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ORIGINAL RESEARCH

Silencing NEDD4L Effectively Inhibits the Malignant Behaviors of Hepatocellular Carcinoma

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Background: NEDD4L, an E3 ubiquitin ligase, has served a pivotal function in the malignant progression of different cancers. However, research focusing on its involvement in hepatocellular carcinoma (HCC) remains relatively scarce.

Methods: This investigation examined NEDD4L's expression, survival implications, and regulatory mechanisms of NEDD4L in HCC using RNA-seq and microarray data across multiple databases. Additionally, we investigated the impact of NEDD4L expression on malignant biological behaviors of HCC by in vitro functional assays including Edu, CCK-8, Transwell, and wound healing assay. Finally, single-cell sequencing data from HCC patients were employed to further investigate and validate NEDD4L expression patterns across various stages of HCC and its immune functional states.

Results: NEDD4L was identified as one of the most markedly differentially expressed genes linked to ubiquitination in HCC and was noted to be an independent prognostic marker for overall survival, with higher expression levels correlating with poorer outcomes. Knockdown of NEDD4L significantly inhibited cell proliferation, migration, and scratch healing ability in HCC. Moreover, NEDD4L expression was closely linked to the regulation of the cell cycle and DNA damage repair, potentially driving abnormal cell cycle progression and HCC development by mediating the degradation of inhibitory cell cycle checkpoints or their upstream transcription factors via ubiquitination. Single-cell sequencing analysis revealed that NEDD4L was notably enriched in cancer stem cell populations across different HCC developmental stages and immune states, with subgroups exhibiting high NEDD4L expression sharing substantial co-expressed genes with stem cell subpopulations. Furthermore, an analysis of NEDD4L's relationships with immune infiltration indicated that NEDD4L could facilitate immune evasion in HCC by downregulating stimulatory immune checkpoints and immune cell infiltration, a phenomenon also observed in other types of tumors.

Conclusion: These findings indicate that NEDD4L is upregulated in HCC and strongly associated with unfavorable patient outcomes, pointing to its prospective utility as a biomarker for HCC. Furthermore, silencing NEDD4L could effectively inhibit the malignant behaviors of HCC. Given its dual role in regulating both cell cycle and immune checkpoints, NEDD4L represents a promising therapeutic target for HCC. Targeting NEDD4L could potentially enhance the efficacy of cyclin-dependent kinase inhibitors and immune checkpoint inhibitors.

Keywords: hepatocellular carcinoma, immune suppression, NEDD4L, therapeutic target

Introduction

Primary liver cancer ranks as the sixth most frequently diagnosed malignancy and represents the third principal factor in cancer-associated deaths globally, with roughly 906,000 newly diagnosed instances and 830,000 fatalities documented in

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2020. Furthermore, it is anticipated that the number of new cases will surpass 1 million by 2025.[1,2] Hepatocellular carcinoma (HCC), the most common subtype of liver cancer, represents approximately 85–90% of all liver cancer cases. Currently, the main therapeutic approaches for HCC include surgical resection, radiotherapy, chemotherapy, and targeted therapies. However, these treatments yield suboptimal outcomes. According to data from the SEER database of the National Cancer Institute, the five-year survival rate for HCC patients in the United States is a mere 19.6%, with an even more dismal 2.5% survival rate for those with advanced metastatic disease.[3] Consequently, the identification of alternative molecular targets and innovative treatment approaches remains critically important.

The pathogenesis, progression, and prognosis of HCC are intrinsically associated with genetic alterations and protein post-translational modifications (PTMs), among which, ubiquitination represents a pivotal regulatory mechanism.[4–6] Ubiquitination constitutes a multi-enzymatic cascade mediated sequentially by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). Emerging evidence highlights the critical regulatory roles of E3 ubiquitin ligases - the central components of the ubiquitin-proteasome system (UPS) - in HCC tumorigenesis, progression, and therapeutic responses. These enzymes orchestrate tumor proliferation,[7,8] metastasis,[9] immune evasion,[10] stemness maintenance,[11] metabolic reprogramming,[12] and chemoresistance through substrate-specific ubiquitination of key regulatory proteins.[13] Notable examples include Cullin family ligases (Cullin4A, Cullin7, etc) that promote HCC proliferation via degradation of cell cycle regulators (p53, Cyclin D1).[14,15] Mechanistic studies reveal that E3 ligase DTX2 facilitates CXCL2/CXCL6-mediated recruitment of tumor-associated neutrophils (TANs), establishing an immunosuppressive microenvironment through inhibitory immune cell infiltration and $CD8^+$ T cell suppression, thereby attenuating PD-1 antibody efficacy.[16] Pharmacological inhibition of DTX2 significantly reduces TAN infiltration and enhances immunotherapy response.[17] Additionally, the SCF complex member FBXO28 demonstrates tumor-suppressive effects by ubiquitinating EMT master regulator SNAI2 for proteasomal degradation, thereby inhibiting HCC migration and invasion.[18] The therapeutic potential of ubiquitination modulation is being progressively unlocked. Emerging strategies include: 1) Small-molecule inhibitors targeting oncogenic E3s (eg, mDTX2i showing synergistic effects with PD-1 blockade in murine models);[16] 2) Activators restoring tumor-suppressive E3 functions (eg, FBXO28 activation suppressing metastasis);[18] 3) Poly-pharmacological approaches combining AKT inhibitor capivasertib with USP14 inhibitor IU1-248 to enhance TUBA1A degradation;[19] and 4) Proteolysis-Targeting Chimera (PROTAC) technology leveraging E3 ligases for targeted oncoprotein degradation.[20] Notably, E3 ligase functionality exhibits marked tissue specificity and substrate dependency. For instance, FBXO28 demonstrates context-dependent oncogenic or tumor-suppressive roles across malignancies. FBXO28 demonstrates tumor-suppressive activity in HCC by antagonizing metastatic progression through PKA-dependent proteasomal degradation of SNAI2.[18] Paradoxically, this E3 ubiquitin ligase exhibits oncogenic functions in pancreatic ductal adenocarcinoma (PDAC), where it mediates SMARCC2 ubiquitination to potentiate tumor proliferation, invasion, and metastatic dissemination.[21] Similarly, in ovarian carcinogenesis, FBXO28 orchestrates malignant progression by activating the TGF-β1/SMAD2/3 signaling axis.[22] Therefore, comprehensive clinical validation and multi-omics integration of the ubiquitination process is imperative to elucidate the complex regulatory networks, which will accelerate the discovery of novel therapeutic targets and facilitate the development of precision medicine paradigms in HCC management.

Neural precursor cell expressed developmentally downregulated gene 4-like (NEDD4L) belongs to the NEDD4 family of E3 HECT domain ubiquitin ligases. By mediating the transfer of ubiquitin to substrates, NEDD4L determines substrate specificity, thereby regulating the stability, localization, and function of downstream proteins. Recent investigations[23–27] have suggested that NEDD4L dysfunction notably impacts the onset and development of various malignancies, encompassing lung, breast, gastric, and colorectal cancers, influencing processes such as cell proliferation, apoptosis, cell cycle regulation, migration, invasion, epithelial-mesenchymal transition, and tumor resistance to chemotherapy. Despite these findings, NEDD4L targets distinct substrates and performs varying functions across different cancer types, resulting in diverse tumor characteristics.[28] Furthermore, experimental investigations have shown that NEDD4L is implicated in modulating immune functions.[29,30] Currently, the literature on NEDD4L in HCC remains limited, with discrepancies among various studies. Given its potential involvement in cell cycle regulation and immune modulation, the present study comprehensively examined NEDD4L expression patterns, prognostic significance, and downstream regulatory mechanisms in HCC through publicly available databases, such as The Cancer Genome Atlas

(TCGA), International Cancer Genome Consortium (ICGC), and Gene Expression Omnibus (GEO). This investigation strives to uncover NEDD4L's influence on HCC development and progression, providing a molecular foundation for HCC detection and clinical management.

Materials and Methods

Data Collection and Screening

All sample data and corresponding clinical details in this study were retrieved from publicly available databases, encompassing TCGA (https://portal.gdc.cancer.gov/), ICGC (https://dcc.icgc.org/), and GEO (https://www.ncbi.nlm.nih.gov/geo/). Consequently, no ethical concerns or conflicts of interest were identified. RNA-seq data were initially downloaded from the respective databases and subsequently mapped to official gene symbols. Data rows and columns with over 50% missing values were excluded from the dataset. The remaining missing data were imputed utilizing the impute package in R software (with K = 10 neighbors). The dataset was then normalized using log2(X+1) transformation prior to further analysis. Moreover, patient clinical data were procured from the TCGA and ICGC databases. Samples with missing values for NEDD4L expression, overall survival (OS), and survival outcomes were excluded from the analysis. Detailed information on the datasets following the application of exclusion criteria is depicted in Table 1.

Differential Analysis

Limma (Linear Models for Microarray Data) serves as a method for differential expression analysis grounded in generalized linear models. The ICGC gene expression profile data were subjected to multiple linear regression utilizing the lmFit function, followed by the eBayes function to compute moderated t-statistics, moderated F-statistics, and log-odds for differential expression. This procedure involved empirical Bayes moderation of standard errors towards a common value, ultimately yielding the differential significance of each gene. Differentially expressed genes (DEGs) were ascertained per the criteria of fold change ≥ 2 and false discovery rate ≤ 0.01.

Analysis of Independent Samples and Paired Samples

R software (version 4.3.2) was employed to conduct parametric tests for analyzing the differential expression of NEDD4L between HCC and adjacent normal tissues in the TCGA and GEO databases (GSE36376, GSE25097). Patient data were subsequently matched for paired sample analysis. Paired *t*-tests were utilized for the differential analysis, with outliers identified based on 1.5 times the interquartile range of differences. The Shapiro–Wilk normality test was applied to evaluate the distribution of the data.

