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ORIGINAL RESEARCH

Discover Mutational Differences Between Lung Adenocarcinoma and Lung Squamous Cell Carcinoma and Search for More Effective Biomarkers for Immunotherapy

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Purpose: Lung cancer is a severe malignant tumor. This study aims to more comprehensively characterize lung cancer patients and identify combination markers for immunotherapy.

Patients and Methods: We gathered data from 166 lung cancer patients at the Cancer Hospital Affiliated with Xinjiang Medical University. The collected samples underwent NGS sequencing using a panel of 616 genes associated with cancer. Subsequently, data analysis was conducted to identify markers that are more suitable for lung cancer immunotherapy.

Results: In this study, the most common variant genes in LUAD were TP53, EGFR, MST1, KMT2C, RBM10, LRP1B. Meanwhile, the highest mutation frequency genes in LUSC samples were TP53, KMT2D, LRP1B, FAT1, MST1, KMT2C. Mutation frequencies, tumor mutation burden (TMB), PD-L1 expression, and mutant-allele tumor heterogeneity (MATH) values differed between LUAD and LUSC, with LUSC exhibiting higher values than LUAD. Irrespective of LUAD or LUSC, patients with TMB≥10 demonstrated better immunotherapy efficacy compared to patients with TMB<10. Similarly, when PD-L1≥50%, whether in LUAD or LUSC, the immunotherapy effect was superior to that of patients with PD-L1<50%. Combining TMB≥10 and PD-L1≥50% as immunotherapy markers, in both LUAD and LUSC, resulted in a very favorable immunotherapy effect, with the overall response rate (ORR) reaching 100%.

Conclusion: We observed distinct mutation patterns and clinical factors between LUAD and LUSC, and noted that patients with TMB≥10 and PD-L1≥50% exhibited enhanced immunotherapy effects. Combining TMB≥10 and PD-L1≥50% proved to be a more effective predictor of immunotherapy efficacy.

Keywords: Non-small cell lung cancer, Mutation, Genomic features, Immunotherapy

Introduction

Lung cancer is a severe malignant tumor and remains one of the leading causes of cancer-related deaths globally.[1] The predominant type of lung cancer is non-small cell lung cancer (NSCLC), constituting approximately 85% of all cases. NSCLC is further categorized into three main subtypes: large cell carcinoma (15%), lung squamous cell carcinoma (LUSC, 30%), and lung adenocarcinoma (LUAD, 40%).[2] Treatment and prognosis for LUAD and LUSC vary depending on the subtype. Advances in genetic testing technology have identified key driver genes in different lung cancer types, such as EGFR mutations, ALK fusions, ROS1 fusions, PTEN mutations, FGFR1 amplifications, and KRAS mutations.[3] Compared to LUSC, LUAD benefits from a more responsive mutation-targeted treatment plan. However, the prognosis for patients with advanced NSCLC remains grim, with a 5-year survival rate potentially falling below 15%.[4] Regardless of LUAD or LUSC, beyond chemotherapy and targeted drugs, immunotherapy has demonstrated both safety and efficacy.[5,6]

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The advent of immune checkpoint inhibitory therapies (ICIs) has introduced significant treatment advancements for various cancer types, including NSCLC.[7,8] However, the efficacy of ICIs is limited to a minority of NSCLC patients, prompting efforts to identify therapeutic biomarkers indicative of ICIs sensitivity. While positive PD-L1 protein expression in over 50% of tumor cells is an established biomarker in NSCLC, approximately half of PD-L1-positive patients do not respond to ICIs.[9,10] Several other independently predictive biomarkers have emerged, with tumor mutation burden (TMB) being one of them.[11] Results from multiple clinical studies have indicated that a TMB value of 10 mutations/Mb or higher serves as a reliable cutoff for predicting patient response to initial immunotherapy.[12] TMB is an emerging independent biomarker for predicting immunotherapy outcomes across various tumor types, including lung cancer. In the CheckMate 568 trial, a Phase 2 study of nivolumab plus ipilimumab in NSCLC, a TMB of at least 10 mutations per megabase was identified as an effective cutoff for selecting patients most likely to respond, regardless of their tumor's PD-L1 expression level. Similarly, CheckMate 227, an open-label Phase 3 trial, evaluated the efficacy of nivolumab or nivolumab-based regimens as first-line treatments in a biomarker-selected population of patients with advanced NSCLC. Following new data on TMB, the CheckMate 227 protocol was amended to include a coprimary endpoint for evaluating progression-free survival with nivolumab plus ipilimumab versus chemotherapy in patients with a TMB of at least 10 mutations per megabase, independent of PD-L1 expression levels. Although high TMB may forecast the benefits of ICI in NSCLC, further information is required to optimize its clinical utility.[13] A more thorough exploration of the mutational profiles of LUAD and LUSC can offer better guidance for their respective treatments. Additionally, for advanced lung cancer, it is imperative to explore biomarker information that can more effectively steer immunotherapy strategies.

In this study, we conducted a retrospective analysis of the molecular profiles of lung cancer patients treated between November 2018 and June 2021. Our aim was to explore the correlation between clinical features, molecular characteristics, and treatments to provide a more comprehensive characterization of lung cancer. The ultimate goal is to identify combination markers for immunotherapy that can enhance our understanding and better inform treatment decisions for lung cancer patients undergoing immunotherapy.

Materials and Methods

Patient and Sample Collection

This retrospective study was conducted by the Affiliated Cancer Hospital of Xinjiang Medical University. Tumor samples were collected from 166 lung cancer patients spanning the period from November 2018 to June 2022. The study received approval from our hospital's committee, specifically the Ethics Committee of Cancer Hospital Affiliated to Xinjiang Medical University, with the ethical approval number K-2022041. The study was conducted in adherence to the principles outlined in the Declaration of Helsinki. Given its retrospective nature, the need for informed consent from participants was waived by the ethics committee. Staging was performed in accordance with the 8th edition of the tumor, node, and metastasis (TNM) criteria, and histological classification was assessed following the latest World Health Organization criteria.[14] Progression-free survival (PFS) was computed as the duration from the initiation of treatment to the occurrence of progressive disease (PD) or the last follow-up. Information on all histological subtypes, tumor stages (I–IV), treatments and the expression of PD-L1 was extracted from the medical records.

NGS Library Preparation and Sequencing

Lung cancer samples were performed on NGS test with the pan-cancer panel, which spans 2.2 MB of human genome and consists of all exons and critical introns of 616 cancer regenes (The list of genes was provides in Table S1). DNA was extracted from tissue and centrifugation of hydrothorax samples using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany). DNA concentration was estimated using a Qubit fluorometer and Qubit dsDNA High Sensitivity (HS) Assay Kit (Invitrogen, USA). 50–100ng of sheared genomic DNA or ctDNA was subjected to library construction with an MGIEasy universal DNA library kit (MGI, China), then followed by hybrid capture using an xGen Hybridization and Wash Kit (IDT, USA). The qualified libraries were sequenced with 2×100bp paired-end reads on a MGISEQ-2000 (MGI, China) platform.

Bioinformatics Analysis

The paired-end reads were aligned to human reference genome GRCh37/hg19 using BWA-MEM (v0.7.17).[15] SNVs and InDels were called by VarScan (v 2.4.3) by verified settings.[16] SNVs and InDels from tissue were filtered by mean depths >800X. At least 5 supporting reads were needed for InDels, while 8 supporting reads were needed for SNVs to be called. CNVs were analyzed with in-house algorithm based on sequencing depth of coverage data of capture intervals. The minimum threshold of copy number gain or loss was CN >2.75 or CN <1.75 for hotspot genes, and CN >3 or CN <1.5 for others. Gene fusion was analyzed using FACTERA.[17] TMB was assessed as described by Chalmers and colleagues.[18]

PD-L1 Immunohistochemical Analysis

Standard automatic staining protocols were employed using the fully automated Dako Autostainer Link 48 (National Medical Device No. 20160557). Tissue specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and processed for immunohistochemistry (IHC) following standard procedures. Paraffin-embedded samples were sectioned into 4 μm thick slices, with formalin-fixed tissue sections of the same thickness used for immunohistochemical analysis of NSCLC patient samples. The primary antibody used was a monoclonal mouse anti-PD-L1 antibody, clone 22C3 (Cat. No.: M3666, 1:50, Dako), and the staining was carried out using the Dako Autostainer Link 48. PD-L1 membrane staining, detected by the primary antibody, was considered positive in cancer cells. The proportion of PD-L1 positive cells was calculated as the percentage of total cancer cells. PD-L1 immunostaining results were categorized according to the tumor proportion score (TPS): (1) negative, when <1% of cells exhibited absent or detectable staining; (2) low expression, when 1%–49% of cells showed membrane staining; and (3) high expression, when ≥50% of cells displayed membrane staining. Two authors, blinded to the clinical data, independently assessed the immunostaining. Discrepancies were resolved by reviewing the corresponding tissue sections and discussing the findings (Figure S1).

