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ORIGINAL RESEARCH

Integrative Multi-Omics and Functional Characterization Reveal MCM4 as a Key Oncogenic Regulator in Hepatocellular Carcinoma

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Background: Hepatocellular carcinoma (HCC) is a highly lethal malignancy with limited therapeutic options and poor prognosis. Identifying robust prognostic biomarkers and therapeutic targets is essential for improving patient outcomes. Minichromosome maintenance complex component 4 (MCM4), a DNA replication licensing factor, has been associated various malignancies, yet its involvement in HCC remains underexplored.

Methods: We performed integrative bioinformatics analyses on three public HCC datasets (GSE14520, GSE56545, and GSE84402) to identify consistently dysregulated genes. Functional enrichment analyses were conducted using GO, KEGG, and Reactome databases. PPI networks were constructed via STRING. The expression and prognostic value of MCM4 were evaluated using GEPIA, Human Protein Atlas, and KM-Plotter. Single-cell and spatial transcriptomics from the HCCDB were analyzed to explore MCM4 localization. Functional roles of MCM4 were validated in vitro using siRNA-mediated knockdown and plasmid-based overexpression in HepG2 and Huh7 cells.

Results: MCM4 was identified as a consistently upregulated gene in HCC and was associated with poor overall, disease-free, recurrence-free, and disease-specific survival. Single-cell and spatial transcriptomic analyses revealed MCM4 enrichment in proliferative tumor regions. Functional assays demonstrated that MCM4 promotes HCC cell growth, motility, invasiveness, and enhances EMT and stemness. Conversely, MCM4 knockdown attenuated these malignant phenotypes.

Conclusion: Our study establishes MCM4 as a key regulator of HCC progression and a potential prognostic biomarker. These findings suggest that MCM4 may serve as a potential target and underscore integrative and spatial transcriptomic approaches in cancer biomarker discovery.

Keywords: HCC, MCM4, prognostic biomarker, spatial transcriptomics, gene expression analysis, tumor progression

Introduction

Hepatocellular carcinoma (HCC) ranks as the sixth most prevalent malignancy worldwide and stands as the third leading contributor to cancer-related mortality, accounting for over 900,000 newly diagnosed cases and more than 800,000 deaths each year.[1,2] HCC frequently develops against a backdrop of chronic hepatic conditions and cirrhosis, with primary risk factors encompassing hepatitis B and C virus infections, excessive alcohol consumption.[3,4] Despite advancements in targeted therapies and immunotherapies such as sorafenib, lenvatinib, and immune checkpoint inhibitors, outlook for individuals with advanced HCC remains bleak, with a five-year survival rate falling below 20%.[5,6] Tumor heterogeneity, high recurrence rates, and late-stage diagnosis contribute to the limited therapeutic efficacy, underscoring the urgent need for novel biomarkers and therapeutic targets to improve disease management and patient outcomes.

High-throughput transcriptomic technologies, including microarray and RNA sequencing (RNA-seq), have significantly advanced our understanding of the molecular landscape of HCC. These approaches could reveal differentially expressed genes (DEGs) and dysregulated signaling pathways involved in tumor initiation and progression.[7] The integration of multi-cohort gene expression datasets using bioinformatics tools enhances the robustness of DEG

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identification by minimizing batch effects and increasing statistical power. Meta-analyses of GEO and TCGA datasets have proven valuable for uncovering clinically relevant molecular signatures and prognostic biomarkers in HCC.[8–10] Functional enrichment analyses offer deeper understanding of the dysregulated biological processes and signaling pathways involved in HCC, facilitating the prioritization of candidate genes for experimental validation.

Minichromosome maintenance complex component 4 (MCM4) is a core member of the MCM2–7 helicase complex, which plays a pivotal role in DNA replication origin licensing and elongation.[11] Dysregulation of MCM family members has been implicated in various malignancies, including lung, ovarian, and liver cancers, where they are frequently associated with increased proliferation, genomic instability, and poor prognosis.[12,13] MCM4, in particular, has been reported to exhibit oncogenic properties through its involvement in cell cycle regulation and DNA replication stress response.[14] However, the functional role and prognostic value of MCM4 in HCC remain insufficiently explored, necessitating systematic investigation using integrative bioinformatics and experimental approaches.

This study performed a comprehensive bioinformatics analysis for three independent HCC datasets (GSE14520, GSE56545, and GSE84402) to identify robust DEGs and characterize their functional implications through GO, KEGG, and Reactome pathway enrichment analyses. Among the identified DEGs, MCM4 emerged as a consistently upregulated gene across datasets.

We further evaluated the expression and clinical relevance of MCM4 in HCC using GEPIA, the Human Protein Atlas, and KM-Plotter databases. Single-cell and spatial transcriptomic analyses were employed to map the localization and cell-type specificity of MCM4 in HCC tissues. Finally, functional validation was conducted via siRNA-mediated knockdown and plasmid-driven overexpression of MCM4 in HCC cell lines to assess its role in proliferation, invasion, migration, and the regulation of epithelial–mesenchymal transition (EMT) and stemness-associated genes. Collectively, our results shed light on the molecular mechanisms underlying the tumor-promoting function of MCM4 and highlight its promise as both a diagnostic biomarker and a therapeutic target in HCC.

Materials and Methods

Data Acquisition and Identification of DEGs

Gene expression profiles of HCC datasets, namely GSE14520, GSE56545, and GSE84402, were obtained from the Gene Expression Omnibus. Differential analysis was performed using Limma package in R software. Genes were considered significantly differentially expressed based on the criteria of |log2 fold-change (FC)| >1 and adjusted p-value <0.05. Volcano plots were constructed using the ggplot2 package in R to illustrate the overall distribution and significance of DEGs. Subsequently, a Venn diagram illustrating common DEGs among datasets was constructed using the VennDiagram R package.

GO Enrichment Analysis

To explore the functional roles of common DEGs, GO analysis was conducted via the DAVID database (https://david.ncifcrf.gov/). GO terms encompassing biological processes, cellular components, and molecular functions with an adjusted p-value below 0.05 were deemed significantly enriched. The enrichment outcomes were visualized using bar plots generated through the EnrichR platform.

KEGG and Reactome Pathway Analysis

KEGG and Reactome pathway analyses were conducted using EnrichR tool. Enriched pathways ($p<0.05$) were identified and visualized using bar plots, depicting the key signaling pathways implicated by the common DEGs.

Protein-Protein Interaction (PPI) Network

A PPI network was constructed using the STRING database (https://string-db.org/) with a minimum interaction score of 0.4. Cytoscape software was used to visualize the network and further identify key hub genes via the CytoHubba plugin. Most prominent hub genes were identified based on their connectivity degrees.

Analysis of MCM4 Expression and Prognosis

Profiling MCM4 across various cancer types, including hepatocellular carcinoma (HCC), was assessed using the GEPIA web tool (http://gepia.cancer-pku.cn/) and the Human Protein Atlas (https://www.proteinatlas.org/). Prognostic analyses—covering overall survival (OS), disease-free survival (DFS), recurrence-free survival (RFS), and disease-specific survival (DSS)—were performed using GEPIA, the Kaplan–Meier Plotter (https://kmplot.com/analysis/), and the HCCDB resource. Statistical significance was evaluated using Log rank tests.

Single-Cell and Spatial Transcriptomic Analysis

Single-cell RNA sequencing and spatial transcriptomic data were analyzed using the HCCDB tool (http://lifeome.net/database/hccdb/home.html). Expression and localization of MCM4 within distinct cellular populations and spatial compartments of HCC tissues were visualized through integrated single-cell and spatial transcriptomic maps.