Table 1 The Basic Information of the Datasets

Database Sources	Dataset Name	Platform	Sequencing Data	Sample Size (HCC/ Control)	Type of Clinical Information
TCGA	Liver Hepatocellular Carcinoma (LIHC)	–	RNA-seq	373 (370)*/50	1
ICGC	Hepatocellular carcinoma (LIRI-JP)	–	RNA-seq	243/202	1
GEO	GSE36376	GPL10558	Microarray	240/193	0
	GSE25097	GPL10687	Microarray	268/243	0
	GSE14520	GPL3921	Microarray	214/214	0
	GSE146115	C1	scRNA-seq	–	0
	GSE125449	10x Genomics	scRNA-seq	–	0

Notes: *After screening, there were 373 samples in the TCGA database with NEDD4L expression values that could be used for analyses in outcomes 3.1 and 3.2 below, but only 370 of these samples had detailed clinical information, and were therefore only used for analyses of independent prognostic factors in 3.2. 0 indicates no clinical information; 1 indicates detailed clinical information with OS and survival outcomes.

Table 2 The Sequences of the Primers

Gene Name	Sequences	
NEDD4L	Forward	GACATGGAGCATGGATGGGAA
	Reverse	GTTCGGCCTAAATTGTCCACT
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

RT-qPCR was executed to measure NEDD4L mRNA expression levels in clinical HCC tissue samples. Total RNA was extracted from the tissue samples utilizing TRIzol reagent (Invitrogen). Complementary DNA was generated utilizing the PrimeScript RT Reagent Kit (TaKaRa, Japan). RT-qPCR was then performed with ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd, Nanjing, China) and specific primers on an ABI 7500 HT system. The mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method. GAPDH was employed as a normalization control. The primer sequences used in RT-qPCR are provided in Table 2.

Cell Lines and Cell Culture

The HCC cells and immortalized human liver THLE-2 cells utilized in this study represent widely used human cell models. Specifically, the HCC cell lines Huh7, MHCC97H, Hep3B, and HepG2 were sourced from Haixing Company. THLE-2 cells were obtained from Wuhan Procell Life Science & Technology Co. The HCC cell lines were cultured in DMEM, MEM, or RPMI-1640 medium (Gibco, Carlsbad, USA), supplemented with 10% fetal bovine serum (FBS) (ExCell Bio) and 1% penicillin-streptomycin (HyClone, Logan, USA) at 37°C in a 5% CO_2 atmosphere. THLE-2 cells were cultured in a Complete THLE-2 medium containing BEGM and 10% FBS.

Western Blot Analysis

Total protein was procured from cells utilizing radioimmunoprecipitation assay lysis buffer (Beyotime) comprising protease inhibitor (HY-K0010; MCE), phosphatase inhibitor (HY-K0021; MCE), and phenylmethylsulfonyl fluoride (Beyotime, Shanghai, China). The protein content was determined utilizing the Pierce bicinchoninic acid Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. The proteins underwent separation through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred onto polyvinylidene difluoride membranes (Merck, China) by electrophoresis. The membranes were blocked with 5% bovine serum albumin (BSA) (Servicebio, China) to prevent non-specific binding, succeeded by washing steps. The membranes underwent overnight incubation with primary antibodies at 4°C. Following washing, the membranes were treated with horseradish peroxidase-conjugated goat anti-rabbit (1:10,000, Abcam, ab6789) or goat anti-mouse (1:10,000, Abcam, ab6721) immunoglobulin G antibodies for 1.5 hours. Immunoreactive bands were visualized using enhanced chemiluminescence. Primary antibodies, anti-NEDD4L (Proteintech, 13690-1-AP) and anti-Beta Actin (Proteintech, 66009-1-Ig), were obtained from Proteintech Technology (Proteintech, China). Western blot analysis was conducted independently in triplicate.

Small Interfering RNA (siRNA) Transfection

NEDD4L-targeting siRNA was purchased from Obio Technology (Shanghai, China). Cells were trypsinized, centrifuged, and uniformly seeded into 6-well cell culture plates. Transfection was performed using Lipofectamine™ 3000 (Thermo Fisher Scientific, #L3000008), when cell confluence reached 60–70%, following the manufacturer's protocol with optimized siRNA-to-reagent ratios. siRNA sequences are provided in Table S1.

5-Ethynyl-2′-Deoxyuridine (EdU) Incorporation Assay

Different groups of cells were inoculated in 96-well plates (5×10^3 cells/well) and cultured for 24 h. Cells were incubated with 10 μM EdU riboside for 2 h at 37°C protected from light, fixed with 4% paraformaldehyde, permeabilised with

0.5% Triton X-100, and stained according to the manufacturer's protocols using the VaClick 488-EdU Cell Proliferation Test Kit (Vazyme, #A411-01) for staining. Hoechst 33342 (1 μg/mL) was used. Fluorescence images were captured using an inverted fluorescence microscope and quantitatively analysed using ImageJ software. Experiments were repeated independently 3 times.

CCK-8 Cell Proliferation Assay

Different groups of cells were trypsin digested, centrifuged, resuspended, counted and then inoculated in 96-well cell culture plates at a density of 5000 cells per well. At 0, 24, 48, 72 and 96 h after inoculation, 10 μL of CCK-8 (Biosharp, #BS350A) solution was added to each well. After incubation for 2 h at 37°C protected from light, the absorbance at 450 nm was measured using a microplate reader and three technical replicates were averaged for each time point. Cell proliferation kinetic curves were plotted using 0 h optical density as a standard. Experiments were repeated independently 3 times.

Cell Migration Assay

Cell migration ability was assessed using Transwell® chambers (Corning, #3422). Cells from different subgroups were trypsin-digested, centrifuged and resuspended in 200 μL of serum-free medium and inoculated in the upper chamber. The lower chamber was inoculated with 700 μL of complete medium containing 10% FBS. After 48 h of incubation, cells migrating to the submembrane surface were fixed with 4% paraformaldehyde (PFA), stained with 0.1% crystal violet and imaged under an inverted microscope. Uninvaded cells in the upper chamber were removed by wiping with a cotton swab before staining. Experiments were repeated independently 3 times.

Wound Healing Assay

Would healing assay was performed by scratch closure analysis. Different subgroups of cells were trypsin-digested, centrifuged, resuspended, counted and inoculated into 6-well cell culture plates. When the tumor cells reached 95% ~ 100% fusion, mechanical injury was performed using a sterile 200 μL pipette tip. The original medium was replaced with fresh medium containing 2% FBS. The trauma was labelled and imaged under a phase contrast microscope at 0, 24 and 48 h post-injury, respectively. Experiments were repeated independently 3 times.

Functional Enrichment Analysis

Pearson correlation analysis (PCA) was employed to identify genes most strongly associated with NEDD4L, which were subsequently uploaded to the Database for Annotation, Visualization, and Integrated Discovery (DAVID, v6.8). Official gene symbols were employed as identifiers, with humans selected as the species of interest. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were executed, yielding relevant results. The top six findings, ranked by *P*-value in descending order, are presented in this study.

Gene Set Variation Analysis (GSVA)

Gene sets associated with cell cycle processes were retrieved from the AmiGO 2 portal (https://amigo.geneontology.org/amigo). Using default parameters, the GSVA package in R software was utilized to compute functional enrichment scores for each HCC sample, and the pheatmap package was employed to generate a heatmap displaying the enrichment results. PCA was then conducted to assess the relationships between NEDD4L and cell cycle processes.

Single-Cell RNA Sequencing Analysis

The Seurat package in R software was employed for data quality control, normalization, dimensionality reduction, clustering, and differential expression analysis. Initially, the appropriate data dimensions were determined using the JackStraw algorithm, followed by clustering analysis conducted with the FindNeighbors and FindClusters functions. The clustering outcomes were visualized through UMAP or tSNE algorithms. Subsequently, DEGs between each cluster and all other clusters were identified using the FindAllMarkers function. The expression profiles of specific genes across

different clusters were visualized using the VlnPlot and FeaturePlot functions. Cell clusters exhibiting high expression levels of ANPEP, ICAM1, and KLF8 were classified as malignant cells and extracted for further analysis.

Cell Cluster Similarity Analysis

The similarity between cell clusters was determined based on the number of feature genes shared across different clusters. A larger count of shared feature genes suggests a higher degree of similarity between the cell clusters. The results of the similarity analysis are visualized using a Sankey Diagram.

Analysis of Immune Cell Infiltration (ICI), Immune Checkpoints, and Immune Regulatory Genes in Pan-Cancer

The ICI scores for each sample in Liver Hepatocellular Carcinoma and 38 other cancer types from the TCGA database were computed based on NEDD4L expression levels using the ESTIMATE (ESTIMATE algorithm) and IOBR (xCELL algorithm) packages in R software. Pearson's correlation coefficients between NEDD4L expression and immune infiltration scores across different cancer types were computed with the corr.test function from the psych package and the outcomes were visualized through heatmaps. Furthermore, Pearson's correlations between NEDD4L and markers of stimulatory (36) and inhibitory (24) immune checkpoint pathways, along with 80 immunomodulatory genes—including chemokines (41), receptors (18), and Major Histocompatibility Complex (MHC) (21)—were also computed to investigate the relationships between NEDD4L and immune regulation, specifically focusing on immune checkpoints. These results were similarly displayed in specific heatmap format.

Ethics Approval and Informed Consent

This investigation was sanctioned by the Ethics Committee of Liaoning Cancer Hospital & Institute. Informed written consent was acquired from all participants. Also, our study complied with the Declaration of Helsinki.