Statistical Analysis

Statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Fisher's exact test was used for the association analysis of categorical variables. The associations of normally and non-normally distributed data were analyzed using Student's *t*-test and Wilcoxon rank test respectively. Kaplan-Meier survival analysis was used to evaluate the association between TMB and PFS. P<0.05 was considered statistically significant.

Results

Clinical Characteristics of Patients

The cohort of 166 lung cancer patients comprised 101 males and 65 females, with a median age of 62 years (range, 26–93 years), and a mean age of 60 years. Among them, 87.95% (146/166) were diagnosed with LUAD, while 12.05% (20/166) were classified as LUSC. More than half of the samples originated from patients at the metastatic stage (clinical stage IV, 133/166, 80.12%), and 19.88% (33/166) were from patients at stages III. Furthermore, we observed correlations between patient sex and age at diagnosis with tumor subtypes (p<0.001). The proportion of male patients with LUSC was significantly higher than that of female patients compared to LUAD. Additionally, the age at diagnosis for LUSC was significantly higher than that for LUAD (see Tables 1 and S2 for a summary of other characteristics in this cohort).

Overview of Genomic Alterations in This Cohort

In this study, the predominant genomic alterations were single nucleotide variations (SNVs) at 84.8%, followed by Indel (12.5%), splice site mutations (2.3%), and fusion events (0.3%). Among the 166 samples, 98.1% exhibited at least one detected variant. In LUAD samples, the most prevalent variant genes were TP53 (60%, 88/146), EGFR (55%, 80/146), MST1 (37%, 54/146), KMT2C (27%, 39/146), RBM10 (17%, 25/146), LRP1B (17%, 25/146), SPTA1 (16%, 23/146), CDH4 (14%, 20/146), and KRAS (10%, 14/146) (Figure 1A). In contrast, in LUSC samples, the genes with the highest mutation frequencies were TP53 (75%, 15/20), KMT2D (40%, 8/20), LRP1B (40%, 8/20), FAT1 (30%, 6/20), MST1 (30%, 6/20), KMT2C (30%, 6/20), ARID1A (25%, 5/20), AR (25%, 5/20), and NOTCH1 (25%, 5/20) (Figure 1B). Simultaneously, we identified numerous co-mutations and mutually exclusive mutations in LUAD. Among them, the

Table 1 Clinical Characteristics of LUAD and LUSC

Characteristic	All patients (n=166)	LUAD (n=146)	LUSC (n=20)
Age			
≥60	97	80	17
<60	69	66	3
Gender			
male	101	82	19
female	65	64	1
Stage			
III	33	29	4
IV	133	117	16
TMB			
≥10	67	53	14
<10	99	93	6
PD-L1			
≥1	117	103	14
<1	35	31	4
≥50	40	33	7
<50	112	101	11
NA	14	12	2
Smoking history			
Nosmokers	96	89	7
Smokers	70	57	13

gene with the highest frequency of co-mutations is RET. Genes co-mutating with RET mainly include PIK3CA, ALK, MET, PTEN, FGFR1, NRAS, ROS1, and AKT1. Additionally, AKT1 and PTEN exhibit several co-mutated genes. Notably, EGFR shows the highest number of mutually exclusive mutations, with KRAS and EGFR mutations being mutually exclusive and particularly significant (Figure 1C). Conversely, LUSC displays fewer co-mutated genes. Major co-mutated genes in LUSC include PTEN, KRAS, and ERBB2. Interestingly, there is no significant pattern of reciprocal suppression among mutated genes in LUSC(Figure 1D).

Among the mutated genes we detected, most are genes closely related to the occurrence and development of cancer. The TP53 gene encodes a tumor suppressor protein with transcriptional activation, DNA binding, and oligomerization domains. This protein responds to various cellular stresses, regulating the expression of target genes to induce processes such as cell cycle arrest, apoptosis, senescence, DNA repair, or metabolic changes. Mutations in TP53 are linked to many human cancers.[19] EGFR is a member of the epidermal growth factor receptor (HER) family, and studies have shown that it is highly or abnormally expressed in several solid tumors. EGFR plays a role in tumor cell proliferation, angiogenesis, tumor invasion, metastasis, and apoptosis inhibition. Research on EGFR's connection to tumor angiogenesis, invasiveness, and metastasis suggests that EGFR can influence tumor angiogenesis by regulating factors such as Ang-1 and VEGF.[20] The KRAS protein operates downstream of the EGFR signaling pathway. Normally, activation of the EGFR pathway triggers transient KRAS activation, which is promptly followed by inactivation. However, mutations in the KRAS gene result in continuous pathway activation, which accelerates tumor cell proliferation. These KRAS mutations encode abnormal proteins that drive the growth and spread of malignant cells.[21] KMT2C, encoding histone lysine methyltransferase 2C, is an important epigenetic regulator. It is frequently mutated in various cancers, playing a crucial role in cancer development.[22] The RBM10, an RNA-binding motif protein, is a splicing regulator involved in diverse biological processes, including mRNA splicing.[23] Recently, RBM10 has been recognized as a cancer-related gene, with its downregulation or mutation observed in cancers such as lung adenocarcinoma, colorectal, and cervical cancers.[24] KMT2D encodes a histone methyltransferase that methylates the Lys-4 position of histone H3 and regulates transcription factors like estrogen receptor (ER) and FOXA1.[25]

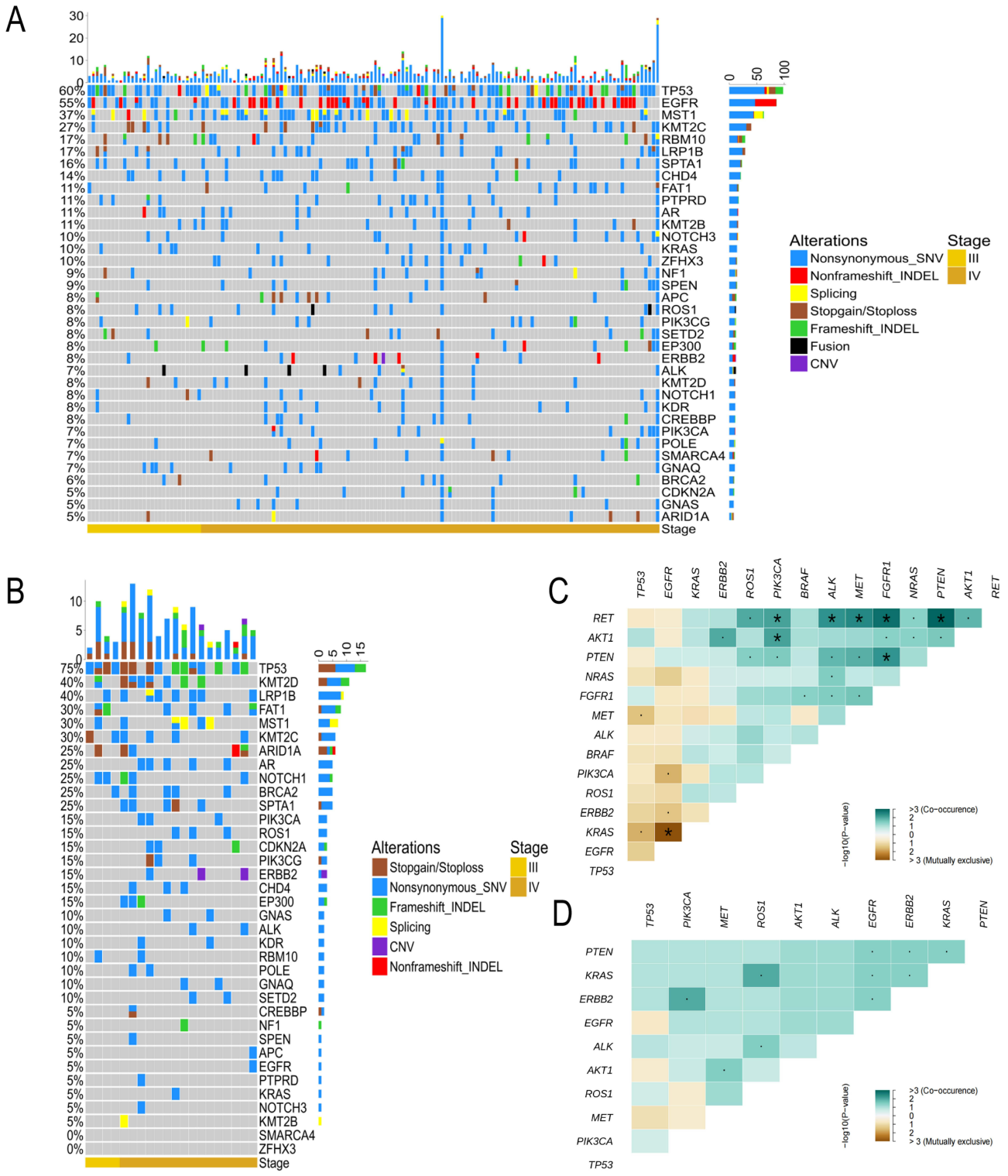

Figure 1 Mutational landscape. (**A**) LUAD mutational landscape. (**B**) LUSC mutational landscape. (**C**) Heatmap of exclusivity and co-occurrence analysis in LUAD. (**D**) Heatmap of exclusivity and co-occurrence analysis in LUSC.(* P < 0.01 and • P < 0.05).

