

NOL11 is a novel prognostic biomarker associated with immune infiltration and tumor progression in hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) remains a highly lethal malignancy, and the lack of reliable biomarkers for prognosis assessment and therapeutic guidance continues to limit effective clinical management. Nucleolar protein 11 (NOL11), an important regulator of ribosomal biogenesis and cellular homeostasis, has remained to be fully characterized in HCC. The present study aimed to systematically investigate the expression pattern, prognostic significance, immune relevance and biological function of NOL11 in HCC using transcriptomic and clinical data from The Cancer Genome

Atlas, together with *in vitro* functional assays. NOL11 was significantly upregulated in HCC tissues and was associated with advanced clinicopathological features and poor overall survival. Immune infiltration analysis revealed that NOL11 expression was correlated with distinct immune cell populations, particularly macrophage subsets, and functional enrichment analysis indicated involvement in tumor-related pathways, including the PI3K-Akt and MAPK signaling pathways. In addition, an eight-gene prognostic risk model based on NOL11-related genes was established using LASSO Cox regression, which effectively stratified patient survival and served as an independent prognostic factor. High-risk patients exhibited elevated tumor mutational burden and increased expression of immune checkpoint molecules, including programmed cell death 1 and cytotoxic T-lymphocyte antigen 4, suggesting potential relevance to immunotherapy responsiveness. Furthermore, NOL11 knockdown significantly inhibited HCC-cell proliferation, migration and invasion *in vitro*. Collectively, these findings suggest that NOL11 plays an important role in HCC progression and tumor immune microenvironment remodeling, and that the NOL11-related risk model may serve as a useful prognostic tool, highlighting NOL11 as a potential biomarker and therapeutic target in HCC.

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Abbreviations: AUC, area under the curve; CIBERSORT, Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts; CNV, copy number variation; CTLA-4, cytotoxic t-lymphocyte associated protein 4; GO, gene ontology; HCC, hepatocellular carcinoma; HPA, Human Protein Atlas; IDO1, indoleamine 2,3-dioxygenase 1; KDR, kinase insert domain receptor; KEGG, Kyoto Encyclopedia of Genes and Genomes; LAG-3, lymphocyte activation gene 3; LASSO, Least Absolute Shrinkage and Selection Operator; NOL11, nucleolar protein 11; OS, overall survival; PCA, principal component analysis; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; ROC, receiver operating characteristic; shRNA, short hairpin RNA; STRING, Search Tool for the Retrieval of Interacting Genes; TCGA, The Cancer Genome Atlas; TMB, tumor mutational burden; t-SNE, t-distributed stochastic neighborhood embedding

Key words: hepatocellular carcinoma, NOL11, prognostic biomarker, tumor immune microenvironment, risk model, nucleolar protein, bioinformatics, nomogram

Introduction

Hepatocellular carcinoma (HCC), as a highly prevalent primary malignant liver tumor worldwide, has long ranked among the leading causes of cancer incidence and mortality, posing a significant public health challenge (1,2). Chronic hepatitis C virus infection represents a major etiological factor for HCC development, with genetic polymorphisms affecting both treatment outcomes and disease progression toward cirrhosis and hepatocarcinogenesis (3-5). Despite advances in surgical techniques, local regional therapies and systemic treatments, the long-term prognosis for patients with HCC remains poor due to frequent tumor recurrence, early metastasis and significant molecular heterogeneity (6). Furthermore, the lack of reliable biomarkers for early diagnosis and precise prognosis assessment continues to hinder the effective management of

HCC (7,8). Therefore, identifying novel molecular biomarkers closely associated with tumor progression, immune micro-environment remodeling and patient prognosis is crucial for improving HCC risk stratification and developing personalized treatment strategies.

Nucleolar proteins play a central role in ribosomal biosynthesis and regulate key processes such as cell cycle progression, stress responses and genomic stability (9). The function of nuclear proteins has been increasingly recognized as a hallmark feature of tumorigenesis and malignant transformation (10,11). Nucleolar protein 11 (NOL11), as a conserved nucleolar protein, participates in ribosomal RNA transcription and processing, playing a crucial role in maintaining nucleolar integrity and cellular homeostasis (12). Research indicates that abnormal expression of NOL11 can lead to the development of various diseases, including liver cirrhosis (13-15). However, its expression pattern, biological function and clinical significance in HCC remain poorly understood.

The present study aimed to systematically investigate the expression pattern, prognostic significance, immune relevance and biological function of NOL11 in HCC using transcriptomic and clinical data from The Cancer Genome Atlas (TCGA). Specifically, the study sought to analyze the association between NOL11 expression and clinicopathological characteristics, patient survival, immune cell infiltration and immune-related signaling pathways. The present study further aimed to construct and validate a NOL11-related prognostic risk model and to evaluate its potential clinical value in survival prediction. In addition, the associations of the risk model with tumor mutational burden (TMB) and immune checkpoint molecule expression were explored to assess its possible relevance to immunotherapy responsiveness. Finally, *in vitro* experiments were performed to verify the effects of NOL11 on HCC cell proliferation, migration and invasion. These analyses were expected to provide new insight into the role of NOL11 in HCC progression and to support its potential utility as a prognostic biomarker and therapeutic target.

Materials and methods

Data acquisition and processing. The data retrieval and bioinformatics analyses were conducted between December 2024 and April 2025. RNA transcriptome data, clinical information and somatic mutation data were obtained from the TCGA HCC project (<https://portal.gdc.cancer.gov/>) (16). Transcriptome data were uniformly converted to fragments per kilobase million format and standardized to ensure comparability across samples (17). During clinical information integration, samples with missing or incomplete key survival time were excluded to guarantee the reliability of subsequent analyses. The final research cohort comprised 370 HCC tissue samples and 50 adjacent normal tissue samples. In addition, the infiltration levels of 22 immune cell types in the HCC samples were estimated using the CIBERSORT algorithm (<https://cibersort.stanford.edu/>), and NOL11 expression across clinicopathological subgroups was analyzed using the UALCAN database (<https://ualcan.path.uab.edu/>). The protein expression of NOL11 in normal liver and HCC tissues was examined using the Human Protein Atlas (HPA) database (<https://www.proteinatlas.org/>).

Functional enrichment and protein interaction network analysis. To elucidate the potential biological functions of NOL11 in HCC, this study comprehensively employed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) for functional annotation and pathway enrichment analysis using the clusterProfiler package in R (<https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>). All analyses set a P-value <0.05 as the threshold for statistically significant enrichment, with results visualized using the ggplot2 package. Additionally, the protein interaction network for NOL11 was constructed via the search tool for the retrieval of interacting genes (STRING) database (<http://string-db.org>), setting a minimum interaction confidence of 0.6 to identify highly associated protein interactions (18).

Construction and validation of risk model. This analysis first identified NOL11 co-expressed genes significantly associated with HCC prognosis through univariate Cox regression analysis. To optimize model stability and prevent overfitting, these genes were further incorporated into a least absolute shrinkage and selection operator (LASSO) Cox regression analysis performed using the glmnet package in R (<https://cran.r-project.org/web/packages/glmnet/index.html>), to construct a risk model. Based on the expression levels of the selected genes and their corresponding coefficients in the LASSO regression, a risk score was calculated for each patient using the following formula: Risk score = $\sum$ (Gene expression level $\times$ Coefficient). Patients were then stratified into high-risk or low-risk groups using the median risk score across the entire cohort as the cutoff point. To further evaluate the prognostic discriminatory power of this risk model, survival analysis was performed on the TCGA-HCC cohort to compare overall survival (OS) differences between the two groups. Additionally, receiver operating characteristic (ROC) curves and calibration curves were used to assess the model's accuracy and consistency in predicting 1-, 3- and 5-year survival rates. Principal component analysis (PCA) and t-distributed stochastic neighborhood embedding (t-SNE) were employed to visualize distribution differences between the high- and low-risk groups. Finally, univariate and multivariate Cox regression analyses were performed to validate whether the risk score could serve as an independent prognostic factor for HCC, with P<0.05 set as the threshold for statistical significance.

Construction and prediction of nomograms. Based on risk scores and other independent clinical prognostic factors, this study utilized the regplot package in R to construct a clinical prognostic nomogram for HCC patients. This nomogram quantifies individual patient risk and predicts 1-, 3- and 5-year OS rates. The accuracy and consistency of the nomogram in predicting survival probability were evaluated by plotting ROC curves and calibration curves.

