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Original Research

LRRC3-DT Inhibits Malignant Behaviors of Hepatocellular Carcinoma and is Associated With CCDC81 Expression

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Abstract

Background: Enhancer RNAs (eRNAs) are important regulators of tumor development and progression. Nevertheless, the diagnostic utility of circulating eRNAs in hepatocellular carcinoma (HCC) remains unclear. **Methods:** This was a retrospective study. The recently identified eRNA, leucine-rich repeat-containing 3 divergent transcript (LRRC3-DT), was analyzed in serum samples, tissue specimens, and various cell lines. Its diagnostic performance was evaluated alone and in combination with alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) using receiver operating characteristic (ROC) curve analysis. Functional investigations were performed to assess the biological role of LRRC3-DT by overexpressing and knocking down this eRNA in HCC cell lines. Furthermore, using transcriptome sequencing, potential target genes of LRRC3-DT were identified. **Results:** The serum levels of LRRC3-DT were significantly lower in patients diagnosed with HCC than in healthy controls. When LRRC3-DT was assessed alongside AFP and PIVKA-II, the combination improved sensitivity for the detection of HCC. Despite earlier research indicating that LRRC3-DT is present primarily in the nucleus and is strongly associated with tumor proliferation, invasion, and apoptosis, the serum levels of LRRC3-DT were similar between patients with HCC and those with benign liver diseases in the present study. These findings indicate limited specificity in distinguishing between these two conditions. **Conclusions:** Serum LRRC3-DT represents a potential auxiliary biomarker for early HCC screening; its combined use with other indicators improved diagnostic performance. However, it cannot individually discriminate HCC from benign liver disorders, and additional validation studies are warranted to confirm its differential diagnostic value.

Keywords: hepatocellular carcinoma; long noncoding RNA; LRRC3-DT; CCDC81; biomarker

1. Introduction

Hepatocellular carcinoma (HCC) is a prevalent and lethal tumor that significantly contributes to cancer-related mortality [1,2]. In clinical settings, curative approaches for this disease continue to focus predominantly on surgical removal and liver transplantation (LT) [3,4,5]. Nevertheless, it is undeniable that individuals undergoing these interventions face substantial recurrence likelihood postoperatively [6,7]. The development of this particular type of carcinoma is characterized by complex pathological mechanisms that involve a variety of changes in genetic activity [8]. These mechanisms highlight the multifaceted nature of the disease, indicating that several distinct genetic alterations contribute to its progression and characteristics [9,10]. In the realm of clinical diagnostics, the effectiveness of current serum markers such as alpha-fetoprotein and protein induced by vitamin K absence or antagonist-II has been shown to be limited [11,12,13,14]. Specifically, these markers exhibit suboptimal sensitivity and specificity, making them insufficient for reliable diagnosis and monitoring of carcinoma. Therefore, the development of novel biomarkers is highly necessary.

Enhancers are essential components of the intricate process of transcriptional regulation and play a pivotal role in gene expression [15,16]. These regulatory sequences can interact with specific transcription factors, thereby influencing the expression of target genes located at considerable distances. A fascinating aspect of enhancer regions is their ability to be transcribed, resulting in the production of enhancer RNAs (eRNAs) [17,18]. Recent research highlights the critical involvement of eRNAs in tumorigenesis, suggesting that these molecules may have far-reaching implications for cancer development and progression [19,20]. For instance, the long noncoding RNA colon cancer-associated transcript 1 (CCAT1) has been shown to facilitate both the initiation and progression of prostate cancer [21]. Additionally, superenhancer RNA (seRNA) marked by N6-

methyladenosine (m6A) modification plays a notable role in the progression of pancreatic ductal adenocarcinoma (PDAC) by influencing the cofilin 1-methyltransferase-like 3 (CFL1-METTL3)-seRNA m6A-YTH domain containing 2 (YTHDC2)/mixed-lineage leukemia 1 (MLL1) signaling axis [22]. Another illustrative example is the enhancer that mediates the expression of miR-483-5p, which has been implicated in hastening the tumor progression of HCC by modulating the insulin-like growth factor 2 (IGF2)/H19 axis [23]. Collectively, these studies reveal an essential function of eRNAs in the processes underlying tumor formation and advancement. In light of this emerging evidence, our study focuses on the eRNA known as Leucine Rich Repeat Containing 3 Divergent Transcript (LRRC3-DT) and explores its potential role as both a biomarker and a tumor suppressor in HCC. Through this investigation, we aim to contribute to the understanding of how eRNAs can affect cancer biology and assist in the identification of novel therapeutic targets.

Multiple circulating long noncoding RNAs have been validated as noninvasive diagnostic candidates for HCC liquid biopsy, among which highly upregulated in liver cancer (HULC) and HOX transcript antisense RNA (HOTAIR) represent the two most extensively characterized serum biomarkers. The oncogenic long noncoding RNA (lncRNA) HULC is markedly enriched in serum from patients with HCC and achieves reliable diagnostic efficacy when distinguishing patients with HCC from healthy individuals; however, its specificity is compromised in cohorts with chronic hepatitis or liver fibrosis, since sustained hepatic inflammation independently triggers HULC secretion into peripheral blood. Similarly, serum HOTAIR is overexpressed in hepatitis B virus (HBV)-associated HCC, but overlapping inflammatory liver injury blunts its ability to separate malignant tumors from benign hepatic lesions across clinical subgroups. Notably, both HULC and HOTAIR exert protumorigenic effects to facilitate HCC proliferation and metastasis [24]. In contrast, the LRRC3-DT identified in the current study functions as a tumor-suppressive eRNA predominantly localized to cell nuclei. Consistent with the limitations observed for HULC and HOTAIR, serum LRRC3-DT cannot differentiate HCC from inflammatory benign liver disease, which is attributable to the shared release of inflammation-associated cells from nonmalignant liver cells. Existing circulating liver cancer lncRNAs are mostly cancer-promoting molecules, and the serum diagnostic value of nucleus-localized tumor suppressor eRNAs has rarely been studied.

LRRC3-DT expression is significantly downregulated in the serum, tissues, and cells of patients with HCC. Furthermore, it is proposed that HCC initiation and progression may be suppressed by this molecule through regulation of its downstream target gene coiled-coil domain containing 81 (*CCDC81*), with the aim of exploring its potential role in HCC pathogenesis.

2. Materials and Methods

2.1 Clinical Specimens

The samples for this study were sourced from the laboratories of Nantong University Affiliated Hospital and Nantong Third People's Hospital and included 105 serum samples from patients with liver cancer, 69 samples from individuals with benign liver conditions collected from 2018 to 2021, and 96 serum samples from healthy participants who underwent routine physical check-ups. All patients with liver cancer had their tumors surgically removed, with the exception of individuals diagnosed with other gastrointestinal malignancies or those who had received prior treatments such as ultrasound-guided radiofrequency ablation or transarterial chemoembolization (TACE) before surgery. Those with benign liver diseases were identified as positive for hepatitis B surface antigen and were excluded if they had other gastrointestinal tumors (e.g., pancreatic cancer, gastric cancer, or colorectal cancer) or if they tested positive for antibodies related to other hepatitis types (e.g., hepatitis A, C, or E). A total of 52 pairs of HCC tissues and adjacent normal tissues were obtained from the pathology department of Nantong Third People's Hospital. All tissue specimens were pathologically verified as HCC. Immediately following excision, the tissues were frozen in liquid nitrogen and stored at –80 °C. Informed consent was obtained from all participants before the clinical trial, and they agreed to the publication of the findings. Ethical approval number: EK2022023.

