

Prognostic value of M2 macrophage-related genes and their importance in the immunotherapy response in hepatocellular carcinoma

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Abstract. Liver hepatocellular carcinoma (LIHC) is a major contributor to cancer-associated mortality worldwide, with a poor prognosis due to late-stage diagnosis and limited therapeutic options. M2 macrophages serve crucial roles in the tumor microenvironment (TME), contributing to tumor progression, immune evasion and resistance to therapy. However, the prognostic importance of M2 macrophage-related genes in LIHC and their potential for predicting responses to immunotherapy remain underexplored. A bioinformatics analysis was performed using The Cancer Genome Atlas data to identify M2 macrophage-related genes in LIHC. Differential expression and weighted gene co-expression network analysis were used to identify key gene modules and a prognostic model was developed and validated using Kaplan-Meier analysis and receiver operating characteristic curves. The immune therapy response was assessed using tracking of indels by

decomposition and Submap analyses. Killer cell lectin like receptor B1 (KLRB1) was knocked down in HuH-7 cells and overexpressed in HuH-1 cells to evaluate its effect on tumor cells and macrophage regulation. The effect on human umbilical vein endothelial cell tube formation was also assessed. A total of two M2 macrophage-related genes (KLRB1 and phosphatidylinositol-5-phosphate 4-kinase type 2α) were identified as notable prognostic biomarkers for LIHC. The prognostic model demonstrated certain predictive power, with high-risk patients exhibiting markedly worse overall survival. This model was validated in external datasets and associated with immune infiltration patterns. Furthermore, low-risk patients were more likely to respond to immune checkpoint blockade therapy. The inhibition of KLRB1 enhanced LIHC cell activity and increased macrophage polarization from the M0 phenotype to the M2 phenotype by regulating LIHC cell secretions. In conclusion, M2 macrophage-related genes are valuable prognostic biomarkers in LIHC. The prognostic model effectively stratifies patients by survival risk and predicts immunotherapy responses, thereby highlighting the potential for improved TME-targeted therapies in LIHC. The mechanism of KLRB1 regulation in LIHC-macrophage interactions and its impact on LIHC activities were also evaluated.

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Abbreviations: LIHC, liver hepatocellular carcinoma; TME, tumor microenvironment; NK, natural killer; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; ICGC, International Cancer Genome Consortium; WGCNA, weighted gene co-expression network analysis; ROC, receiver operating characteristic; scRNA-seq, single-cell RNA sequencing; Tex, CD8⁺ exhausted T cells; OS, overall survival; CCK-8, Cell Counting Kit-8; PI, propidium iodide; RT-qPCR, reverse transcription quantitative PCR; LUAD, lung adenocarcinoma

Key words: liver hepatocellular carcinoma, M2 macrophages, prognostic risk model, bioinformatics analysis, immunotherapy prediction, tumor immune microenvironment

Introduction

Liver hepatocellular carcinoma (LIHC) is a major contributor to cancer-associated mortality worldwide, accounting for a notable proportion of global cancer mortalities with a 5-year-survival probability of ~18%; of these occurrences, ~90% are classified as HCC (1). Despite advancements in surgical techniques, liver transplantation and systemic therapies, the prognosis for patients with LIHC remains poor (2). This poor prognosis is primarily due to late-stage diagnoses, high recurrence rates and resistance to conventional treatments (3,4). Furthermore, the lack of reliable early detection biomarkers and effective therapeutic options exacerbates the challenges in managing LIHC (5). Consequently, there is an urgent need to identify novel molecular biomarkers that can serve as prognostic indicators and guide therapeutic decision-making, thus ultimately improving patient outcomes.

The tumor microenvironment (TME) is a crucial driver of cancer progression, with macrophages (particularly M2 macrophages) serving a central role in modulating immune responses (6). M2 macrophages promote tumor growth, metastasis and immune evasion by secreting cytokines such as IL-10 and TGF- β , which suppress the activation of cytotoxic T cells and natural killer (NK) cells (7,8). Research has demonstrated that M2 macrophages notably influence the progression of LIHC by promoting tumor growth and metastasis (9). M2 macrophages are generally considered to possess immunosuppressive properties, which enable them to create a tumor-supportive microenvironment. These cells achieve this effect by secreting a variety of cytokines and chemokines that facilitate tumor cell survival and dissemination (10,11). Previous studies have revealed that M2 macrophages contribute to the initiation and progression of liver cancer via interactions with hepatic stellate cells within the LIHC microenvironment (12,13). This crosstalk not only enhances the polarization of M2 macrophages but also promotes tumor immune evasion by inhibiting the activity of CD8⁺ T cells. Consequently, therapeutic strategies targeting M2 macrophages may offer novel approaches for the treatment of LIHC (11,14). Furthermore, M2 macrophages contribute to resistance to chemotherapy and immunotherapy, thus positioning these cells as critical targets for novel therapeutic strategies (15). The elucidation of the molecular mechanisms underlying M2 macrophage polarization in LIHC may provide valuable insights into improving prognostic assessment and enhancing treatment efficacy.

The present study aimed to explore the prognostic significance of M2 macrophage-associated genes in LIHC and create a predictive model for patient outcomes. Using data from The Cancer Genome Atlas (TCGA) database, key genes associated with M2 macrophage infiltration were identified using the weighted gene co-expression network analysis (WGCNA) algorithm. A risk model was constructed based on these genes and validated using survival analysis. Additionally, the potential of these genes as therapeutic targets was examined by assessing their relationships with immune checkpoint expression and responses to immunotherapy. The findings offer valuable insights into the role of M2 macrophages in LIHC and identify potential biomarkers for prognosis and immunotherapy strategies.

Materials and methods

Data collection and preprocessing. The primary dataset used for data analysis included the LIHC cohort from the TCGA database, which included gene expression profiles and clinical data for patients with LIHC. The raw RNA sequencing (RNA-seq) data were processed and normalized to the transcripts per million format to standardize the gene expression levels across all samples. In addition, samples with a reported survival time of 0 days were excluded from the analysis to ensure the integrity and reliability of the survival data. To validate the prognostic model, two independent external validation datasets were used. The first dataset (GSE76427) was retrieved from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>), and the second dataset (LIRI-JP) was obtained from the International Cancer Genome Consortium (ICGC) database (<https://dcc.icgc.org/>).

Evaluation of macrophage immune infiltration in LIHC. To assess macrophage immune infiltration in LIHC and normal tissue samples, six widely used immune infiltration estimation methods were applied: CIBERSORT (16), xCell (17), TIMER (18), QUANTISEQ (19), EPIC (20) and MCPCOUNTER (21). These algorithms, which leverage transcriptomic data, were used to quantify the relative levels of macrophage infiltration in both LIHC and normal samples. Additionally, the present study focused on the differential expression of M2 macrophages and performed comparative analyses of gene expression between patients with LIHC and normal controls.

Network construction, module preservation and correlation analysis. WGCNA was employed to identify gene modules associated with M2 macrophages (22). After excluding outlier samples, the top 25% most variable genes were selected for subsequent analysis. The optimal soft threshold for constructing the network was determined by evaluating the scale-free topology model fit. A power value that resulted in a fit close to 0.9 was chosen. The gene co-expression network was subsequently constructed and modules were identified based on hierarchical clustering. Preservation analysis was performed to assess the reproducibility of the identified modules. The modules were categorized as being strongly preserved ($Z > 10$), weakly preserved ($2 < Z < 10$) or not preserved ($Z < 2$). Modules with poor preservation were excluded from further analysis. Finally, the correlation between M2 macrophage expression levels and the identified gene modules was assessed using various immune infiltration methods. The modules with the greatest correlations with M2 macrophages were selected for further modelling.

Prognostic gene selection and M2 macrophages-related model development. Univariate Cox regression analysis was performed on 292 genes to identify those associated with prognosis in LIHC. Genes with P-values < 0.05 were selected for further investigation. To refine this selection, Least Absolute Shrinkage and Selection Operator (LASSO) regression was performed with 10-fold cross-validation to identify the optimal regularization parameter (λ). A λ -value that optimized the model fit while minimizing overfitting was chosen. Multivariate Cox regression was subsequently performed to evaluate the independent prognostic value of the selected genes. A prognostic risk score model was developed based on the expression levels of these genes, which were weighted by their respective regression coefficients. The risk score formula was calculated by multiplying the expression level of each gene by its corresponding coefficient. To assess the model's predictive accuracy, Kaplan-Meier survival analysis and time-dependent receiver operating characteristic (ROC) curves were used to evaluate overall survival (OS) and model performance in both the training cohort (GSE76427) and independent validation cohorts (LIRI-JP).

Functional enrichment analysis. To examine the differential activation of biological pathways between the two risk groups, gene set variation analysis (GSVA) was performed on the hallmark gene sets (23). GSVA scores were computed for each gene set and differential expression analysis was performed to

identify the pathways with significant differential activation between the two groups. In addition, Gene Ontology (GO) analysis was performed to identify biological processes and cellular components that were differentially enriched in the two risk groups. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis with the package 'clusterProfiler' of R studio (24) was also performed to examine differences in signaling pathway activation between the groups. For all the analyses, statistical significance was assessed using the Benjamini-Hochberg method to correct for multiple comparisons.

