

Prognostic biomarker SCN1 is associated with immune infiltration and therapeutic response, and promotes tumor migration in hepatocellular carcinoma

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Abstract. Sodium channel modifier 1 (SCN1) is involved in biological processes including RNA splicing and the regulation of primary cilium length; however, its role in hepatocellular carcinoma (HCC) remains largely unclear. The present study aimed to investigate the expression pattern, clinical relevance and functional significance of SCN1 in HCC. Clinical and transcriptomic data were obtained from The Cancer Genome Atlas, Genotype-Tissue Expression, International Cancer Genome Consortium and Gene Expression Omnibus databases. SCN1 expression, DNA methylation status and immune infiltration characteristics were systematically analyzed. A SCN1-related prognostic gene signature was constructed using machine learning approaches. Cell Counting Kit-8, colony formation and Transwell migration assays were performed to evaluate the effects of SCN1 knockdown on Huh1 cell proliferation, clonogenic capacity and migration, respectively. SCN1 protein expression was also evaluated by immunohistochemistry in clinical tissue specimens obtained from 10 patients with HCC. SCN1 was significantly upregulated in HCC tissues and was associated with unfavorable survival outcomes. Immunohistochemical staining further confirmed higher SCN1 protein expression in HCC tissues than in non-tumor liver tissues. Gene set enrichment analysis indicated that SCN1 expression was closely associated with metabolic pathways, including oxidative phosphorylation and immune-related processes. Elevated SCN1 expression was associated with increased estimated infiltration of immune cells, including B cells and macrophages, and positively

correlated with the expression of antigen processing and presentation-related genes, including human leukocyte antigen-A, transporter associated with antigen processing (TAP)1 and TAP2. Functional experiments demonstrated that SCN1 knockdown suppressed HCC cell proliferation, clonogenic capacity and migratory ability. In addition, the SCN1-based prognostic model effectively stratified patients with distinct survival outcomes. In conclusion, the results indicated that SCN1 is upregulated in HCC and is associated with poor prognosis and immune-related characteristics. The SCN1-related prognostic model may provide a useful tool for patient risk stratification and offer insights into the clinical relevance of SCN1 in HCC.

Introduction

In 2022, ~865,000 new liver cancer cases and 758,000 related deaths were estimated worldwide (1). Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer (2). According to the National Cancer Center of China, an estimated 367,700 new liver cancer cases occurred in China in 2022, making liver cancer the fourth most frequently diagnosed malignancy and the second leading cause of cancer-related death nationally (3). Previously reported 5-year survival estimates summarized by Shan *et al* (4) were 12.1% in China and 17.4% in the USA, highlighting the poor prognosis of liver cancer and differences in outcomes between populations. These observations support the need to better characterize the molecular features associated with HCC progression and patient survival.

The immune microenvironment provides an additional context for investigating molecular biomarkers in HCC. Single-cell sequencing has revealed distinct populations of tumor-infiltrating T cells, including enriched exhausted CD8⁺ T cells and regulatory T cells, demonstrating the diversity of immune states within HCC tissues (5). These findings provide a rationale for examining candidate biomarkers in relation to immune cell populations rather than considering tumor tissue as a uniform cellular compartment. Combining bulk transcriptomic analyses with single-cell expression profiles may help clarify the cellular distribution and immune-related associations of candidate genes.

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Sodium channel modifier 1 (SCNM1) encodes a zinc finger protein involved in pre-mRNA splicing. In mice, *Scnm1* was identified as a modifier of disease severity through its effects on the splicing of a mutant *Scn8a* sodium channel transcript (6). Subsequent experiments demonstrated an interaction between SCNMI and the spliceosomal protein U1-70K, supporting a direct role in RNA splicing (7). Thus, the relationship of SCNMI with sodium channel biology involves transcript processing rather than direct regulation of channel gating. In the major spliceosome, splicing factor 3a (SF3a) contributes to the formation of functional U2 small nuclear ribonucleoprotein particles and prespliceosome assembly (8). Structural studies identified SCNMI as a component of the minor spliceosome, where it functionally mimics SF3a and contributes to stabilization of the catalytic center during U12-type intron splicing (9). Furthermore, biallelic loss-of-function mutations in the SCNMI gene cause orofaciocigital syndrome through defective minor intron splicing affecting primary cilia (10).

Evidence also supports a role for SCNMI in HCC. Ofuchi *et al* (11) reported that elevated SCNMI expression was associated with chromosome 1q amplification and poor prognosis, and their functional experiments implicated SCNMI in tumor growth and resistance to apoptosis through splicing-related regulation involving Derlin-2 and B-cell-associated gene 6. These findings establish a basis for further investigation of SCNMI in liver cancer. Building on this work, the present study focused on the associations of SCNMI with immune infiltration, metabolic pathways and DNA methylation, together with the prognostic information provided by SCNMI-related gene expression patterns. These complementary analyses were intended to extend the characterization of SCNMI beyond its previously reported associations with tumor growth and survival.

The present study integrated multiple public datasets to examine SCNMI expression and its associations with clinicopathological characteristics, survival and immune-related features in HCC. Bulk transcriptomic and single-cell analyses were combined with pathway enrichment and DNA methylation analyses. A prognostic signature based on SCNMI-related genes was developed and evaluated in an independent cohort. Clinical tissue specimens and Huh1 cell experiments were used to assess SCNMI protein expression and the effects of SCNMI knockdown on proliferation, colony formation and migration. The aim was to further characterize the clinical and biological relevance of SCNMI and evaluate the potential of its related gene signature for prognostic risk stratification.

Materials and methods

Collection and organization of clinical data and gene expression. Clinical and transcriptomic data for liver hepatocellular carcinoma (LIHC) were obtained from multiple publicly available databases. The Cancer Genome Atlas (TCGA)-LIHC dataset, including tumor samples and corresponding adjacent normal tissues, were collected from the TCGA Genomic Data Commons (GDC) portal (<https://portal.gdc.cancer.gov/>), and were primarily used for paired and unpaired differential expression analyses, survival analysis and model construction. For differential expression analysis, significance was defined as log fold change >1 and adjusted $P < 0.05$. Expression values

were normalized to transcripts per million (TPM) format for subsequent analyses. Illumina HumanMethylation450 (450K) β -value data for TCGA-LIHC were also retrieved from the TCGA GDC data portal. Using the Illumina 450K annotation incorporated in the ChAMP workflow (version 2.38.1), cg probes were mapped to their corresponding genes, and all 450K-covered CpG sites annotated to SCNMI, together with their β -values, were extracted. These data, along with clinical information from TCGA-LIHC, were used for subsequent differential methylation analysis, correlation analysis and Kaplan-Meier survival analysis.

To increase the number of normal controls for tumor vs. normal comparisons, normal liver tissue data from the Genotype-Tissue Expression (GTEx) database were integrated with TCGA tumor samples. The combined TCGA + GTEx TPM expression matrix was obtained from the University of California, Santa Cruz Xena database and log₂-transformed as log₂(TPM+1) prior to analysis.

The transcriptome expression profiles and associated clinical data of the International Cancer Genome Consortium-liver cancer-RIKEN (ICGC-LIRI) cohort were obtained from the ICGC Data Coordination Centre portal (<https://dcc.icgc.org/>). This cohort was used as an external validation set for survival analysis and prognostic model evaluation. The transcriptomic data were obtained in normalized expression format from the ICGC data portal.

For protein-level validation of the SCNMI levels, the Clinical Proteomic Tumor Analysis Consortium (CPTAC)-HCC cohort was used. Given the unavailability of the complete raw proteomic dataset from the CPTAC-HCC cohort, SCNMI protein levels in normal and tumor tissues, together with the corresponding statistical results, were retrieved from the University of Alabama at Birmingham Cancer-CPTAC portal (<https://ualcan.path.uab.edu/>). SCNMI protein expression levels in tumor and normal liver tissues were reported as Z-values derived from two-sided tests, with an unpaired Student's t-test performed by the platform's built-in analysis module.