Although not fully understood, KMT2D may function as a tumor suppressor, helping to maintain genome stability.[26] Frequent somatic mutations occur in the FAT1 gene, located at 4q35.2, which encodes a member of the FAT protocadherin family—a group of transmembrane proteins generally expressed in epithelial tissues. As a tumor

suppressor, FAT1 mutations in various cancers suggest it may activate the Wnt pathway, a common mechanism in human cancers.[27] Subunits of the SWI/SNF chromatin remodeling complex, including ARID1A, are implicated as tumor suppressor genes. Mutations in ARID1A are recurrent across many cancers, where its loss affects signaling pathways via dysregulated transcription, potentially activating major pathways that enhance tumor cell proliferation and survival.[28] Lastly, NOTCH proteins, single-pass transmembrane receptors, regulate various oncogenes and tumor suppressors such as c-myc, PI3K, EGFR, PTEN, and TP53. Disruptions in NOTCH can contribute to tumorigenesis by both overexpression and downregulation, as observed across several human cancers.[29]

Comparisons of the LUAD Group and the LUSC Group

In our tumor sample analysis, the mutation frequencies in the LUAD group and the LUSC group exhibited a statistically significant difference ($p<2.2e-16$, Figure 2A). The LUAD group demonstrated a higher propensity for C > A mutations, while the LUSC group exhibited a higher likelihood of C > G and C > T mutations (Figure 2B). Additionally, the TMB in the LUSC group was significantly higher than that in the LUAD group ($p=0.00021$, Figure 2C). While there was no significant difference in the expression of PD-L1 between LUSC and LUAD, it was relatively high in LUSC ($p=0.13$, Figure 2D).

Analysis of tumor heterogeneity revealed no significant difference in the cancer cell fraction (CCF) value of the mutations ($p=0.21$, Figure 2E). However, the mutant-allele tumor heterogeneity (MATH) value in the LUSC group was higher than that in the LUAD group ($p<2.2e-16$, Figure 2F), indicating greater tumor heterogeneity in the LUSC group.

Clinical Differences Between LUAD and LUSC

In this study, the majority of patients were diagnosed with LUAD, followed by LUSC. Consequently, we assessed the clinical differences between these two groups. In the LUAD group, the gender distribution is fairly balanced, with males comprising 56.16% (82/146) and females 43.84% (64/146). In contrast, the LUSC group is predominantly male, constituting 95% (19/20), with females representing only 5% (1/20) (Figure 3A).

Our findings indicate that patients with LUSC are generally older, with 85% of them being over 60 years old, while the age distribution of patients with LUAD shows relatively less variation (Figure 3B). Additionally, a higher percentage of smokers is observed in the LUSC group, accounting for 65% (13/20), while there are more non-smokers in the LUAD group, constituting 60.9% (89/146) (Figure 3C). Importantly, there is no significant difference in tumor stage between LUAD and LUSC (Figure 3D).

Effect of TMB on LUAD and LUSC Immunotherapy

Firstly, we analyzed the impact of immunotherapy on the survival of LUAD and LUSC. The results revealed that when both LUAD and LUSC underwent immunotherapy, the pathological subtype of NSCLC had no discernible impact on the outcomes of immunotherapy. There was no significant difference in either progression-free survival (PFS) or overall survival (OS) (Figure 4A and B). Despite the significant efficacy of immunotherapy, some patients still exhibited a poor response. Therefore, we compared the effects of TMB≥10 and <10 on LUAD and LUSC.

The results indicated that when TMB <10, LUSC's response to immunotherapy significantly decreased, with an ORR of only 16.7%, whereas LUAD still achieved a 50% ORR. However, when TMB≥10, both LUAD and LUSC showed robust responses to immunotherapy. In such cases, the ORR of LUSC reached 80%, and the ORR of LUAD was 87.5% (Figure 4C and D). Prognostic analysis of immunotherapy patients in LUAD and LUSC revealed that in the LUAD group, the TMB size had a substantial impact. LUAD patients with TMB ≥ 10 exhibited significantly longer PFS (mean PFS: 9.125 vs 3.16) and OS (mean OS: 17.5 vs 6.4) compared to LUAD patients with TMB < 10. There was a notable difference in the survival curves (Figure 4E and F). Similarly, among LUSC patients, those with TMB ≥ 10 also experienced a longer survival time than those with TMB < 10. The average PFS and average OS of patients with TMB ≥ 10 were 7.2 months and 14.8 months, respectively, while for patients with TMB < 10, the average PFS was 6.5 months, and the average OS was 11.9 months (Figure 4G and H).

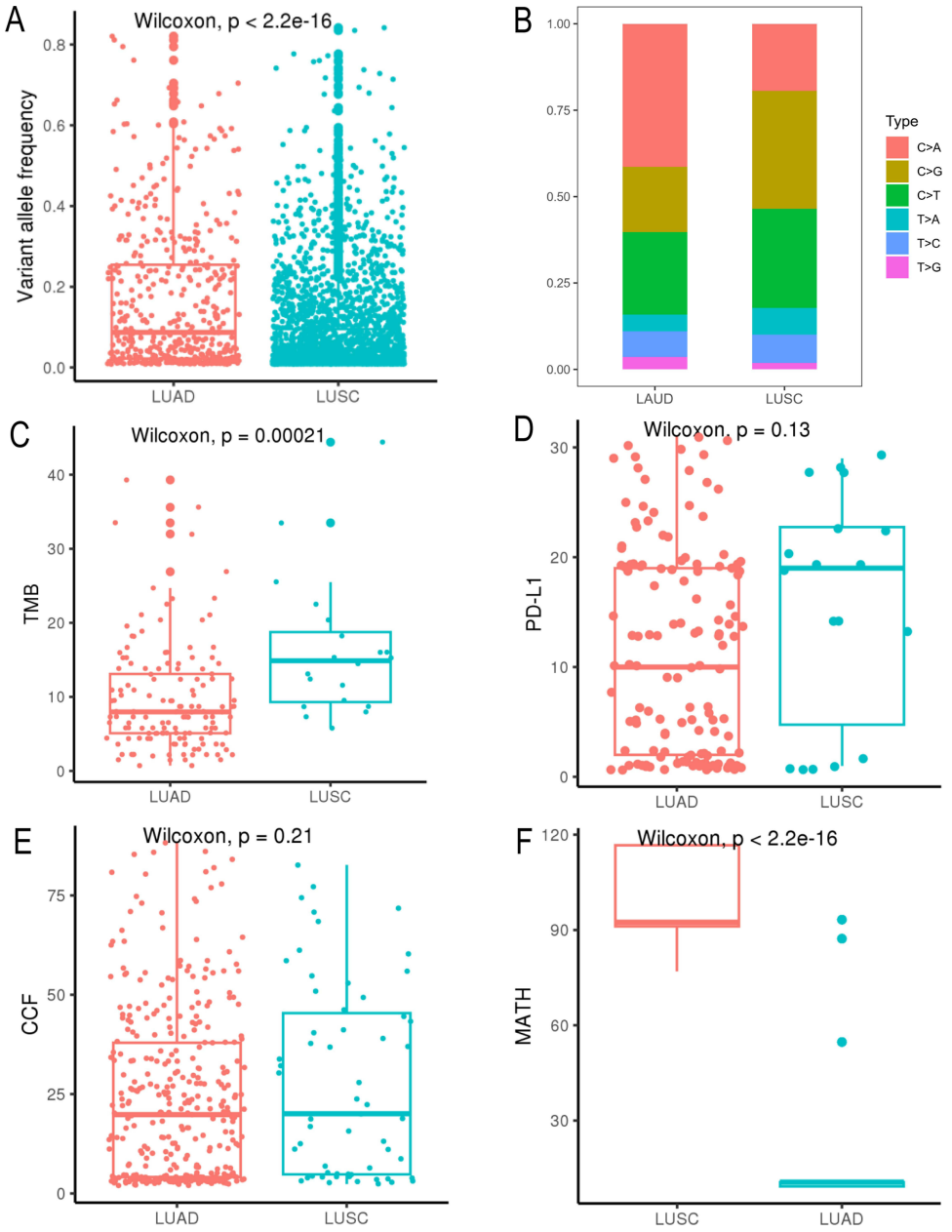

Figure 2 Difference analysis of mutations between LUAD and LUSC. (**A**) LUAD and LUSC mutation frequency analysis. (**B**) Analysis of the difference in base composition of the LUAD and LUSC. (**C**) TMB difference analysis between LUAD and LUSC. (**D**) PD-L1 difference analysis between LUAD and LUSC. (**E**) Analysis of CCF difference between two groups. (**F**) Analysis of the difference between the MATH value of the LUAD group and LUSC group.