Results

NEDD4L was Identified as One of the Most Significant DEGs Associated with Protein Ubiquitination Modification in Patients with HCC

Initially, 4565 DEGs (up = 3472, down = 1093) were identified from 243 HCC samples and 202 normal samples in the ICGC database. The original data was presented in Supplementary File 1. Visual representations of these DEGs were generated using volcano plots and heatmaps (Figure 1A and B). The ubiquitin-proteasome system, a finely regulated pathway responsible for protein degradation, serves an essential function in maintaining cellular homeostasis. It has been extensively documented that this system is implicated in various cellular processes, encompassing DNA damage repair, cell cycle checkpoint regulation, immune response modulation, and the regulation of aberrant cancer-related protein expressions.[31–35] Recently, substantial evidence has been accumulated regarding the key molecules and regulatory mechanisms governing this system.[36–40] This highlights the significance of exploring key targets within protein ubiquitination pathways in HCC, which may offer deeper insights into carcinogenesis and guide therapeutic strategies. Gene sets related to ubiquitin-mediated proteolysis were subsequently retrieved from the Human Gene Database (Supplementary File 2) and the Molecular Signatures Database (Supplementary File 3). A Venn diagram was constructed using the DEGs identified from the ICGC database and these datasets, leading to the identification of ten ubiquitination-related DEGs (Figure 1C). The expression heatmap of these ten DEGs, based on the ICGC database, is shown in Figure 1D. After a comprehensive background review and literature analysis, NEDD4L was selected as the target gene for further investigation.

NEDD4L is Highly Expressed in HCC and Serves as an Independent Prognostic Factor for OS in Patients with HCC

In the TCGA database, a markedly higher expression of NEDD4L was observed in HCC tissues compared to adjacent normal tissues (P = 5.5e-30, Figure 2A). Further paired sample analyses also confirmed elevated NEDD4L expression in cancerous tissues (P = 3.0e-14, Figure 2B). This observation was consistently validated across multiple GEO datasets

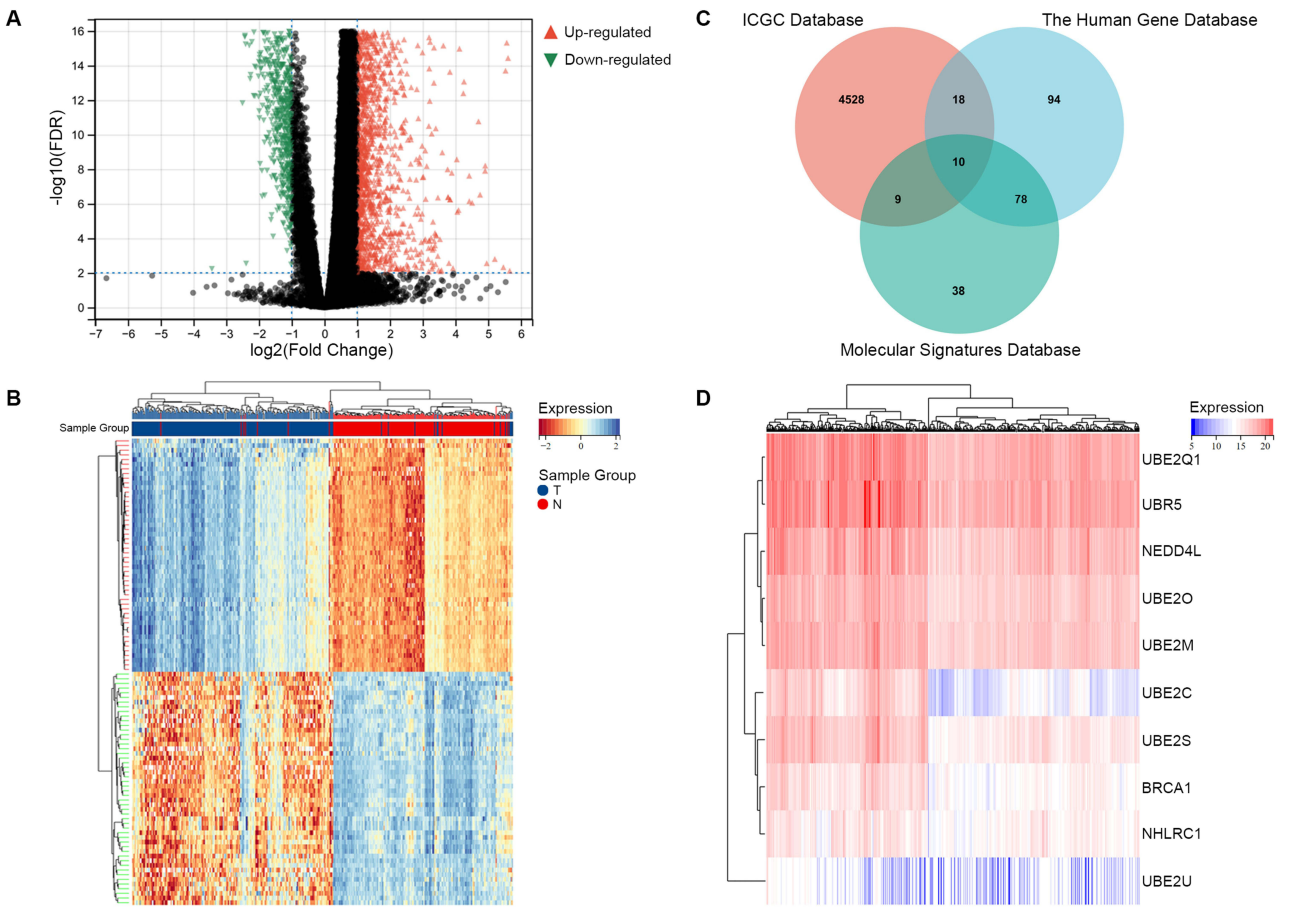

Figure 1 NEDD4L is one of the top DEGs which were most associated with ubiquitination in patients with HCC. (**A** and **B**) Volcano and heat maps of HCC differential genes based on the ICGC database. (**C**) A Wayne diagram showing the intersection of HCC DEGs with ubiquitination-related genes in The Human Gene Database (https://www.genecards.org/) and Molecular Signatures Database (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp). In the Human Gene Database, we searched for "UBIQUITATION" and selected the top 200 genes related to ubiquitination according to the Relevance Scores. Also, we searched the Molecular Signatures Database for "UBIQUITIN MEDIATED PROTEOLYSIS". (**D**) Heatmap of the expression levels of these 10 DEGs which were most relevant to ubiquitination based on the ICGC database.

(GSE36376, GSE25097, GSE14520), showing statistically significant differences (P = 2.4e-39, P = 5.2e-33, P = 1.1e-44, Figure 2C–E). The expression of NEDD4L in 10 pairs of fresh clinical specimens was also analyzed, and HCC tumor tissues were compared with corresponding adjacent normal tissues. RT-qPCR analysis revealed a marked increase in NEDD4L expression in tumor tissues (P = 1.2e-3, Figure 2F). Additionally, when compared to the immortalized human liver cell line THLE-2, NEDD4L protein expression was notably upregulated in HCC cell lines (Figure 2G). Furthermore, distinct clinical characteristics were associated with varying levels of NEDD4L expression. As NEDD4L expression increased, asymmetric distributions were observed in patient age, gender, and TNM staging (Supplementary Figure 1A and B). Subsequent subgroup analyses were performed; however, no statistically significant associations were found between NEDD4L expression and the aforementioned clinical features (Supplementary Figure 1C–H). Notably, higher NEDD4L expression was observed in advanced TNM stages compared to early stages (Supplementary Figure 1C and F), indirectly suggesting that more aggressive forms of HCC may express elevated levels of NEDD4L. To further investigate the prognostic significance of NEDD4L in patients with HCC, Kaplan-Meier survival analyses, along with univariate and multivariate Cox proportional hazards model analyses, were conducted using samples from both the TCGA and ICGC databases. In the TCGA dataset, patients exhibiting high NEDD4L expression (median survival: 780 days) demonstrated markedly shorter OS when compared to those with low NEDD4L expression (median survival: 2102 days) (Figure 2H). This trend was also evident in the ICGC dataset (Figure 2I), further validating the prognostic value of NEDD4L. Additionally, in both TCGA and ICGC databases, NEDD4L was identified as an independent prognostic predictor for OS in HCC patients, as summarized in Tables 3 and 4.

