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LncRNA NCK1-AS1 Aggravates Hepatocellular Carcinoma by the miR-22-3p/YARS Axis to Activate PI3K/AKT Signaling

Weixin Zhou, Jie Wang, Jie Zhang, Yuhan Wang, Ling Jiang, Tianhong Guo, Binrui Luo, Qi Xu, Yuanshuai Huang

Department of Transfusion, the Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, China

Address for correspondence:
Yuanshuai Huang
Affiliated Hospital of Southwest Medical University, No. 25, Taiping Street, Jiangyang District, Luzhou 646000, Sichuan, China;
hysys@126.com

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is frequently diagnosed at late stages when curative treatments are no more appliable. Many studies have proved the active role of long non-coding RNAs (lncRNAs) in cancers' biology; here, the functional role of lncRNA NCK1-AS1 in HCC was identified.
Methods: Gene expression in tumor tissues of HCC was evaluated by examining online databases and 88 collected HCC samples from our hospital. The interactions of miR-22-3p with NCK1-AS1 and tyrosyl-tRNA synthetase (YARS) were tested by conducting bioinformatics analysis, luciferase report, and RNA pulldown experiments. CCK-8, colony formation, flow cytometry, wound healing, transwell experiments were used to dissect the role of the NCK1-AS1/miR-22-3p/YARS axis in HCC.
Results: NCK1-AS1 was overexpressed in HCC cells and tissues. Functional assays depicted that depletion of NCK1-AS1 hampered malignant character of HCC cells. NCK1-AS1 controlled the availability of miR-22-3p, resulting in YARS upregulation. YARS was found to have a clinical value for HCC diagnosis. Moreover, rescue experiments revealed that miR-22-3p inhibition or YARS overexpression partially blocked the function of NCK1-AS1 deficiency in HCC cells. As for the downstream signaling pathway, we discovered that NCK1-AS1 activated PI3K/AKT signaling by the miR-22-3p/YARS axis.
Conclusion: The present study verified that NCK1-AS1 could promote HCC progression via the miR-22-3p/YARS axis to activate PI3K/AKT signaling.

Key words: NCK1-AS1 – YARS - miR-22-3p – hepatocellular carcinoma – PI3K/AKT.

Abbreviations: ceRNA: competitive endogenous RNA; HCC: hepatocellular carcinoma; lncRNA: long non-coding RNA; miRNA: microRNA; NCK1-AS1: noncatalytic region of tyrosine kinase adaptor protein 1 antisense RNA 1; YARS: tyrosyl-tRNA synthetase.

INTRODUCTION

Due to high mortality and increasing incidence, hepatocellular carcinoma (HCC) is the most malignant primary liver cancer all over the world [1, 2]. Every year, more than 500,000 people suffer from HCC, with low long-term survival rates [3]. Over the past decades, some progress in HCC treatment such as liver transplantation or curative surgery has been made; however, the clinical outcomes for patients remain less than satisfactory [4]. Most of the HCC patients have a poor prognosis due to the drug resistance and tumor reoccurrence [5]. In recent years, many studies have explored various biomarkers in HCC, but the underlying molecular mechanisms of many of these biomarkers need to be further uncovered. Hence, it is urgent to develop more biological targets associated with the prohibition of HCC development and figure out the molecular mechanisms regarding their regulation.

Nowadays an increasing number of studies showed that long noncoding RNAs (lncRNAs), comprising over 200 nucleotides, were the most common non-protein-coding transcripts [6], widely regulating the cell survival, growth, metastasis, invasion, differentiation, and apoptosis [7, 8]. MicroRNAs (miRNAs) are short endogenous ncRNA transcripts, regulating both intracellular physiologic and pathologic processes [9]. Mechanically, lncRNAs can perform as competitive endogenous RNAs (ceRNAs) to decoy specific miRNAs, followed by the prevention of the miRNA-mediated inhibition of downstream target expression [10]. Numerous lncRNAs display either oncogenic or anti-oncogenic roles by acting as

ceRNAs in cancer, including in HCC. For example, lncRNA MYLK-AS1 contributes to angiogenesis and tumorigenesis in HCC by the miR-424-5p/E2F7 axis [11]. LncRNA SNAI3-AS1 decoys miR-27a-3p to promote the PEG10-mediated HCC proliferation and metastasis [12]. LncRNA ZFPM2-AS1 works as a miRNA-139 sponge to accelerate HCC cell invasion through upregulating GDF10 [13]. Previous studies verified that lncRNA NCK1-AS1 functions as a cancer-promoting molecule in the regulation of cancer development [14, 15]. Intriguingly, NCK1-AS1 contributes to tumor progression by sponging miRNAs through a ceRNA network. NCK1-AS1 increases melanoma cell survival via decoying miR-526b-5p to overexpress ADAM15 [16]. NCK1-AS1 absorbs miR-22-3p to positively regulate BCL9 in gastric cancer [17]. Nevertheless, the role of NCK1-AS1 in HCC is not elucidated.

Based on the aforementioned information, we hypothesized that NCK1-AS1 could affect HCC progression through a ceRNA network. We determined the expression status of NCK1-AS1 in HCC samples and adjacent non-tumor tissues and determined its functional role in HCC cells. The relevant mechanisms were investigated to verify our hypothesis.

METHODS

Eighty-eight HCC samples and paired adjacent non-tumor tissues were collected in HCC patients after surgical resection at the Affiliated Hospital of Southwest Medical University. Tissue samples confirmed by experienced pathologists were stored at -80^{0}C. Informed consent for this study was obtained from all patients and this research was conducted with the approval from the Ethics Committee of the Affiliated Hospital of Southwest Medical University. None of these patients had previously received HCC treatment.

Cells and Transfection

Human HCC cell lines Hep-3B, Huh7, HepG2, SK-Hep1 and normal liver cell line L02, provided by the ATCC (VA, USA), were cultured in RPMI 1640 medium enriched with 10% FBS (Gibco, Invitrogen, USA). Cells were maintained in the humid conditions with 5% CO_2 at 37^{0}C.

Hep-3B and Huh7 cells in 6-well plates were transfected with shRNAs for NCK1-AS1 (sh-NCK1-AS1#1, GGATACAAATACAAAGTTA; sh-NCK1-AS1#2, GGTTCTGTGTTTCAAATTA), sh- tyrosyl-tRNA synthetase (YARS) (GGTTTGTAGTTATATGCTA), negative control (sh-NC, ATTTGTATTTTAGTGGGAC), NC inhibitor (AUUGAUUCCCUGUGACAAUCGC), miR-22-3p inhibitor (ACAGUUCUUCAACUGGCAGCUU), NC mimics (CCGGGACCAAUUAUGUAGAGAU), miR-22-3p mimics (AAGCUGCCAGUUGAAGAACUGU) using Lipofectamine RNAiMAX (Gibco) following the manufacturer's protocols. Overexpression plasmid of YARS was acquired by inserting full-length YARS into the pcDNA3.1 vector. Empty vector pcDNA3.1 acted as a NC. All plasmids were obtained from Sangon Biotech (Shanghai, China). Transfection lasted for 48 h.

