

Bulk and single-cell analysis of FAM72D reveals its prognostic and functional relevance in hepatocellular carcinoma

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Abstract. Family with sequence similarity 72 member D (FAM72D) has been implicated in proliferative programs in several malignancies, but its clinical significance, cellular distribution and underlying biological programs in hepatocellular carcinoma (HCC) remain insufficiently characterized. FAM72D expression and its clinical relevance were evaluated using The Cancer Genome Atlas-liver hepatocellular carcinoma (TCGA-LIHC) cohort and three independent GEO cohorts. Cox regression, patient-level bootstrap validation, calibration analysis and decision curve analysis were used to assess its prognostic contribution. Single-cell and spatial transcriptomic analyses were performed to define the cellular distribution of FAM72D. Differential-expression, functional-enrichment, protein-protein interaction and SCENIC analyses were used to characterize FAM72D-centered transcriptional programs. Immune-related analyses were conducted using tumor immune estimation resource and tumor immune dysfunction and exclusion (TIDE) and the functional effects of FAM72D silencing were examined in HuH7 cells. FAM72D was consistently upregulated in HCC tissues in TCGA and three independent GEO cohorts and was associated with adverse clinicopathological characteristics. Higher FAM72D expression was independently associated with unfavorable overall survival in the multivariable TCGA model (hazard ratio 2.02; 95% confidence interval 1.45-2.81; $P < 0.001$). Adding FAM72D increased the optimism-corrected C-index from 0.611 to 0.662, while independent tissue-level

validation yielded area under the curve of 0.861, 0.926 and 0.832 in GSE76427, GSE39791 and GSE54236, respectively. Single-cell analysis identified a FAM72D-high malignant-cell population with pronounced G₂/M, mitotic, DNA-replication and DNA-repair activity. Bulk and single-cell transcriptomic analyses converged on a coordinated proliferative program characterized by enhanced cell-cycle signaling and forkhead box M1 (FOXM1) regulon activity. High FAM72D expression was also coupled to increased immune-checkpoint expression, reduced estimated CD8⁺ T-cell abundance and higher TIDE scores. Silencing FAM72D reduced HuH7-cell viability, colony formation and migration and increased apoptosis. FAM72D expression is associated with unfavorable prognosis and a proliferative malignant-cell state in HCC. The combined clinical, transcriptomic, single-cell, immune-related and functional findings support further evaluation of FAM72D as a candidate prognostic marker and a feature of proliferative HCC-cell programs.

Introduction

Liver cancer is the sixth most common malignancy worldwide. According to Global Cancer Statistics, it caused an estimated 757,948 deaths in 2022, making it the third leading cause of cancer-related death (1). Hepatocellular carcinoma (HCC) accounts for 75-85% of primary liver cancers (2). Current treatment options include surgical resection, local ablative and interventional therapy, systemic targeted therapy, immunotherapy and liver transplantation (3). However, potentially curative treatments are largely restricted to selected patients with early-stage disease. As early symptoms are often nonspecific, numerous patients are diagnosed at an intermediate or advanced stage and continue to experience poor long-term survival (4). Identifying molecular markers associated with HCC biology and patient outcome may therefore improve disease characterization and risk assessment (5).

The family with sequence similarity 72 (FAM72) gene family comprises FAM72A, FAM72B, FAM72C and FAM72D and has been studied in neural progenitor-cell self-renewal and cancer biology (6). FAM72D has been identified in prognostic or proliferation-related transcriptional signatures in prostate cancer, renal cell carcinoma and multiple myeloma (7-9). FAM72A has also been associated with clinicopathological

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characteristics, prognosis and drug sensitivity in HCC (10). More recently, Kong *et al* (11) systematically examined the expression profiles and prognostic values of the FAM72 family, including FAM72D, in liver cancer. Pan-cancer and experimental analyses have further connected FAM72D with aggressive tumor-cell phenotypes (12). These findings suggest that FAM72D may participate in a broader proliferative program, but its malignant-cell distribution, regulatory context, prognostic contribution and functional relevance in HCC require integrated investigation.

The present study combined bulk RNA sequencing, independent tissue-level cohorts, single-cell RNA sequencing, spatial transcriptomics, immune-related analyses, tissue immunohistochemistry and *in vitro* experiments to characterize FAM72D in HCC. The present study first evaluated its expression and prognostic significance, followed by patient-level bootstrap validation of a locked clinical model. It then examined the malignant-cell distribution of FAM72D and defined its associated transcriptional, regulatory and immune context. Finally, transient FAM72D silencing was used to determine its contribution to HCC-cell proliferation, apoptosis and migration.

Materials and methods

Clinical and pathological data sources. mRNA expression data generated by RNA sequencing and corresponding clinical information were obtained from The Cancer Genome Atlas liver hepatocellular carcinoma (TCGA-LIHC) cohort (<https://portal.gdc.cancer.gov/>) (13). Independent tissue-level validation was conducted using datasets deposited in the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) (14). GSE76427 included 167 tissues from 115 patients, comprising 115 HCC and 52 adjacent non-tumor tissues (15). GSE39791 included 144 tissues from 72 patients, with one paired HCC and non-tumor tissue from each patient (16). GSE54236 initially contained 161 arrays from 77 patients; repeated arrays representing the same patient-tissue combination were averaged, yielding 154 patient-level tissue measurements comprising 77 HCC and 77 non-tumor tissues (17).

Survival analysis. Overall-survival data were obtained from TCGA-LIHC. The locked prognostic analysis included 341 patients with complete data for the prespecified model variables, among whom 117 mortalities were observed. The multivariable Cox model included age centered at the cohort mean and modeled per 10-year increase, sex with female as the reference, pathologic stage with stage I as the reference and continuous FAM72D expression measured as $\log_2(\text{TPM}+1)$, where TPM denotes transcripts per million. Stage II and stage III/IV were represented by separate indicator variables.

Proportional-hazards assumptions were evaluated using Schoenfeld-residual-based diagnostics and spline analyses were used to assess potential nonlinearity of the continuous predictors. Median-based FAM72D groups were retained for descriptive Kaplan-Meier analyses but were not used in the locked prognostic model.

Establishment and evaluation of the HCC survival nomogram. A nomogram was constructed from the locked Cox model to

estimate 1-, 3- and 5-year overall-survival probabilities. Model discrimination was quantified using Harrell's C-index. Internal validation was performed using 1,000 patient-level bootstrap resamples. In each resample, the model was refitted, optimism was calculated as the difference between bootstrap-sample performance and performance in the original cohort and mean optimism was subtracted from the apparent C-index. Percentile bootstrap 95% confidence intervals (CI) were calculated for the apparent C-index.

Calibration was assessed at 1, 3 and 5 years by comparing mean predicted mortality risk with Kaplan-Meier-observed mortality across tertiles of predicted risk, followed by bootstrap optimism correction. Decision curve analysis compared the net benefit of the clinical model and the clinical-plus-FAM72D model with treat-all and treat-none strategies. Threshold ranges were 0.05-0.40 for 1-year mortality, 0.10-0.70 for 3-year mortality and 0.20-0.77 for 5-year mortality.

Independent tissue-level Receiver Operating Characteristic (ROC) validation was conducted in GSE76427, GSE39791 and GSE54236. Official platform annotations identified ILMN_1719870 on GPL10558 and A_32_P151800 on GPL6480 as the corresponding FAM72D probes. Area Under the Curve (AUC) CIs were calculated using 2,000 patient-cluster bootstrap resamples, thereby preserving the dependence between paired tissues and repeated measurements from the same patient.

Gene set enrichment analysis (GSEA) and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of bulk RNA-sequencing data. In TCGA-LIHC, GSEA was used to compare samples with high and low FAM72D expression. Differentially expressed genes (DEGs) were identified using thresholds of $|\log_2\text{FC}| > 1.5$ and adjusted $P < 0.05$. GO and KEGG enrichment analyses were performed using the R packages clusterProfiler and org.Hs.eg.db (18-20). For GSEA, $|\text{NES}| > 1$, $P < 0.05$ and FDR < 0.25 were considered statistically significant.