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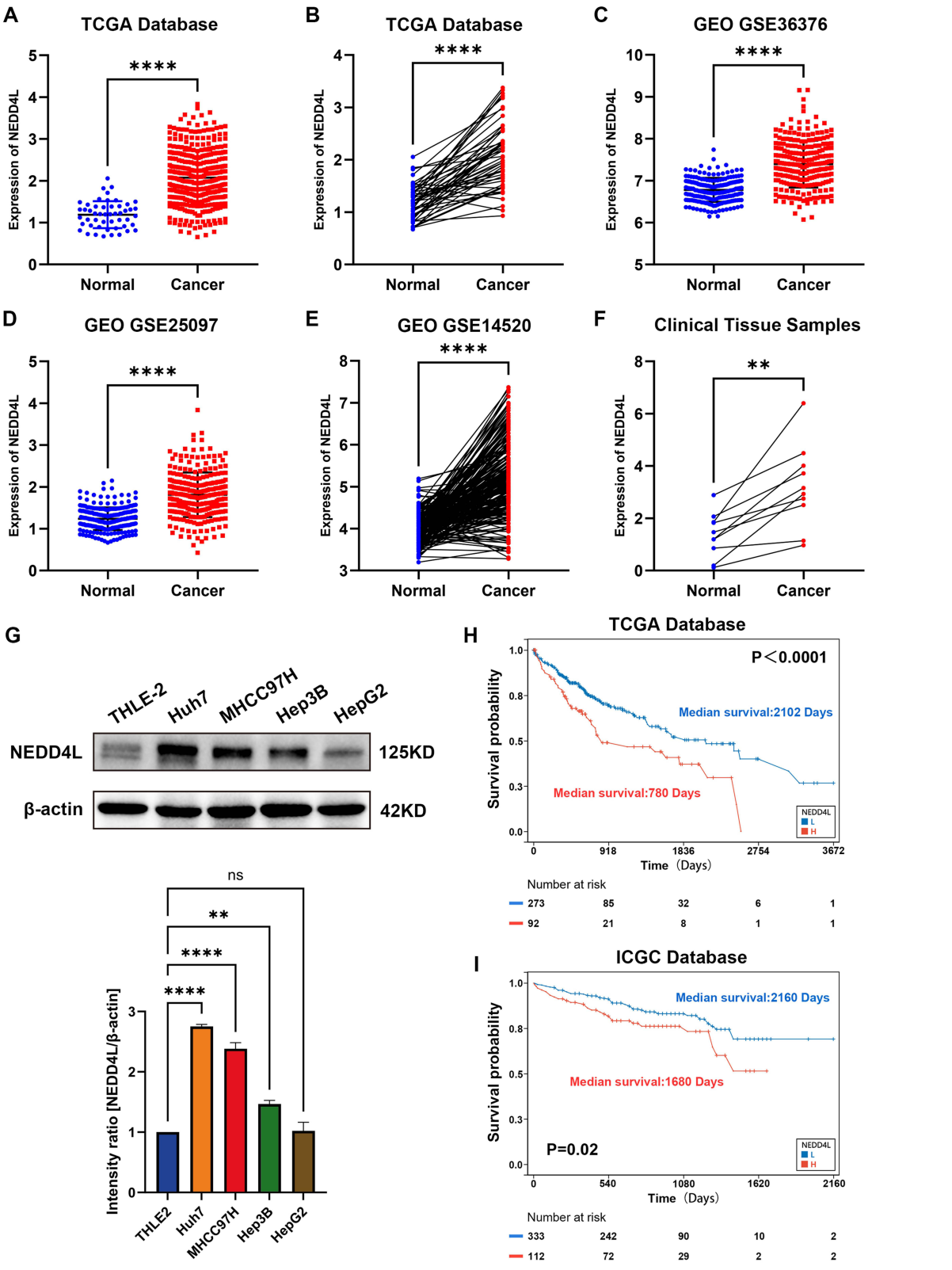

Figure 2 NEDD4L expression was detected in normal tissues and HCC tissues. (**A**) Differential expression of NEDD4L in independent 50 normal tissues and 373 HCC tissues from the TCGA database. (**B**) Differential expression of NEDD4L in normal tissues adjacent to HCC and paired 50 HCC tissues from the TCGA database. (**C**) Differential expression of NEDD4L in independent 193 normal tissues and 240 HCC tissues from the GEO database (GSE36376). (**D**) Differential expression of NEDD4L in independent 243 normal tissues and 268 HCC tissues from the GEO database (GSE25097). (**E**) Differential expression of NEDD4L in normal tissues adjacent to HCC and paired 214 HCC tissues from the GEO database (GSE14520). (**F**) The validation of NEDD4L mRNA expression in 10 pairs clinical tissue samples from patients with HCC. (**G**) Expression of NEDD4L in the human hepatic immortalized cell line THLE-2 and human HCC cell models. (**H** and **I**) Kaplan-Meier survival analysis of NEDD4L expression in the patients with HCC based on the data from the TCGA and ICGC databases. The significance of prognostic value was assessed using Log rank test method. (**P <0.01, ****P <0.0001).

Table 3 Univariate and Multivariate Cox Regression Analysis of Prognostic Parameters in OS Based on the TCGA Database

Variable	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
NEDD4L expression	1.742 (1.208–2.511)	0.003	1.628 (1.099–2.412)	0.015
Age	1.013 (0.999–1.027)	0.073	/	/
Sex	0.815 (0.572–1.161)	0.257	/	/
TNM state		0.000		0.000
State (II→I)	1.423 (0.872–2.323)	0.158	1.393 (0.853–2.274)	0.186
State (III→I)	2.675 (1.754–4.081)	0.000	2.534 (1.657–3.877)	0.000
State (IV→I)	5.497 (1.695–17.823)	0.005	6.182 (1.897–20.148)	0.003

Table 4 Univariate and Multivariate Cox Regression Analysis of Prognostic Parameters in OS Based on the ICGC Database

Variable	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
NEDD4L expression	1.590 (1.010–2.503)	0.045	1.753 (1.106–2.778)	0.017
Age	1.005 (0.983–1.027)	0.673	/	/
Sex	0.537 (0.344–0.839)	0.006	0.411 (0.258–0.654)	0.000
TNM state		0.000		0.000
State (II→I)	5.223 (1.255–21.747)	0.023	5.719 (1.373–23.830)	0.017
State (III→I)	7.246 (1.729–30.359)	0.007	9.288 (2.198–39.251)	0.002
State (IV→I)	21.214 (4.916–91.537)	0.000	28.874 (6.621–125.915)	0.000

Silencing NEDD4Linhibits the Malignant Biological Behaviors of HCC Cells

In the aforementioned results, we observed that NEDD4L expression was significantly elevated in HCC Huh7 cell lines compared to other cancer cell lines (Figure 2G). Consequently, Huh7 cells were selected for siRNA transfection to further investigate the potential biological functions of NEDD4L in HCC. Through RT-qPCR and WB analyses (Figure 3A and B), we selected si#NEDD4L-C as the most effective sequence for subsequent investigations. For simplicity in subsequent experiments, si#NEDD4L-C was designated as si#NEDD4L. To examine whether NEDD4L could regulate HCC cell proliferation, we initially assessed the impact of NEDD4L silencing on Huh7 proliferative capacity using EdU incorporation and CCK-8 assays. The results demonstrated that suppression of NEDD4L expression significantly attenuated both proliferative activity and cell viability in Huh7 cells (Figure 3C and D). Furthermore, given previous reports that NEDD4L promotes migratory capacities in various malignancies including prostate cancer,[41] invasive gallbladder carcinoma,[42] and melanoma,[43] we further validated its role in HCC cell migration. Both wound healing and Transwell assays revealed that si#NEDD4L-transfected cells exhibited significantly impaired migratory ability compared to normal control groups, with statistically significant differences (Figure 3E and F). Collectively, these findings demonstrate that silencing NEDD4L inhibits malignant biological behaviors of HCC, including proliferation and migration.

NEDD4L Regulates HCC Progression Through Modulating Cell Cycle and DNA Damage Repair

To investigate the biological functions associated with NEDD4L, PCA was performed to identify the 500 genes most strongly correlated with NEDD4L ($P < 0.05$) in both the TCGA and ICGC databases. GO and KEGG analyses were then conducted using these gene sets. In the TCGA database, the biological processes most strongly associated with NEDD4L were found to include cell division, DNA replication, and chromosome segregation (Figure 4A).

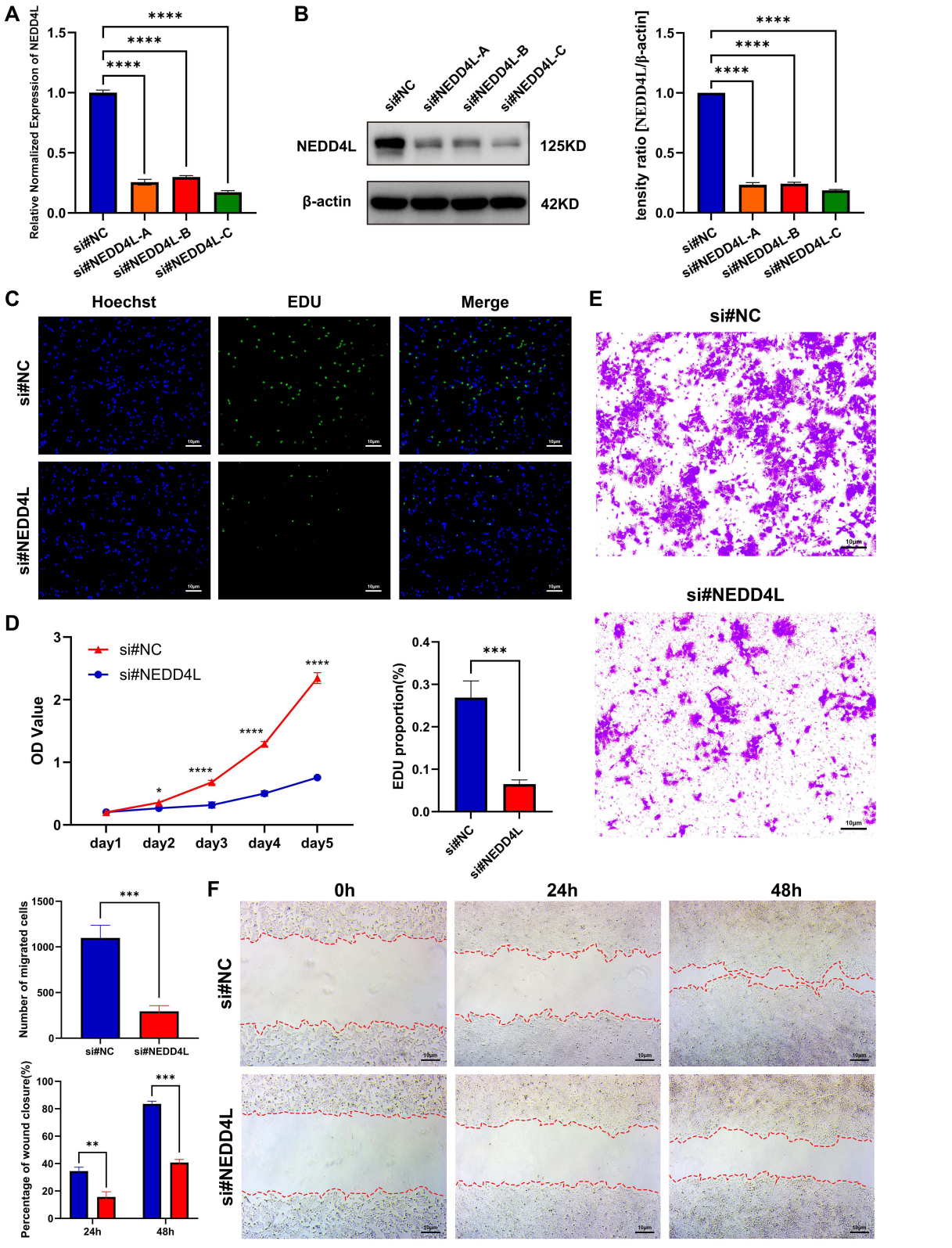

Figure 3 Silencing NEDD4L significantly inhibited the malignant biological behaviors of HCC cells. (**A** and **B**) Silencing efficiency of NEDD4L was validated in Huh7 cell lines using RT-qPCR and Western blotting analysis. (**C** and **D**) EdU incorporation assay and CCK-8 assay demonstrated that silencing NEDD4L inhibited the proliferative capacity and cellular viability of Huh7 cells. Error bars represent mean ± SD. (**E**) Transwell assay revealed that NEDD4L downregulation significantly suppressed the migrative potential of Huh7 cells. (**F**) Wound healing assay showed that NEDD4L knockdown markedly inhibited the wound healing ability of Huh7 cells. (*P <0.05, **P <0.01, ***P <0.001, ****P <0.0001, si#NEDD4L: small interfering RNA-NEDD4L).