TMB and immune checkpoint analysis. This study first employed the maftools software package (19) to conduct systematic analysis and visualization of somatic mutation data from patients with HCC, revealing their genetic mutation spectrum characteristics. Building upon this foundation, differential analysis, correlation analysis and survival

analysis methods were further integrated to systematically evaluate the association between risk scores and TMB, and explore their synergistic role in HCC prognosis. Additionally, by comparing expression differences of key immune checkpoint molecules [such as programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1), cytotoxic T-lymphocyte antigen (CTLA)-4, lymphocyte activating (LAG)-3, indoleamine 2,3-dioxygenase 1 (IDO1) and kinase insert domain receptor (KDR)] between high- and low-risk groups, the correlation between risk scores and immune checkpoint expression levels was analyzed to explore the potential value of the risk model in predicting responses to immunotherapy.

Short hairpin RNA (shRNA) plasmid transfection and establishment of NOL11-knockdown cell lines. shRNA expression plasmids targeting human NOL11 (shNOL11-1 and shNOL11-2), together with a non-targeting negative-control plasmid (shCtrl), were designed and synthesized by GenePharma. The target sequences of the shRNAs, excluding the plasmid vector backbone region, were as follows: shNOL11-1, 5'-CCAAACGCATGTGCTTTCTTACAGT-3'; shNOL11-2, 5'-GTCTACTTCTGGATGCGAATT-3'; and shCtrl, 5'-TTCTCCGAACGTGTCACGT-3'. The shCtrl sequence was a scrambled non-targeting sequence with no significant homology to known human genes. The shRNA constructs carried a puromycin-resistance cassette to allow the selection of stably transfected cells.

The human HCC cell lines Huh-7 and Hep3B were obtained from Procell Life Science & Technology Co., Ltd. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biotechnology Co., Ltd.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO₂. Huh-7 and Hep3B cells were seeded in six-well plates and cultured in antibiotic-free complete medium until they reached ~70-80% confluence. Cells were then transfected with shNOL11-1, shNOL11-2 or shCtrl plasmids using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, 2 µg of plasmid DNA and 4 µl of Lipofectamine 2000 were separately diluted in Opti-MEM reduced-serum medium (Gibco; Thermo Fisher Scientific, Inc.). After incubation for 5 min at room temperature, the diluted DNA and Lipofectamine 2000 solutions were combined and incubated for an additional 20 min to allow complex formation. The DNA-lipid complexes were then added dropwise to the cells. After 4-6 h of incubation at 37°C in a humidified atmosphere containing 5% CO₂, the transfection medium was replaced with fresh complete culture medium. At 48 h after transfection, cells were cultured in complete medium supplemented with puromycin for the selection of stably transfected cells. The puromycin-containing medium was replaced every 2-3 days until non-transfected control cells were completely eliminated. The resulting puromycin-resistant cell populations were expanded and used for subsequent experiments. The knockdown efficiency of NOL11 was confirmed by western blotting.

Western blot analysis. Western blotting was performed to determine NOL11 protein expression in Huh-7 and Hep3B cells. Total cellular proteins were extracted using RIPA lysis buffer (cat. no. P0013B; Beyotime Biotechnology) supplemented with protease inhibitors (phenylmethylsulfonyl fluoride; cat. no. ST506; Beyotime Biotechnology). Protein concentrations were quantified using a bicinchoninic acid protein assay kit (cat. no. P0010; Beyotime Biotechnology). Equal amounts of protein (35 µg per lane) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred onto polyvinylidene difluoride membranes (MilliporeSigma). After transfer, the membranes were blocked with 5% bovine serum albumin (cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 1 h and incubated overnight at 4°C with the following primary antibodies: Anti-NOL11 antibody (1:2,000 dilution; cat. no. ab180132; Abcam) and anti-β-actin antibody (1:25,000 dilution; cat. no. ab49900; Abcam). After washing with Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, 1:3,000 dilution; cat. no. A0208; and goat anti-mouse IgG, 1:3,000 dilution; cat. no. A0216; both from Beyotime Institute of Biotechnology) for 1 h at room temperature. Immunoreactive bands were visualized using a chemiluminescence detection system (MilliporeSigma). β-actin was used as the internal loading control.

Cell functional assays. To evaluate the function of NOL11 in HCC cells, a series of experiments including cell proliferation, colony formation, migration and invasion assays were performed. Cell viability was assessed using the MTT assay (cat. no. M8180; Beijing Solarbio Science & Technology Co., Ltd.); Huh-7 and Hep3B cells were seeded at 1×10³ cells per well in a 96-well plate. MTT solution (20 µl; 5 mg/ml) was added to designated wells on each of 5 consecutive days, with a separate set of wells assayed at each time-point, and absorbance was measured at 570 nm following dissolution of the formazan crystals in dimethyl sulfoxide. For the colony-formation assay, equal numbers of cells (1,000 cells per well) were seeded into 6-well plates. After 10 days of culture, cells were stained with 0.1% crystal violet (cat. no. C8470; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 20 min and colonies (≥50 cells) were counted. Cell migration and invasion capabilities were evaluated using Transwell chambers (8-µm pore size; cat. no. 3422; Corning, Inc.). The migration assay: cells (1×10⁵ cells per well) were resuspended in 200 µl serum-free medium and seeded into the upper chamber with uncoated 8-µm pores, while 600 µl complete medium containing 10% FBS was added to the lower chamber; the invasion assay utilized a pre-coated Matrigel matrix (cat. no. M8370; Beijing Solarbio Science & Technology Co., Ltd.). After incubation for 24 and 48 h, respectively, cells were fixed with 4% paraformaldehyde at room temperature for 15 min and stained with 0.1% crystal violet at room temperature for 20 min and the number of cells that had transgressed through the membrane was counted under a light microscope (x200 magnification) using ImageJ software (version 1.54; National Institutes of Health; <https://imagej.net/ij/>) to assess metastatic potential.