Immune infiltration, tumor mutational burden (TMB) and immune checkpoint analysis. To assess the relationships between immune characteristics and risk stratification, the ESTIMATE algorithm (ESTIMATE: Home) was first applied to quantify the scores for each patient with LIHC. Pearson correlation analyses were subsequently performed to examine the associations between these scores and the prognostic risk score. The TMB was also evaluated for each patient and a correlation analysis was performed with the risk score to determine whether the TMB was associated with the risk profile. To further explore immune responses, we employed the 'limma' package of R studio to conduct differential expression analysis was performed on common inhibitory and stimulatory immune checkpoint genes with the threshold of $\log_2$ fold change >1.5 and $P < 0.05$. The expression of these genes was compared between the two risk groups to investigate their potential roles in immune evasion and therapeutic resistance. Additionally, the Tumor Immune Dysfunction and Exclusion (TIDE) algorithm was employed to assess the immune escape potential in both risk groups (25). Finally, the Submap algorithm was applied to predict the likelihood of patient response to immune checkpoint blockade (ICB) therapy based on the gene expression signatures (26).

Single-cell (sc)RNA-seq based on the tumor immune single cell Hub 2 (TISCH2) database. The killer cell lectin like receptor B1 (KLRB1), with the smallest P-value in the multivariate Cox regression, was analyzed across multiple single-cell datasets to investigate its role in the TME. Using the TISCH2 database (27), the expression of KLRB1 was assessed in six independent scRNA-seq datasets (<http://tisch.compbio.cn/home/>). The expression levels of KLRB1 were compared across various immune cell types, including CD8⁺ T cells, CD8⁺ exhausted T cells (Tex) and NK cells. Ligand-receptor interactions were also examined between different cell populations using a heatmap to visualize the strength of these interactions. The interactions were further represented as a network diagram to illustrate the intensity or activity of ligand-receptor signaling, specifically between CD8⁺ T cells and other cell types in the TME.

Pan-cancer expression and prognostic analysis of KLRB1. To investigate the potential role of KLRB1 in cancer biology, a pan-cancer analysis was performed using data from TCGA. First, KLRB1 expression across various cancer types (including LIHC) was assessed to identify significant differences in expression. The prognostic value of KLRB1 was subsequently examined by analyzing its expression in relation

to clinical outcomes, including the disease-free interval (DFI), disease-specific survival (DSS), OS and platinum-free interval (PFI) parameters across various cancer types. A forest plot analysis was performed to further investigate the relationship between KLRB1 expression and OS across various cancers. Finally, the samples were categorized into high- and low-KLRB1 groups and differential expression analysis was performed. Gene Set Enrichment Analysis (GSEA) was subsequently used to identify enriched biological pathways in the high- and low-expression groups, thus providing insights into the potential mechanisms through which KLRB1 influences tumor progression.

Cell culture. The HuH-1 (CL-0811), HuH-7 (CL-0120), and THP-1 (CL-0233) cell lines were purchased from Procell Life Science & Technology Co., Ltd. The HuH-1 and HuH-7 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from GIBCO; Thermo Fisher Scientific, Inc.). The THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (all from GIBCO; Thermo Fisher Scientific, Inc.). Cultures were maintained in a humidified incubator at 37°C with a 5% CO₂ atmosphere to ensure optimal growth conditions.

Construction of KLRB1 knockdown and overexpression cell lines. A lentivirus-based short hairpin RNA (shRNA) strategy was employed using three distinct shRNA sequences to down-regulate KLRB1 expression in HuH-7 cells. These shRNA sequences were cloned into the lentiviral vector pLKO.1. Simultaneously, full-length KLRB1 complementary DNA (cDNA) was synthesized by PCR using total RNA extracted from HuH-1 cells and cloned into the lentiviral overexpression vector pLV-CMV-MCS-PGK-Puro to induce KLRB1 overexpression in HuH-1 cells. Both vectors were obtained from Addgene (Beijing Zhongyuan Co.). For lentivirus production, 10 µg of pLV-CMV-MCS-PGK-Puro was co-transfected with 7.5 µg of packaging plasmid (pMDLg/pRRE; Addgene; Beijing Zhongyuan Co.), 2.5 µg of envelope plasmid (VSV-G; Addgene; Beijing Zhongyuan Co.) and 15 µg of a helper plasmid (pRSV-Rev; Addgene; Beijing Zhongyuan Co.) using the calcium phosphate transfection method as part of a 3rd generation lentiviral system. Transfection was performed at 37°C for 48 h. Lentiviral particles were generated by co-transfecting 293T cells (Procell Life Science & Technology Co., Ltd.) with a packaging plasmid mix (REV: VSVG: PMDL=2:3:5) using TurboFect transfection reagent (cat. no. R0532; Fermentas; Thermo Fisher Scientific, Inc.), with virus supernatant harvested 36-48 h post-transfection and filtered for use in infection. HuH-7 and HuH-1 cells were seeded in 6-well plates until reaching 60-70% confluency before infection with lentiviral particles at a multiplicity of infection of 10, supplemented with poly-L-lysine (8 µg/ml) to enhance transduction. Following 12-16 h of co-culture with the virus, the medium was replaced with fresh culture medium. After 48 h, stable transductants were selected using 2 µg/ml puromycin (the original concentration was 1 mg/ml) to confirm successful transduction. All procedures were conducted at 37°C with 5% CO₂. The sequences utilized for KLRB1 knockdown and overexpression are shown in Table SI.

RNA extraction and reverse transcription quantitative PCR (RT-qPCR). According to the manufacturer's protocol, total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, a cDNA synthesis kit (cat. no. R212-01; Vazyme Biotech Co., Ltd.) was used to convert the RNA into cDNA. The qPCR experiments were conducted using SYBR Green qRT-PCR Master Mix (Vazyme Biotech Co., Ltd.), with GAPDH serving as the internal control for normalization and the $2^{-\Delta\Delta C_q}$ method (28) was used to analyze the difference. The thermocycling protocol consisted of an initial pre-denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 20 sec. The primer sequences used are listed in Table SII.

Cell proliferation assay. Cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. A total of 2,500 cells were seeded into 96-well plates and cultured for 3 days. A total of 10 μ l of CCK-8 solution was added to each well for 1 h before measurement of the absorbance at 450 nm at room temperature.

Transwell migration assay. A Transwell chamber (pore size, 8 μ m; Corning, Inc.) was used to assess the migratory ability of the cells. Target cells (typically 1×10^5 cells per well) were seeded into the upper chamber of the Transwell system, with serum-free medium used as the inducer. The lower chamber contained complete culture medium supplemented with 10% serum (GIBCO; Thermo Fisher Scientific, Inc.) as a chemoattractant to guide cell migration. The cells were incubated at 37°C in a 5% CO₂ incubator for 24 to 48 h, thus allowing the cells to migrate through the membrane into the lower chamber. After incubation, non-migrating cells in the upper chamber were gently removed with a cotton swab. The cells in the lower chamber were subsequently washed with PBS, fixed with methanol at room temperature for 30 min and stained with 0.1% crystal violet solution at room temperature for 10 min. Finally, the stained cells in the lower chamber were observed and counted under an inverted microscope to assess the migratory ability of the cells through the software of Image J (version 1.8.0_345; National Institutes of Health).

Annexin V/propidium iodide (PI) staining assay. To assess cell apoptosis, the Annexin V-FITC/PI (BioLegend, Inc.) dual-staining method was used. First, sh-KLRB1-2 and OE-KLRB1 cells were seeded into culture dishes and cultured until they reached 70-80% confluence. After treatment, the cells were collected and washed twice with PBS to remove residual culture medium. The cells were then resuspended in 1x binding buffer and stained with annexin V-FITC and PI solution. The mixture was gently vortexed and incubated at room temperature in the dark for 15 min. Annexin V-FITC binds to phosphatidylserine exposed on the surface of the cell membrane, thereby marking early apoptotic cells, whereas PI stains the nuclei of late apoptotic or necrotic cells with compromised membrane integrity. After staining, apoptosis was analyzed by flow cytometry (FACSAria III; BD Biosciences) and evaluation using FlowJo (version 10.8.1). The dual fluorescence signals from annexin V-FITC and PI allowed for the

distinction between healthy cells, early apoptotic cells, late apoptotic cells and necrotic cells, thereby providing a comprehensive assessment of the apoptotic status of the cells.

Cell tube formation assay. Human umbilical vein endothelial cells (HUVECs) (PUMC-HUVEC-T1; cat. no. CL-0675) were obtained from Procell Life Science & Technology Co., Ltd. The third to the fifth passage of HUVECs in the logarithmic growth phase were used for the subsequent experiments. The cell tube formation assay involved seeding HUVECs onto a solidified layer of Matrigel® and incubation for 4 to 6 h at 37°C to allow tube formation in a 96-well plate. After the cells were incubated with the coculture supernatant of HuH-7 or HuH-1 cells for 8 h at 37°C, tube-like structures formed. After the incubation period, the formation of tube-like structures was examined under a fluorescence microscope. The degree of tube formation was quantified by assessing parameters such as the number of branches and total branching length through Image J (version 1.8.0_345).

qPCR detection of macrophage cell polarization. To evaluate the polarization status of M0 cells, the expression levels of polarization marker genes were assessed using qPCR. The THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (all from GIBCO; Thermo Fisher Scientific, Inc.). Cultures were maintained in a humidified incubator at 37°C with a 5% CO₂ atmosphere to ensure optimal growth conditions. THP-1 cells were first treated with 100 nM phorbol 12-myristate 13-acetate for 48 h to induce differentiation into M0 cells. The culture supernatants from sh-KLRB1-2 and OE-KLRB1 cells were subsequently added to the M0 cells for 24 h at 37°C, after which the gene expression of the M1 and M2 markers was measured. According to manufacturer's protocol, total RNA was extracted from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcription was performed to generate cDNA according to the manufacturer's instructions using a reverse transcription kit (cat. no. R212-01; Vazyme Biotech Co., Ltd.). SYBR Green fluorescence dye (Vazyme Biotech Co., Ltd.) was used for qPCR. Specifically, the first stage involved pre-denaturation at 95°C for 5 min, for one cycle. Then the second stage began, which involved denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 20 sec, for 40 cycles. The relative expression levels of the M1 and M2 marker genes were calculated using the $2^{-\Delta\Delta C_q}$ method (28), with GAPDH serving as the internal control. The expression levels of both M1 markers (CD86 and IL-12) and M2 markers (IL-10 and CD206) were measured. The sequences of the primers utilized are listed in Table SII.

