

Role of lncRNA NR2F1-AS1 and lncRNA H19 Genes in Hepatocellular Carcinoma and Their Effects on Biological Function of Huh-7

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Objective: This research was designed to probe into the expression and related mechanism of lncRNA NR2F1-AS1 and H19 in hepatocellular carcinoma (HCC).

Methods: Forty-two HCC patients who came to our hospital from February 2018 to August 2019 were included into a research group (RG). Meanwhile, 46 healthy controls were regarded as a control group (CG). BEL-7402, Huh-7 human hepatoma cells and HL-7702 human normal liver cells were purchased, and the NR2F1-AS1 and H19 levels in serum and tissues of HCC patients were detected. pcDNA3.1-NR2F1-AS1, si-NR2F1-AS1, NC, pcDNA3.1-H19 and si-H19 were transfected into BEL-7402 and Huh-7 cells. The NR2F1-AS1 and H19 levels in samples were detected via qRT-PCR, and the expression of apoptosis-related proteins in cells was tested through WB. Cell proliferation, invasion, or apoptosis was detected by CCK8, Transwell or flow cytometry, respectively.

Results: The NR2F1-AS1 and H19 levels were high in human hepatoma cells, and AUCs of lncRNA NR2F1-AS1 and lncRNA H19 were both >0.8. The lncRNA NR2F1-AS1 and lncRNA H19 were associated with HCC staging. After transfection of pcDNA3.1-NR2F1-AS1, si-NR2F1-AS1, NC, pcDNA3.1-H19, si-H19 BEL-7402 and Huh-7 cells, silencing NR2F1-AS1 and H19 expression can promote apoptosis and inhibit cell growth, while silencing their over-expression can inhibit the EMT process of Huh-7 cells.

Conclusion: lncRNA NR2F1-AS1 and lncRNA H19 genes are abnormally expressed in HCC. Furthermore, the two can suppress the EMT process of Huh-7 cells and promote apoptosis effectively.

Keywords: lncRNA H19, lncRNA NR2F1-AS1, hepatocellular carcinoma, HCC, trophoblast cells, invasion, apoptosis

Introduction

Hepatocellular carcinoma (HCC) is one of the complications of advanced cirrhosis. Its morbidity and mortality make it the sixth most common cancer around the world.¹⁻⁴ At present, HCC is usually treated by liver transplantation clinically. However, due to the shortage of suitable liver organs, most advanced HCC patients are getting worse without a suitable liver. So, improving their survival rate is still a challenge for clinicians.^{5,6} With the deepening of research on long non-coding RNA (lncRNA) in tumor drug resistance, clinical medicine began to analyze lncRNA's role as a molecular marker for detection and diagnosis of various human cancers and its abnormally expressed lncRNA in HCC.⁷⁻⁹

With the development of next-generation sequencing technology, genome-wide transcriptome analysis has become an effective method to detect new

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lncRNA in various disease states.^{10,11} A large number of studies have analyzed the disorders of lncRNAs related to liver cancer (LC) by genome-wide transcriptome; it is found that the lncRNA NR2F1-AS1 and lncRNA H19 levels are abnormally up-regulated.^{12,13} And it has been proved that lncRNA NR2F1-AS1 regulates miR-17/SIK1 axis to inhibit invasion and migration of cervical squamous cell carcinoma cells.¹⁴ Long non-coding RNA H19 is involved in sorafenib resistance of HCC by up-regulating miR-675.¹⁰ The molecular mechanism of lncRNA NR2F1-AS1 and lncRNA H19 in HCC has not been completely characterized. Thus, this paper aims to provide a new theoretical basis for HCC diagnosis and treatment in molecular biology, influencing the role of lncRNA NR2F1-AS1 and lncRNA H19 genes in HCC and the biological function of Huh-7.

Data and Methods

General Data

Forty-two HCC patients diagnosed and treated in our hospital were included in the research group (RG). Simultaneously, 46 healthy controls were enrolled in the healthy control group (CG). Inclusion and exclusion criteria: (1) All subjects were diagnosed as HCC by our hospital, and the clinical diagnosis was made according to the International Health Organization (WHO) standard;¹⁵ patients did not receive neoadjuvant radiotherapy or chemotherapy before operation. (2) Patients who complicated with infectious diseases, other primary malignancy diseases, or complications affecting the level of target genes were excluded. All of them voluntarily took part in the experiment, signed an informed consent form, and cooperated with medical staff to complete relevant diagnosis and treatment work. This research was conducted in accordance with the "Declaration of Helsinki". This research was approved by the Ethics Committee of our hospital and National Cancer Center.

