

Characterization of the Different Subtypes of Immune Cells Infiltration to Aid Immunotherapy

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Abstract

Background: PD-1 ablation or PD-L1 specific monoclonal antibody (mAb) against PD-1 can recruit the accumulation of functional T cells, leading to tumor rejection in microenvironment and significantly improving the prognosis of various cancers. Despite these unprecedented clinical successes, intervention remission rates remain low after treatment, rarely exceeding 40%. The observation of PD-1/L1 blocking in patients is undoubtedly multifactorial, but the infiltrating degree of CD8+T cell may be an important factor for immunotherapeutic resistance.

Method: We proposed two computational algorithms to reveal the immune cell infiltration (ICI) landscape of 1646 lung adenocarcinoma patients. Three ICI patterns were defined and the relative ICI scoring were depended on principal-component analysis.

Result: A high ICI score was associated with the increased tumor mutation burden (TMB) and cell proliferation-related signaling pathways. Different cellular signaling pathways were observed in low ICI score subtypes, indicating cell proliferation active, and may be associated with poor prognosis.

Conclusion: Our research identified that the ICI scores worked as an effective immunotherapy index, which may provide a promising therapeutic strategies on immune therapeutic for lung adenocarcinoma.

Introduction

Lung cancer is the leading factor of death and causes about 1.6 million people death¹. On the basis of histological types of tumor tissues, lung cancer can be classified as small cell lung cancer (SLC) and non-small cell lung cancer (NSCLC), while NSCLC accounts the about 87% in total lung cancer patients².

Among these lung cancer, the

5-year overall survival rate of NSCLS is only about 27% and most of NSCLC is diagnosed at the advanced stage, which also leads the poor survival rate.³ To extend the overall survival of NSCLC patients, many advanced diagnosis and treatment methods have been introduced in clinical interventions, including the targeted molecular therapy⁴⁻⁵, immunotherapy⁶⁻⁷. To precisely provide the therapeutic interventions, the precise molecular classification and diagnosis can significantly improve the overall survival of NSCLC patients, for example Osimertinib for EGFR T790M patient therapy⁸.

Recently, many investigations have proved that the immune evasion is deeply affected the immune therapy⁹⁻¹⁰. However, expansive landscapes of immune cell infiltration and the microenvironment (TME) of LUAD has not been well investigated. Thus, it is crucial to identify the roles of various TME in LUAD to conduct the individual immune therapies. The over-activated inflammatory pathways plays an crucial hallmark in the tumorigenesis, which involved into various of cancer-related oncogenic pathways¹¹. These heterogeneities of cancers lead the failure of therapy and suggested that the individual immunotherapy strategies may be helpful to improve the overall survival of cancer patients. In the tumorigenesis stage, the immune surveillance mechanisms have been well invalidated, and indicated that

the immunotherapies can lead the immune response for improving the anticancer therapy¹². To perform these therapeutic strategies, various types of immunotherapeutic approaches have been developed, for example vaccine therapy¹³, chimeric antigen receptor (CAR) T cells¹⁴, programmed cell death 1 (PD-1) & programmed cell death ligand-1 (PD-L1)¹⁵⁻¹⁷.

Despite these unprecedented clinical successes in anticancer, the immunotherapy remission rates of various types of cancers still remain low. Meanwhile, the immune tolerance is obtained after receiving the immunotherapy, which is owing to cross-talks between tumorigenesis and immune-response¹⁸. In the past decades, next-generation sequencing (NGS) have promoted the development of biomedical field and obtained the great achievement¹⁹. Moreover, the NGS algorithm technology also revealed the huge biological information about cancer tissue²⁰. To identify the percentage of immune cell infiltration, two algorithm have been well introduced, which are CIBERSORT²¹ and ESTIMATE²², respectively. By independently utilizing these algorithms, different combinations of biomarkers can be well-related with the prognosis of cancer patients²²⁻²³.

In this research, we utilized the both of CIBERSORT and ESTIMATE to analyze the gene expression information of tumor tissues, including the immune cell infiltration and spatial distribution, so-called intra-tumoral immune landscape. Here, we

identified that the LUAD can be divided into three subtypes based on the immune cell-infiltration patterns. Finally, we conducted the correlation analysis between ICI score and PD-1/PD-L1 immunotherapy response or various immune landscapes, which indicated that ICI score could be as the clinical prediction indexes for LUAD patient to receive immunotherapy.

Materials And Methods

Datasets and Samples

A total of 1646 patients mRNA expression with detail survival information was

download from TCGA-LUAD from TCGA database (<https://portal.gdc.cancer.gov/>) and GEO datasets of GSE31210, GSE30219, GSE68465 and GSE72094. Expression values were log-transformed and meanwhile we use the "ComBat" algorithm for reducing the possibility of batch effects due to non-biotech bias between different data sets²⁴.