Western blot analysis. The total protein of cells was obtained using radioimmunoprecipitation assay buffer for 30 sec (cat. no. P0013B; Beyotime Institute of Biotechnology). The total protein was separated using 10% SDS-PAGE. The separated proteins were subsequently transferred to a polyvinylidene fluoride membrane (GE Healthcare). Following a 2-h blocking step at room temperature with 5% non-fat dry milk (Yili), the membranes were incubated with the

primary antibody overnight at 4°C. The primary antibodies were as follows: Bax (1:500 dilution; cat. no. A12009), Bcl-2 (1:1,000 dilution; cat. no. A19693), C-caspase-3 (1:500 dilution; cat. no. A11319) and β -actin (1:10,000 dilution; cat. no. AC038; all from ABclonal Biotech Co., Ltd.). Subsequently, the membranes were incubated with the secondary antibody (1:10,000 dilution; cat. no. AB_1185567; goat anti-rabbit IgG; Invitrogen; Thermo Fisher Scientific, Inc.) for 2 h at room temperature. The blots were visualized using an enhanced chemiluminescence system (Beyotime Institute of Biotechnology) and analyzed densitometrically using ImageJ software (version 1.8.0_345).

Statistical analysis. All bioinformatics analyses in the present study were performed using R Studio version 4.3.1 (<https://cran.rstudio.com/bin/windows/base/old/4.3.1/>), while data were processed with GraphPad Prism 9.0 (Dotmatics). Statistical comparisons between two groups were assessed using an unpaired Student's t-test, whereas differences among multiple groups were evaluated by one-way ANOVA and the Bonferroni's test was used as the post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. To ensure reproducibility, all experiments were repeated at least three times, with results expressed as the mean $\pm$ standard deviation.

Results

Differential expression of M2 macrophages in patients with LIHC and normal controls. Macrophage infiltration levels in LIHC and normal tissue samples were evaluated using six immune infiltration analysis methods. The heatmap shown in Fig. 1A illustrates the distribution of infiltrating macrophages in both LIHC and normal samples across the different methods. Differential expression analysis of M2 macrophages (Fig. 1B) revealed significant differences between patients with LIHC and normal controls. Notably, all analysis methods indicated relevant findings, except for the MCPCOUNTER method, which did not show a statistically significant difference. Specifically, quantTiseq exhibited a significant decrease, while the other methods demonstrated increases in expression. Consequently, the results are inconsistent across the different approaches. These results underscore the potential involvement of M2 macrophages in the immune microenvironment of LIHC.

Identification of M2 macrophage-related gene modules. The optimal soft threshold for constructing the gene co-expression network was determined to be 14, which yielded a scale-free topology model fit value approaching 0.9 (Fig. 2A). A total of 16 distinct gene modules were identified using WGCNA (Fig. 2B). Preservation analysis indicated that four modules (grey, gold, midnight blue and black) demonstrated Z scores < 2 , thereby indicating poor preservation; thus, these modules were excluded from further analysis (Fig. 2C). Correlation analysis between M2 macrophage expression levels and the gene modules revealed that the light cyan and yellow modules were the most strongly correlated with M2 macrophages (Fig. 2D). Genes from these two modules were selected for subsequent prognostic modelling.

Development and validation of the M2 macrophage-related genes prognostic model. Univariate Cox regression analysis identified 14 genes with significant associations with patient prognosis ($P < 0.05$) in the LIHC cohort (Fig. 3A). LASSO regression analysis (with a λ value of 0.0514) revealed three genes for inclusion in the final model, including KLRB1 (Fig. 3B). Multivariate Cox regression analysis validated the independent prognostic value of these three genes. The prognostic risk score model was constructed based on the following formula: RiskScore = phosphatidylinositol-5-phosphate 4-kinase type 2 α (PIP4K2A) $\times$ 1.0165 + KLRB1 $\times$ 0.8897 (Fig. 3C). Kaplan-Meier survival analysis revealed significant differences in survival between the two risk groups (Fig. 3D). The model's performance was validated using time-dependent ROC analysis, which demonstrated a certain predictive ability across three separate cohorts, including TCGA LIHC, GSE76427 (GEO) and LIRI-JP (ICGC) (Fig. 3E).

Differential pathway and functional enrichment between the two risk groups. GSVA of the hallmark gene sets revealed distinct differences in pathway activation between the two risk groups. The high-risk group exhibited upregulation of pathways associated with coagulation, allograft rejection and myogenesis, thus suggesting an active inflammatory and tissue remodeling environment. By contrast, the low-risk group exhibited increased activity in pathways involved in cell cycle regulation, including 'E2F_TARGETS', 'G2M_CHECKPOINT' and 'MITOTIC_SPINDLE', thereby indicating increased cell proliferation and division (Fig. 4A). GO enrichment analysis further highlighted functional differences between the groups. The high-risk group was significantly enriched for pathways associated with cellular polarity and membrane organization, including the 'apical plasma membrane' and 'apical part of the cell' (Fig. 4B). Conversely, the low-risk group was enriched in immune-related pathways, particularly those associated with immunoglobulin antigen binding, thus reflecting an active immune response (Fig. 4C). KEGG pathway analysis also revealed several signaling pathways, such as 'Primary immunodeficiency', 'Graft-versus-host disease' and 'Hippo signaling pathway', that were differentially enriched between the two risk groups. The KEGG pathways with similar biological functions were further categorized to highlight shared themes across the risk groups (Fig. 4D).

Immunotherapy prediction analysis based on the TIDE and Submap algorithms. A strong negative correlation was observed between the risk score and all three ESTIMATE scores (immune, stromal and overall ESTIMATE scores), thereby indicating that higher scores were associated with lower levels of immune and stromal cell infiltration (Fig. 5A). By contrast, no significant correlation was observed between the risk score and the TMB, thereby suggesting that the TMB is not a major factor influencing risk stratification in the present cohort (Fig. 5B). Subsequently, differential expression analysis of common inhibitory immune checkpoint genes was performed. The results revealed that most inhibitory checkpoint genes (including IL2, CD40 ligand and CD70) were significantly differentially expressed, with higher expression levels being observed in the high-risk group (Fig. 5C). Conversely, the expression of stimulatory immune