2.2 Cell Culture

The HCC cell lines HepG2, HCCLM3, SK-HEP-1, PLC/PRF/5, Hep3B, and MHCC97-H, as well as normal human liver cells (THLE2), were acquired from the Cell Bank of the Chinese Academy of Sciences. HepG2, HCCLM3, Hep3B, MHCC97-H, and THLE2 cells were cultured in DMEM, whereas SK-HEP-1 and PLC/PRF/5 cells were maintained in MEM. The complete culture medium contained 10% fetal bovine serum (Lonsa Science SRL, Montevideo, Uruguay) and 1% penicillin–streptomycin-amphotericin B (NCM Biotech, Suzhou, China). All the cell cultures were incubated at 37 °C in a 5% CO_2 atmosphere. All cell lines were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Address: No.320 Yueyang Road, Xuhui District, Shanghai) and were authenticated by short tandem repeat (STR) profiling. All the cell lines were routinely tested and confirmed to be free of mycoplasma contamination.

2.3 Total RNA Extraction and Complementary DNA Synthesis

Total RNA was extracted from the serum of patients with HCC, patients with benign liver diseases, and healthy individuals using a spin column isolation kit (BioTeke, Beijing, China). After serum RNA was eluted, RNA purity was determined by a NanoDrop spectrophotometer by measur-

ing the A260/A280 ratio; only samples with A260/A280 values ranging from 1.8 to 2.1 were retained for subsequent reverse transcription. Additionally, total RNA from HCC tissues and cells was extracted using FreeZol Reagent (Vazyme, Nanjing, China). PrimeScript RT Master Mix (Takara, Kyoto, Japan) was used to reverse transcribe total RNA into complementary DNA (cDNA) under the following conditions: 37 °C for 15 minutes, 85 °C for 5 seconds, and indefinitely at 4 °C.

2.4 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total reaction volume for quantitative PCR was 20 µL, which contained 10 µL of ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.), 5 µL of complementary DNA (cDNA), 1 µL of primers, and 4 µL of nuclease-free water. The primers used included both forward and reverse primers for LRRC3-DT and 18S. The reactions were conducted using an ABI QuantStudio 5 (Thermo Fisher Scientific). The expression levels of LRRC3-DT were quantified using the $2^{-\Delta\Delta CT}$ method, with 18S serving as an internal reference. All primers were synthesized by RiboBio (Guangzhou, China).

2.5 Cell Transfection

The LRRC3-DT inhibitor (designated as sh) and the overexpression vector (designated as oe), along with their corresponding negative controls, were procured from GenePharma (Shanghai, China). The HCC cell lines HCCLM3 and HepG2 were evenly seeded into 6-well plates and cultured until they reached 70–80% confluence. The medium was subsequently replaced with serum-free basal medium, and the cells were transfected with plasmids using Lipofectamine 3000 (Thermo Fisher Scientific, Carlsbad, CA, USA). The cells were cultured overnight to facilitate transfection. After 48 hours, the cells were harvested, and the transfection efficiency was verified using quantitative polymerase chain reaction.

2.6 Colony Formation Assay

In each well of a 6-well plate, 1000 to 2000 cells were seeded at 48 hours post-transfection and cultured in complete medium supplemented with 10% fetal bovine serum. The medium was replaced every 3 to 4 days, and the size of the cell colonies was monitored. After approximately one week of culture, the cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes, stained with crystal violet dye for 8 to 10 minutes, and air-dried to eliminate residual moisture from the surface of the plate. Statistical analysis was subsequently conducted.

2.7 CCK-8 Cell Proliferation Assay

In a 96-well plate, 100 µL of a cell suspension containing 1500 to 2000 viable cells was added to each well. Following a 24-hour preincubation period, 10 µL of Cell Counting Kit-8 (CCK-8) solution was added to each well. The plate was then incubated in the dark for 2 hours, after which the absorbance at 450 nm was measured. Measurements were conducted daily over a span of four consecutive days, and the data were subsequently analyzed.

2.8 Scratch Assay

Once the cells in a 6-well plate had adhered and achieved confluence, a cross-shaped scratch was created using a 10 µL pipette tip. Images of the scratch were captured under a microscope at both 0 hours and 24 hours, and the results were subjected to statistical analysis.

2.9 Transwell Cell Migration and Invasion Assay

A PET membrane chamber was placed in each well of a 24-well plate. The lower chamber was filled with 800 µL of complete medium containing 20% fetal bovine serum, while 300 µL of basal medium and 50,000 cells were added to the upper chamber. For the invasion assay, prediluted Matrigel (Corning, Bedford, MA, USA) was used to coat the chamber one day in advance. The mixture was then incubated at 37 °C in a 5% CO_2 incubator. After 4 to 5 days of culture, the chambers were removed, fixed with 4% paraformaldehyde at room temperature for 20 minutes, and stained with crystal violet dye for 10 minutes. The inner side of the chamber was cleaned with a Q-tip, air-dried, and photographed using an upright microscope (Olympus, Japan).

2.10 Apoptosis Assay

The transfected cells were collected, washed with phosphate-buffered saline (PBS, NCM Biotech, Suzhou, Jiangsu, China), and resuspended. Following the protocol, Annexin V-fluorescein isothiocyanate (FITC) binding solution and propidium iodide staining solution were added. The cells were incubated at room temperature in the dark for 10 to 15 minutes. The reaction was then terminated using an ice bath, and apoptosis was analyzed using a flow cytometer (BD FACSCalibur, USA). A C1062L-Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology, Shanghai, China) was used for this assay. Cells were first gated on FSC-A versus SSC-A dot-plot to exclude cell debris and noise; single-cell events were subsequently identified using FSC-H versus FSC-A to remove doublets and cell aggregates, and the singlet population was further analyzed in the Annexin-V-FITC versus PI dot-plot to distinguish viable (Annexin-V^- PI^-), early apoptotic (Annexin-V^+ PI^-), late apoptotic (Annexin-V^+ PI^+), and necrotic (Annexin-V^- PI^+) cells, with all data processed by FlowJo software.

2.11 Fluorescence In Situ Hybridization (FISH)

HCC cells were evenly seeded onto sterile coverslips placed at the bottom of the wells in a 24-well plate and incubated overnight at 37 °C in a 5% CO_2 incubator. The following day, the medium was discarded, and the cover-

slips were washed twice with PBS for 5 minutes each. Two hundred microliters of 4% paraformaldehyde were added to each well, and the samples were fixed at room temperature for 15 minutes. Subsequent procedures were performed using the reagents provided by GenePharma (Shanghai, China). Finally, the cell nuclei were stained with 4′,6-Diamidino-2-Phenylindole (DAPI) in the dark for 10 to 20 minutes. Place the side of the coverslip with cells onto a slide that has been pretreated with Antifade Mounting Medium and observe the cells under a fluorescence microscope (Olympus, Japan), capturing images as necessary.