Cell Culture and Transfection Experiments

Human HCC cell lines (HepG2 and Huh7) were purchased from ATCC (Manassas, USA) and a normal liver cell line (LO2) were purchased from Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). They were cultured under standard conditions in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA) at 37°C in a 5% CO2 atmosphere. For knockdown experiments, MCM4-specific siRNAs (si-MCM4-a: 5′- GCAGAAGAUAUAGUGGCAATT-3′ and si-MCM4-b: 5′- GCCAGUACACGUCUGGGAATT-3′) and control siRNAs (5′-GGAAACTTAGAGGATCGTAAT-3′; GenePharma, Shanghai, China) were transiently transfected into HepG2 and Huh7 cells using Lipofectamine 3000 (Invitrogen, USA) based on manufacturer's protocol. For overexpression experiments, cells were transfected with pcDNA3.1-MCM4 plasmids or control vectors (#V790-20; GenePharma, Shanghai, China) based on supplier's protocol. Functional assays were performed 24 hours post-transfection.

RNA Extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA), and cDNA synthesis was performed using the PrimeScript RT reagent kit (Takara, Japan). Quantitative real-time PCR (qRT-PCR) was conducted using SYBR Green qPCR Master Mix (Takara, Japan) on an ABI 7500 Real-Time PCR System (Applied Biosystems, USA). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH serving as the internal control. Primers were presentedin Supplemental Table S1.

Cell Proliferation

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan). Cells were seeded into 96-well plates, transfected accordingly, and incubated for 24, 48, and 72 hours. Cell viability was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, USA).

Cell Migration and Invasion

Cell migration and invasion were assessed using Transwell chambers (Corning, USA) without or with Matrigel coating (BD Biosciences, USA). Briefly, transfected cells were seeded into the upper chambers in serum-free medium, while the lower chambers contained medium with 10% FBS. After incubation for 24 hours, cells that migrated or invaded were fixed, stained with crystal violet, and counted under a microscope.

Statistical Analysis

All experiments were performed independently in triplicate. Statistical analysis was conducted using GraphPad Prism (version 9.0). Data were expressed as means ± standard deviation. Statistical analyses were performed using Student's *t*-test for comparisons between two groups or one-way ANOVA followed by Tukey's post hoc test for multiple-group comparisons. A p-value < 0.05 was considered statistically significant.

Results

Analysis of DEGs in GSE14520, GSE5645 and GSE84402 Datasets

Differentially expressed genes (DEGs) were analyzed across three independent datasets (GSE14520, GSE56545, and GSE84402), with findings summarized in Figure 1. Volcano plots visually represented the distribution and magnitude of DEGs within each dataset. For dataset GSE14520, 1308 DEGs were revealed, comprising 378 significantly upregulated and 930 significantly downregulated ones (Figure 1A). Similarly, dataset GSE56545 exhibited 2437 DEGs, including 1326 upregulated and 1111 downregulated genes (Figure 1B), while dataset GSE84402 revealed 1218 DEGs, among which 435 were upregulated and 783 downregulated (Figure 1C).

Venn diagram further illustrated uniqueness of DEGs among datasets, identifying 153 common DEGs shared across all three datasets (Figure 1D). This intersectional analysis highlights conserved gene expression alterations, potentially pinpointing critical genes consistently involved in the biological processes under investigation.

GO Enrichment Analysis for Common DEGs

GO enrichment analysis was conducted to decipher biological functions associated with common DEGs identified in Figure 1. GO enrichment analysis is presented in Figure 2. Most significantly enriched GO terms within the category of biological processes included "monocarboxylic acid metabolic process" "zymogen activation" and "acute-phase response" (Figure 2A).

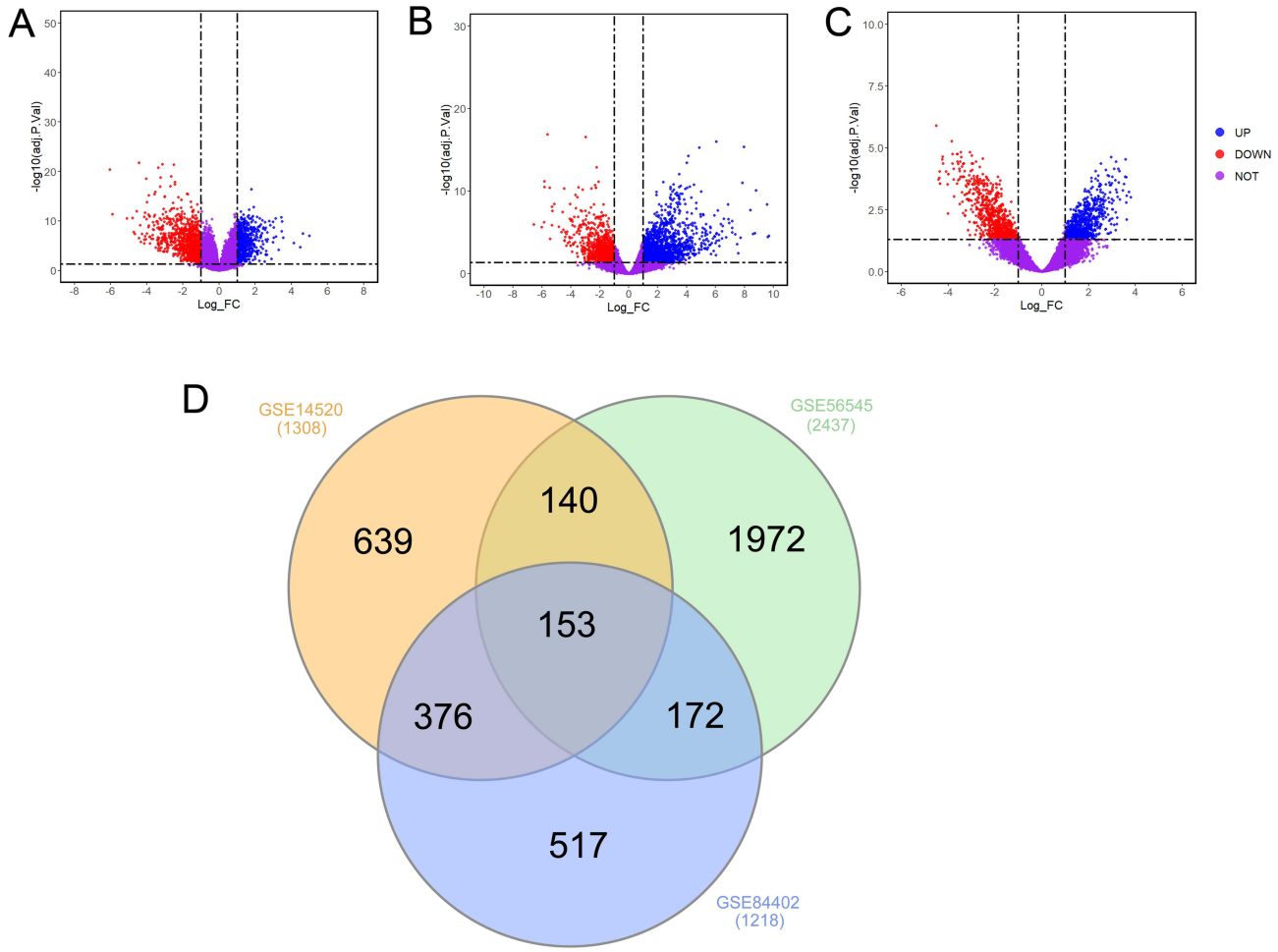

Figure 1 Analysis of DEGs in GSE14520, GSE56545 and GSE84402 datasets. Volcano plot elucidating DEGs for GSE14520 (**A**), GSE56545 (**B**) and GSE84402 (**C**). (**D**) Venn diagram elucidating common DEGs for GSE14520, GSE56545 and GSE84402.