Consensus Clustering for Tumor-Infiltrating Immune Cells

The gene expression matrix data was uploaded to the CIBERSORT with employing the LM22 signature and set 100 permutations to obtain the immune cell infiltration matrix²⁵. Meanwhile we use the ESTIMATE package to evaluate the immune and stromal score in per LUAD sample²⁶. These samples was stratified and clustered based on the ICI pattern. In the analysis, we use "hc" method of the unsupervised clustering with pearson, and both innerLinkage and finalLinkage was set Ward.D2, executed

by using the "ConsensusClusterPlus" R package²⁷. In order to make the classification steady, the maxK was set 3 and the reps was set 100 times.

DEGs Associated with the ICI Phenotype

Based on the ConsensusClusterPlus analysis, these patients was grouped into three ICI groups. Meanwhile, we use the limma package to identify the differential expression genes (DEGs) in those ICI cluster with significance cutoff criteria $p < 0.05$ (adjusted) and absolute fold-change > 1.5 .

Dimension Reduction and Generation of ICI Score

Basing on the three ICI clusters, we used consensus clustering (parameters: reps:100, pltem:1, pFeature:1; Ward.D2 and euclidean distance, k:4) using expression values of those DEGs to identified the gene clusters, meanwhile in order to reduce the relevant variables and unnecessary noise, we use the Boruta package to reduce dimension and when the DGEs were positive with cluster marked as signatures A and as it were negatively marked signatures B. Then we identified that the principal component 1 as the signature score by using the PCA analysis. Finally, we constructed that an approach which is similar to the gene expression grade index to identify the ICI score of various patient: ICI score = PC1A- PC1B:

Collection of Somatic Alteration Data

To confirm the tumor mutational burden of lung adenocarcinoma, we download the mutation data from cBioPortal (<https://www.cbioportal.org/>; luad_tcga_pan_can_atlas 2018), and evaluated the entire number of non-synonymous mutations in LUAD. In the both high ICI score and low ICI score groups, we used the "maftool" R package to analysis the somatic alterations especially the top 25 driver genes with the highest alteration frequency in lung adenocarcinoma.

Gene Expression Data with Immunotherapy

For further immunotherapy research, we download the IMvigor210 dataset to analyze the value of ICI scores in the PD-1 response predicted, which can be acquired from <http://research-pub.gene.com/IMvigor210CoreBiologies> with completed information about the response to PD-L1 blockade.

Analysis of the validation set

We downloaded a data set including 535 lung adenocarcinoma patients from TCGA database(<https://portal.gdc.cancer.gov/cart>), next used TIDE website³⁷ to predict the cancer immunotherapy response for the 535 patients, and then the 535 lung adenocarcinoma patients were divided into 2 groups according to the TIDE prediction:Response group and Non-response group. Finally, we performed the ICI score analysis for these 2 groups and compared the results with the TIDE results,

Statistical Analyses

We utilized Graphpad prism and R 4.0.0 software to conduct all statistical analyses. The Kruskal-Wallis test and Wilcoxon test was used to compare two groups and more than two groups. In each dataset, survival curves for subgroups were analyzed by using the Kaplan-Meier plotters. Chi-square test was used to analyze the correlation between ICI score subgroup and somatic mutation frequency, and Spearman analysis was used to calculate the correlation coefficient. And when the $p < 0.05$ was considered as statistically significant.

Result

The Landscape of Immuno-cell Infiltration in the TME of LUAD

Firstly, the CIBERSORT and ESTIMATE algorithms were employed to identify the immuno-cell infiltration in LUAD tumor tissues. Based on 1646 tumor samples with matched immune cell infiltration (ICI) profiles from the meta-cohort (Array express database: GSE31210, GSE30219, GSE68465 and GSE72094; The Cancer Genome Atlas TCGA-LUAD), unsupervised clustering of LUAD patients into different subtypes was performed as described in the MATERIAL and METHOD section.

To our knowledge, the immune cell infiltration condition is highly associated with the patients' prognosis. To identify the role of specific immune cells in tumor progression, classification of LUAD based on the immune cell infiltration was employed. Here, there are three ICI clusters were identified with various infiltrated immune cells (Figure 1A). Meantime, we also found that the ICI cluster 2 has a favorable prognosis(Figure 1B). Considering the immune landscape of LUAD (Figure 1A), the infiltration percentage of Dendritic cells resting, Dendritic cells activated, Macrophages M2, Mast cells resting, NK cells activated and T cells CD4 memory resting in the ICI cluster 2 were significantly higher than ICI cluster 1 and 3.

Meanwhile, the landscape of immune cell interaction in TME are pictured in the Figure 1C. We can observe that the correlation between T CD8+ cell and T CD4+ cell memory resting and activated more than other infiltration immune cells. Moreover, we also tested the PD-1 and CLAT4 expression level in each ICI clusters by using the Kruskal-Wallis test, and the results showed that the cluster 3 has higher expression level with significant difference (Figures 1D and 1F). While, we cannot observe any difference between cluster 1 and 2, in which the expression level of PD-1 and CTLA4 is similar.

Identified Immune Gene Subtype

For confirming the underlying biological functions in different immune-phenotypes, we employed the Limma R package to identify the differential expression genes. Owing that the clinical information of TCGA-LUAD cohort is not entire, we mainly use LUAD patients with entire clinical information to further perform the analysis in the following study.