Main Reagents and Detection Methods

Cell Culture

Human LC cells BEL-7402, Huh-7 and normal liver cells HL-7702 [L-02] were purchased from Bena Culture Collection (provided by frozen storage tube/T25 culture bottle resources), No. BNCC338237, BNCC337690, BNCC100012, respectively. Growth conditions: All cells were cultured at 37°C and 5%CO₂ in a CM2-1 culture medium (90%RPMI-1640+10%FBS).

Construction and Transfection of Expression Vectors

All expression vectors were designed by Thermo Fisher Scientific, including NR2F1-AS1 low expression vector (si-NR2F1-AS1) and H19 low expression vector (si-H19). As to NR2F1-AS1 and H19 over-expression, the coding sequence (CDS) was amplified and inserted into pcDNA3.1 vector (pcDNA3.1-NR2F1-AS1, pcDNA3.1-H19) (constructed by FitGene Co., Ltd.), and miRNA targeted inhibitory sequence (miRNA-inhibitor), miRNA mimic sequence (miRNA-mimics) and miR negative control group (miR-NC, Guangzhou, Guangdong, China). Cells were transfected by Lipofectamine™ 2000 kit, and the operation steps were strictly in line with the instructions. siRNA sequence: siH19-1: 5'-ACGAGGCACUGCGGCCAG-3'.

qRT-PCR

Under fasting condition, 5 mL blood was drawn from elbow vein of each participant. Serum samples were prepared by conventional methods. Total RNA in serum, tissue samples and cells was separated by Trizol reagent (Invitrogen, carlsbad, California, USA), and its concentration and quality were measured by Nanodrop. Next, it was reverse transcribed into cDNA using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). PCR was performed on qRT-PCR detection system (Applied Biosystems, Foster City, CA). All Ct values were measured by 2^{-ΔΔCt} method. The primer sequences are shown in Table 1.

CCK8 Cell Proliferation Experiment

After logarithmic growth phase cells were digested with trypsin and re-suspended, the cell concentration was adjusted to 2×10⁴/mL. They were inoculated into the

Table 1 Primer Sequence

	Forward Primer	Reverse Primer
NR2F1-AS1	5'-CAGCGGTGCAAACCATGTGC-3'	3'-GCAAGTTGGCTGAACCAAATG-5'
H19	5'-ACTGCACTACCTGACTCAGGAAT-3'	5'-AAGAGACAGAAGGATGAAAAAGA-3'
GAPDH	5'-TGCACCACCAACTGCTTAG-3'	5'-GATGCAGGGATGATGTTTC-3'

plate and transfected with siRNA, miRNA or plasmid based on appropriate schemes. At each time point, CCK-8 reagent was added to each hole. Subsequently, the absorbance at 450 nm was measured by microplate reader to reflect the cytoactive.

Transwell Invasion Experiment

The cells were re-suspended in DMEM medium comprising 1% FBS at 4×10^5 cells/mL. Then, 200 μ L cell suspension was added to the upper chamber, and 700 μ L DMEM medium containing 10% FBS was added to the lower chamber. After the cells were incubated for 24 h to 48 h, those in the upper chamber were taken out and stained 20 min with 0.5% crystal violet. Five visual fields were observed randomly under an optical microscope, and the cells passing through the basement membrane of the chamber were counted.

Flow Cytometry Experiment

After the Huh-7 cells were transfected for 48 h, the apoptosis was measured. Collected by EDTA-trypsin, the cells were washed with PBS. Next, a 1×10^6 cells/mL suspension was prepared after cells were re-suspended by 100 μ L AnnexinV Binding Buffer. Afterwards, 5 μ L Annexin-V/FITC solution was added and incubated 15 min at 4°C, and 5 μ L propidium iodide PI staining solution was added and incubated 5 min at 4°C. To take the average value, the test was repeated three times, and we analyzed it through flow cytometry (BD Biosciences, USA).

Western Blot (WB) Analysis

Protein samples were extracted by radioimmunoassay supplemented with protease inhibitors. Then, the protein was separated on 10% SDS-PAGE gel and transferred to PVDF membrane. The membrane was incubated with primary antibody and cultivated at 4°C all night. Then, the members were incubated with the secondary antibody. Finally, the protein was visualized by ECL detection system and quantified by ImageJ Software. β -actin was used as loading control. The primary antibody and secondary antibody goat anti-rabbit (HRP cross-linking) were bought from Abcam, Shanghai.