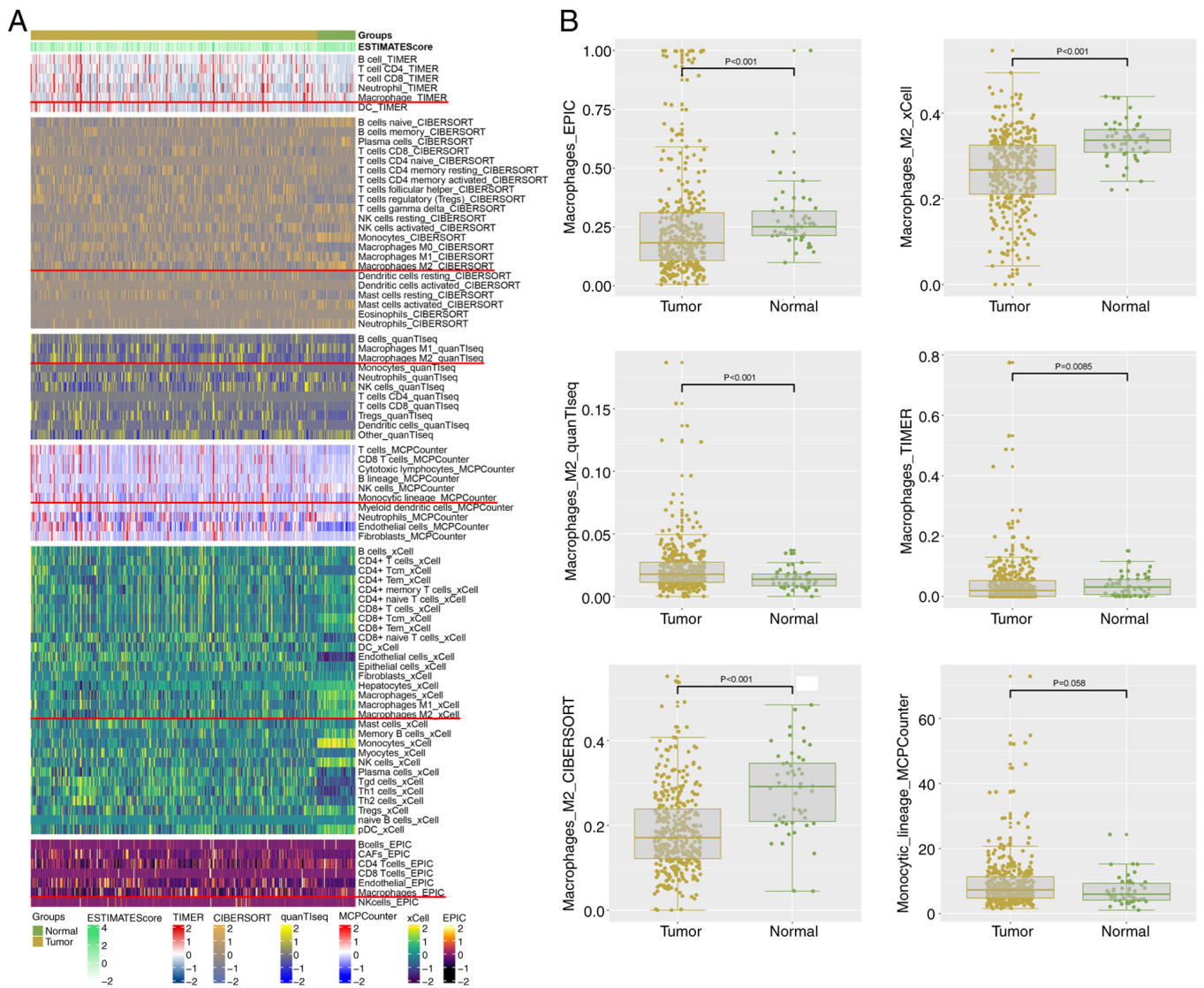


Figure 1. Macrophage infiltration and M2 macrophage differential expression in LIHC. (A) Heatmap illustrating macrophage infiltration levels in LIHC and normal tissue samples, as quantified by six different immune infiltration analysis methods. The heatmap provides a visual representation of macrophage levels across all methods. The red lines indicate the distribution of infiltrating macrophages in both LIHC and normal samples across the different methods. (B) Differential expression analysis of M2 macrophages between patients with LIHC and normal controls. Significant differences in M2 macrophage expression were observed in LIHC samples compared with normal controls across all methods, with the exception of the MCPCounter method. LIHC, liver hepatocellular carcinoma.

checkpoint genes did not significantly differ between the two groups (Fig. 5D). TIDE analysis revealed that low-risk patients exhibited significantly lower TIDE and dysfunction scores, thus indicating a potentially stronger immune response and a lower risk of immune escape. The exclusion score of low-risk patients was significantly higher, which was inconsistent with the overall prediction result. This may be due to reasons such as sample differences. Due to sample differences and other reasons, dysfunction and exclusion alone may not be able to make good predictions. Therefore, the TIDE total score, which combined the scores of dysfunction and exclusion, was usually adopted as the final prediction result of the TIDE analysis. (Fig. 5E). Finally, Submap analysis revealed that patients in the low-risk group were more likely to respond favorably to ICB therapy, thereby further suggesting that the low-risk group demonstrates greater potential for immunotherapy responsiveness (Fig. 5F).

Expression and ligand-receptor interactions of KLRB1 in single-cell datasets. To understand the role of KLRB1 in immune cell populations, its expression was analyzed in six distinct single-cell datasets from the TISCH2 database. KLRB1 was observed to be predominantly expressed in CD8⁺ T cells, CD8⁺ Tex and NK cells, thus indicating its potential involvement in immune regulation and response within these cell types (Fig. 6A). The heatmap displays strong ligand-receptor interactions between various immune cell types, with notable interaction intensities being observed between CD8⁺ T cells and other immune populations (Fig. 6B). Finally, the network diagram depicts the dynamic interactions between CD8⁺ T cells and surrounding cell populations, thus highlighting the strength and activity of these interactions. The analysis demonstrated that CD8⁺ T cells exhibit significant signaling crosstalk with other immune cell populations (Fig. 6C).

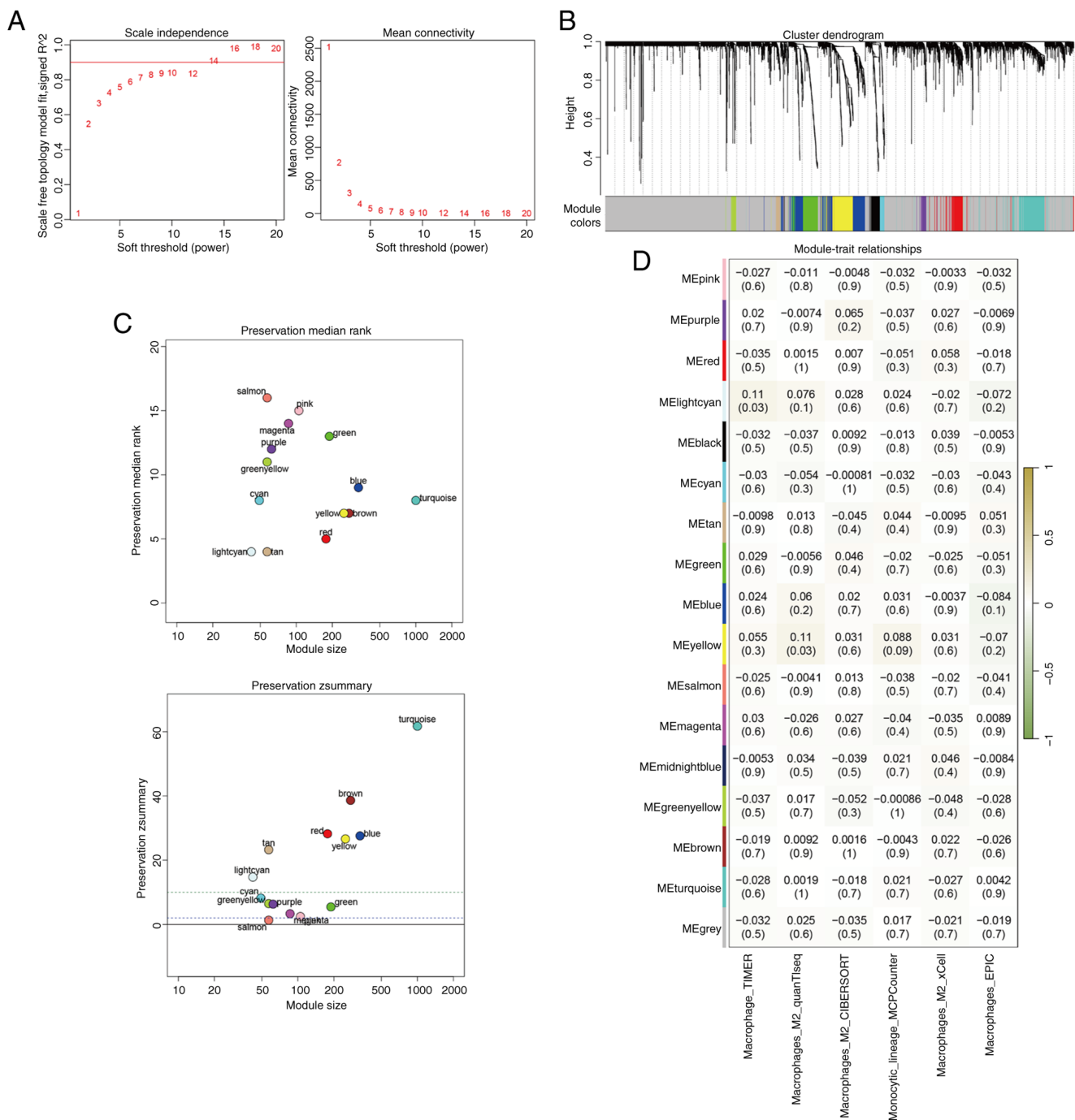


Figure 2. Identification and preservation of gene modules associated with M2 macrophages. (A) Determination of the optimal soft threshold for network construction based on the scale-free topology model fit. A power value of 14 was selected, achieving a model fit close to 0.9. (B) Dendrogram showing the identification of 16 gene modules using weighted gene co-expression network analysis. (C) Preservation analysis of the identified gene modules. Modules with Z scores <2 (grey, gold, midnight blue and black) were excluded due to poor preservation. (D) Correlation analysis between M2 macrophage expression levels and the gene modules. ME, module eigengene.

Expression and prognostic importance of KLRB1. KLRB1 expression was first assessed in multiple cancer types using TCGA pan-cancer data. KLRB1 was observed to be significantly differentially expressed in a majority of cancer types, including LIHC, thereby highlighting its relevance in tumor biology (Fig. 7A). Prognostic analysis revealed that KLRB1 expression was associated with favorable outcomes (including DFI, DSS, OS and PFI) in most cancer types (Fig. 7B). Furthermore, high expression of KLRB1 was found to be closely related to poor OS (Fig. 7C). The LIHC samples were then stratified

into high- and low-KLRB1 expression groups. GSEA of these groups revealed significant enrichment of specific biological pathways such as wnt- β -catenin signaling, TGF- β signaling and PI3K-AKT-mTOR signaling in both the high- and low-expression groups, thus suggesting that KLRB1 may serve a key role in regulating various tumor-related processes (Fig. 7D).