Public Database Analysis

StarBase (ENCORI) (http://starbase.sysu.edu.cn/index.php) was examined to investigate the NCK1-AS1 level in 374 HCC samples and 50 adjacent non-tumor tissues samples, the expression association among miR-22-3p, NCK1-AS1, and YARS in HCC samples, and the survival of HCC patients with aberrant miR-22-3p or YARS expression. The YARS level in HCC in TCGA and prognostic values of NCK1-AS1 expression for HCC were retrieved by examining GEPIA database (http://gepia.cancer-pku.cn/). UALCAN (http://ualcan.path.uab.edu/index.html) and Kaplan Meier plotter (http://kmplot.com/analysis/) were used to evaluate the YARS level and prognostic values of YARS in HCC.

Table I. Correlations between NCK1-AS1 expression and clinical parameters in HCC patients

Characteristic	Cases	NCK1-AS1 expression		p
	88	Low (n=44)	High (n=44)	
Age (years)				0.647
<60	60	31	29	
≥60	28	13	15	
Gender				0.088
Male	46	27	19	
Female	42	17	25	
Tumor size (cm)				0.375
<5	32	14	18	
≥5	56	30	26	
Tumor number				0.83
Solitary	39	20	19	
Multiple	49	24	25	
TNM stage				0.031
I-II	50	20	30	
III-IV	38	24	14	
AFP (ng/mL)				0.647
<400	40	22	18	
≥400	44	22	22	
Liver cirrhosis				0.393
Absent	42	23	19	
Present	46	21	25	
HBV				0.085
Yes	50	29	21	
No	38	15	23	
Histological differentiation				0.20
Well	46	20	26	
Moderate/Poor	42	24	18	
BCLC stage				0.018
A	39	25	14	
B-C	49	19	30	
Survival status				0.001
Live	37	11	26	
Dead	51	33	18	

HCC: hepatocellular carcinoma; AFP: alpha-fetoprotein; TNM: tumor-node-metastasis; HBV: hepatitis B virus; BCLC: Barcelona clinic liver cancer stage.

RT-qPCR

Total RNA was extracted using TRIzol (Invitrogen). For cDNA synthesis, extracted RNA was reverse transcribed with the

High-Capacity RNA-to-cDNA Kit (Applied Biosystems, USA). The SYBR® Premix Ex TaqTM II Kit (Takara, Japan) was applied to perform RT-qPCR with Thermal Cycler Dice Real-Time PCR System (Takara). Relative expression of genes was determined with the comparative $2^{-\Delta\Delta Ct}$ method, using GAPDH and U6 as internal controls. Primer sequences are as follows: NCK1-AS1 forward: 5'-GGAGAAATGGGAACTCAAACC-3', NCK1-AS1 reverse: 5'-CCTGATTCTGGTTAGTCCCT-3', miR-22-3p forward: 5'-AAGCTGCCAGTTGAAGAACTGTA-3', miR-22-3p reverse: 5'-CAATTCAGTTGAGACAGTTCT-3', YARS forward: 5'-ATCACTGTGGAGAAGCACC-3', YARS reverse: 5'-GACTCCTCTCATCTTCTGGG-3', GAPDH forward: 5'-TCAAGATCATCAGCAATGCC-3', GAPDH reverse: 5'-CGATACCAAAGTTGTCATGGA-3', U6 forward: 5'-ATACAGAGAAAGTTAGCACGG-3', U6 reverse: 5'-GGAATGCTTCAAAGAGTTGTG-3'.

Cell Proliferation Assays

Hep-3B and Huh7 cells seeded in 96-well plates (2×10^3 cells/well) were maintained overnight. At 0, 24, 48 and 72 h, 10 µl CCK-8 reagent (Beyotime, Haimen, China) was added to the plates and cells were grown for 4 h at 37°C. A microporous plate reader (Tecan Company) at 450 nm was used to observe the absorbance values. For the colony formation experiment, Hep-3B and Huh7 cells (500 cells/well) were plated in 6-well plates. The fresh medium was used, and cells were cultured for continuous 10 days. After washing with PBS, the colonies were fixed with 70% methanol before staining with 0.5% crystal violet, and then counted under a microscope. Assays were performed in duplicate.

Cell Apoptosis Assay

Cell apoptosis was analyzed with the Annexin-V FITC Kit (C1063, Beijing, China) following the manufacturer's protocols. Briefly, Hep-3B and Huh7 cells were centrifuged at 1000 r/min, suspended, and centrifuged again. The supernatant was removed and suspended in 5 ml binding buffer. Afterwards, 5 µl Annexin V FITC and PI staining solution was used to stain cells for 10 min in the dark. Apoptosis was analyzed by flow cytometry (BD Biosciences, NJ, USA).

Wound Healing Assay

Hep-3B and Huh7 cells were seeded in 6-well plates (5×10^5 cells/well) and incubated overnight in FBS-free medium. When cells grew in full monolayer, a wound line was created by scratching the surface of plates using a 200 µL sterile plastic tip. Cellular debris was washed with PBS. Cells were incubated for 48 h in serum-free medium. Wound images at 0 and 48 h were taken with a phase-contrast microscope. The migrating distance was measured using Image J software.

Transwell Assay

Migration and invasion of cells with different treatment were measured with 24-well chambers with 8 µm pore size (Corning, NY, USA). The chambers were precoated with (for invasion assay) or without (for migration assay) Matrigel. Cells (1×10^5) with serum-free medium were placed in the upper chamber. Then, the medium with 10% FBS was added to the lower chamber. Forty-eight hours later, non-invaded or non-migrated cells were carefully wiped off. The remaining cells were fixed and stained with 0.1% Giemsa for 10 min. Then, 5 fields were picked out randomly, and cells was counted under a microscope.

Western Blotting

Total protein in cells was extracted by RIPA lysis buffer. Next, the proteins were separated by 12% SDS-PAGE electrophoresis and transferred onto PVDF membranes. Membranes were then blocked with 5% skim milk powder and incubated with the primary antibodies overnight at room temperature. Membranes were then incubated with the secondary antibody for 2 hours at 4°C. The protein bands were detected using the ECL Kit (ThermoFisher, USA). Primary antibodies are presented as follows: E-cadherin (ab40772, 1:10000, Abcam, USA), N-cadherin (ab76011, 1:5000), YARS (ab150429, 1:1000), P-mTOR (#5536, 1:1000, Cell Signaling Technology), mTOR (#2983, 1:1000), P-ERK (#4370, 1:1000), ERK (#4695, 1:1000), P-AKT (#4060, 1:1000), AKT (#4691, 1:1000), P-PI3K (#9655, 1:1000), PI3K (#4249, 1:1000), and GAPDH (#5174, 1:1000).

FISH

The localization of NCK1-AS1 in Hep-3B and Huh7 cells was assessed using FISH Kit (RiboBio, Guangdong, China). NCK1-AS1 probe or control probe was synthesized and labeled with Cy3 by Sangon Biotech (Shanghai, China). Cells were fixed, permeabilized, and hybridized with 20 µM Cy3-labeled probes overnight. The nuclei were stained with DAPI (Sigma-Aldrich, USA) and recorded with a fluorescence microscope.

Luciferase Reporter Assay

The biological prediction website starBase was used to seek the predicted binding site between genes. NCK1-AS1 or YARS sequence containing wild-type (Wt) or mutant (Mut) miR-22-3p response elements was inserted into the pmirGLO (Promega) to produce NCK1-AS1-Wt/Mut or YARS-Wt/Mut plasmids, which were provided by GeneCreate (Hubei, China). NCK1-AS1-Wt/Mut or YARS-Wt/Mut plasmids were synchronously transfected with miR-22-3p mimics into Hep-3B and Huh7 cells, respectively, for 2 days. Luciferase activity was analyzed with luciferase assay system (Promega).