Impact of PD-L1 on LUAD and LUSC Immunotherapy

We conducted an analysis of the impact of PD-L1 expression on the efficacy of LUAD and LUSC immunotherapy. The results indicated that the positive (PD-L1≥1%) or negative (PD-L1<1%) expression of PD-L1 had little impact on the efficacy of LUAD immunotherapy, but it did have a certain impact on LUSC (Figure 5A). Furthermore, irrespective of PD-L1 expression being positive (PD-L1≥1%) or negative (PD-L1 < 1%), the efficacy of LUAD immunotherapy was superior to that of LUSC (Figure 5B).

When considering the influence of high PD-L1 expression (PD-L1≥50%) on immunotherapy in LUAD and LUSC, it was observed that patients with PD-L1≥50%, whether in LUAD or LUSC, exhibited a better response to immunotherapy compared to patients with PD-L1<50% (Figure 5C). Simultaneously, we observed that when PD-L1<50%, immunotherapy was less effective in LUSC patients, with an objective response rate (ORR) of only 33.3%, which is lower than that

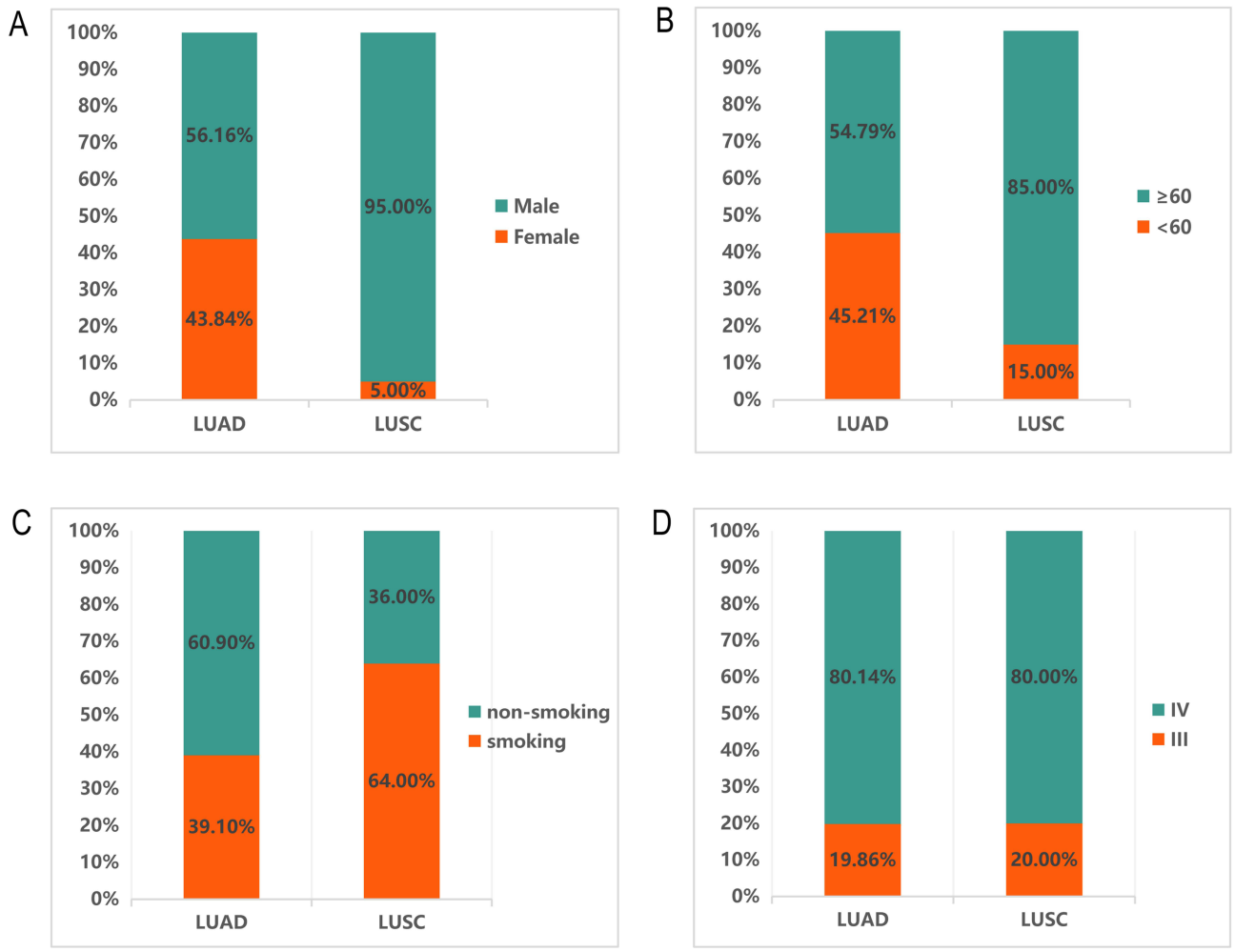

Figure 3 Analysis of clinical differences between LUAD and LUSC. (**A**) Analysis of the proportion of men and women in the LUAD group and LUSC group. (**B**) Age analysis in LUAD group and LUSC group. (**C**) Analysis of smoking status in LUAD group and LUSC group. (**D**) Analysis of tumor satge situation in LUAD group and LUSC group.

of LUAD. However, when PD-L1≥50% in LUSC patients, the efficacy of immunotherapy was significant, with the ORR reaching 100%, surpassing the 80% of LUAD patients (Figure 5D).

Stratifying the survival of LUAD and LUSC according to PD-L1 expression, it was found that when PD-L1 was set at 1% as the cutoff value for patient grouping, the immunotherapy effects of LUAD and LUSC patients could not be clearly distinguished. However, when a stratified analysis was performed with a PD-L1 cutoff value of 50%, it was found that the survival curves of patients with LUAD and LUSC could be differentiated (Figure S2).

NSCLC patients with TMB≥10 and PD-L1≥50% have better outcomes with immunotherapy.

Through the preceding analysis, it was observed that whether it is LUAD or LUSC, immunotherapy can yield positive outcomes when TMB≥10 and PD-L1≥50%. Subsequently, our analysis of NSCLC with TMB≥10 and PD-L1≥50% revealed that all these patients achieved a partial response through immunotherapy, with an objective response rate (ORR) of 100%. In contrast, only 41% of other patients achieved a partial response, resulting in a lower ORR compared to patients with TMB≥10 and PD-L1≥50% (Figure 6A and B).

Survival analysis further indicated that NSCLC patients with TMB≥10 and concurrent PD-L1≥50% had longer PFS and OS than other patients. The mean PFS and OS for patients with TMB ≥10 and concurrent PD-L1≥50% were 9 months and 16.75 months, whereas the mean PFS and OS for other patients were 5.59 months and 11.18 months (Figure 6C and D).

