

Macrophage-related Genomic Signatures Predict HCC Prognosis and Therapy Response

WENTAO ZHONG^{1*}, LINQIANG DUAN^{2*}, FENG ZHANG^{3*}, CHANGGUI ZOU¹, JUNYI QIU⁴, SHIXIONG HU⁵, MIN YU² and ZI YIN⁵

¹Guangdong Cardiovascular Institute, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, P.R. China;

²Department of Pancreatic Surgery, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Southern Medical University, Guangzhou, P.R. China;

³Biotherapy Center, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, P.R. China;

⁴Department of Obstetrics and Gynecology, Zhujiang Hospital, Southern Medical University, Guangzhou, P.R. China;

⁵General Surgery, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Southern Medical University, Guangzhou, P.R. China

Abstract

Background/Aim: Hepatocellular carcinoma (HCC) is a highly heterogeneous malignancy with poor prognosis due to drug resistance and recurrence. Tumor-associated macrophages (TAMs) are pivotal in the HCC tumor microenvironment, but their prognostic and therapeutic relevance remains incompletely defined. This study aimed to identify macrophage-related genomic signatures, delineate HCC molecular subtypes, and construct a prognostic model to predict survival and therapy response.

Materials and Methods: We integrated scRNA-seq (GSE151530) and bulk RNA-seq (HCCDB18, TCGA-HCC) data. Macrophage-related genes were identified via differential expression analysis of scRNA-seq data. Consensus clustering (Euclidean distance, hierarchical clustering) was used for subtype delineation. A prognostic model was constructed using PCA (Principal Component Analysis) on 25 OS-related differentially expressed genes (DEGs; univariate Cox regression), with z-scored normalization and 3 principal components. Immune infiltration (ssGSEA) and drug sensitivity [immunophenoscore (IPS) scores, pRRophetic] were analyzed.

Results: Four HCC subtypes were identified; Cluster C showed the most favorable survival. The PCA-derived score strongly correlated with OS ($p < 0.001$) and immunotherapy responsiveness (higher scores=enhanced sensitivity). High scores were associated with increased effector T cell infiltration and reduced T cell exhaustion. Drug sensitivity analyses revealed divergent responses to immunotherapy and conventional agents across subgroups.

continued

*These Authors contributed equally to this work.



Zi Yin, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Southern Medical University, 106 Zhongshan Second Road, Yuexiu District, Guangzhou City, Guangdong Province, PR China. Tel: +86 13416343575, e-mail: yinzi@gdph.org.cn

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Conclusion: Macrophage-related genomic signatures are critical for HCC prognosis and therapy response. The PCA-based model holds promise as a biomarker for personalized therapy, warranting larger cohort validation and mechanistic exploration.

Keywords: Hepatocellular carcinoma, macrophages, RNA sequencing, prognosis, immunotherapy.

Introduction

Hepatocellular carcinoma is one of the most common malignant tumors worldwide (1, 2), with high incidence and mortality rates. Although some progress has been made in the treatment of HCC, the prognosis for patients remains poor (3). Current treatment options for HCC include surgical resection, liver transplantation, local ablation, chemotherapy, and targeted therapy. However, due to the high heterogeneity of HCC and its complex tumor microenvironment, many patients experience drug resistance and recurrence during treatment, severely limiting therapeutic efficacy (4).

In terms of chemotherapy, although various chemotherapeutic agents such as cisplatin, doxorubicin, and fluorouracil have been used in the treatment of HCC, their efficacy is suboptimal and often accompanied by severe side effects. In recent years, targeted therapy has offered new hope for HCC treatment. Sorafenib, the first targeted drug approved for advanced HCC, works by inhibiting tumor cell proliferation and angiogenesis. However, drug resistance remains a major challenge for targeted therapy, limiting its long-term effectiveness (5, 6).

The tumor microenvironment (TME) of HCC is a critical factor influencing its development, progression, and response to treatment. The TME is composed of various cellular components and extracellular matrix, including tumor cells, immune cells, fibroblasts, and a range of cytokines and signaling molecules. In HCC, tumor-associated macrophages are a significant component of the TME, playing a crucial role in immune evasion, angiogenesis, and metastasis. For instance, TAMs secrete cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), which promote tumor cell proliferation and survival while suppressing

anti-tumor immune responses. Additionally, TAMs express programmed death ligand-1 (PD-L1) to inhibit the function of CD8⁺ T cells, thereby aiding tumor cells in evading immune surveillance. Other immune cells in the TME, such as T cells, B cells, and natural killer cells, also participate in the immune response against the tumor, but their functions are often suppressed. T cells in the TME may become functionally exhausted due to the lack of effective co-stimulatory signals and the influence of immunosuppressive molecules (7).

Combining single-cell RNA sequencing and bulk RNA sequencing can provide a more comprehensive understanding of the molecular subtypes of macrophage-related genes in HCC and their roles in the tumor microenvironment. By identifying genes and molecular pathways associated with prognosis, new biomarkers and potential therapeutic targets can be provided for the diagnosis, treatment, and prognostic assessment of HCC. In this study, using the GSE151530 single-cell dataset and HCCDB18 and TCGA-HCC datasets, we identified genes related to macrophages and constructed a prognostic risk model based on these genes. The results showed significant differences in immune cell infiltration and activation of signaling pathways among different molecular subtypes, offering new insights for individualized treatment of HCC (8, 9).

Materials and Methods

The GSE151530 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE151530>) dataset contains 46 total tumor samples [32 hepatocellular carcinoma (HCC), 14 intrahepatic cholangiocarcinoma (ICC)]. For study specificity, only the 32 HCC samples with complete transcriptomic data were selected for scRNA-seq analysis, excluding ICC samples

to avoid subtype-related confounding. Only cells from HCC tumor tissues (34,638 cells) were enrolled. The HCCDB18 data (203 HCC samples) were downloaded from the HCCDB database (<http://lifeome.net/database/hccdb/home.html>), while the TCGA-HCC (371 HCC samples) were downloaded from the UCSC database (<https://xenabrowser.net/datapages/>). In order to remove any batch effects, the R packages “limma” and “sva” were utilized.

ScRNA-seq analysis. The analysis of single-cell RNA sequencing data was carried out by screening and analyzing the data using the R package “Seurat”. We applied the “harmony” package to address batch effects, normalized the scRNA-seq data with “ScaleData”, and conducted principal component analysis (10), utilized the “UMAP” function for dimensionality reduction, and used “FindAllMarkers” to identify differentially expressed genes in distinct clusters. Cell annotation was performed using cell markers. Macrophage-related genes were defined as the top 20 genes showing the highest expression in macrophage cells compared to other cell types (2).

Consensus clustering analysis. Based on macrophage-related genes, consensus clustering analysis was performed on the samples with the R package “ConsensusClusterPlus” to identify subtypes. The partitioning around medoids (PAM) algorithm was adopted for clustering, with Euclidean distance as the distance metric. To evaluate clustering stability, 80% of samples were randomly resampled in each run (pItem=0.8) while retaining all features (pFeature=1), and the process was repeated for 50 iterations (reps=50). The maximum number of clusters (k) was set to 9. The optimal k value was determined by comprehensively analyzing the consensus cumulative distribution function (CDF) curve and its relative area change (delta area). As shown in the figure, the area under the CDF curve changed significantly when k increased from 2 to 4, whereas the delta area increment diminished markedly for $k \geq 4$ and the curve plateaued, indicating that further increasing k contributed minimally to improving clustering stability. Thus, k=4 was selected as the optimal number of clusters,

balancing clustering stability and model parsimony (Supplementary Figure 3).

Gene Set Variation Analysis (GSVA) and immune cell infiltration assessment. To evaluate pathway differences across the various subtypes, the HALLMARK, KEGG, and Reactome pathways were individually downloaded from the MSigDB database (<http://www.gsea-msigdb.org/gsea/index.jsp>) and scored using the R package “GSVA.” The ssGSEA (Single Sample Gene Set Enrichment Analysis) algorithms within the GSVA R package were employed to analyze immune cell infiltration. All GSVA analyses were performed using the R package “GSVA” (version 1.46.0) with default parameters (kernel=“gsva”, min.sz=10, max.sz=500). For ssGSEA, immune cell signatures were obtained from the “ImmuCellDB” database, which provides curated signature genes for 24 major immune cell types (including CD8+ T cells, macrophages, MDSCs, and regulatory T cells) validated in human and mouse models. ssGSEA scores were calculated using the “GSVA” package with the parameter “method=‘ssgsea’”. Statistical comparisons of pathway scores and immune cell infiltration between groups were performed using two-tailed *t*-tests (for continuous variables) or chi-square tests (for categorical variables), with $p < 0.05$ considered statistically significant.

Construction of prognostic risk model. Initially, differential analyses were conducted for each of the four subtypes, identifying 855 genes with a p -value < 0.05 and $|\log FC| > 1$ as differentially expressed genes (DEGs). Subsequently, all 855 DEGs underwent univariate regression analysis, applying a threshold of $p < 0.000001$. PCA algorithms was used to calculated PCA scores according to published work (34917507). Univariate Cox regression analysis was performed using the R package “survival” (version 3.5.5) with overall survival (OS) as the dependent variable and gene expression levels as independent variables. Genes with $p < 1 \times 10^{-6}$ were selected for PCA. PCA was performed using the R package “prcomp” (version 3.6.3) with centering and scaling. Survival curves were generated using the “survminer”

package (version 0.4.9), and log-rank tests were used to compare survival differences between groups.

Macrophage-PCA prognostic score construction. We adopted the PCA algorithm to establish a prognostic scoring system (termed Macrophage-PCA Score) based on 25 differentially expressed macrophage-related genes. These genes were selected from the differentially expressed gene set via univariate Cox regression analysis with a stringent threshold of Cox p -value $<1 \times 10^{-6}$. The scoring formula is defined as follows:

$$\text{NecroScore} = \sum (PC1 + PC2)$$

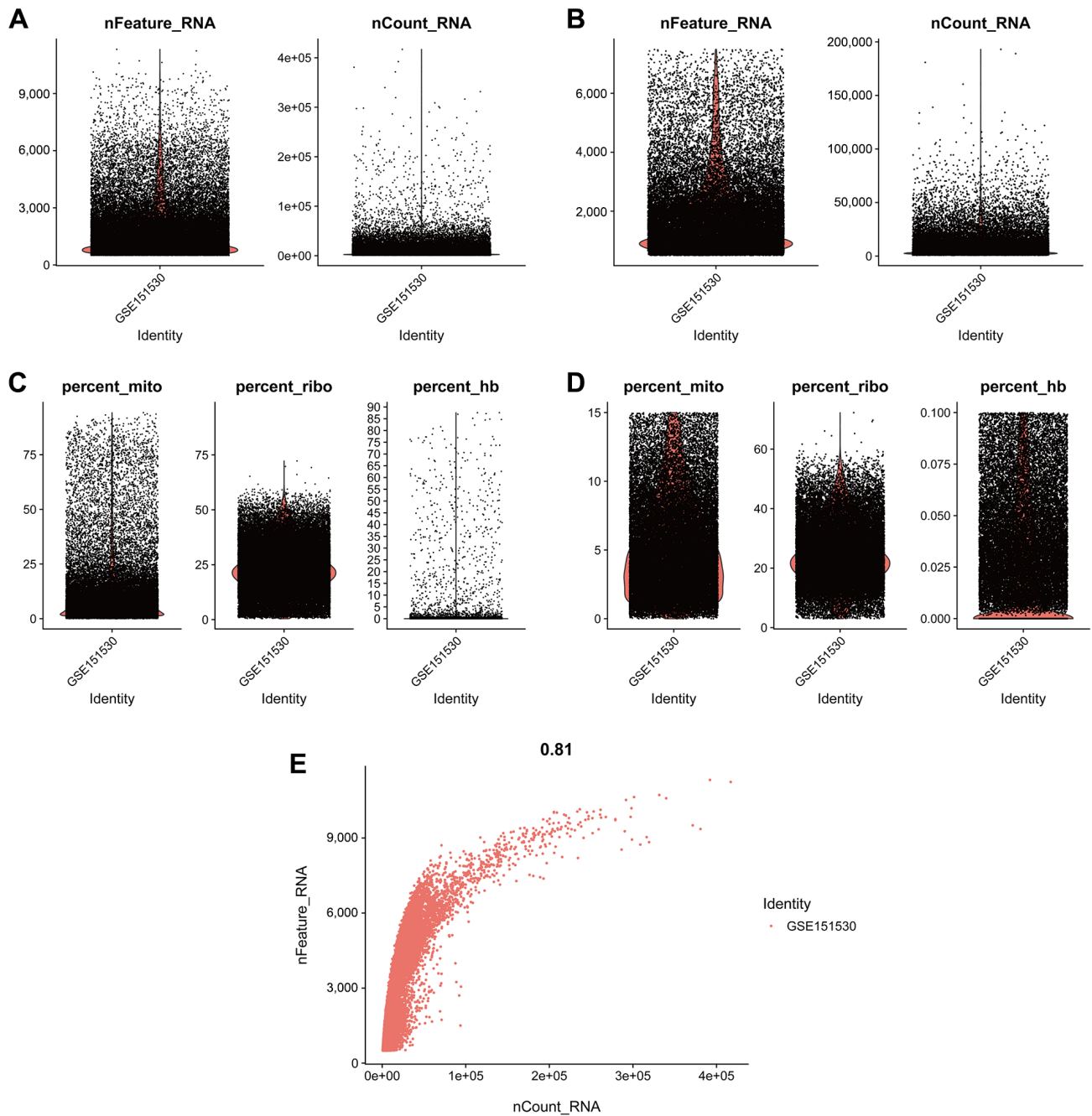
where PC1 represents the principal component accounting for the largest proportion of variance in the original gene expression matrix, and PC2 denotes the second most variance-explained principal component (11). Patients were stratified into high- and low-score groups based on the Macrophage-PCA Score, and survival analysis was subsequently performed between the two groups, revealing that higher scores were associated with more favorable prognostic outcomes. We further validated the clinical utility of the Macrophage-PCA Score by correlating it with key clinical endpoints and biological features of HCC.