Firstly, we analyzed the aforementioned differentially expressed genes (DEGs) by using the ConsensusClusterPlus package with the unsupervised clustering analysis functions, and these genes can

be classified into three genomic clusters: gene clusters

A, B and C. To reduce the noises of cluster genes, we used the Boruta package to reduce the dimension in both signature A and B. Here, we identified 144 genes, which were positively correlated with the gene cluster A. And, the residual 64 genes were identified as the signature B. All the gene expression in LUAD patients were pictured in Figure 2A mainly based on the genecluster types. To analyze by the expression level, the genecluster A is significantly different with other two types, in which ICI signature gene A is lower and ICI signature B is higher. These results are consistent with the prognosis of LUAD patients based on the genecluster classification (Figure 2B).

Moreover, we also performed the Gene Ontology (GO) analysis to identify the role of differentially expressed genes. As shown in Figure 2C-D, some signal pathways associated with T regulation processes and cytokines secretion were enriched, for example positive regulation of T cell activation and T cell activation. These results indicated that the T cell regulation related pathways played a critical role in promoting the tumorigenesis.

The previous investigations have proved that the immune system may display the different roles with both favorable and adverse outcomes, which is determined by the role of immune cells as the pro-tumor or the anti-tumor as we observed in our previous analysis. As shown in Figure 2E, the gene cluster A exhibited the high B cells memory, Dendritic cells activated, Dendritic cells resting, Macrophages M2, Mast cells resting, Monocytes and T cells CD4 memory resting. These immune cells displayed the higher level of infiltration, and may involve into the improving the therapy. PD-1 is the critical biomarker for immunotherapeutic intervention and is always expressed on activated T, natural killer ad B lymphocytes, macrophages, dendritic cells and monocytes²⁸. In addition, the expression level of PD-1 was significantly different among the three genomic clusters (Kruskal-Wallis, $P < 0.01$; Figure 2F). ICI gene clusters of A and B were associated with lower PD1/PD-1 expression levels, while ICI gene clusters C were associated with higher PD1/PD-L1 expression levels. Our investigation showed that the consistency of immune-profile of different gene groups indicated the different prognostic profile.

Construction of the ICI Score

We used the principal component analysis to count the total score of both the ICI score A and B for getting the appropriate indicators, and this score were regard as the summed indexes of each patient. We utilized the X-tile software to divide the TCGA cohort into the high and low ICI scores, which was showed in the Figure 3A. Here,

we chosen six biomarkers (CD274, CTLA4, HAVCR2, IDO1, LAG3, and PDCD1) as immune-checkpoint-relevant signatures, while other eight biomarkers (CD8A, CXCL10, CXCL9, GZMA, GZMB, IFNG, PRF1, TBX2, and TNF) is treated as immune-activity-related signatures. As showed in Figure 3B, these biomarkers are overexpressed in the high ICI group by using the Wilcoxon test. Then, we used the gene set enrichment analysis (GSEA) to explore the underlying biology functions. The GSEA results indicated that the enriched pathways in high ICI score group was associated with the various cell proliferation signaling

(Figure 3C) and the low ICI score group was attributed to cell cycle pathways (Figure 3D). Meanwhile, the Kaplan-Meier plotter showed that the high ICI score group had much better survival rate than the low group (Figure 3E).

The Correlation between the ICI Scores and Somatic Variants

More evidences have demonstrated that high mutation load (non-synonymous variation) in tumors were associated with increasing infiltration of CD8+ T cells, which showed that the tumor load mutation (TMB) may play an important role in the immunotherapy response. Based on the important role of TMB in clinical diagnosis, we aimed to study the correlation between TMB and ICI score to clarify the genetic imprinting of each ICI subgroup. Thus, we investigated the TMB between the high and low ICI scores, which showed that the high ICI score groups suffered the significantly higher TMB than the low ICI score group (Wilcoxon test $p < 0.001$, Figure 4A). Meanwhile, the correlation results (Figure 4B) showed that the ICI score was positively correlated with the TMB ($R = 0.359$, $P < 0.001$). Further study about TMB in survival was employed, and the result showed that the survival predication of high TMB was better than the low TMB group, to some extent (Figure 4C). In order to consider the association between the TMB and ICI scores, we analyzed the synergistic effect of these scores in prognostic stratification and the result showed that the overall survival of high ICI score (including ICI^{high}-TMB^{high} and ICI^{high}-TMB^{low}) was much better than the low ICI score (including ICI^{low}-TMB^{high} and ICI^{low}-TMB^{low}), and the overall survival of ICI^{high}-TMB^{high} was comparable with that of ICI^{high}-TMB^{low} (Figure 4D), which indicated that high ICI score played a leading role in predicted survival. Meanwhile, the various ICI score groups in different TMB group had significantly survival differences. In conclusion, our study identified that the ICI score may be an important diagnosis index in the predicted survival and evaluates the immunotherapy response.

Moreover, we assessed the driver genes distribution in both high ICI score and low ICI score using the MAFtools. The top 25 genes with highest frequency were pictured in the both high ICI score group and low ICI score group (Figure 4E and F), which those genes were significantly different in both groups. These results provide one new

analysis approach for studying the mechanism of tumor ICI components and gene mutations in immune therapy.