In addition, Gene Expression Omnibus (GEO) datasets (<https://www.ncbi.nlm.nih.gov/geo/>) were included for independent validation. Expression matrices and clinical data for liver cancer were sourced from the GEO database. These GEO datasets were mainly used for independent validation of SCNMI expression patterns and its association with clinical outcomes. Specifically, data were obtained from the following datasets: GSE54236 (12), GSE14520 (13), GSE116174 (14), GSE104580 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104580>) and GSE109211 (15). GSE54236 and GSE14520 include HCC and adjacent normal liver tissues, GSE116174 contains transcriptomic profiles of HCC samples, while GSE104580 and GSE109211 are treatment-related cohorts associated with transarterial chemoembolization and sorafenib therapy, respectively. Further information on the datasets used is provided in Tables I, II and SI.

Clinical samples. All specimens used in the present study were formalin-fixed paraffin-embedded HCC tumor tissues and their paired adjacent non-tumorous liver tissues. The paired adjacent non-tumorous specimens were collected from macroscopically normal liver parenchyma >2 cm away

Table I. Detailed information of datasets used in the present study.

Data source	Dataset	Platform	Sequencing data	Tumor samples	Normal samples	Purpose
UCSC Xena TCGA	TCGA + GTEx	Illumina HiSeq	RNA-seq	9,379	6,397	DEA
	TCGA-LIHC	Illumina HiSeq	RNA-seq	374	50	DEA, prognostic model construction, model training
ICGC GEO	TCGA-LIHC	HumanMethylation450	Microarray	379	50	DEA and prognosis
	ICGC-LIRI	Illumina HiSeq	RNA-seq	243	202	External validation
	GSE112271	Illumina HiSeq	scRNA-seq	-	-	Hub gene/biomarker expression characterization
	GSE140228	Illumina HiSeq	scRNA-seq	-	-	Hub gene/biomarker expression characterization
IMvigor210CoreBiologies	GSE54236	GPL6480	Microarray	81	80	DEA and prognosis
	GSE14520	GPL3921	Microarray	222	212	DEA and prognosis
	GSE116174	GPL13158	Microarray	64	-	Prognosis
	GSE104580	GPL570	Microarray	147	-	TACE response evaluation
	GSE109211	GPL13938	Microarray	140 (sorafenib, 67; placebo, 73)	-	Sorafenib response evaluation
	GSE173839 (Wolf)	GPL20078	Microarray	105 (control, 34; treatment, 71)	-	Immunotherapy response and prognosis
	GSE78220 (Hugo)	Illumina HiSeq	RNA-seq	27	-	Immunotherapy response and prognosis
	GSE135222 (Kim)	Illumina HiSeq	RNA-seq	27	-	Immunotherapy response and prognosis
	Invigor210	Illumina HiSeq	RNA-seq	348 (not evaluable, 50; non-responder, 230; responder, 68)	-	Immunotherapy response and prognosis

UCSC, University of California, Santa Cruz; TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression; LIHC, liver hepatocellular carcinoma; ICGC, International Cancer Consortium; GEO, Gene Expression Omnibus; DEA, differential expression analysis; TACE, transarterial chemoembolization; RNA-seq, RNA-sequencing; scRNA-seq, single-cell RNA-seq; LIRI, liver cancer-RIKEN.

Table II. Clinical characteristics of the International Cancer Genome Consortium-liver cancer-RIKEN cohort (n=243).

Characteristic	Distribution, n (%)
Status	
Alive	199 (81.9)
Deceased	44 (18.1)
Sex	
Male	184 (75.7)
Female	59 (24.3)
Age, years	
≤65	94 (38.8)
>65	149 (61.2)
Stage	
I	36 (14.8)
II	110 (45.3)
III	76 (31.3)
IV	21 (8.6)

from the tumor margin after surgical resection. All specimens were obtained from patients with HCC who underwent surgical treatment at Hebei General Hospital (Shijiazhuang, China). Tissue specimens were collected between January 2024 and June 2024. Histopathological confirmation of HCC was required for inclusion. No additional exclusion criteria were applied. A total of 10 patients were enrolled, including 7 men and 3 women. The median age was 54 years (range, 41-68 years). The study protocol was approved by the Ethics Committee of Hebei General Hospital (approval no. 2024-43), and written informed consent was obtained from all participating patients.

SCNM1 enrichment analysis. To conduct a gene co-expression analysis of SCNM1, the LinkedOmics platform (www.linkedomics.org/login.php) was utilized. For this analysis, the ‘HiSeq RNA’ platform and the ‘TCGA_LIHC’ cohort were selected. To identify correlations between SCNM1 and the co-expressed genes, the Spearman’s correlation test was employed. The results of this analysis were visually represented using heat maps. Furthermore, the Gene Set Enrichment Analysis (GSEA) tool within the LinkInterpreter module was used to perform GSEA of Kyoto Encyclopedia of Genes and Genomes and Gene Ontology pathways based on genes ranked by their correlation with SCNM1 expression. Significant enrichment was defined as a false discovery rate <0.05 and an absolute normalized enrichment score >1. Additionally, Gene Set Variation Analysis (GSVA) was performed using the GSVA R package (version 2.2.0; <https://github.com/rcastelo/GSVA>).

Analysis of immune infiltration levels. To estimate the relative abundance of immune cell populations within the HCC tumor microenvironment, immune infiltration analysis was conducted in the TCGA-LIHC cohort using the immunedeconv R package (version 2.1.4; <https://github.com/icbi-lab/immunedeconv>). Using the quanTIseq and TIMER algorithms

implemented in the immunedeconv R package, the levels of immune cell infiltration in the tumor microenvironment were assessed, with TCGA-LIHC transcriptomic expression data as the input. These algorithms infer the relative proportions of multiple immune cell types based on gene expression signatures. Comparisons of immune cell infiltration levels were performed between subgroups dichotomized by the median SCNM1 expression level and the median risk score, respectively. The use of two independent algorithms aimed to enhance the robustness of immune infiltration estimation.

Single-cell RNA sequencing (scRNA-seq) data analysis. The IMMUcan platform (<https://immucanscdb.vital-it.ch>), a comprehensive database of the tumor microenvironment (TME), serves as a large-scale scRNA-seq database that facilitates the visualization of TME at the single-cell level across various cancer types. For the present study, the scRNA datasets GSE140228 (16) and GSE112271 (17) were selected for data analysis and the construction of Uniform Manifold Approximation and Projection plots. Utilizing ‘annotation immune’ for annotation, visualizations were generated depicting the expression of SCNM1 in different types of immune cells within the LIHC TME from both datasets.

Prediction of the immunotherapeutic response. The molecular predictive performance of SCNM1 for immunotherapy response was evaluated in two immunotherapy-treated cohorts, namely Imvigor210 and GSE173839, using receiver operating characteristic (ROC) curve analysis. Data for the IMvigor210 cohort were retrieved from the ‘IMvigor210CoreBiologies’ R package. Additionally, GSE78220 (18), GSE135222 (19) and Imvigor210 were used for prognostic survival analysis. The Kaplan-Meier method with the log-rank test was applied to evaluate differences in overall survival using the ‘survival’ and ‘survminer’ R packages.

Prognosis signature based on SCNM1-related genes. Based on the expression levels of SCNM1 in the TCGA-LIHC cohort, patients were stratified into high- and low-expression groups using the median expression value as the cut-off. To identify SCNM1-related prognostic genes, the Gradient Boosted Decision Tree (GBDT) algorithm (implemented via the gbm R package; <https://github.com/gbm-developers/gbm>) and the AdaBoost algorithm were applied for prognostic signature screening. Subsequently, a predictive model was developed using Least Absolute Shrinkage and Selection Operator-Cox regression. For the present study, TCGA-LIHC was employed as the training dataset, whereas ICGC-LIRI served as the validation dataset. The associations between SCNM1 expression and clinicopathological characteristics were analyzed using clinical data from the TCGA-LIHC cohort. Furthermore, a prognostic nomogram integrating SCNM1 expression and key clinicopathological features was constructed using the R package (version 6.7-1; <https://cran.r-project.org/package=rms>).