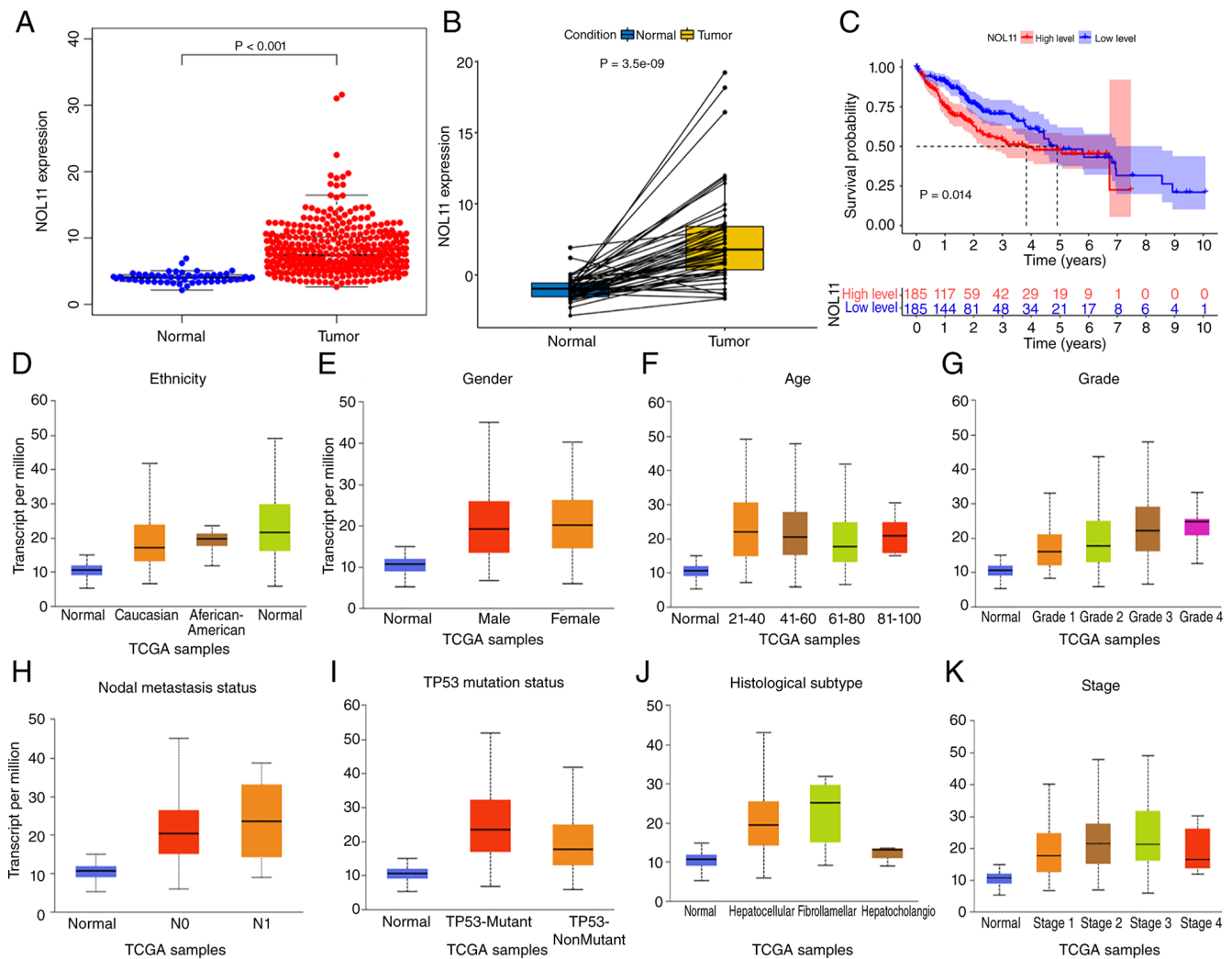


Figure 1. High expression of NOL11 is associated with poor prognosis in HCC. (A) Differential expression of NOL11 between HCC and normal liver tissues ($P < 0.001$). (B) Differential expression of NOL11 in paired HCC and adjacent normal tissues ($P < 0.001$). (C) Survival analysis was conducted to compare OS rates between patients with HCC with high and low NOL11 expression ($P = 0.014$). (D-K) NOL11 expression in different clinico-pathological subgroups of patients with HCC. (D) Ethnicity; (E) gender; (F) age; (G) grade; (H) nodal metastasis status; (I) TP53 mutation status; (J) histological subtype; and (K) stage. NOL11 expression increased gradually with higher tumor grade, nodal metastasis status and TP53-mutant status. With respect to clinical stage, NOL11 expression increased progressively from stage 1 to stage 3 but was lower at stage 4 than at stage 3. TCGA, the Cancer Genome Atlas; HCC, hepatocellular carcinoma; NOL11, nucleolar protein 11; OS, overall survival.

Statistical analysis. Bioinformatics analyses were performed using R software (version 4.2.3). Differential gene expression analysis was conducted using the limma package. Survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. Univariate and multivariate Cox proportional hazards regression analyses were performed to evaluate the independent prognostic value of clinicopathological variables and the risk score. Comparisons of immune cell infiltration levels among the four NOL11 copy number states were performed using the Kruskal-Wallis test. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant. *In vitro* experiments were performed as three independent replicates. Quantitative data are presented as the mean $\pm$ standard deviation. Statistical analyses for *in vitro* experiments were performed using GraphPad Prism version 10.4 (Dotmatics). Comparisons among shCtrl, shNOL11-1 and shNOL11-2 groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's

multiple-comparisons test, with the shCtrl group designated as the reference group. For MTT cell-growth curves involving multiple groups measured over multiple time-points, two-way ANOVA followed by Dunnett's multiple-comparisons test was used. A two-sided $P < 0.05$ was considered to indicate statistical significance.

Results

High expression of NOL11 is associated with poor prognosis in HCC. Based on HCC transcriptome and clinical data from the TCGA database (including 50 normal tissues and 370 tumor tissues), NOL11 expression and its clinical significance in HCC were assessed. Results revealed significantly higher mRNA expression levels of NOL11 in HCC tissues compared to normal tissues (Fig. 1A). This trend was further validated in paired homologous patient samples, where NOL11 expression was markedly elevated in tumor tissues relative to adjacent

normal tissues (Fig. 1B). Survival analysis demonstrated that patients with high NOL11 expression exhibited shorter OS compared to those with low expression, with statistically significant differences (Fig. 1C). Subgroup analysis using the UALCAN database revealed that NOL11 transcription levels in HCC were significantly higher than those in normal controls across different ethnic groups, genders, ages, grades, nodal metastasis statuses, TP53 mutation statuses, histological subtypes and stages (Fig. 1D-K). Additionally, NOL11 expression increased gradually with higher tumor grade, lymph node metastasis and TP53-mutant status. With respect to clinical stage, NOL11 expression increased progressively from stage 1 to stage 3 but was lower at stage 4 than at stage 3.

Correlation between NOL11 and tumor-infiltrating immune cells in HCC. To elucidate the relationship between NOL11 and the immune microenvironment, a systematic analysis of the composition of tumor-infiltrating immune cells in HCC samples was conducted. The results revealed that five immune cell types exhibited significant differences between the NOL11 low-expression and high-expression groups (Fig. 2A). Additionally, NOL11 expression was positively correlated with the infiltration of B cells naive, Macrophages M0 and Eosinophils, while it was negatively correlated with T cells CD4 memory resting, T cells CD8 and Macrophages M2 (Fig. 2C). Combining the differential and correlation analyses, the immune cell types identified as differentially infiltrated and those significantly correlated with NOL11 expression were intersected, and three cell types, namely M0 macrophages, M2 macrophages and eosinophils, were found to overlap between the two analyses, suggesting that NOL11 may primarily regulate immune activity in the tumor microenvironment by modulating the infiltration levels of these three cell types (Fig. 2B). Further analysis revealed that the CNV of NOL11 was also closely associated with the infiltration levels of multiple immune cell types. In general, the infiltration levels of CD8+ T cells, CD4+ T cells, macrophages, neutrophils and dendritic cells were lower in tumors with arm-level gain or high-level amplification of NOL11 than in those with arm-level deletion, with stepwise decreases being most evident for dendritic cells, macrophages, CD8+ T cells and CD4+ T cells ($P < 0.05$ for CD8+ T cells, macrophages and dendritic cells; $P < 0.01$ for neutrophils; $P < 0.001$ for CD4+ T cells), whereas B-cell infiltration did not differ evidently among the NOL11 copy number states (Fig. 2D). GO enrichment analysis indicated that NOL11 participates in multiple immune-related biological processes (Fig. 3A). KEGG pathway enrichment analysis further suggested that NOL11 may be associated with tumor-related pathways such as the PI3K-Akt signaling pathway and the MAPK pathway (Fig. 3B). Collectively, these results indicate that NOL11 expression levels may serve as a potential indicator reflecting the immune status of the tumor microenvironment.

Construction and validation of risk model. A protein interaction network for NOL11 was constructed based on the STRING database, from which the 30 genes most closely interacting with NOL11 were identified (Fig. 3C). To further evaluate the potential prognostic value of these co-expressed genes

in HCC, univariate Cox regression analysis was performed, revealing that 19 of these genes were significantly associated with HCC patient prognosis (Fig. 3D). Subsequently, a risk prediction model comprising eight genes was constructed using LASSO-Cox regression analysis (Fig. 3E and F). The risk score calculation formula is as follows: Risk score = (XRN2 expression $\times$ 0.0748) + (BOP1 expression $\times$ 0.0277) + (RRP9 expression $\times$ 0.0426) + (NCL expression $\times$ 0.2772) + (WDR12 expression $\times$ 0.1253) + (MPHOSPH6 expression $\times$ 0.1213) + (NOP58 expression $\times$ 0.1833) + (NHP2 expression $\times$ 0.1482). Patients were stratified into high-risk and low-risk groups based on median risk scores. The results demonstrated that high-risk patients exhibited higher mortality rates and significantly shorter OS compared to low-risk patients (Fig. 4A and B). ROC curve and calibration curve analyses further confirmed that this NOL11-related gene-based risk model possesses high predictive accuracy for patient prognosis (Fig. 4C and D). PCA and t-SNE dimensionality reduction revealed that the risk score effectively distinguished patients with HCC across subgroups, with significant differences in clinicopathological characteristics and prognosis between groups (Fig. 4E and F). Finally, both univariate and multivariate Cox regression analyses confirmed that the risk score represents an independent prognostic factor for patients with HCC (Fig. 4G and H).

Construction and prediction of nomograms. To develop a clinical tool capable of quantitatively predicting 1-, 3- and 5-year OS in patients with HCC, a predictive nomogram was constructed based on independent prognostic factors (Fig. 5A). By accumulating the scores corresponding to each variable in the nomogram, a total score for each patient is obtained. This total score is then projected onto the survival probability axis to derive the patient's expected survival rates at 1, 3 and 5 years. ROC analysis revealed area under curve values of 0.779, 0.765 and 0.757 for predicting 1-, 3- and 5-year survival rates, respectively (Fig. 5B). Compared with other clinical prognostic factors, this nomogram demonstrated higher accuracy in predicting HCC patient survival rates (Fig. 5C). Calibration curves further demonstrated that the predicted survival rates at 1-, 3- and 5-year time-points closely aligned with the ideal reference line, indicating excellent predictive consistency and reliability (Fig. 5D). In summary, this nomogram serves as an intuitive and accurate prognostic assessment tool with potential clinical utility, facilitating personalized survival prediction for patients with HCC.