The Role of ICI Scores in the Prediction of Immunotherapeutic Benefits

The immune therapy was applied to improve the tumor therapy outcomes, especially by using PD-1 or PD-L1 specific monoclonal antibody (mAb). However, the remission rate remains lower than expected, rarely exceeding 40 percent. In order to analyze the application of ICI score in the assessment of immune therapy, we use the IMvigor210 cohort, in which these patients received the anti-PD-L1 immunotherapy, to be divided into the high ICI score group and low ICI score group. Then, we assessed the survival rates in

the different ICI score group, and the results showed that the high score had much better survival than the low score group (Figure 5B). The objective response rate of anti-PD-L1 therapy was higher in the high ICI score group than in the low ICI group (Figure 5A). We also found that higher ICI scores are correlated with objective response to anti-PD-L1 therapy (Figure 5C).

We also performed a validation set, and we used TIDE website³⁷ to predict the cancer immunotherapy response for the 535 patients from TCGA database, and our ICI score is consistent with TIDE index from the aspect of predictive results (Figure 5D), which can be an effective immunotherapy index for identifying the suitable candidates to receive the immunotherapy.

Discussion

The cancer immunotherapy of PD-1/PD-L1 have significantly improved the NSCLC therapy²⁹. However, the loss of predictive biomarkers has limited the further clinical application in precise medicine³⁰⁻³¹. Aimed to resolve these problems in immunotherapy, how to build one method for identifying the suitable LUAD patients to further receive PD-1/PD-L1 therapy. In this investigation, we established a methodology to evaluate the tumor immune cell infiltration in LUAD. To our knowledge, the patient survival should be associated with the gene expression of tumor tissues¹¹. However, to simply attribute the patients to specific subtypes, e.g. oncogenic genes, did not significantly improve the therapeutic benefits. As a result, more precise attribution may be more useful for applying the patient-specific tailored therapy.

Firstly, the landscape of immune cell infiltration in LUAD was established. In this attribution, the immunological status of tumor can be well-identified. Based on the overall survival, these patients can be divided into three subtypes and patients in ICI Cluster 2 has a favorable prognosis. By analyzing the infiltration of immune cells, we found that the infiltration percentage of several immunological cells in ICI cluster 2 is higher than other groups, including Dendritic cells resting, Dendritic cells activated, Macrophages M2, Mast cells resting, NK cells activated and T cells CD4 memory resting. Moreover, the immunoscore and stromalscore is also higher. Considering the favorable prognosis of ICI Cluster 2, these results indicates that the overall survival of

LUAD patients may be benefited from the higher immune cell infiltration and implied that the immunological status of these tumors are “hot”. The “hot” tumor tissue may be more sensitive for immune-therapy³².

Then, we also fetched the immune-related genes expression on the basis of aforementioned clusters. Difference expression genes (DEGs) were further utilized for characterizing the expression condition and classified as two subtypes, ICI signature genes A (144 DEGs) and B (64 DEGs). Interestingly, the gene profiles of these DEGs displayed the difference based on the classification of ICI signature. And the expression levels of ICI signature gene A in ICI gene cluster B and C displayed the higher ICI signature score and immune cell infiltration. While the gene cluster A exhibits the higher ICI signature gene B. Consistent with these findings, the patients in gene cluster A exhibits the better prognosis than gene

cluster B and C. The anti-cancer immune response in gene cluster A is associated with favorable prognosis and we believed that the patients in gene cluster A may benefit from the immunotherapy. In order to identify the role of higher immune cell infiltration in improving the patient survival, the GSEA analysis was performed and the results showed that the immunological response-related pathways have been well activated, such as pathway related to positive regulation of cytokine production³³⁻³⁴, regulation of immune effector process³⁵, leukocyte proliferation³⁶ etc. And, the relative biomarker expression of cytokines also confirmed this result. These results demonstrated that the immune cell infiltration have significantly involved into the immune response.

Previous studies suggested high mutation load in tumors were associated with increasing infiltration of CD8+ T cells. We also explore the immunological infiltration on genome stability, we also tested the TMB frequency based on the ICI score. By the analysis, the higher ICI score indicated the higher tumor mutation burden and meant the worse prognosis but high ICI score but not high TMB played a leading role in predicted survival. Recent study claimed that High tumor mutation burden fails to predict immune checkpoint blockade response across all cancer types³⁸, which supported our conclusion.

To identify the correlation between ICI score and immunological response, we also tested the association between anti-PD-L1 response and ICI score. The higher ICI score of LUAD patients suffered the better responder. We can find that the response rate of anti-PD-L1 therapy in high ICI score is much higher than low ICI group, confirming that the ICI scoring can be as the predictive indexes for LUAD before receiving the PD-1/PD-L1 therapy.

There is a limitation in this study, and this study only used public data for analysis, and did not involve in vivo and in vitro experimental verification. The experimental verification are required in the future to promote our understanding about the landscape of immune cell infiltration and tumor immune response .