2.12 Statistical Analysis

All the statistical analyses in this study were performed using SPSS Statistics version 29.0.1.0 (IBM SPSS Statistics), while the graphs were generated using GraphPad Prism 10.0 software (GraphPad Software Inc., CA, USA). The expression of LRRC3-DT across different groups is presented as the mean ± standard deviation (SD). Normality tests were conducted on all the data in this study. Survival outcomes of patients with HCC from The Cancer Genome Atlas (TCGA) cohort were analyzed via Kaplan–Meier curves. Subjects were stratified into high- and low-LRRC3-DT groups on the basis of median transcript expression, and intergroup survival disparities were assessed with the log-rank test. The Wilcoxon signed-rank test was used to compare the expression levels of LRRC3-DT in HCC tissue and adjacent tissue. For comparisons across three or more independent experimental groups, one-way analysis of variance (one-way ANOVA) was used to detect overall intergroup differences, followed by Dunnett's post hoc test. Dunnett's test was used for targeted pairwise comparisons only between each intervention group and its corresponding negative control (sh-LRRC3-DT vs. sh-NC, oe-LRRC3-DT vs. oe-NC), with built-in correction for multiple comparisons to control familywise type I error, which was applied to Transwell migration/invasion and flow cytometry apoptosis assays. The chi-square test was used to analyze the correlation between LRRC3-DT expression levels and the clinicopathological features of patients with HCC. The diagnostic performance of LRRC3-DTs in serum from patients with HCC was evaluated by constructing receiver operating characteristic curves and calculating the area under the curve (AUC). Statistical significance was defined as $p < 0.05$.

3. Results

3.1 Basic Information and Prerequisites for Clinical Application of LRRC3-DT

According to GeneCards (https://www.genecards.org), LRRC3-DT is located on the long arm of human chromosome 21 (Fig. 1A). Analysis of the data from the TCGA database revealed that LRRC3-DT is downregulated in patients with HCC (Fig. 1B). Moreover, patients with high expression levels of LRRC3-DT had significantly better survival rates ($p = 0.027$) (Fig. 1C). To evaluate the potential of LRRC3-DT as a reliable serum biomarker, amplification results were used to construct a single-peak melting curve and a smooth amplification curve, indicating the specificity of the primer design (Fig. 1D,E). The qRT–PCR products were further validated through agarose gel electrophoresis and Sanger sequencing. Agarose gel electrophoresis revealed a single amplification band of approximately 135 bp, which is consistent with the designed amplification length for the LRRC3-DT gene (Fig. 1F). Sanger sequencing confirmed that the product contained the full-length sequence of LRRC3-DT, which completely matched the National Center for Biotechnology Information (NCBI) reference sequence (Fig. 1G).

3.2 Characterization of LRRC3-DT Expression in Serum From Patients With HCC and Its Clinical Correlations

In this study, qRT–PCR was used to assess the serum expression levels of LRRC3-DTs in three groups: 105 patients with HCC, 69 patients with benign liver diseases, and 96 healthy controls. The results demonstrated that the relative expression of LRRC3-DT in patients with HCC was significantly lower than that in healthy controls ($p < 0.0001$) and marginally lower than that in patients with benign liver disease. However, no statistically significant difference was observed between the HCC and benign liver disease groups ($p = 0.5439$) (Fig. 2A). Additionally, experiments conducted at room temperature, along with repeated freeze–thaw cycles, demonstrated that LRRC3-DT maintained excellent stability in serum samples (Fig. 2B,C).

To evaluate the diagnostic efficacy of serum LRRC3-DTs, we conducted a receiver operating characteristic (ROC) curve analysis. LRRC3-DT demonstrated diagnostic efficacy comparable to that of alpha-fetoprotein (AFP) (Fig. 2D). We further analyzed the serum levels of AFP and protein induced by vitamin K absence or antagonist-II (PIVKA-II) in patients with early-stage HCC (tumor node metastasis (TNM) Stages I and II) compared with those in healthy controls, and the results indicated that the diagnostic efficacy of LRRC3-DTs for early HCC was comparable to that of AFP (Fig. 2E). In the exploration of multi-indicator joint diagnosis (Fig. 2F,G), the AUCs of all the joint schemes were significantly greater than the corresponding independent diagnostic AUCs of the single indicators. These results strongly confirm the diagnostic value of multibiomarker combinations—by integrating the information dimensions of different biomarkers, the accuracy and stability of diagnosis can be effectively enhanced, providing better strategic choices for early precise diagnosis of clinical diseases.

Finally, we analyzed the relationships between clinicopathological parameters and serum LRRC3-DT expression levels using the χ^2 test. The results indicated that LRRC3-DT expression was significantly associated with TNM stage ($p < 0.001$) and nerve/vascular invasion (p