Estimation of TMB and immune checkpoint expression levels by risk model. Fig. 6A and B display significantly enriched signaling pathways in the high-risk and low-risk groups. Analysis of the HCC mutation spectrum based on the TCGA cohort revealed that although mutation frequencies varied across genes between the high-risk and low-risk groups, TP53, CTNNB1 and TTN were consistently the three most frequently mutated genes in both groups (Fig. 6C and D). Further calculation of TMB scores and comparison between groups revealed significantly higher TMB in the high-risk group compared to the low-risk group, with risk scores positively correlated with TMB (Fig. 6E and F). Survival analysis revealed significantly

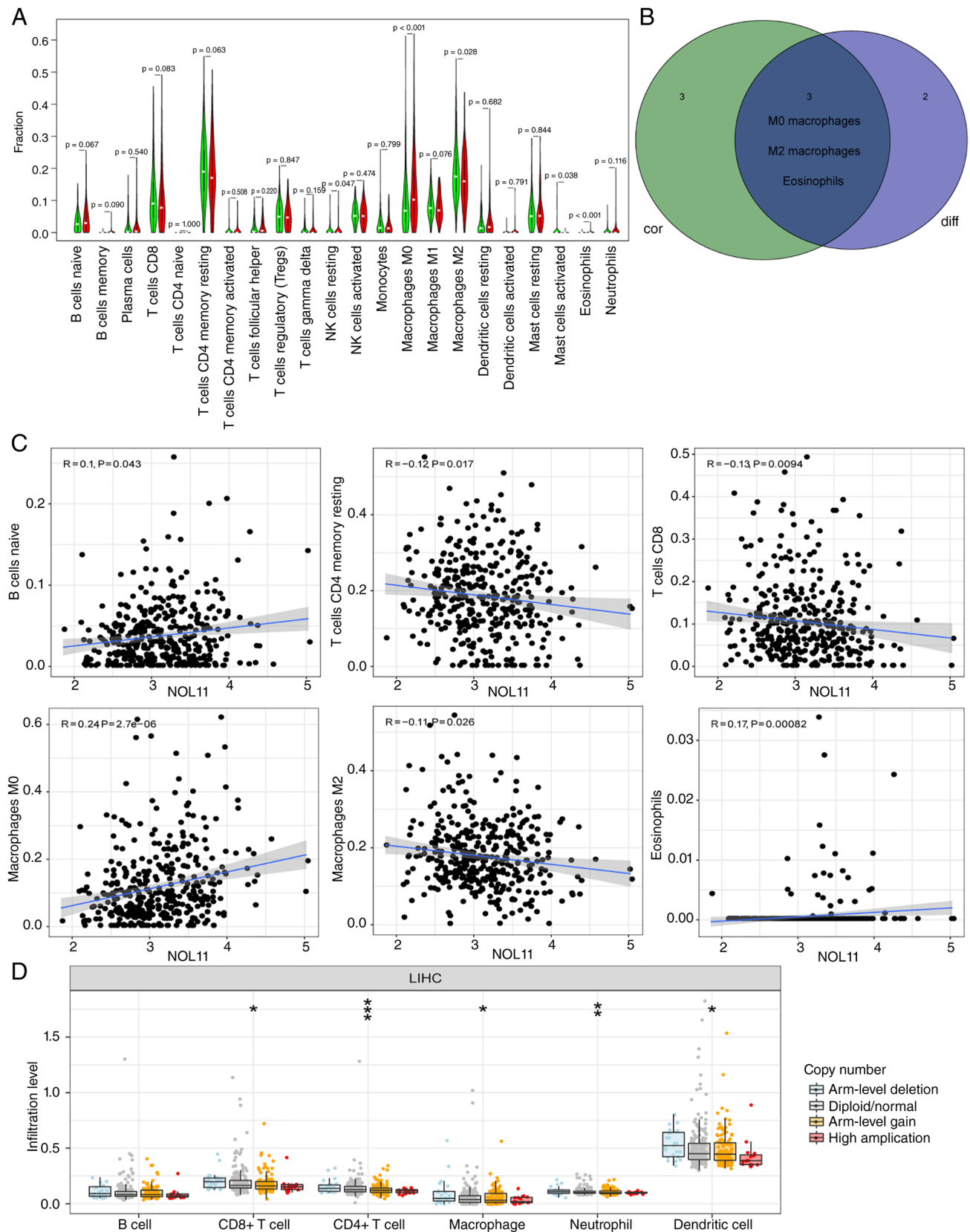


Figure 2. Correlation between NOL11 and tumor-infiltrating immune cells in HCC. (A) The violin plot shows the difference in immune cell types in the NOL11 low expression group vs. the high expression group. (B) The Venn diagram shows the overlap between the immune cell types identified by the differential analysis and those significantly correlated with NOL11 expression; the three cell types within the overlap are labelled in the figure and are M0 macrophages, M2 macrophages and eosinophils. (C) The scatter plots show 6 immune cell types positively or negatively correlated with NOL11 expression ($P < 0.05$). The specific cell types and the direction of each correlation are indicated within the figure. (D) Correlation analysis between NOL11 copy number states (arm-level deletion, diploid/normal, arm-level gain and high amplification) and the infiltration levels of immune cells in HCC; differences among the four NOL11 copy number states were assessed using the Kruskal-Wallis test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ among the NOL11 copy number states. HCC, hepatocellular carcinoma; NOL11, nucleolar protein 11; diff, differential analysis; cor, correlation analysis.

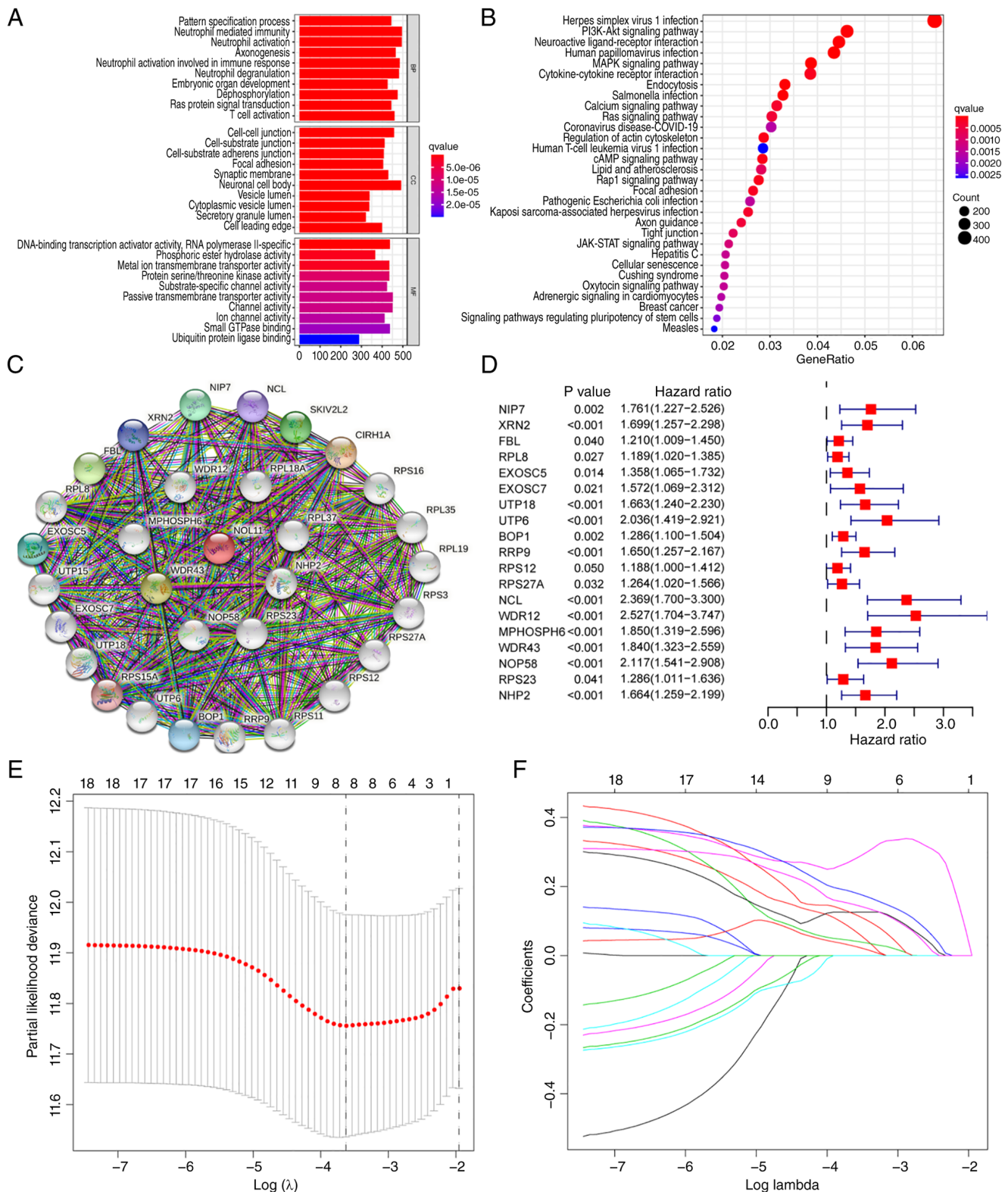


Figure 3. Construction of a risk model based on NOL11-related genes. (A) Bar charts and (B) bubble charts respectively show the results of Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses. (C) The network view displays the predicted protein interaction network highly correlated with NOL11. (D) The forest plot displays 19 genes identified through univariate cox regression analysis as being closely associated with hepatocellular carcinoma prognosis. (E) Partial likelihood deviance and (F) coefficient profiles of LASSO regression model. NOL11, nucleolar protein 11.