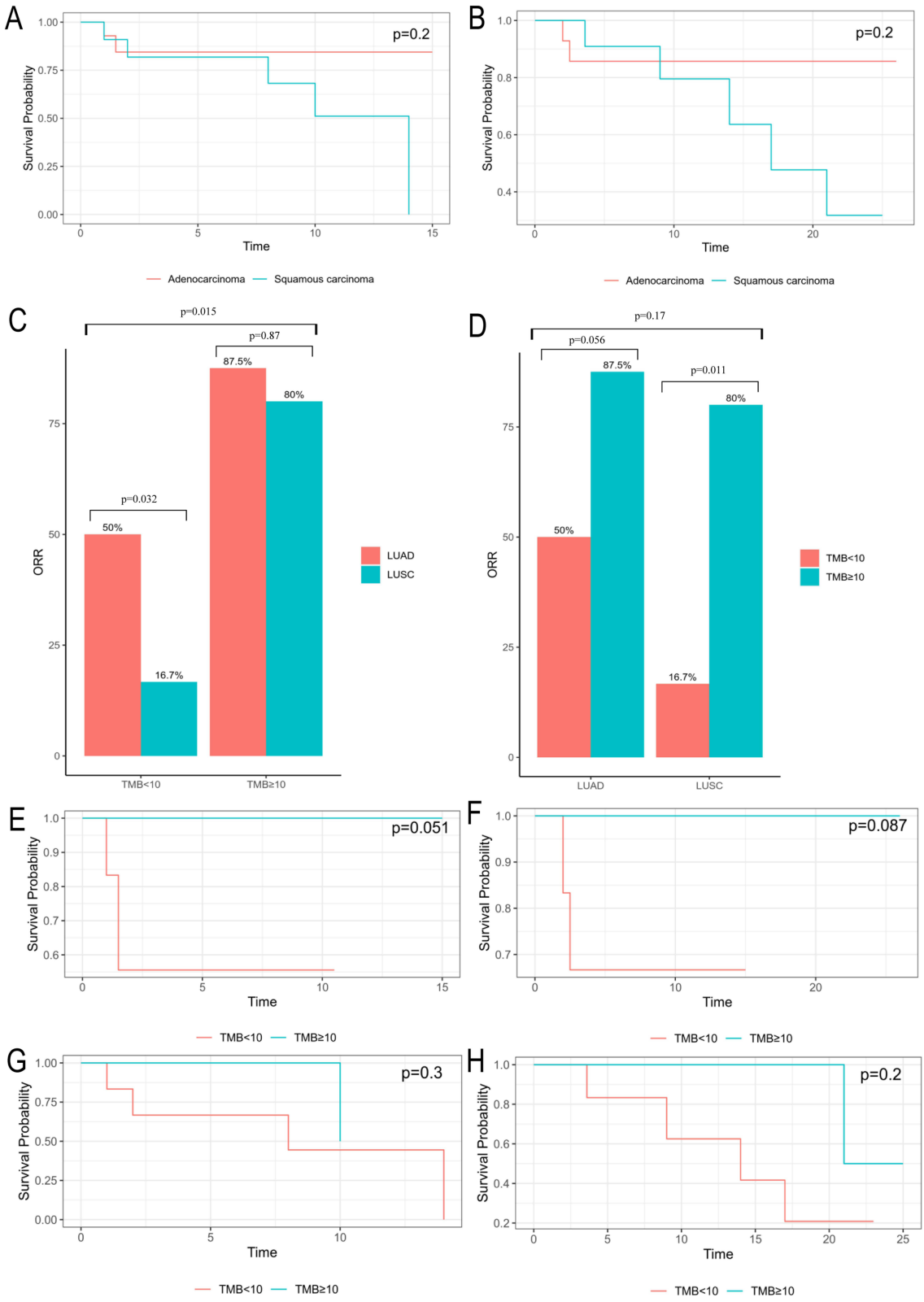

Figure 4 Analysis of the impact of TMB on LUAD and LUSC immunotherapy. (**A**) Analysis PFS of LUAD and LUSC during immunotherapy. (**B**) Analysis OS of LUAD and LUSC during immunotherapy. (**C** and **D**) ORR analysis of TMB≥10 and TMB<10 in patients with LUAD and LUSC. (**E**) PFS analysis of patients with TMB≥10 and TMB<10 in LUAD. (**F**) OS analysis of patients with TMB≥10 and TMB<10 in LUAD. (**G**) PFS analysis of patients with TMB≥10 and TMB<10 in LUSC. (**H**) OS analysis of patients with TMB≥10 and TMB<10 in LUSC.

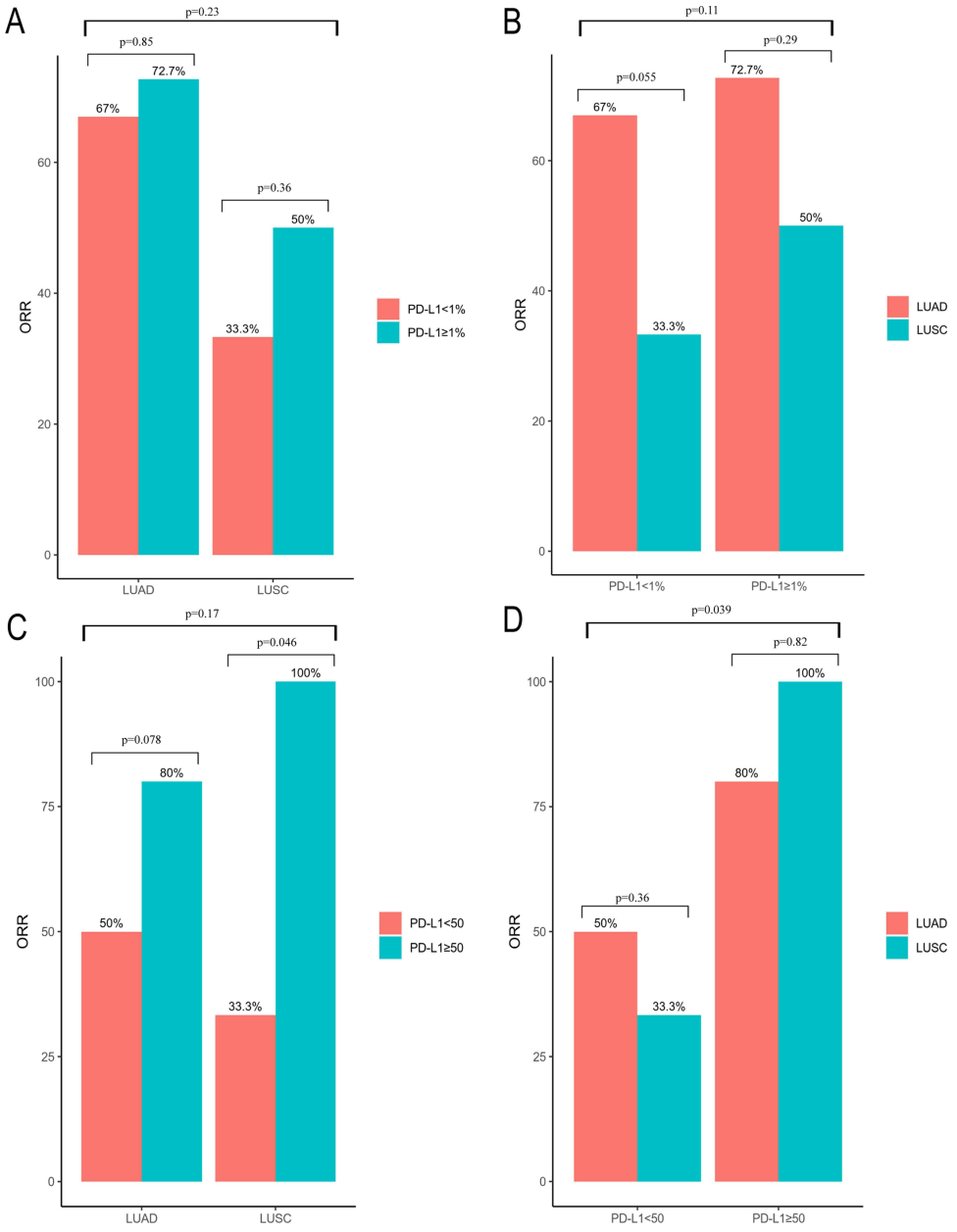

Figure 5 Analysis of the impact of PD-L1 on LUAD and LUSC immunotherapy. ORR analysis of PD-L1≥1 and PD-L1<1 in patients with LUAD and LUSC (**A** and **B**). ORR analysis of PD-L1≥50 and PD-L1<50 in patients with LUAD and LUSC (**C** and **D**).

Discussion

It is widely acknowledged that LUAD and LUSC are not only tumors with distinct histological types but also differ in biological characteristics and clinical significance.[30,31] Previous studies have suggested that LUAD and LUSC may originate from different epithelial cells, express different cellular markers, and exhibit different genomic profiles.[32,33] In our study, we identified TP53 as the most frequently mutated gene in both LUAD and LUSC, with a prevalence of 60% in LUAD and 75% in LUSC. Additionally, we characterized the differences in molecular alterations specific to each tissue type.