Statistical Methods

SPSS 20.0 (SPSS, Inc, Chicago, IL, USA) was used for statistical analysis. The normal distribution data were expressed by means \pm standard deviation (meas \pm SD), analyzed by Bonferroni post hoc test, and then assessed via two-tailed *t*-test and one-way or two-way analysis of

variance. $p < 0.05$ values were considered to be remarkably different.

Results

Expression of Target lncRNA Gene in HCC

Quantitative detection of gene expression by qRT-PCR:

(1) Compared with healthy controls, the lncRNA NR2F1-AS1 and lncRNA H19 levels in serum of HCC patients increased dramatically (all $p < 0.001$). Receiver operating characteristic (ROC) curve found that AUCs of the two lncRNA were greater than 0.8.

(2) Compared with the paracancerous tissues, the lncRNA NR2F1-AS1 and lncRNA H19 levels in HCC tissues increased dramatically (all $P < 0.001$).

(3) Compared with normal human normal liver cells HL-7702 [L-02], the lncRNA NR2F1-AS1 and lncRNA H19 levels in Huh-7 cells and BEL-7402 cells increased markedly (all $P < 0.001$).

(4) In view of the median expression of the two target genes lncRNA, patients were divided into high expression group (HEG) and low expression group (LEG). It showed that the lncRNA NR2F1-AS1 and lncRNA H19 levels in the serum of stage III/IV HCC patients were higher and higher (all $P < 0.001$) (Figures 1 and 2).

Transfection of Cell Lines

(1) After Huh-7 cell transfection, the NR2F1-AS1 expression in the si-NR2F1-AS1 interference group was markedly lower than that in NC group, and the expression in the pcDNA3.1-NR2F1-AS1 group was markedly higher than that in NC group (all $P < 0.001$); the H19 expression in the si-H19 interference group was markedly lower than that in NC group, and the expression in the pcDNA3.1-H19 group was markedly higher than that in NC group (all $p < 0.001$) (Figures 3 and 4).

(2) Compared with NC group, the proliferation and invasion of the si-NR2F1-AS1 interference group and the si-H19 interference group were obviously weakened, while those of the pcDNA3.1-NR2F1-AS1 group and the pcDNA3.1-H19 group were obviously enhanced ($P < 0.001$); the apoptosis rate of the si-NR2F1-AS1 interference group and the si-H19 interference group decreased obviously, and the rate of the pcDNA3.1-NR2F1-AS1 group increased dramatically ($P < 0.001$) (Figures 3 and 4).

(3) Compared with NC group, N-cadherin, SNAIL, Slug, CK18 and Vimentin in the si-NR2F1-AS1 interference