shorter OS in patients with high TMB compared to those with low TMB (Fig. 6G). Subgroup analysis further demonstrated the poorest prognosis in patients with high TMB combined with the high-risk subgroup, while the best prognosis was

observed in those with low TMB combined with the low-risk subgroup (Fig. 6H). Considering that patients with elevated TMB may exhibit greater sensitivity to immune checkpoint inhibitor therapy, the relationship between risk scores and

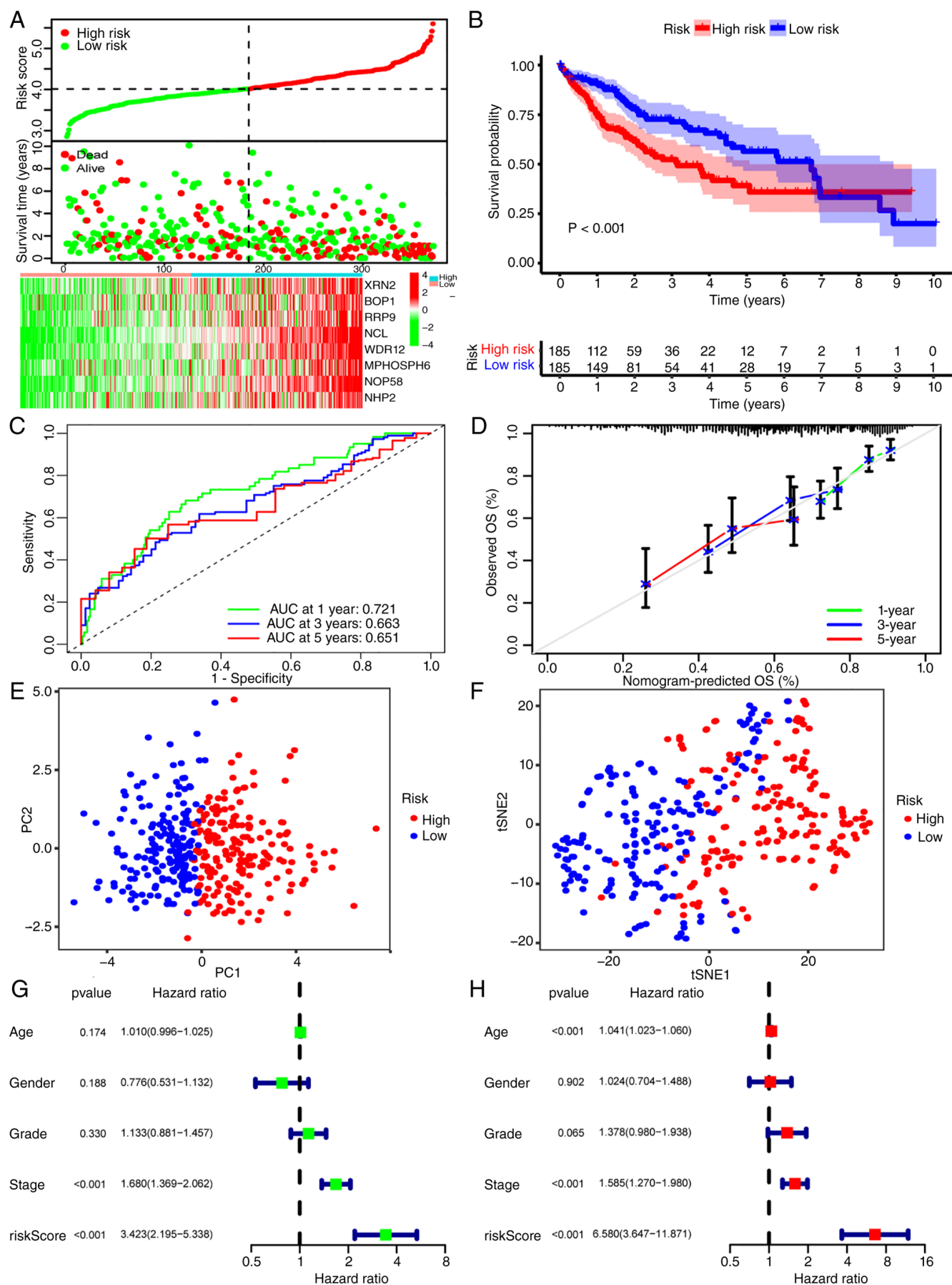


Figure 4. Validation of the risk model. (A) Distribution of risk score, survival status and gene expression among patients with hepatocellular carcinoma. (B) OS was compared between the high-risk and low-risk groups. The 1-, 3- and 5-year (C) ROC curves and (D) calibration curve of the risk model. (E) The PCA and (F) t-SNE of two risk groups. (G) Univariate and (H) multivariate Cox regression analysis of prognostic factors. OS, overall survival; AUC, area under the ROC curve; ROC, receiver operating characteristic; PCA, principal component analysis; t-SNE, t-distributed stochastic neighborhood embedding.

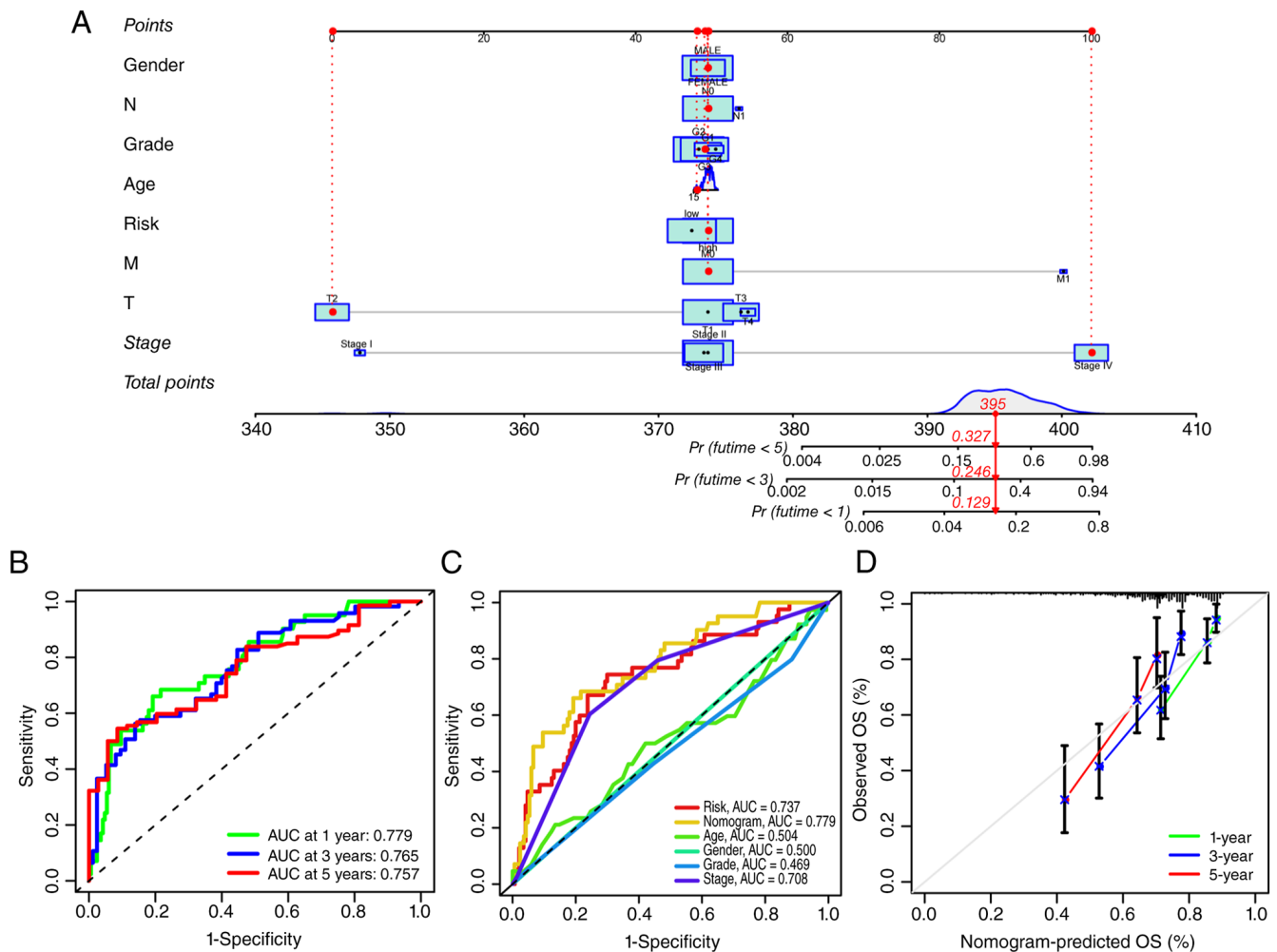


Figure 5. Construction and prediction of nomograms. (A) A nomogram predicting 1-, 3- and 5-year OS in patients with hepatocellular carcinoma was constructed based on independent factors. (B) The 1-, 3- and 5-year ROC curves of the nomogram. (C) ROC curves of different clinical factors were compared. (D) The calibration curve of the nomogram for the prediction of 1-, 3- and 5-year survival probability. AUC, area under the ROC curve; ROC, receiver operating characteristic; OS, overall survival.