RNA Pulldown Assay

Pulldown assay was used to detect the potential binding capacity of NCK1-AS1 and miR-137, miR-1197, and miR-22-3p. Bio-NCK1-AS1 and Bio-NC that were purchased from Sangon (Shanghai, China) were cultured with streptavidin agarose beads (Life Technologies, USA). The cell lysates of Hep-3B and Huh7 cells were added with Bio-NCK1-AS1 or Bio-NC probe-covered beads at 4°C. Afterwards, the RNA complexes in the beads were eluted. The bound RNAs were subjected to RT-qPCR for measurement.

Statistical Analysis

Statistical analysis was conducted with SPSS 22.0 (SPSS, USA). Data were displayed as the mean ± standard deviation (SD). Each assay was performed in triplicate. Spearman's correlation method was applied to show the correlation between genes. Student's *t* test, one-way and two-way ANOVA

were employed to evaluate group differences. A p value < 0.05 was considered significant.

RESULTS

NCK1-AS1 Expression in HCC Samples

The starBase v3.0 online database shows that NCK1-AS1 has a markedly high level in 374 HCC samples (Fig. 1A). We further analyzed the NCK1-AS1 level in the collected HCC tissues for this research. As shown in Fig. 1B, NCK1-AS1 expression in HCC tissues (n=88) was much higher than in normal tissues (n=88). Furthermore, the patients in advanced stages of HCC (n=46) had higher NCK1-AS1 expression than those in early stages of HCC (n=42) (Fig. 1C). Then we analyzed the effect of NCK1-AS1 expression on the overall survival of patients, and the data of GEPIA show that HCC patients with high NCK1-AS1 expression tend to have significantly poor overall survival (Fig. 1D). Correlation analysis of NCK1-AS1 expression with HCC clinicopathological characteristics revealed that NCK1-AS1 expression was associated with TNM stage (p=0.031), BCLC stage (p=0.018), survival status (p=0.001), but not related to age, gender, tumor size, tumor number, AFP, liver cirrhosis, HBV and histological differentiation (Table I). Univariate and multivariate COX regression analyses demonstrated that TNM stage, BCLC stage, survival status and high NCK1-AS1 expression were independent predictive factors of the overall survival rate of HCC patients (Table II). Additionally, we detected NCK1-AS1 expression in HCC cells, and normal liver cell line L02 served as a control. NCK1-AS1 expression in HCC cell lines was notably upregulated (Fig. 1E). To understand the function and mechanisms of NCK1-AS1 in HCC cells, its subcellular location in Hep-3B and Huh7 cells was determined. NCK1-AS1 was chiefly located in the cytoplasm (Fig. 1F), implying the role of NCK1-AS1 at the post-transcriptional level.

NCK1-AS1 Functions in HCC Progression

Next, we explored whether NCK1-AS1 functions in HCC progression. The sh-NCK1-AS1#1/#2 was transfected into Hep-3B and Huh7 cells (Fig. 2A). The two NCK1-AS1 shRNAs significantly downregulated NCK1-AS1 expression in HCC cells, in which sh-NCK1-AS1#1 showed a relatively higher interference effect (Fig. 2A). Then, to reveal the impact of NCK1-AS1 in cell viability, we performed CCK-8 and found that HCC cell viability was markedly prohibited by NCK1-AS1 knockdown (Fig. 2B). Similarly, the colonies in

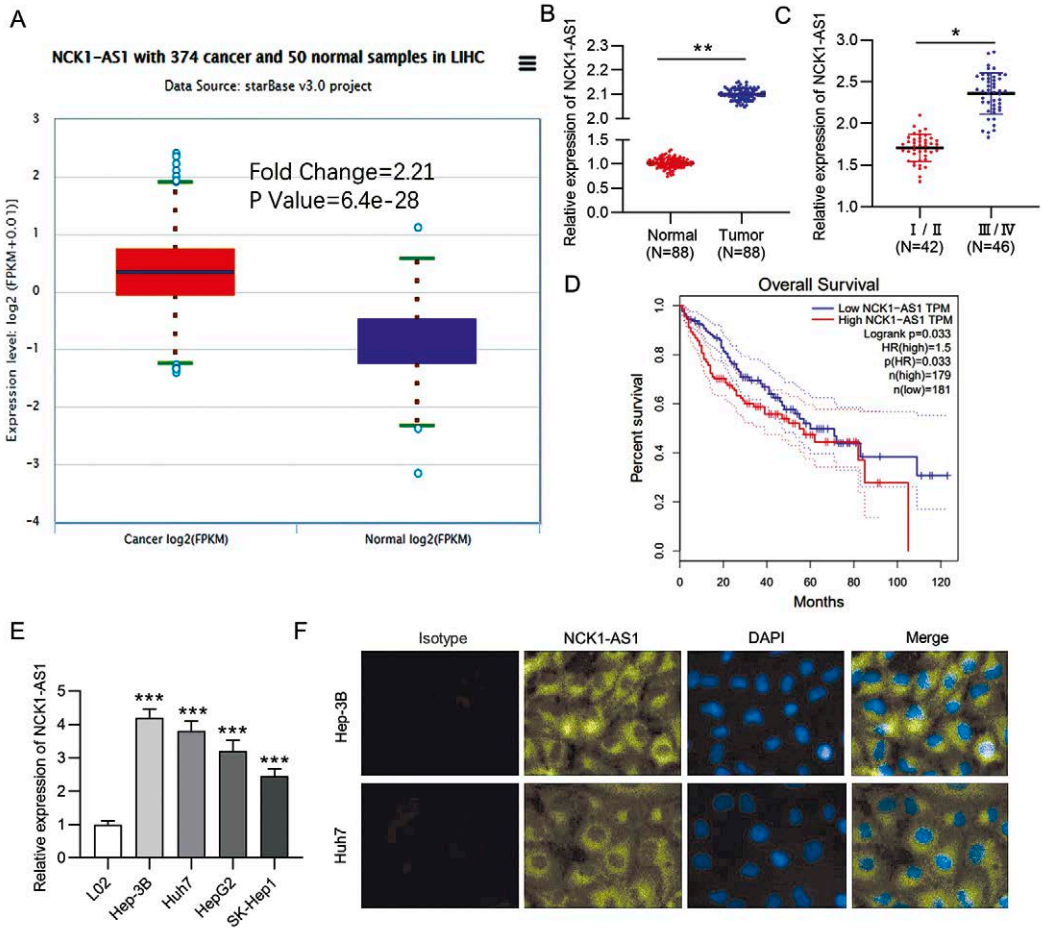

Fig.1. NCK1-AS1 is upregulated in HCC. (A) StarBase data for the NCK1-AS1 level in 374 HCC tissues and 50 normal samples; (B) RT-qPCR of NCK1-AS1 expression in human HCC tissues (n = 88); (C) NCK1-AS1 expression in different clinical stages of HCC patients; (D) GEPIA data for the overall survival of HCC patients with high and low NCK1-AS1 level; (E) NCK1-AS1 expression in HCC cells and L02 cell line; (F) Subcellular location of NCK1-AS1 in Hep-3B and Huh7 cells detected by FISH *p < 0.05, **p < 0.01, ***p < 0.001.

