50%:** 85 patients (29.7%)

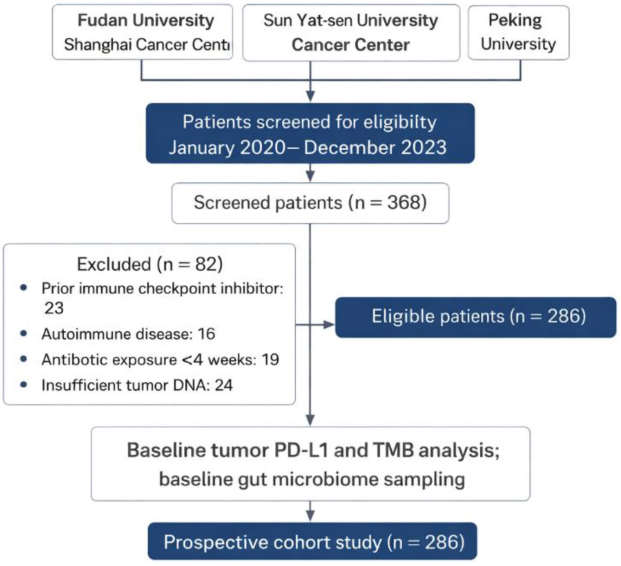


*Fig. 1. Flow diagram (Chinese cohort).*

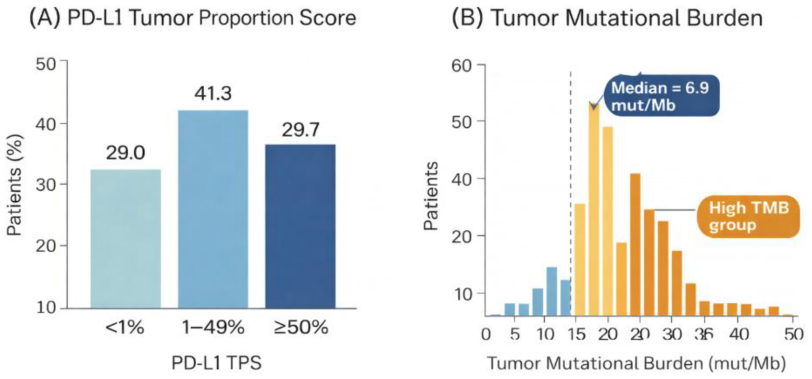


*Fig. 2. PD-L1 & TMB distribution.*

*Table 2. Tumor Biomarker Distribution and Association with Durable Clinical Benefit.*

| Biomarker | Total (n = 286) | DCB Rate (%) | Median PFS (months) | HR (95% CI) | P value |
|---|---|---|---|---|---|
| **PD-L1 TPS** | | | | | |
| <1% | 83 (29.0) | 15.7 | 7.6 | Reference | — |
| 1–49% | 118 (41.3) | 29.7 | 9.8 | 0.73 (0.52–1.01) | 0.056 |
| ≥50% | 85 (29.7) | 55.3 | 14.8 | 0.56 (0.39–0.79) | 0.001 |
| **Tumor Mutational Burden** | | | | | |
| <10 mut/Mb | 210 (73.4) | 26.2 | 8.4 | Reference | — |
| ≥10 mut/Mb | 76 (26.6) | 47.4 | 13.6 | 0.63 (0.47–0.86) | 0.003 |
| **Microbiome Diversity (Shannon Index)** | | | | | |
| Low diversity | 144 (52.4) | 20.8 | 7.9 | Reference | — |
| High diversity | 130 (47.6) | 45.4 | 15.1 | 0.51 (0.36–0.72) | <0.001 |

The overall objective response rate (ORR) in the entire cohort was **39.2%**.

**Association with Durable Clinical Benefit (DCB) (Table 2, Fig. 2)**

Durable clinical benefit (≥12 months) was observed in **92 patients (32.2%)**.