Immunohistochemistry. Resected liver tissues were fixed in 4% paraformaldehyde at room temperature for 24 h, followed by paraffin embedding. Tissue blocks were cut into 4-μm-thick sections. For immunohistochemical staining, paraffin sections were first dewaxed in xylene and rehydrated through a

descending ethanol series. Antigen retrieval was performed by heating the slides in Tris-EDTA buffer (pH 9.0) using an autoclave high-pressure procedure at 121°C for 10 min. After cooling down to room temperature, 3% hydrogen peroxide solution was used to quench endogenous peroxidase activity. Blocking was performed with 5% goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at 37°C. The primary SCNM1 antibody (cat. no. 15490-1-AP; Proteintech Group, Inc.) was diluted to a ratio of 1:200 and incubated overnight at 4°C. An HRP-conjugated goat anti-rabbit secondary antibody was obtained from a ready-to-use IHC detection kit (cat. no. PV-6000; OriGene Technologies, Inc.), and incubated for 30 min at 37°C without further dilution. 3,3'-Diaminobenzidine staining was performed for 1 min. Sections were counterstained with hematoxylin at room temperature for 2 min. Slides were finally observed and photographed under a light microscope.

Cell culture and cell lines. The human hepatocellular carcinoma cell line Huh1 (accession no. CVCL_2956) was obtained from Procell Life Science & Technology Co., Ltd. The human liver epithelial cell line THLE-2 (accession no. CVCL_3803) was obtained from Shanghai Fuheng Biotechnology Co., Ltd. THLE-2 and Huh1 were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (100 U/ml penicillin, 100 µg/ml streptomycin; Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Cell transfection. For short hairpin RNA (shRNA)-mediated knockdown of SCNM1, shRNA expression plasmids (pLVX-shRNA1) targeting human SCNM1 and negative control (sh-NC) were purchased from VectorBuilder, Inc. It should be noted that the circular plasmid DNA, rather than packaged lentiviral particles, was used for transient plasmid transfection in the present study. The core targeting sequences of shRNA were as follows: SCNM1-sh1: 5'-GCTAACTCA GAGACACGACTTATT-3'; SCNM1-sh2: 5'-AGACGAGCC CTTGGACCATTAT-3'; and sh-NC: 5'-TTCTCCGAACGT GTCACGT-3'. Huh1 cells were transfected with the indicated shRNA plasmids using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. For transfection in 6-well plates, 2 µg of plasmid DNA per well was used. The cells were maintained at 37°C in a humidified incubator with 5% CO₂ during transfection. Cells were incubated for 48 h post-transfection before being harvested for subsequent experiments.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from Huh1 cells using TRIzol® reagent (Invitrogen, Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA using PrimeScript RT Reagent Kit (Takara Bio, Inc.) according to the manufacturer's protocol. RT-qPCR was performed using TB Green® Premix Ex Taq™ II (Takara Bio, Inc.) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following thermocycling conditions: 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 30 sec at 60°C. The relative expression level of SCNM1 was calculated

using the 2^{-ΔΔC_q} method (20), with GAPDH as the internal reference. Primer sequences (5'-3') were as follows: SCNM1 forward, AGAAGACGAGCCCTGGACCATT and reverse, AGGTGGTTCCTCCTCATCAGAG; GAPDH forward, GTC TCCTCTGACTTCAACAGCG and reverse, ACCACCCTG TTGCTGTAGCCAA.

Western blot analysis. Total protein was extracted from human THLE-2 and Huh1 cells using RIPA lysis buffer (Beyotime Biotechnology) containing protease inhibitors. Protein concentration was determined using a BCA protein assay kit (Beyotime Biotechnology). Equal amounts of protein (30 µg per lane) were separated by 10% SDS-PAGE and transferred to a PVDF membrane (MilliporeSigma). The membrane was blocked with 5% non-fat milk at room temperature for 1 h. Primary antibodies against SCNM1 (rabbit polyclonal; 1:1,000; cat. no. 15490-1-AP; Proteintech Group, Inc.) and β-actin (mouse monoclonal; 1:5,000; cat. no. 66009-1-Ig; Proteintech Group, Inc.) were incubated overnight at 4°C. Following washing with Tris-buffered saline containing 0.1% Tween-20, the membrane was incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5,000; cat. no. SA00001-2; Proteintech Group, Inc.) and HRP-conjugated goat anti-mouse IgG secondary antibody (1:5,000; cat. no. SA00001-1; Proteintech Group, Inc.). Protein bands were visualized using an enhanced chemiluminescence kit (cat. no. P0018AS; Beyotime Biotechnology). Band densitometry analysis was performed using ImageJ software (version 1.54v; National Institutes of Health).

Functional assays. Cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8) assay kit (Dojindo Laboratories, Inc.) according to the manufacturer's instructions. Briefly, transfected Huh1 cells were seeded at a density of 3,000 cells per well in 96-well plates. Cell viability was detected at 24, 48 and 72 h after seeding. A total of 10 µl CCK-8 reagent was added to each well and incubated for 2 h at 37°C. The optical density value at 450 nm was measured using a microplate reader.

For colony formation assays, transfected Huh1 cells were seeded at a density of 800 cells per well in 6-well plates and cultured for 14 days. Colonies were fixed with 4% paraformaldehyde at room temperature for 20 min and stained with 0.1% crystal violet at room temperature for 15 min. Colonies containing >50 cells were defined as positive colonies, and colony numbers were determined by manual visual counting under a light microscope.

For the Transwell migration assay, 24-well Transwell inserts were applied without Matrigel pre-coating. Transfected Huh1 cells (4x10⁴ cells) were suspended in serum-free culture medium and seeded into the upper chamber of the insert. The lower chamber was filled with culture medium supplemented with 10% FBS as a chemoattractant. The Transwell system was incubated at 37°C in a humidified incubator with 5% CO₂ for 24 h. After incubation, non-migrated cells remaining on the upper surface of the membrane were gently wiped off. Migrated cells on the lower membrane surface were fixed with 4% paraformaldehyde at room temperature for 15 min, followed by staining with 0.1% crystal violet at room temperature for 15 min. Migratory cells were observed and photographed under a light microscope.

Statistical analysis. Data from the *in vitro* experiments are presented as the mean $\pm$ SD of three independent experiments. Statistical analyses were performed using R software (version 4.4.0; R Foundation for Statistical Computing). Normally distributed continuous variables were analyzed using Student's t-test, where paired t-test was used for matched tumor and adjacent normal tissues from the same patients (TCGA-LIHC cohort), and unpaired t-test was used for two independent groups. One-way ANOVA with Tukey's post hoc test (equal variance) or Welch's ANOVA with Dunnett T3 post hoc test (unequal variance) was used for the comparison of multiple groups in *in vitro* experiments. The non-parametric Wilcoxon rank-sum test was used to assess the relationship between two groups for non-continuous variables. The Kruskal-Wallis test followed by Dunn's post hoc test was employed for comparisons among >2 clinical subgroups. Kaplan-Meier analysis followed by the log-rank test was used to evaluate survival; for survival curves with late-stage crossover, the follow-up time was truncated before the crossover point to meet the proportional hazards assumption. The Cox proportional hazards regression model was applied to screen for independent prognostic predictors. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SCNM1 is consistently upregulated in HCC. Given the limited knowledge surrounding SCNMI in HCC, the expression of SCNMI was investigated across both tumor and normal tissues. Pan-cancer analysis of TCGA and GTEx data revealed significantly higher SCNMI expression in several tumor types, including LIHC, compared with the corresponding normal tissues (Fig. 1A). Within the TCGA-LIHC cohort, SCNMI expression was significantly higher in tumor tissues than in matched adjacent non-tumor tissues in the paired analysis (Fig. 1B). The unpaired comparison also showed significantly higher SCNMI expression in tumor tissues than in normal liver tissues (Fig. 1C). Notably, ROC curve analysis indicated that SCNMI expression effectively differentiated HCC tissues from non-tumor samples, highlighting its strong potential as a diagnostic marker (Fig. 1D). Supporting these transcriptomic results, independent GEO datasets (GSE54236 and GSE14520) corroborated the increased expression of SCNMI in HCC (Fig. 1E). In these studies, samples were categorized into tumor and adjacent normal tissues, with expression differences assessed using the Wilcoxon rank-sum test, revealing significantly higher SCNMI levels in tumor samples. Immunohistochemical analysis of the clinical tissue specimens collected from the 10 patients with HCC enrolled in the present study showed significantly higher SCNMI protein expression in tumor tissues than in adjacent non-tumor liver tissues (Fig. 1F). Analysis of the CPTAC dataset further supported the upregulation of SCNMI protein expression in HCC tissues compared with normal liver tissues (Fig. 1G). Western blot analysis revealed that SCNMI expression was significantly upregulated in HCC Huh1 cells compared with that in normal hepatic THLE-2 cells (Fig. 1H). Overall, these findings showed that SCNMI was consistently upregulated in HCC at both the mRNA and protein levels.