In conclusion, we have analyzed the landscape of immune cell infiltration and established one approach for evaluating immune response condition of LUAD. The

higher ICI score was identified to be correlated with tumor immune response and this evaluating tool can be as the ideal index for identify the suitable candidates to receive the immunotherapy.

Declarations

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CONFLICTS OF INTEREST

The authors declare no competing interests.

Data Availability Statement

Publicly available datasets were analyzed in this study. This data can be found here: <https://portal.gdc.cancer.gov/>; <https://www.cbioportal.org/>; <https://cistrome.shinyapps.io/timer/>.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors approved for publication.

Competing interests

The authors have no conflicts of interest to declare.

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Author Contributions

ZL conceived and designed the study, obtained funding, and drafted the manuscript. ZL and KM acquired the data and drafted the manuscript. ZL critically revised the manuscript. KM and ZL performed statistical analysis and technical support. All authors contributed to the article and approved the submitted version.

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Figures

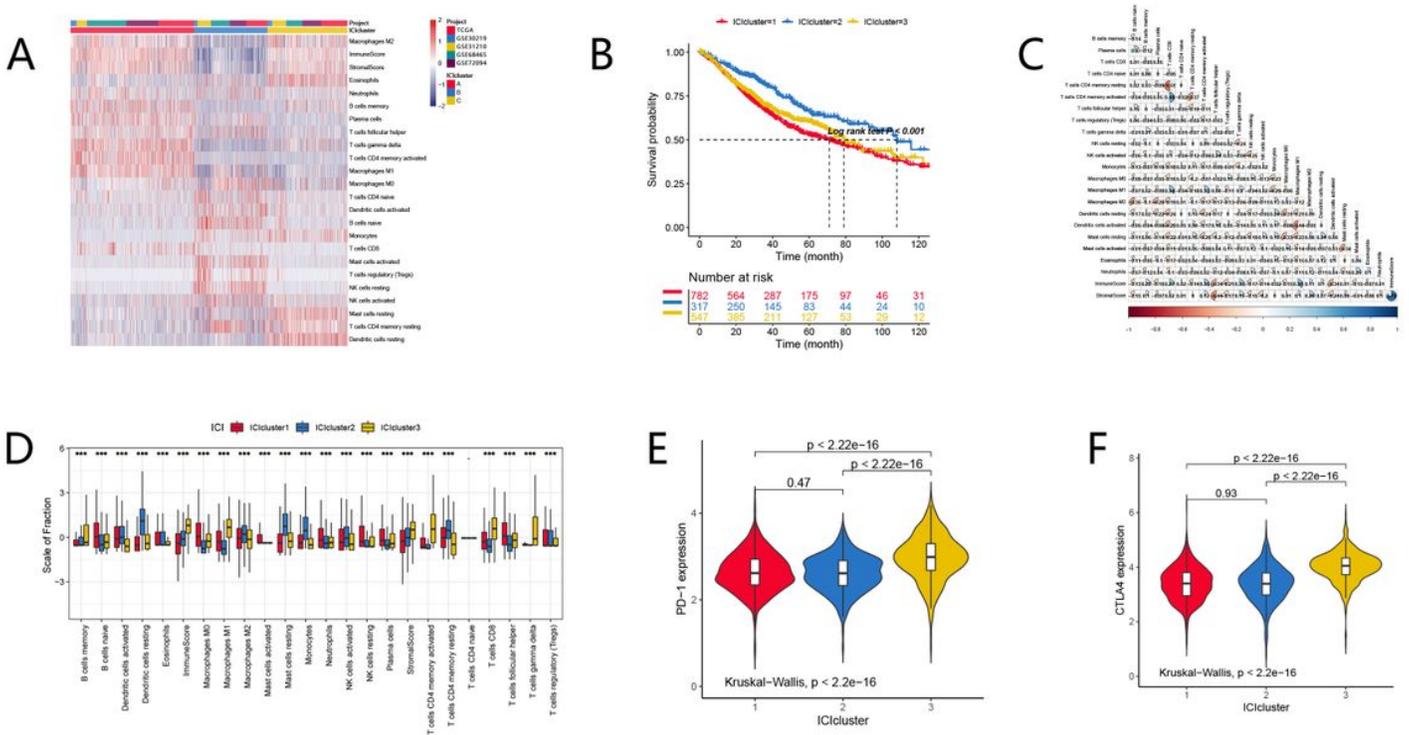


Figure 1

(A):The landscape immune cell infiltration in unsupervised clustering analysis with 1646 samples from the data sets described in the methods . (B):Survival curves in three clusters of LUAD patients with different immune cell infiltration classes($P < 0.001$). (C):The correlation in different immune cell in the tumor patients . (D): The distribution of tumor-infiltrating immune cells in three ICI clusters.(E): PD-1 expression level of immune cells in different ICI clusters.