Protein-protein interaction (PPI) network. A PPI network of genes coexpressed with FAM72D was constructed using the STRING database (<https://string-db.org/>) (21). Hub genes were selected and visualized using Cytoscape version 3.10.1 (<https://cytoscape.org/>) (22).

Tumor Immune Dysfunction and Exclusion (TIDE) analysis. The TIDE algorithm was used to compare computationally inferred patterns of immune dysfunction and exclusion between the FAM72D expression groups in the TCGA-HCC cohort (23).

Tumor immune estimation resource (TIMER) analysis. The TIMER web server (<https://cistrome.shinyapps.io/timer/>) was used to estimate immune-cell abundance in HCC tissues (24). Associations between FAM72D mRNA expression and the estimated abundance of B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages and dendritic cells were examined. Associations with the expression of immune-checkpoint-related genes were also assessed. Correlation coefficients and P-values were obtained from the specified public resources.

Cell clustering and annotation. A gene-by-cell UMI count matrix for GSE151530 was obtained from GEO (25). Cells

with <200 detected genes, >6,000 detected genes, or >20% mitochondrial transcripts were excluded. The matrix contained 56,721 cells and 18,667 genes before filtering and 51,759 cells and 18,667 genes after filtering.

The 46 sample identifiers were C25, C26a, C26b, C29, C35, C39, C42, C46a, C46b, C52, C56, C60, C66, C76, H08, H18, H21, H23, H28, H30, H34a, H34b, H34c, H37, H38, H41, H43, H49a, H49b, H54, H55, H58a, H58b, H58c, H62, H63, H65, H68a, H68b, H70, H72, H73a, H73b, H74, H75 and H77.

Single-cell analyses were performed using Seurat version 5.0.3 (<https://satijalab.org/seurat/>). Data were log-normalized and scaled and the percentage of mitochondrial transcripts was regressed out. The 2,000 most variable genes were selected using FindVariableFeatures with the vst method. Principal component analysis was performed and the first 50 principal components were retained. A shared nearest-neighbor graph was constructed from these principal components and cells were clustered using the Louvain algorithm with FindClusters at a resolution of 1. No additional cross-sample batch-correction algorithm was applied. Uniform manifold approximation and projection (UMAP) was performed using the RunUMAP function in Seurat, with principal components 1-50 as input, to visualize cell populations and malignant-cell subclusters.

Cell identities were adopted from the dataset annotations. Cells in the malignant-cell compartment were reclustered at a resolution of 0.6. Cluster-specific DEGs were identified using FindAllMarkers function in Seurat, with a Wilcoxon test, $\log_2\text{FC} > 0.25$ and Benjamini-Hochberg-adjusted $P < 0.05$. FAM72D-high and FAM72D-low cells were separated using the central-tendency threshold implemented in the original analytical workflow.

Gene regulatory network analysis. Regulon activity was reconstructed with SCENIC version 1.3.1 (<https://github.com/aertslab/SCENIC>). Raw UMI counts were used as input for the runCorrelation and runGENIE3 functions to generate gene coexpression modules. Candidate regulons were refined by motif-enrichment analysis using RcisTarget version 1.22.0 (<https://bioconductor.org/packages/RcisTarget/>) and their activity in each cell was scored using AUCell version 1.24.0 (<https://bioconductor.org/packages/AUCell/>). The resulting AUC scores were z-normalized across the dataset to facilitate comparisons.

Spatial transcriptomic analysis. Spatially resolved gene-expression profiles were analyzed using SpatialTME, a web-based platform integrating histologic images with single-cell and spatial transcriptomic data (26). The HRA000437 HCC dataset was analyzed using the platform's workflow (27), which included spot-level expression generation, exclusion of spots with <200 detected genes or >15% mitochondrial transcripts, SCTransform normalization (<https://satijalab.org/seurat/reference/sctransform>), principal component analysis and Louvain clustering. FAM72D transcript signals were compared between tumor and adjacent-tissue regions using the supplied annotations.

Gene set variation analysis (GSVA) and GO/KEGG analyses of single-cell RNA-sequencing data. GSVA was performed to

visualize functional programs across cell clusters. The resulting enrichment-score matrix was z-scaled by row and displayed as a heatmap summarizing pathway-activation patterns across the identified cell populations. Differential expression among malignant-cell subclusters was assessed using FindMarkers. Genes with adjusted $P < 0.05$ and $\log_2\text{FC} > 1.44$ were considered markedly upregulated in individual subclusters and were included in downstream enrichment analyses.

Immunohistochemistry (IHC). Written informed consent was obtained from all patients who provided tissue samples for this study. The IHC cohort comprised 10 patients, of 5 males and 5 females, recruited between January 2023 and December 2023. The median age was 62 years (range, 48-72 years) and demographic data were available for all 10 patients. Paired HCC and adjacent non-tumor tissues were collected at Hunan Provincial People's Hospital/The First Affiliated Hospital of Hunan Normal University in Changsha, China. Adjacent non-tumor tissues were obtained at least 2 cm from the tumor margin and were confirmed by histopathological examination to be free of tumor involvement. The inclusion criteria were age ≥ 18 years, histopathologically confirmed primary HCC, availability of paired tumor and adjacent non-tumor tissue specimens, and no anticancer treatment before surgery. The exclusion criteria were recurrent HCC, a concurrent malignancy and insufficient or inadequately preserved tissue for IHC analysis.

Specimens were fixed in 10% neutral buffered formalin (~4% formaldehyde) for 24 h at room temperature, dehydrated through 70, 80 and 95% ethanol for 1 h each and absolute ethanol twice for 1 h each, and cleared in xylene twice for 30 min each. Tissues were infiltrated with paraffin at 60°C twice for 1 h each, embedded and sectioned at 4 μm .

Sections were deparaffinized in xylene and rehydrated through graded ethanol. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) at 95-98°C for 15 min. No separate permeabilization step was used. Endogenous peroxidase activity and nonspecific binding were blocked with 3% hydrogen peroxide for 10 min and 5% bovine serum albumin for 30 min, respectively, at room temperature.

Sections were incubated with a rabbit FAM72-family polyclonal antibody (1:100; catalog no. 21203-1-AP; Proteintech Group, Inc.) for 16 h at 4°C, followed by an HRP-conjugated goat anti-rabbit IgG secondary antibody (1:200; catalog no. 111-035-003; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. Staining was developed with 3,3'-diaminobenzidine, followed by hematoxylin counterstaining for 1 min at room temperature. Sections were examined using a bright-field light microscope (BX53; Olympus Corporation). Because the primary antibody recognizes multiple FAM72 family members, staining was evaluated as FAM72-family immunoreactivity. Slides were examined under an optical microscope (Nikon E1000; Nikon Corporation) and independently evaluated by at least two pathologists. Five representative fields from each specimen were assessed at magnification, $\times 400$. Positive FAM72-family immunoreactivity was defined as distinct brown chromogenic signals in the cytoplasmic and membranous compartments. Images were acquired using a Nikon DS-Fi3 microscope imaging system (Nikon Corporation).

Cell transfection and proliferation assays. FAM72D was knocked down in HuH7 cells using three small interfering (si)RNAs targeting FAM72D and a negative-control siRNA (si-NC; Shanghai GenePharma Co., Ltd.). Cells were seeded in 6-well plates at 2×10^5 cells per well 24 h before transfection and transfected at approximately 50–60% confluence. Each targeting siRNA and si-NC were used at a final concentration of 50 nM. For each well, 100 pmol siRNA was complexed with 5 μ l Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) in Opti-MEM reduced-serum medium and added to the cells in a final volume of 2 ml. P3000 reagent was not used. Cells were incubated with the transfection complexes at 37°C with 5% CO₂ for 6 h, after which the medium was replaced with fresh complete culture medium. Knockdown efficiency was assessed 48 h after transfection. The siRNA sequences are provided in Table SI. The reagents designated _001, _002 and _003 corresponded to si-FAM72D-1, si-FAM72D-2 and si-FAM72D-3, respectively. si-FAM72D-3 showed the greatest knockdown efficiency and was selected for subsequent experiments.