Elevated SCNMI expression is associated with adverse clinical outcomes. Following the consistent upregulation of SCNMI in HCC, the potential connection between SCNMI expression and patient outcomes was investigated. In several independent studies, Kaplan-Meier survival analyses revealed that individuals with elevated SCNMI levels had significantly reduced overall survival compared with those with lower levels (Fig. 2A). Alongside these survival disparities, clinicopathological association analyses indicated that high SCNMI expression was significantly associated with advanced tumor stages, increased histological grades, vascular invasion and elevated serum alpha-fetoprotein levels (Fig. 2B). SCNMI expression was also significantly higher in the lower prothrombin time category (≤ 4 vs. > 4 ; $P = 0.0062$), in patients aged ≤ 60 years compared with those aged > 60 years ($P = 0.0093$), and in patients with a body mass index (BMI) of ≤ 25 kg/m² compared with those with a BMI of > 25 kg/m² ($P < 0.0001$; Fig. 2B). Collectively, these results showed that SCNMI expression was associated with advanced clinicopathological features and poorer overall survival in patients with HCC.

SCNM1 provides prognostic information independent of conventional clinical factors. Considering its strong link to patient outcomes, the prognostic relevance of SCNMI beyond established clinical factors was investigated. A prognostic nomogram that integrated SCNMI expression with essential clinicopathological variables was developed (Fig. 3A). Calibration assessments showed a strong alignment between the predicted and actual survival rates at various intervals (Fig. 3B), while time-dependent ROC curve analyses demonstrated consistent predictive accuracy (Fig. 3C). Notably, survival analyses across different clinical subgroups, such as tumor stage, histological grade and age groups, confirmed that high SCNMI expression was consistently associated with poor prognosis (Fig. 3D). These findings indicated that SCNMI expression retained prognostic relevance across multiple clinical subgroups.

SCNM1 dysregulation is linked to immune remodeling, metabolic pathways and epigenetic alterations. scRNA-seq analysis demonstrated that SCNMI was expressed in diverse immune cell types and tumor cells within the HCC microenvironment (Figs. 4A and B and S1). Among the cell populations with the highest SCNMI expression were plasma cells and dendritic cells in GSE112271 (Fig. 4A), and CD8⁺ T cells and natural killer cells in GSE140228 (Fig. 4B). Bulk transcriptomic analysis revealed positive correlations between SCNMI expression and major histocompatibility complex (MHC) molecules (Fig. 4C). Infiltration of certain immune cell types was significantly higher in the SCNMI high-expression group than in the low-expression group (Fig. 4D and E).

Correlation analysis identified numerous genes positively and negatively correlated with SCNMI expression (Fig. S2A-C). GSEA of genes ranked by their correlation with SCNMI expression revealed significant positive enrichment of the biological process 'protein localization to endoplasmic reticulum' (Fig. S2D), the molecular function 'structural constituent of ribosome' (Fig. S2E) and the cellular component 'ribosome' (Fig. S2F). Kyoto Encyclopedia of Genes and

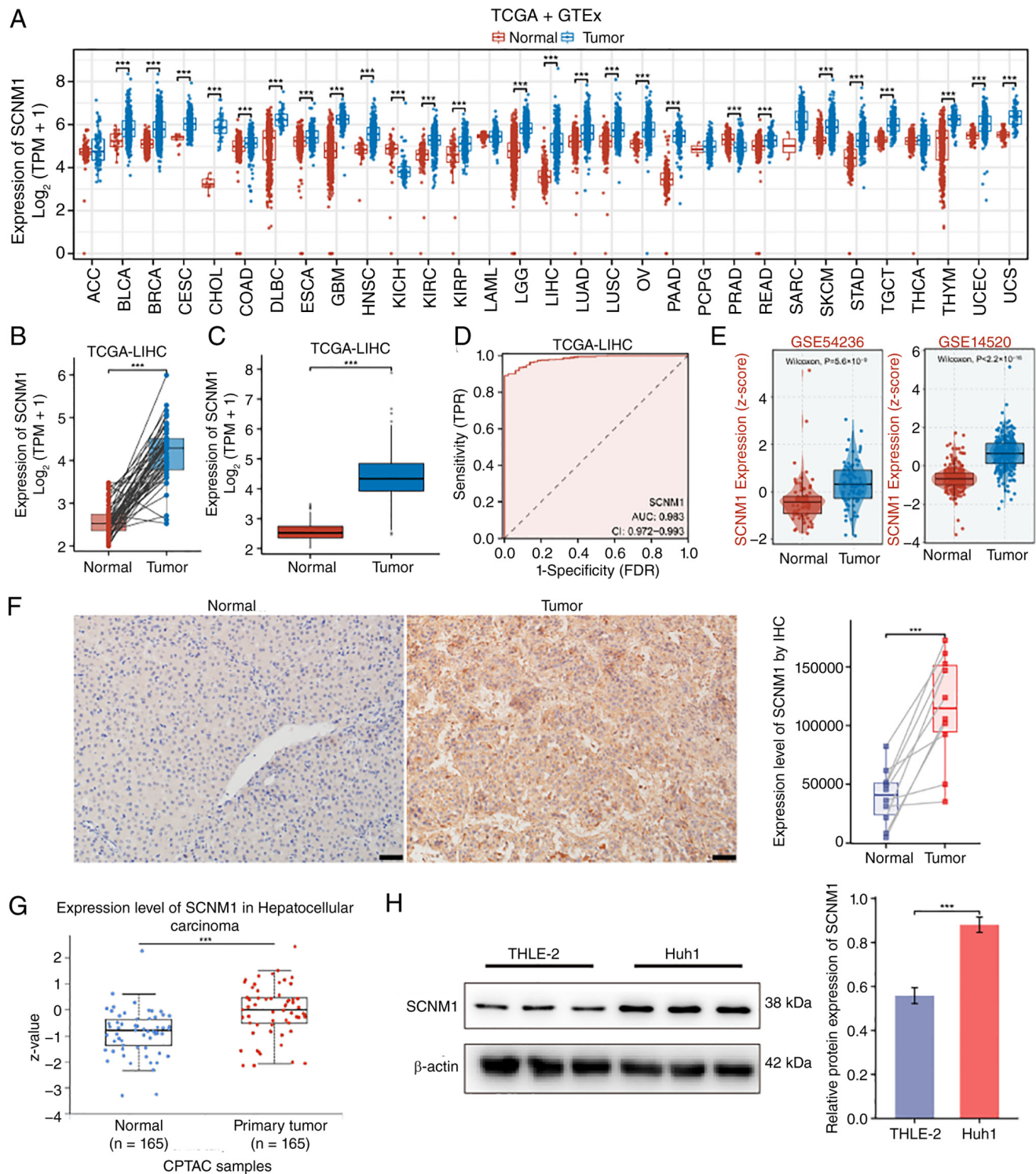


Figure 1. SCN1 expression in HCC. (A) Pan-cancer SCN1 expression (TCGA and GTEx). (B) Paired comparison of SCN1 mRNA expression between HCC and matched adjacent non-tumor liver tissues in the TCGA-LIHC cohort. (C) Unpaired comparison of SCN1 mRNA expression between HCC and normal liver tissues in the TCGA-LIHC cohort. (D) Receiver operating characteristic curve analysis of SCN1 for HCC diagnosis. (E) Independent validation of increased SCN1 mRNA expression in HCC tissues compared with non-tumor liver tissues in the GSE54236 and GSE14520 datasets. (F) Representative IHC images and quantification of SCN1 protein expression in HCC and non-tumor liver tissue specimens collected in the present study. Scale bar, 50 μ m. (G) Protein expression analysis of SCN1 in the CPTAC database. (H) Representative western blots and quantification of SCN1 protein expression in THLE-2 liver epithelial cells and Huh1 HCC cells. β -actin was used as a loading control. *** $P < 0.001$. SCN1, sodium channel modifier 1; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression; LIHC, liver hepatocellular carcinoma; TPM, transcripts per million; AUC, area under the curve; IHC, immunohistochemistry.

Genomes (KEGG) analysis also showed significant positive enrichment of 'ribosome' and 'oxidative phosphorylation' (Fig. S2G).