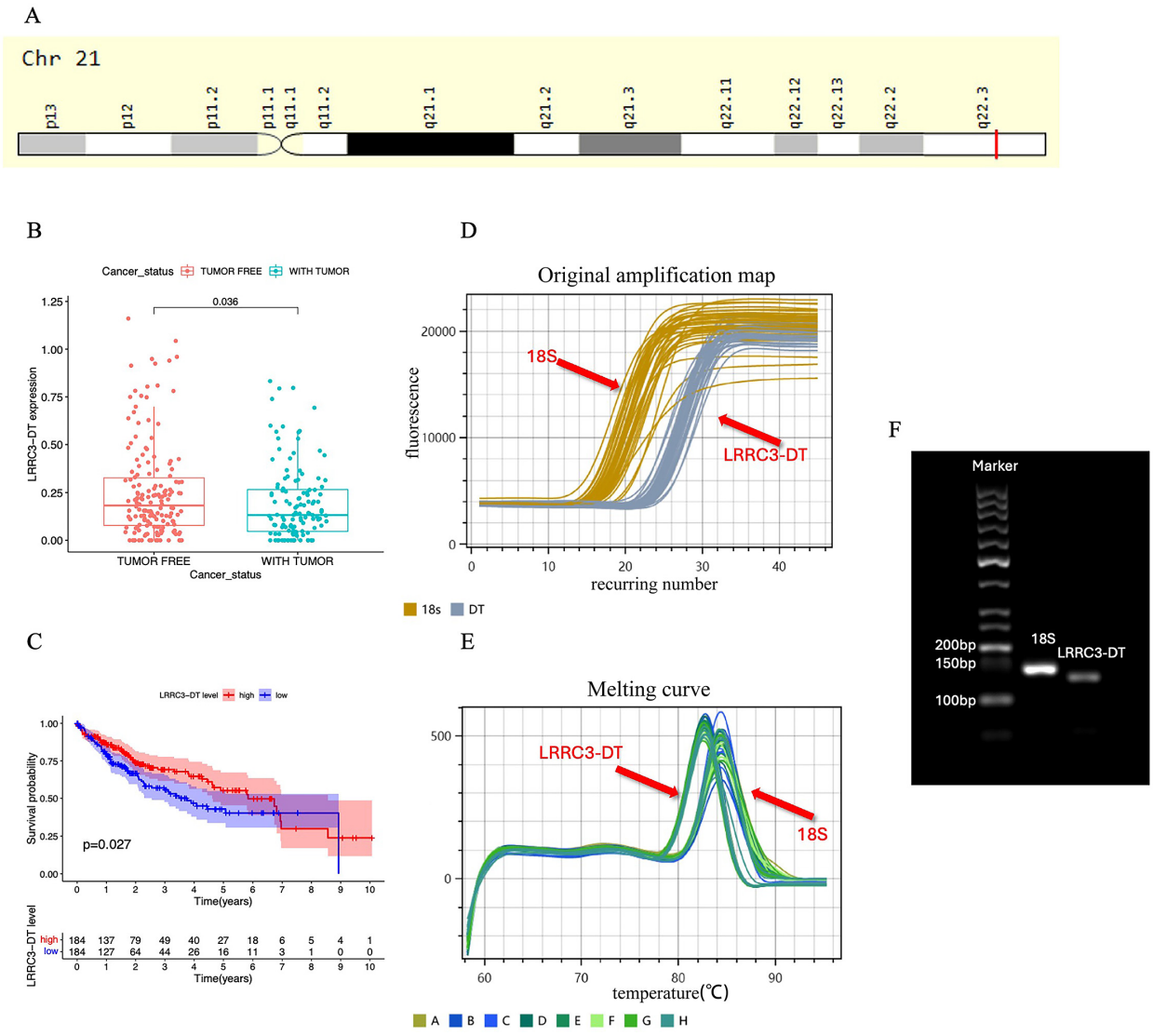

Fig. 1. LRRC3-DT expression is low in HCC. (A) Localization of LRRC3-DT chromosomes. (B) The expression of LRRC3-DT was significantly lower in tissues in patients with tumor than those without tumors, according to data from the TCGA database (https://www.cancer.gov/ccg/research/genome-sequencing/tcga). (C) The correlation between LRRC3-DTs and patient survival outcomes was determined from the TCGA database. (D,E) Amplification curve and melting curve of LRRC3-DT. (F) Agarose gel electrophoresis revealed a single amplification band corresponding to the expected fragment size (~135 bp). (G) Sanger sequencing confirmed the identity of the amplified product, which encompassed the full-length LRRC3-DT sequence. TCGA, The Cancer Genome Atlas; LRRC3-DT, Leucine Rich Repeat Containing 3 Divergent Transcript; HCC, hepatocellular carcinoma.

< 0.05). However, no statistically significant differences were observed concerning sex, age, HBV infection status, tumor size, or differentiation grade ($p > 0.05$) (Table 1).

3.3 LRRC3-DT Inhibits HCC Development In Vitro

The results of the qRT–PCR experiments demonstrated that LRRC3-DT was expressed at low levels in both HCC tissues and cells (Fig. 3A,B). Furthermore, pathologi-

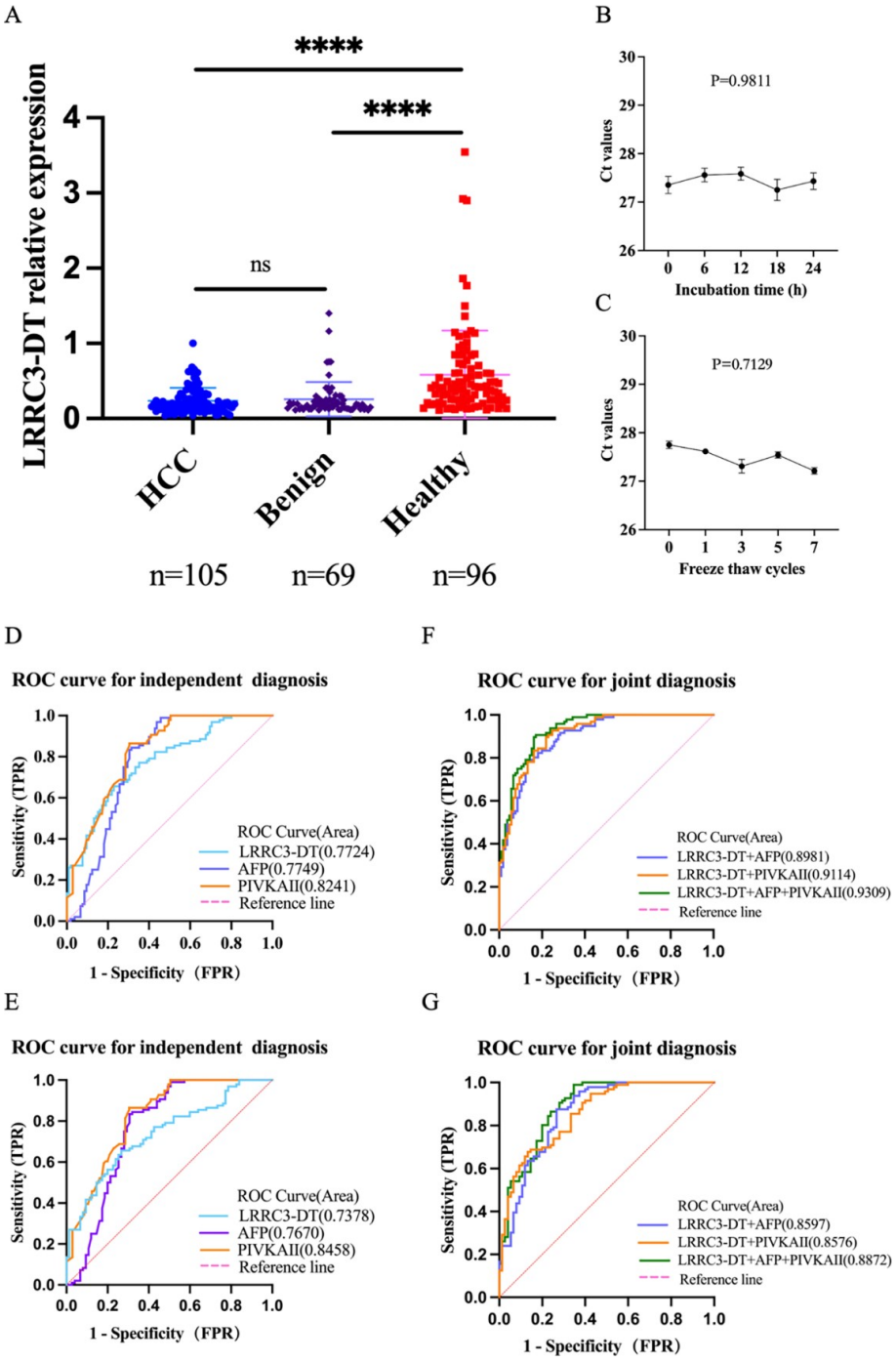

Fig. 2. Assessing LRRC3-DT as a serum biomarker for HCC detection. (A) Quantitative analysis of LRRC3-DTs in serum samples from patients with HCC (n = 105), patients with benign liver disease (n = 69), and healthy donors (n = 96). (B,C) Stability assessment under room temperature storage and repeated freeze–thaw cycles revealed no significant alterations in LRRC3-DT expression levels ($p > 0.05$). (D,F) Comparison of the performance of LRRC3-DT with that of conventional biomarkers (AFP/PIVKA-II) in individual and combined diagnostic approaches for distinguishing patients with HCC from healthy controls. (E,G) Comparison of the performance of LRRC3-DT with that of conventional biomarkers (AFP/PIVKA-II) in individual and combined diagnostic approaches for distinguishing patients with early-stage HCC from healthy controls. ****$p < 0.0001$, ns, not significant. AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II.

cal parameter analysis revealed that LRRC3-DT expression was not significantly correlated with sex, age, tumor size, or degree of differentiation ($p > 0.05$). However, a significant correlation was observed between LRRC3-DT expression and the TNM stage of patients ($p < 0.05$) (Table 2).

Table 1. Pathological parameter analysis of serum LRRC3-DTs.

Parameter	No. of patients	LRRC3-DT expression		*p* value
		(high = 52)	(low = 53)	
Gender				
Male	72	38	34	0.325
Female	33	14	19	
Age (years)				
≤60	40	22	18	0.379
>60	65	30	35	
HBV infection				
Positive	74	39	35	0.314
Negative	31	13	18	
Serum AFP (ng/mL)				
≤200	80	42	38	0.275
>200	25	10	15	
Tumor size (cm)				
≤5	68	35	33	0.589
>5	37	17	20	
TNM stage				
I+II	75	46	29	<0.001***
III+IV	30	6	24	
Differentiation grade				
Well	24	12	12	
Moderate	55	28	27	0.922
Poor	26	12	14	
Nerve/vascular invasion				
Positive	45	17	28	0.037*
Negative	60	35	25	

Note: TNM, tumor node metastasis; HBV, hepatitis B virus; *$p < 0.05$; ***$p < 0.001$.