Cell proliferation was evaluated using CCK-8 (Dojindo Laboratories, Inc.) and colony-formation assays. For the CCK-8 assay, cells were seeded in 96-well plates at 2×10^3 cells per well and absorbance at 450 nm was measured with a microplate reader (Thermo Fisher Scientific, Inc.) at 0, 24, 48 and 72 h after transfection. For the colony-formation assay, 200 transfected cells per well were cultured in 6-well plates at 37°C with 5% CO₂ for 14 days. Colonies were fixed with 75% ethanol for 15 min at room temperature and stained with 0.1% crystal violet for 20 min at room temperature. Colonies containing at least 50 cells were counted manually under a light microscope.

RNA extraction, reverse transcription and reverse transcription-quantitative (RT-q) PCR. HuH7 cells were collected 48 h following transfection. Total RNA was extracted using the FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme Biotech Co., Ltd.). Total RNA (1 μ g) was reverse-transcribed using HiScript III RT SuperMix for qPCR (+gDNA wiper; Vazyme Biotech Co., Ltd.), with genomic DNA removal at 42°C for 2 min, reverse transcription at 37°C for 15 min and enzyme inactivation at 85°C for 5 sec.

RT-qPCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd) on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions were 95°C for 30 sec, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. GAPDH was used as the reference gene and si-NC was used as the calibrator. Relative FAM72D expression was calculated using the $2^{-\Delta\Delta C_q}$ method (28). Primer sequences are provided in Table SII. Three independent biological experiments were performed, with three technical replicates per biological replicate.

Wound-healing and migration assays. For the wound-healing assay, standardized scratches were made in confluent monolayers using sterile 200- μ l pipette tips. Migration distances were measured after 24 h of serum-free incubation. Transwell migration assays were performed in uncoated chambers with 8- μ m pores (Corning, Inc.); neither Matrigel nor another

extracellular-matrix coating was used. A total of 2×10^5 cells in serum-free medium were added to the upper chamber, while medium containing 10% fetal bovine serum was added to the lower chamber. After 24 h, migrated cells were fixed with 4% paraformaldehyde for 15 min at room temperature, stained with 0.1% crystal violet for 20 min at room temperature and counted at x200 magnification.

Flow cytometry. Cells (5×10^4 per well) were harvested 48 h after transfection by trypsinization without EDTA. Apoptotic cells were quantified by flow cytometry (BD FACSCanto II; BD Biosciences) after staining with 5 μ l of Annexin V-FITC and 10 μ l of propidium iodide for 20 min at room temperature. Flow cytometry data were analyzed using FlowJo version 10.8.1 (BD Biosciences). The apoptotic rate was calculated as the sum of the percentages of early apoptotic cells (Annexin V-positive/propidium iodide-negative) and late apoptotic cells (Annexin V-positive/propidium iodide-positive) within the gated single-cell population following exclusion of debris and doublets.

Statistical analysis. Statistical analyses included Wilcoxon tests, unpaired Student's t-tests, χ^2 tests, Kruskal-Wallis tests, Kaplan-Meier analysis, Cox regression, ROC analysis, bootstrap internal validation, calibration analysis and decision curve analysis, as appropriate. All tests were two-sided and $P < 0.05$ was considered to indicate a statistically significant difference.

The locked Cox model used continuous FAM72D expression rather than a data-dependent cutoff. Patient-level resampling was used for TCGA internal validation and patient-cluster resampling was used for the GEO ROC analyses to account for paired tissues. The original transcriptomic analyses were performed primarily using R version 4.3.3 (<https://www.r-project.org/>), and the additional validation analyses were performed using Python (<https://www.python.org/>).

Results

FAM72D is consistently upregulated in HCC and associated with disease progression. The present study first characterized FAM72D mRNA expression in TCGA and three independent GEO cohorts. In TCGA-LIHC, FAM72D expression was significantly higher in HCC tissues than in adjacent non-tumor tissues ($P < 0.001$; Fig. 1A and B). Consistent expression differences were observed in GSE76427, GSE39791 and GSE54236 (Fig. 1C-E), demonstrating reproducible FAM72D upregulation across independent patient cohorts and two microarray platforms.

In TCGA-LIHC, FAM72D expression varied according to pathologic T category, pathologic N category, pathologic stage and overall-survival event status (Fig. 1F-I). Spatial transcriptomic analysis further showed stronger FAM72D transcript signals in tumor-labeled regions than in adjacent-tissue-labeled regions in HRA000437 (Fig. 1J-M).

Tissue-level staining also revealed stronger FAM72-family immunoreactivity in HCC tissues than in paired adjacent non-tumor tissues. Among the 10 paired samples, 9 HCC tissues showed high FAM72-family immunoreactivity, whereas all 10 adjacent non-tumor tissues showed low immunoreactivity