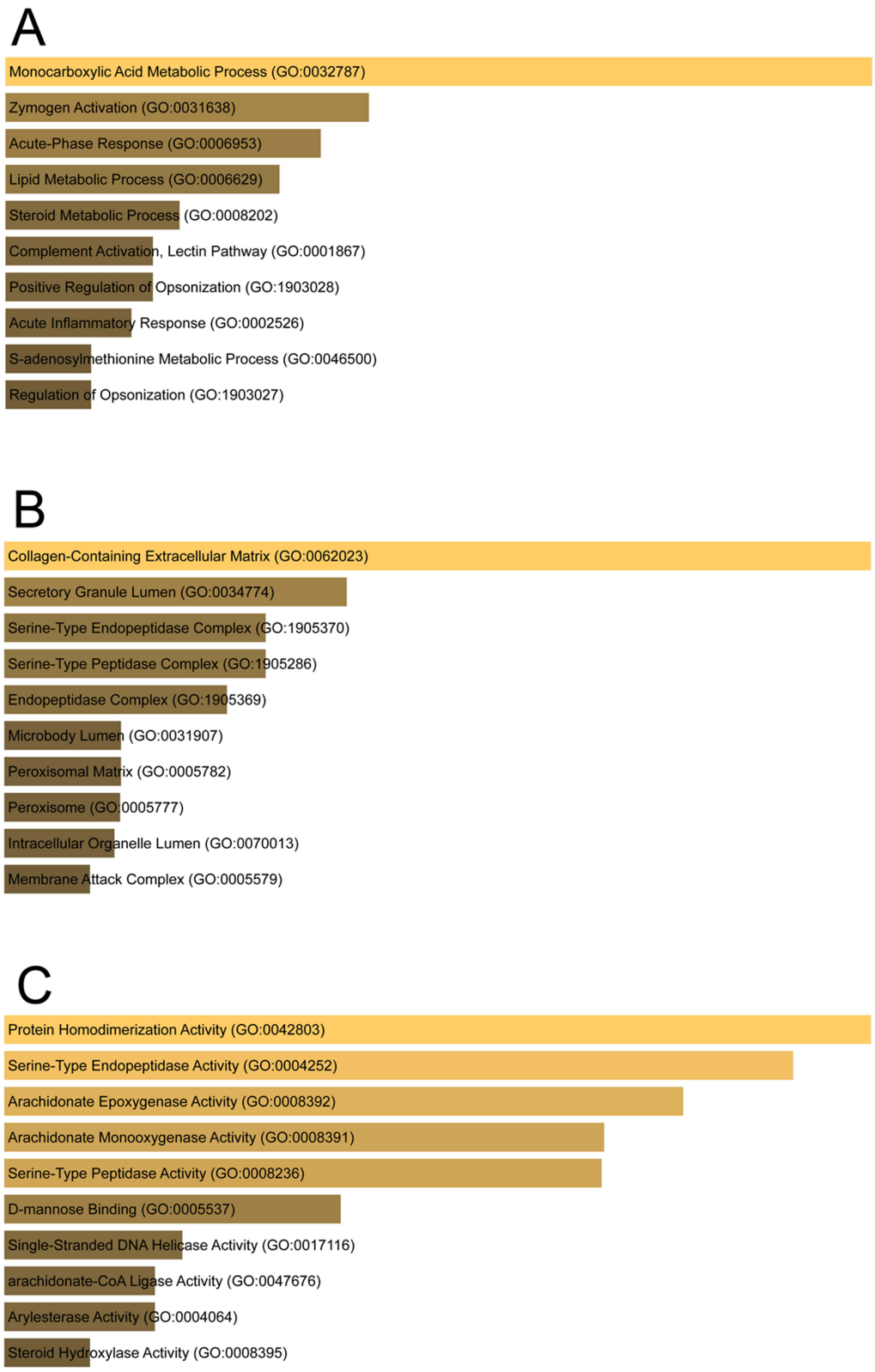

Figure 2 GO enrichment analysis for common DEGs. (**A**) Common DEGs enriched in GO_biological process. (**B**) Common DEGs enriched in cellular component. (**C**) Common DEGs enriched in molecular function.

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In the cellular component category, common DEGs primarily enriched in "collagen-containing extracellular matrix", "secretory granule lumen" and "serine-type endopeptidase complex" indicating the cellular locations most impacted by these gene expression changes (Figure 2B).

Furthermore, molecular function analysis revealed significant enrichment in terms such as "protein homodimerization activity", "serine-type endopeptidase activity" and "arachidonate epoxygenase activity" highlighting the functional attributes and activities predominantly associated with the identified common DEGs (Figure 2C).

KEGG and Reactome Analysis for Common DEGs

Pathway enrichment analyses using KEGG and Reactome databases were performed to assess signaling pathways associated with the identified common DEGs. KEGG analysis revealed enrichment in pathways such as "complement and coagulation cascades", "retinal metabolism", "fatty acid degradation", and "DNA replication" (Figure 3A). Additionally, Reactome analysis demonstrated substantial enrichment for the common DEGs within pathways including