Table II. Univariate and multivariate Cox analysis of clinical parameters for overall survival in HCC patients

Characteristic	Univariate analysis			Multivariate analysis		
	HR	95%CI	p	HR	95%CI	p
Age (<60 vs. ≥60)	0.917	0.363-2.318	0.855	0.593	0.129-2.732	0.502
Gender (Male vs. Female)	0.630	0.255-1.559	0.318	0.525	0.146-1.889	0.324
Tumor size (cm) (<5 vs. ≥5)	2.142	0.764-6.003	0.147	0.384	0.050-2.943	0.357
Tumor number (Solitary vs. Multiple)	0.617	0.200-1.903	0.400	0.274	0.057-1.321	0.107
AFP (ng/mL) (<400 vs. ≥400)	1.205	0.477-3.044	0.694	4.958	0.347-70.826	0.238
Liver cirrhosis (absent vs. present)	1.326	0.499-3.519	0.571	0.302	0.063-1.450	0.135
HBV (Yes vs. No)	1.657	0.624-4.403	0.311	3.148	0.498-19.913	0.223
Histological differentiation (Well vs. Moderate/Poor)	0.984	0.307-3.156	0.979	0.392	0.071-2.155	0.281
TNM stage (I-II vs. III-IV)	3.898	1.262-12.036	0.018	12.808	2.490-65.887	0.002
BCLC stage (A vs. B-C)	3.464	1.300-9.232	0.013	25.199	3.294-192.778	0.002
Survival status (live vs dead)	3.464	1.300-9.230	0.01	25.2	3.294-192.780	0.002
NCK1-AS1 expression (low vs. high)	0.156	0.050-0.487	0.001	0.026	0.002-0.277	0.003

HCC: hepatocellular carcinoma; HR: hazard ratio; CI: confidence interval; AFP: alpha-fetoprotein; TNM: tumor-node-metastasis; HBV: hepatitis B virus; BCLC: Barcelona clinic liver cancer stage.

the sh-NCK1-AS1#1 group were notably attenuated (Fig. 2C). After that, we examined cell apoptosis by performing flow cytometry and found Hep-3B and Huh7 cell apoptosis was promoted by NCK1-AS1 silencing (Fig. 2D). Moreover, the results of wound healing and Transwell assays showed that NCK1-AS1 silencing blocked the migration and invasion of HCC cells (Fig. 2E-2G). As shown by western blotting, the sh-NCK1-AS1#1 group had markedly upregulated E-cadherin and downregulated N-cadherin compared to the sh-NC group (Fig. 2H). Overall, we concluded that NCK1-AS1 may be an oncogene in HCC.

Interactions between Specific MiRNA-22-3p and NCK1-AS1

Considering the localization of NCK1-AS1, we supposed that NCK1-AS1 may interact with specific miRNAs. The starBase website was utilized, which showed that there are 3 miRNAs that might bind to NCK1-AS1: miRNA-22-3p, miRNA-137 and miRNA-1197 (Condition: Pan-Cancer ≥ 5 cancer types). By RNA pulldown assay, we found that in the group of NCK1-AS1 probe-biotin, miRNA-22-3p showed the most enrichment in Hep-3B and Huh7 cells compared with miRNA-137 and miRNA-1197 (Fig. 3A). NCK1-AS1 harbors a binding site for miRNA-22-3p (Fig. 3B). We successfully upregulated miRNA-22-3p in Hep-3B and Huh7 cells by transfection of miRNA-22-3p mimics (Fig. 3C). As seen in Fig. 3D, the luciferase activity of NCK1-AS1-Wt was significantly decreased by miRNA-22-3p mimics, and NCK1-AS1-Mut presented no marked change in the miRNA-22-3p mimics and NC mimics groups, indicating that NCK1-AS1 binds to miRNA-22-3p. Moreover, miRNA-22-3p was significantly upregulated by sh-NCK1-AS1#1 (Fig. 3E). It was detected that miRNA-22-3p had a low level in HCC cell lines and clinal tissues (Fig. 3F-3G). Based on the online website, the survival of HCC patients with low miRNA-22-3p expression is poorer than that of patients with high miRNA-22-3p expression ($p<0.05$) (Fig. 3H). Additionally, a negative association between NCK1-AS1 and miRNA-22-3p in HCC tissues was found, which was demonstrated in our study (Fig. 3I) and online database (Fig. 3J).

Role of MiRNA-22-3p in the Regulation of NCK1-AS1 in HCC

To test whether miRNA-22-3p is involved in the regulation of NCK1-AS1 in HCC, we transfected Hep-3B and Huh7 cells with sh-NC, sh-NCK1-AS1#1, and sh-NCK1-AS1#1 plus miR-22-3p inhibitor. Efficiency of silencing miR-22-3p was verified (Fig. 4A). MiR-22-3p downregulation notably prevented the suppressive role of silencing NCK1-AS1 in HCC cell proliferation (Fig. 4B-4C). Flow cytometry analysis manifested that miR-22-3p inhibitor decreased the number of apoptotic cells induced by sh-NCK1-AS1#1 (Fig. 4D). Then, miR-22-3p inhibitor restored the migration and invasion in the sh-NCK1-AS1#1 plus miR-22-3p inhibitor group, as shown by wound healing and Transwell assays (Fig. 4E-4G). Similarly, E-cadherin upregulation and N-cadherin downregulation caused by the knockdown of NCK1-AS1 were partially reversed after silencing miR-22-3p (Fig. 4H).

Target Genes of miRNA-22-3p in HCC

The bioinformatics analysis was applied to search the putative targets of miRNA-22-3p in starBase (search category: Clip-Data >=5; PicTar, RNA22, microT). As Fig. 5A showed, we got 5 target genes (YARS, RCOR1, WRNIP1, BCL9, FBXL19). After miRNA-22-3p overexpression in HCC cells, their expression levels were determined, with the results showing that YARS presented the most downregulation among five genes (Fig. 5B). There is a binding region between YARS and miR-22-3p (Fig. 5C). In the luciferase reporter experiment, a markedly inhibited luciferase activity of YARS-Wt was detected in cells with increased miR-22-3p expression, which indicated that YARS could bind to miR-22-3p (Fig. 5D). In Fig. 5E-5F, YARS expression in HCC cell lines and clinical tissues was higher than in normal controls. Additionally, NCK1-AS1 was positively related to YARS in the expression in HCC tissues (Fig. 5G). The relationship among NCK1-AS1, miR-22-3p, and YARS was also detected. The YARS level was markedly lowered in the sh-NCK1-AS1#1 group, while it was restored in the sh-NCK1-AS1#1 plus miR-22-3p inhibitor group (Fig. 5H-5I), suggesting that NCK1-AS1 positively modulates YARS through controlling miR-22-3p availability.