We performed DNA methylation analyses in the TCGA-LIHC cohort. Differential methylation analysis identified distinct methylation patterns of SCN1-associated

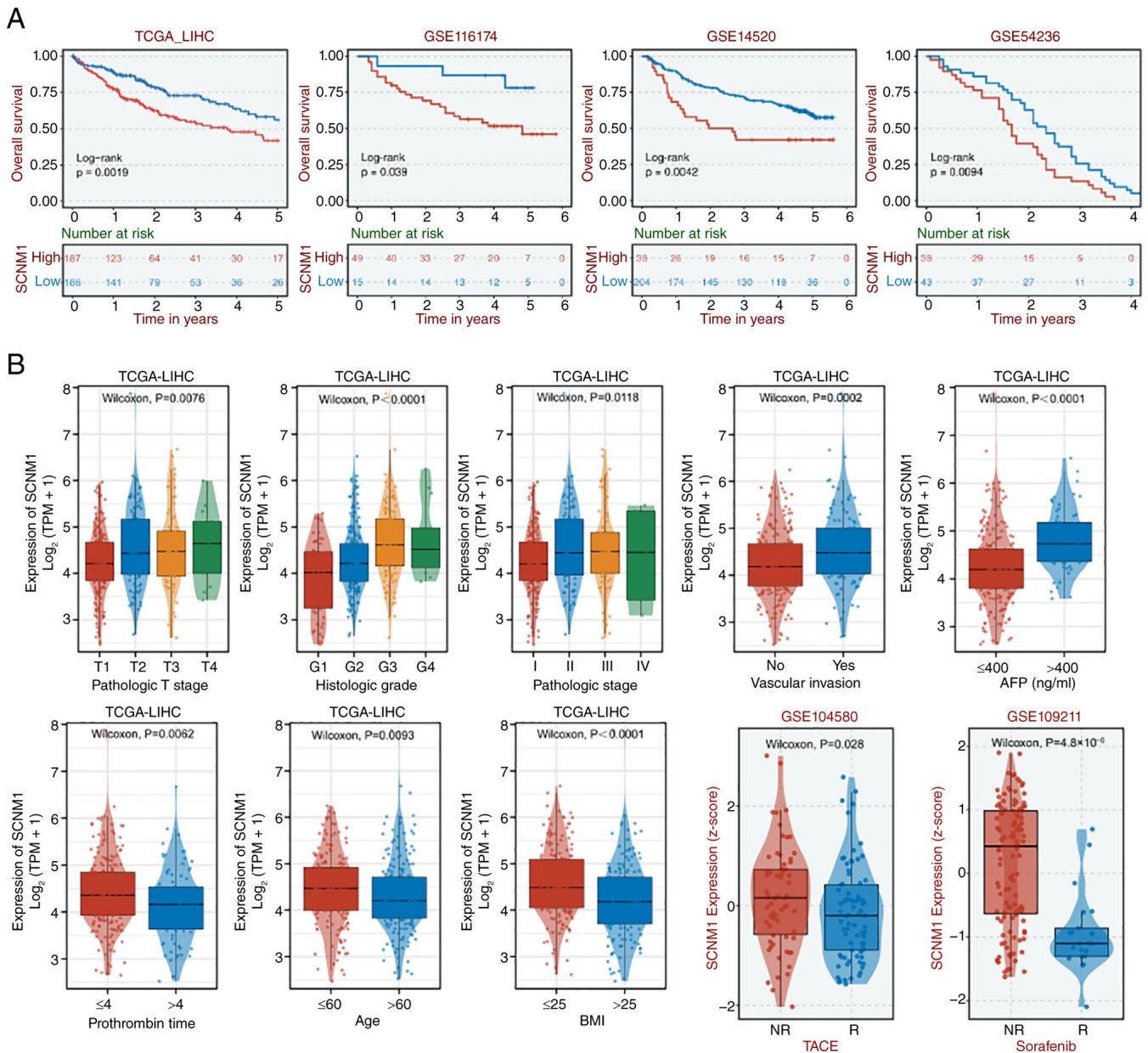
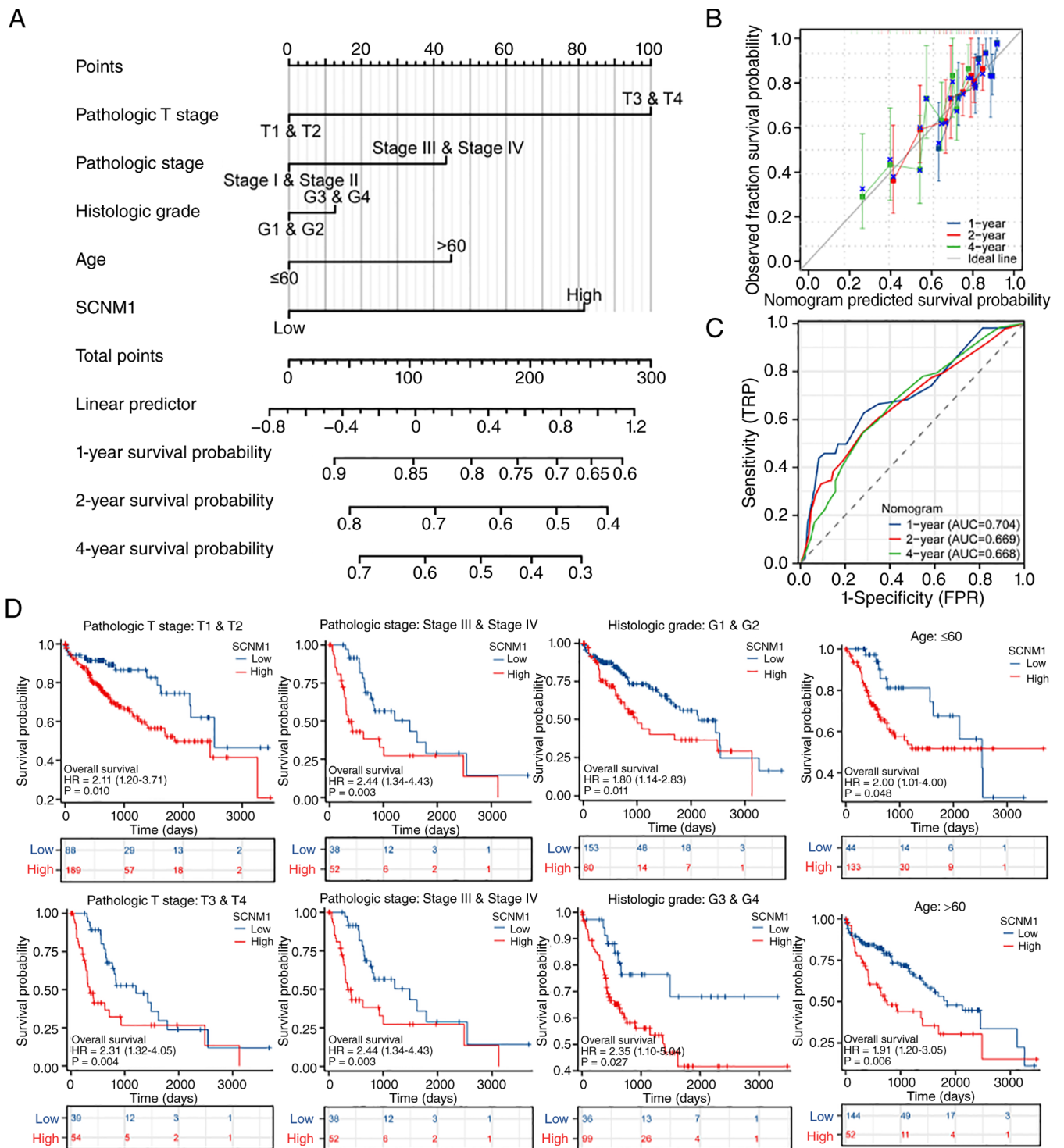


Figure 2. Clinical associations of SCN1M1 in hepatocellular carcinoma. (A) Overall survival stratified by SCN1M1 expression. (B) SCN1M1 expression across clinicopathological subgroups. Comparison of R and NR in treatment cohorts is also shown. SCN1M1, sodium channel modifier 1; LIHC, liver hepatocellular carcinoma; TPM, transcripts per million; TACE, transarterial chemoembolization; TCGA, The Cancer Genome Atlas; AFP, alpha-fetoprotein; NR, non-responders; R, responders; ns, not significant.