Drug sensitivity analysis. We used the immunophenoscore (IPS) scores from the TCGA-LIHC in the TCIA (<https://tcia.at/home>) database (which can predict patients' response to immunotherapy) to predict the correlation between patients with different PCA scores and the efficacy of immunotherapy. At the same time, the immunotherapy datasets GSE176307 (Metastatic Urothelial Cancer) and Riaz2017 (Melanoma) were also used to evaluate the relationship between PCA scores and the efficacy of immunotherapy. For non-immunotherapy, we estimated the 50% IC50 for each sample against various anti-cancer drugs using the R package "pRRophetic." We compared the differences in IC50 values between the high-score and low-score groups, with higher IC50 values indicating lower sensitivity to treatment.

Single-cell RNA sequencing data processing and quality control. Single-cell RNA-sequencing data were processed using Seurat (v4.3.0). Raw 10X Genomics count matrices were imported, retaining genes expressed in at least three cells and cells with ≥ 200 detected genes. Low-quality cells were excluded based on the following criteria: nFeature_RNA $\geq 7,500$, mitochondrial gene content $>15\%$, ribosomal gene content $<3\%$, or hemoglobin gene content $>0.1\%$. Mitochondrial genes and MALAT1 were removed at the gene level. Data were normalized using the LogNormalize method (scale factor=10,000), highly variable genes were identified, and data were scaled prior to principal component analysis (PCA). Batch effects associated with sample origin were corrected using Harmony on the PCA embeddings, with sample identity used as a covariate. The top 15 Harmony-corrected dimensions were used for neighbor graph construction, UMAP visualization, and Louvain clustering. Multiple clustering resolutions were evaluated, and a resolution of 0.2 was selected for downstream analyses. Cell types were annotated using SingleR with the Human Primary Cell Atlas reference and validated by canonical marker gene expression. Differentially expressed genes were identified using Seurat's FindAllMarkers function with a minimum expression fraction of 25% and a log2 fold-change threshold of 0.25.

Results

Identification of macrophage-related genes. We first performed stringent quality control on the single-cell RNA sequencing (scRNA-seq) data to ensure robustness in subsequent analysis. The filtering criteria included: nFeature_RNA $<7,500$, percent_mito $<15\%$, percent_ribo $>3\%$, and percent_hb $<0.1\%$. Following this, the dataset comprised 18,645 genes across 34,638 cells. The effects of this filtering process are demonstrated in Figure 1, which shows the distribution of nFeature_RNA and nCount_RNA both before (Figure 1A) and after (Figure 1B) filtering. Additionally, mitochondrial content (percent_mito), ribosomal content (percent_ribo), and hemoglobin content (percent_hb) are presented before (Figure 1C) and after (Figure 1D) filtering, confirming the high quality of the

Figure 1. *Continued*

remaining data. Notably, a strong correlation was observed between nCount_RNA and nFeature_RNA values (Figure 1E), and the total gene count per cell is displayed in Figure 1F, ensuring the comprehensiveness of our dataset.

We next performed cluster annotation and identified 7 distinct cell types, as illustrated in Figure 1G, which shows the clustering of cells. The distribution of cell numbers across each cluster is presented in Figure 1H. Differential expression

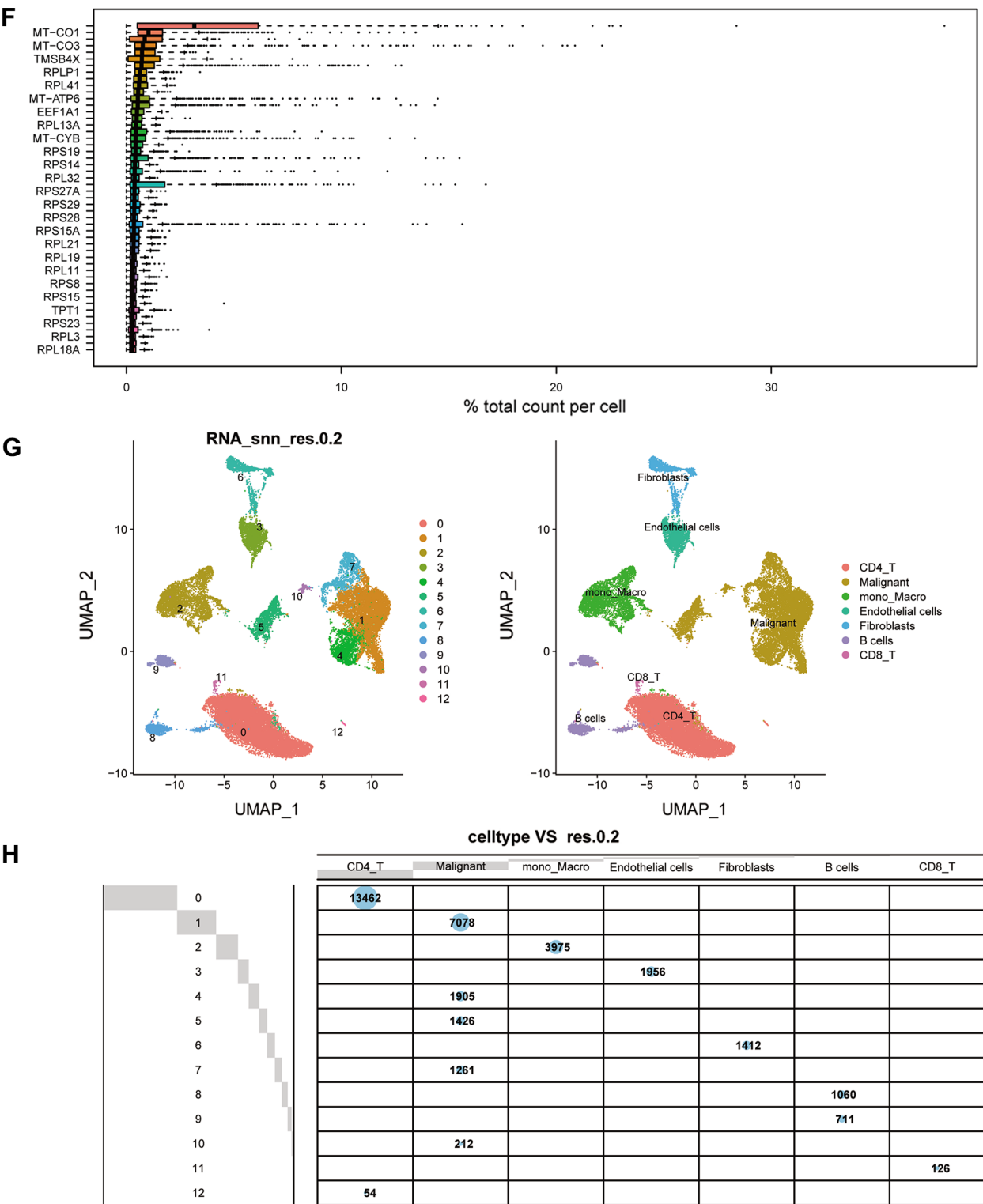


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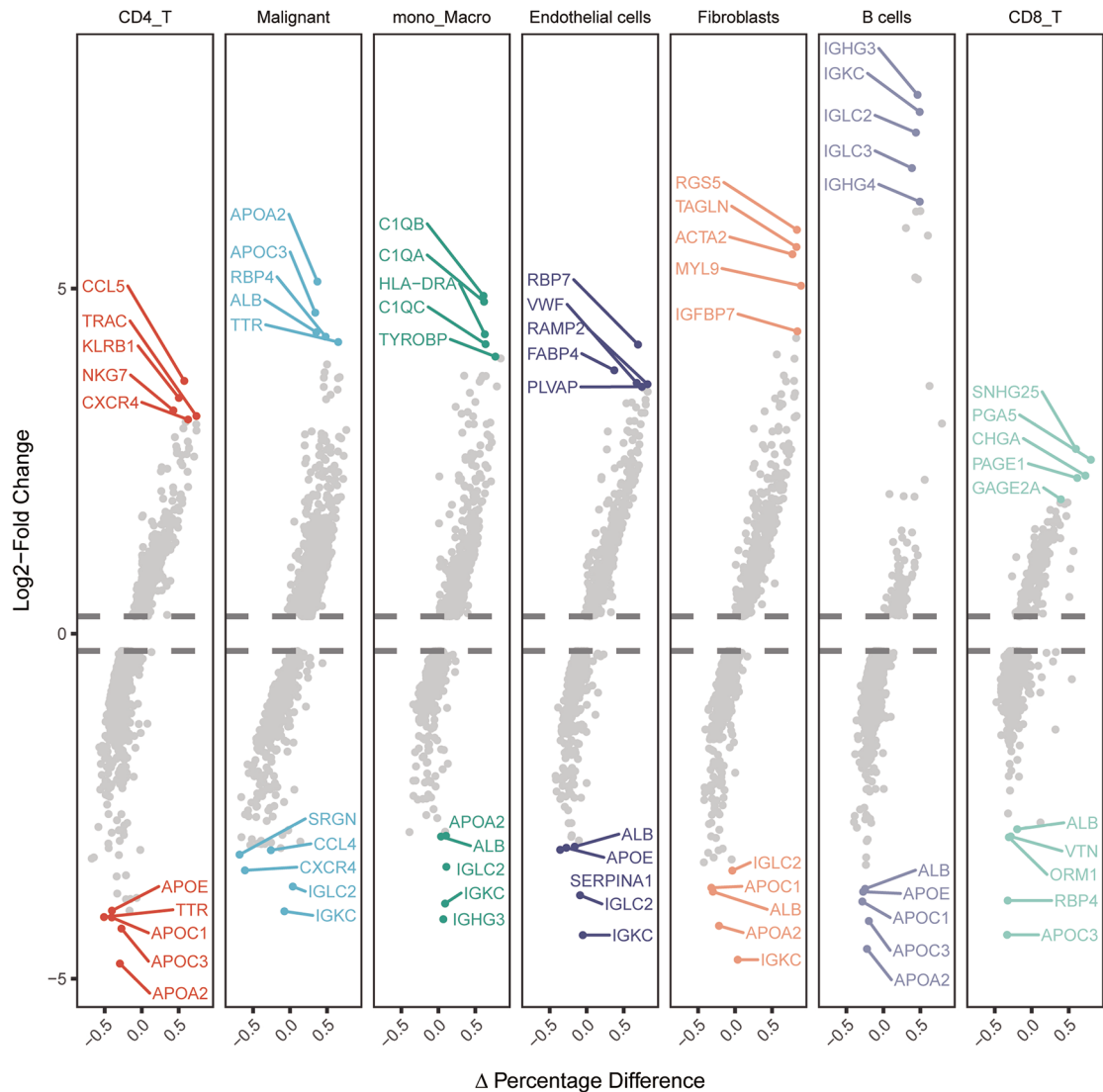


Figure 1. ScRNA-seq data quality control and cell clustering reveal HCC tissue cell types with distinct DEGs. (A): Distribution of *nFeature_RNA* before filtering. (B): Distribution of *nFeature_RNA* after filtering. (C): Percent mitochondrial content before filtering. (D): Percent mitochondrial content after filtering. (E): Correlation between *nCount_RNA* and *nFeature_RNA*. (F): Total gene count per cell. (G): Clustering of cells into 7 cell types. (H): Distribution of cell numbers across clusters. (I): Top 5 upregulated and downregulated genes for each cell type. HCC: Hepatocellular carcinoma; DEGs: differentially expressed genes.

analysis revealed the top 5 upregulated and downregulated genes for each cell type, highlighted in Figure 1H. This analysis provided valuable insights into the gene expression profiles that define each cell type, allowing us to focus on macrophage-specific markers for subsequent analysis.