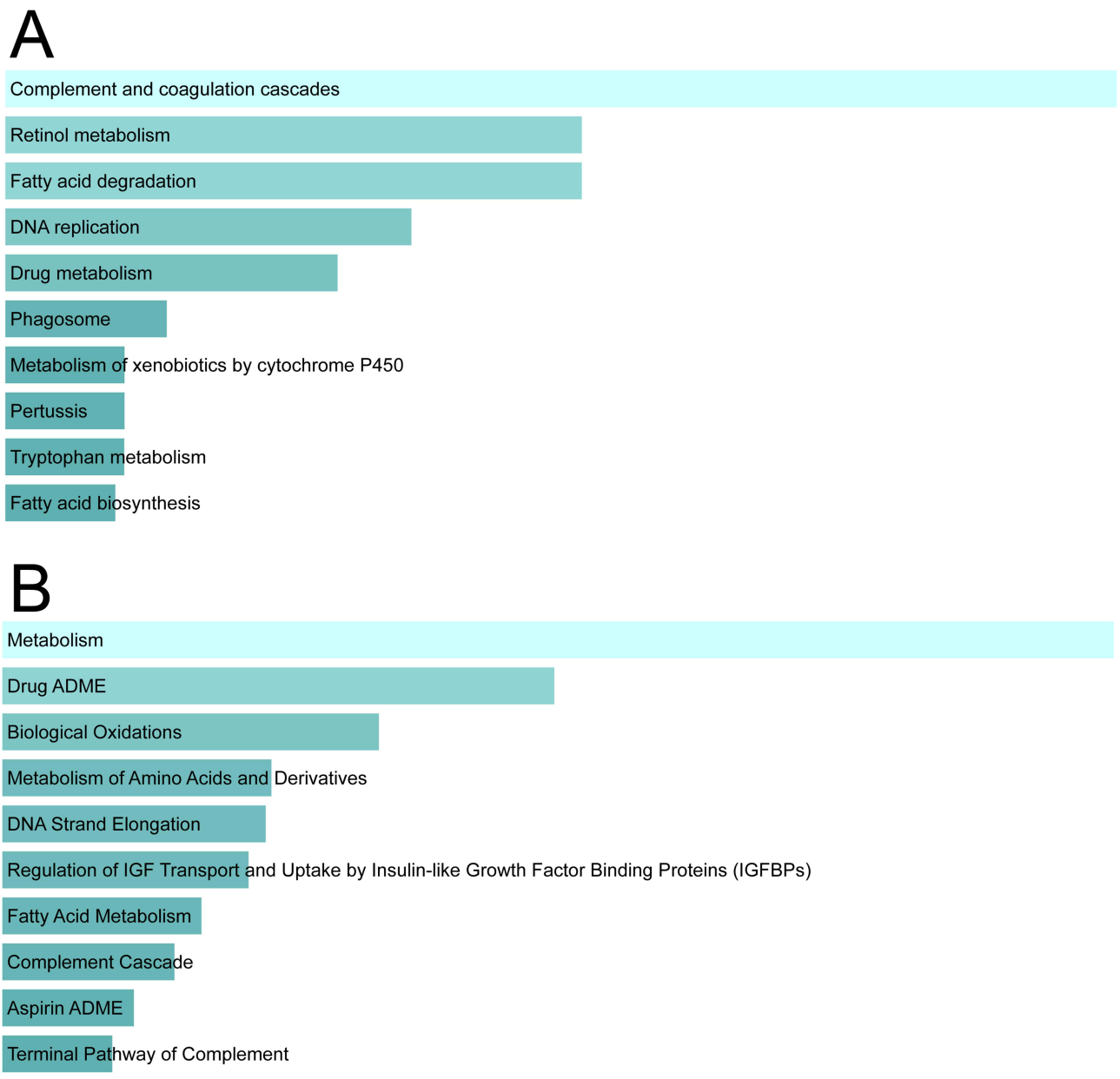

Figure 3 KEGG and Reactome analysis for common DEGs. (**A**) Common DEGs enriched in KEGG pathway. (**B**) Common DEGs enriched with Reactome.

"metabolism" "drug ADME" and "biological oxidations" further underscoring their potential roles in regulating or contributing to essential molecular mechanisms (Figure 3B).

PPI Network Construction of DEGs

To explore potential interactions among the identified DEGs, PPI network analysis was conducted. Figure 4A illustrates the comprehensive PPI network constructed using STRING database. Network comprised a total of 88 nodes representing the DEGs, interconnected through 250 interactions, indicating significant connectivity and interaction potential among the proteins.

Cytoscape CytoHubba was utilized to reconstruct and identify core sub-network, highlighting hub genes within the PPI network (Figure 4B). The sub-network analysis identified 20 key hub genes, notably including " MCM6" "MCM4" and "FEN1" which exhibited the highest degrees of connectivity and may represent critical regulators or markers central to the biological processes (Figure 4B). Based on the literature research and our findings in the subnetwork analysis, we selected MCM4 for further investigation.

Expression and Survival Analysis of MCM4

The expression and prognostic significance of MCM4 were comprehensively evaluated across multiple analyses (Figure 5). Pan-cancer analysis revealed varying levels of MCM4 expression across different tumor types, notably showing high expression in cancers such as "glioblastoma" "lung cancer", "colon cancer", "pancreatic cancer" and "HCC" (Figure 5A). Further, using the GEPIA database, MCM4 mRNA expression was elevated in HCC tissues comparing to non-cancerous ones ($p<0.05$; Figure 5B). Consistently, protein expression analysis derived from the Human Protein Atlas database confirmed higher MCM4 levels in HCC tissues relative to adjacent normal ones (Figure 5C).

Subsequently, survival analyses were conducted to evaluate the clinical implications of MCM4 expression in HCC patients. OS analyses using GEPIA demonstrated that high MCM4 level correlated with poorer prognosis ($p=0.0083$; Figure 5D). Validation through Kaplan–Meier plotter also indicated significantly reduced OS in HCC patients exhibiting

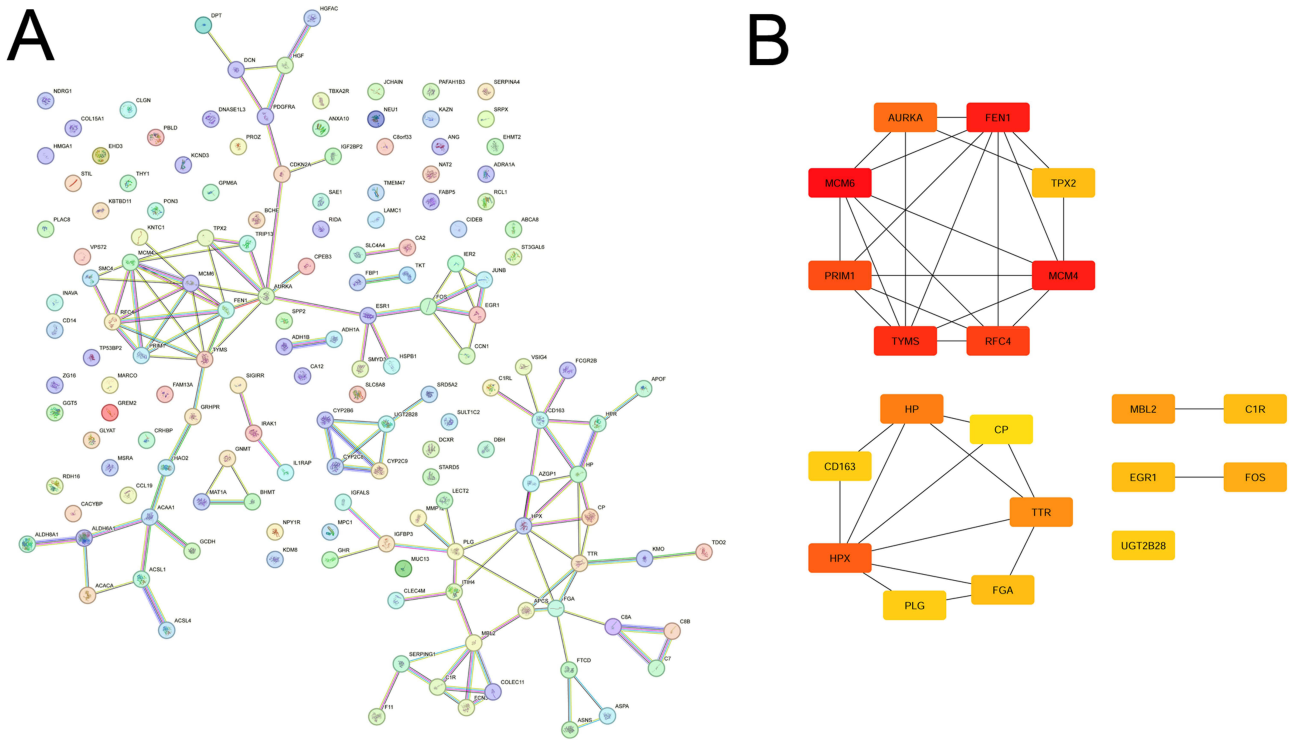

Figure 4 PPI network construction of DEGs. (**A**) PPI network analysis of DEGs as constructed by DB String. (**B**) Sub-PPI network reconstruction by Cytohuba tool in Cytoscape.

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elevated MCM4 expression (p=0.00092; Figure 5E). Moreover, DFS (Figure 5F), RFS (Figure 5G), and DSS (Figure 5H) analyses demonstrated that higher MCM4 level was significantly associated with adverse survival outcomes (DFS p=0.01; RFS p=0.031; DSS p=0.00043). Consistently, OS analysis of MCM4 using HCCDB tool revealed similar results (Supplemental Figure S1).

Single Cell Analysis and Spatial Transcriptomic Analysis of MCM4

Single-cell analysis and spatial transcriptomic profiling were conducted to delineate the cellular distribution and localization of MCM4 expression within HCC tissues (Figure 6). Single-cell transcriptomic analysis using the HCCDB tool indicated heterogeneous expression patterns of MCM4, predominantly enriched within specific cell populations, notably "endothelial cells", "malignant cells" and "NK/T" cells, suggesting its cell-specific regulatory roles in the tumor microenvironment (Figure 6A). Spatial transcriptomic analysis further revealed distinct localization patterns of MCM4 expression in HCC tissue samples, with higher transcript abundance primarily concentrated in tumor regions compared to adjacent non-tumorous regions (Figure 6B and C).