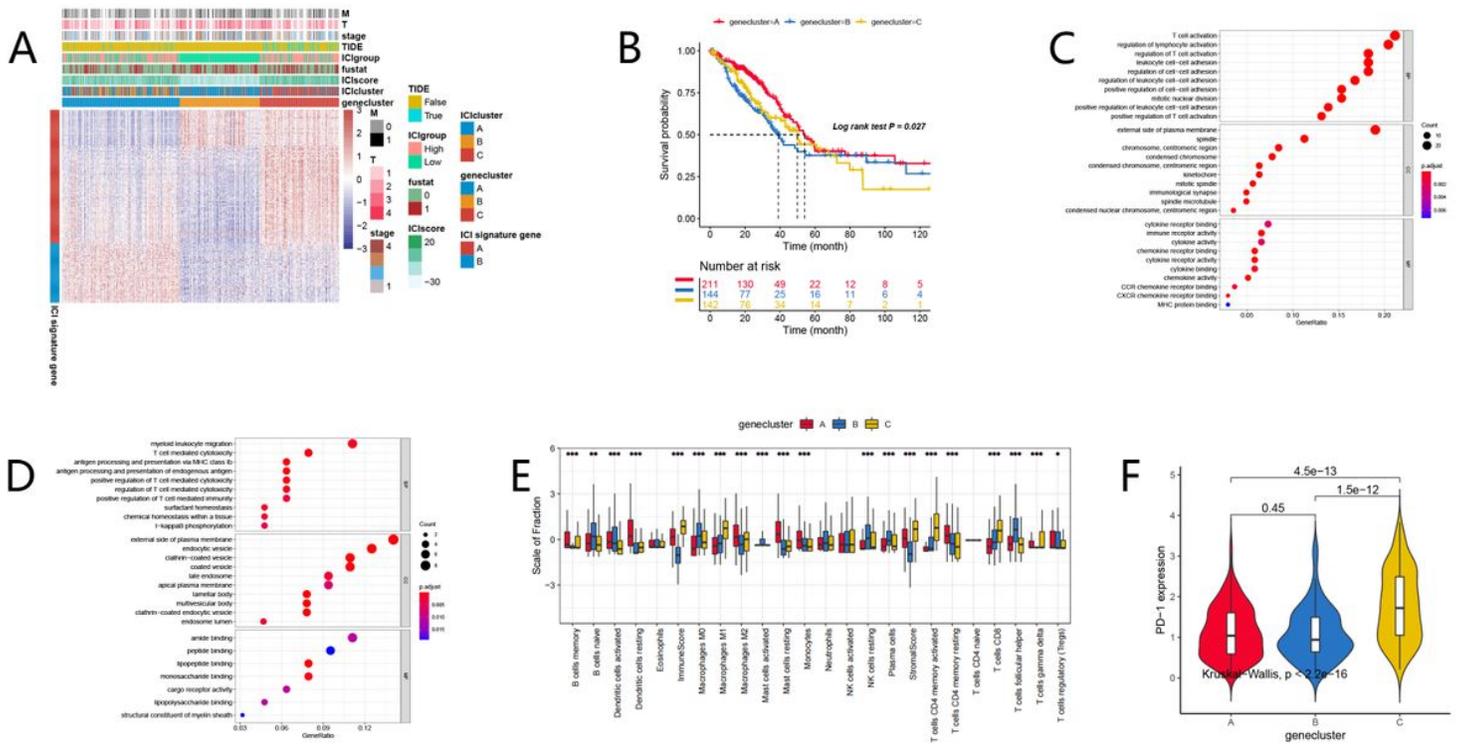


Figure 2

(A): Unsupervised clustering analysis in DEGs of three ICI clusters into three groups: gene clusters A,B,C ,535 patients in the TCGA-LUAD cohort with entire clinical information were included into the analysis. (B): Survival curves for three gene clusters. (C,D): The biological function of two signature clusters: signature A and B. (E): The distribution of immune cell infiltration in three gene clusters. (G):PD-1 expression level in different gene clusters.