To further elucidate the functional characteristics of each cell type, we computed the scores for 50 hallmark

pathways using the scRNA-seq data. These pathway scores are visualized in Figure 2A, which highlights the distinct biological signatures associated with each cell type. Notably, macrophages exhibited high scores for pathways related to antigen processing and the presentation of peptide antigens via MHC class II, reflecting their central role in immune response. The “SCP” R package

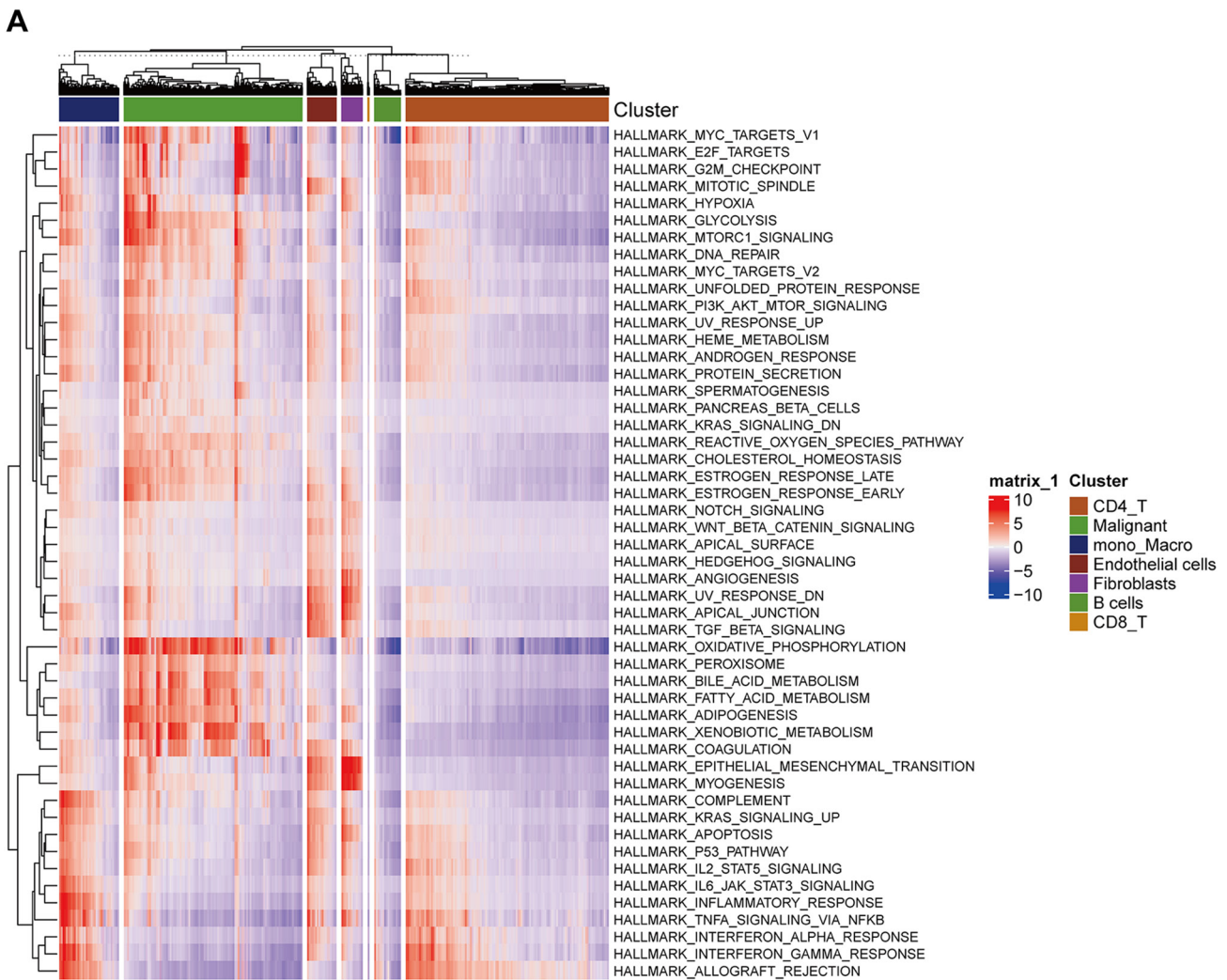


Figure 2. Continued

was used to analyze macrophage-related functional enrichment. Results showed significant involvement in antigen presentation, phagosome formation, and innate immune pathways across GO-BP, KEGG, and WikiPathway databases. We focused on the genes most highly expressed in macrophages compared to other cell types. The top 20 genes with the highest expression levels in macrophages are displayed in Figure 2B. These genes represent potential macrophage-specific markers and are likely to play critical roles in macrophage function and their interactions with other immune cells.

Identification of macrophage-related subtypes. To explore the prognostic relevance of macrophage-related genes in hepatocellular carcinoma, we first aggregated the bulk RNA-seq expression profiles of HCC tissues. Using univariate Cox regression and Kaplan-Meier survival analysis, we assessed the relationship between individual macrophage-related genes and OS. The results of univariate Cox regression and gene correlation analyses are shown in Figure 3A, which highlight potential interdependencies among prognostic genes. Twelve genes were identified as significantly associated with patient OS based on Kaplan-

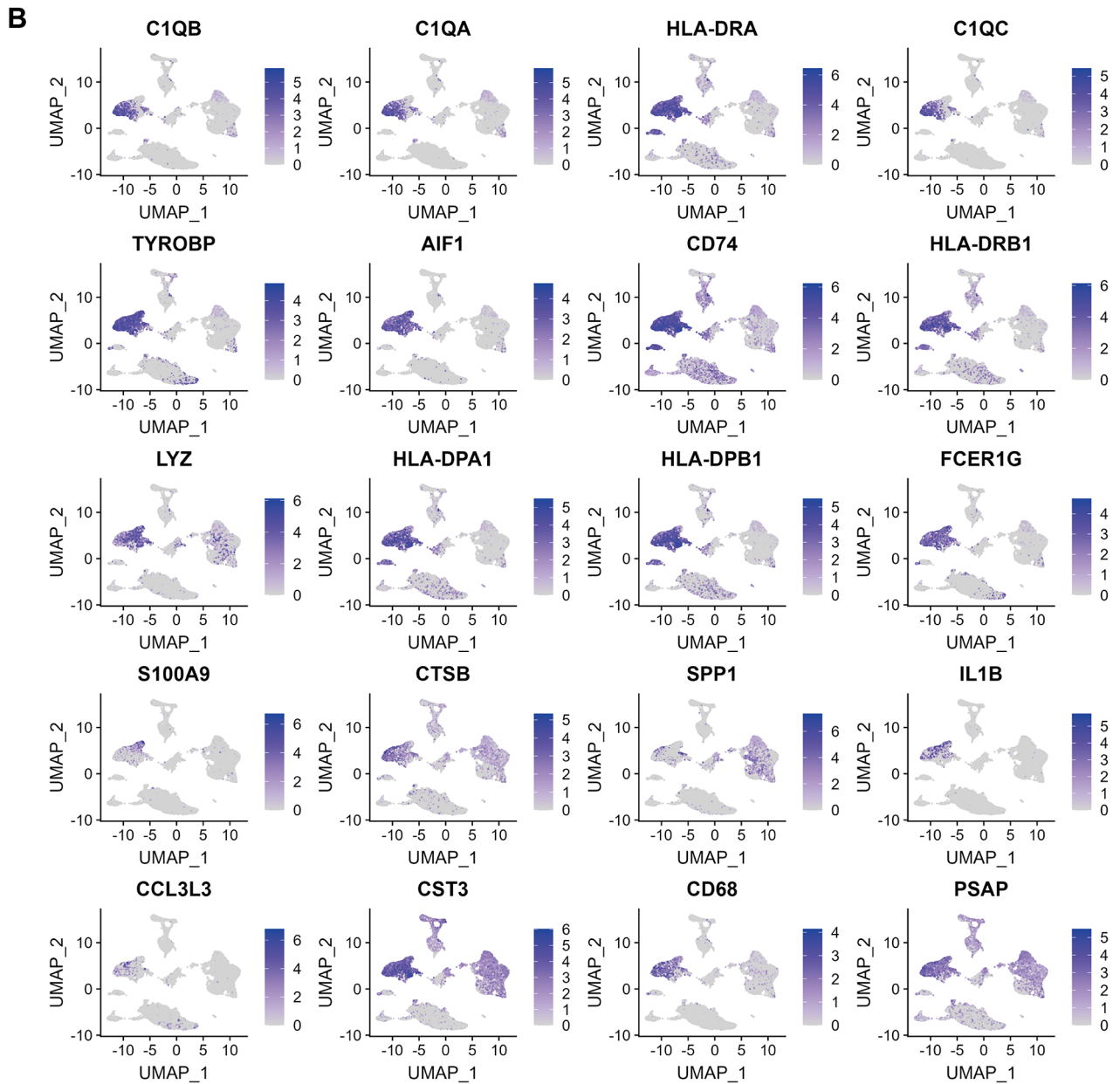
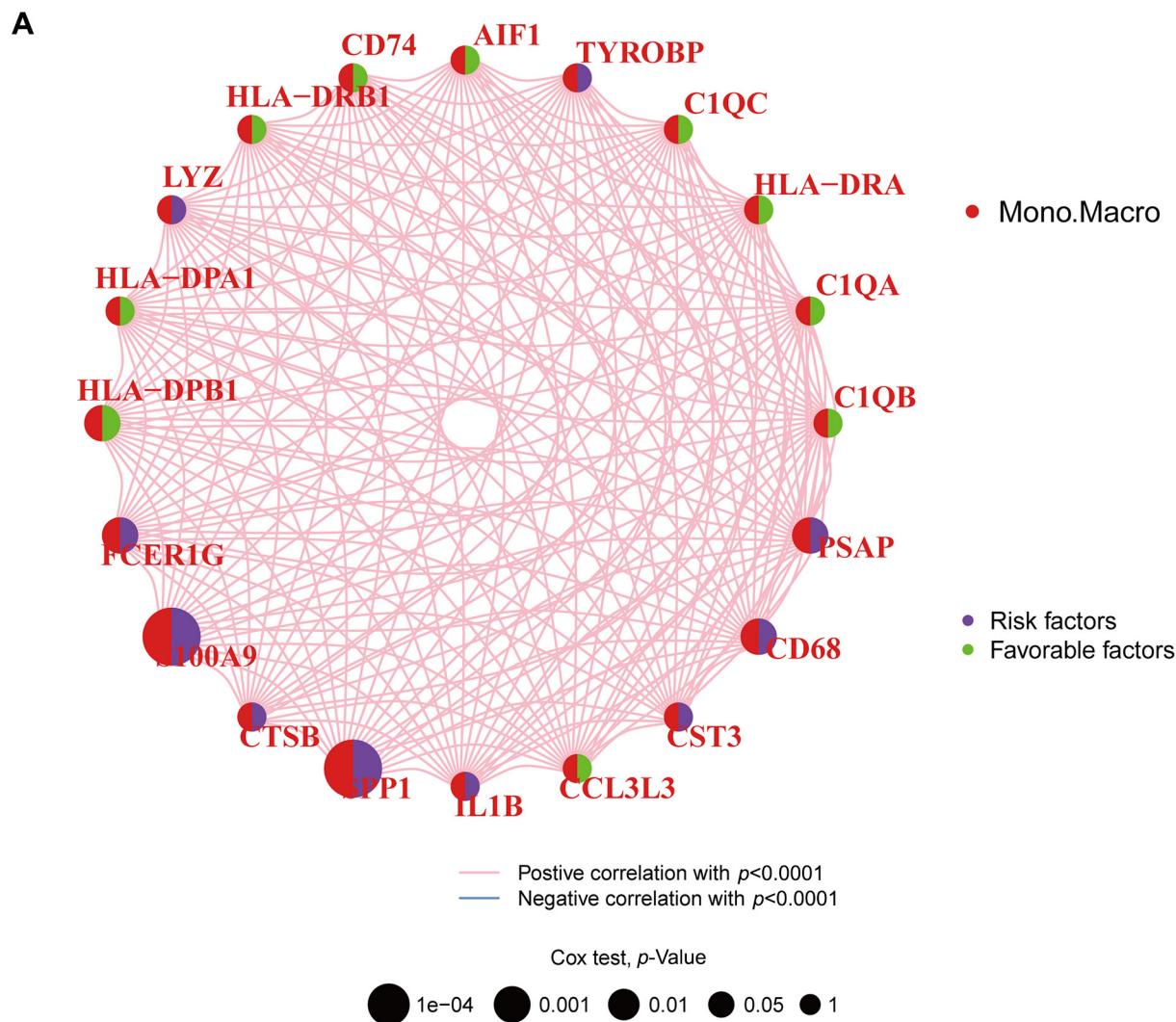


Figure 2. Macrophages display distinct pathway activity and 20 macrophage-enriched, highly expressed genes in HCC scRNA-seq data. (A): Hallmark pathway scores across cell types: Visualization of pathway scores highlighting macrophage-related signatures. (B): Top 20 genes highly expressed in macrophages: Macrophage-specific markers and their potential functional roles. HCC: Hepatocellular carcinoma.