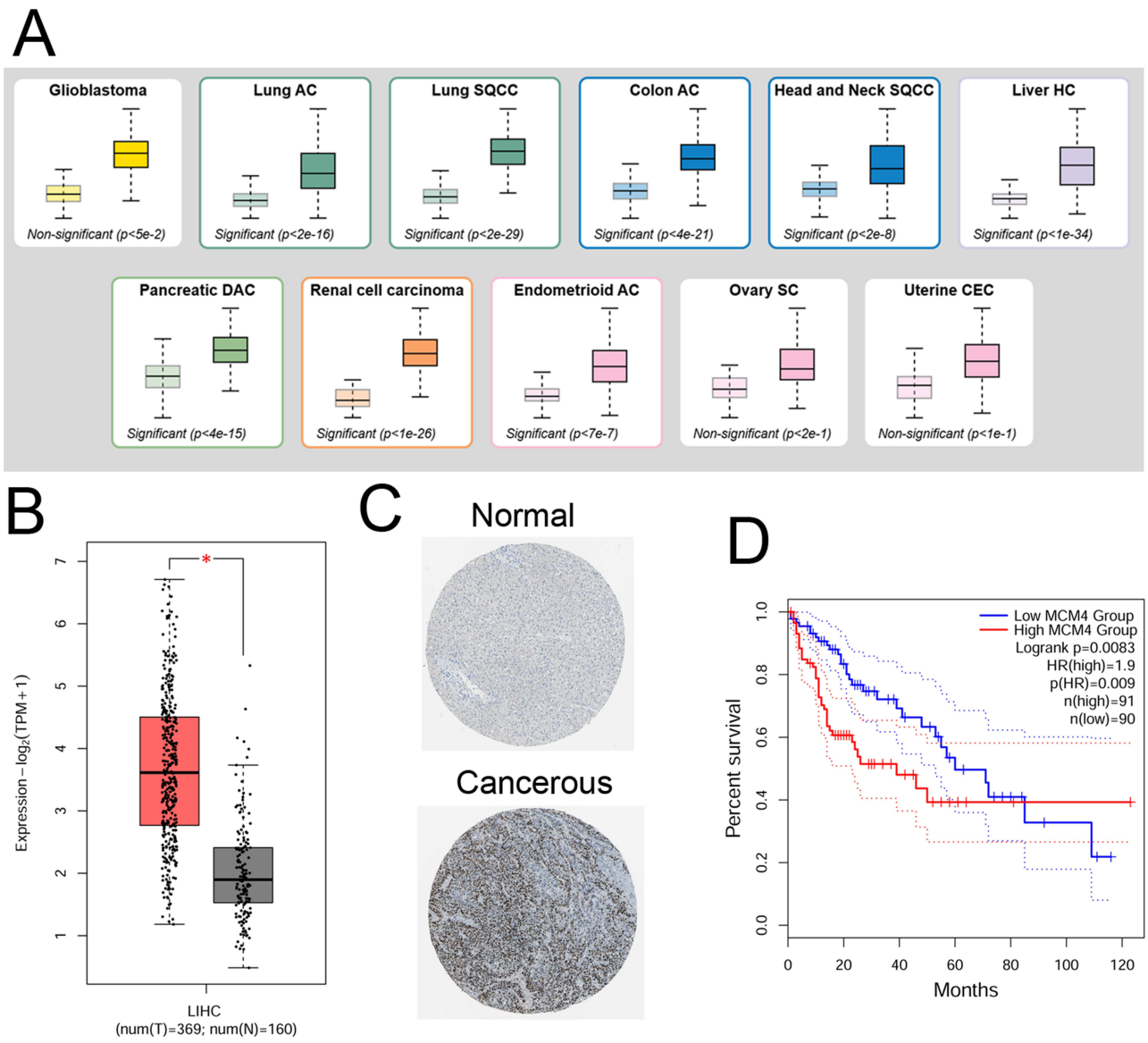

Figure 5 Continued.

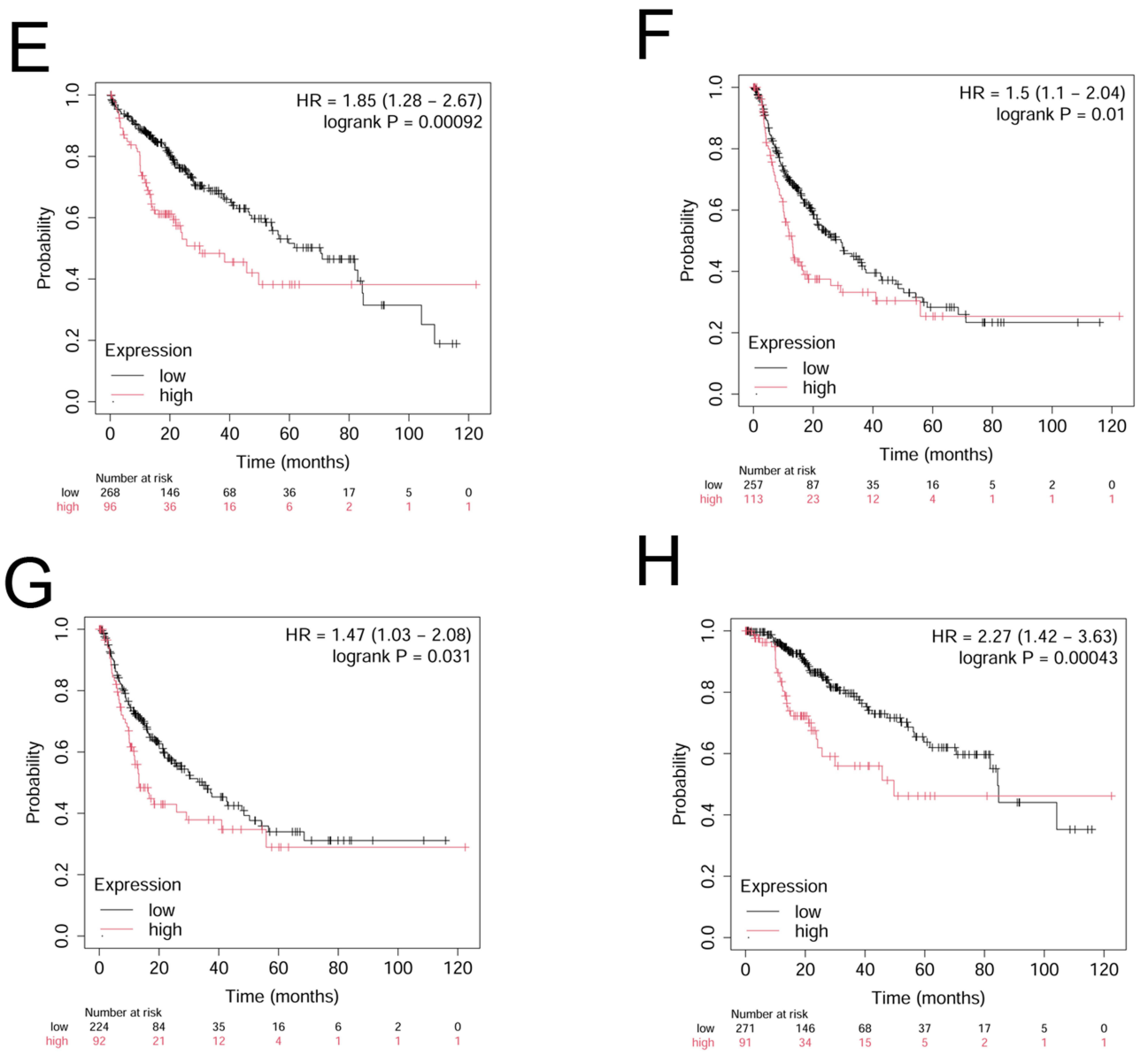

Figure 5 Expression and survival analysis of MCM4. (**A**) Pan-cancer expression of MCM4. (**B**) MCM4 mRNA expression in non-cancerous liver tissue and HCC tissues as determined by GEPIA tool. (**C**) MCM4 protein expression in non-cancerous liver tissue and HCC tissues as determined by Human Protein Atlas database. (**D**) OS analysis of MCM4 in HCC patients as determined in GEPIA tool. (**E**) OS analysis of MCM4 in HCC patients as determined in KM-plotter. (**F**) DFS analysis of MCM4 in HCC patients as determined in KM-plotter. (**G**) RFS analysis of MCM4 in HCC patients as determined in KM-plotter. (**H**) DDS analysis of MCM4 in HCC patients as determined in KM-plotter. *P<0.05.