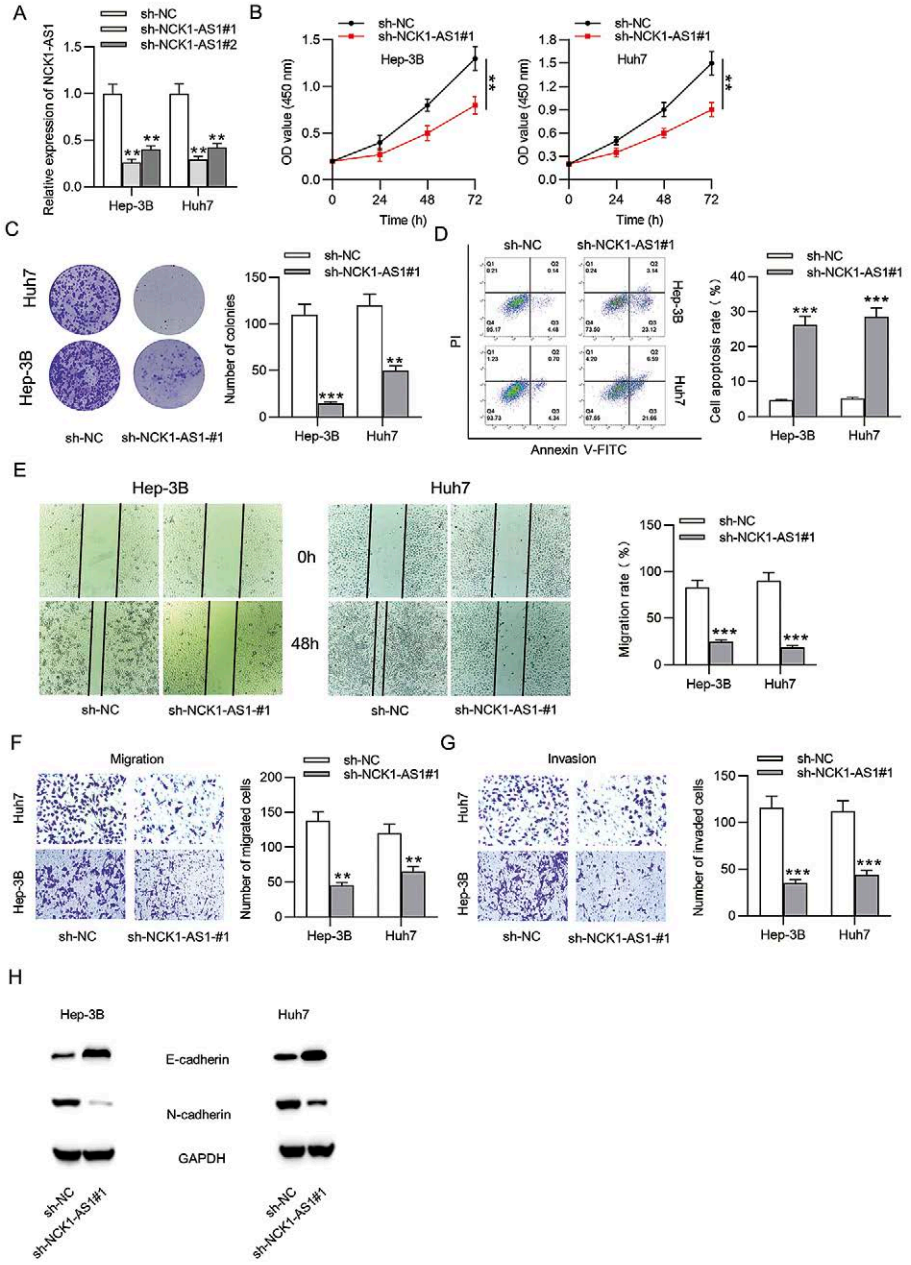

Fig. 2. NCK1-AS1 knockdown attenuates malignant character of HCC cells. (A) NCK1-AS1 expression in Hep-3B and Huh7 cells transfected with sh-NCK1-AS1 or control. (B) CCK-8 assay of the viability after silencing NCK1-AS1. (C) Colony formation assay of the proliferation of Hep-3B and Huh7 cells after silencing NCK1-AS1. (D) Flow cytometry analysis of HCC cell apoptosis after silencing NCK1-AS1. (E-G) Migrated and invaded cells after silencing NCK1-AS1 measured by wound healing and Transwell assays. (H) The E-cadherin and N-cadherin expression after silencing NCK1-AS1 examined by western blotting. $^{**}p < 0.01$, $^{***}p < 0.001$.

Correlation between YARS Expression and Prognosis in HCC Patients

To analyze the possible contribution of the target gene YARS to HCC, we took advantage of the bioinformatics tools to assess whether YARS has clinical values for HCC patients. The UALCAN database reveals that YARS expression in 371 HCC samples is significantly higher than in 50 normal controls (Supplementary Fig. 1A). The UALCAN, Kaplan Meier Plotter, and starBase databases with prognostic information collectively show that increased level of YARS is positively related to poor survival of HCC patients (all $p < 0.05$) (Supplementary Fig. 1B-1D). Additionally, the starBase database exhibits a negative correlation between miRNA-22-3p and YARS and positive correlation between NCKA-AS1 and YARS in the expression in HCC samples (Supplementary Fig. 1E).

NCK1-AS1 Biological Function through YARS Modulation on HCC Phenotypes

Finally, we explored whether NCK1-AS1 exerts biological function through modulating YARS. The overexpression efficiency of pcDNA3.1/YARS was verified (Fig. 6A). After co-transfection of pcDNA3.1/YARS into sh-NCK1-AS1#1