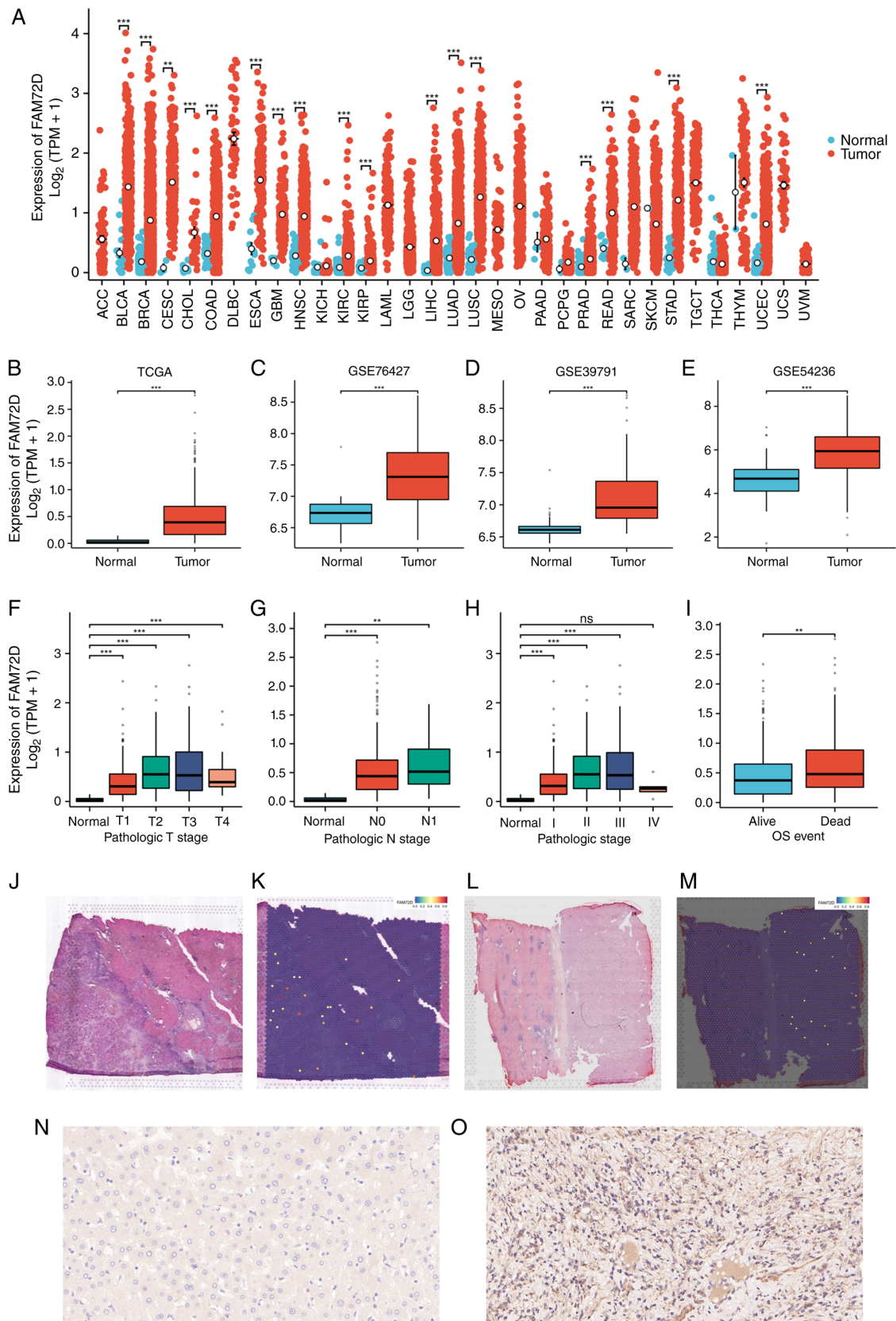


Figure 1. FAM72D expression and clinical associations in HCC. (A) FAM72D expression across cancer types compared with normal tissues in TCGA. (B) FAM72D mRNA expression in HCC and adjacent non-tumor tissues in TCGA-LIHC. FAM72D mRNA expression in HCC and normal or adjacent liver tissues in (C) GSE76427, (D) GSE39791 and (E) GSE54236. (F) FAM72D mRNA expression in normal tissue and across pathologic T categories T1-T4. (G) FAM72D mRNA expression in normal tissue and across pathologic N categories N0 and N1. (H) FAM72D mRNA expression in normal tissue and across pathologic stages I-IV. (I) FAM72D mRNA expression according to OS event status. (J and L) H&E-stained HCC sections. (K and M) Spatial transcriptomic maps showing the distribution of FAM72D transcripts in the analyzed HCC tissue sections. Representative IHC images of FAM72-family immunoreactivity in (N) adjacent non-tumor and (O) HCC tissues. FAM72D, family with sequence similarity 72 member D; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; LIHC, liver hepatocellular carcinoma; H&E, hematoxylin and eosin; IHC, immunohistochemistry; OS, overall survival. **P<0.01 and ***P<0.001; ns, not significant. Comparisons are indicated by brackets.

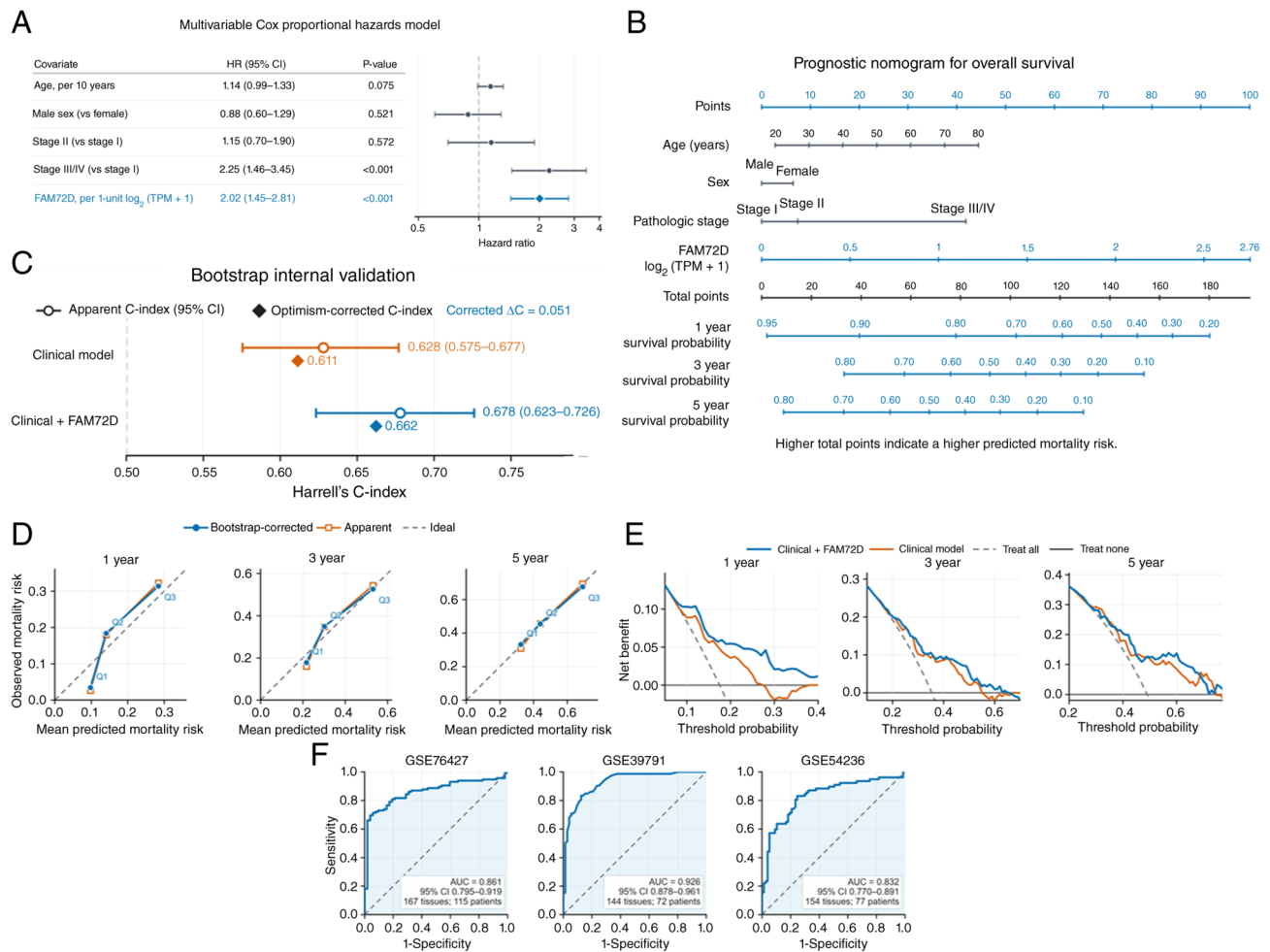


Figure 2. Prognostic modeling, internal validation and independent tissue-level validation of FAM72D in HCC. (A) Forest plot of the locked multivariable Cox model including age per 10 years, sex, pathologic stage and continuous FAM72D expression. Hazard ratios are shown with 95% confidence intervals. (B) Nomogram derived from the same locked model for estimation of 1-, 3- and 5-year overall-survival probabilities. (C) Harrell's C-indices for the clinical model and clinical-plus-FAM72D model. Open circles and horizontal bars represent apparent C-indices and bootstrap 95% CI; diamonds represent optimism-corrected C-indices obtained from 1,000 bootstrap resamples. (D) Bootstrap-corrected calibration of 1-, 3- and 5-year mortality risk. Orange squares indicate apparent Kaplan-Meier estimates, blue circles indicate optimism-corrected estimates and dashed lines indicate ideal calibration. (E) Decision curve analysis of the clinical-plus-FAM72D model, clinical model, treat-all strategy and treat-none strategy at 1, 3 and 5 years. (F) Independent tissue-level ROC validation in GSE76427, GSE39791 and GSE54236. AUC confidence intervals were calculated using 2,000 patient-cluster bootstrap resamples. FAM72D, family with sequence similarity 72 member D; HCC, hepatocellular carcinoma; HR, hazard ratio; CI, confidence interval; ROC, Receiver Operating Characteristic; AUC, Area Under the Curve.

(Fig. 1N and O). Collectively, these findings establish elevated FAM72D expression as a reproducible molecular feature of HCC closely linked to disease progression.