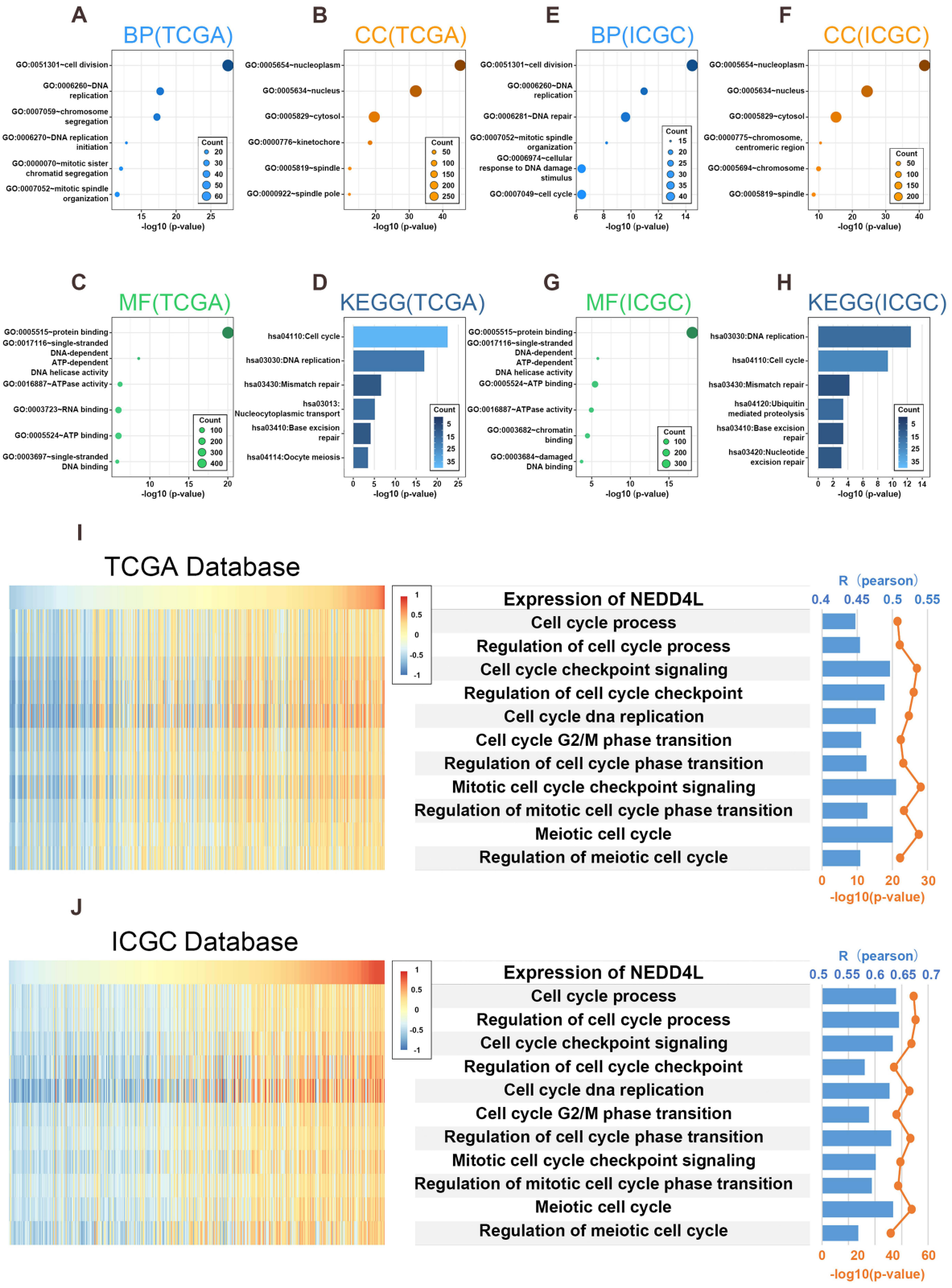

Figure 4 NEDD4L is closely associated with aberrant cell cycle regulation and DNA damage repair in HCC. (**A**–**C**) The most relevant BP, CC, and MF for NEDD4L in the TCGA database. (**D**) KEGG pathway analysis of NEDD4L in the TCGA database. (**E**–**G**) The most relevant BP, CC and MF for NEDD4L in the ICGC database. (**H**) KEGG pathway analysis of NEDD4L in the ICGC database. (**I** and **J**) The heatmaps show the enrichment scores of NEDD4L expression and cell cycle regulatory function for each HCC patient in TCGA and ICGC databases. The samples were arranged in ascending order of NEDD4L expression. The rightmost bar and line plots represent the R-value and -log10(p-value) of the correlation analysis, respectively.

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Moreover, the cellular components related to NEDD4L were primarily localized in the nucleoplasm (Figure 4B). Molecular functions identified included protein binding, single-stranded DNA-dependent ATP-dependent DNA helicase activity, and adenosine triphosphatase activity (Figure 4C). KEGG pathway analysis revealed that the signaling pathways most markedly linked to NEDD4L were those involved in the cell cycle, DNA replication, and mismatch repair (Figure 4D). In the ICGC database, the biological processes, cellular components, molecular functions, and signaling pathways associated with NEDD4L mirrored those found in the TCGA dataset (Figure 4E–H). These results suggest that NEDD4L may serve a pivotal function in regulating the cell cycle and facilitating DNA damage repair, potentially acting as a cell cycle checkpoint in the development and progression of HCC.

NEDD4L Expression in Tumor Cells Shows a Significant Positive Correlation with Cell Cycle Pathway Activation

Aberrant progression of the cell cycle is considered a fundamental mechanism driving tumor initiation and advancement, positioning cell cycle regulation as a promising target for cancer therapy.[44,45] In light of these observations, the impact of NEDD4L expression on cell cycle pathways, including checkpoint regulation, signaling pathways, and phase transitions, was investigated. GSVA was utilized to assess the enrichment scores for cell cycle processes within both the TCGA and ICGC databases. A correlation analysis between the enrichment scores and NEDD4L expression revealed a significant positive relationship between NEDD4L levels and the activation of cell cycle pathways, as well as the regulation of cell cycle functions (Figure 4I). These findings were subsequently corroborated in the ICGC database (Figure 4J). Taken together, these results suggest a strong association between NEDD4L and aberrant cell cycle progression and checkpoint regulation in HCC.

NEDD4L Overexpression is Markedly Associated with Cell Cycle Checkpoint Dysregulation and Enhanced Transcription Factor Activity

Given the pivotal roles of NEDD4L in the regulation of the tumor cell cycle, the associations between NEDD4L and key cell cycle checkpoint-related proteins were further explored in both TCGA and ICGC databases. These included various cyclins (Cyclin A, Cyclin B, Cyclin D, Cyclin E), CDC25A, PLK1, GADD45, Cyclin-dependent kinases (CDKs), and CDK inhibitors (p21, p57, p130). The analysis revealed that, with the exception of Cyclin D1, NEDD4L exhibited robust positive correlations with other cyclins, CDKs, CDC25A, and PLK1, while showing negative correlations with CDK inhibitors and inhibitory checkpoints such as GADD45 (Figure 5A and B). Furthermore, seven gene sets associated with transcription factor activity were selected as markers of cell cycle status. These included transcription coactivator activity, transcription coregulator activity, transcription elongation factor activity, basal transcription machinery binding, general transcription initiation factor activity, RNA polymerase II general transcription initiation factor activity, and RNA polymerase III general transcription initiation factor activity. Correlation analysis demonstrated varying degrees of positive associations between NEDD4L and these gene sets across both TCGA and ICGC databases, with stronger correlations observed in the ICGC database (Figure 5C and D). Taken together, these results suggest that NEDD4L may facilitate abnormal cell cycle progression and the development of HCC by mediating the ubiquitination and degradation of inhibitory cell cycle checkpoints or their upstream transcription factors, thus upregulating the activity of cyclins, CDKs, and transcription factors.