Regulation of tumors by KLRB1 and its associated secretory factors on macrophage polarization. qPCR was used to assess the mRNA levels of KLRB1 in HuH-7 and HuH-1

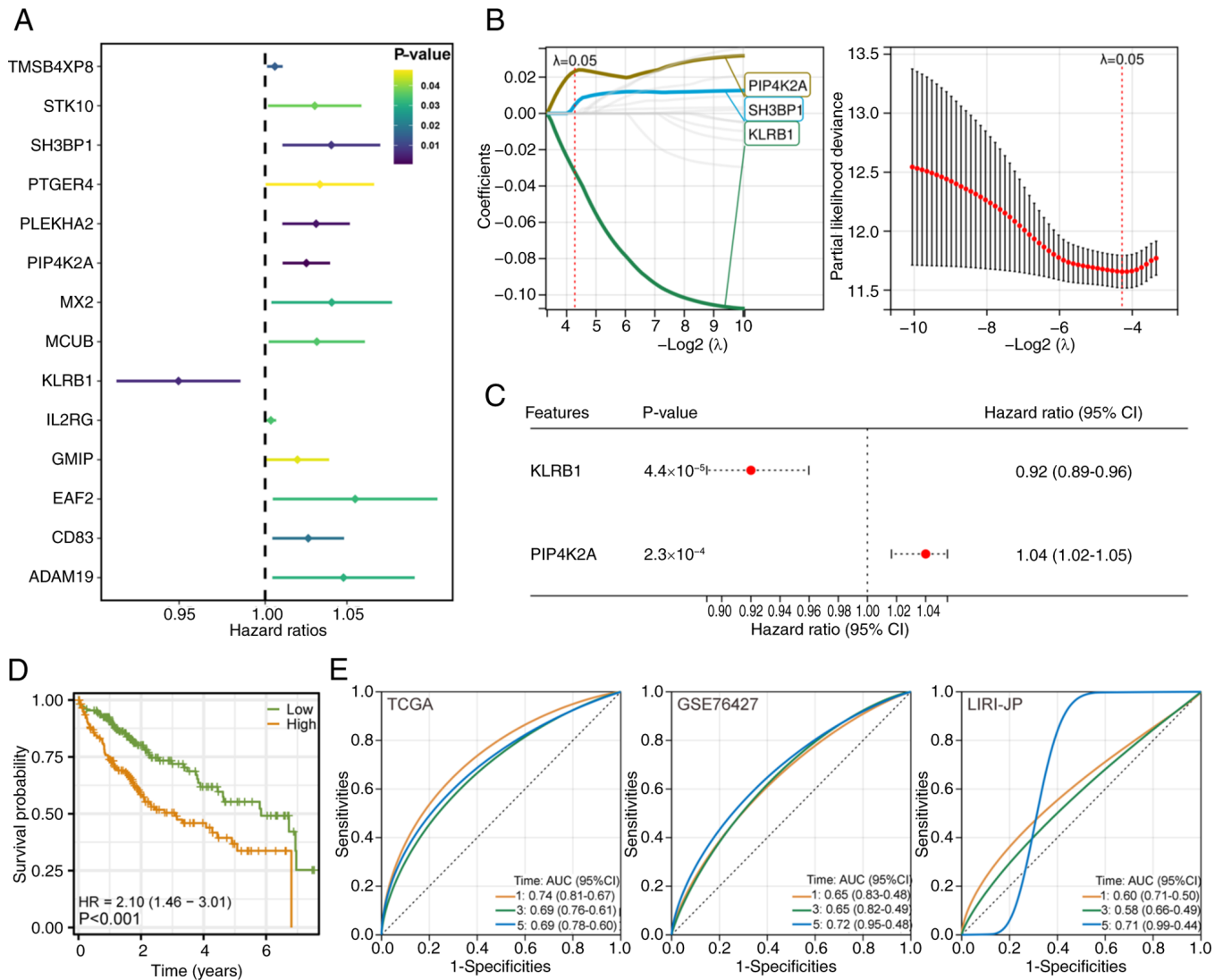


Figure 3. Development and validation of the M2 macrophage-related prognostic model. (A) Univariate Cox regression analysis identified 14 genes with significant prognostic value ($P < 0.05$) in patients with LIHC. (B) Least Absolute Shrinkage and Selection Operator-Cox regression was applied to refine the gene set, selecting three key genes based on a λ value of 0.0514 (10-fold cross-validation). (C) Multivariate Cox regression confirmed the independent prognostic significance of the two selected genes. A prognostic risk score model was constructed using the following formula: RiskScore = PIP4K2A $\times$ 1.0165 + KLRB1 $\times$ 0.8897. (D) Kaplan-Meier survival curves comparing two risk groups in the TCGA-LIHC cohort. A significant difference in overall survival was observed between the groups. (E) Time-dependent receiver operating characteristic curve analysis of the prognostic risk score model, showing certain predictive performance across three independent cohorts: TCGA-LIHC, GSE76427 (Gene Expression Omnibus) and LIRI-JP (International Cancer Genome Consortium). LIHC, liver hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; PIP4K2A, phosphatidylinositol-5-phosphate 4-kinase type 2 α ; SH3BP1, SH3 domain binding protein 1; KLRB1, killer cell lectin like receptor B1; AUC, area under the curve; TMSB4XP8, TMSB4X pseudogene 8; STK10, serine/threonine kinase 10; PTGER4, prostaglandin E receptor 4; PLEKHA2, pleckstrin homology domain containing A2; MX2, MX dynamin like GTPase 2; MCUB, mitochondrial calcium uniporter dominant negative subunit β ; IL2RG, IL2 receptor subunit γ ; GMIP, GEM interacting protein; EAF2, ELL associated factor 2; ADAM19, ADAM metalloproteinase domain 19; HR, hazard ratio.

cells. The results revealed that KLRB1 expression was higher in HuH-7 cells compared with HuH-1 cells (Fig. 8A). To knock down KLRB1 in HuH-7 cells (sh-KLRB1-2), shRNA was used and the knockdown cell line was selected using puromycin. For stable overexpression of KLRB1 in HuH-1 cells (OE-KLRB1), lentiviral vectors were used, followed by puromycin selection to generate stable overexpression cell lines (Fig. 8B). To evaluate the impact of KLRB1 on tumor cell proliferation, CCK-8 assays were performed. The results demonstrated that KLRB1 knockdown significantly increased cell proliferation compared with the control group, whereas overexpression significantly decreased cell proliferation (Fig. 8C). Transwell assays were performed to assess the effect of KLRB1 on cell

migration. KLRB1 knockdown enhanced cell migration, whereas KLRB1 overexpression reduced migration (Fig. 8D and E). Additionally, the influence of KLRB1 on cell apoptosis was examined. The flow cytometry results indicated that KLRB1 knockdown inhibited apoptosis, whereas KLRB1 overexpression promoted apoptosis (Fig. 8F). And through WB experiments, it was found that knocking out KLRB1 could significantly inhibit the expression of Bax and c-caspase-3, and enhance the expression of Bcl-2 and caspase-3, while overexpression achieved the opposite result (Fig. S1). Furthermore, the tube formation assay demonstrated that, compared with the controls, the culture supernatant from KLRB1 knockdown cells increased the tube forming ability of HUVECs, whereas

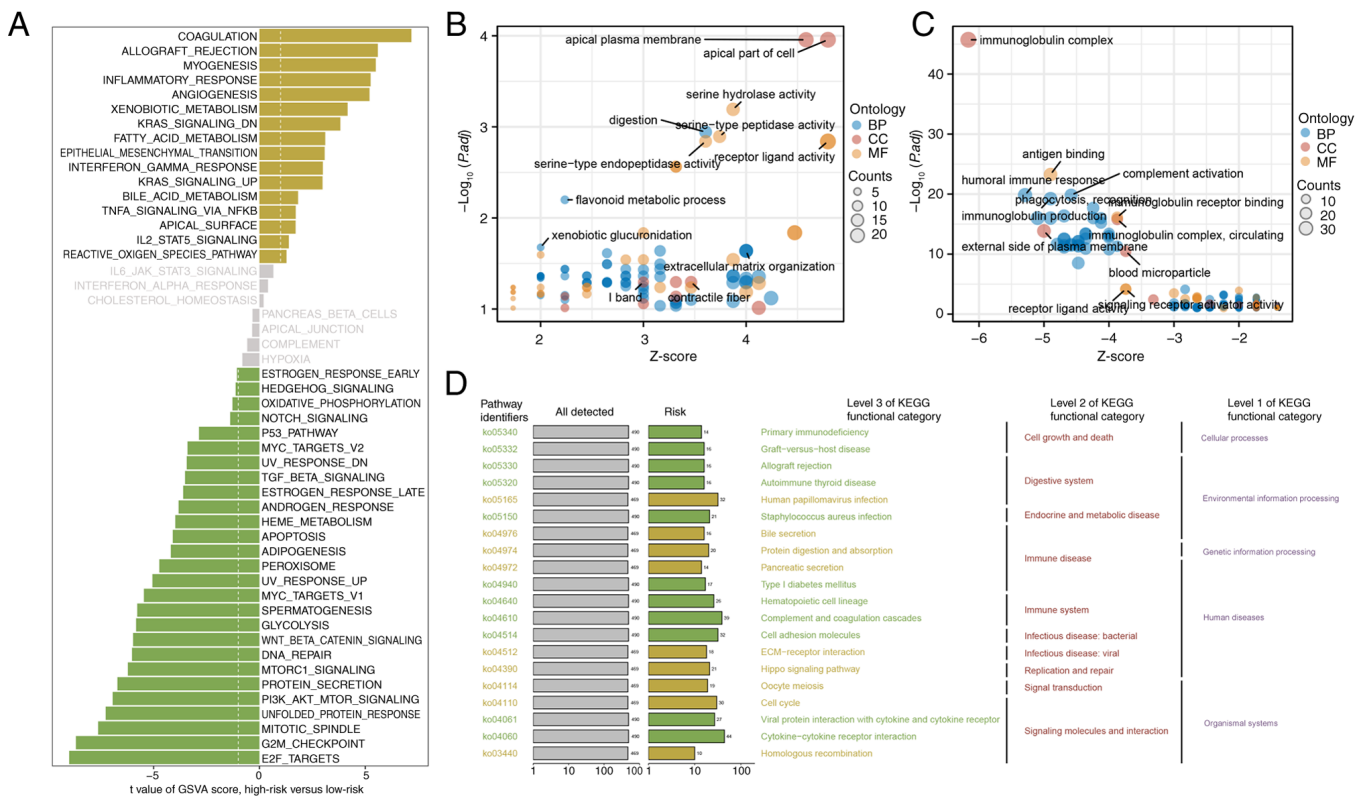


Figure 4. Differential pathway and functional enrichment analysis. (A) GSEA of the hallmark gene sets revealed significant differences in pathway activation between the two risk groups. (B) GO functional enrichment analysis identified significant enrichment for pathways related to cellular polarity and apical membrane organization in the high-risk group. (C) GO analysis of the low-risk group showed enrichment for immune response-related pathways, including immunoglobulin antigen binding. (D) KEGG pathway enrichment analysis identified differential activation of signaling pathways between the two risk groups. Pathways were categorized based on shared biological functions to reveal common themes across the groups. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Variation Analysis; BP, biological process; CC, cellular component; MF, molecular function.

the supernatant from KLRB1-overexpressing cells reduced the tube forming ability of HUVECs (Fig. 8G).