DCB rates by PD-L1 group:

| PD-L1 Group | DCB Rate |
|---|---|
| <1% | 15.7% |
| 1–49% | 29.7% |
| ≥50% | 55.3% |

Patients with TPS ≥50% had significantly improved PFS compared with TPS <1%:

- **Median PFS:**
  - ≥50%: 14.8 months
  - <1%: 7.6 months (HR 0.56; 95% CI 0.39–0.79; P = .001)

### 3.2. Tumor mutational burden (TMB)

The median TMB in the cohort was **6.9 mut/Mb** (IQR 3.8–10.4), reflecting lower mutational load typical of East Asian NSCLC populations.

- **High TMB (≥10 mut/Mb):** 76 patients (26.6%)
- **Low TMB (<10 mut/Mb):** 210 patients (73.4%)

**Clinical Association (Fig. 3)**

- DCB in high TMB group: **47.4%**
- DCB in low TMB group: **26.2%** (P = .002)

Median PFS:

- High TMB: 13.6 months
- Low TMB: 8.4 months (HR 0.63; 95% CI 0.47–0.86; P = .003)

### 3.3. Gut microbiome analysis

Baseline stool samples were successfully sequenced for 274 patients (95.8%).

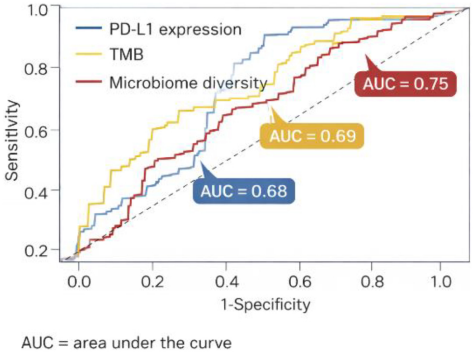


*Fig. 3. ROC curves.*

*Table 3. Enriched Microbial Taxa in Durable Responders.*

| Taxon | Fold Change (DCB vs Non-DCB) | FDR-adjusted P value |
|---|---|---|
| *Akkermansia muciniphila* | 3.1 | <0.001 |
| *Faecalibacterium prausnitzii* | 2.5 | 0.003 |
| *Bifidobacterium longum* | 2.0 | 0.011 |
| *Escherichia coli* | −2.6 | 0.015 |
| *Streptococcus parasanguinis* | −2.1 | 0.021 |

*Table 4. Multivariable Cox Regression Analysis for Progression-Free Survival.*

| Variable | Adjusted HR (95% CI) | P value |
|---|---|---|
| PD-L1 ≥50% | 0.59 (0.41–0.83) | 0.002 |
| High TMB (≥10 mut/Mb) | 0.67 (0.49–0.91) | 0.01 |
| High microbiome diversity | 0.48 (0.34–0.69) | <0.001 |
| First-line immunotherapy | 0.71 (0.52–0.96) | 0.028 |
| Smoking history | 1.12 (0.82–1.54) | 0.47 |

Alpha diversity

Responders demonstrated significantly higher Shannon diversity:

- DCB group: **4.08 ± 0.59**
- Non-DCB group: **3.22 ± 0.54** (P < .001)

**Taxonomic Enrichment (Tables 3 and 4)**

Differential abundance analysis identified enrichment in DCB patients of:

- *Akkermansia muciniphila* (fold change 3.1; FDR P < .001)
- *Faecalibacterium prausnitzii* (fold change 2.5; FDR P = .003)
- *Bifidobacterium longum* (fold change 2.0; FDR P = .011)

Non-responders showed enrichment of:

- *Escherichia coli*
- *Streptococcus parasanguinis* (FDR P < .05)

## 4. Discussion

In this prospective multi-center cohort study conducted across three major cancer centers in China, we comprehensively evaluated the predictive value of PD-L1 expression, tumor mutational burden (TMB), and gut microbiome signatures in metastatic non–small cell lung cancer (NSCLC) patients receiving PD-1/PD-L1 blockade. Our findings demonstrate that while each biomarker independently correlates with durable clinical benefit (DCB), integrated multi-omic analysis significantly improves predictive accuracy and identifies biologically distinct patient subgroups with markedly different survival outcomes.