NEDD4L is Highly Enriched in Tumor Stem Cell Subpopulations During Different Stages of HCC Development and Various Immune Function States in Patients with HCC

Given the notably elevated expression of NEDD4L in HCC tissues and its marked correlation with cell cycle checkpoints, a more in-depth exploration of its enriched cell types was undertaken using scRNA-seq data obtained from the GEO database (GSE146115, GSE125449). Analysis of the scRNA-seq dataset from the training cohort, GSE146115, revealed the presence of 12 distinct cell clusters in HCC tissue samples (Figure 6A). Based on the

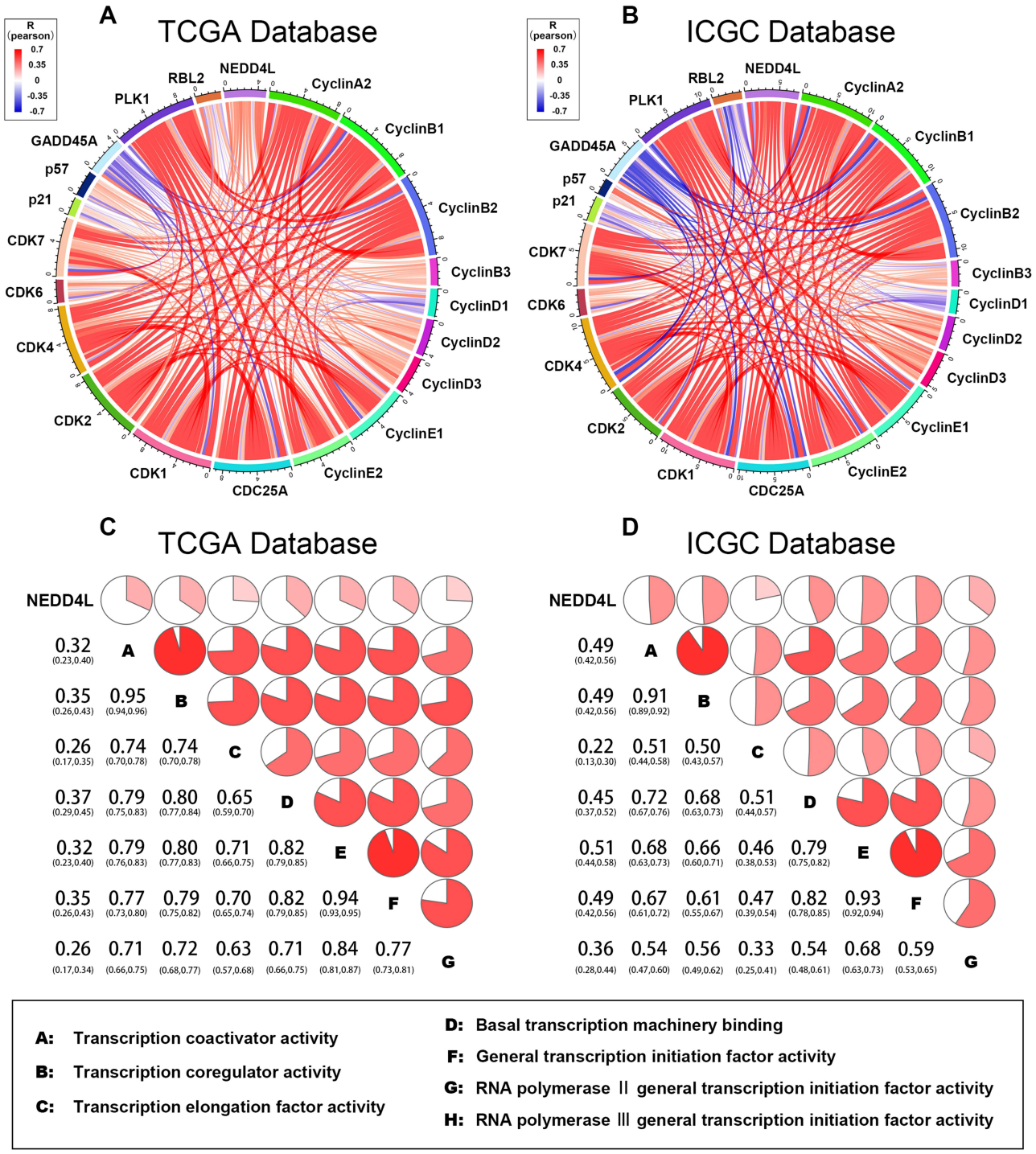

Figure 5 Correlations between NEDD4L expression and cell cycle checkpoints and transcription factor activity. (**A**) Pearson correlation between NEDD4L and cell cycle checkpoint genes and checkpoint-associated genes internally based on the TCGA database. The width of the bands represents the R-value, where the larger the width, the larger the R-value. The bar color represents the *P* value, where red represents positive correlation and blue represents negative correlation. The correlation was tested by Pearson correlation analysis. (**B**) Pearson correlation between NEDD4L and cell cycle checkpoint genes and checkpoint-associated genes internally based on the ICGC database. (**C**) Correlation matrix of NEDD4L with the set of genes related to transcription factor activity based on the TCGA database. The correlation coefficients are shown on the lower left and the visualization of the correlation coefficients is shown as a pie chart on the upper right. Red represents a positive correlation. The correlation was tested by Pearson correlation analysis. (**D**) Correlation matrix of NEDD4L with the set of genes related to transcription factor activity based on the ICGC database.

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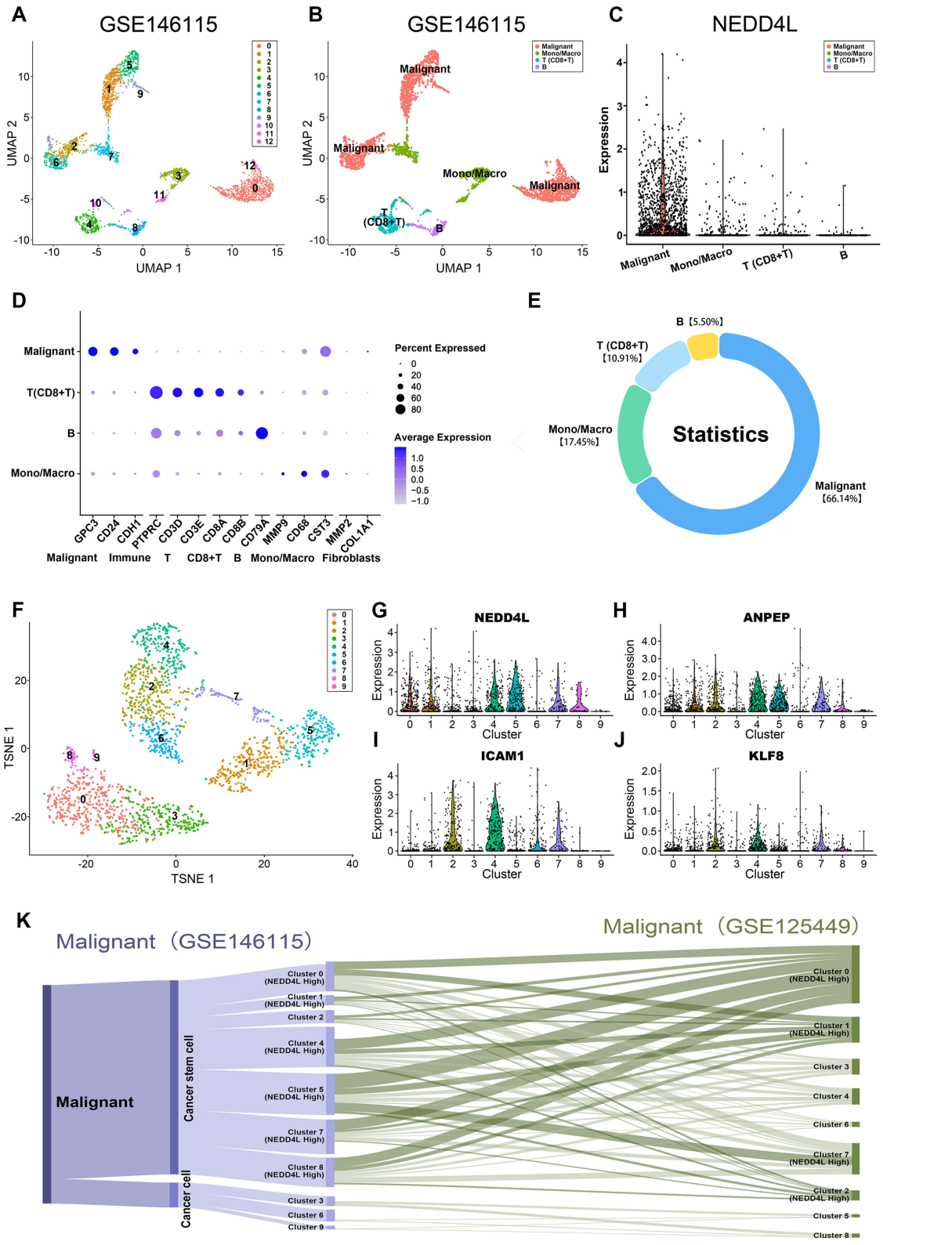

Figure 6 Identification of NEDD4L expression in HCC by scRNA-seq based on GSE146115 dataset. (**A**) Clusters of cells identified in HCC tissues based on the GSE146115 dataset. (**B**) Cell types identified in HCC tissues based on the GSE146115 dataset. (**C**) Differential expression of NEDD4L in cell types identified in HCC tissues. (**D**) Representative marker genes of identified cell types in HCC tissues based on the GSE146115 dataset. (**E**) Percentage of each cell type in HCC tissues based on the GSE146115 dataset. (**F**) Clusters of further identified cells in malignant/tumor cell subpopulations based on the GSE146115 dataset. (**G**–**J**) Differential expression of NEDD4L in cell clusters identified in different malignant/tumor cell subpopulations. (**K**) Similarity analysis between cell clusters identified in different malignant/tumor cell subpopulations based on GSE146115 dataset and GSE125449 dataset. The width of the bands represents the counts of genes co-expressed between different cell clusters.