In this study, we systematically examined the expression of PD-L1 and TMB to explore their relationship with ICIs and the effect of various biomarker combinations on immunotherapy outcomes. We also evaluated the response of LUAD and LUSC to immunotherapy, comparing the predictive roles of PD-L1 and TMB as ICIs biomarkers in NSCLC within the Chinese population. Additionally, we analyzed the immunotherapy responses of LUAD and LUSC under different stratifications of PD-L1 and TMB. Our findings revealed a modest correlation between PD-L1 and TMB, indicating that

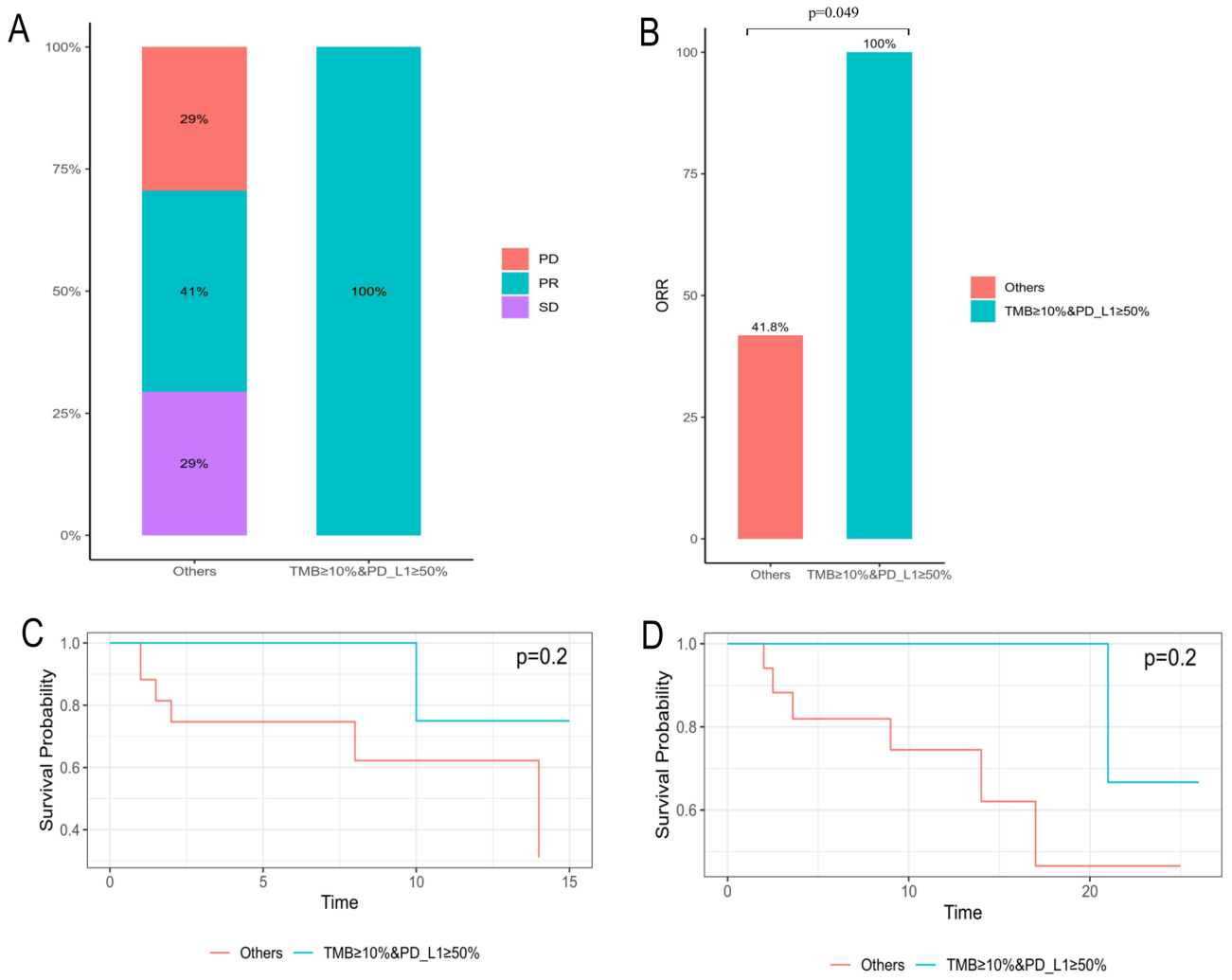

Figure 6 NSCLC patients with TMB≥10 and PD-L1≥50% have better outcomes with immunotherapy. (**A**) Analysis of immunotherapy effects in patients with TMB≥10 and PD-L1≥50% and other patients. (PR: Partial Response SD: StableDisease PD: progressive disease) (**B**) ORR analysis of the patients with TMB≥10 and PD-L1≥50% and other patients. (**C**) PFS analysis of the patients with TMB≥10 and PD-L1≥50% and other patients. (**D**) OS analysis of the patients with TMB≥10 and PD-L1≥50% and other patients.

each biomarker can guide NSCLC immunotherapy individually. However, their combination provides a stronger predictive value for treatment outcomes. For both LUAD and LUSC, patients with TMB ≥ 10 and PD-L1 ≥ 50% exhibited favorable responses to immunotherapy.

Previous studies have also confirmed the role of PD-L1 and TMB as guiding biomarkers for the efficacy of immunotherapy in NSCLC. For example, Hellmann et al demonstrated that nivolumab combined with ipilimumab showed promising therapeutic effects in lung cancer with high tumor mutation burden.[34] Shi et al integrated the analysis of genomic mutations, TMB, and PD-L1 expression to evaluate the role of biomarkers in lung cancer treatment.[35] Additionally, Chen et al examined the unique distribution of PD-L1 expression in East Asian NSCLC and its impact on immunotherapy outcomes.[36] However, most prior studies have focused on a single biomarker, with few combining PD-L1 and TMB for analysis across NSCLC subtypes.

The relationship between PD-L1 and TMB has been debated in the literature, with some studies reporting no correlation.[37] However, several recent studies have shown a small but positive association between PD-L1 and TMB in NSCLC and other cancers.[12] In certain tumor types responsive to ICIs, TMB and PD-L1 expression have shown independence. Our study reflects similar findings, demonstrating that both PD-L1 and TMB have a significant and complementary guiding role in immunotherapy.

In LUAD, the most altered genes (excluding TP53) were EGFR (55%), MST1 (37%), KMT2C (27%), RBM10 (17%), LRP1B (17%), SPTA1 (16%), and CHD4 (14%). In the LUSC dataset, the most altered genes were KMT2D (40%), LRP1B (40%), FAT1 (30%), MST1 (30%), and KMT2C (30%). These findings highlight histological type-

specific mutated gene patterns, revealing differences in common versus mutually exclusive mutations in LUAD and LUSC within these two datasets.

Mounting evidence indicates that, alongside traditional chemotherapy and gene-targeted therapy, immunotherapy holds promise in enhancing both survival and quality of life for patients with LUAD or LUSC. Regarding TMB, the effectiveness of immunotherapy across different cancers can be anticipated by establishing a threshold value for TMB. In recent years, TMB has garnered substantial attention as a novel biomarker quantifying the number of mutations within a tumor.[38] As a prospective biomarker in oncology, TMB holds potential applications in clinical immunotherapy.[39] Establishing cancer-specific TMB thresholds that reliably predict treatment responses across various cancers could contribute to advancing immuno-oncology within the realm of precision medicine.[40] It is presumed that tumors with a higher TMB possess an increased number of neoantigens, which are effectively recognized by the immune system in response to immune checkpoint inhibition.[41] TMB was evaluated in the Checkmate 026 clinical study involving patients with metastatic NSCLC who underwent either nivolumab treatment or platinum-doublet chemotherapy as their initial therapy. Those with elevated TMB demonstrated superior response rates and enhanced progression-free survival compared to those receiving nivolumab monotherapy (9.7 months vs 5.8 months).[42] In a separate study, outcomes from whole-exome sequencing revealed that NSCLC patients undergoing pembrolizumab treatment exhibited a heightened durable clinical benefit and an overall increased burden of somatic nonsynonymous mutations, correlating with a higher remission rate.[43] In 2019, Chen et al discovered that the PD-L1 positivity rate in LUSC is notably higher than that observed in LUAD.[36] This implies that, in LUAD, patients were more inclined to exhibit PD-L1 negativity in contrast to those with LUSC, a trend consistent with our study findings. Additionally, our investigation revealed higher PD-L1 expression in LUSC. Research has demonstrated that patients with positive PD-L1 expression, especially those with PD-L1≥50%, experience enhanced immune efficacy and are more responsive to immunotherapy.[44] Moreover, in the majority of tumor types, there is a relative independence observed between PD-L1 expression and TMB. This suggests that each biomarker can independently offer valuable guidance for ICIs therapy.[45,46] In our investigation, it was observed that when TMB was elevated, both LUAD and LUSC exhibited a higher ORR compared to patients with low TMB. Simultaneously, individuals in the high TMB group demonstrated prolonged PFS and OS compared to those in the low TMB group. Similarly, within the high PD-L1 expression group (PD-L1≥50%), both LUAD and LUSC patient cohorts displayed an increased ORR. When contrasted with patients expressing low levels of PD-L1, those with high PD-L1 expression exhibited a significantly improved ORR.