To investigate how KLRB1 affects the secretion of tumor cell-derived factors involved in macrophage polarization, culture supernatants were collected from KLRB1-knockdown and KLRB1-overexpressing cells and incubated with THP-1 cell-derived M0 macrophages. The polarization status of M1 and M2 macrophages was then assessed using qPCR. The results demonstrated that KLRB1 knockdown culture supernatant increased the expression of M2 markers CD206 and IL-10, whereas KLRB1-overexpressing cell culture supernatant reduced the expression of CD206 and IL-10. By contrast, the M1 markers CD86 and IL-12 were downregulated following KLRB1 knockdown culture supernatant treatment and upregulated after overexpression supernatant treatment (Fig. 8H). These findings suggest that KLRB1 may inhibit the polarization of M0 macrophages towards the M2 phenotype. In summary, the upregulation of KLRB1 inhibits the activity of LIHC cells and facilitates the polarization of macrophages from the M0 phenotype to the M1 phenotype through changes in the cell secretome.

Discussion

LIHC is one of the most prevalent and fatal malignancies worldwide, with a poor prognosis being observed, primarily due to late-stage diagnoses, high recurrence rates and

resistance to conventional treatments (29). The TME serves a crucial role in the progression of LIHC, with macrophages (particularly M2 macrophages) being key contributors to tumor growth, metastasis and immune evasion (30). M2 macrophages are characterized by their immunosuppressive properties, which promote tumor progression through the cells, thereby fostering immune escape (31). Additionally, M2 macrophages facilitate angiogenesis and ECM remodeling, thus further enhancing the pro-tumorigenic environment in LIHC (32). Previous advances in bioinformatics have enabled the integration of large-scale genomic data, thus allowing for the identification of key molecular signatures associated with immune infiltration and tumor progression (33,34). Using techniques such as WGCNA, researchers have identified M2 macrophage-related genes as potential biomarkers for prognosis in LIHC (35). Given the pivotal role of M2 macrophages in modulating the TME and influencing immune responses, a deeper understanding of their gene expression profiles could offer novel insights into prognostic prediction and therapeutic strategies for LIHC.

In the present study, M2 macrophage-related genes that serve significant roles in the prognosis of LIHC were identified. Using bioinformatics analyses, a prognostic risk model was constructed based on these genes, which demonstrated promising predictive power in both the training and external validation datasets. These findings reinforce the growing body of evidence suggesting that M2 macrophages are key

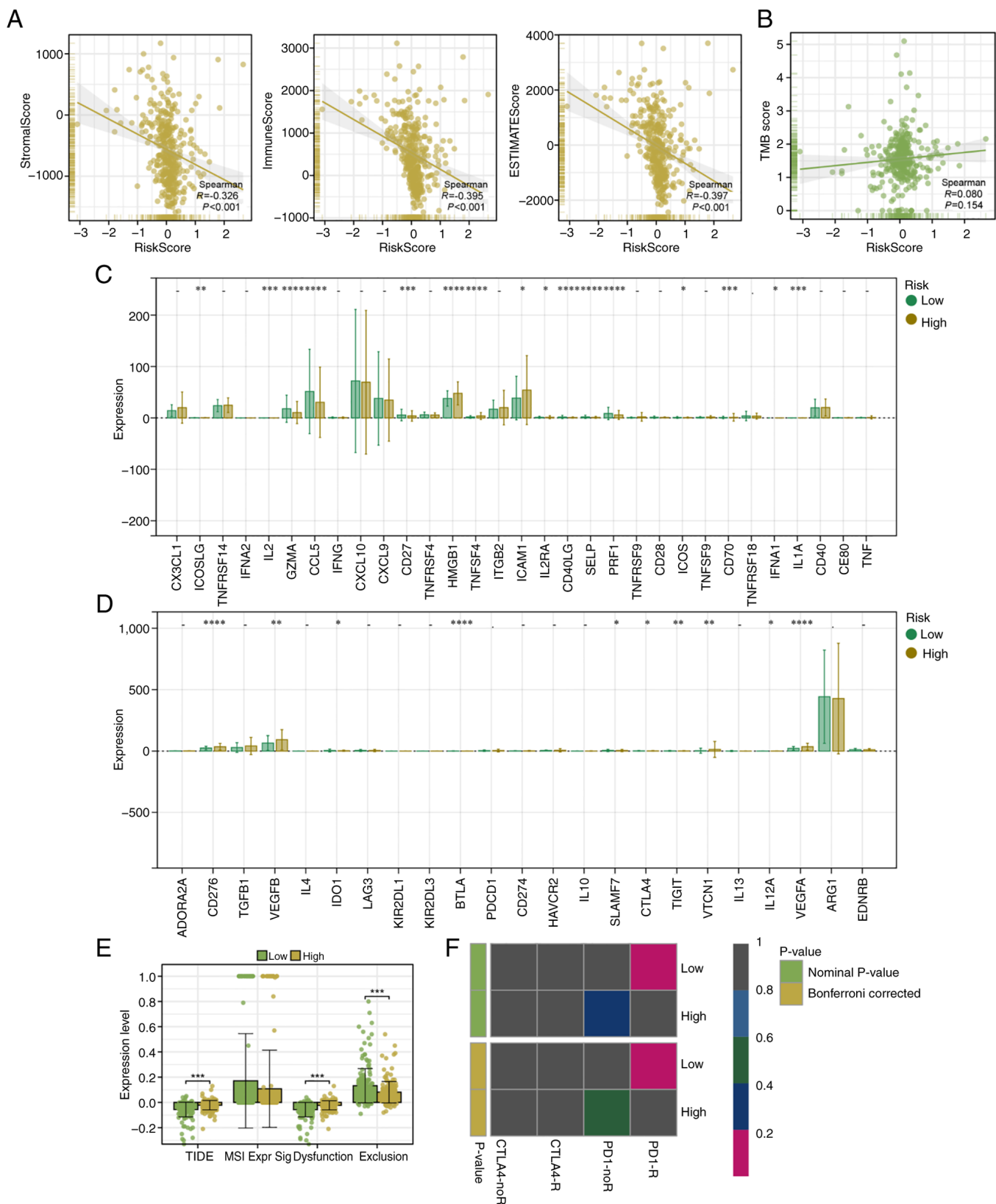


Figure 5. Immune infiltration, TMB, immune checkpoint expression and immunotherapy response in two risk groups. (A) Correlation between the prognostic risk score and the immune, stromal and ESTIMATE scores, calculated using the ESTIMATE algorithm. (B) Correlation analysis between the risk score and TMB showed no significant association. (C) Differential expression analysis of inhibitory immune checkpoint genes revealed significant differences between two risk groups. (D) Differential expression of stimulatory immune checkpoint genes showed no significant differences between the two groups. (E) TIDE analysis demonstrated that low-risk patients had significantly lower TIDE scores, suggesting they have an improved immune response and a reduced potential for immune escape. (F) SubMap analysis predicted that low-risk patients are more likely to respond to immune checkpoint blockade therapy, highlighting their greater potential for benefiting from immunotherapy. PD1-R indicates a response to PD1 treatment, while PD1-noR indicates no response to PD1 treatment. CTLA4-R indicates a response to CTLA4 treatment, while CTLA4-noR indicates no response to CTLA4 treatment. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$; t-test based P-value. TMB, tumor mutational burden; TIDE, Tumor Immune Dysfunction and Exclusion; PD1, programmed cell death protein 1; CTLA4, cytotoxic T-lymphocyte associated protein 4.