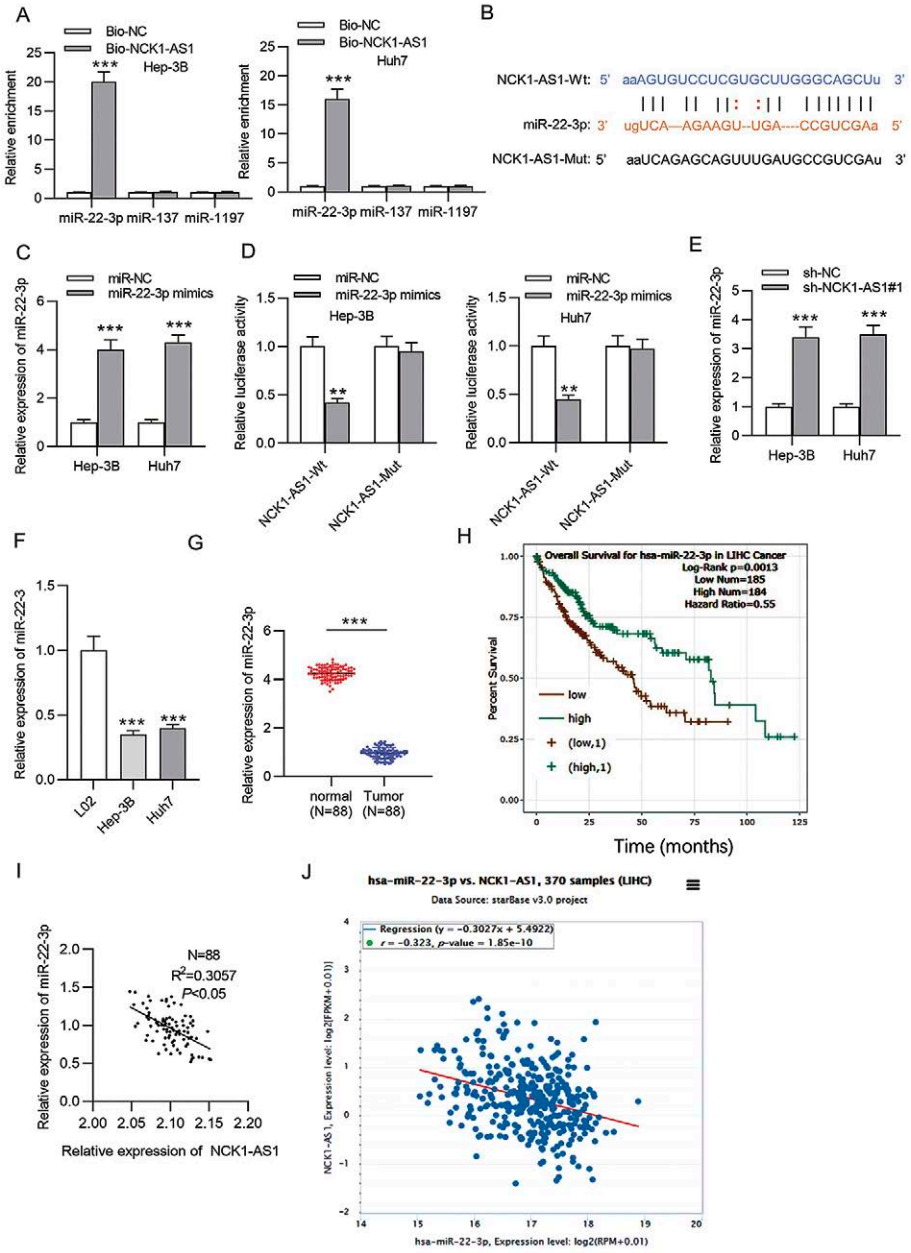

Fig. 3. MiR-22-3p is sponged by NCK1-AS1. (A) The binding affinity of miRNAs to NCKA-AS1 in HCC cells detected by RNA pulldown. (B) NCK1-AS1 harbors a binding site for miR-22-3p. (C) The overexpression efficiency of miR-22-3p mimics. (D) Luciferase report experiment of the binding of NCK1-AS1 to miR-22-3p by co-transfecting NCK1-AS1-Wt/Mut along with NC-mimics or miR-22-3p mimics into HCC cells. (E) MiR-22-3p expression in Hep-3B and Huh7 cells transfected with sh-NCK1-AS1 or control. (F-G) MiR-22-3p expression in HCC cells and tissues. (H) StarBase data for the overall survival of HCC patients with high and low miR-22-3p level. (I-J) RT-qPCR and starBase data for the expression correlation between miR-22-3p and NCK1-AS1 in HCC samples. **p < 0.01, ***p < 0.001.

transfected Hep-3B and Huh7 cells, the impaired proliferation ability induced by NCK1-AS1 knockdown was restored (Fig. 6B-6C). In apoptosis assay, YARS overexpression eliminated the effect of silenced NCK1-AS1 on HCC cell apoptosis (Fig. 6D). Wound healing and Transwell assays revealed that YARS upregulation restored the migration and invasion prevented by NCK1-AS1 knockdown (Fig. 6E-6G). Additionally, the elevated E-cadherin and reduced N-cadherin caused by knockdown of NCK1-AS1 were partially reversed by YARS overexpression (Fig. 6H).

Regulatory Function of the NCK1-AS1/miRNA-22-3p/YARS Axis

Due to the role of YARS in PI3K/AKT pathway activation [18], we determined the regulatory function of the NCK1-AS1/miRNA-22-3p/YARS axis in this pathway in HCC.

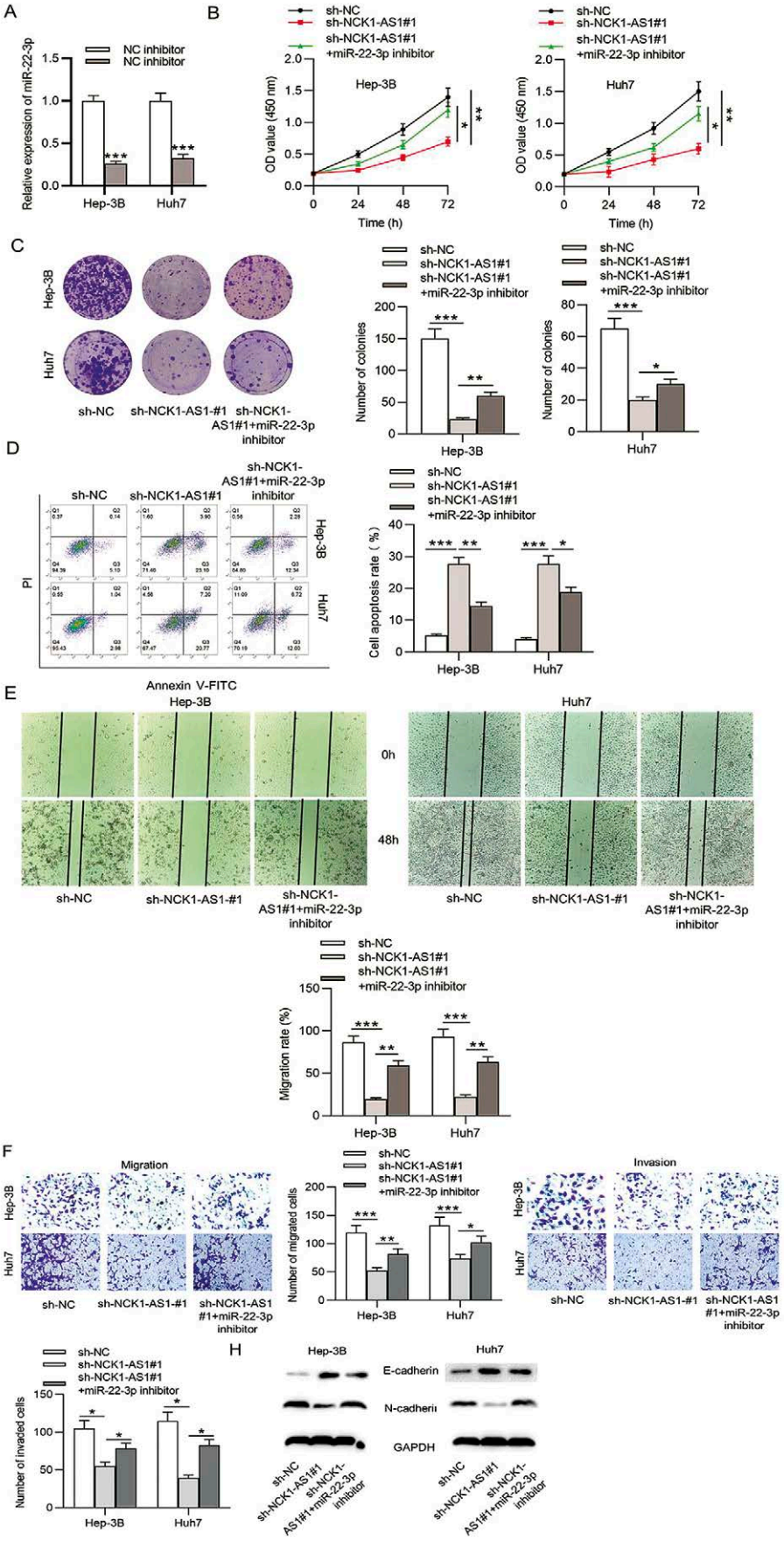

Fig. 4. MiR-22-3p countervails the NCK1-AS1-mediated regulation in HCC. (A) The silencing efficiency of miR-22-3p inhibitor. Hep-3B and Huh7 cells were transfected with sh-NC, sh-NCK1-AS1#1, and sh-NCK1-AS1#1 plus miR-22-3p inhibitor; (B) CCK-8 assay of cell viability; (C) Colony formation assay of cell proliferation; (D) Flow cytometry of cell apoptosis; (E-G) Migrated and invaded cells measured by wound healing and Transwell assays; (H) The expression of E-cadherin and N-cadherin detected by western blotting. *p < 0.05, **p < 0.01, ***p < 0.001.