FAM72D expression is independently associated with unfavorable overall survival in TCGA-LIHC. A locked multivariable Cox model was developed using age, sex, pathologic stage and continuous FAM72D expression in 341 patients with 117 mortalities. Stage III/IV disease was associated with poorer overall survival than stage I disease [hazard ratio (HR)=2.25; 95% confidence interval (CI) 1.46-3.45; $P<0.001$]. Higher FAM72D expression remained independently associated with poorer overall survival after adjustment for the clinical variables (HR per 1-unit increase in $\log_2$ [TPM + 1]=2.02, 95% CI 1.45-2.81; $P<0.001$; Fig. 2A).

A nomogram based on the same locked model was constructed to estimate 1-, 3- and 5-year overall-survival probabilities (Fig. 2B). Descriptive Kaplan-Meier and subgroup

analyses consistently linked higher FAM72D expression to unfavorable survival and are presented in Fig. S1.

The clinical model had an apparent C-index of 0.628 (bootstrap 95% CI 0.575-0.677) and an optimism-corrected C-index of 0.611. After FAM72D was added, the apparent C-index increased to 0.678 (bootstrap 95% CI 0.623-0.726) and the optimism-corrected C-index increased to 0.662. The resulting optimism-corrected increment in C-index was 0.051, while mean optimism remained small for both models (Fig. 2C).

Bootstrap-corrected calibration showed favorable agreement between predicted and observed mortality risks at 1, 3 and 5 years (Fig. 2D). The group-weighted mean absolute deviations were 0.045, 0.030 and 0.013, respectively. Decision curve analysis showed incremental net benefit from adding FAM72D, with the most consistent advantage observed for 1-year mortality prediction across thresholds of 0.06-0.40 (Fig. 2E). The maximum incremental net benefits were 0.048, 0.044 and 0.054 at 1, 3 and 5 years, respectively.

Independent tissue-level validation further demonstrated consistent discrimination between HCC and non-tumor liver tissues. The AUC was 0.861 in GSE76427 (patient-cluster bootstrap 95% CI 0.795-0.919), 0.926 in GSE39791 (95% CI 0.878-0.961) and 0.832 in GSE54236 (95% CI 0.770-0.891) (Fig. 2F). All 2,000 bootstrap resamples were successful in each cohort. Together, these analyses demonstrate that FAM72D provides prognostic information beyond conventional clinical variables and reproducibly distinguishes HCC from non-tumor liver tissue.

FAM72D defines a highly proliferative malignant-cell population in HCC. Single-cell RNA-sequencing data from GSE151530 were analyzed to determine the cellular distribution of FAM72D. Uniform manifold approximation and projection (UMAP) visualization identified epithelial, immune and stromal populations based on the dataset annotations (Fig. 3A). The malignant-cell compartment was subsequently divided into nine transcriptional subclusters, designated C0-C8 (Fig. 3B).

FAM72D expression was strongly enriched in subcluster C5 (Fig. 3C). Cell-cycle analysis showed that C5 contained a higher proportion of cells in G₂/M phase and had higher G₂/M scores than the other malignant-cell subclusters (Fig. 3D and E). C5 also exhibited pronounced activation of cell-cycle, DNA-repair and mitotic programs (Fig. 3F-H).

GO enrichment analysis showed that FAM72D-high malignant cells were enriched in cell-cycle and mitotic processes, including positive regulation of the cell cycle, mitotic cell-cycle phase transition, G₂/M-phase transition and mitotic spindle organization. Enriched molecular functions and cellular components included kinase binding and spindle/microtubule-cytoskeleton-related terms (Fig. 3I). These results identified FAM72D as a defining molecular feature of a highly proliferative malignant-cell population in HCC.

FAM72D-centered transcriptional programs converge on cell-cycle regulation and FOXM1 activity. To define the transcriptional architecture associated with FAM72D, DEGs were identified between TCGA-LIHC samples with high and low FAM72D expression ($|\log\text{FC}| > 1.5$ and adjusted $P < 0.05$; Fig. 4A). GO and KEGG enrichment analyses showed that the FAM72D-high phenotype was dominated by programs involving nuclear division, cell-cycle progression, chromosome organization, humoral immune responses, channel activity and neuroactive ligand-receptor interactions (Fig. 4B).

GSEA showed positive enrichment of Reactome pathways related to sister-chromatid cohesion resolution, mitotic prometaphase, sister-chromatid separation, mitotic G₂-G₂/M phases and mitotic metaphase/anaphase in samples with high FAM72D expression (Fig. 4C). By contrast, bio-oxidation, biotransformation and metabolic signatures were relatively reduced in the FAM72D-high group (Fig. 4D).

SCENIC analysis revealed marked enrichment of proliferative regulons in C5. FOXM1 was among the regulons showing increased activity in C5 relative to the other malignant-cell subclusters (Fig. 4E). FOXM1 regulon activity was concentrated in C5 and closely aligned with the G₂/M transcriptional score (Fig. 4F). PPI analysis of FAM72D-associated DEGs

further revealed a densely connected gene network dominated by proliferation-related components (Fig. 4G).

The agreement among differential-expression analysis, pathway enrichment, single-cell scoring, regulon activity and PPI connectivity places FAM72D within a tightly coordinated FOXM1-centered proliferative network in HCC malignant cells.

The FAM72D-high phenotype is coupled to an altered immune context in HCC. TIMER-based analysis showed that FAM72D expression was positively correlated with the immune-checkpoint-related genes PDCD1 ($R=0.324$; $P<0.001$), CD274 ($R=0.114$; $P=0.027$) and CTLA4 ($R=0.369$; $P<0.001$). By contrast, FAM72D expression was negatively correlated with estimated CD8⁺ T-cell abundance ($R=-0.233$; $P<0.001$; Fig. 5A).

The FAM72D-high group had higher TIDE scores than the FAM72D-low group ($P<0.05$; Fig. 5B), connecting elevated FAM72D expression with transcriptional features of T-cell dysfunction and exclusion. In GSE151530, FAM72D expression was additionally visualized alongside the treatment-outcome annotations provided for the selected samples (Fig. 5C). Together, these findings indicated that the FAM72D-high proliferative phenotype is coupled to increased immune-checkpoint expression, reduced estimated CD8⁺ T-cell abundance and an immune-dysfunction-associated tumor context.

FAM72D silencing suppresses proliferative and migratory phenotypes in HCC cells. To examine the functional contribution of FAM72D, HuH7 cells were transiently transfected with three si-FAM72D reagents. RT-qPCR confirmed significant reductions in FAM72D mRNA expression, with si-FAM72D-3 producing the greatest knockdown efficiency (Fig. 6A). si-FAM72D-3 was therefore selected for the subsequent experiments.

Compared with si-NC, FAM72D silencing markedly reduced HuH7-cell viability and colony formation (Fig. 6B and C). Flow-cytometric analysis further showed an increase in Annexin V-FITC/PI-positive apoptotic cells following FAM72D knockdown (Fig. 6D).

Wound-healing and uncoated Transwell assays both demonstrated markedly reduced HuH7-cell migration after FAM72D silencing (Fig. 6E and F). These functional changes were concordant with the cell-cycle and proliferative programs identified by the bulk and single-cell transcriptomic analyses. Collectively, the experimental findings support an active contribution of FAM72D to HCC-cell proliferation, survival and migration.