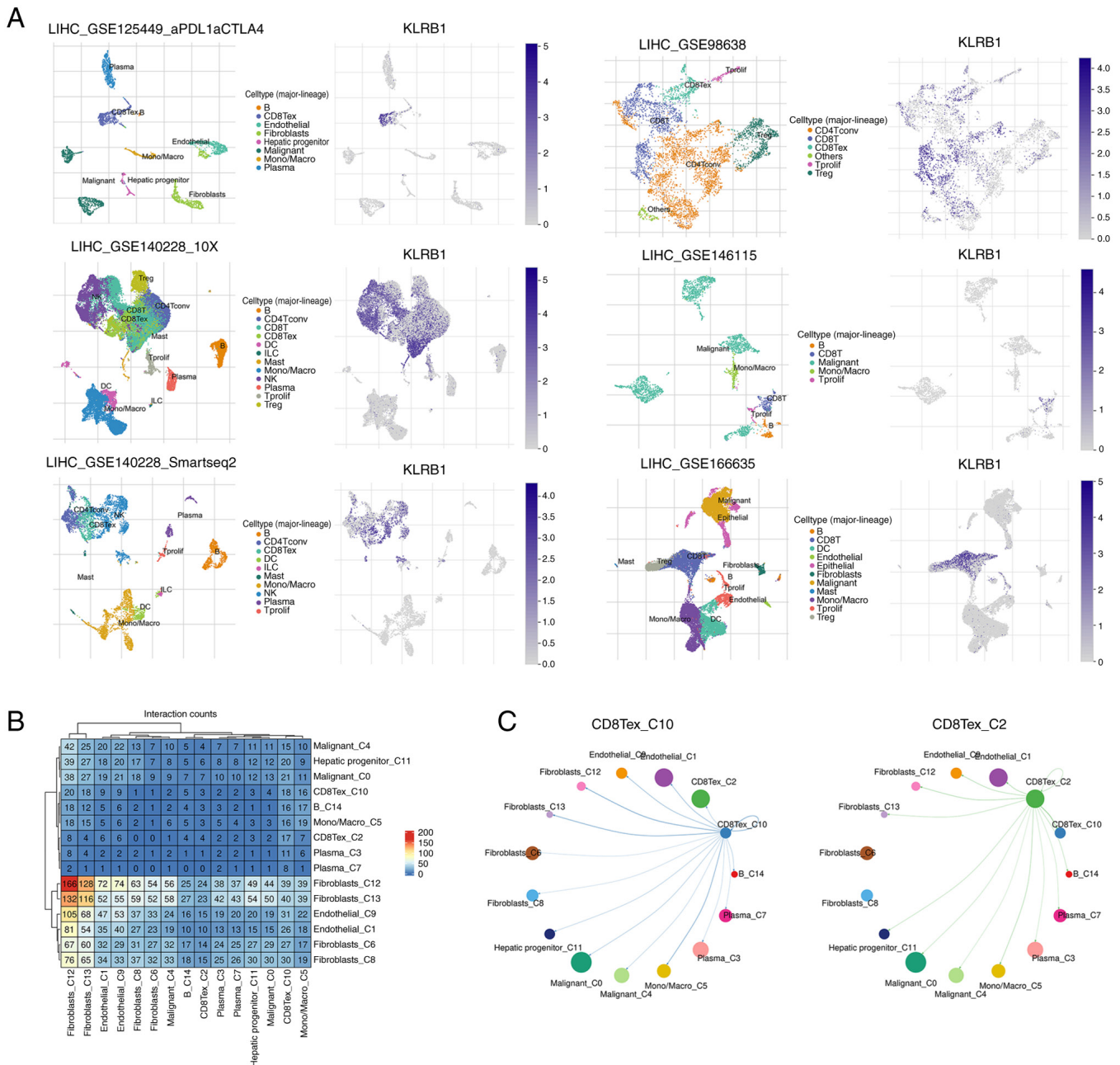


Figure 6. KLRB1 expression and ligand-receptor interactions in the TME. (A) Expression of KLRB1 across six single-cell RNA-sequencing datasets from the Tumor Immune Single Cell Hub 2 database. KLRB1 was predominantly expressed in CD8⁺ T cells, CD8⁺ Tex and NK cells. (B) Heatmap showing the strength of ligand-receptor interactions between different immune cell populations. (C) Network diagram illustrating the intensity of ligand-receptor interactions between CD8⁺ T cells and other cell populations in the TME. KLRB1, killer cell lectin like receptor B1; TME, tumor microenvironment; NK, natural killer; Tex, exhausted T cells; LIHC, liver hepatocellular carcinoma; DC, dendritic cell; Treg, T-regulatory cell.

players in the TME of LIHC and contribute to tumor progression (9,36). However, unlike some studies that have established associations between high M2 macrophage infiltration and poor clinical outcomes, the present results focused on the identification of gene expression patterns associated with M2 macrophages that may provide more reliable prognostic information for patients with LIHC.

Although increased infiltration of M2 macrophages has been associated with poor prognoses in various cancers, the present study did not identify direct correlations between M2 macrophage infiltration and various clinical outcomes (such as OS or progression-free survival) in patients with LIHC.

This discrepancy may be due to the complex nature of the liver TME, where multiple factors, including hepatic fibrosis, viral infections and cirrhosis, contribute to disease progression beyond macrophage infiltration. Therefore, although M2 macrophage-related genes demonstrate potential as biomarkers for predicting disease progression, additional studies are necessary to elucidate the precise mechanisms through which M2 macrophages influence the clinical course of LIHC.

Notably, the present study suggests that M2 macrophages may influence the efficacy of immunotherapies in LIHC. Although immune checkpoint inhibitors have demonstrated

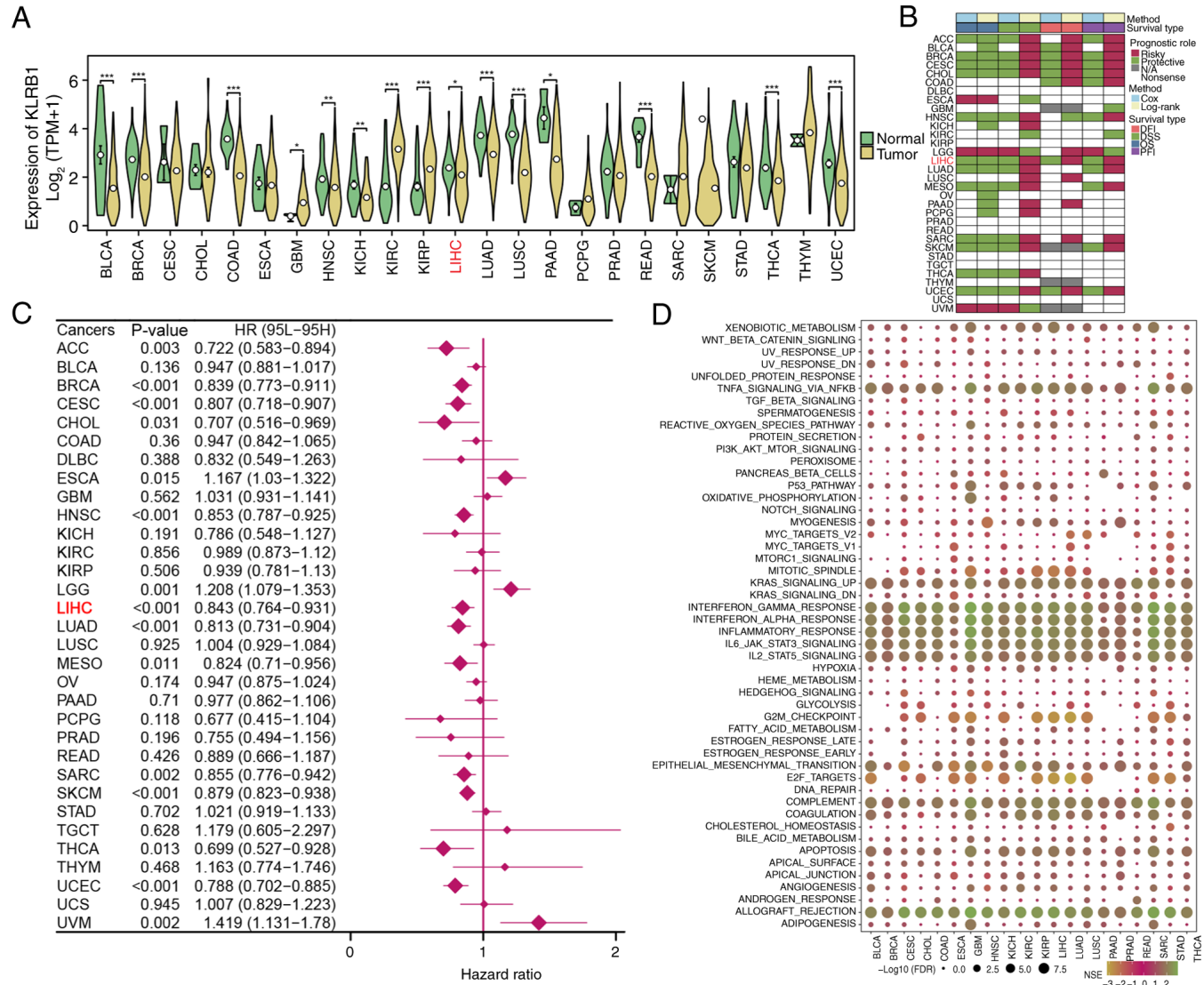


Figure 7. Pan-cancer analysis of KLRB1 expression and prognostic impact. (A) Expression of KLRB1 across multiple cancer types in The Cancer Genome Atlas. Significant differences in KLRB1 expression were observed in several cancer types, including LIHC. (B) Prognostic analysis of KLRB1 expression in pan-cancer datasets, including DFI, DSS, OS and PFI. (C) Forest plot analysis of OS based on KLRB1 expression in pan-cancer. (D) Gene Set Enrichment Analysis revealed significant biological pathways enriched in both high- and low-expression groups, suggesting that KLRB1 may regulate important tumor-related processes. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t-test based P-value. KLRB1, killer cell lectin like receptor B1; LIHC, liver hepatocellular carcinoma; DFI, disease-free interval; DSS, disease-specific survival; OS, overall survival; PFI, platinum-free interval; FDR, false discovery rate; NES, normalized enrichment score; ACC, adrenocortical carcinoma; BLCA, bladder cancer; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumor; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrioid carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

promising results in various cancers, their effectiveness in LIHC is often hindered by the immunosuppressive TME, which is partly shaped by M2 macrophages (37). The analysis revealed that the preoperative risk model, which was constructed based on M2 macrophage-related genes, was associated with improved immune responses and improved outcomes in the low-risk group. Therefore, the targeting of M2 macrophages or their associated signaling pathways may provide a promising strategy to increase the effectiveness of immunotherapy in LIHC, particularly in low-risk patients who are identified using this model.