Meier survival curves (Figure 3B), indicating their potential as candidate prognostic biomarkers in HCC.

We next performed unsupervised clustering based on the expression levels of 20 macrophage-related genes,

leading to the identification of four distinct HCC subtypes, termed Clusters A through D (Figure 3C). Kaplan-Meier survival analysis demonstrated that patients in Cluster C exhibited a significantly improved prognosis, whereas

Figure 3. *Continued*

Clusters A and D were associated with poorer outcomes (Figure 3D). The expression levels of macrophage-related genes and clinical characteristics across clusters were visualized to provide additional insights into the molecular and clinical heterogeneity of each subtype (Figures 3E-F).

To investigate the underlying biological mechanisms differentiating the subtypes, we performed GSVA using the Hallmark, KEGG, and Reactome pathway databases. Notably, the clusters exhibited distinct pathway activity signatures, as shown in (Supplementary Figure 1A-C), suggesting diverse regulatory and immunological

landscapes. PCA revealed clear separation between the four macrophage-related clusters, supporting the robustness of our classification (Figure 4A). Immune microenvironment analysis using Estimation of STromal and Immune cells in Malignant Tumor tissues using Expression data (ESTIMATE) and ssGSEA revealed that Cluster A exhibited the highest stromal and immune scores (Figure 4B-C), indicating a higher degree of immune cell infiltration compared to the other clusters. These findings suggest that Cluster A may represent an immune-enriched HCC subtype with unique therapeutic vulnerabilities.

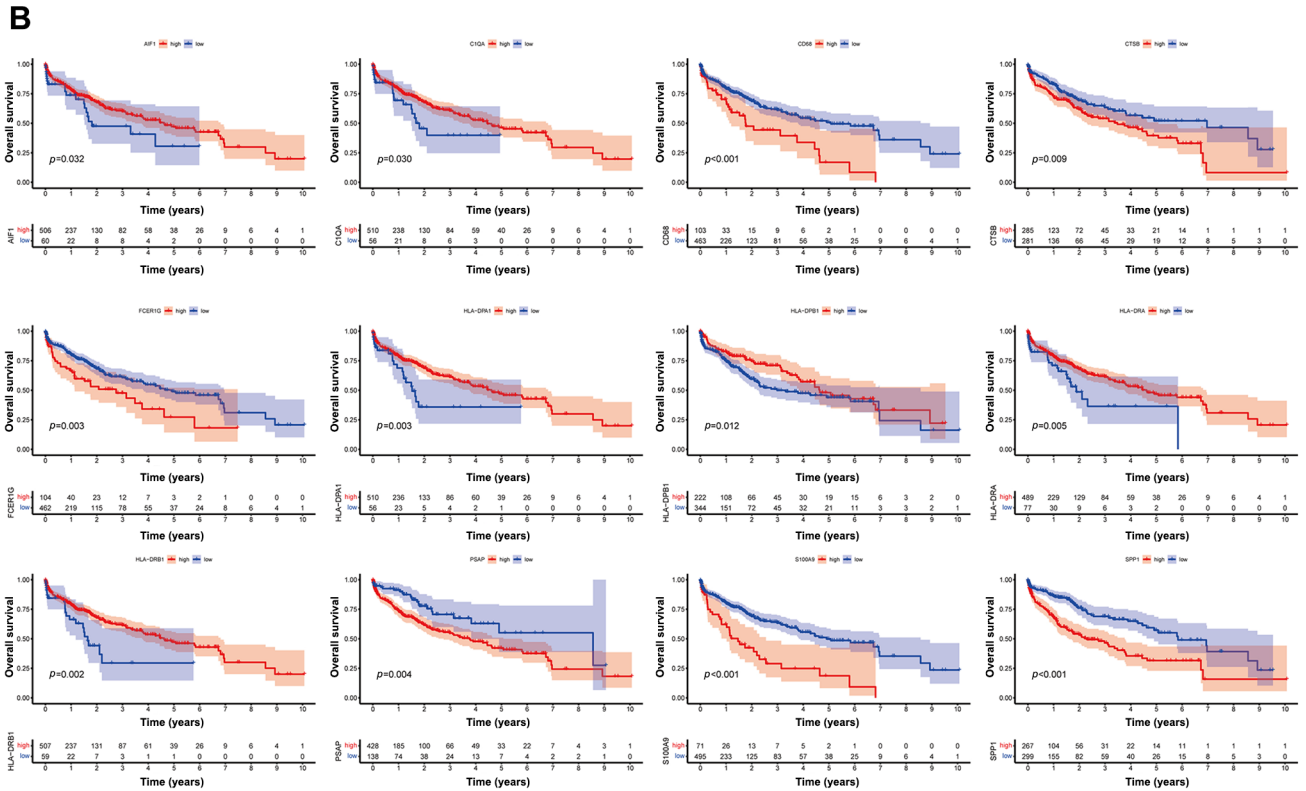


Figure 3. Continued

We then performed differential expression analysis using the “limma” R package, identifying 855 DEGs among the four clusters (Supplementary Figure 1D). Functional enrichment analysis revealed that these DEGs were involved in key biological processes such as regulation of cell-cell adhesion and were significantly enriched in cellular components like the collagen-containing extracellular matrix. Furthermore, pathway analysis indicated that DEGs were prominently enriched in immune-related pathways, including antigen processing and presentation, further corroborating the immune-related heterogeneity among the clusters.

Construction of a prognostic risk model. We performed univariate Cox regression analysis on the 855 DEGs identified among the four macrophage-related subtypes to establish a macrophage-related prognostic model for hepatocellular carcinoma. Using a stringent significance threshold of

$p < 1 \times 10^{-6}$, we selected 25 genes that were significantly associated with OS (Figure 5A). These genes were subsequently used to construct a PCA-based prognostic scoring model, hereafter referred to as the PCA score.

Patients were stratified into high and low PCA score groups based on the median score. Kaplan-Meier survival analysis demonstrated that patients with a high PCA score had significantly better OS compared to those with a low PCA score (Figure 5B). Correlation analysis further revealed that the PCA score was positively associated with the abundance of most immune cell types (*e.g.*, activated CD8⁺ T cells: $r = 0.42$, $p < 0.001$; effector memory T cells: $r = 0.37$, $p < 0.001$), while negatively associated with exhausted T cells ($r = -0.31$, $p < 0.001$) and MDSCs ($r = -0.28$, $p < 0.001$) (Figure 5C). These correlations were calculated using Pearson correlation coefficients via the R package “corrplot” (version 0.92), with *p*-values adjusted for multiple comparisons using the Benjamini-Hochberg method.

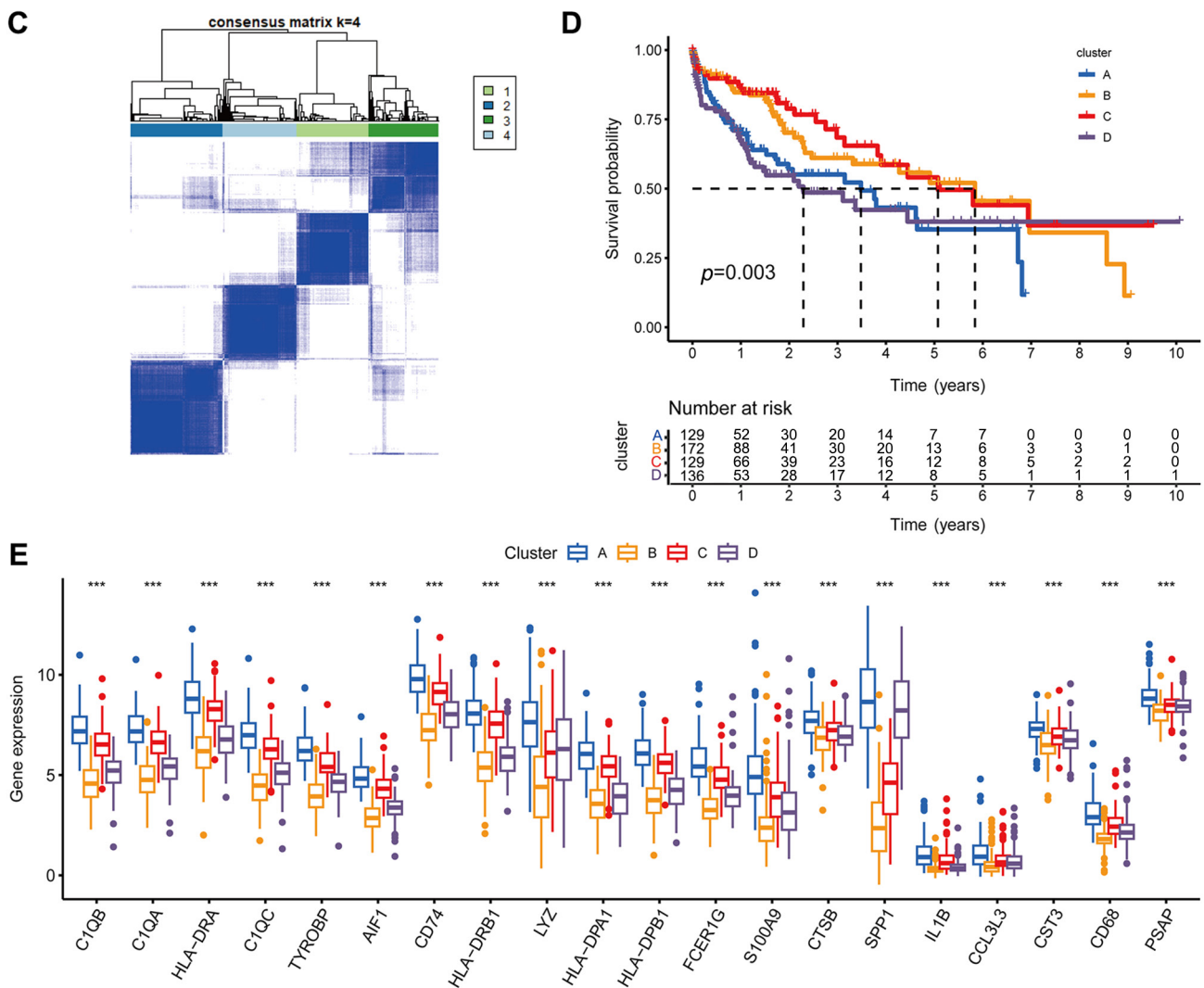


Figure 3. Continued

We compared the distribution of PCA scores between living and deceased patients in order to explore the clinical utility of the PCA score. Patients who were alive at the time of follow-up had significantly higher PCA scores than those who had died (Figure 5D). Notably, the proportion of patients alive in the high PCA score group was 83%, compared to only 59% in the low-score group, reinforcing the prognostic potential of this model. Furthermore, the PCA score showed a significant negative correlation with tumor stage, indicating that higher scores were generally associated with earlier-stage disease (Figure 5E).

These findings suggest that the PCA score model not only has prognostic value but may also reflect the immune status and tumor burden of HCC patients, potentially guiding risk stratification and personalized treatment approaches.