To investigate the functional significance of LRRC3-DT in HCC, we constructed two recombinant vectors: a short hairpin RNA (shRNA) targeting LRRC3-DT (sh-LRRC3-DT) and an LRRC3-DT overexpression plasmid (oe-LRRC3-DT). Transfection efficiency analysis revealed that the knockdown efficiency of sh-LRRC3-DT was approximately 74% and 75% in the HepG2 and HCCLM3 cell lines, respectively, whereas the oe-LRRC3-DT plasmid induced 33-fold and 10-fold upregulation, respectively, of LRRC3-DT expression in these two cell lines (Fig. 3C,D), confirming the successful construction of the vectors.

Functional analyses performed through colony formation assays (Fig. 3E,F) and CCK-8 assays (Fig. 3G,H) revealed significant increases in the clonogenic capacity and proliferation rates following the knockdown of LRRC3-DT. These findings indicate that a reduction in LRRC3-DT expression leads to enhanced cell growth and survival, highlighting its role in promoting oncogenic characteristics. In contrast, the overexpression of LRRC3-DT resulted in a substantial decrease in these oncogenic properties, suggesting that higher levels of LRRC3-DT may inhibit cell proliferation and colony formation, thereby resulting in a tumor-suppressive effect.

Transwell assays provided additional evidence that the silencing of LRRC3-DT, achieved through sh-LRRC3-DT, effectively promoted both the migratory and invasive capabilities of HCC cells. In contrast, the overexpression of LRRC3-DT, indicated by oe-LRRC3-DT, had opposite effects, impairing the aggressive traits of the cancer cells (Fig. 4A–C). Furthermore, flow cytometry analysis revealed that the silencing of LRRC3-DT resulted in a significant suppression of apoptosis among the HCC cells. Conversely, the overexpression of LRRC3-DT was associated with increased apoptosis (Fig. 4D). Collectively, these findings substantiate the role of LRRC3-DT as a tumor suppressor gene, highlighting its function in the inhibition of HCC progression *in vitro*.

3.4 LRRC3-DT Inhibits HCC Development via Positive Regulation of CCDC81

To elucidate the molecular basis of the role of LRRC3-DT in HCC pathogenesis, we first mapped its subcellular distribution. FISH revealed that the majority of the LRRC3-DTs were localized in the nucleus (Fig. 5A). The results of RNA FISH verified that LRRC3-DT is predominantly localized in the nucleus of HCC cells. Combined with the published literature, these findings suggest that

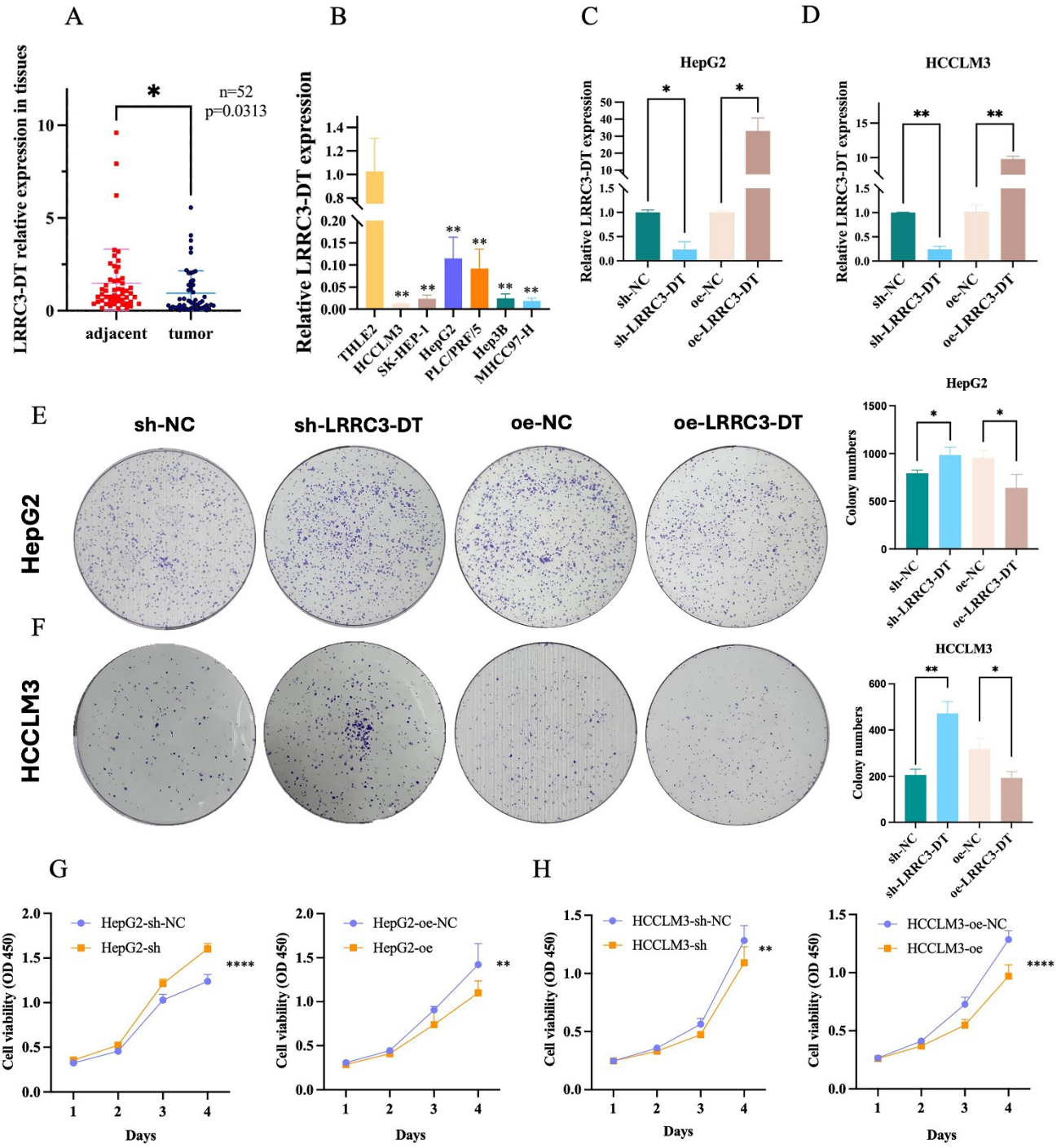

Fig. 3. LRRC3-DT inhibits the proliferation of HCC cells. (A) Comparison of the LRRC3-DT levels in HCC and adjacent normal tissues. (B) qRT-PCR was used to quantify LRRC3-DT expression in HCC cell lines (HCCLM3, SK-HEP-1, HepG2, PLC/PRF/5, Hep3B and MHCC97-H) and the normal cell line THLE2. (C,D) RT–qPCR detection of LRRC3-DT expression in HepG2 and HCCLM3 cells transfected with the shRNA and overexpression vector (oe). Colony formation assays (E,F) and Cell Counting Kit-8 (CCK-8) assays (G,H) were used to examine the effects of LRRC3-DT knockdown and overexpression on the proliferation of HepG2 and HCCLM3 cells. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

the presence of LRRC3-DTs in serum may be due to two distinct pathways. First, extensive apoptosis triggered by inflammation and tumor lesions disrupts the nuclear and plasma membranes, leading to the passive release of nuclear LRRC3-DTs into the bloodstream alongside cellular debris [25]. Second, viable tumor cells partially export LRRC3-DT from the nucleus to the cytoplasm via nuclear pores; subsequently, this transcript is sorted into ex-

Table 2. Clinicopathological analysis of tissue LRRC3-DTs.