immune checkpoint gene expression was further analyzed. The results demonstrated that PD-1, CTLA-4 and IDO1 were significantly upregulated in the high-risk group, with their expression levels positively correlated with risk scores. The expression levels of PD-L1, LAG3 and KDR did not differ significantly between the two groups, and the correlation for LAG3 was not significant. KDR expression was numerically higher in the low-risk group (Fig. 7).

Knockdown of NOL11 inhibits the proliferation and migration of HCC cells in vitro. To further validate the expression level of NOL11 in HCC, immunohistochemical images were viewed in the HPA database, revealing obviously elevated NOL11 protein expression in HCC tissues compared with normal liver tissues (Fig. 8). To investigate the biological role of NOL11 in HCC cells, NOL11 expression was silenced in Huh-7 and Hep3B cells using two independent shRNA plasmids, shNOL11-1 and shNOL11-2. Western blotting confirmed that both shNOL11 constructs substantially reduced NOL11 protein expression compared with the non-targeting shCtrl (Fig. 9A). In functional assays, NOL11 knockdown markedly impaired cell proliferation (Fig. 9B). MTT assays, which

reflect the number of viable cells and thus cell proliferation rather than cell death, further confirmed that NOL11 knockdown significantly reduced the number of viable HCC cells (Fig. 9C). Colony-formation assays revealed markedly reduced colony formation in both Huh-7 and Hep3B cells following NOL11 knockdown (Fig. 9D and E). Given the clinical association between NOL11 overexpression and HCC metastasis (Fig. 1), cell migration and invasion assays were subsequently performed. The results indicated that NOL11 knockdown significantly inhibited the migration capacity in both Huh-7 and Hep3B cells (Fig. 9F and G). Similarly, the cell invasion assays demonstrated markedly reduced invasive ability of tumor cells following NOL11 interference (Fig. 9H and I). These findings collectively demonstrated that NOL11 plays a crucial regulatory role in the proliferation, migration, and invasion processes of HCC cells.

Discussion

In the present study, the expression pattern, prognostic significance, immune relevance and biological function of NOL11 in HCC were comprehensively characterized. Through

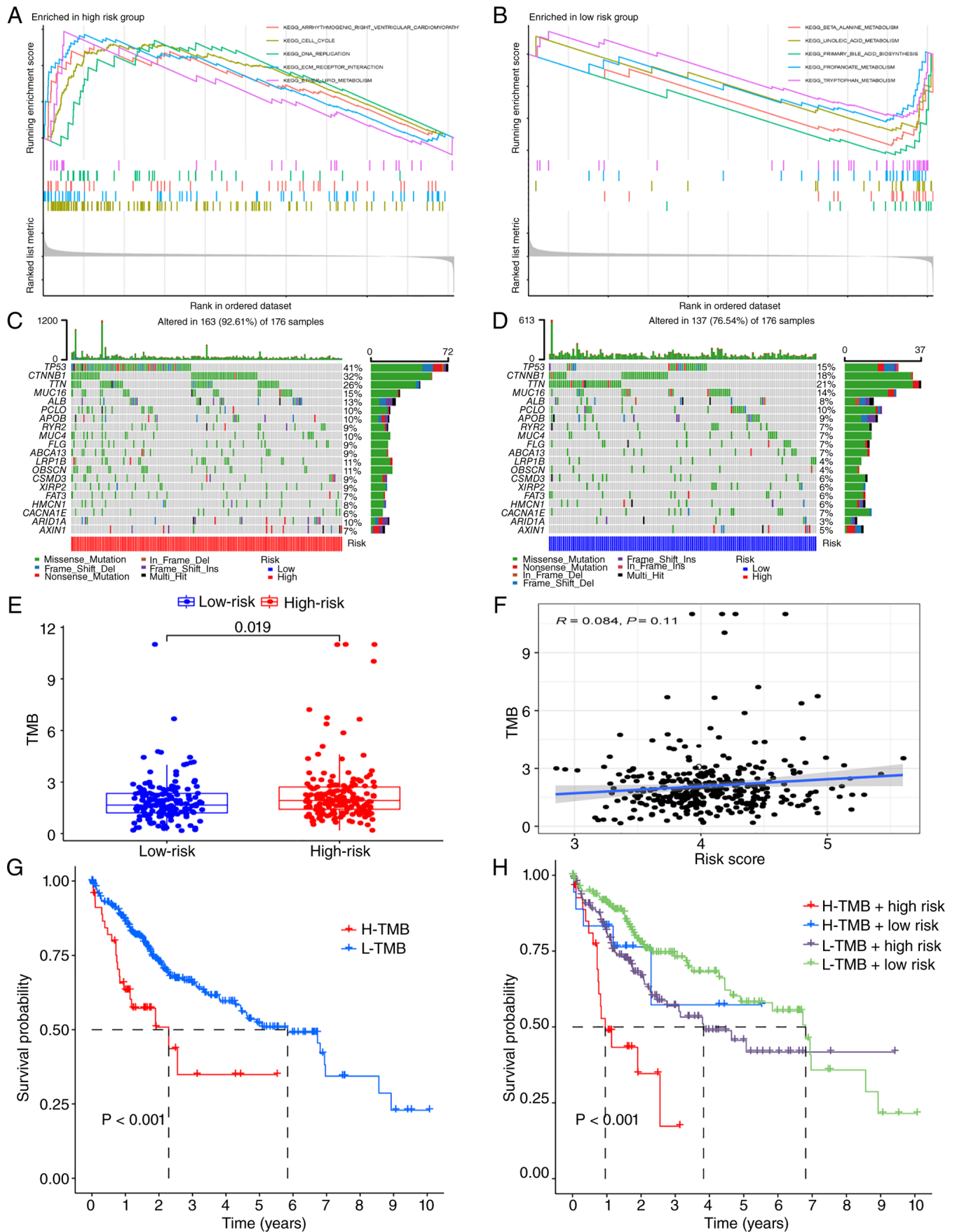


Figure 6. Estimation of TMB in the high-risk and low-risk groups. (A and B) Kyoto Encyclopedia of Genes and Genomes enrichment analysis in (A) the high-risk group and (B) in the low-risk group. (C and D) Waterfall plots display the types and proportions of mutated genes in (C) the high-risk and (D) low-risk groups. (E) Differences in TMB between high-risk and low-risk groups. (F) Correlation analysis between risk scores and TMB. (G) Comparison of OS between high-TMB and low-TMB groups in patients with HCC. (H) Differences in OS among HCC patients across different TMB levels and risk score groups. OS, overall survival; H-TMB, high tumor mutation burden; HCC, hepatocellular carcinoma; Ins, insertion; Del, deletion.