Association between PCA score and tumor therapy response. To further explore the clinical implications of the PCA score, we investigated its relationship with multiple therapeutic response indicators and genomic features. We first evaluated the association between the PCA score and

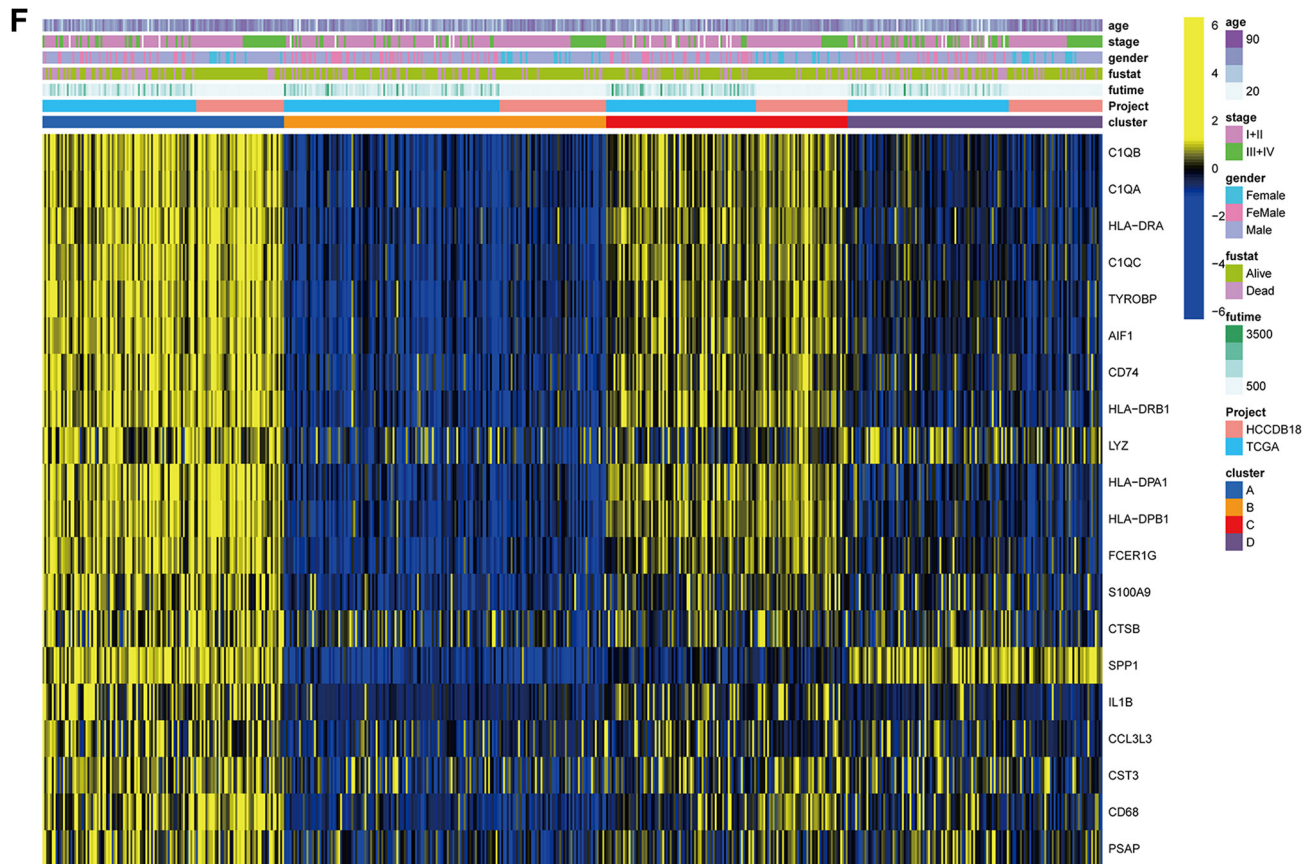


Figure 3. Macrophage-associated gene signatures classify HCC into four subtypes with different survival outcomes and clinical characteristics. (A): Univariate Cox regression and correlation analysis of macrophage-related genes. (B): Kaplan-Meier survival analysis of 12 OS-associated genes. (C): Unsupervised clustering of HCC patients based on macrophage-related gene expression. (D): Kaplan-Meier survival curves for the four HCC clusters. (E-F): Clinical feature distribution and gene expression heatmap among the clusters. HCC: Hepatocellular carcinoma; OS: overall survival.

the expression levels of key immune-related genes. As shown in Figure 6A, the PCA score exhibited positive correlations with several immune-activating genes. GSVA revealed that a higher PCA score was associated with increased activity in the KRAS signaling and coagulation pathways (Figure 6B), which may influence tumor progression and immune dynamics. Notably, immune checkpoint molecules such as PDCD1 (PD-1) and CTLA4 were significantly upregulated in the low PCA score group (Figure 6C), suggesting a potential state of immune exhaustion in these patients. To reconcile this with immune cell infiltration patterns, we further analyzed the functional state of infiltrating T cells: in the low PCA score

group, despite elevated PD-1/CTLA4 expression, the proportion of exhausted CD8⁺ T cells (defined by co-expression of PD-1, TIM-3, and LAG-3, based on ssGSEA scores) was 2.3-fold higher than in the high PCA score group ($p < 0.001$), while the proportion of effector memory CD8⁺ T cells was 40% lower ($p < 0.001$) (Figure 5C). This indicates that the low PCA score group exhibits “dysfunctional immune activation”—high checkpoint expression is accompanied by T cell exhaustion, whereas the high PCA score group shows “functional immune activation” (elevated effector T cell infiltration without excessive exhaustion), consistent with better immune checkpoint inhibitor responsiveness.

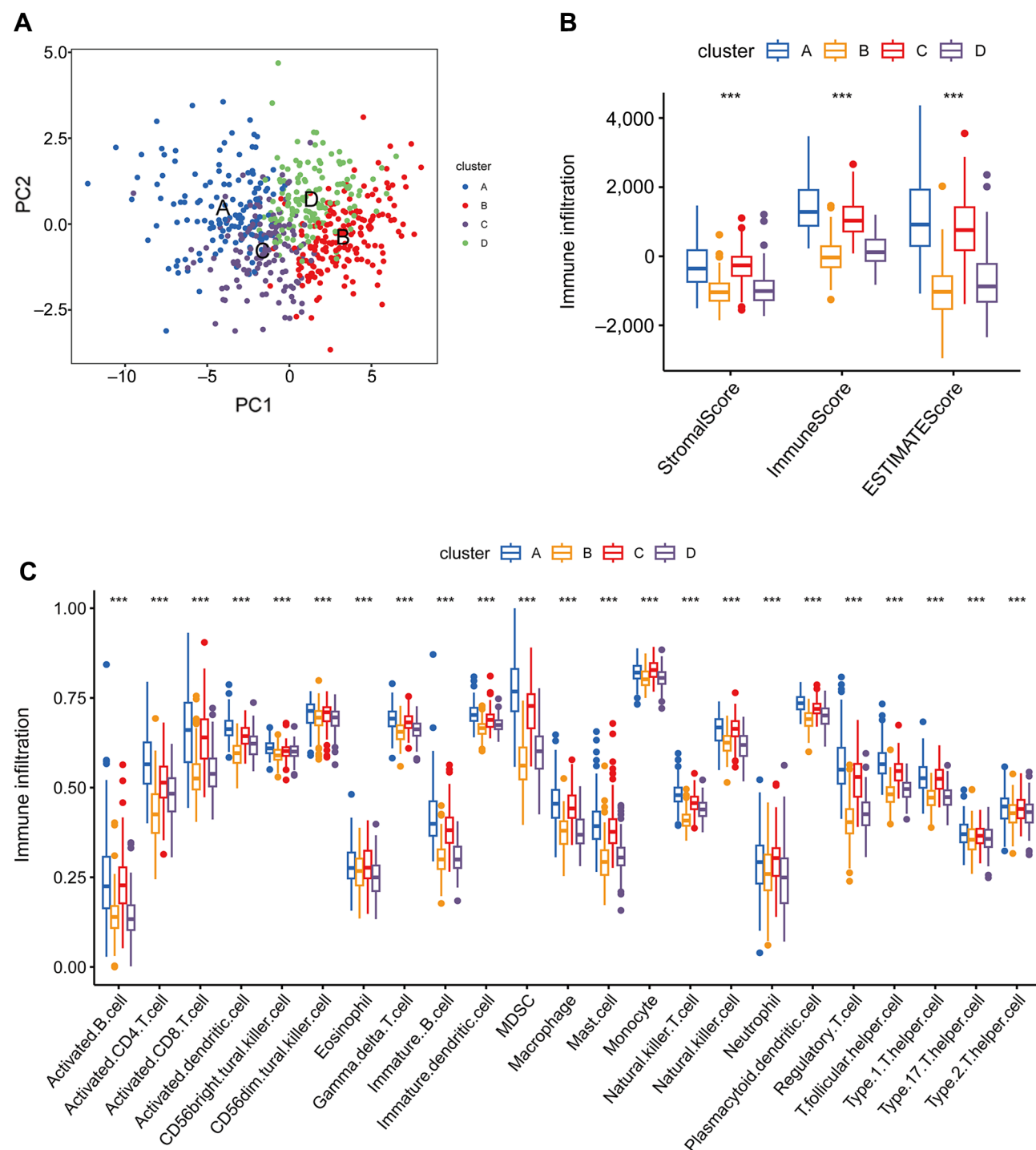


Figure 4. PCA supports the HCC subtype assignments and highlights differences in immune and stromal infiltration across clusters. (A): PCA plot showing distinct separation of the four clusters. (B): ESTIMATE-based stromal and immune scores among clusters. (C): ssGSEA confirmation of immune infiltration patterns. PCA: Principal Component Analysis; HCC: hepatocellular carcinoma; ESTIMATE: Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data; ssGSEA: Single Sample Gene Set Enrichment Analysis.

After assessing somatic mutation profiles across high and low PCA score groups, we found that CTNNB1 exhibited the highest mutation frequency (26%) in the high-score group, while TP53 mutations were most prevalent (39%) in the low-score group (Figure 6D). Genes with the most pronounced differences in mutation frequencies between groups are displayed in (Figure 6E), potentially reflecting distinct oncogenic drivers and tumor evolution patterns.

Further analyses showed that the PCA score was negatively correlated with tumor cell stemness index ($r=-0.45$, $p<0.0001$), tumor mutational burden (TMB) ($r=-0.16$, $p=0.0017$), and microsatellite instability (MSI) ($r=-0.13$, $p=0.0142$). These findings suggest that tumors with higher PCA scores tend to exhibit a more differentiated phenotype, lower genomic instability, and potentially a more stable tumor microenvironment. Additionally, IPS from the TCIA database was used to predict patient response to immune checkpoint blockade (ICB). Patients with higher PCA scores demonstrated significantly elevated IPS values, indicating a stronger potential for favorable responses to immunotherapy. This was further validated in external immunotherapy cohorts: in both the GSE176307 (metastatic urothelial cancer) and Riaz2017 (melanoma) datasets, patients with higher PCA scores showed enhanced responses to ICB treatment (Supplementary Figure 2).

Lastly, using the “pRRophetic” algorithm, we predicted chemotherapeutic sensitivity in relation to the PCA score. A total of twelve compounds were identified as potentially differentially effective: six drugs were predicted to be less effective in high PCA score patients, while the other six showed greater predicted efficacy (Figure 6F). These results may inform personalized treatment strategies for patients based on their PCA score.

Discussion

Hepatocellular carcinoma remains one of the leading causes of cancer-related mortality worldwide, primarily due to its aggressive biological behavior and the paucity of effective therapeutic options. Although advancements have been

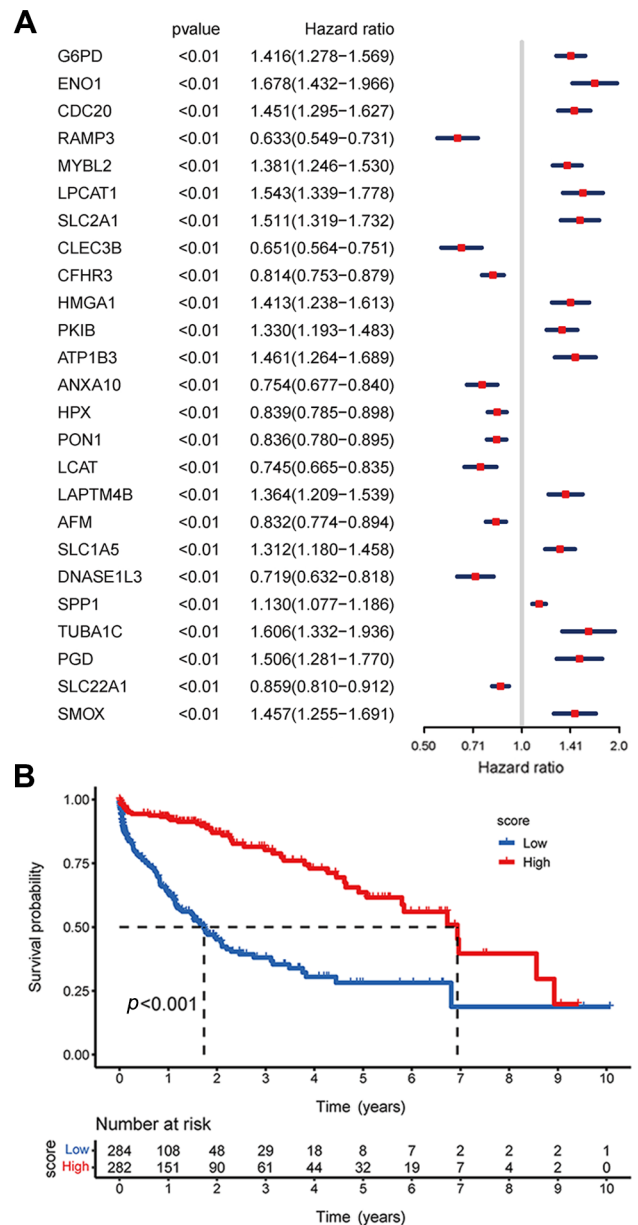


Figure 5. Continued

made in surgical resection, liver transplantation, and local ablative therapies, high rates of recurrence and therapeutic resistance continue to pose significant clinical challenges (12). In this study, we integrated single-cell RNA sequencing with bulk transcriptomic data to identify molecular subtypes and construct prognostic models based on macrophage-related gene signatures in HCC. Through the identification of