Characteristic	n	LRRC3-DT expression		*p* value
		(high = 26)	(low = 26)	
Gender				
Male	38	21	17	0.211
Female	14	5	9	
Age (years)				
≤60	20	12	8	0.254
>60	32	14	18	
Tumor size (cm)				
≤5	37	19	18	0.760
>5	15	7	8	
TNM stage				
I+II	41	24	17	0.017*
III+IV	11	2	9	
Histological differentiation				
Well	15	7	8	
Moderate	29	17	12	0.231
Poor	8	2	6	

Note: TNM, tumor node metastasis; *$p < 0.05$.

osomes for secretion. Encapsulation within extracellular vesicles protects LRRC3-DTs from degradation by circulating RNases [26,27,28]. Notably, activated hepatic stellate cells and immune cells also secrete LRRC3-DT transcripts, which accounts for the poor discriminative ability of serum LRRC3-DT to distinguish HCC from benign liver diseases [29]. RNA sequencing analysis revealed LRRC3-DT as a potential regulator of downstream targets, including ankyrin repeat domain-containing protein 1 (ANKRD1), CCDC81, FMC1, homeobox D4 (HOXD4), and solute carrier family 16 member 12 (SLC16A12) (Fig. 5B). Subsequent qRT–PCR validation revealed that CCDC81 expression was reduced in HepG2 and HCCLM3 cells following LRRC3-DT knockdown compared with sh-NC controls, whereas compared with overexpression-negative control (oe-NC) controls, LRRC3-DT overexpression conversely increased CCDC81 levels (Fig. 5C,D). Bioinformatics analysis via the TIMER2.0 and UALCAN databases revealed elevated CCDC81 expression in HCC tissues (Fig. 5E,F). Compared with that in normal tissues, the expression of the CCDC81 gene in tumor tissues in HCC was significantly greater, and it increased with increasing tumor grade (Fig. 5G). It is speculated that this gene may be involved in the occurrence and development of HCC, and its expression level could serve as a potential biomarker reflecting tumor malignancy. High expression of CCDC81 may be associated with poor prognosis in patients with liver hepatocellular carcinoma (LIHC) (Fig. 5H). However, owing to insufficient statistical significance ($p = 0.26$), this association requires validation through larger sample sizes or more in-depth studies. These data collectively demonstrate that CCDC81 is a functional target of LRRC3-DT in HCC cells.

4. Discussion

HCC is a heterogeneous malignancy triggered by multiple risk factors, including hepatitis B virus infection, aflatoxin exposure, environmental hazards, and heritable and epigenetic alterations. Although conventional therapeutic regimens such as surgical resection, radiotherapy, and chemotherapy confer certain clinical benefits, patients diagnosed with advanced or metastatic HCC have a 5-year survival rate of less than 10% [1,30]. Unraveling the oncogenic cascades of HCC and constructing novel prognostic signatures may facilitate the development of innovative therapeutic strategies [31]. Currently, multiomics platforms covering genomics, proteomics, and metabolomics have been extensively adopted to screen HCC biomarkers, and advances in diverse biotechnologies have greatly accelerated the discovery of molecular indicators for HCC diagnosis and prognosis.

Regulation of enhancer activity mediated by microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) at the transcriptional level has emerged as a central research frontier in gene expression regulation [32,33,34]. Accumulating evidence indicates that various noncoding RNAs assemble intricate regulatory circuits to cooperatively govern metastatic progression in patients with HCC [35,36,37]. A recent comprehensive review systematically summarized the predominant pro-metastatic roles of miRNAs in HCC, revealing that dysregulated miRNAs restructure cellular motility, facilitate extracellular matrix degradation, and enable distant tumor colonization, thereby driving widespread tumor dissemination [38]. Enhancer RNAs (eRNAs) represent another novel subclass of noncoding transcripts exclusively transcribed from genomic enhancer loci [39,40]. The majority of eRNAs are divergent single-exon tran-

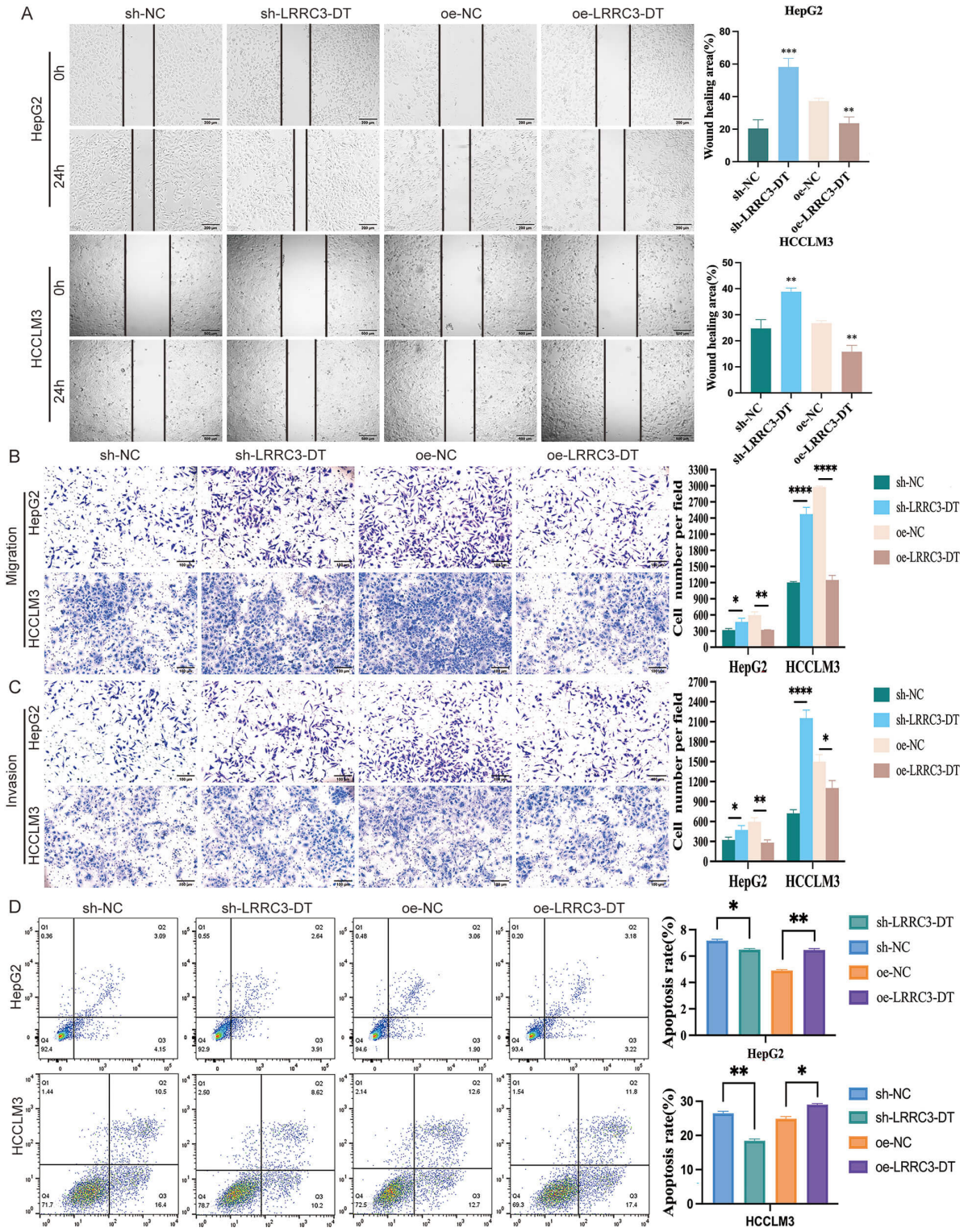

Fig. 4. LRRC3-DT inhibits the invasion ability of HCC cells. Cell scratch (A), migration (B), and invasion (C) assays and flow apoptosis analysis (D) were carried out in HCC cells with LRRC3-DT knockdown or overexpression. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Scale bars: 200 μm (upper part of A), 500 μm (lower part of A), 100 μm (B,C). Original magnification: ×10 (upper part of A), ×4 (lower part of A), ×20 (B,C).