expression profiles of marker genes, clusters 0, 1, 2, 5, 6, 9, and 12 were classified as malignant cells, while clusters 3, 7, and 11 were identified as monocytes/macrophages. Clusters 4 and 10 were designated as $CD8^+$ T cells, and cluster 8 was assigned to B cells (Figure 6B). NEDD4L was observed to be predominantly enriched in the malignant tumor cells, as depicted in Figure 6C. The marker genes corresponding to each cell type are shown in Figure 6D, with the proportions of the different cell types displayed in Figure 6E. To further investigate the roles of NEDD4L in HCC, malignant cells were isolated from the aforementioned clusters for additional analysis. These malignant cells were subsequently divided into nine distinct clusters (Figure 6F). The proteins ANPEP (CD13), ICAM1, and KLF8, established as reliable markers for tumor stem cells in HCC,[46–50] were selected for further analysis. As illustrated in Figure 6G–J, NEDD4L was found to be markedly enriched in the subgroups of tumor stem cells expressing high levels of ANPEP, ICAM1, and KLF8. This suggests that NEDD4L may also serve as a potential marker for tumor stem cells. To confirm these results, scRNA-seq data from the GSE125449 cohort were analyzed. The analysis revealed that cells in this dataset were organized into 16 subgroups (Supplementary Figure 2A), which were classified into six cell types (Supplementary Figure 2B). Consistently, NEDD4L remained highly enriched in malignant tumor cell clusters (Supplementary Figure 2C). The marker genes and cell type proportions are depicted in Supplementary Figure 2D and E. Subsequent analysis of single malignant tumor cells revealed similar findings to those observed in GSE146115, with NEDD4L being markedly enriched in the HCC stem cell subgroups (Supplementary Figure 2F–J). Moreover, as shown in Figure 6E and Supplementary Figure 2E, NEDD4L exhibited consistent enrichment across various stages of HCC progression and in different immune status backgrounds, further supporting the above conclusions.

Since NEDD4L exhibited similar characteristics as a cancer stem cell marker in both the GSE146115 and GSE125449 datasets, a deeper analysis of the transcriptomic similarities between malignant cell clusters in these datasets was conducted. As illustrated in Figure 6K, a significant number of co-expressed genes were identified across the cancer stem cell subpopulations in the two dataset samples. Notably, clusters 0, 5, and 8, which displayed high levels of NEDD4L expression in the training cohort, shared almost all genes with those in the validation cohort. This finding not only confirmed the accuracy of the previously identified cell type annotations but also suggested that NEDD4L likely facilitates HCC progression through interactions with these co-expressed genes within tumor cells. These results further supported the notion that HCC stem cell subpopulations with elevated NEDD4L expression represented highly analogous cell populations at the transcriptomic level, highlighting the potential of NEDD4L as a cancer stem cell marker.

NEDD4L May Promote HCC Immune Evasion by Inhibiting Stimulatory Immune Checkpoints and ICI

A growing body of evidence suggests that cell cycle regulatory pathways are intricately linked with other cancer-associated characteristics, such as metabolic reprogramming and immune evasion.[51] In the context of NSCLC, NEDD4L has been implicated in these processes.[52] To explore the relationships between NEDD4L expression and immune infiltration in HCC, correlation analyses were conducted using data from the TCGA database. Stromal scores (−0.22), immune scores (−0.12), and comprehensive ESTIMATE scores (−0.18) were calculated for NEDD4L expression in HCC samples using the ESTIMATE algorithm (Figure 7A–C). The analysis revealed a significant negative correlation between NEDD4L expression levels and ICI in the HCC tumor microenvironment ($P < 0.05$). To further elucidate the immune cell types affected by NEDD4L expression and to assess whether similar immune effects occur in other cancers, the xCELL algorithm was applied to evaluate the correlations between 64 immune cell types (ICI) and NEDD4L expression across 39 different tumor types. In HCC, elevated NEDD4L expression was associated with a reduction in the infiltration of 23 immune cell types, including aDC, $CD8^+$ T cells, and $CD4^+$ T cells, while promoting the infiltration of 6 immune cell types, such as Basophils, NKT cells, and Th1 cells. Notably, this suppressive effect on immune cell infiltration was also observed in 21 other cancer types, including esophageal, gastric, and pancreatic cancers, with the most pronounced effects seen in testicular and thymic cancers (Supplementary Figure 3A). Moreover, stromal scores derived from both algorithms indicated that high NEDD4L expression was markedly associated with reduced stromal cell presence in the HCC tumor microenvironment ($P < 0.0001$, $P < 0.05$). A reduction in stromal cell proportion is well-established as a marker of accelerated tumor cell growth, increased invasiveness, and heightened malignancy. These findings are

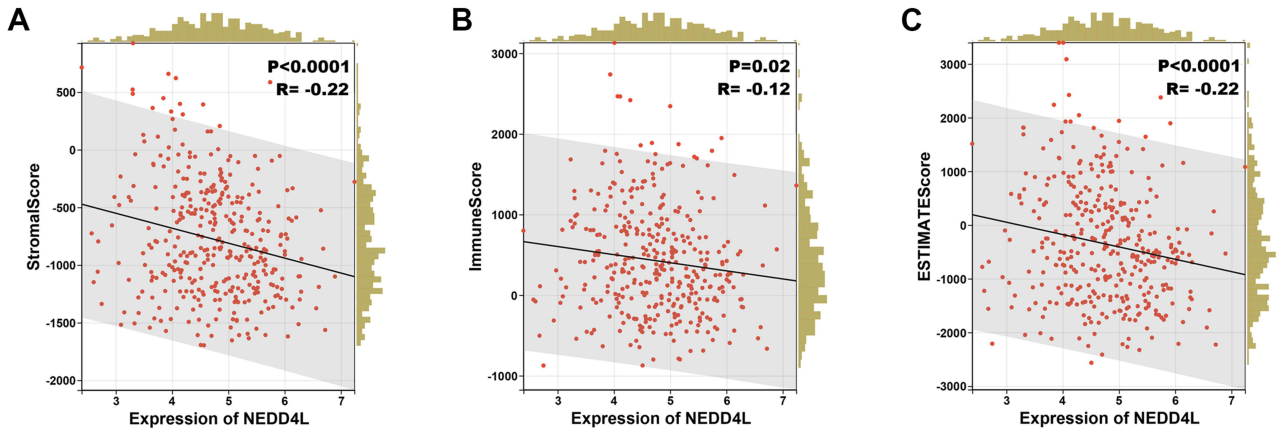

Figure 7 Correlations between NEDD4L expression and tumor immunity. (**A**–**C**) Pearson's correlation coefficient scatterplot of NEDD4L versus immune infiltration score in HCC. The bars at the edge of the scatterplot represent the data distribution.

consistent with the results from the single-cell sequencing analysis (NEDD4L was highly enriched in malignant cell clusters) and with the analysis in Section 3.5 (where high NEDD4L expression was positively correlated with dysregulation of cell cycle checkpoints).

Tumor cells frequently inhibit ICI and immune responses through interactions with immune regulatory genes and immune checkpoint molecules, such as PD-1 and CTLA-4, ultimately contributing to immune evasion.[53–55] Building on the aforementioned analysis, the Pearson correlation between NEDD4L expression in various tumor cells and the marker genes associated with Chemokines, Chemokine Receptors, MHC,[56] as well as both stimulatory and inhibitory immune checkpoint pathways, was subsequently assessed.[57] The analysis revealed that in HCC, NEDD4L expression was predominantly negatively correlated with a range of Chemokines, Chemokine Receptors, MHC, and stimulatory immune checkpoints (Supplementary Figure 3B and C). This suggests that the suppression of ICI in cases with elevated NEDD4L expression may be, in part, attributed to these correlations. Similar patterns of immune modulation have been observed in other cancer types, including esophageal cancer, pancreatic cancer, and clear cell renal cell carcinoma.

Discussion

HCC is recognized as one of the most prevalent solid tumors, with global mortality rates continuing to rise.[58] A significant proportion of patients are diagnosed at advanced stages, limiting therapeutic options to systemic treatments. The evolution of systemic therapies has progressed from single-target approaches, such as sorafenib and lenvatinib, to combinations of checkpoint inhibitors with targeted therapies, such as atezolizumab combined with bevacizumab.[59] Despite substantial progress in combination therapies, only a small subset of patients experiences durable clinical responses, and overall outcomes remain unsatisfactory, posing significant therapeutic challenges. As a result, there is an urgent need to explore alternative therapeutic strategies and identify novel molecular targets to inform clinical treatment decisions. The initiation, progression, and prognosis of cancer are strongly linked to genetic alterations and post-translational modifications.[60–62] Protein ubiquitination, a pivotal post-translational modification process facilitated by E3 ubiquitin ligases, serves a crucial function in regulating nearly all cellular processes.[63] Recently, NEDD4L, the prototypical member of the NEDD4 family, has emerged as a key factor in an increasing number of cancers.[64–66] The findings of this study further support the notion that NEDD4L may serve as a potential therapeutic target for HCC.

Transcriptome data from 1783 HCC patients across the TCGA, ICGC, and GEO databases were utilized to categorize the samples into training and validation cohorts, in accordance with the objectives of the analysis. A comprehensive evaluation and validation of NEDD4L expression levels in HCC samples from various sources were performed. The results revealed that NEDD4L was upregulated in HCC and predominantly enriched in cancer stem cell subpopulations, thereby suggesting that NEDD4L could serve as a potential marker for HCC stem cells. Furthermore, in vitro functional assays also found that silencing NEDD4L significantly inhibited the cell proliferation, migration, and wound healing

ability in HCC, which indicated that NEDD4L could significantly affect the malignant behaviors of HCC. However, these findings are in contrast to those reported by Zhao et al, which indicated NEDD4L downregulation and tumor-suppressive functions in HCC.[67] This discrepancy may be attributed to the relatively small sample size in Zhao's study and the limited geographic representation of Chinese populations. Consequently, the conclusions drawn by Zhao et al may have been influenced by sampling bias and inaccuracies. Considering the limitations of Zhao's study, as well as recent advancements in high-throughput omics technologies (including genomics and transcriptomics), the present analysis incorporated a larger, more diverse cohort that spanned multiple nationalities, regions, and ethnicities. This approach not only addressed the existing gaps in understanding the roles of NEDD4L in HCC but also contested previous conflicting findings. Moreover, through GO analysis, KEGG pathway analysis, and GSVA analysis, it was found that NEDD4L predominantly regulates cell cycle signaling pathways involved in processes such as cell division and DNA replication. Further investigation suggested that NEDD4L might downregulate inhibitory cell cycle checkpoints via ubiquitination modification, thereby enhancing the activity or expression of cyclins, CDKs, and transcription factors, ultimately contributing to aberrant cell cycle progression.