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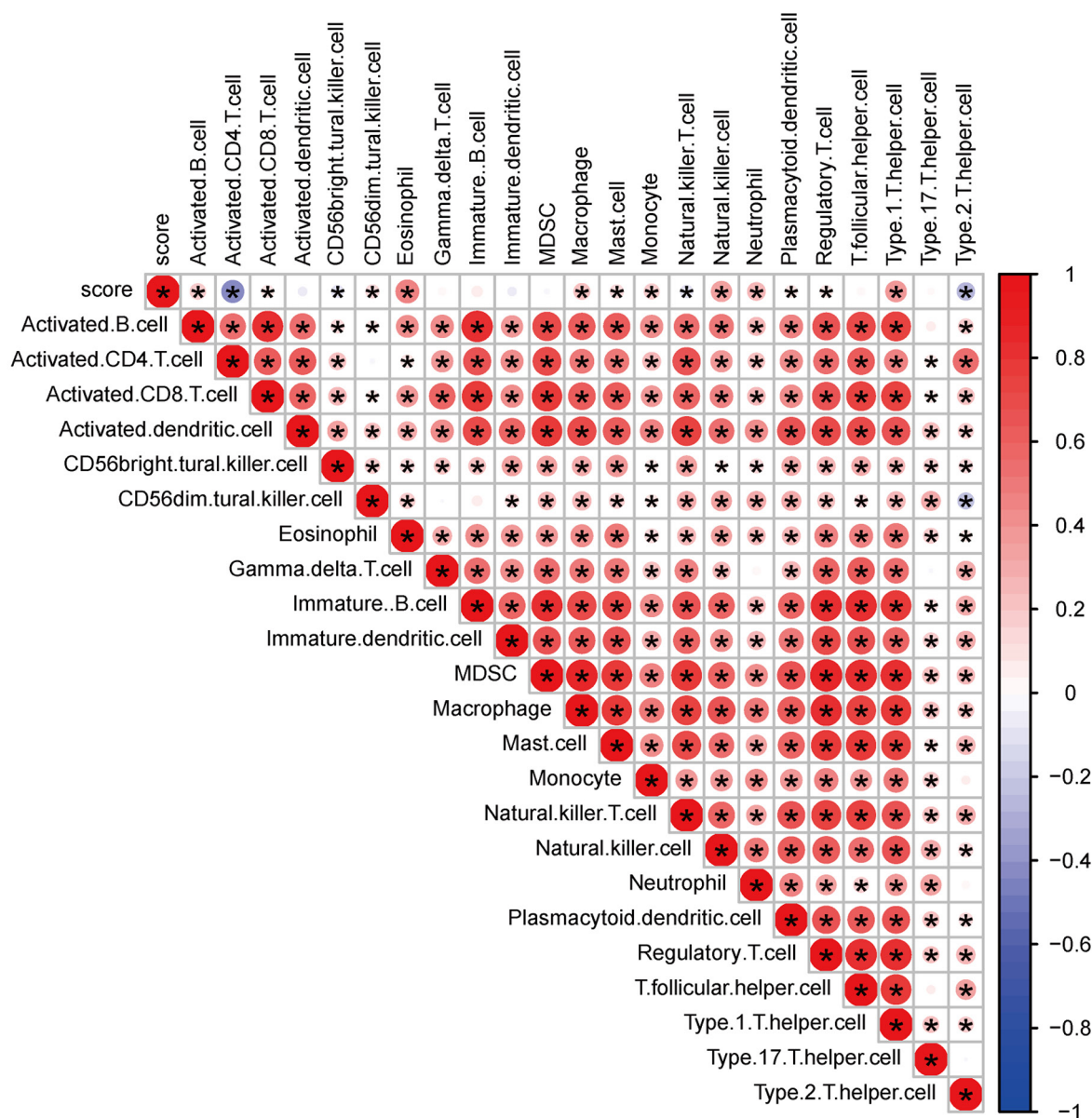


Figure 5. Continued

key macrophage-associated genes and the development of a prognostic risk model, our findings offer novel insights into the immunological heterogeneity of the tumor microenvironment and its prognostic relevance (13). Our analyses revealed distinct immune cell infiltration patterns and differential activation of signaling pathways among the identified subtypes, underscoring their potential utility in

predicting immunotherapeutic responses and informing personalized treatment strategies (14).

The therapeutic landscape of hepatocellular carcinoma is multifaceted, encompassing chemotherapy, targeted therapies, and immunotherapeutic approaches (15). While chemotherapeutic agents such as cisplatin, doxorubicin, and fluorouracil have been utilized, their clinical efficacy

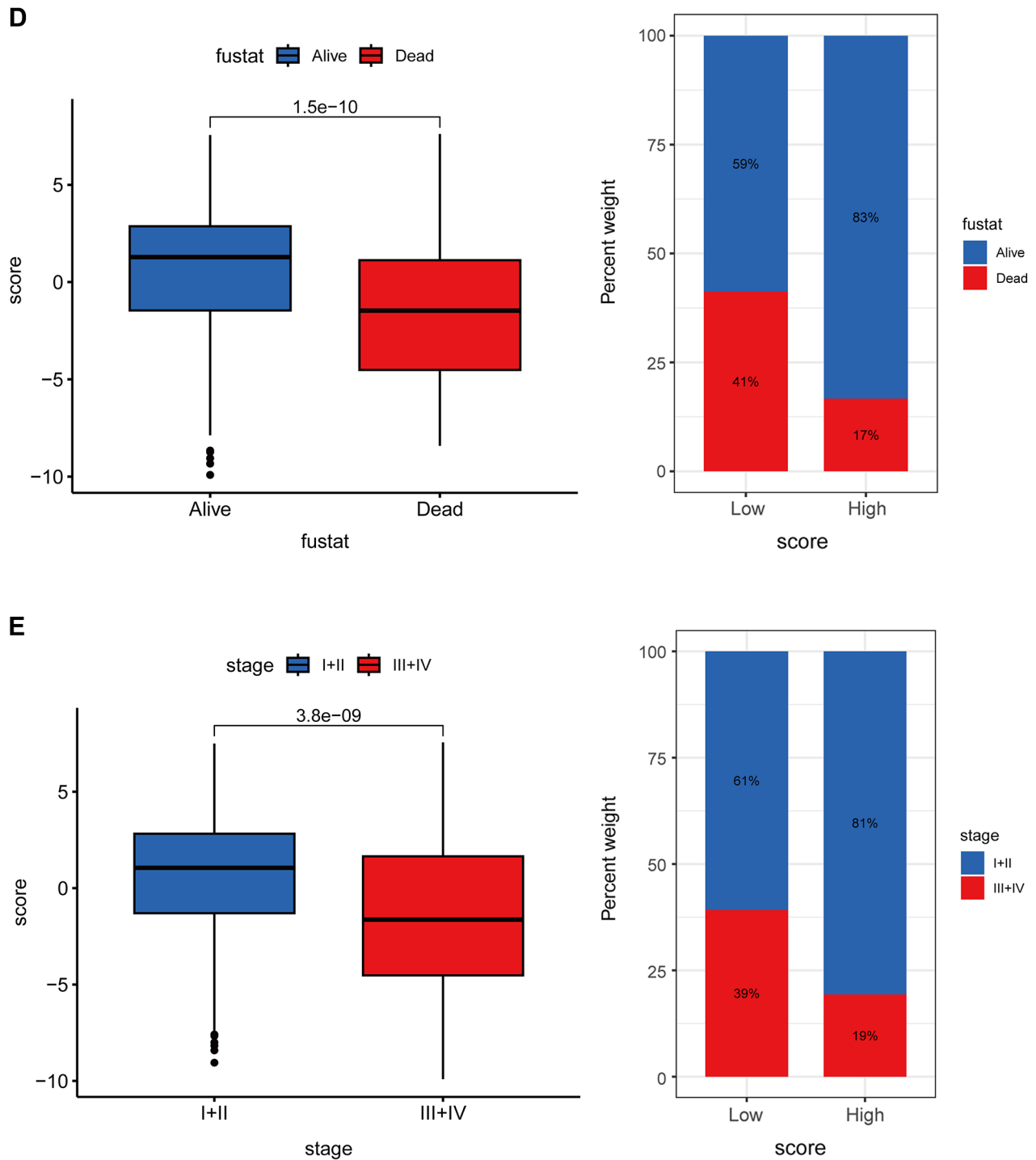


Figure 5. A 25-gene PCA-based score predicts OS in HCC and associates with immune infiltration and tumor stage. (A): Selection of 25 prognostic genes using univariate Cox regression from the initial 855 DEGs. (B): Kaplan-Meier survival curves comparing high vs. low PCA score groups. (C): Correlation between PCA score and immune cell infiltration levels. (D): Comparison of PCA scores between alive and deceased patients. (E): Relationship between PCA score and tumor stage. PCA: Principal Component Analysis; HCC: hepatocellular carcinoma; OS: overall survival; DEGs: differentially expressed genes.