scripts (2D-eRNAs) lacking polyadenylation modification [41]. As a special subset of lncRNAs transcribed from DNA enhancer regions, eRNAs modulate the transcriptional activity of neighboring protein-coding genes [42]. For instance, PSA eRNA originates from an enhancer element of the PSA gene; it increases androgen receptor (AR) ex-

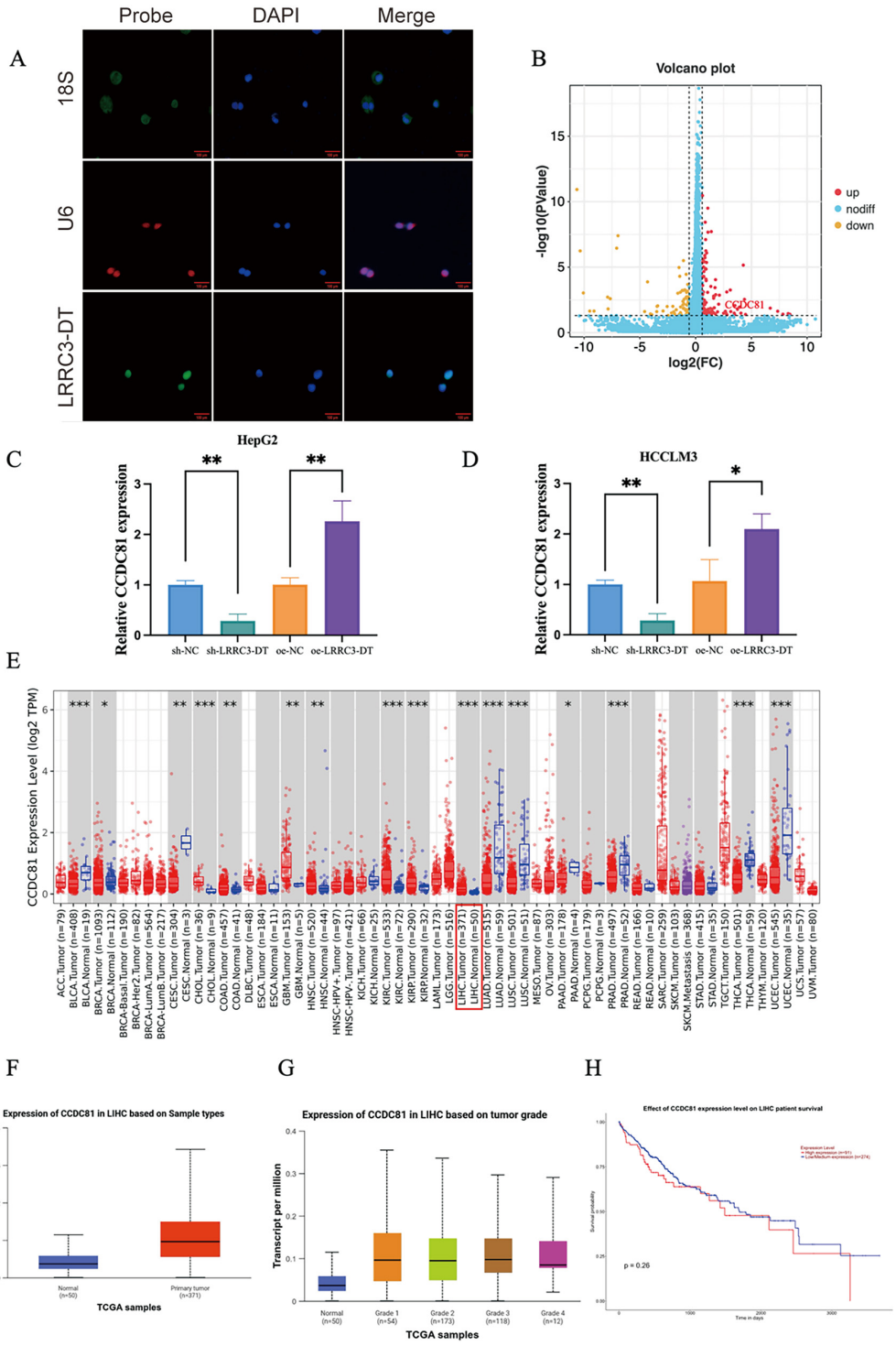

Fig. 5. LRRC3-DT inhibits HCC progression by positively regulating CCDC81 expression. (A) FISH was used to determine the localization of LRRC3-DT in HCCLM3 cells. 18S rRNA and U6 were utilized as cytoplasmic and nuclear controls, respectively. (B) Identification of LRRC3-DT-regulated genes and transcripts via RNA-seq (RNA sequencing). (C,D) RT–qPCR was used to detect the expression of CCDC81 in HepG2 and HCCLM3 cells after transfection. (E) TIMER 2.0 database analysis of CCDC81 expression levels in HCC tissues. (F,G) Analysis of the expression level of CCDC81 in HCC tissues and different pathological stages in the UALCAN database. (H) There was no difference in the survival of patients with HCC between those with low and high expression of CCDC81 according to the TIMER 2.0 database. CCDC81, coiled-coil domain containing 81; FISH, fluorescence *in situ* hybridization. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bars: 100 μm (A). Original magnification: ×20 (A).

pression in tumor cells and promotes disease progression in castration-resistant prostate cancer (CRPC) [43]. Such observations suggest that eRNAs are promising candidate biomarkers for malignancies. In the present research, we identified LRRC3-DT, an eRNA with tumor-suppressive properties, which robustly represses the migration and invasion of HCC cells and is strongly correlated with the expression of CCDC81. Collectively, these findings expand our understanding of noncoding RNA-mediated regulatory axes that underpin malignant transformation in HCC.

Numerous oncogenic lncRNAs with high evolutionary conservation across mammals, whose biological functions in diverse cellular events have been thoroughly characterized, have been identified in HCC [44,45]. The lncRNA high expression in hepatocellular carcinoma (HEIH), initially discovered in HBV-positive HCC, physically interacts with enhancer of zeste homolog 2 (EZH2), a core subunit of the polycomb repressive complex 2 (PRC2) complex. Via an EZH2-dependent signaling axis, HEIH counteracts PRC2-induced transcriptional repression of downstream targets, accelerates cell cycle transition and increases tumor proliferation, and its intratumoral expression level serves as a prognostic marker for patients with HCC [46]. The oncogenic lncRNA HULC is aberrantly overexpressed in HCC, gastric cancer and colorectal cancer, and its upregulation is correlated with poor clinical outcomes in patients with HCC and pancreatic cancer. Circulating HULC in peripheral blood can act as a noninvasive diagnostic marker for HCC, and this transcript modulates multiple liver metabolic and signaling pathways to shape diverse malignant phenotypes of HCC cells [47]. Another HCC-inducible lncRNA, HOXA transcript at the distal tip (HOTTIP), is located adjacent to homeobox A13 (HOXA13) and orchestrates transcription of the entire HOXA locus; abnormal activation of embryonic development-related transcription factors encoded by this locus is tightly linked to HCC tumorigenesis [48].