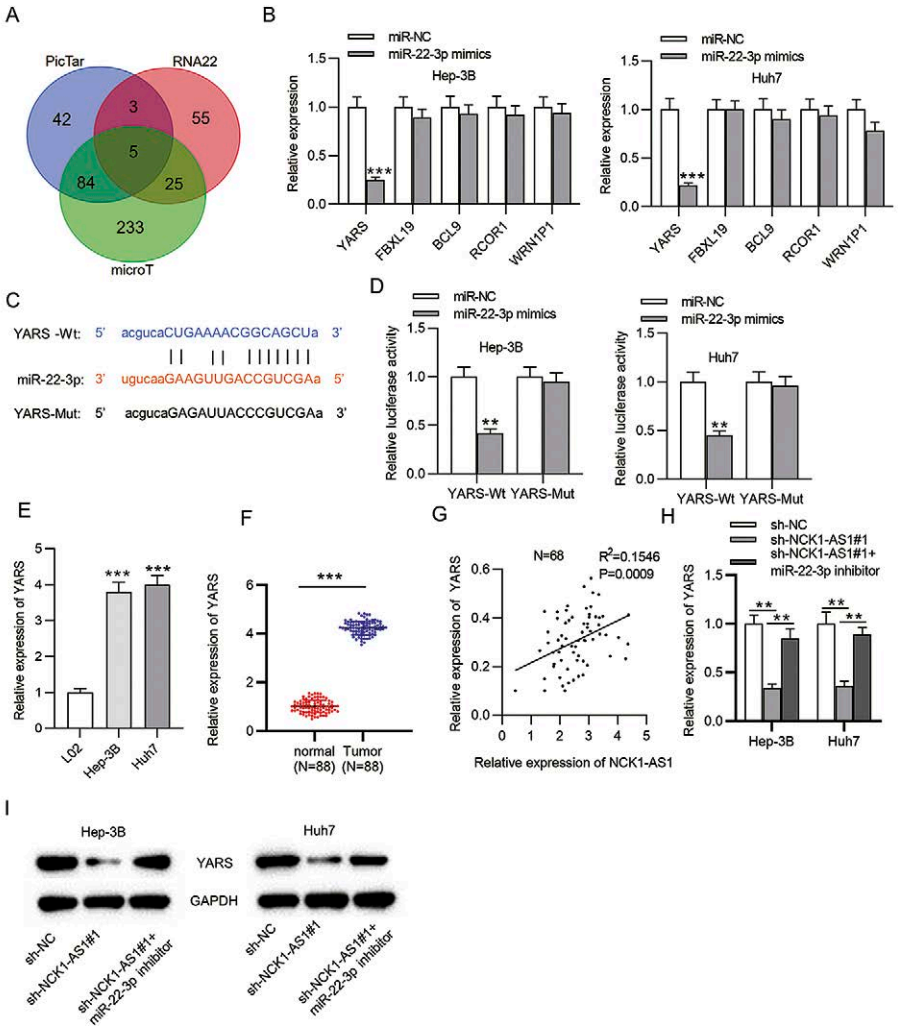

Fig. 5. YARS binds to miR-22-3p. (A) The potential mRNAs of miR-22-3p obtained in starBase (search category: Clip-Data >=5; PicTar, RNA22, microT); (B) The expression of candidate mRNAs in Hep-3B and Huh7 cells after overexpressing miR-22-3p; (C) YARS harbors a binding site for miR-22-3p; (D) Luciferase report experiment of the binding of YARS to miR-22-3p by co-transfecting YARS-Wt/Mut along with NC-mimics or miR-22-3p mimics into HCC cells; (E-F) YARS expression in HCC cells and tissues; (G) The relationship between miR-22-3p and YARS in HCC tissues. (H-I) RT-qPCR and western blotting of YARS expression in Hep-3B and Huh7 cells transfected differently. **p < 0.01, ***p < 0.001.

The interference efficiency of sh-YARS in Hep-3B and Huh7 cells was confirmed (Supplementary Fig. 2A). The levels of PI3K/AKT pathway members (PI3K, AKT, ERK, mTOR) in sh-YARS transfected cells were tested using western blotting. The phosphorylation of these proteins was repressed by YARS deficiency (Supplementary Fig. 2B). Interestingly, NCK1-AS1 knockdown repressed the phosphorylation of these proteins, while co-transfection of miRNA-22-3p knockdown or YARS overexpressed restored the phosphorylation in HCC cells (Supplementary Fig. 2C).

DISCUSSION

Many researchers proved that lncRNAs acted actively in numerous cancers such as breast cancer, cervical cancer, and lung cancer [19-21]. Moreover, it was documented that many lncRNAs function as oncogenes in HCC, such as lncRNA CRNDE [22], lncRNA FOXP4-AS1 [23], and lncRNA ILF3-AS1 [24]. Thus, it is worthwhile to investigate the function of lncRNAs in HCC. We chose NCK1-AS1 as the research object due to its oncogenic property and significantly high expression in HCC samples based on bioinformatics database. Although NCK1-AS1 was shown to be a tumor driver in several cancers [25-27], its biological function in HCC remained unclear and deserves further detection.

In our study, the NCK1-AS1 level was markedly increased in HCC cells and tissues. Also, its high level related to a poor prognosis. Furthermore, we investigated the clinicopathological value of NCK1-AS1 expression in HCC patients; our results indicated that high expression of NCK1-AS1 was correlated with TNM stage, BCLC stage and survival status in HCC patients (Table I). Similarly, Li et al. [28] reported that highly expressed NCK1-AS1 was significantly associated with lymph node status and histological type in cervical cancer. Zha et al. [29] also suggested that NCK1-AS1 overexpression in non-small cell lung cancer was positively correlated with TNM stage,