While PD-L1 expression and TMB demonstrated a degree of independence, patients exhibiting both high TMB and high PD-L1 expression consistently achieved the most favorable outcomes. Conversely, individuals with low TMB and low PD-L1 expression experienced the least favorable outcomes.[47] Hence, the combination of PD-L1 expression and TMB proves to be a more effective predictor of ICIs efficacy. Our dataset revealed that when patients satisfy both TMB≥10 and PD-L1≥50% criteria, immunotherapy exhibits the most favorable outcomes, achieving a 100% Partial Response (PR) and an Overall Response Rate (ORR) of 100%. In contrast, only 41% of other patients achieved PR. Additionally, patients meeting the TMB≥10 and PD-L1≥50 % criteria demonstrated prolonged PFS and OS compared to their counterparts. Therefore, the combined assessment of PD-L1 expression and TMB offers superior predictive capabilities for immunotherapy outcomes, with these patients experiencing enhanced immunotherapy responses and longer survival durations.

While our study provides valuable insights, it is important to acknowledge certain limitations. Firstly, in the survival analysis, the observed differences are not statistically significant. Although variations exist in both PFS and OS among different groups, the p-values exceed 0.05, indicating a lack of statistical significance. This could be attributed to our relatively small sample size. In our survival analysis, when comparing the PFS and OS across different subgroups, we found that the p-values for some results were not significant. However, this does not imply that the findings are meaningless. For instance, in LUAD samples, patients with TMB≥10 exhibited significantly longer PFS (mean PFS: 9.125 vs 3.16 months) and OS (mean OS: 17.5 vs 6.4 months) compared to those with TMB < 10, with notable differences in the survival curves (Figure 4E and F). The PFS was extended by nearly 6 months, and the OS was extended by 10 months, which has important clinical implications. The lack of statistical significance can be attributed to several factors. First, the small sample size may limit the statistical power, preventing the detection of significant differences even if they exist. Increasing the sample size in future studies would help improve the ability to detect meaningful differences. Second, the fine subdivision of subgroups may also dilute the statistical significance. Therefore, future research should aim to include a larger sample size and explore these findings further. For instance, the group meeting both TMB ≥ 10 and PD-L1 ≥ 50% criteria comprises only 8 patients, potentially influencing the results. Another limitation lies in the grouping process,

where some groups have fewer participants, leading to imbalances that may impact the outcomes. While the conclusions drawn from our current data remain accurate, incorporating a larger number of patients in future analyses will strengthen and refine our perspectives.

With the advancement of science and technology, alongside continuous progress in medicine, additional ICIs biomarkers have emerged beyond PD-L1 and TMB. The efficacy of immune checkpoint inhibitors is influenced by a combination of factors, including tumor genomics, host germline genetics, PD-L1 levels, characteristics of the tumor microenvironment, and even the intestinal microbiome. One of the most studied biomarkers is microsatellite instability (MSI), which has been shown to be highly predictive of ICIs responsiveness. Tumors exhibiting mismatch repair deficiency (MMRd) and MSI are particularly sensitive to ICIs therapy, regardless of their tissue of origin.[48] Additionally, the tumor microenvironment has emerged as a promising area for immunotherapy biomarker research, with numerous studies underway to explore its potential.[49] It is believed that ICIs therapy, particularly anti-PD1 and anti-PD-L1 treatments, works in part by restoring pre-existing immune responses within the tumor. Consequently, tumor-infiltrating lymphocyte (TIL) density is another potential predictor of ICI response.[50] Other promising ICIs biomarkers include copy number variations, HLA class I diversity, T-cell inflamed microenvironments, and STK11 mutations.[51] However, further research is needed to better understand and validate these biomarkers in clinical settings.

Conclusion

In summary, our study reveals distinct mutation patterns between LUAD and LUSC, encompassing differences in mutation types, frequencies, and immunotherapy-related markers. Additionally, clinical insights indicate that LUSC is characterized by a higher proportion of male, older, and smoking patients, suggesting potential influences on the pathological subtypes of NSCLC. Notably, TMB≥10 and PD-L1 ≥50% emerge as promising immunotherapy markers. Across both LUAD and LUSC, patients with TMB ≥ 10 and/or PD-L1 ≥ 50% exhibit enhanced immunotherapy responses. Moreover, the simultaneous presence of TMB ≥ 10 and PD-L1 ≥ 50% yields the most favorable outcomes, resulting in a 100% ORR with all patients achieving partial remission. Of course, there are also many new ICIs biomarkers are researching and developing, such as MSI, immune micro-environment, Copy Number Variations, HLA Class I Diversity, T-Cell Inflamed Microenvalits, and Stk11 Mutations, etc. NSCLC immunotherapy biomarkers need more attention and exploration, and NSCLC immunotherapy will become more and more effective.

Data Sharing Statement

The datasets generated and analyzed during this study are not publicly available due to privacy restrictions of ethical review bodies but are available from the corresponding author upon reasonable request. If related data is needed, please contact the corresponding author.

Ethics Approval and Consent to Participate

This study have been approved by the Ethics Committee of Cancer Hospital Affiliated to Xinjiang Medical University, and this study was conducted in accordance with the Declaration of Helsinki. The study was carried out in accordance with the applicable guidelines and regulations. We have protected the privacy of patients, and all patients' data were anonymized and maintained with confidentiality. Informed consent of participants was waived by the ethics committee.