CpG sites between HCC and normal liver tissues (Fig. S3A). Methylation levels at several CpG sites were significantly correlated with SCN1M1 mRNA expression (Fig. S3B and C). To further investigate the association between CpG methylation and overall survival in TCGA-LIHC cohort, Kaplan-Meier analyses followed by log-rank tests for all CpG sites were performed. Six sites showed prognostic significance. Kaplan-Meier curves for these sites indicated that higher methylation was associated with improved survival (Fig. S3D).

SCN1M1-related gene signature enables robust prognostic stratification. Building on the molecular associations of SCN1M1, these findings were next translated into a clinically applicable prognostic framework. The intersection of the gene sets identified by AdaBoost and GBDT with the overall

survival-related gene set yielded 39 candidate genes (Fig. 5A). These candidates were subsequently subjected to least absolute shrinkage and selection operator-Cox regression in the TCGA-LIHC cohort. Cross-validation was used to evaluate the partial likelihood deviance across different values of the penalty parameter λ for model selection (Fig. 5B). The coefficient trajectories showed progressive shrinkage of the candidate gene coefficients toward zero as λ increased, enabling selection of genes with non-zero coefficients for the prognostic signature (Fig. 5C). In TCGA cohort, patients were stratified into high- and low-risk groups based on these scores, which exhibited significantly different overall survival outcomes (Fig. 5D, F and G). Time-dependent area under the curve (AUC) analysis (1- and 3-year) demonstrated superior predictive performance compared with existing HCC prognostic



models (Figs. S4 and S5). External validation was further assessed in the ICGC-LIRI cohort (Table II), where consistent risk score distributions and survival separation were observed between risk groups (Fig. 5E, H and I). Further analyses were performed in the TCGA-LIHC cohort to characterize the biological and immune-related features associated with the SCNM1-related risk score. GSVA showed differences in pathway scores between the high- and low-risk groups across

gene sets from KEGG, Reactome and WikiPathways, including B-cell receptor signaling, T-cell receptor signaling, chemokine signaling and cytokine signaling pathways (Fig. S6A). GSEA highlighted biological process terms involving both cell proliferation and immune regulation, including 'DNA replication initiation', 'regulation of mitotic nuclear division', 'regulation of B cell activation', 'positive regulation of B cell activation' and 'B cell receptor signaling pathway' (Fig. S6B). The cellular

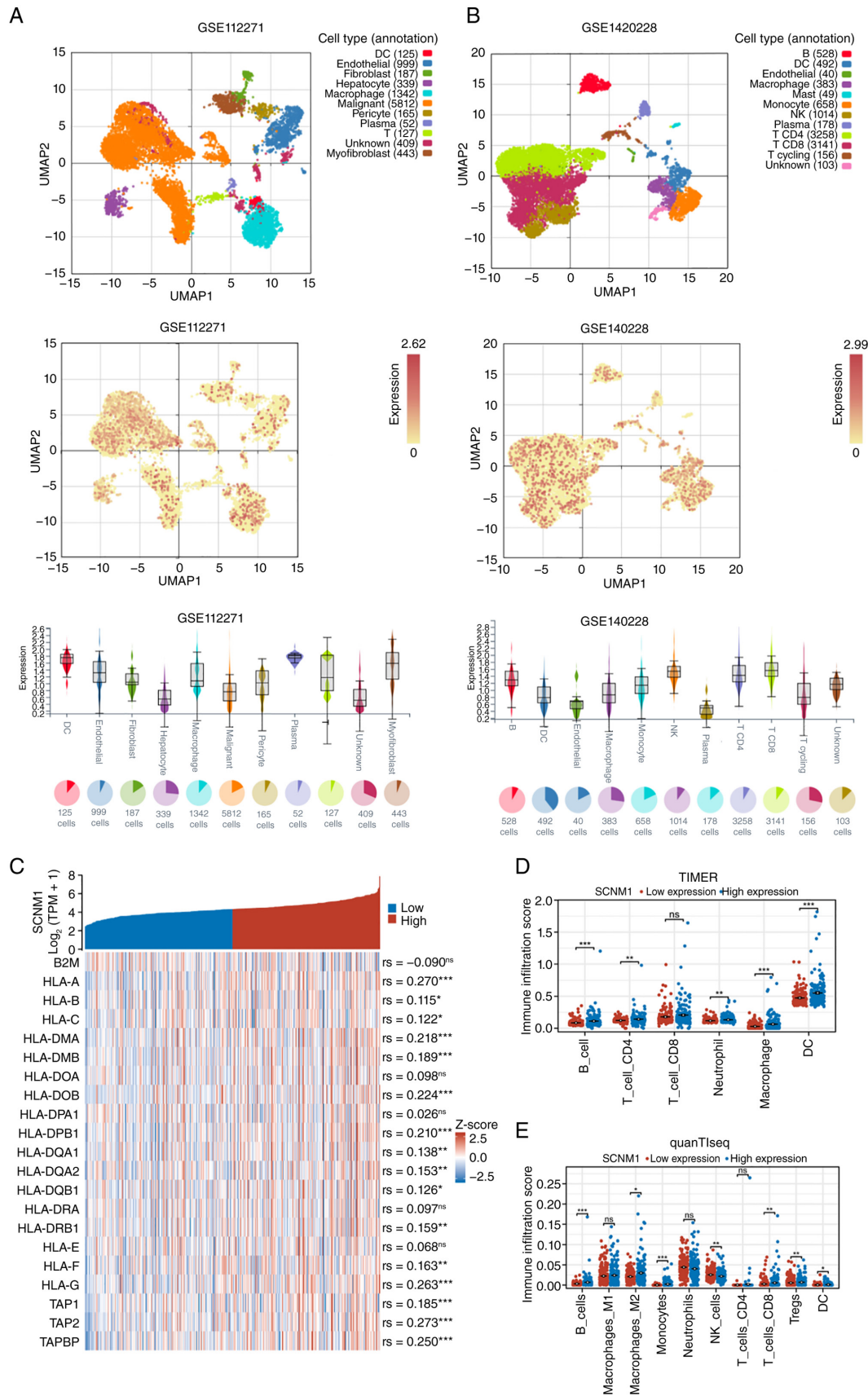


Figure 4. SCN1M1 and immune microenvironment in hepatocellular carcinoma. (A) UMAP plots showing cell-type annotations and SCN1M1 expression in GSE112271. (B) UMAP plots showing cell-type annotations and SCN1M1 expression in GSE140228. The violin plots with overlaid box plots below the UMAP plots show the distribution of SCN1M1 expression across the annotated cell types in each dataset. (C) Correlation of SCN1M1 with major histocompatibility complex molecules. (D) Immune infiltration in SCN1M1 high- and low-expression groups (TIMER). (E) Immune infiltration in SCN1M1 high- and low-expression groups (quanTiseq). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. UMAP, Uniform Manifold Approximation and Projection; SCN1M1, sodium channel modifier 1; TPM, transcripts per million; DC, dendritic cells; NK, natural killer; TIMER, Tumor Immune Estimation Resource; TAP, transporter associated with antigen processing 1; TAPBP, TAP-binding protein; HLA, human leukocyte antigen; ns, not significant.