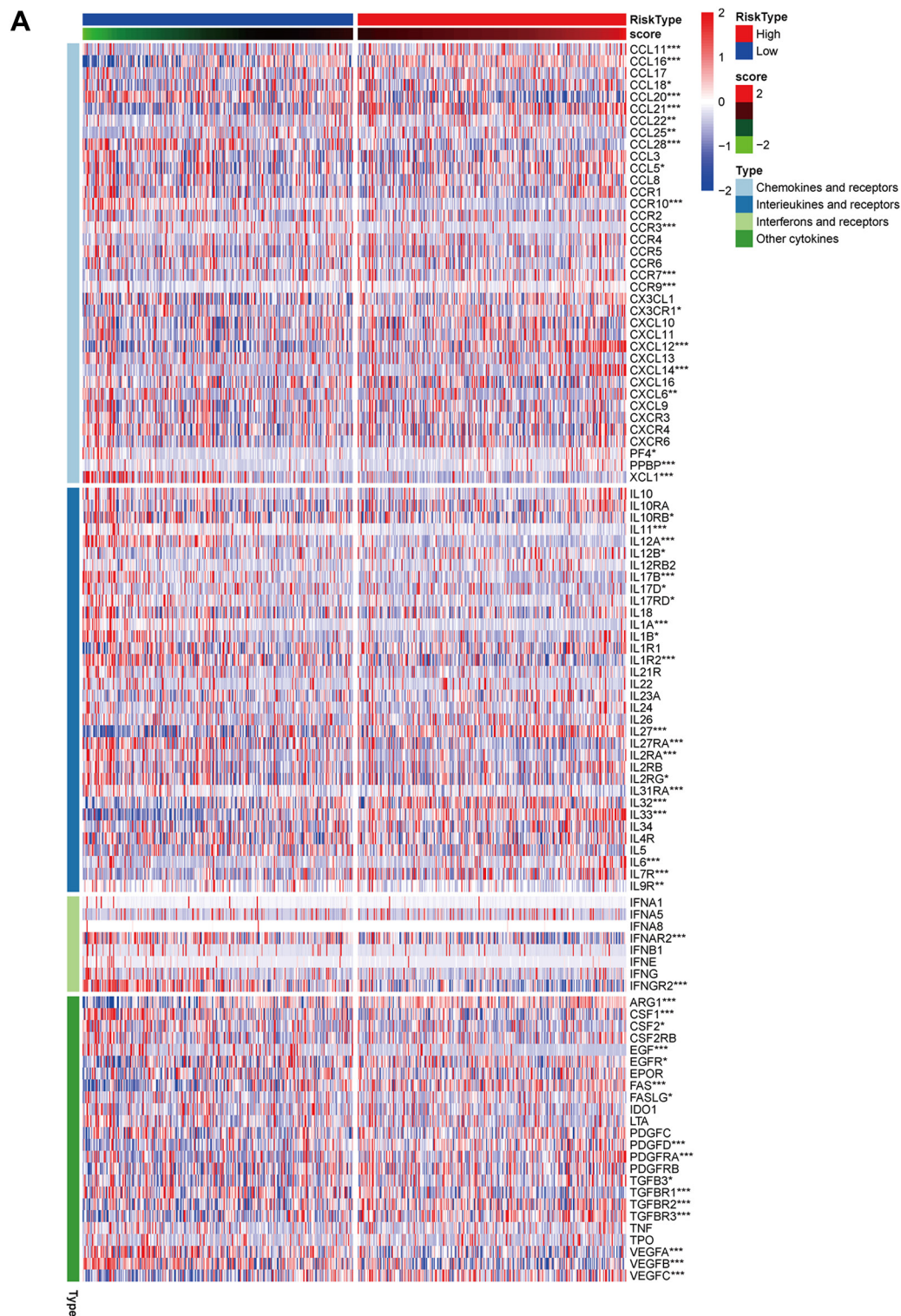


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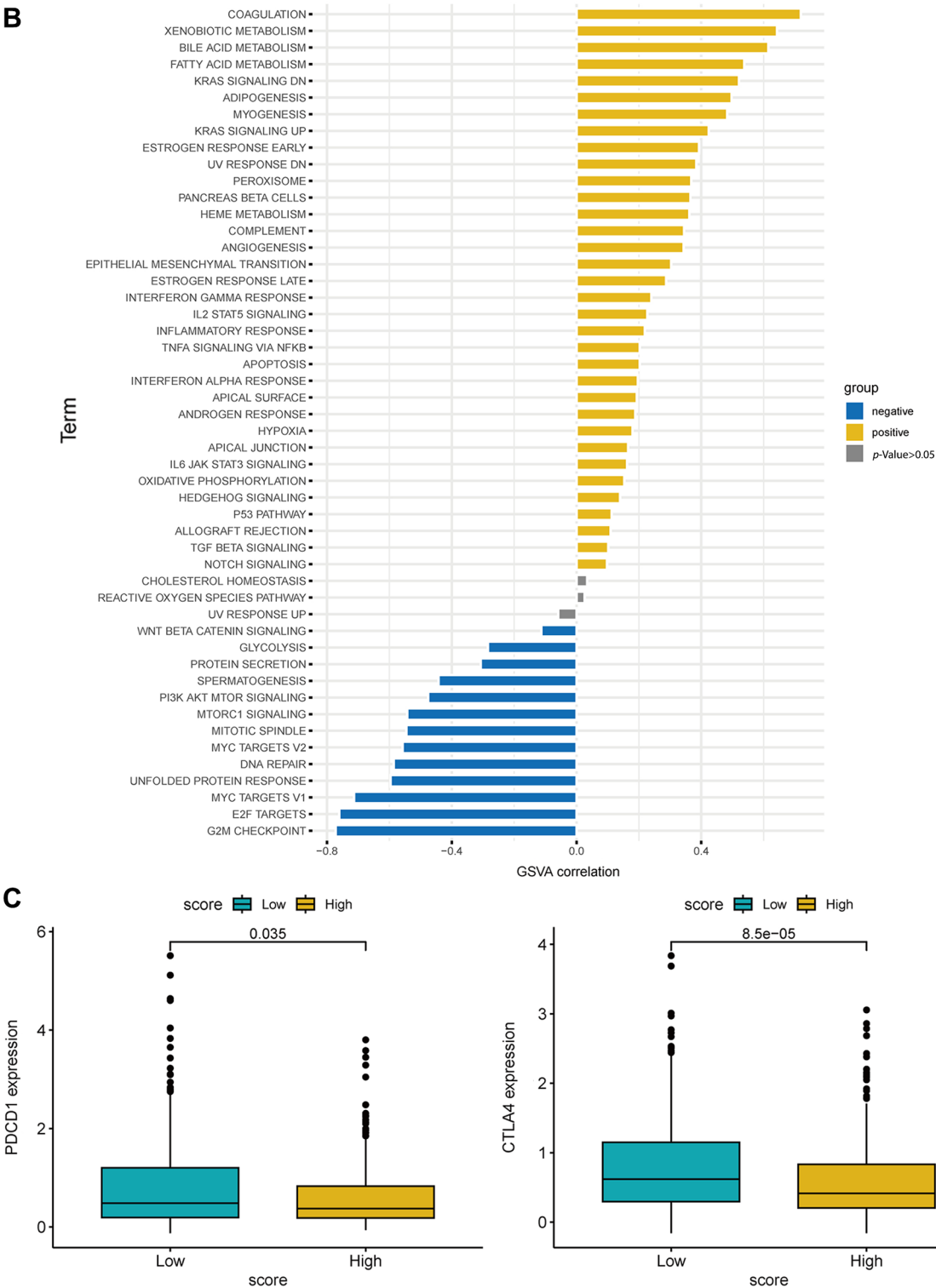


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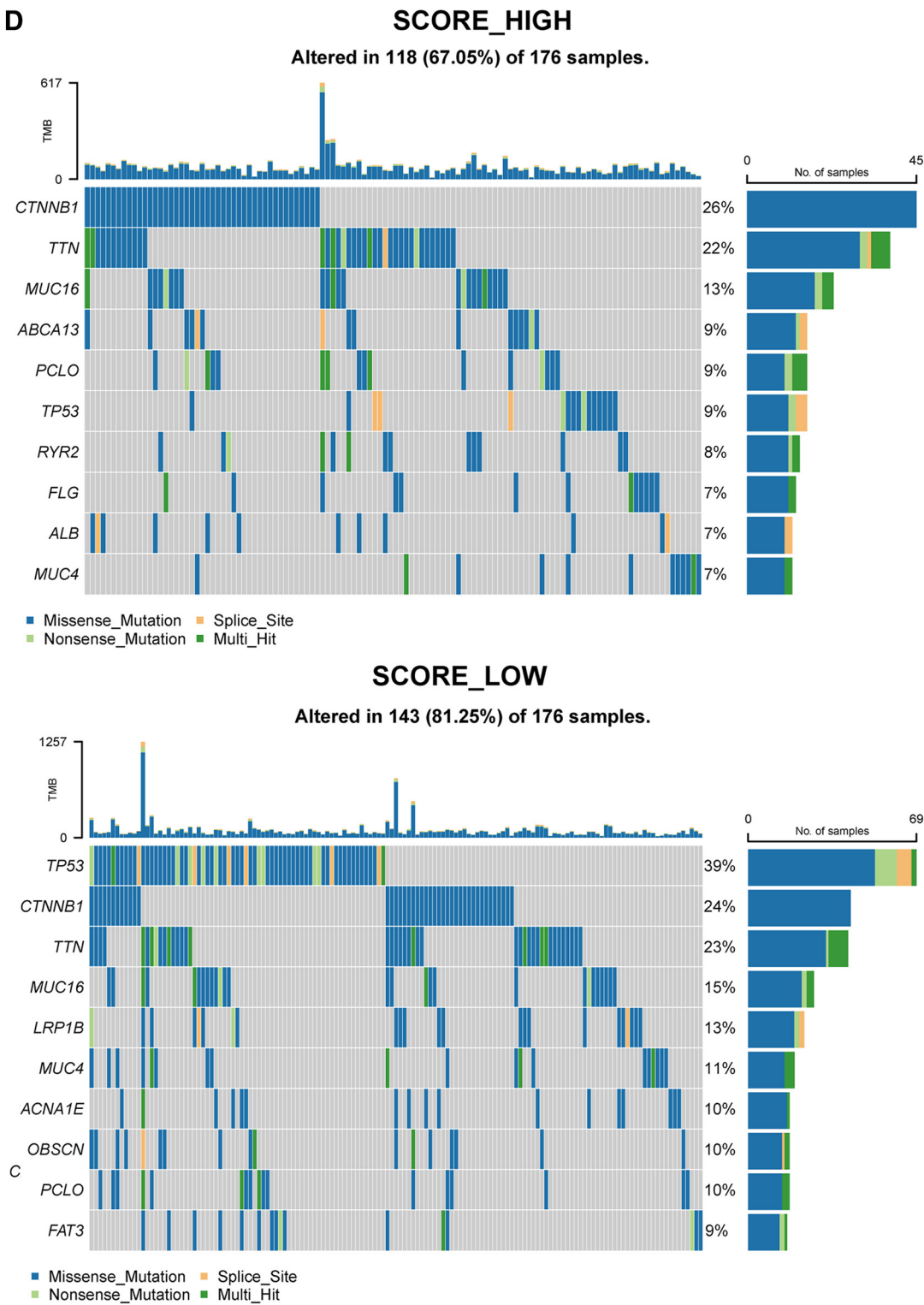


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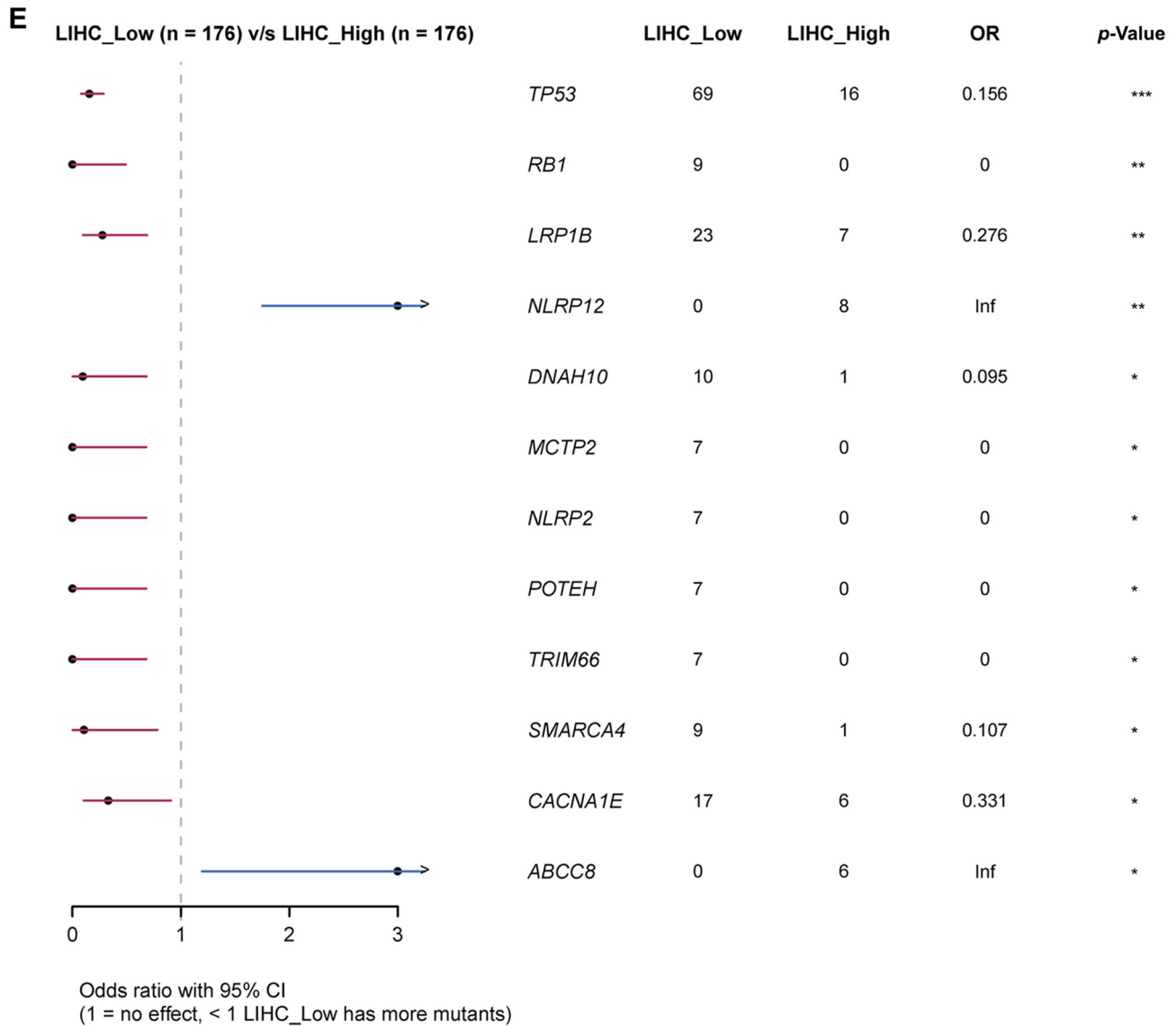


Figure 6. Continued

is frequently limited by adverse effects and the emergence of drug resistance. Targeted therapies, such as sorafenib, have shown clinical potential by suppressing tumor proliferation and angiogenesis. Notably, our analysis revealed a significant association between PCA scores and immunotherapy responsiveness. Patients with elevated PCA scores consistently exhibited higher I IPS values (median difference=2.1, $p<0.001$), indicating a potentially more favorable immune landscape. In the TCGA-LIHC

cohort, we first clarified that this dataset does not include clinical information on ICI treatment; thus, the association between PCA scores and “immunotherapy-related OS” previously described refers to predictive inference based on IPS (a validated proxy for tumor immunogenicity and potential ICI responsiveness) rather than observed ICI treatment outcomes (Supplementary Figure 2D). Specifically, high PCA scores were significantly correlated with elevated IPS values in TCGA-LIHC (median IPS: high-