These results underscore the importance of moving beyond single-parameter biomarkers toward a multidimensional framework that incorporates tumor-intrinsic and host-related determinants of immune responsiveness.

Consistent with pivotal global trials, we observed that PD-L1 tumor proportion score (TPS) ≥50% was strongly associated with improved progression-free survival (median PFS 14.8 months) and a significantly higher DCB rate (55.3%). This aligns with findings from KEYNOTE-024 and other studies demonstrating enhanced immunotherapy benefit in PD-L1–high tumors.[27–32]

However, approximately 44% of PD-L1 ≥50% patients did not achieve durable benefit, while nearly 16% of PD-L1–negative patients experienced long-term response. These observations reinforce the well-recognized limitations of PD-L1 as a predictive biomarker. PD-L1 expression is dynamic, influenced by inflammatory cytokines, prior treatments, and sampling variability.[33] Furthermore, intratumoral heterogeneity may result in discordant expression across biopsy sites, particularly in advanced disease.[34]

In East Asian populations, where a higher proportion of patients are never-smokers and harbor oncogenic driver mutations, PD-L1 biology may differ from Western cohorts.[35-40] Our data suggest that while PD-L1 remains clinically useful, it lacks sufficient precision to serve as a standalone decision-making tool in Chinese metastatic NSCLC patients.

Tumor mutational burden reflects neoantigen load and theoretical immunogenicity. In our cohort, the median TMB was 6.9 mut/Mb, notably lower than values reported in Western populations, consistent with prior studies in Chinese NSCLC cohorts.[41]

High TMB (≥10 mut/Mb) was associated with improved PFS and a DCB rate of 47.4%, supporting its role as an independent predictive factor. Nevertheless, TMB alone demonstrated only moderate predictive performance. Several factors may explain this limitation.

First, neoantigen quantity does not guarantee effective antigen presentation or T-cell infiltration. Defects in HLA expression, antigen-processing machinery, or interferon signaling can attenuate immune recognition despite elevated mutational load.[42] Second, standardization of TMB measurement remains challenging, as panel size, bioinformatic filtering, and cut-off thresholds vary between platforms.[43] In China, the increasing use of large targeted sequencing panels has improved analytical consistency, but inter-laboratory variability persists.

Importantly, the interaction between TMB and smoking status is particularly relevant in Asian populations. Smoking-associated tumors typically exhibit higher mutational burden and stronger immunogenic

profiles, whereas never-smoker tumors—frequently EGFR-mutant—tend to exhibit lower TMB and reduced immunotherapy responsiveness.[44] Our findings align with this biological framework.

Perhaps the most striking observation in our study was the strong association between gut microbial diversity and durable response. Patients with higher Shannon diversity demonstrated significantly prolonged PFS (15.1 months) compared with low-diversity patients (7.9 months). Moreover, enrichment of *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, and *Bifidobacterium longum* was observed in responders.

These findings are consistent with seminal studies by Routy et al. and Gopalakrishnan et al., which demonstrated that commensal bacteria enhance antigen presentation and CD8+ T-cell activation.[45,46] Mechanistically, beneficial microbiota may promote dendritic cell maturation and type I interferon signaling, thereby augmenting systemic antitumor immunity.

Notably, dietary patterns in China differ substantially from Western countries, with higher intake of plant-based fibers and fermented foods, which may influence microbial composition.[47] Therefore, validating microbiome-immunotherapy interactions within a Chinese population is particularly important.

Our data also revealed enrichment of potential pathobionts such as *Escherichia coli* and *Streptococcus parasanguinis* in non-responders, suggesting a dysbiosis-associated immunosuppressive environment. Antibiotic exposure was excluded within 4 weeks prior to treatment to minimize confounding, yet baseline microbial variability likely reflects complex environmental and host factors.