Discussion

The analyzed datasets consistently showed higher FAM72D expression in HCC. In TCGA-LIHC, higher FAM72D expression was independently associated with unfavorable overall survival, while single-cell analysis showed enrichment of FAM72D in an annotation-defined malignant-cell population with a highly proliferative transcriptional state. Integrated bulk and single-cell analyses further linked FAM72D to a coordinated transcriptional program involving cell-cycle

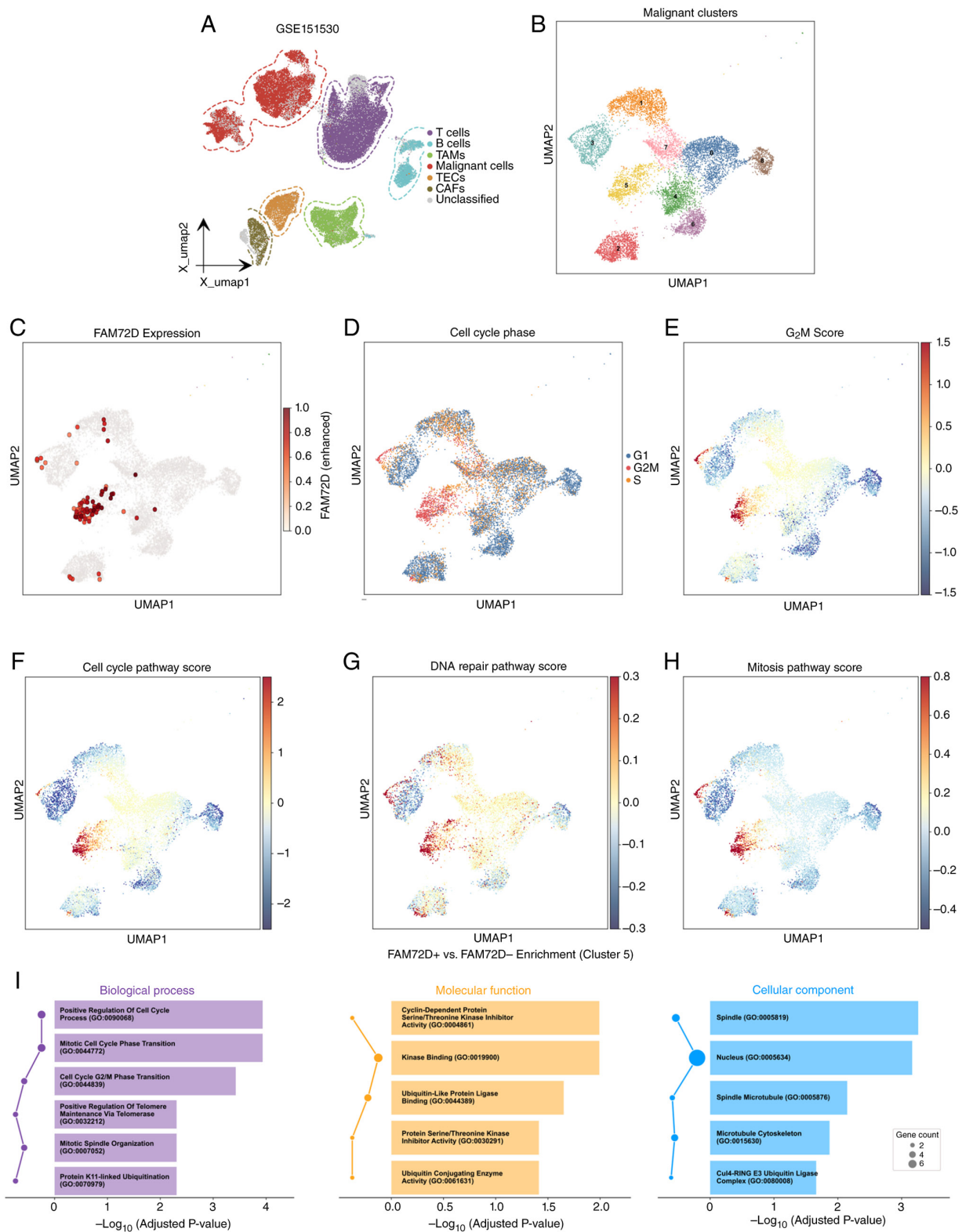


Figure 3. Malignant-cell subclusters and proliferative features associated with FAM72D in GSE151530. (A) UMAP visualization of the cell populations annotated in the dataset. (B) UMAP visualization of the malignant-cell compartment divided into nine transcriptional subclusters (C0-C8). (C) Density distribution of FAM72D expression across the malignant-cell subclusters, with the strongest signal in C5. (D) Cell-cycle-phase distribution. (E) G₂M-score distribution. (F) Cell-cycle pathway scores. (G) DNA-repair pathway scores. (H) Mitosis pathway scores. (I) GO enrichment analysis comparing FAM72D-high and FAM72D-low malignant cells using the central-tendency threshold implemented in the original analytical workflow. FAM72D, family with sequence similarity 72 member D; UMAP, uniform manifold approximation and projection; GO, Gene Ontology.

progression, DNA replication, mitosis and FOXM1 regulon activity. Elevated FAM72D expression was also associated with increased immune-checkpoint expression, lower

estimated CD8⁺ T-cell abundance and higher TIDE scores. In HuH7 cells, FAM72D silencing was accompanied by reduced proliferation and migration and increased apoptosis. Together,