Effects of MCM4 Silence on HCC Cell Progression

To explore functional role of MCM4 in HCC cell progression, we performed loss-of-function experiments using siRNA-mediated silencing (Figure 7). Initially, baseline expression of MCM4 was examined across different cell lines. MCM4 expression was significantly elevated in HCC cell lines HepG2 and Huh7 compared to normal ones (Figure 7A).

Subsequent knockdown experiments confirmed efficient silencing of MCM4, with significantly decreased MCM4 mRNA levels observed in HepG2 (Figure 7B) and Huh7 cells (Figure 7C) after respective siRNA transfections. Functional assays demonstrated that silencing MCM4 markedly suppressed proliferation in both HepG2 (Figure 7D) and Huh7 cells (Figure 7E) as measured by CCK-8 assays. Additionally, transwell invasion assays (Figure 7F) and migration assays (Figure 7G) revealed significant impairment of invasive and migratory capabilities in HCC cells following MCM4 knockdown. Furthermore, silencing MCM4 resulted in significant alterations in the expression of markers associated with EMT and stemness. Specifically, HepG2 cells showed significantly reduced expression of

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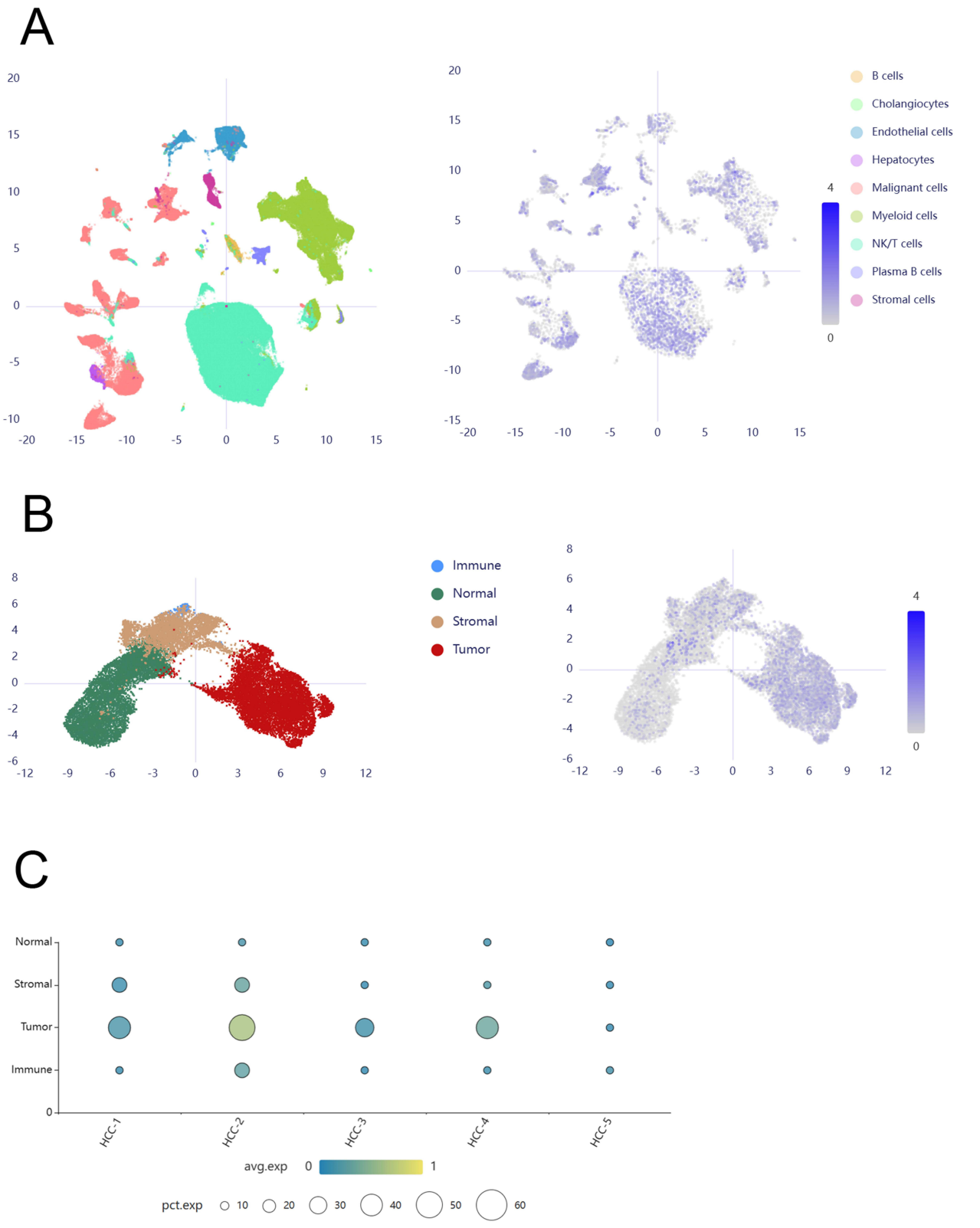

Figure 6 Single cell analysis and spatial transcriptomic analysis of MCM4. (**A**) Single cell analysis of MCM4 in HCC using HCCDB tool. (**B**) spatial transcriptomic analysis of MCM4 in HCC using HCCDB tool. (**C**) MCM4 expression levels in different tissue types based on the spatial transcriptomic analysis using HCCDB tool.