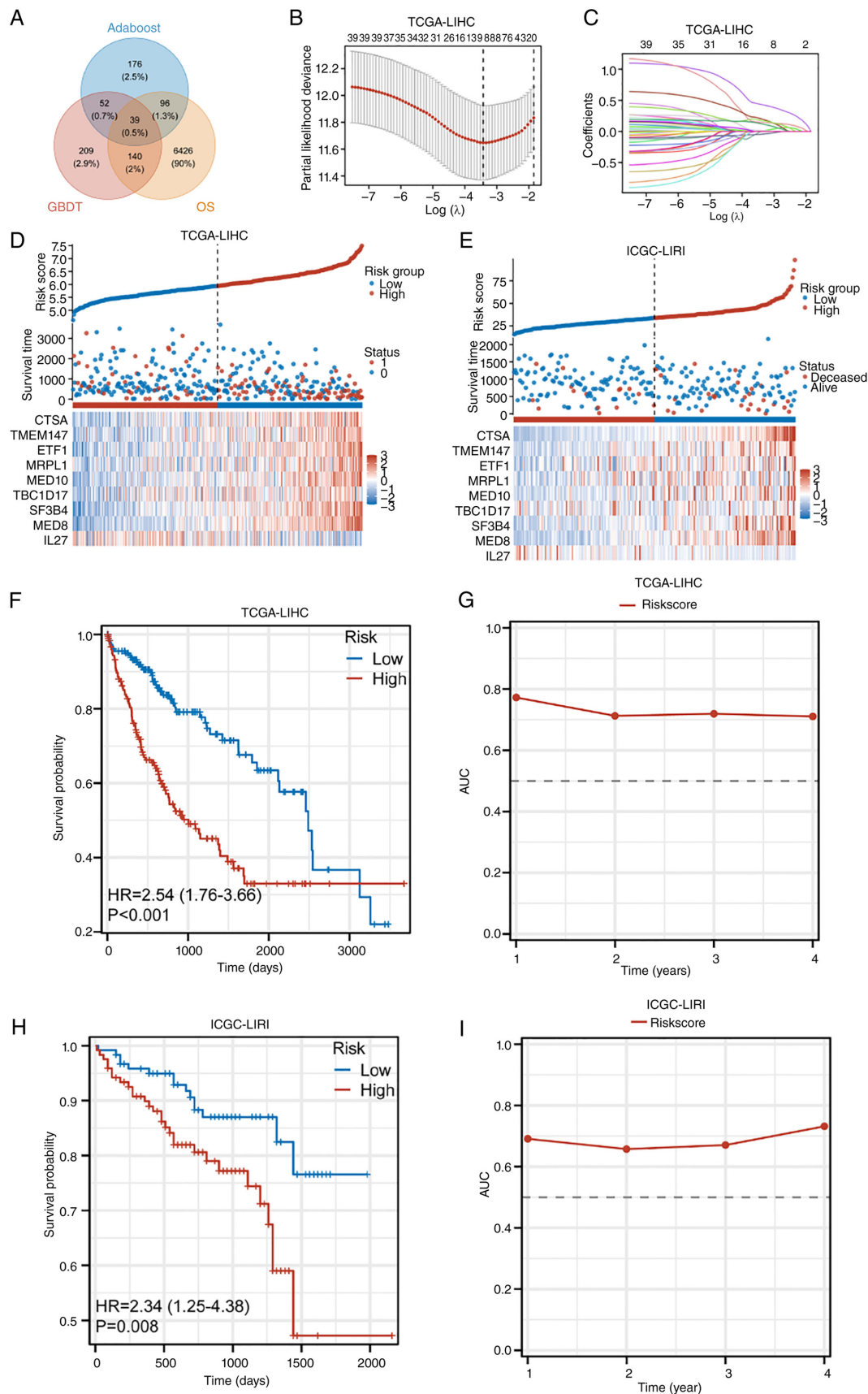


Figure 5. Sodium channel modifier 1-related prognostic signature. (A) Venn diagram of prognostic genes selected by GBDT and AdaBoost from univariate Cox-identified OS-related genes (B) LASSO coefficients. (C) Cross-validation of the LASSO model. (D) Gene expression heatmap (TCGA-LIHC). (E) Gene expression heatmap (ICGC-LIRI). (F) Survival comparison (TCGA-LIHC). (G) AUC values (1-4 year) in TCGA-LIHC cohort. (H) Survival comparison (ICGC-LIRI). (I) AUC values (1-4 year) in ICGC-LIRI cohort. TCGA, The Cancer Genome Atlas; LIHC, liver hepatocellular carcinoma; ICGC, International Cancer Genome Consortium; OS, overall survival; LIRI, liver cancer-RIKEN; AUC, area under the curve; HR, hazard ratio; LASSO, least absolute shrinkage and selection operator; GBDT, Gradient Boosted Decision Tree.

component terms included 'T cell receptor complex', 'outer kinetochore', 'spindle midzone', 'immunoglobulin complex, circulating' and 'immunoglobulin complex' (Fig. S6C). The molecular function terms included 'ATP-dependent activity, acting on DNA', 'DNA helicase activity', 'single-stranded DNA helicase activity', 'antigen binding' and 'immunoglobulin receptor binding' (Fig. S6D).

The risk score showed a weak positive correlation with tumor mutational burden (TMB; Spearman's $\rho=0.131$, $P=0.014$; Fig. S6E). Spearman's correlation analysis further identified significant correlations between the risk score and the expression of MHC-related genes and multiple immune checkpoint-related genes. The accompanying correlation matrices also illustrated relationships among genes within these two groups (Fig. S6F).

TIMER analysis showed significantly higher estimated infiltration scores for B cells, CD4⁺ T cells, CD8⁺ T cells, neutrophils, macrophages and dendritic cells in the high-risk group than in the low-risk group (Fig. S6G). In the quanTIseq analysis, significantly higher estimated infiltration scores were observed in the high-risk group for several populations, including B cells, monocytes, M2 macrophages, CD8⁺ T cells and regulatory T cells. No significant differences were observed for dendritic cells, CD4⁺ T cells, natural killer cells or neutrophils in this analysis (Fig. S6H).

SCNM1 promotes malignant phenotypes of HCC cells. Stable knockdown of SCNM1 in HCC cell lines effectively suppressed its expression at both mRNA and protein levels (Fig. 6A-C). Functional assays demonstrated that SCNM1 silencing significantly impaired cell proliferation, clonogenic capacity and migratory ability (Fig. 6D-H).

In parallel, translational analyses in immunotherapy-treated cohorts provided clinical context for SCNM1 dysregulation. In the TCGA-LIHC cohort, SCNM1 expression was positively correlated with the expression of immune checkpoint-related genes, including LGALS9, CTLA4, LAG3 and PDCD1, in both the pathological T1-T2 and T3-T4 subgroups (Fig. S7A). In the IMvigor210 cohort, SCNM1 expression was significantly higher in responders to anti-programmed death-ligand 1 therapy than in non-responders ($P=0.0009$), and ROC analysis yielded an AUC of 0.633 for distinguishing the two groups (Fig. S7B). Similarly, in the Wolf cohort (GSE173839), responders had significantly higher SCNM1 expression than non-responders ($P=0.0068$), with an AUC of 0.664 (Fig. S7C). Higher SCNM1 expression was associated with longer overall survival in the Hugo cohort (GSE78220; $P=0.038$) and the IMvigor210 cohort ($P=0.0094$), and with longer progression-free survival in the Kim cohort (GSE135222; $P=0.046$; Fig. S7D). However, these findings should be interpreted cautiously given their retrospective nature and require prospective validation.

Discussion

The present study focused on the function of SCNM1 in HCC and created a prognostic model utilizing genes associated with SCNM1. Recently, Ofuchi *et al* (11) emphasized the clinical and biological importance of SCNM1 in HCC, noting its involvement in the minor spliceosome. The present study builds upon these insights by incorporating multi-cohort

transcriptomic data, thoroughly examining the link between SCNM1 expression and the immune microenvironment, and formulating a prognostic model centered on SCNM1-related genes. The results revealed that SCNM1 levels are significantly higher in liver cancer tissues compared with those in normal tissues and are associated with unfavorable clinical outcomes, indicating the potential of SCNM1 as a prognostic biomarker for HCC. However, single-cell analysis did not reveal distinct tumor cell-specific enrichment of SCNM1 expression, suggesting that the increased expression observed in bulk transcriptomic data may reflect contributions from multiple cell types within the tumor microenvironment. The heightened SCNM1 expression in advanced tumor stages, such as elevated T stages and vascular invasion, was associated with adverse clinicopathological characteristics; however, this observation was based on correlative evidence, and further experimental studies are needed to clarify its direct role in tumor progression or metastasis. The high diagnostic accuracy of SCNM1 (AUC=0.983) underscores its potential role in HCC diagnosis (21-24). Additional mechanistic investigations are necessary to uncover the pathways regulating SCNM1 in HCC.