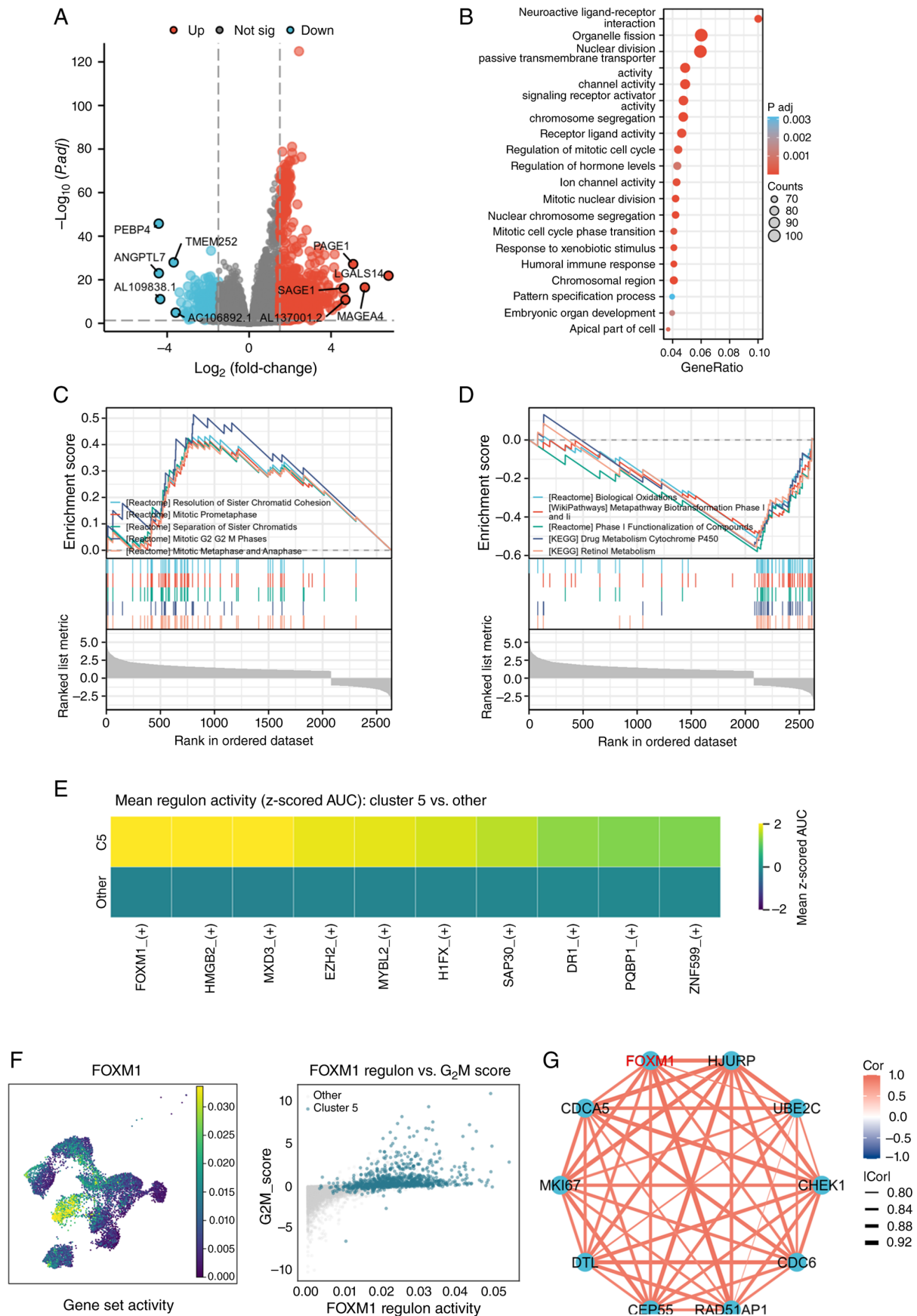


Figure 4. FAM72D-centered transcriptional features and FOXM1 regulon activity. (A) Volcano plot of DEGs between FAM72D-high and FAM72D-low TCGA-LIHC samples, with selected highly altered genes highlighted. (B) KEGG and GO enrichment analyses of DEGs ($\log_{2}FC > 1.5$ and adjusted $P < 0.05$). (C and D) GSEA results. (E) Heatmap of regulon activity in C5 vs. the other malignant-cell subclusters. (F) FOXM1 regulon-activity feature plot and association between FOXM1 regulon activity and the G_2/M score. (G) PPI network of FAM72D-associated DEGs. FAM72D, family with sequence similarity 72 member D; FOXM1, forkhead box M1; DEGs, differentially expressed genes; TCGA, The Cancer Genome Atlas; LIHC, liver hepatocellular carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; GSEA, gene set enrichment analysis; PPI, protein-protein interaction.

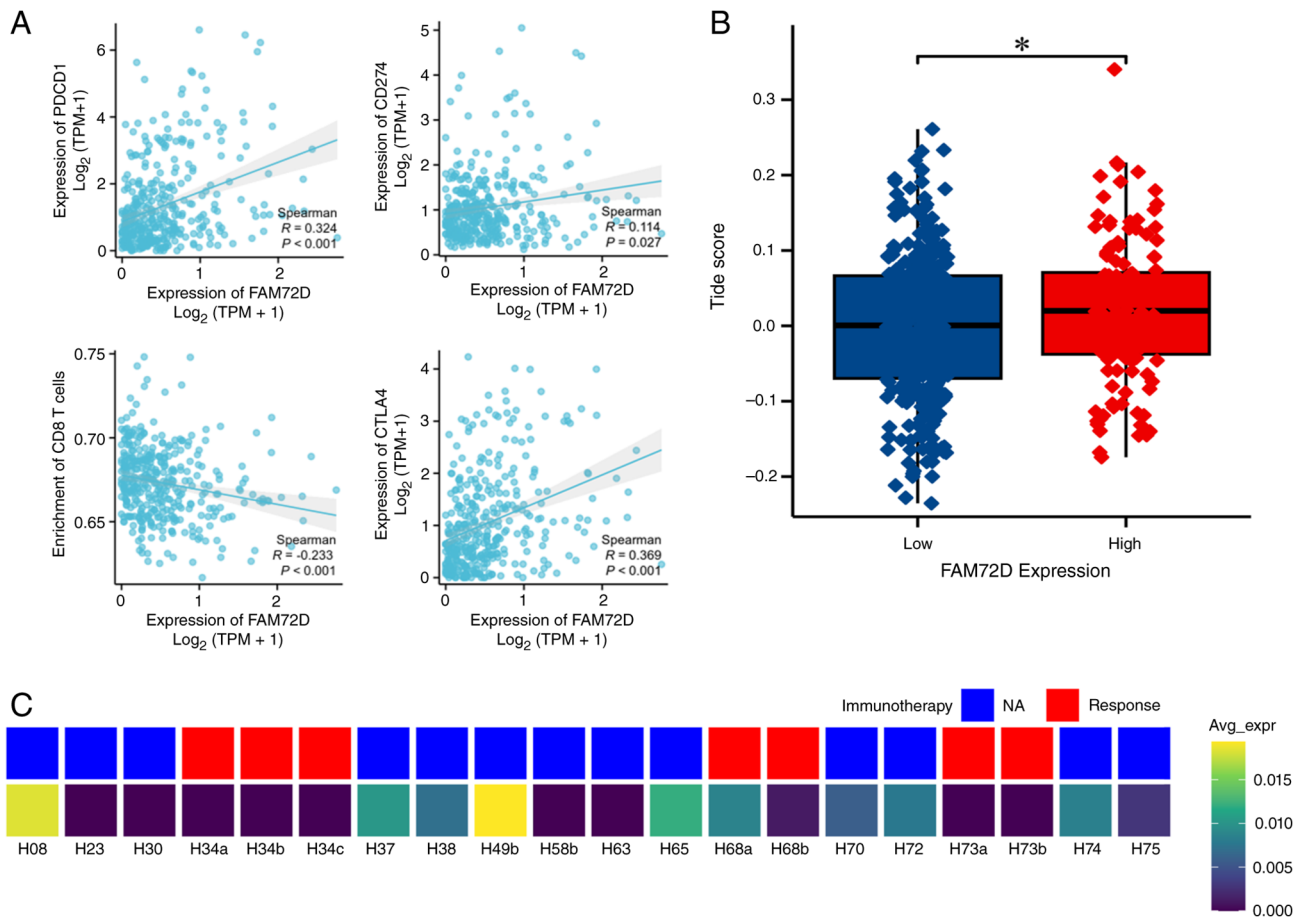


Figure 5. FAM72D expression and immune-related features in HCC. (A) Correlations of FAM72D mRNA expression with PDCD1, CD274, CTLA4 and estimated CD8+ T-cell abundance. (B) Comparison of TIDE-related scores between the FAM72D expression groups. (C) Heatmap of selected GSE151530 samples. The upper tile shows the treatment-outcome annotation provided with the dataset and the lower tile shows mean normalized FAM72D expression. FAM72D, family with sequence similarity 72 member D; HCC, hepatocellular carcinoma; TIDE, tumor immune dysfunction and exclusion. *P<0.05, as indicated by the bracket.

these findings supported an association between FAM72D, unfavorable prognosis and proliferative malignant-cell states in HCC.

The FAM72 family has previously been linked to neuronal progenitor-cell self-renewal and cancer-associated proliferation (6). FAM72D has also emerged in prognostic or proliferation-related signatures in prostate cancer, renal cell carcinoma, multiple myeloma and lung adenocarcinoma (7-9,12). In HCC, earlier studies primarily focused on FAM72A or on the expression and prognostic profiles of the FAM72 family (10,11). The present study extended this work by integrating FAM72D expression with patient-level prognostic modeling, malignant-cell heterogeneity, regulon activity, immune context and experimentally measured tumor-cell phenotypes.

Contemporary HCC management incorporates surveillance, prevention, locoregional therapy, systemic treatment, immunotherapy and liver transplantation (29-31). At the same time, recent studies have emphasized the importance of spatially organized stromal niches and exhausted T-cell-macrophage interactions in shaping HCC progression and treatment response (32,33). Against this heterogeneous clinical background, FAM72D remained independently associated with overall survival after adjustment for age, sex and

pathologic stage. Its addition increased the optimism-corrected C-index by ~0.05 and the increment persisted after patient-level bootstrap correction. The calibration and decision curve analyses further supported the contribution of FAM72D to prognostic stratification, while the three GEO cohorts independently reproduced its marked tumor-versus-normal expression difference.

The concentration of FAM72D in the C5 malignant-cell population provides a cellular explanation for its elevated bulk-tissue expression and adverse clinical association. Transcriptome-based risk studies in other tumors have similarly highlighted recurrence-associated proliferative programs (34). Cell-cycle progression is coordinated by interacting E2F, mTORC1, MYC and FOXM1 networks (35-38). In HCC, FOXM1 upregulation has been associated with metastatic progression and unfavorable prognosis (39). In the present analysis, FAM72D-high malignant cells displayed concordant activation of G₂/M, mitotic, DNA-replication, DNA-repair, chromosome-segregation and spindle-related programs. Rather than representing a nonspecific tumor-associated transcript, FAM72D therefore delineates a malignant-cell population with a pronounced proliferative identity.