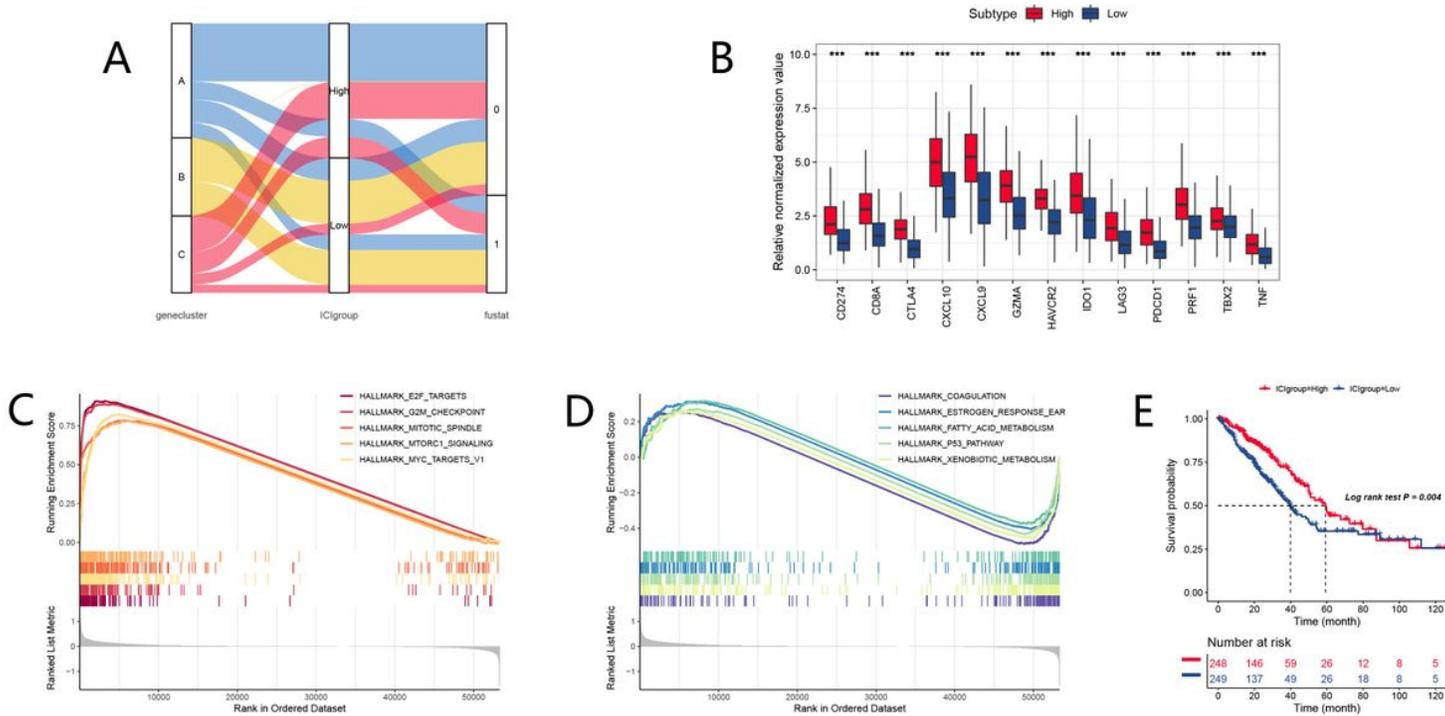


Figure 3

(A) The alluvial plot of ICI gene cluster distribution, ICI score and survival outcome in different ICI clustering groups. (B) the immune checkpoint relevant genes and immune-activation-relevant genes expressed in high and low ICI score subgroups. (C,D) The biological function of low and high ICI score groups. (E) Survival curve for high and low ICI score groups in the TCGA-LUAD cohort .

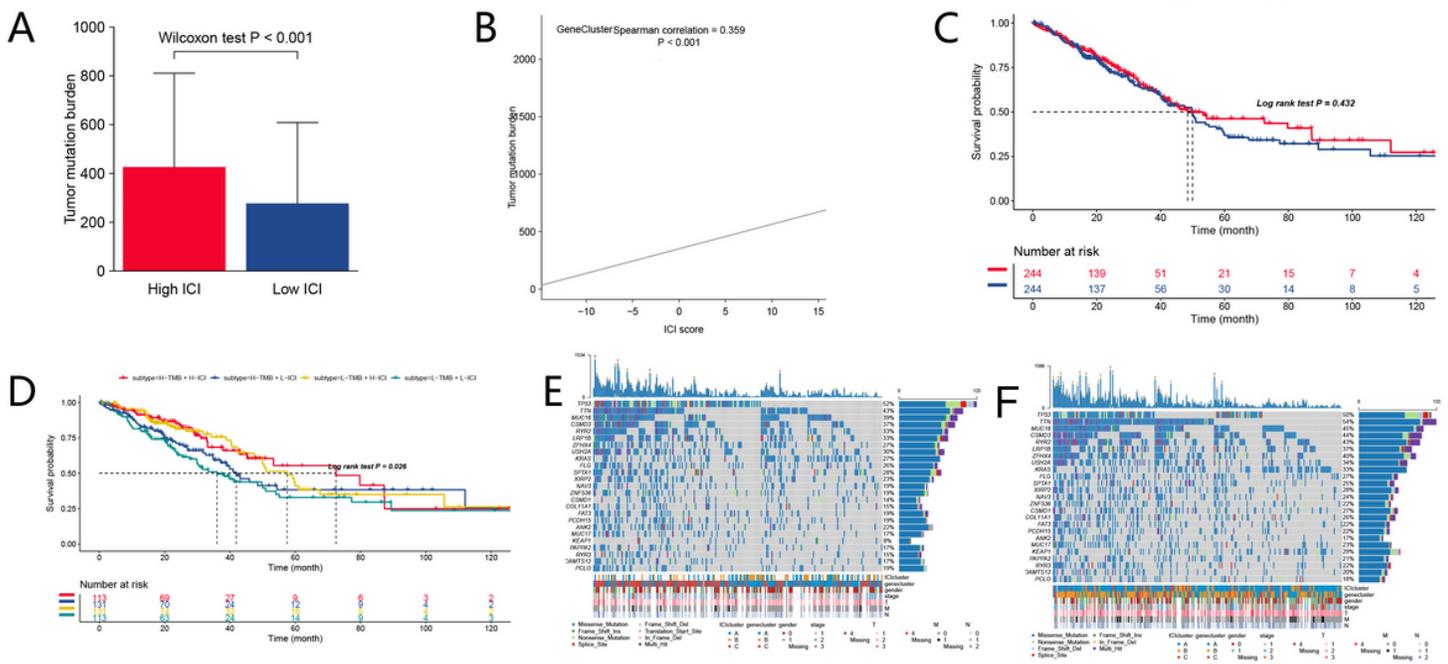


Figure 4

(A) The distribution of tumor burden mutational in different ICI score cluster. (B) The correlation between ICI score and TMB. (C) Survival curves for different TMB groups in TCGA-LUAD cohort. (D) Survival curves for different subgroups stratified by both TMB and ICI scores. (E-F) The top 25 genes mutation frequency in the high ICI scores (left) (E) and low ICI scores on the right (F).