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We thank all the patients for consenting to participate.Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Sung H, Ferlay J, Siegel R, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca a Cancer J Clinicians*. 2021;71(3):209–249. doi:10.3322/caac.21660
2. Zheng M. Classification and Pathology of Lung Cancer. *Surg Oncol Clin North Am*. 2016;25(3):447–468. doi:10.1016/j.soc.2016.02.003
3. Wang Y, Gorlova O, Gorlov I, et al. Association analysis of driver gene-related genetic variants identified novel lung cancer susceptibility loci with 20,871 lung cancer cases and 15,971 controls. *Cancer Epidemiol Biomarkers Prevent*. 2020;29(7):1423–1429. doi:10.1158/1055-9965.EPI-19-1085
4. Mao Y, Yang D, He J, Krasna M. Epidemiology of lung cancer. *Surg Oncol Clin North Am*. 2016;25(3):439–445. doi:10.1016/j.soc.2016.02.001
5. Bersanelli M, Tiseo M, Banna G. Nivolumab plus ipilimumab in non-small-cell lung cancer. *New Engl J Med*. 2020;382(9):874–875.
6. Nagasaka M, Sexton R, Alhasan R, Rahman S, Azmi A, Sukari A. Gut microbiome and response to checkpoint inhibitors in non-small cell lung cancer-A review. *Crit rev oncol/hematol*. 2020;145:102841. doi:10.1016/j.critrevonc.2019.102841
7. Dercle L, Fronheiser M, Rizvi N, et al. Baseline radiomic signature to estimate overall survival in patients with NSCLC. *J Thorac Oncol*. 2023;18(5):587–598. doi:10.1016/j.jtho.2022.12.019
8. Brahmer J, Lee J, Ciuleanu T, et al. Five-year survival outcomes with nivolumab plus ipilimumab versus chemotherapy as first-line treatment for metastatic non-small-cell lung cancer in checkmate 227. *J Clin Oncol*. 2023;41(6):1200–1212. doi:10.1200/JCO.22.01503
9. Nishino M, Ramaiya N, Hatabu H, Hodi F. Monitoring immune-checkpoint blockade: response evaluation and biomarker development. *Nat Rev Clin Oncol*. 2017;14(11):655–668. doi:10.1038/nrclinonc.2017.88
10. Hong L, Negrao M, Dibaj S, et al. Programmed death-ligand 1 heterogeneity and its impact on benefit from immune checkpoint inhibitors in NSCLC. *J Thorac Oncol*. 2020;15(9):1449–1459. doi:10.1016/j.jtho.2020.04.026
11. Anagnostou V, Bardelli A, Chan T, Turajlic S. The status of tumor mutational burden and immunotherapy. *Nat Cancer*. 2022;3(6):652–656. doi:10.1038/s43018-022-00382-1
12. Yarchoan M, Albacker L, Hopkins A, et al. PD-L1 expression and tumor mutational burden are independent biomarkers in most cancers. *JCI Insight*. 2019;4(6). doi:10.1172/jci.insight.126908.
13. Westcott P, Muyas F, Hauck H, et al. Mismatch repair deficiency is not sufficient to elicit tumor immunogenicity. *Nature Genet*. 2023;55(10):1686–1695. doi:10.1038/s41588-023-01499-4
14. Travis WD, Brambilla E, Nicholson AG, et al. The 2015 world health organization classification of lung tumors: impact of genetic, clinical and radiologic advances since the 2004 classification. *J Thorac Oncol*. 2015;10(9):1243–1260. doi:10.1097/JTO.0000000000000630
15. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *ArXiv*. 2013;1303.
16. Koboldt DC, Zhang Q, Larson DE, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res*. 2012;22(3):568–576. doi:10.1101/gr.129684.111
17. Newman AM, Bratman SV, Stehr H, et al. FACTERA: a practical method for the discovery of genomic rearrangements at breakpoint resolution. *Bioinformatics (Oxford, England)*. 2014;30(23):3390–3393. doi:10.1093/bioinformatics/btu549
18. Chalmers ZR, Connelly CF, Fabrizio D, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Med*. 2017;9(1):34. doi:10.1186/s13073-017-0424-2
19. Peuget S, Zhou X, Selivanova G. Translating p53-based therapies for cancer into the clinic. *Nat Rev Cancer*. 2024;24(3):192–215. doi:10.1038/s41568-023-00658-3
20. Senft D. Developing multiple EGFR-mutant lung cancers. *Nat Rev Cancer*. 2024;24(12):826. doi:10.1038/s41568-024-00773-9
21. Cuevas-Navarro A, Pourfarjam Y, Hu F, et al. Pharmacologic restoration of GTP hydrolysis by mutant RAS. *Nature*. 2024. doi:10.1038/s41586-024-08283-2
22. Chen X, Zhang G, Chen B, et al. Association between histone lysine methyltransferase KMT2C mutation and clinicopathological factors in breast cancer. *Biomed Pharmacothe*. 2019;116:108997. doi:10.1016/j.biopha.2019.108997
23. Wang E, Pineda J, Kim W, et al. Modulation of RNA splicing enhances response to BCL2 inhibition in leukemia. *Cancer Cell*. 2023;41(1):164–180.e168. doi:10.1016/j.ccell.2022.12.002
24. Zhang Y, Ma Z, Li C, et al. The genomic landscape of cholangiocarcinoma reveals the disruption of post-transcriptional modifiers. *Nat Commun*. 2022;13(1):3061. doi:10.1038/s41467-022-30708-7
25. Froimchuk E, Jang Y, Ge K. Histone H3 lysine 4 methyltransferase KMT2D. *Gene*. 2017;627:337–342. doi:10.1016/j.gene.2017.06.056
26. Kandoth C, McLellan M, Vandin F, et al. Mutational landscape and significance across 12 major cancer types. *Nature*. 2013;502(7471):333–339.
27. Morris L, Kaufman A, Gong Y, et al. Recurrent somatic mutation of FAT1 in multiple human cancers leads to aberrant Wnt activation. *Nature Genet*. 2013;45(3):253–261. doi:10.1038/ng.2538
28. Wiegand K, Lee A, Al-Agha O, et al. Loss of BAF250a (ARID1A) is frequent in high-grade endometrial carcinomas. *J Pathol*. 2011;224(3):328–333. doi:10.1002/path.2911
29. Weng A, Ferrando A, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*. 2004;306(5694):269–271.

30. Herbst R, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. *Nature*. 2018;553(7689):446–454.
31. Relli V, Trerotola M, Guerra E, Alberti S. Abandoning the Notion of Non-Small Cell Lung Cancer. *Trends Mol Med*. 2019;25(7):585–594. doi:10.1016/j.molmed.2019.04.012
32. Campbell J, Alexandrov A, Kim J, et al. Distinct patterns of somatic genome alterations in lung adenocarcinomas and squamous cell carcinomas. *Nature Genet*. 2016;48(6):607–616. doi:10.1038/ng.3564
33. Anusewicz D, Orzechowska M, Bednarek A. Lung squamous cell carcinoma and lung adenocarcinoma differential gene expression regulation through pathways of Notch, Hedgehog, Wnt, and ErbB signalling. *Sci Rep*. 2020;10(1):21128. doi:10.1038/s41598-020-77284-8
34. Hellmann M, Ciuleanu T, Pluzanski A, et al. Nivolumab plus Ipilimumab in Lung Cancer with a High Tumor Mutational Burden. *New Engl J Med*. 2018;378(22):2093–2104. doi:10.1056/NEJMoa1801946
35. Shi Y, Lei Y, Liu L, et al. Integration of comprehensive genomic profiling, tumor mutational burden, and PD-L1 expression to identify novel biomarkers of immunotherapy in non-small cell lung cancer. *Cancer Med*. 2021;10(7):2216–2231. doi:10.1002/cam4.3649
36. Chen Q, Fu Y, Yue Q, et al. Distribution of PD-L1 expression and its relationship with clinicopathological variables: an audit from 1071 cases of surgically resected non-small cell lung cancer. *Int J Clin Exp Pathol*. 2019;12(3):774–786.
37. Hellmann M, Nathanson T, Rizvi H, et al. Genomic features of response to combination immunotherapy in patients with advanced non-small-cell lung cancer. *Cancer Cell*. 2018;33(5):843–852.e844. doi:10.1016/j.ccell.2018.03.018
38. Liu L, Bai X, Wang J, et al. Combination of TMB and CNA stratifies prognostic and predictive responses to immunotherapy across metastatic cancer. *Clin Cancer Res*. 2019;25(24):7413–7423. doi:10.1158/1078-0432.CCR-19-0558
39. Hellmann M, Callahan M, Awad M, et al. Tumor mutational burden and efficacy of nivolumab monotherapy and in combination with ipilimumab in small-cell lung cancer. *Cancer Cell*. 2018;33(5):853–861.e854. doi:10.1016/j.ccell.2018.04.001
40. Guo X, Zhang Y, Zheng L, et al. Global characterization of T cells in non-small-cell lung cancer by single-cell sequencing. *Nature Med*. 2018;24(7):978–985. doi:10.1038/s41591-018-0045-3
41. Steuer C, Ramalingam S. Tumor mutation burden: leading immunotherapy to the era of precision medicine? *J Clin Oncol*. 2018;36(7):631–632. doi:10.1200/JCO.2017.76.8770
42. Hellmann M, Paz-Ares L, Bernabe Caro R, et al. Nivolumab plus Ipilimumab in advanced non-small-cell lung cancer. *New Engl J Med*. 2019;381(21):2020–2031. doi:10.1056/NEJMoa1910231
43. Rizvi N, Hellmann M, Snyder A, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science*. 2015;348:6230):124–128. doi:10.1126/science.aaa1348
44. Ohri N, Jolly S, Cooper B, et al. Selective personalized radioimmunotherapy for locally advanced non-small-cell lung cancer trial. *J Clin Oncol*. 2023;JCO2300627.
45. Wu S, Wu S, Liao X, et al. Pembrolizumab combined with anlotinib improves therapeutic efficacy in pulmonary sarcomatoid carcinoma with TMB-H and PD-L1 expression: a case report and literature review. *Front Immunol*. 2023;14:1274937. doi:10.3389/fimmu.2023.1274937
46. Wang L, Yang Z, Guo F, et al. Research progress of biomarkers in the prediction of anti-PD-1/PD-L1 immunotherapeutic efficiency in lung cancer. *Front Immunol*. 2023;14:1227797. doi:10.3389/fimmu.2023.1227797
47. Paz-Ares L, Garassino M, Chen Y, et al. Durvalumab ± tremelimumab + platinum-etoposide in extensive-stage small-cell lung cancer (CASPIAN): outcomes by PD-L1 expression and tissue tumor mutational burden. *Clin Cancer Res*. 2023;30(4).
48. Le D, Durham J, Smith K, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science*. 2017;357(6349):409–413.
49. Vétizou M, Pitt J, Daillère R, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science*. 2015;350(6264):1079–1084. doi:10.1126/science.aad1329
50. Topalian S, Drake C, Pardoll D. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell*. 2015;27(4):450–461. doi:10.1016/j.ccell.2015.03.001
51. Havel J, Chowell D, Chan T. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. *Nat Rev Cancer*. 2019;19(3):133–150. doi:10.1038/s41568-019-0116-x