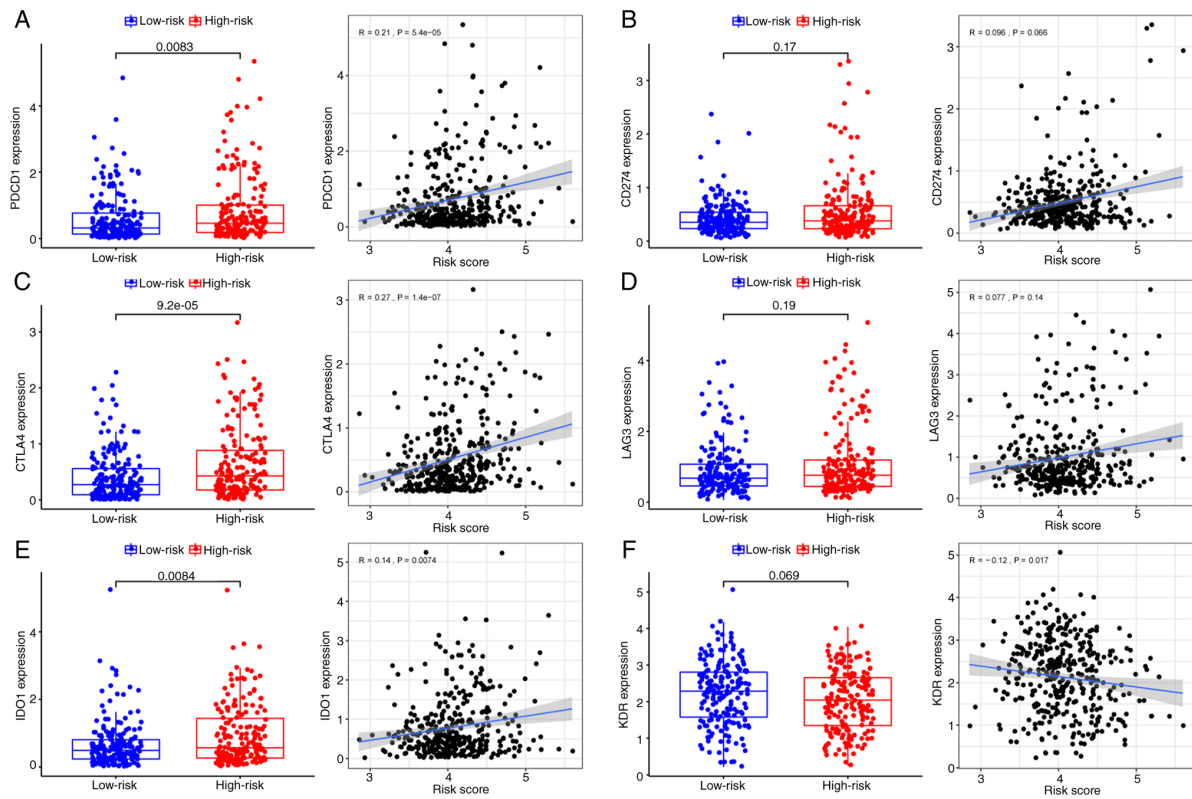


Figure 7. Estimation of immune checkpoint expression levels by risk model. High risk scores are correlated with upregulated (A) PD-1, (B) PD-L1, (C) CTLA4, (D) LAG3 and (E) IDO1 in patients with HCC. Low risk scores correlate with upregulated (F) KDR in patients with HCC. HCC, hepatocellular carcinoma; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; CTLA, cytotoxic T-lymphocyte antigen; LAG3, lymphocyte activating 3; IDO1, indoleamine 2,3-dioxygenase 1; KDR, kinase insert domain receptor.

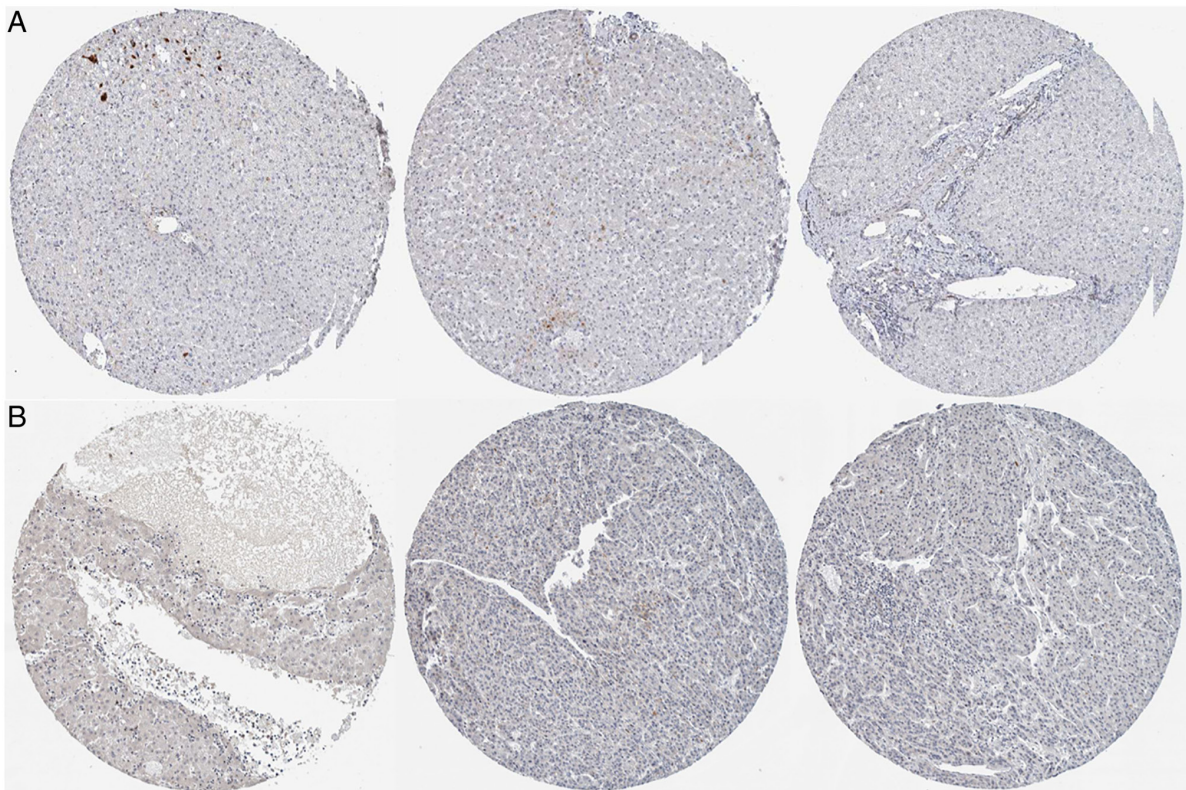


Figure 8. NOL11 protein expression in HCC and normal liver tissue in the Human Protein Atlas database. Immunohistochemistry reveals the expression levels of NOL11 protein in (A) normal liver tissue samples and (B) HCC tissue samples. Positive NOL11 staining is indicated by brown (3,3'-diaminobenzidine) staining, and nuclei were counterstained with hematoxylin (blue). The images were viewed in the Human Protein Atlas database (<https://www.proteinatlas.org/>); the database does not provide scale bars or objective magnification for these images. NOL11, nucleolar protein 11; HCC, hepatocellular carcinoma.