FOXM1 regulon activity was elevated in the FAM72D-high C5 population and was associated with the G₂/M program.

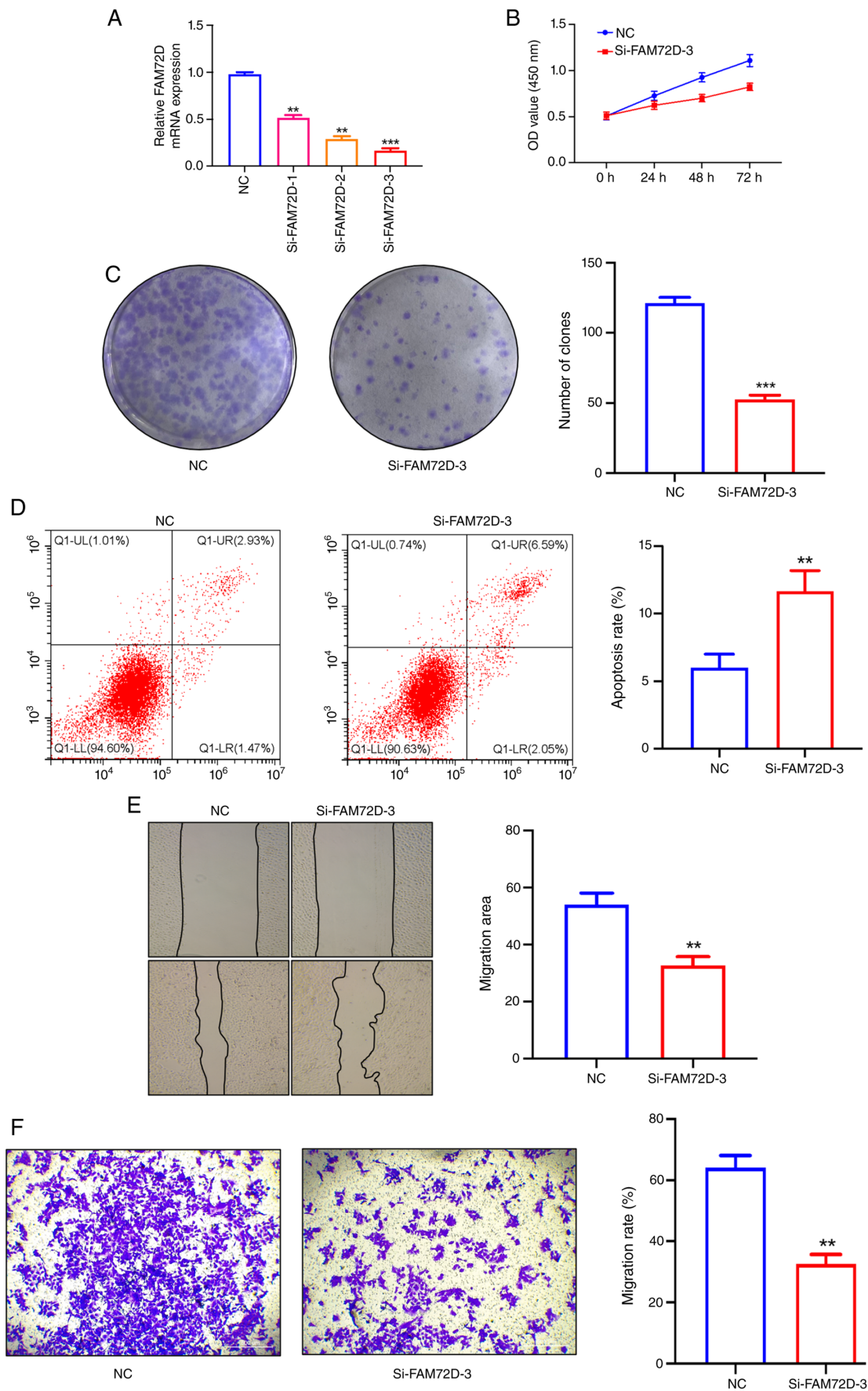


Figure 6. Effects of siRNA-mediated FAM72D silencing in HuH7 cells. (A) RT-qPCR measurement of FAM72D mRNA expression following transfection with si-FAM72D reagents. (B) CCK-8 cell-viability assay. (C) Colony-formation assay. (D) Flow-cytometric analysis of apoptosis. (E) Wound-healing migration assay. Original magnification, x10. (F) Uncoated Transwell migration assay. Original magnification, x200. Neither Matrigel nor another extracellular-matrix coating was used in the Transwell assay. **P<0.01, ***P<0.001. si, short interfering; FAM72D, family with sequence similarity 72 member D; RT-qPCR, reverse transcription-quantitative PCR; NC, negative-control.

This finding was reinforced by the convergence of bulk-tissue GSEA, single-cell pathway scores, SCENIC regulon activity and PPI-network connectivity. The consistency across these independent analytical layers positions FAM72D within a proliferative program associated with FOXM1 regulon activity and provides a coherent transcriptional framework linking elevated FAM72D expression to aggressive HCC-cell behavior.

HCC progression is also shaped by immune escape, lymphocyte recruitment, angiogenic remodeling, oncogenic signaling and inflammatory cell-death pathways (40-44). In the present study, elevated FAM72D expression was accompanied by higher PDCD1, CD274 and CTLA4 expression, reduced estimated CD8+ T-cell abundance and higher TIDE scores. FOXM1 has also been connected to PD-L1 expression and antitumor immune responses in other tumor systems (45,46). These findings place the FAM72D-high proliferative phenotype within a broader immune-dysfunction-associated tumor context and suggest coordination between malignant-cell proliferation and immune remodeling in HCC.

The *in vitro* experiments provide functional support for the transcriptomic findings. FAM72D silencing reduced cell viability and colony formation, increased apoptosis and impaired migration in both wound-healing and uncoated Transwell assays. These phenotypic effects align closely with the enrichment of cell-cycle and mitotic programs in FAM72D-high malignant cells and indicate that FAM72D is not merely a passive marker of proliferative HCC cells. Instead, the combined evidence supports an active contribution of FAM72D to the maintenance of proliferative, survival and migratory phenotypes.

Several limitations should be acknowledged. The survival model was internally validated but was not evaluated in an independent cohort with fully compatible survival and clinical variables. The IHC reagent recognized the FAM72 protein family and therefore did not provide FAM72D-specific protein quantification. The *in vitro* experiments were performed in HuH7 cells using transient siRNA-mediated knockdown and validation in additional HCC models, rescue experiments and *in vivo* systems would extend the functional findings. In addition, the retained single-cell and spatial-transcriptomic outputs did not preserve all sample-level quality-control and section-level metadata. Future studies incorporating prospective clinical cohorts, FAM72D-specific protein-level assays and direct perturbation of the FOXM1-associated program will help define the translational and mechanistic scope of these findings.

In conclusion, FAM72D is consistently upregulated in HCC, independently predicts unfavorable overall survival and defines a highly proliferative malignant-cell population. Integrated bulk, single-cell and regulatory-network analyses place FAM72D within a coordinated program involving cell-cycle progression, DNA replication, mitosis and FOXM1 activity. The suppression of HCC-cell proliferation and migration and induction of apoptosis following FAM72D silencing further support its functional contribution to aggressive tumor-cell phenotypes. Collectively, these findings establish FAM72D as a biologically relevant prognostic marker and an integral component of the proliferative program of HCC malignant cells.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

QJ and QY conceptualized the study. QJ, YL and WD developed the methodology. CZ and JY validated the findings using independent datasets and experimental assays. JY prepared the figures and graphical representations of the analytical results. CZ and QY wrote the original draft. CZ, QY and WD reviewed and edited the manuscript. CZ and QJ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All the human tissue samples involved in this study have been approved by the Ethics Committee of Hunan Provincial People's Hospital [(2024) -108] and with the informed consent of the patients themselves or their guardians and have followed the ethical guidelines of the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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