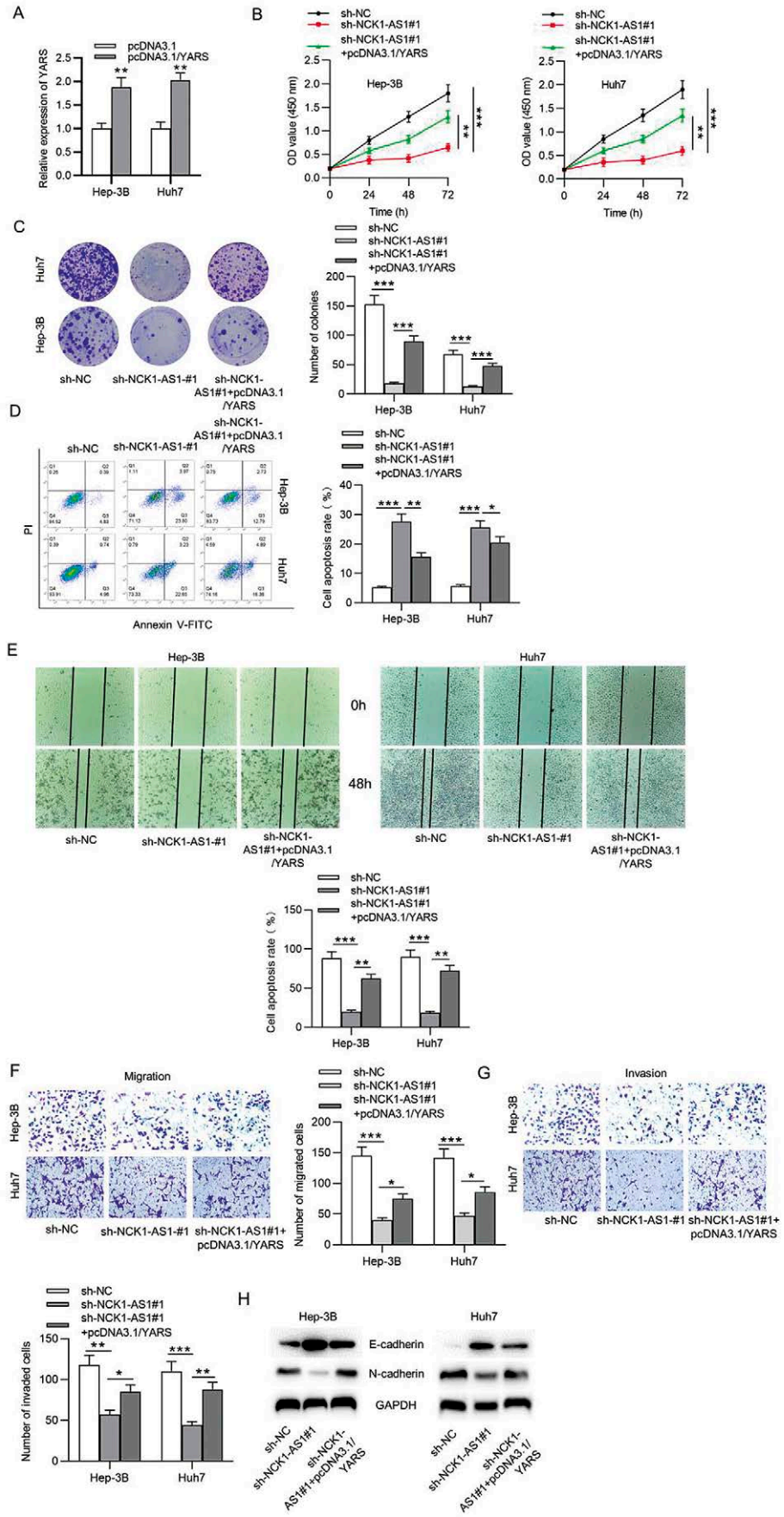

Fig. 6. YARS is correlated with poor prognosis in HCC patients. (A) UALCAN data for the YARS level in 371 HCC tissues and 50 normal samples; (B-D) The UALCAN, Kaplan Meier Plotter, and starBase databases with prognostic information for the association between the YARS level and the survival of HCC patients; (E) StarBase data for the expression correlation between miR-22-3p and NCK1-AS1, and YARS and NCK1-AS1 in HCC samples.

tumor size, and lymph node metastasis. In addition, our results of both univariate and multivariate COX regression analyses identified TNM stage, BCLC stage, survival status and high NCK1-AS1 expression as risk factors for poor HCC prognosis (Table II). Moreover, the knockdown experiments showed that NCK1-AS1 silencing prevented HCC cell proliferation, migration, invasion and contributed to apoptosis. Therefore, NCK1-AS1 might aggravate the progression of HCC.

MicroRNAs are short noncoding RNA molecules, which can regulate gene expression at posttranscriptional level [30]. Nowadays, many studies proved the roles of lncRNAs in cancer progression through ceRNA pattern [31]. Here, we identified that NCK1-AS1 was mainly distributed in cytoplasm, showing that NCK1-AS1 potentially sponged miRNAs post-transcriptionally through a ceRNA pattern. Bioinformatics predictions revealed that miR-22-3p may bind to NCK1-AS1. We further demonstrated their binding affinity in HCC cells. As previously reported, deficiency of miRNA-22-3p attenuates the inhibitory role of catalpol in the tumorigenesis of HCC cells [32]. Berberine suppresses HCC cell proliferation via upregulating miRNA-22-3p [33]. Additionally, it was recorded that NCK1-AS1 served as a miRNA-22-3p sponge in other cancer types [17, 34]. In this work, reduced expression level of miRNA-22-3p in HCC samples and cells was verified. NCK1-AS1 was negatively associated with miRNA-22-3p in the expression in HCC sample. Moreover, downregulated miRNA-22-3p reduced the anti-tumor function of NCK1-AS1 knockdown in HCC cells. We concluded that NCK1-AS1 might regulate HCC cell processes by miRNA-22-3p.

Previously, NCK1-AS1 was reported to sponge miR-22-3p and to modulate Bcl9 expression, thereby promoting cell proliferation, migration and invasion in gastric cancer [17]. Bcl9 was also predicted as a candidate target gene of miRNA-22-3p by using informatic tools in our study. However, in HCC cells, miRNA-22-3p overexpression led to a significant reduction in the expression of YARS, while exerted no remarkable influence on the expression of Bcl9. Furthermore, the binding between miRNA-22-3p and YARS in HCC cells was validated in our study. YARS can promote the binding of tyrosine to homologous tRNA and modulate intracellular signaling [35]. The YARS mutation is closely associated with genetic diseases, including neuropathy and autosomal recessive multi-organ disease [36, 37]. The elevated expression of YARS was detected in some cancer types [38, 39]. To date, YARS's association with cancer has been still largely unknown. A study indicated that YARS was a novel tumorigenic factor due to its upregulation and its function in driving gastric cancer development by activating PI3K/AKT pathway [18]. In this study, by consulting online databases and performing experiments, we confirmed the upregulation of YARS in HCC. Moreover, its upregulation reflects poor survival of HCC patients. We demonstrated that NCK1-AS1 positively modulated YARS by controlling miRNA-22-3p availability. Rescue assays showed that YARS overexpression partially abated the NCK1-AS1 deficiency-mediated inhibition of HCC progression. The significance and potential of PI3K/AKT pathway for human cancer progression have been widely reported [40, 41]. PI3K/AKT pathway displays a pivotal role in hepato-carcinogenesis and is hyperactivated in most HCC cases [42]. Here, we further demonstrated that NCK1-AS1 activated PI3K/AKT pathway by the miRNA-22-3p/YARS axis.

CONCLUSIONS

We demonstrated that NCK1-AS1 upregulated YARS to drive hepato-carcinogenesis by controlling the availability of miRNA-22-3p, which may be achieved by activating the PI3K/AKT pathway. This may provide an instructive theoretic basis for deeper investigation on HCC treatment strategies. Further studies are required to consolidate our results, and to investigate the regulatory mechanisms of NCK1-AS1 in HCC pathogenesis.

Conflicts of interest: None to declare.

Authors' contribution: W.Z., J.W. and J.Z conceived and designed the experiments. W.Z., J.W., J.Z. and Y.H drafted the paper. W.Z., J.W., J.Z., Y.W, L.J., T.G., B.L., Q.X. and Y.H. carried out the experiments. W.Z., J.W., J.Z. and Y.H analyzed the data. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

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