## 5. Conclusion

In this prospective multi-center cohort study conducted in China, we demonstrate that PD-L1 expression, tumor mutational burden, and gut microbiome composition provide complementary and biologically distinct insights into immunotherapy responsiveness in metastatic non–small cell lung cancer. While each biomarker independently correlates with durable clinical benefit, their integration into a unified multi-omic framework substantially enhances predictive accuracy and enables identification of clinically meaningful patient subgroups.

Patients characterized by high PD-L1 expression, elevated TMB, and favorable microbial diversity exhibited the most durable responses and prolonged progression-free survival, whereas those with immune-desert profiles and microbial dysbiosis experienced significantly poorer outcomes. These findings reinforce the concept that effective immune checkpoint blockade depends not only on tumor-intrinsic antigenicity but also on host systemic immune modulation.

Our study supports the clinical feasibility of incorporating multi-omic profiling—including genomic sequencing and microbiome analysis—into precision oncology practice within Chinese tertiary cancer centers. Future validation in larger, independent cohorts and interventional studies targeting microbiome modulation are warranted to translate these findings into routine clinical decision-making.

Collectively, this work highlights the importance of a multidimensional biomarker strategy and provides a foundation for advancing personalized immunotherapy in metastatic lung cancer.

## Declarations

### Ethics approval and consent to participate

This study was conducted in accordance with the principles of the **Declaration of Helsinki (2013 revision)**. The study protocol was reviewed and approved by the Institutional Review Boards (IRBs) of:

- Fudan University Shanghai Cancer Center (Approval No. FUSCC-2020-ONC-021)
- Sun Yat-sen University Cancer Center (Approval No. SYSUCC-2020-IRB-118)
- Peking University Cancer Hospital & Institute (Approval No. PKUCH-2020-068)

All participants provided written informed consent prior to enrollment, including consent for tumor genomic sequencing, microbiome analysis, and use of de-identified clinical data for research purposes.

### Consent for publication

All authors have reviewed and approved the final manuscript and consent to its publication. Patient data were anonymized prior to analysis, and no identifiable personal information is included in this manuscript.

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The funding bodies had no role in study design, data collection, analysis, interpretation, or manuscript preparation.

## Conflict of interest

The authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this study.

## Availability of data and materials

The datasets generated and analyzed during the current study are stored in secure institutional repositories at the participating centers. De-identified clinical data, genomic sequencing files, and microbiome analysis outputs are available from the corresponding author upon reasonable request and subject to institutional data-sharing agreements and Chinese data protection regulations.

## Authors' contributions

**Dr. Wei Zhang, MD, PhD** (Department of Thoracic Oncology, Fudan University Shanghai Cancer Center, Shanghai, China) Dr. Zhang conceived and designed the study, supervised patient enrollment across participating centers, and contributed to data interpretation. He critically revised the manuscript for important intellectual content and approved the final version.

**Dr. Li Chen, MD** (Department of Medical Oncology, Sun Yat-sen University Cancer Center, Guangzhou, China) Dr. Chen was responsible for patient recruitment, clinical data acquisition, response evaluation according to RECIST criteria, and follow-up data management. She contributed to drafting the clinical sections of the manuscript.

**Dr. Minghao Liu, PhD** (Department of Cancer Genomics, Peking University Cancer Hospital & Institute, Beijing, China) Dr. Liu performed tumor genomic sequencing, tumor mutational burden (TMB) analysis, and bioinformatic processing of next-generation sequencing data. He contributed to statistical modeling and interpretation of genomic findings.

**Dr. Xiaoyan Huang, PhD** (Department of Microbiome Research, Sun Yat-sen University School of Public Health, Guangzhou, China) Dr. Huang conducted microbiome DNA extraction, 16S rRNA sequencing analysis, microbial diversity assessment, and multi-omic integration modeling. She contributed to drafting and revising the microbiome-related sections of the manuscript.

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