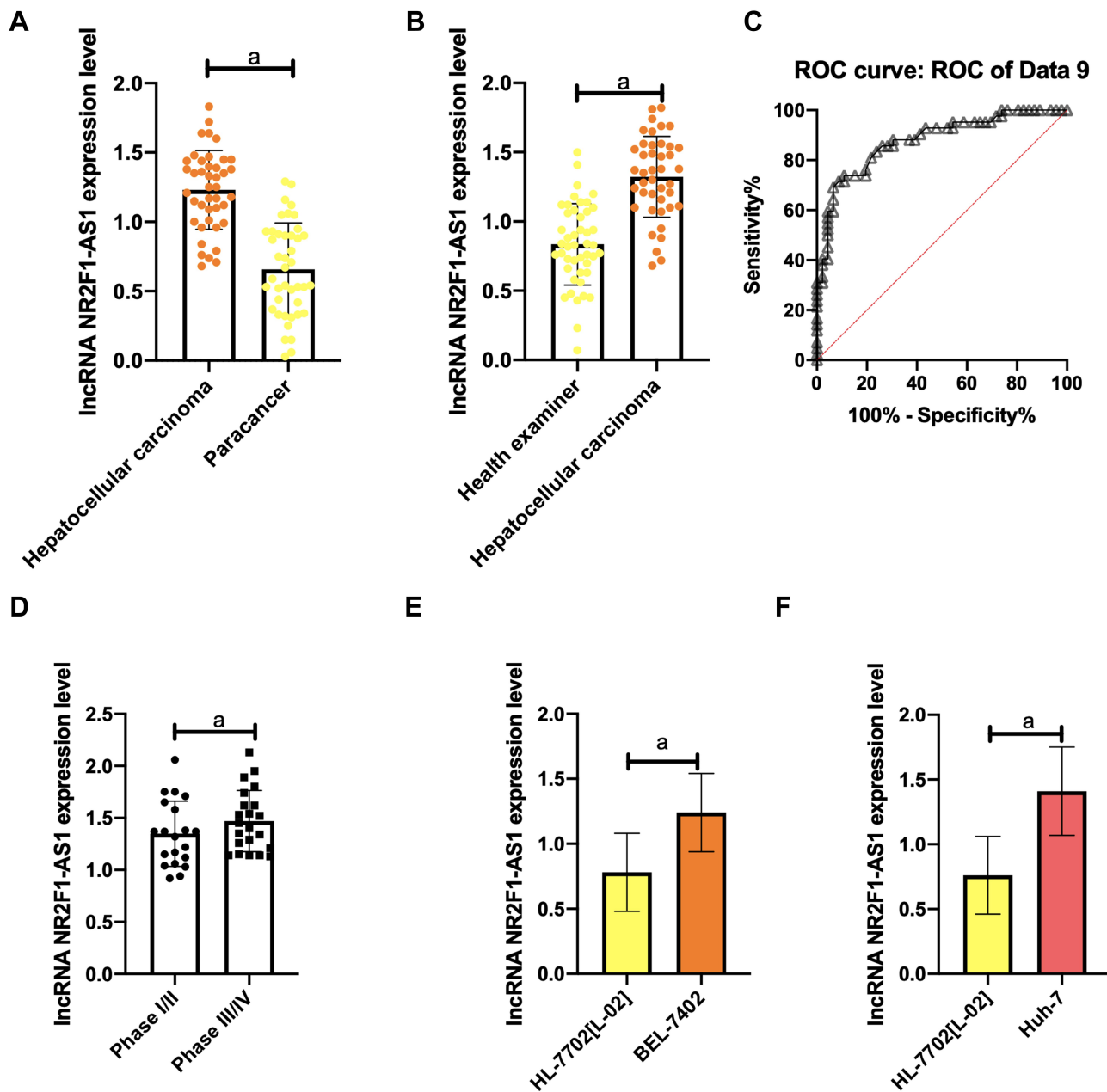


Figure 1 Gene expression of lncRNA NR2F1-AS1. (A) Compared with paracancerous tissues, lncRNA NR2F1-AS1 is markedly up-regulated in HCC tissues. (B) lncRNA NR2F1-AS1 expression in serum of HCC patients; (C) ROC curve analysis shows that AUC of lncRNA NR2F1-AS1 is > 0.8; (D) lncRNA NR2F1-AS1 expression in stage III/IV patients; (E) lncRNA NR2F1-AS1 expression in Huh-7; (F) lncRNA NR2F1-AS1 expression in BEL-7402. **Note:** ^aP<0.001.

group and the si-H19 interference group were dramatically down-regulated (P<0.001); E-cadherin in the si-NR2F1-AS1 interference group and the si-H19 interference group increased dramatically (P<0.001) (Figures 3 and 4).

Functional Target mRNA of lncRNA is Predicted by TargetScan, Starbase Database

(1) It was predicted that miR-363 had complementary binding sequence with NR2F1-AS1 in its 3'-UTR, and

the luciferase reporter plasmid of WT-H19 and mut-H19 binding site are shown in Figure 6A.

The luciferase reporter plasmid containing WT-H19 and miR-140-5p-mimics in Huh-7 cells reduced reporter gene activity (P<0.001). The miR-363 expression was detected by WB. miR-363 was down-regulated in Huh-7 cells after pcDNA3.1-NR2F1-AS1 transfection, and up-regulated after si-NR2F1-AS1 transfection (P<0.001). PcDNA3.1-NR2F1-AS1 and miR-363-mimics were