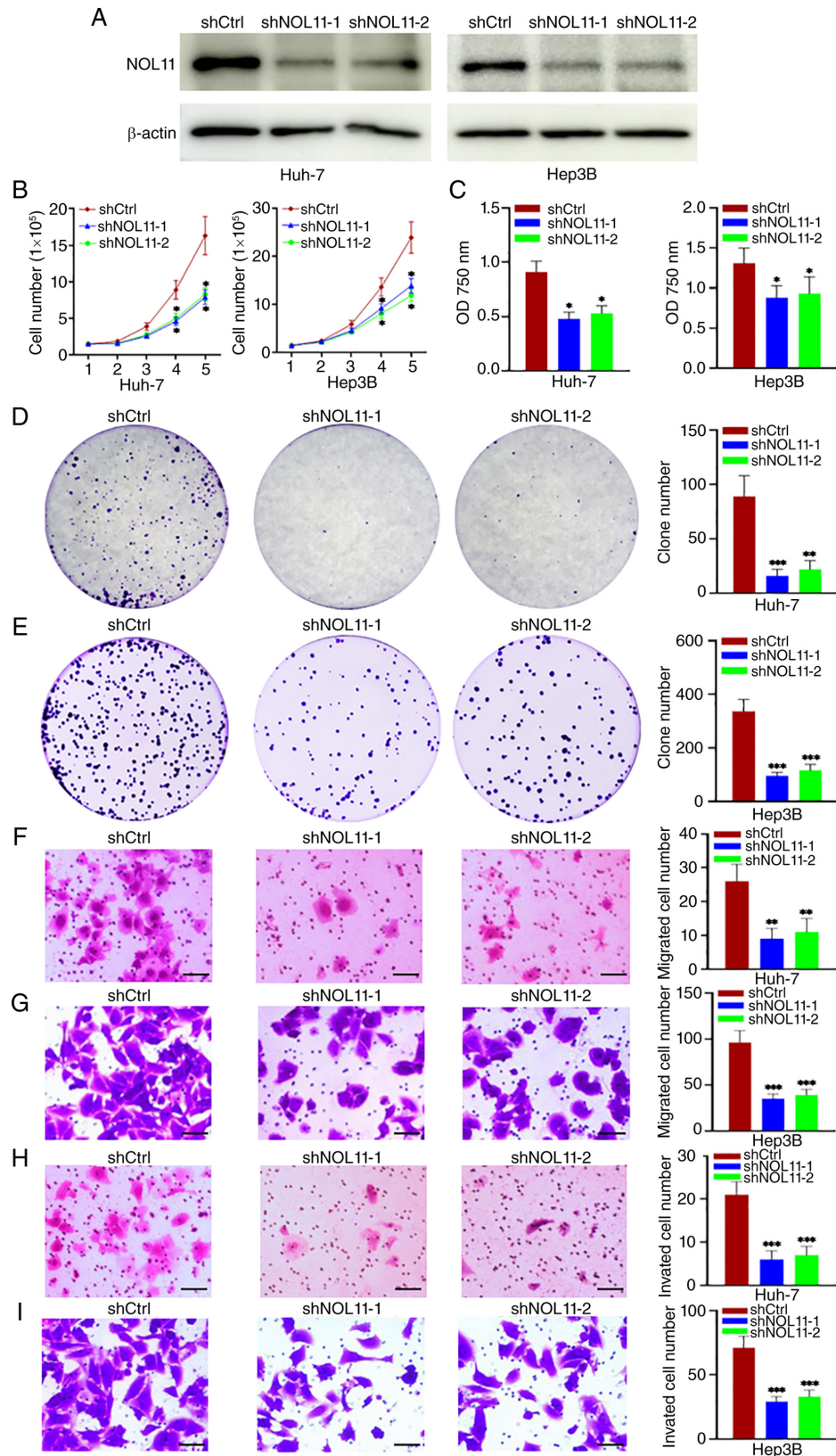


Figure 9. Knockdown of NOL11 inhibits the proliferation and migration of HCC cells *in vitro*. (A) Western blot analysis of NOL11 expression in Huh-7 and Hep3B cells transfected with shNOL11-1, shNOL11-2, or the non-targeting control plasmid shCtrl. β -actin was used as the loading control. (B) Cell proliferation (cell-growth curves) of HCC cells in the control and experimental groups. (C) Cell viability of HCC cells in the control and experimental groups, as determined using the MTT assay. Cell colony formation assay was performed in control and experimental (D) Huh-7 and (E) Hep3B HCC cells. Transwell migration assays in control and experimental (F) Huh-7 and (G) Hep3B HCC cells. Transwell invasion assays were performed in control and experimental (H) Huh-7 and (I) Hep3B HCC cells. Representative Transwell images were captured at x200 magnification (scale bars, 100 μ m). Data are presented as the mean $\pm$ SD from three independent experiments. One-way ANOVA followed by Dunnett's multiple-comparisons test was used for comparisons among three groups in panels C-I, with shCtrl as the reference group. Two-way ANOVA followed by Dunnett's multiple-comparisons test was used for the cell-growth curves in panel B. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. shCtrl. NOL11, nucleolar protein 11; HCC, hepatocellular carcinoma; shNOL11, short hairpin RNA targeting shNOL11; Ctrl, control; OD, optical density.

integrative analyses of transcriptomic and clinical data from the TCGA cohort, it was demonstrated that NOL11 is significantly upregulated in HCC tissues and closely associated with aggressive clinicopathological characteristics and unfavorable OS. Furthermore, a robust NOL11-related risk model was constructed and validated, which effectively stratifies patient prognosis and reflects distinct immune and mutational landscapes. Functional experiments further confirmed that NOL11 promotes HCC cell proliferation, migration and invasion, supporting its oncogenic role in HCC progression.

Nucleolar proteins, as core participants in ribosomal biosynthesis, cell cycle regulation and stress responses, are increasingly recognized for their regulatory roles in tumorigenesis (20). NOL11, an evolutionarily conserved nucleolar protein, is known to play a critical role in the transcription and processing of precursor rRNA and is associated with hepatic pathological processes such as cirrhosis. In recent years, multiple studies have progressively revealed the potential role of NOL11 in the development and progression of various malignant tumors (21,22). However, its role in HCC has not been fully elucidated. The present study confirms that NOL11 is abnormally overexpressed in HCC and is closely associated with tumor grade, lymph node metastasis and clinical staging, suggesting that this protein may be involved in the development and progression of HCC. Mechanistically, functional enrichment analyses revealed that NOL11-associated genes are involved in multiple tumor-related signaling pathways, including the PI3K-Akt and MAPK pathways (23), which are well-established drivers of HCC proliferation, survival and metastasis. Consistent with these bioinformatics findings, *in vitro* functional assays demonstrated that silencing NOL11 significantly suppressed HCC cell proliferation, colony formation, migration and invasion. These results provide direct experimental evidence that NOL11 exerts pro-tumorigenic effects and contributes to the malignant phenotype of HCC cells.

The tumor immune microenvironment plays a pivotal role in HCC progression, therapeutic response and patient prognosis (24,25). HCC typically develops in a background of chronic inflammation and immune dysregulation, resulting in a complex and often immunosuppressive microenvironment characterized by dysfunctional T cells, tumor-associated macrophages and impaired antitumor immunity (26). Increasing evidence indicates that immune cell composition and functional status within the tumor microenvironment critically influence tumor growth, metastatic potential and resistance to therapy (27-29). In recent years, immune checkpoint inhibitors targeting PD-1/PD-L1 and CTLA-4 pathways have emerged as promising treatment options for advanced HCC (24,30). However, only a subset of patients achieves durable clinical benefit, highlighting the urgent need for reliable biomarkers to predict immunotherapy responsiveness. TMB, immune checkpoint expression and immune cell infiltration patterns have been proposed as predictive indicators, yet their clinical utility remains limited when considered individually (30,31).

In this context, the present study revealed a close association between the NOL11-related risk model and the immune landscape of HCC. High-risk patients exhibited significantly higher TMB and poorer survival outcomes,

and those with concomitant high TMB and high-risk scores showed the worst prognosis. Furthermore, key immune checkpoint molecules, including PD-1, CTLA-4 and IDO1, were markedly upregulated in the high-risk group, whereas PD-L1, LAG3 and KDR did not differ significantly between the two groups, with KDR expression being numerically higher in the low-risk group. These findings suggest that the NOL11-related risk model may reflect an immunologically active but functionally suppressed tumor microenvironment and may have potential value in identifying patients who could benefit from immune checkpoint blockade therapy. To facilitate clinical translation, the risk score was further integrated with independent prognostic factors to construct a nomogram for individualized survival prediction. This nomogram demonstrated superior predictive accuracy and excellent calibration for 1-, 3- and 5-year OS compared with traditional clinical parameters alone (32), underscoring its potential utility as a practical prognostic assessment tool in clinical settings. Future research should focus on validating the prognostic and immunological relevance of NOL11 in larger independent and multicenter HCC cohorts, as well as clarifying the molecular mechanisms underlying its role in tumor progression and immune regulation. From a translational perspective, exploration of NOL11-associated molecular networks may help identify potential therapeutic targets. In this context, drug repurposing may represent a practical, cost-effective and potentially sustainable strategy for accelerating the discovery of clinically relevant compounds and facilitating the translation of molecular findings into therapeutic applications (33-35).

The present study has several notable strengths. First, it integrated large-scale transcriptomic and clinical data with multiple bioinformatics approaches to systematically evaluate the expression pattern and prognostic significance of NOL11 in HCC. Second, beyond conventional survival analyses, this study further explored the associations of NOL11 with immune cell infiltration, immune-related pathways, tumor mutational burden and immune checkpoint molecules, thereby providing a broader view of its potential biological and clinical relevance. Third, a NOL11-related prognostic model was constructed to assess its potential value in individualized risk stratification. Finally, the bioinformatics findings were supported by *in vitro* experiments, which strengthened the biological relevance of the study. Despite these strengths, several limitations should be acknowledged. First, all transcriptomic and clinical analyses were based on retrospective public datasets, primarily from TCGA, and lacked independent multicenter external validation (36). Second, although the present *in vitro* experiments confirmed the role of NOL11 in HCC cell proliferation, migration and invasion, the underlying molecular mechanisms were not fully elucidated. Therefore, future studies incorporating large prospective cohorts, external validation datasets and *in vivo* functional experiments are warranted to further confirm the prognostic significance and therapeutic potential of NOL11 in HCC.

In conclusion, the present study demonstrates that NOL11 plays a critical role in HCC progression by promoting tumor aggressiveness and shaping the immune microenvironment. The NOL11-related risk model provides a powerful tool for

prognostic stratification and may aid in predicting immunotherapy responsiveness. These findings expand the current understanding of nucleolar protein dysregulation in HCC and support NOL11 as a promising prognostic biomarker and potential therapeutic target.

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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

HX designed the study. SW and XS performed the bioinformatics analysis and data visualization. LY conducted the *in vitro* experiments. HX and SW drafted the manuscript. HX and SW checked and confirmed the authenticity of all the raw data. All authors 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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