GSEA identified notable enrichment in pathways related to protein localization, oxidative phosphorylation and electron transport activity, suggesting a possible association between SCNM1 and metabolic or redox-related processes in HCC. Since metabolic reprogramming is a defining characteristic of cancer, the participation of SCNM1 in these pathways may contribute to its oncogenic capabilities in liver cancer (25-28). A previous study by Gao *et al* (29) developed a prognostic model based on m6A- and ferroptosis-associated long non-coding RNAs in HCC, further illustrating the potential prognostic relevance of these molecular features and protein targeting to the endoplasmic reticulum. However, this finding does not establish a role for SCNM1 in regulating endoplasmic reticulum stress or promoting tumor survival. Furthermore, the investigation into SCNM1 methylation revealed that certain CpG site methylation patterns were associated with poorer survival rates, implying that epigenetic factors may play a role in SCNM1 dysregulation. Nevertheless, the precise relationship between CpG methylation and the overexpression of SCNM1 remains unclear, and further studies are needed to determine whether promoter hypomethylation or other epigenetic processes are responsible for its increased expression (30-32).

Non-resolving inflammation contributes to HCC development and progression through mechanisms involving inflammatory signaling and interactions within the tumor microenvironment, as reviewed by Yu *et al* (33). Analysis of the immune microenvironment in the present study revealed positive associations between SCNM1 expression and the estimated infiltration of selected immune cell populations, as well as the expression of MHC-related and immune checkpoint-related genes. These findings suggest an association with immune-related characteristics but do not establish a direct regulatory role for SCNM1. Shi *et al* (34) developed a Notch signaling pathway-related prognostic signature associated with survival and distinct immune characteristics in HCC. Their findings provide a rationale for examining prognostic gene signatures alongside immune features. However, single-cell

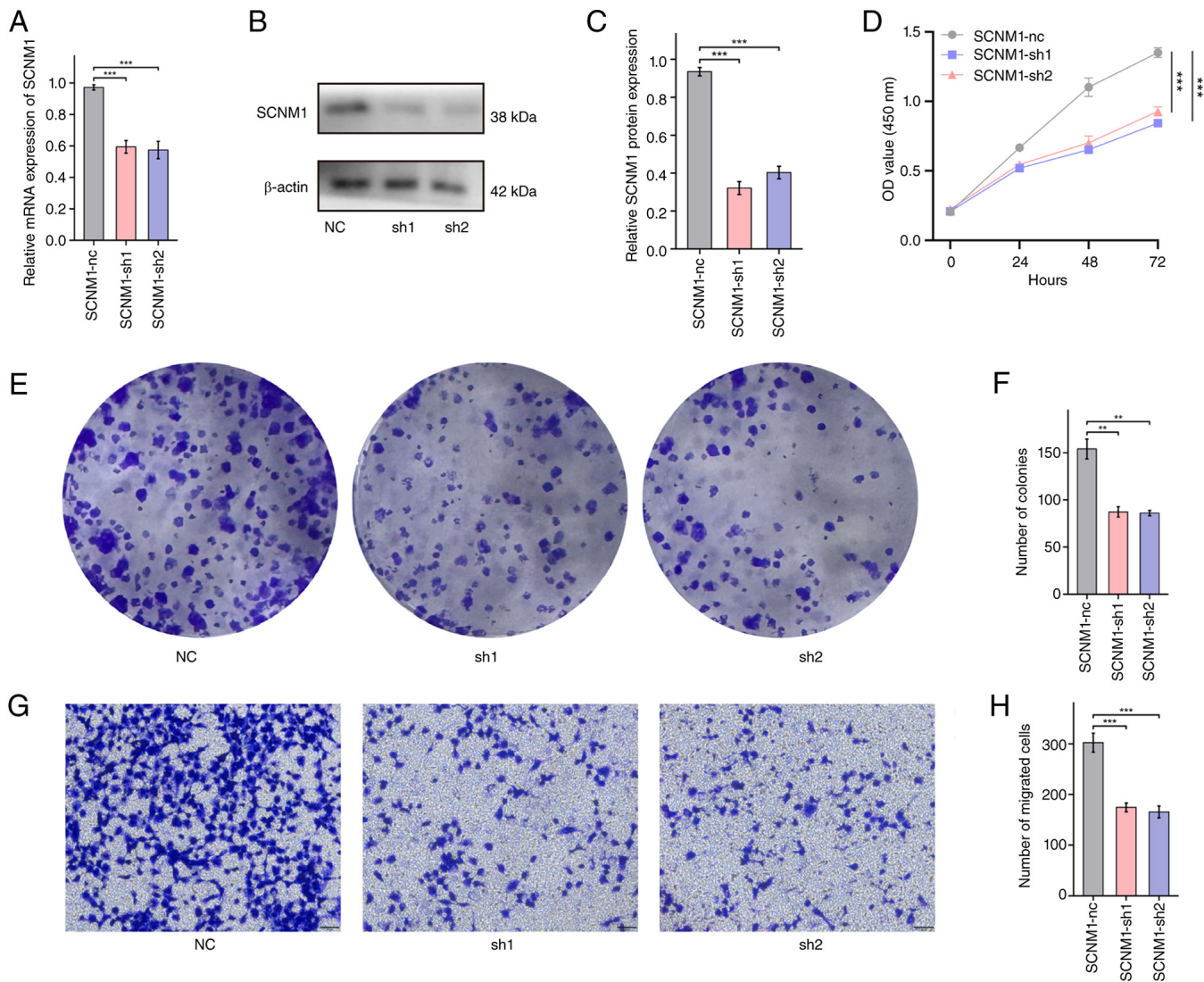


Figure 6. Functional validation of SCNMI1. (A) Reverse transcription-quantitative PCR analysis of SCNMI1 knockdown groups. (B) Western blot analysis and (C) protein quantification of SCNMI1 knockdown groups. (D) Cell proliferation assay. (E) Colony formation assay and (F) quantification. (G) Migration assay and (H) quantification. ** $P < 0.01$; *** $P < 0.001$. SCNMI1, sodium channel modifier 1; NC, negative control; sh, short hairpin.

analyses in the present study did not demonstrate distinct tumor cell-specific enrichment of SCNMI1 expression, suggesting that these associations may reflect the combined influence of SCNMI1 expression across both malignant and non-malignant cell populations. The SCNMI1-related prognostic model stratified patients with different survival outcomes and was associated with immune-related pathway enrichment and a weak positive correlation with TMB. These associations alone do not establish predictive value for immunotherapy response. In the CheckMate 040 trial, El-Khoueiry *et al* (35) reported an objective response rate of 20% in the dose-expansion phase among patients with advanced HCC receiving nivolumab, demonstrating clinical activity in a subset of patients. This provides clinical context for investigating factors associated with immunotherapy outcomes, but does not directly validate SCNMI1 as a predictive biomarker. The associations between SCNMI1 expression and immunotherapy outcomes observed in the present retrospective analyses require further validation in prospective HCC cohorts receiving immunotherapy. Overall, the present study extends the characterization of

SCNMI1-related genes by examining their prognostic relevance and associated immune features.

While the current results are encouraging, the present study has certain limitations. A deeper exploration of the specific molecular pathways associated with SCNMI1 in HCC is necessary, utilizing both *in vivo* and *in vitro* approaches. Furthermore, the reliance of the present study on publicly available databases may introduce potential bias in sample selection, which underscores the need for further confirmation through prospective clinical trials. In summary, SCNMI1 appears to be a promising candidate as a prognostic biomarker and potential therapeutic target in HCC, offering preliminary insights into immunotherapy stratification. Future research should aim to elucidate the functional roles of SCNMI1 and assess its clinical relevance in the management of HCC.

In conclusion, the present study identified SCNMI1 as a clinically relevant biomarker in HCC, with its upregulation associated with poor prognosis, immune-related features and metabolic alterations. The SCNMI1-based prognostic model shows strong potential for patient risk stratification.

While further validation is required, these findings suggest that SCN1M1 may represent a promising candidate for future translational studies in HCC, particularly in the context of immune-related therapeutic strategies.

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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

LJ contributed to the study conception, methodology establishment and original draft writing. ZL contributed to validation of analytical methods, formal data analysis and original draft writing. JW contributed to experimental investigation and original draft writing. HG contributed to data curation, data visualization, interpretation of the results and original draft writing. SH contributed to project administration, funding acquisition, interpretation of the obtained data and original draft writing. YP contributed to study conceptualization, overall supervision of the work, and manuscript review-editing. LJ and YP confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Hebei General Hospital (approval no. 2024-43). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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