CDKs inhibitors constitute a class of therapeutic agents that impede the progression of cancer cells through abnormal cell cycles, thereby curbing their proliferation and reinstating orderly cell cycle regulation. Examples include abemaciclib, which targets CDK4/6,[68] and WEE1 inhibitors, which target CDC2,[69,70] both of which have demonstrated efficacy in treating a variety of cancers. While CDK inhibitors are effective in halting cancer cell growth, they do not induce apoptosis or result in cancer cell death to markedly reduce tumor size.[71] Consequently, numerous clinical trials have been conducted to evaluate the potential of combining CDK inhibitors with other therapeutic strategies in order to achieve improved treatment outcomes.[72–74] Cancer immunotherapy represents an emerging therapeutic approach that seeks to restore or augment immune function, enabling more effective detection and elimination of tumor cells. Antibodies targeting inhibitory immune checkpoints, such as PD-1/PD-L1 inhibitors[75,76] and CTLA-4 inhibitors,[77] are currently at the forefront of cancer treatment. These inhibitors have shown significant success in non-small cell lung cancer (NSCLC),[78–80] colorectal cancer,[81,82] and melanoma.[83,84] However, clinical trials to date indicate that immunotherapy proves effective for merely a limited subset of HCC patients, and the molecular mechanisms underlying their immune response and immune evasion remain poorly understood.[85,86] As a result, there is an urgent need for further research on the role of cell cycle checkpoints and immune checkpoints in HCC to enhance treatment efficacy. Moreover, with the increasing approval of both first-line and second-line therapies and the integration of immunotherapy into treatment guidelines, the therapeutic landscape for advanced HCC patients has grown markedly more diverse. Despite this expansion, the optimal sequencing of these treatments remains to be clarified.[87] Simultaneously, the development of predictive biomarkers to guide clinical treatment decisions has become a critical issue that demands resolution.

Meanwhile, increasing evidence suggests that kinase inhibitors and immune checkpoint inhibitors represent promising therapeutic strategies for advanced HCC.[88] Furthermore, studies have demonstrated that NEDD4L serves an essential function in immune modulation and inflammatory processes.[29,30,89,90] In light of this, the relationships between NEDD4L expression, ICI, and immune checkpoints within the tumor microenvironment were further investigated. The results revealed that elevated NEDD4L expression markedly suppressed the infiltration of 23 immune cell types, including aDC and $CD8^+$ T cells, while also displaying negative correlations with stimulatory immune checkpoints such as CD40LG, SELP, and ENTPD1. Similar observations regarding NEDD4L's involvement in tumor immune regulation have been made in other malignancies, including esophageal and pancreatic cancers. These findings suggest that targeting NEDD4L could potentially synergize with immune checkpoint inhibitors to enhance their therapeutic efficacy. Additionally, considering the limitation that CDK inhibitors alone do not markedly induce cancer cell apoptosis or reduce tumor size, the combination of NEDD4L and CDK inhibition may offer substantial therapeutic benefits. This dual-target approach could not only inhibit tumor cell proliferation effectively but also augment immune responses by upregulating stimulatory immune checkpoints, thereby facilitating the elimination of tumor cells.

In addition to its role as a marker that promotes tumor cell proliferation and suppresses the tumor immune microenvironment in HCC, NEDD4L appears to have context-dependent effects across various types of cancers. For instance, Guo et al identified that NEDD4L exhibits tumor-suppressive properties in breast cancer by inhibiting the CTR1-AKT signaling pathway.[25] In line with this, NEDD4L is downregulated in numerous cancers, such as gastric

cancer,[91] esophageal cancer,[92] colorectal cancer,[24,93] and prostate cancer,[94] where it exerts anti-tumor effects through ubiquitination and subsequent degradation of key molecules, including PD-L1, NOTCH2, LGR5, and LSTAT3. This mechanism suppresses tumor growth and metastasis, promotes apoptosis and ferroptosis, and diminishes tumor stemness. Conversely, in certain solid tumors, including pancreatic cancer, NEDD4L has been implicated as an oncogene. Wang et al demonstrated that NEDD4L interacts with ANXA2, facilitating its degradation and activating the FAK/AKT signaling pathway, which in turn promotes the proliferation and metastasis of pancreatic cancer cells.[95] Furthermore, other studies have shown that NEDD4L functions as a tumor suppressor in lung cancer, where it maintains a delicate balance with the oncogene NEDD4-1. This interaction is essential for inhibiting lung cancer metastasis and preserving lung function in both cancer patients and fetuses.[96,97] A similar interaction between NEDD4L and NEDD4-1 has also been observed in intestinal tumors.[98] Consequently, investigating the interactions between NEDD4L and other family members, such as NEDD4-1, could provide valuable insights for the optimization of HCC treatment strategies.

A more noteworthy point is that the diversity of NEDD4L subcellular localization seems to be closely related to its multiple functions in cancers as described above. A large body of evidence suggests that NEDD4L is involved in the regulation of tumorigenesis, immune escape, cell cycle and metabolism through its distribution in different cellular compartments (eg, nucleus, cytoplasm, plasma membrane).[28,99,100] Firstly, in the nucleus, NEDD4L mainly affects the malignant phenotype of tumors by regulating transcriptional and epigenetic modifications. NEDD4L disrupts Hippo pathway key proteins (eg, WW45 and LATS kinase) via k48-linked polyubiquitination, thereby inhibiting Hippo signaling activity. This inactivation further promotes the accumulation of the transcriptional co-activator YAP/TAZ in the nucleus, which in turn drives a pro-proliferative transcriptional program.[26,101] In addition, NEDD4L modifies transcription factors through ubiquitination and thus regulates the TGF-β signaling pathway.[102,103] In lung cancer, low expression of NEDD4L enhanced the stability of Smad2/3 protein and promoted TGF-β-mediated epithelial-mesenchymal transition (EMT) and metastasis.[104] Secondly, in the cytoplasm, NEDD4L mainly regulates oncoprotein stability through ubiquitination-dependent protein degradation processes. In NSCLC, NEDD4L mediates the ubiquitination-dependent degradation of CPNE1 and inhibits tumor cell proliferation and metastasis.[104] In contrast, in A549 lung cancer cells, NEDD4L also reduces the expression of immune checkpoint proteins such as PD-L1 through ubiquitination, thereby enhancing the anti-tumor immune activity of $CD8^+$ T cells.[52] In addition, it has been pointed out that NEDD4L not only relies on the proteasome to degrade target proteins, but also regulates the stability of the substrate ENaC through the autophagy-lysosome pathway, which affects cellular metabolism and the tumor microenvironment.[105] Meanwhile, at the cell membrane, NEDD4L is mainly involved in the homeostatic regulation of ion channels and receptors. NEDD4L can promote endocytosis and degradation of ENaC by ubiquitinating its β/γ subunits, thus maintaining sodium homeostasis.[106,107] In lung and kidney cancers, a study found that NEDD4L deficiency led to the over-activation of ENaC, triggered cellular oedema and fibrosis, and promoted the remodeling of the tumor microenvironment.[108] In addition, in lung cancer, NEDD4L can also affect tumor immune escape by regulating the membrane localization and stability of the immune checkpoint protein PD-L1.[52] Overall, the subcellular localization of NEDD4L is closely related to its functional diversity: intranuclear regulation of transcription and epigenetics, cytoplasmic regulation of protein stability, plasma membrane regulation of ion channels and immune checkpoints, and extracellular regulation of ECM remodeling. However, the predominant and dominant functions played by different subcellular localizations of NEDD4L vary in different cancer types, which leads to the dual functions of inhibition and promotion of NEDD4L in human tumors. Future studies need to further explore the molecular mechanisms underlying the dynamic changes in its localization, especially its role in tumor metabolic reprogramming, signaling and immunotherapy resistance. For example, targeting the subcellular localization of NEDD4L (eg, development of nuclear localization signaling inhibitors) may provide new strategies for cancer therapy.

However, this investigation still has several limitations. First, the clinical data available in different public databases vary, with many values missing. As a result, the correlation analysis between NEDD4L expression and clinicopathological factors was restricted to only three primary parameters: age, gender, and TNM stage, which does not provide a sufficiently comprehensive analysis. Furthermore, additional in vitro and in vivo experiments involving cellular and animal models are required to fully elucidate the specific roles and molecular mechanisms of NEDD4L in the malignant progression of HCC.

Conclusions

In conclusion, the findings demonstrated that NEDD4L is markedly upregulated in HCC and is strongly correlated with unfavorable patient prognosis. Silencing NEDD4L could effectively inhibit cell proliferation, migration and wound healing ability for HCC. Furthermore, NEDD4L may function as a potential marker for cancer stem cells in HCC. In addition, considering its pivotal involvement in regulating cell cycle and immune checkpoints, NEDD4L presents itself as a promising therapeutic target for HCC. Targeting NEDD4L may facilitate a synergistic enhancement of the efficacy of CDK inhibitors and immune checkpoint inhibitors.

Data Sharing Statement

These data of this manuscript can be available from the corresponding author upon reasonable request.

Ethics Approval and Informed Consent

These specimens used in this study were obtained from Liaoning Cancer Hospital & Institute and was approved by the Institutional Review Board (the committee approval number: KY20240413).

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.

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Disclosure

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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