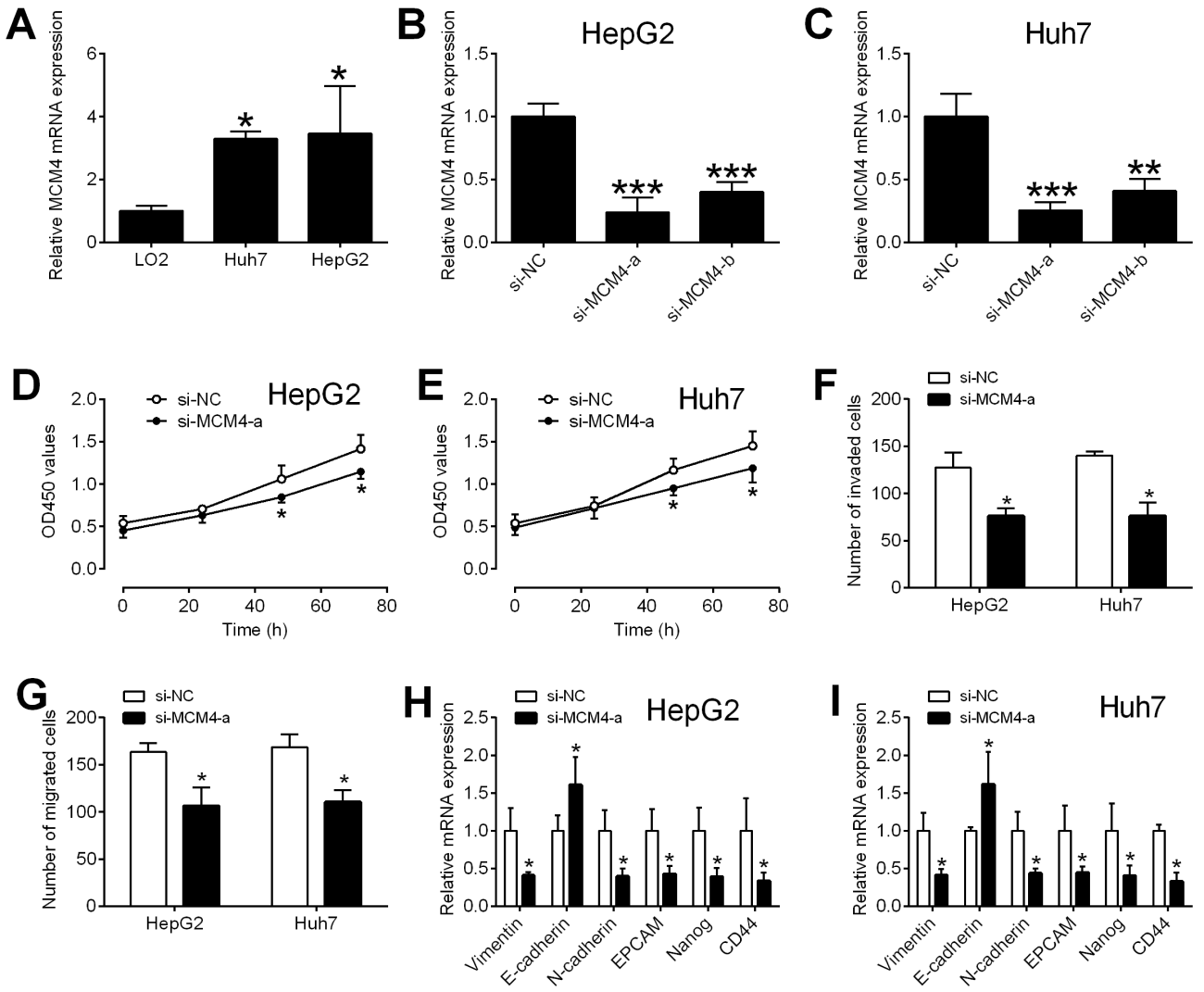

Figure 7 Effects of MCM4 silence on HCC cell progression. (**A**) Expression of MCM4 in normal liver cell line (NC), Huh7 and HepG2 cell lines. (**B**) MCM4 mRNA expression in HepG2 cells after respective siRNA transfection. (**C**) MCM4 mRNA expression in Huh7 cells after respective siRNA transfection. Cell proliferation of (**D**) HepG2 cells and (**E**) Huh7 cells after respective siRNA transfection as determined by CCK-8 assay. (**F**) Cell invasion of HCC cells after respective siRNA transfection. (**G**) Cell migration of HCC cells after respective siRNA transfection. (**H**) mRNA expression of EMT- and stemness-related markers in HepG2 cells after respective siRNA transfection. (**I**) mRNA expression of EMT- and stemness-related markers in Huh7 cells after respective siRNA transfection. N = 3. *P<0.05, **P<0.01 and ***P<0.001 compared to controls.

"vimentin" "N-cadherin", "EPCAM", "Nanog", and "CD44" and increased expression of "E-cadherin" (Figure 7H), while Huh7 cells exhibited similar regulation of these markers (Figure 7I).

Effects of MCM4 Overexpression on HCC Cell Progression

To further investigate oncogenic role of MCM4 in HCC, we examined impact of MCM4 overexpression on cell progression (Figure 8). Overexpression efficiency was confirmed in HepG2 cells transfected with plasmids encoding MCM4, demonstrating a significant increase in MCM4 mRNA expression (Figure 8A). Cell proliferation assays revealed that forced expression of MCM4 significantly promoted cell proliferation in both HepG2 (Figure 8B) and Huh7 cells (Figure 8C), as indicated by CCK-8 assay results. Additionally, transwell assays demonstrated enhanced invasive capability (Figure 8D) and significantly elevated cell migration (Figure 8E) upon MCM4 overexpression compared to control-transfected cells.

Further analysis of EMT and stemness-related markers revealed that MCM4 overexpression significantly elevated the mRNA levels of "vimentin" "N-cadherin", "EPCAM", "Nanog", and "CD44" and down-regulated expression of "E-cadherin" in HepG2 cells (Figure 8F). Similarly, these markers exhibited consistent regulation in Huh7 cells following MCM4 plasmid transfection (Figure 8G).

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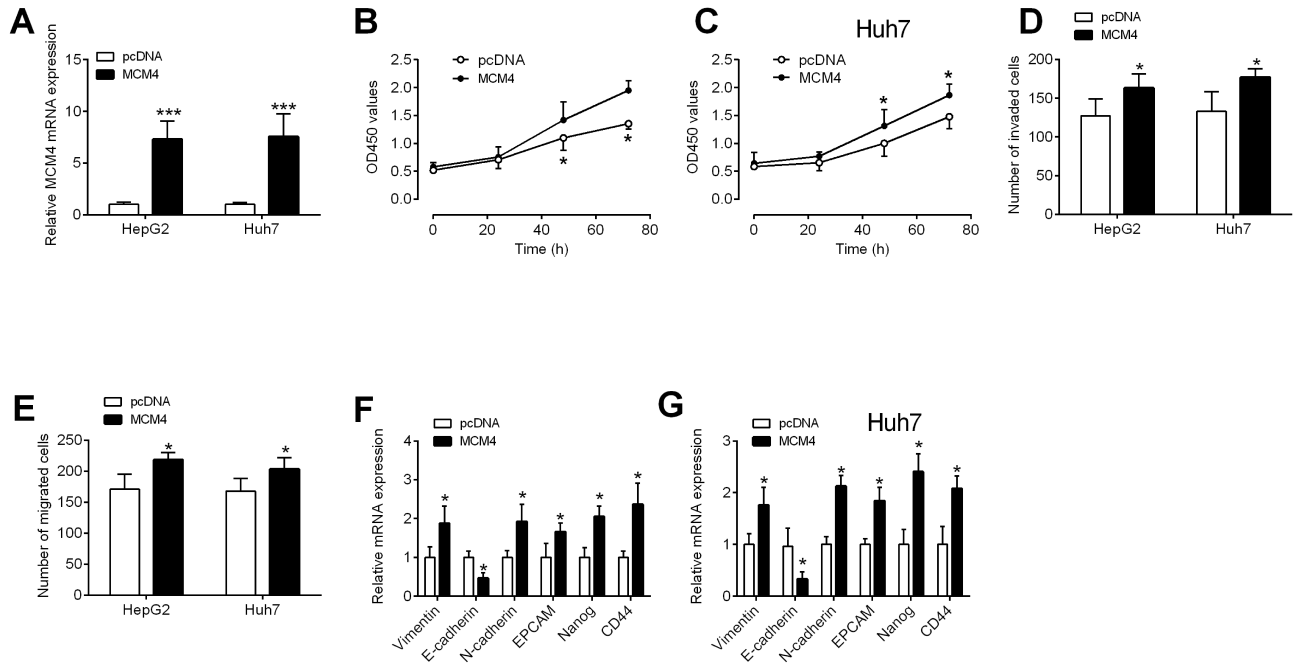

Figure 8 Effects of MCM4 overexpression on HCC cell progression. (**A**) MCM4 mRNA expression in HepG2 cells after respective plasmids transfection. Cell proliferation of (**B**) HepG2 cells and (**C**) Huh7 cells after respective plasmids transfection as determined by CCK-8 assay. (**D**) Cell invasion of HCC cells after respective plasmids transfection. (**E**) Cell migration of HCC cells after respective plasmids transfection. (**F**) mRNA expression of EMT- and stemness-related markers in HepG2 cells after respective plasmids transfection. (**G**) mRNA expression of EMT- and stemness-related markers in Huh7 cells after respective plasmids transfection. N = 3. *P<0.05and ***P<0.001 compared to controls.