In contrast to the transcriptional activation effects exerted by oncogenic noncoding RNAs, the LRRC3-DT identified in this study mediates transcriptional repression and robustly abrogates malignant phenotypes in HCC cells, with its function likely dependent on the downstream effector CCDC81. These findings establish a previously underappreciated regulatory paradigm in which noncoding RNAs negatively modulate enhancer activity, highlighting the core novelty of our work compared with prior studies focusing on miRNA-driven enhancer activation in HCC. The results from clinical specimen detection and cellular functional assays demonstrated decreased LRRC3-DT levels in serum samples from both patients with HCC and individuals with benign liver lesions. Consistent with these findings, LRRC3-DT expression was markedly suppressed in HCC cell lines and tumor tissues, whereas exogenous overexpression of LRRC3-DT effectively restrained malignant progression. Further cohort correlation analyses revealed that low LRRC3-DT expression was significantly associated with poorly differentiated tumors, shortened disease-free survival and reduced overall survival in patients with HCC.

Nevertheless, several limitations exist regarding the serum samples used in this study. First, chronic hepatitis and liver fibrosis independently trigger the release of extracellular RNA into the peripheral circulation. Persistent hepatic inflammatory injury occurred in both the HCC and benign liver disease groups, leading to indistinguishable serum LRRC3-DT concentrations between the two cohorts. These findings suggest that LRRC3-DTs lack sufficient tumor specificity, limiting its standalone value for differentiating benign and malignant hepatic lesions. Second, we did not conduct a priori power analysis to calculate the required sample size before recruitment. Thus, we cannot statistically verify whether the current cohort size possesses adequate statistical power to reliably distinguish HCC from benign liver disorders, and sampling bias caused by insufficient sample numbers cannot be fully ruled out. Future work will perform prospective sample size estimation based on effect sizes derived from preliminary experiments to guarantee sufficient statistical power to rigorously validate the diagnostic and prognostic value of LRRC3-DTs in patients with HCC.

Notably, transcriptome sequencing was performed to screen potential target genes of LRRC3-DT, among which CCDC81 was prioritized. The literature has reported that coiled-coil domain containing 81 (CCDC81) belongs to the CCDC family of proteins, whose nomenclature suggests that it contains a coiled-coil domain, which mainly mediates protein-to-protein interactions [49]. CCDC81 is enriched in the centrosomes of mammalian spermatozoa and may be involved in sperm motility or the stabilization of centrosomes during the process of fertilization [50]. Additionally, CCDC81 may play a role in vascular formation and the recruitment of pericentriolar material (PCM) through interactions with other centrosomal proteins, such as gamma-tubulin [51]. Zhang JZ et al. (2019) [52] reported that CCDC81 is significantly differentially expressed in nasopharyngeal carcinoma (NPC) and may be involved in the regulation of the tumor immune microenvironment. In 2021, Ren J et al. [53] reported that CCDC81 may affect the tumor antigenic response in lung adenocarcinoma (LUAD) through the modulation of cellular adhesion or the Wingless-related integration site (WNT)/phosphoinositide 3-kinase (PI3K) signaling pathway. The methylation level of CCDC81 has also been proposed as a potential biomarker for early diagnosis and prognostic monitoring of LUAD. As an enhancer-derived RNA, LRRC3-DT cis-activates the transcription of its neighboring gene CCDC81. Nevertheless, LRRC3-DT exerts pleiotropic regulatory effects and simultaneously governs multiple tumor-associated signaling pathways beyond CCDC81. Although LRRC3-DT transcriptionally up-

regulates CCDC81, this lncRNA concurrently triggers a series of potent tumor-suppressive signaling cascades that dominate the overall cellular phenotype within our experimental system. CCDC81 only confers mild oncogenic potential in HCC cells, and the robust anti-tumor signals initiated by LRRC3-DT overexpression can fully mask its weak pro-malignant activity. Upon LRRC3-DT knockdown, all downstream tumor-suppressive axes mediated by LRRC3-DT are impaired. Meanwhile, the reduced abundance of CCDC81 disrupts primary cilia stability, and the resulting oncogenic effects become functionally predominant, ultimately leading to markedly aggravated malignant phenotypes in HCC cells.

In conclusion, this study confirms that LRRC3-DT is significantly downregulated in both the tumor tissues and peripheral serum of patients with HCC. Nevertheless, serum LRRC3-DT exhibits insufficient tumor specificity, failing to effectively distinguish hepatic malignant lesions from benign liver diseases. As a nucleus-localized tumor-suppressive eRNA, LRRC3-DT hinders the malignant progression of HCC presumably by transcriptionally upregulating CCDC81. However, the definitive regulatory axis and molecular mechanism underlying the LRRC3-DT/CCDC81-mediated modulation of HCC development remain to be further validated. Collectively, our findings preliminarily reveal the potential clinical and biological value of LRRC3-DT in HCC, providing a novel tentative molecular clue and theoretical reference for subsequent exploration of HCC pathogenesis and targeted therapeutic strategies.

5. Limitations

This study has several limitations that warrant further investigation. First, the inability of serum LRRC3-DTs to discriminate patients with HCC from individuals with benign hepatic lesions can be attributed to multiple confounding factors, including an insufficient sample size and relatively weak tumor specificity of this transcript. Accordingly, expanding the serum sample cohort and implementing pre-experimental statistical power calculations are needed to achieve more reliable intergroup comparisons. Second, we did not collect samples from digestive system tumors other than HCC; thus, we cannot determine whether serum LRRC3-DTs can distinguish HCC from other digestive cancers. Consequently, additional specimens from tumors such as pancreatic, gastric, and colorectal cancers should be included for validation. Third, we did not monitor postoperative serum LRRC3-DT expression levels in surgically resected patients with HCC, nor did we conduct follow-ups to analyze survival differences between patients with HCC with high and low LRRC3-DT expression. Finally, only qRT-PCR was adopted to verify their expression correlation, without rescue functional experiments to confirm the regulatory interaction between LRRC3-DT and CCDC81. The complete upstream and downstream signalling cascades through which LRRC3-DT reverses HCC malignant behaviours remain largely unelucidated. These factors highlight areas for improvement.

6. Conclusions

The results of the present study revealed notably decreased expression of LRRC3-DT in both HCC tissue and serum samples. Additionally, by acting mainly as a tumor suppressor in the nuclear compartment, LRRC3-DT is likely to influence HCC progression by modulating the expression of CCDC81. Nevertheless, additional validation is necessary to determine the clinical relevance of LRRC3-DTs in differentiating HCC from noncancerous liver conditions.

Availability of Data and Materials

The datasets utilized and analyzed in the current study are available from the corresponding author upon reasonable request.

Author Contributions

JL and YL: Methodology, Resources, Writing – original draft; ML: Data curation, Formal analysis; ZY: Methodology, Resources; LY and HW: Data curation, Resources; LC: Formal analysis, Funding acquisition, Writing – review & editing; LJ: Conceptualization, Writing – review & editing. All the authors contributed to editorial changes in the manuscript. All the authors read and approved the final manuscript. All the authors have participated sufficiently in the work and agreed to be accountable for all the aspects of the work.

Ethics Approval and Consent to Participate

The studies involving human participants and the collection of clinical specimens were approved by the Medical Ethics Committee of Nantong Third People's Hospital (Approval Number: EK2022023). Written informed consent was acquired from the legal guardians or next of kin of all participants, in compliance with the principles of the Declaration of Helsinki.

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Conflicts of Interest

The authors declare no conflicts of interest.

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