Although M2 macrophages serve a critical role in the TME and have been associated with poor prognoses in various cancers, the present study identified KLRB1 as an important factor in LIHC. KLRB1 is primarily expressed on NK cells and certain T-cell subsets and has been implicated in immune regulation and tumor immune surveillance (38). In LIHC, KLRB1 may influence the tumor immune landscape by modulating immune cell interactions, thereby potentially affecting tumor progression and patient outcomes. The findings suggest that KLRB1 expression may serve a protective role in LIHC, as higher levels of this gene were observed to

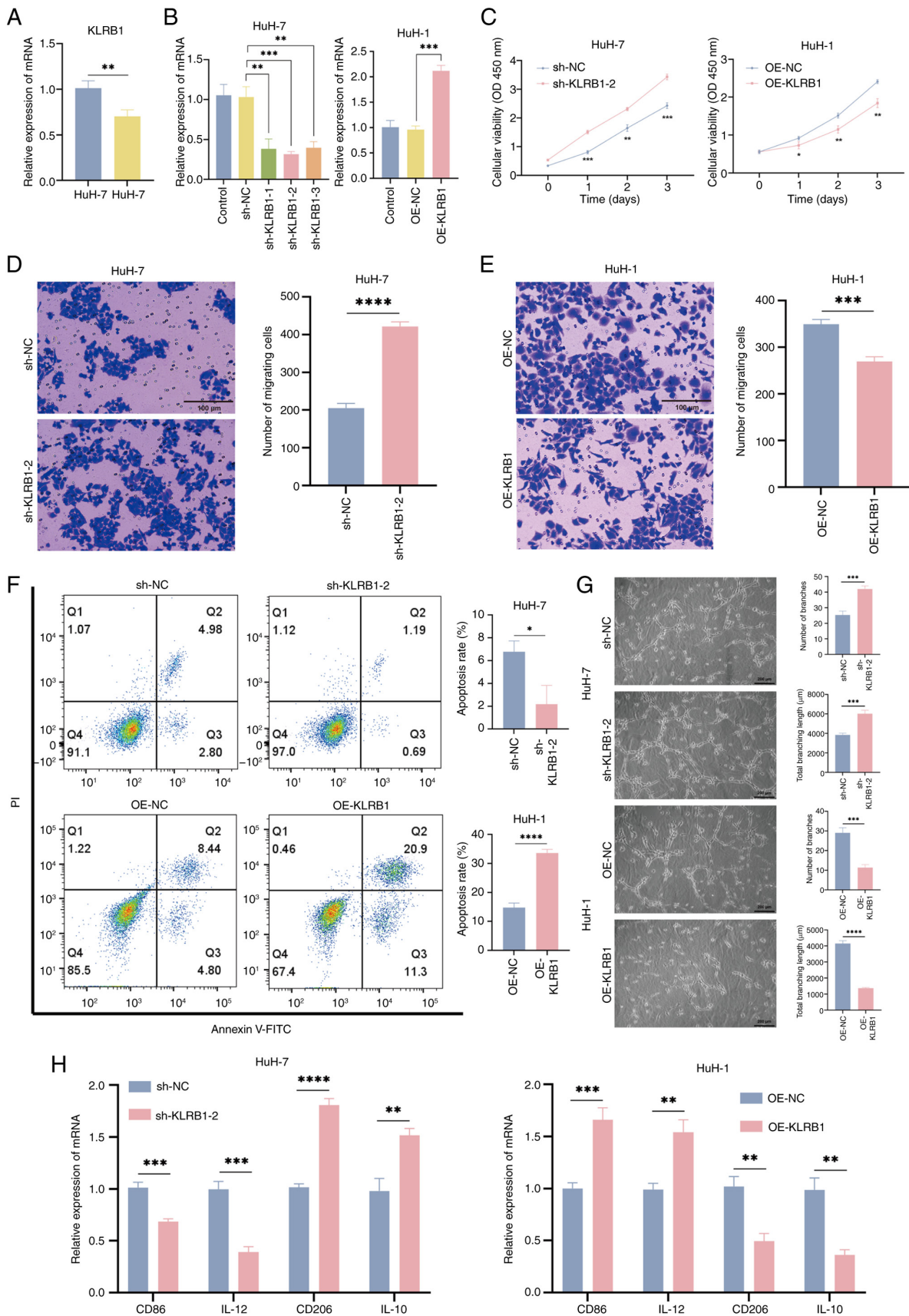


Figure 8. KLRB1 regulates the activity of LIHC cells and its influence on macrophages through LIHC cells. (A) qPCR detection of KLRB1 expression in HuH-7 and HuH-1 cells. (B) Validation of shRNA knockdown KLRB1 in the HuH-7 cell line and lentivirus overexpression of KLRB1 in the HuH-1 cell line. (C) Cell proliferation curve of sh-KLRB1-2 and OE-KLRB1. Cell migration assay of (D) sh-KLRB1-2 and (E) OE-KLRB1 cells (scale bars, 100 μ m). (F) Annexin V-FITC/PI assessed apoptosis in sh-KLRB1-2 and OE-KLRB1 transfected cells. (G) Tube formation assay of HUVECs treated with sh-KLRB1-2 and OE-KLRB1 cell conditioned media (scale bar, 200 μ m). (H) qPCR detection of polarization markers in M0 to M1/M2 cells, treated with sh-KLRB1-2 or OE-KLRB1 cell conditioned media. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; t-test based P-value. LIHC, liver hepatocellular carcinoma; PI, propidium iodide qPCR, quantitative PCR; KLRB1, killer cell lectin like receptor B1; sh, short hairpin; OE, overexpression; NC, negative control; OD, optical density.

be associated with improved survival outcomes. This result indicates the involvement of KLRB1 in promoting antitumor immune responses. However, the precise mechanisms through which KLRB1 modulates tumor progression and immune evasion in LIHC remain to be fully elucidated. Based on the present research findings, it may be speculated that KLRB1 may promote the progression of LIHC disease by regulating the polarity transformation of macrophages. Therefore, the targeting of KLRB1 or its associated signaling pathways may represent a promising therapeutic approach to improve patient prognosis and enhance the efficacy of immunotherapies in LIHC.

KLRB1 serves a crucial role in tumor regulation, with previous studies involving LIHC and lung adenocarcinoma (LUAD) highlighting its complex dual function. The expression levels and functions of KLRB1 vary markedly across different tumor types. For instance, in breast cancer and LUAD, low expression of KLRB1 is typically associated with poor prognosis, and its functions involve immune regulation, cell proliferation and migration (39). Similarly, in other cancer types, low expression of KLRB1 is generally associated with poor survival outcomes. In conclusion, the present study provides strong evidence that KLRB1 serves a multifaceted role in regulating both tumor cell behavior and macrophage polarization. The upregulation of KLRB1 appears to inhibit the proliferation and migration of LIHC cells, promote apoptosis and facilitate the polarization of macrophages towards the M1 phenotype. As KLRB1 is a membrane protein, the conditioned media may also contain exosomes or microvesicles secreted by the KLRB1 knockdown or OE cells. These vesicles can carry not only proteins and lipids but also mRNA and miRNA that can have an impact on recipient cells, including macrophages. For instance, specific miRNAs may influence macrophage gene expression related to polarization. Furthermore, changes in KLRB1 expression can alter the metabolic state of the transfected cells, which in turn could affect the composition of metabolites and extracellular vesicles released into the conditioned media. This is typically more effective in combating tumors. These findings highlight the potential of KLRB1 as a therapeutic target in LIHC and emphasize the importance of understanding the complex interactions between immune cells and cancer cells in the development of novel cancer treatments.

Despite the promising results associated with the M2 macrophage-related prognostic signature, several limitations must be acknowledged in the current study. Firstly, the research primarily relied on data retrieved from public databases, which often contain relatively small sample sizes and may not be promptly updated. In addition, the focal point of the investigation involved M2 macrophage-related genes; however, the specific molecular mechanisms by which these genes regulate the progression of LIHC necessitate further in-depth explorations. Consequently, the interpretation of the results presented requires additional validation and enhancement. Furthermore, the prognostic signature was predominantly verified using public datasets, which may harbor intrinsic biases and do not provide clinical validation within independent, real-world patient cohorts. Secondly, although analyses of immune-cell infiltration and predictions regarding immunotherapy offer valuable insights, the exact mechanisms linking M2 macrophage-related genes

to immune responses in LIHC remain inadequately understood and warrant additional experimental investigations. Furthermore, the present study suggested that KLRB1 may serve a role in modulating immune responses and facilitating the progression of LIHC. A thorough review of the literature regarding KLRB1 indicates that this gene represents a novel therapeutic target; however, the molecular mechanisms through which KLRB1 regulates LIHC remain largely unexplored and should be further investigated. Additionally, the present research did not evaluate potential interactions between other key genes and molecular pathways that could influence the progression of LIHC, thereby highlighting the need for further explorations in this area. Finally, although the prognostic signature demonstrates promise in guiding immunotherapy strategies, its clinical applicability is constrained by the absence of prospective clinical trials to validate its predictive power. Future studies should focus on validating the prognostic signature within larger, more diverse patient populations and elucidating the mechanistic roles of M2 macrophages in the context of LIHC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LC was involved in the study conception and design and reviewed the manuscript. AW, XL and ZW performed the literature search, performed experiments, acquired and analyzed data, performed the statistical analysis and wrote and reviewed the manuscript. LC serves as a guarantor for the integrity of the entire study. LC, AW, XL and ZW confirm the authenticity of the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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