Discussion

In this study, we systematically identified and characterized DEGs across three independent HCC datasets, revealing a set of consistently altered genes with potential diagnostic and therapeutic relevance. Our integrative approach is consistent with prior studies emphasizing the value of multi-dataset analyses to enhance statistical power and reduce false-positive rates. Functional enrichment analysis revealed that the common DEGs were significantly involved in DNA replication, cell cycle progression, and chromatin regulation, pathways frequently reported as hallmarks of HCC and other malignancies.[15,16]

Recent studies have underscored the critical roles of MCM family members in cancer development and therapeutic response. Integrative bioinformatics analyses have revealed that several MCM-related hub genes, including MCM4, CDK1, NUSAP1, and DLGAP5, contribute to hepatocarcinogenesis and may serve as potential drug targets in HCC.[17] Moreover, genes such as CDT1 and CCND1, which participate in DNA replication and cell cycle regulation alongside MCM proteins, have been implicated in the progression of liver diseases from NAFLD to HCC and in promoting cellular proliferation and metastasis.[18,19] In parallel, molecular studies focusing on metabolic regulators, such as ASS1, have linked arginine metabolism and EMT-associated pathways to HCC progression.[20] Together, these findings support the notion that aberrant activation of DNA replication licensing factors and associated regulatory pathways plays a central role in tumor proliferation, invasion, and metabolic adaptation.

Among the identified DEGs, MCM4 was selected for further analysis due to its consistent upregulation in HCC datasets. Our findings align with previous reports showing that MCM family proteins are overexpressed in liver and other solid tumors and are linked to aggressive tumor behavior.[21,22] In gastric cancer, MCM4 expression correlates with cancer stemness, tumor aggressiveness, and unfavorable prognosis, suggesting that its oncogenic activity may partly arise from the enhancement of self-renewal and invasive capabilities in malignant cells.[23] Similarly, in urothelial carcinoma, elevated MCM4 levels are linked to high-grade histology, advanced tumor stage, and poor clinical outcomes, reinforcing its association with aggressive tumor phenotypes.[24] In uterine corpus endometrial carcinoma, MCM4 overexpression has been identified as an independent prognostic indicator, where it is implicated in cell cycle dysregulation and DNA replication stress, further highlighting its role in uncontrolled cellular proliferation.[25] Moreover, in glioma, MCM4

promotes tumor cell growth and correlates with reduced patient survival, emphasizing its broad oncogenic potential across diverse tumor types.[26] Collectively, these findings across multiple malignancies support the concept that MCM4 functions as a universal driver of tumor progression and poor prognosis, thereby warranting detailed investigation of its biological and clinical significance in hepatocellular carcinoma. In our study, MCM4 expression was significantly elevated in both transcriptomic and proteomic datasets and strongly associated with reduced OS, DFS, RFS, and DSS in HCC patients. These results extend the findings of previous pan-cancer studies by demonstrating that MCM4 is also an independent prognostic marker in HCC.

Recent advances in single-cell and spatial transcriptomics have highlighted the importance of tumor heterogeneity in driving disease progression and treatment resistance. Using the HCCDB platform, we showed that MCM4 exhibits heterogeneous expression across cellular subtypes and is spatially enriched in tumor regions. This observation is consistent with emerging literature suggesting that genes involved in cell cycle regulation and DNA replication are often confined to proliferative zones within tumors.[27,28] The cell type-specific expression of MCM4 may reflect its role in maintaining replicative potential in proliferative cancer cells, while its spatial distribution suggests it could contribute to regional differences in treatment response and tumor evolution. These findings provide an additional layer of evidence supporting the oncogenic potential of MCM4 in the tumor microenvironment.

To validate the functional relevance of MCM4 in HCC, we conducted in vitro experiments. Silencing of MCM4 in HepG2 and Huh7 cells resulted in suppressed proliferation, migration, and invasion, as well as reduced expression of EMT and stemness markers, corroborating prior studies that linked MCM4 to cancer cell plasticity and metastatic potential.[29,30] MCM4 upregulation in colorectal cancer was shown to drive tumor progression by modulating the Skp2–p27 signaling axis, leading to uncontrolled cell cycle progression and enhanced proliferation.[31] Similarly, elevated MCM4 expression has been reported in esophageal and breast cancers, where it correlates with advanced pathological grade, lymph node metastasis, and reduced patient survival, underscoring its prognostic significance across epithelial malignancies.[32,33] In pancreatic cancer, MCM4 acts downstream of EZH2 and E2F1, functioning as a key effector in transcriptional networks that sustain DNA replication and tumor growth.[34] Multi-omics pan-cancer analyses have further confirmed MCM4 as a central node in oncogenic pathways associated with DNA replication stress, chromosomal instability, and poor survival outcomes.[30] Moreover, transcriptional activation of MCM4 by E2F2 in ovarian cancer and its modulation by therapeutic agents such as celastrol highlight its broad regulatory and druggable potential across cancers.[35,36] Collectively, our findings underscore essential functions of MCM4 in HCC progression and highlight its promise as a candidate target for therapeutic intervention.

MCM4 plays a pivotal role in DNA replication origin licensing and replication fork progression.[37] Dysregulated MCM4 expression can induce replication stress and activate DNA damage response pathways, including ATR/Chk1 signaling, which has been shown to promote transcriptional programs associated with EMT and stemness.[38] Persistent replication stress and DDR activation may further enhance the activity of E2F1, NF-κB, and β-catenin pathways, which are key transcriptional regulators that drive the expression of EMT markers such as Vimentin, N-cadherin, and ZEB1.[39] Additionally, aberrant MCM4 expression may disrupt cell cycle checkpoints, facilitating uncontrolled proliferation and enabling chromatin remodeling that favors EMT-associated gene expression.[21,25] This mechanistic link highlights that MCM4 is not merely a replication factor but also a potential upstream modulator of transcriptional networks that drive tumor plasticity and metastasis.

Despite the strengths of our work, several limitations should be addressed. First, although our bioinformatics analyses integrated data from multiple independent datasets, inherent differences in sample processing, sequencing platforms, and patient demographics may contribute to residual heterogeneity. Second, our findings are primarily based on retrospective and publicly available data, which may introduce selection bias and limit generalizability. Third, although we validated the functional role of MCM4 in vitro, additional in vivo experiments, such as xenograft tumor models and animal-based mechanistic studies, are warranted to further elucidate its regulatory pathways, biological significance, and therapeutic potential. These follow-up investigations are planned to confirm and extend the current findings in a more physiologically relevant context. Fourth, while GO enrichment analysis was conducted using the single database, validation with additional databases would further enhance the robustness of the functional annotations. Fifth, the functional validation relied mainly on mRNA expression levels of EMT and stemness markers without protein-level confirmation by Western

blotting; therefore, these results should be interpreted with caution. Finally, although single-cell and spatial transcriptomic data provided insights into the localization of MCM4 expression, further high-resolution analyses using clinical specimens are needed to validate these spatial patterns and explore their implications for targeted therapy.

Conclusions

Collectively, our study provides evidence that MCM4 is a consistently upregulated gene in hepatocellular carcinoma and serves as a robust prognostic marker associated with poor clinical outcomes. Through integrative bioinformatics, spatial profiling, and functional validation, we demonstrate that MCM4 plays a pivotal role in promoting HCC progression by enhancing proliferative, invasive, and stemness-related phenotypes. These findings position MCM4 as potential candidates for targeted therapeutic development and highlight the utility of multi-dimensional analyses in advancing precision oncology for HCC.

Data Sharing Statement

Data are available upon reasonable request from the corresponding author.

Ethics Approval and Consent to Participate

In accordance with Item 1 and Item 2 of Article 32 of the Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects (China, February 18, 2023), this study is exempt from ethical approval.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests.

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