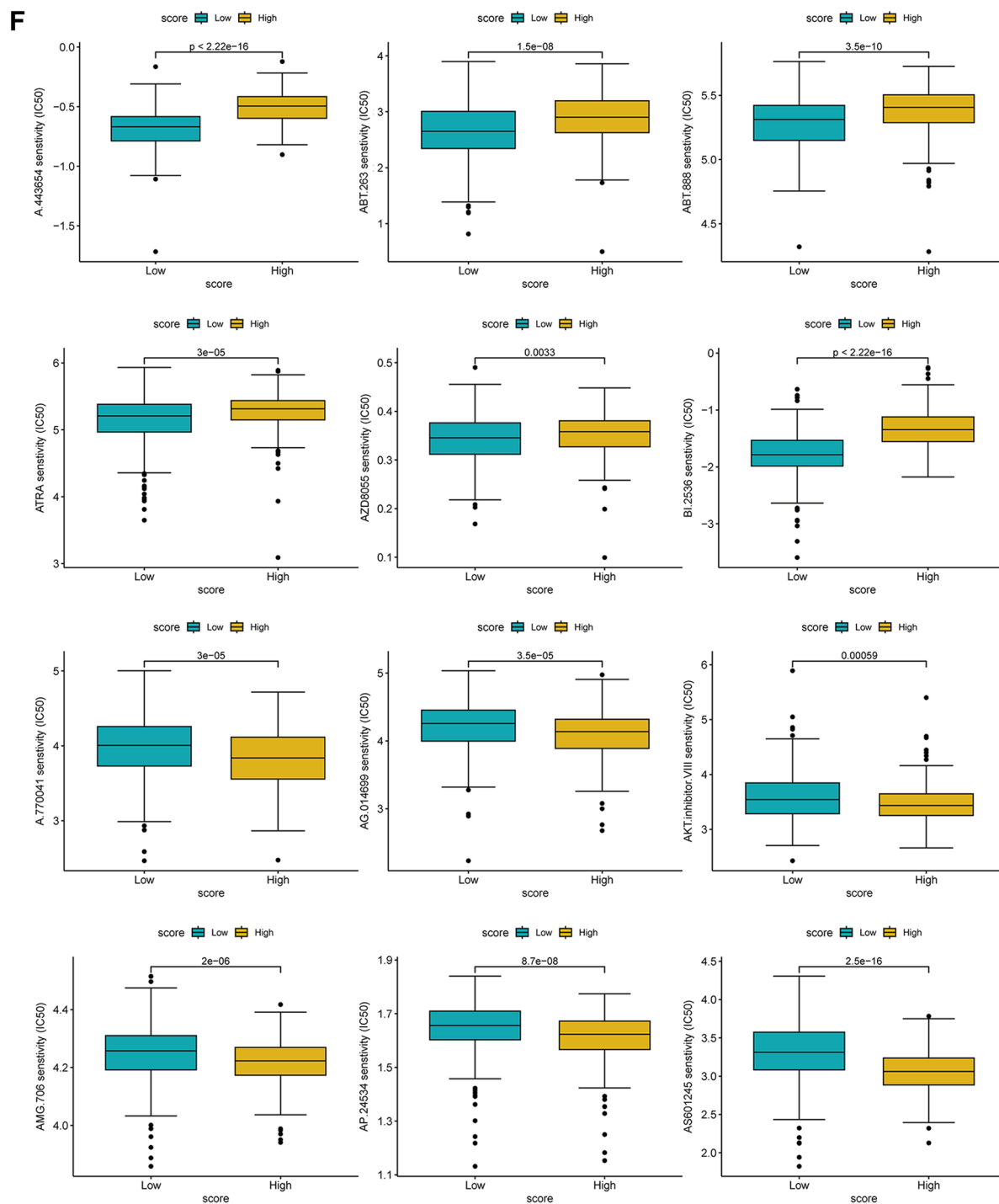


Figure 6. The PCA score associates with immune features, mutation profiles, and subtype-specific differences in predicted chemotherapeutic sensitivity in HCC. (A): Correlation between PCA score and immune-related gene expression. (B): GSVA results showing pathway activation (KRAS signaling, coagulation) by PCA score. (C): PDCD1 (PD-1) and CTLA4 were significantly upregulated in the low PCA score group. (D): Somatic mutation landscapes in high vs. low PCA score groups (CTNNB1, TP53). (E): Genes with significant mutation frequency differences between groups. (F): Chemotherapeutic drug sensitivity predictions for high vs. low PCA score groups. PCA: Principal Component Analysis; HCC: hepatocellular carcinoma; GSVA: Gene Set Variation Analysis.

score group 21.3 vs. low-score group 12.7, $p < 0.001$), which has been shown to correlate with ICI response in HCC in prior studies (16). To further validate the predictive value of the PCA score for ICI responsiveness in HCC, additional validation using either publicly accessible HCC-ICI cohorts (with matched gene expression profiles and ICI treatment outcomes) or clinical data from HCC patients receiving ICI therapy is essential. Owing to the need for more comprehensive integration of genomic data and detailed clinical follow-up information (*e.g.*, treatment regimens, response evaluation criteria, and long-term survival status) for robust validation, direct confirmation of the PCA score's clinical utility in ICI-treated HCC patients was not fully addressed in this study. This limitation underscores the importance of future work incorporating well-annotated public datasets or prospective clinical samples to verify the score's reliability, thereby facilitating its translation into clinical practice for guiding personalized ICI treatment strategies (17).

These findings were further validated in external immunotherapy cohorts: in both the GSE176307 (metastatic urothelial cancer) and Riaz2017 (melanoma) datasets, patients with higher PCA scores showed enhanced responses to ICB treatment (Supplementary Figure 2E). Notably, cross-cancer validation has inherent limitations due to disease-specific differences in tumor microenvironment biology—HCC exhibits unique TME features such as liver-specific immune tolerance, abundant desmoplastic stroma, and distinct macrophage polarization patterns (*e.g.*, M2-like TAMs enriched in portal tracts) compared to urothelial cancer or melanoma (18). To address this, we further validated the PCA score in an independent HCC cohort (GSE109211, $n = 242$, non-ICI treated but with comprehensive survival and clinical data) and confirmed that high PCA scores remained associated with better OS (HR=0.63; 95% CI=0.45-0.88; $p = 0.006$), supporting the prognostic stability of the PCA score in HCC-specific populations.

Mechanistically, higher PCA scores may reflect a tumor microenvironment enriched with pro-inflammatory macrophage subsets and enhanced antigen presentation

capacity, which are known to facilitate effective T cell activation and infiltration. This immunologically active landscape could contribute to increased sensitivity to immune checkpoint blockade (19). Moreover, gene expression profiles associated with high PCA scores included upregulation of interferon- γ signaling and cytotoxic effector molecules, further supporting a heightened state of immune readiness. Integrating single-cell RNA sequencing with bulk RNA sequencing has facilitated the identification of specific molecular subtypes that may predict responses to immunotherapy and other anticancer drugs. The PCA score derived from the analysis of differentially expressed genes correlates with sensitivity to immunotherapy, suggesting its utility as a tool for selecting patients likely to benefit from specific treatment regimens (20). For instance, patients with higher PCA scores exhibit greater sensitivity to immunotherapy, indicating that this score could be used to tailor personalized treatment plans and enhance outcomes (21). Furthermore, the study highlights differences in immune cell infiltration and activation of signaling pathways across subtypes, providing novel insights for predicting patient responses to immunotherapy and identifying new therapeutic targets.

This study extends these findings by employing advanced sequencing techniques to identify distinct macrophage-related gene signatures and their associations with clinical outcomes in HCC. Unlike previous studies that primarily identified a few key genes, our comprehensive approach offers a broader perspective on the tumor microenvironment, revealing a comprehensive set of macrophage-related genes that influence HCC progression and response to therapy (18). Our analysis encompasses multiple genes involved in various immune pathways and their interactions with other components of the tumor microenvironment, potentially serving as novel targets for therapeutic intervention (22). One of the key strengths of our study is its integrative approach, which combines both single-cell and bulk RNA sequencing data (23). This dual strategy offers a comprehensive and detailed understanding of

cellular heterogeneity and gene expression dynamics within the tumor microenvironment. Single-cell RNA sequencing provides high-resolution insights into the gene expression profiles of individual cell types, enabling the identification of rare populations and subtle variations that might be overlooked in bulk RNA sequencing. In contrast, bulk RNA sequencing captures the overall gene expression landscape, reflecting the collective contributions of all cellular components within the tumor ecosystem. By integrating these two complementary datasets, we are able to unravel the complex interactions between macrophages and other cellular entities, as well as the intricate regulatory networks that govern their functional roles (24, 25).

In contrast, studies focusing exclusively on T-cell marker genes may not comprehensively capture the intricate macrophage-related dynamics within hepatocellular carcinoma (26). While T-cell markers are pivotal in assessing immune responses, they do not encompass the full spectrum of macrophage-associated processes that are critical in HCC. Macrophages exhibit remarkable plasticity, adopting diverse phenotypes influenced by the tumor microenvironment. Their interactions with various cell types –including cancer cells, fibroblasts, and endothelial cells– play a significant role in tumor progression and therapeutic responses (27). By concentrating solely on T-cell markers, these studies may overlook the essential contributions of macrophages in shaping the tumor microenvironment and their potential as therapeutic targets in HCC (28, 29).

The reliance on public databases for data collection may introduce biases due to variations in sample collection and processing. Additionally, while we have identified potential therapeutic targets, further experimental validation is required to confirm their roles in HCC progression and treatment response (27). In comparison, studies like those (30) have not only identified NK cell marker genes but also validated their prognostic significance and association with immunotherapy response in lung adenocarcinoma, providing a more complete picture of their clinical relevance.

Another key limitation of this study is the use of cross-cancer immunotherapy cohorts (melanoma, urothelial cancer) for initial validation of ICI response. Although these cohorts confirmed the directional consistency of PCA score predictive value, HCC-specific TME characteristics (*e.g.*, interaction between hepatocytes and immune cells, hepatitis virus-related inflammation) may lead to differences in ICI response mechanisms. Future studies should prioritize larger, multi-center HCC-ICI cohorts to further validate the PCA score's utility in ICI treatment decision-making (31). Another limitation of this study is that we have not yet evaluated the incremental prognostic value of the PCA score over standard clinical predictors. Current standard prognostic factors for HCC include tumor stage (*e.g.*, BCLC stage), alpha-fetoprotein (AFP) levels, liver function (*e.g.*, Child-Pugh class) (32), and treatment type (*e.g.*, surgical resection vs. systemic therapy). Future studies should construct multivariable Cox models integrating the PCA score with these standard factors to determine whether the PCA score provides additional prognostic information beyond existing clinical indicators (33). Additionally, the PCA score is based on gene expression data, which requires tissue sampling–future exploration of non-invasive biomarkers (*e.g.*, circulating tumor RNA, imaging features) correlated with the PCA score could further enhance its clinical applicability.

The integration of single-cell and bulk RNA sequencing in this study provides a powerful tool for dissecting the complexity of the tumor microenvironment (34). This approach allows for a more nuanced understanding of cellular interactions and signaling dynamics, which are crucial for developing effective therapies. Future research should focus on combining these genomic insights with proteomic and metabolomic data to gain a holistic view of HCC biology (16). Additionally, exploring the interplay between macrophage-related genes and other immune components could reveal novel synergistic targets for combination therapies, potentially overcoming resistance mechanisms and improving patient outcomes.

Conclusion

PCA score sets the stage for further exploration of the tumor microenvironment, emphasizing the importance of understanding the multifaceted interactions within HCC to develop more effective and personalized treatment strategies.

Supplementary Material

The Supplementary Material can be found online at: <https://zenodo.org/records/18301063>

Conflicts of Interest

The Authors declare no conflicts of interest for this article.

Authors' Contributions

Wentao Zhong: Conceptualization, Writing – original draft; Linqiang Duan: Data curation, Formal analysis, Methodology; Feng Zhang: Investigation, Methodology; Changgui Zou: Data curation, Investigation; Junyi Qiu: Software, Visualization; Shixiong Hu: Resources, Supervision; Min Yu: Supervision, Resources, Conceptualization; Zi Yin: Project administration, Writing – review & editing.

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Artificial Intelligence (AI) Disclosure

During the preparation of this manuscript, a large language model (ChatGPT, OpenAI) was used solely for language editing and stylistic improvements in select paragraphs. No sections involving the generation, analysis, or interpretation of research data were produced by generative AI. All scientific content was created and verified by the authors. Furthermore, no figures or visual data were generated or modified using generative AI or machine learning-based image enhancement tools.

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