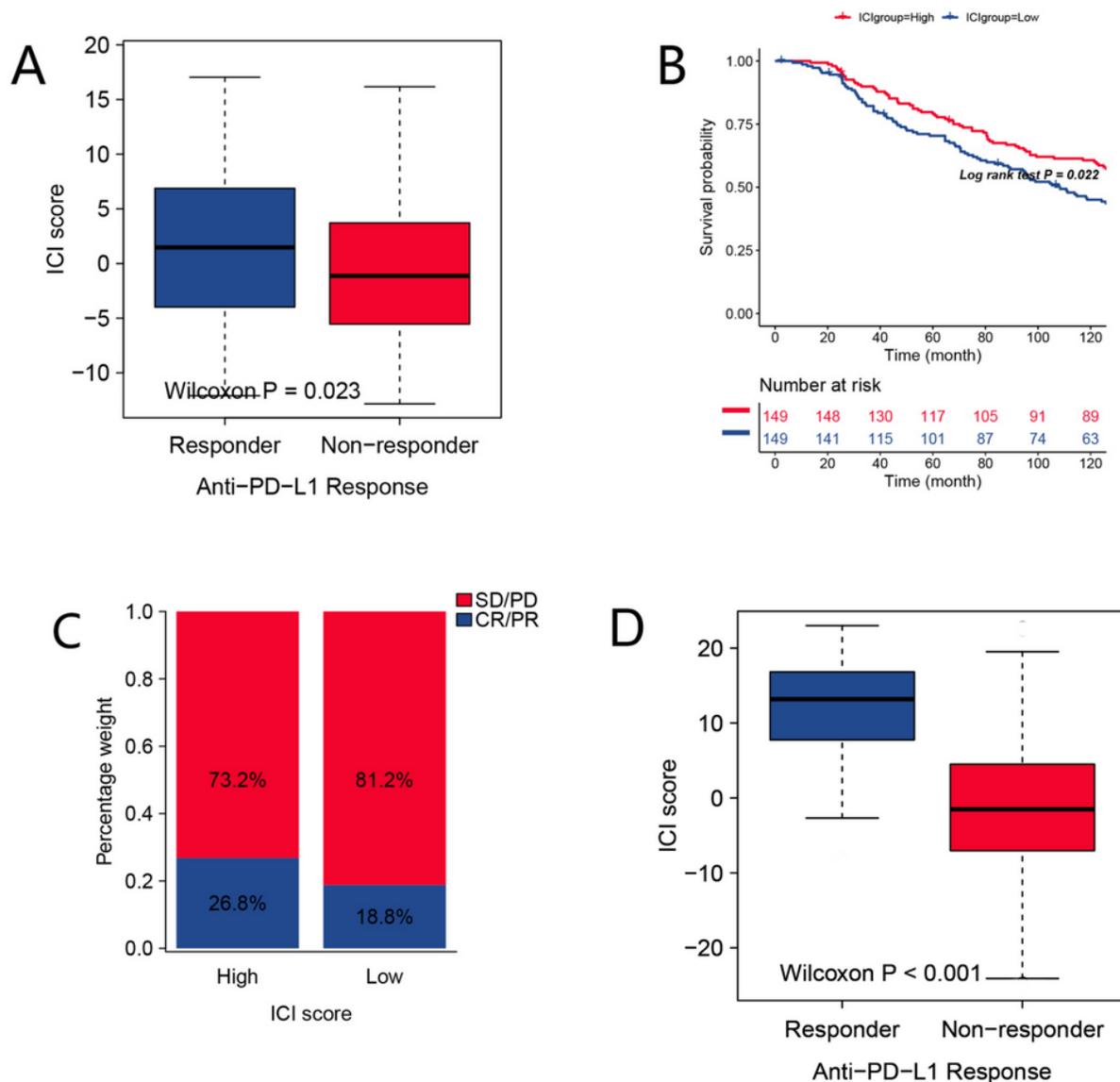


Figure 5

(A) The two ICI score groups with different anti-PD-1 response. (B) Survival curves for different ICI scores in the IMvigor210 cohort. 348 patients in the IMvigor210 cohort were included into the analysis.(C) Clinical response proportion to anti-PD-L1 immunotherapy (response / Non-response and stable disease (SD)/progressive disease (PD)) in the Imvigor210 cohort with high or low ICI groups. (D)The predicted Response group in TIDE analysis showed higher ICI scores than Nonresponse group.