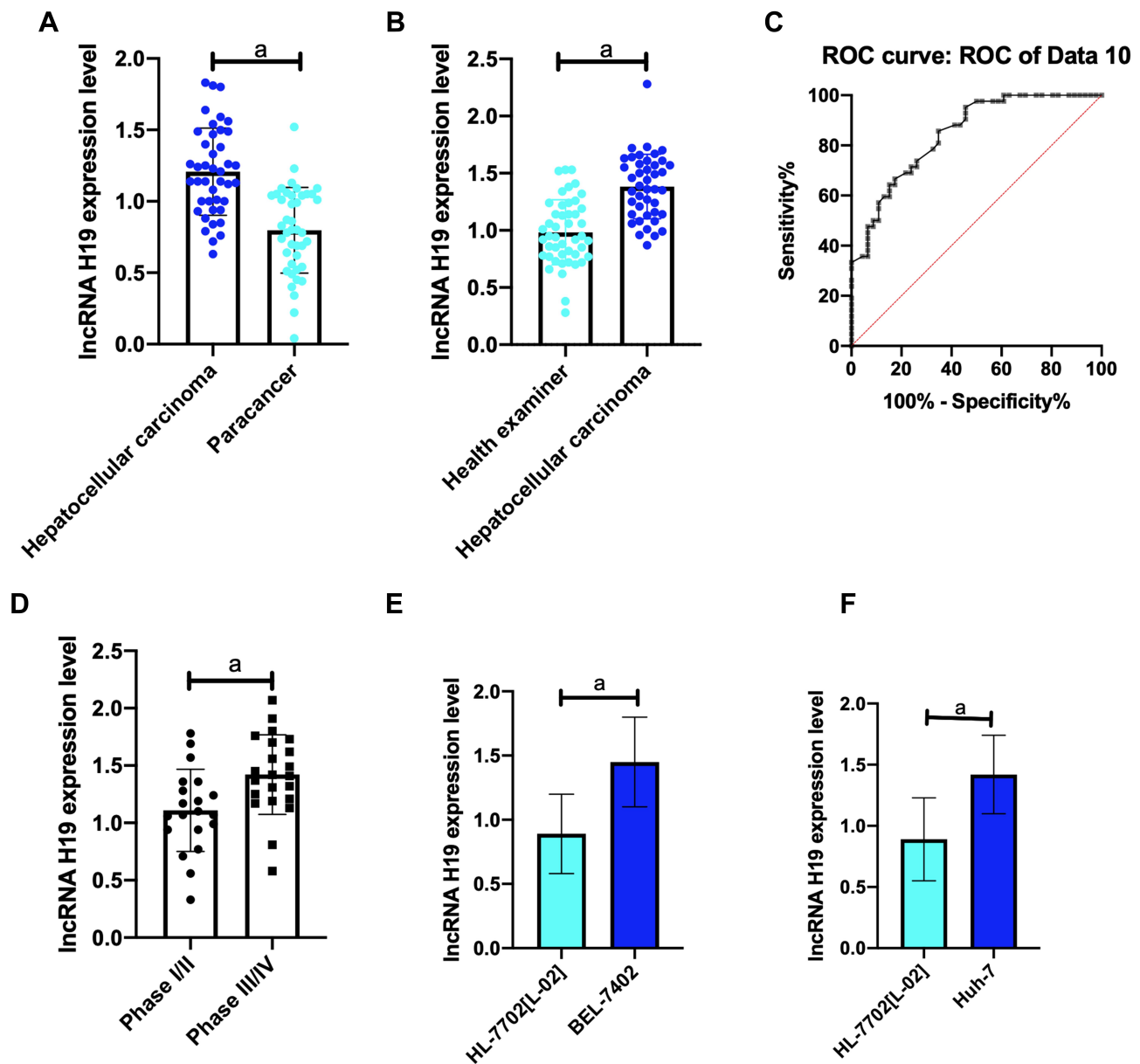


Figure 2 Gene expression of lncRNA H19. (A) The lncRNA H19 expression in HCC is markedly down-regulated compared with that in paracancerous tissues. (B) lncRNA H19 expression in serum of HCC patients; (C) ROC curve analysis shows that AUC of lncRNA H19 is > 0.8 ; (D) lncRNA H19 expression in stage III/IV patients; (E) lncRNA H19 expression in Huh-7; (F) lncRNA H19 expression in BEL-7402.

Note: ^a $P < 0.001$.

co-transfected into Huh-7 cells. Functional analysis identified that after miR-363-mimics transfection, the proliferation and invasion of Huh-7 cells decreased markedly, while the apoptosis increased (all $P < 0.01$). pcDNA3.1-NR2F1-AS1/miR-363-mimics transfection could partially reverse the pro-apoptosis effect of pcDNA3.1-NR2F1-AS1 on Huh-7 cells. These results indicated that miR-363 was the functional target of NR2F1-AS1 in Huh-7 cells (Figure 5).

(2) miR-140-5p had complementary binding sequences with H19 in its 3'-UTR, and the luciferase reporter plasmid and mut-H19 binding site of WT-NR2F1-AS1 are shown in Figure 6A.

The luciferase reporter plasmid comprising WT-NR2F1-AS1 and miR-363-mimics in Huh-7 cells reduced reporter gene activity ($P < 0.001$). The miR-140-5p expression detected by WB showed that miR-140-5p was down-regulated in Huh-7 cells after

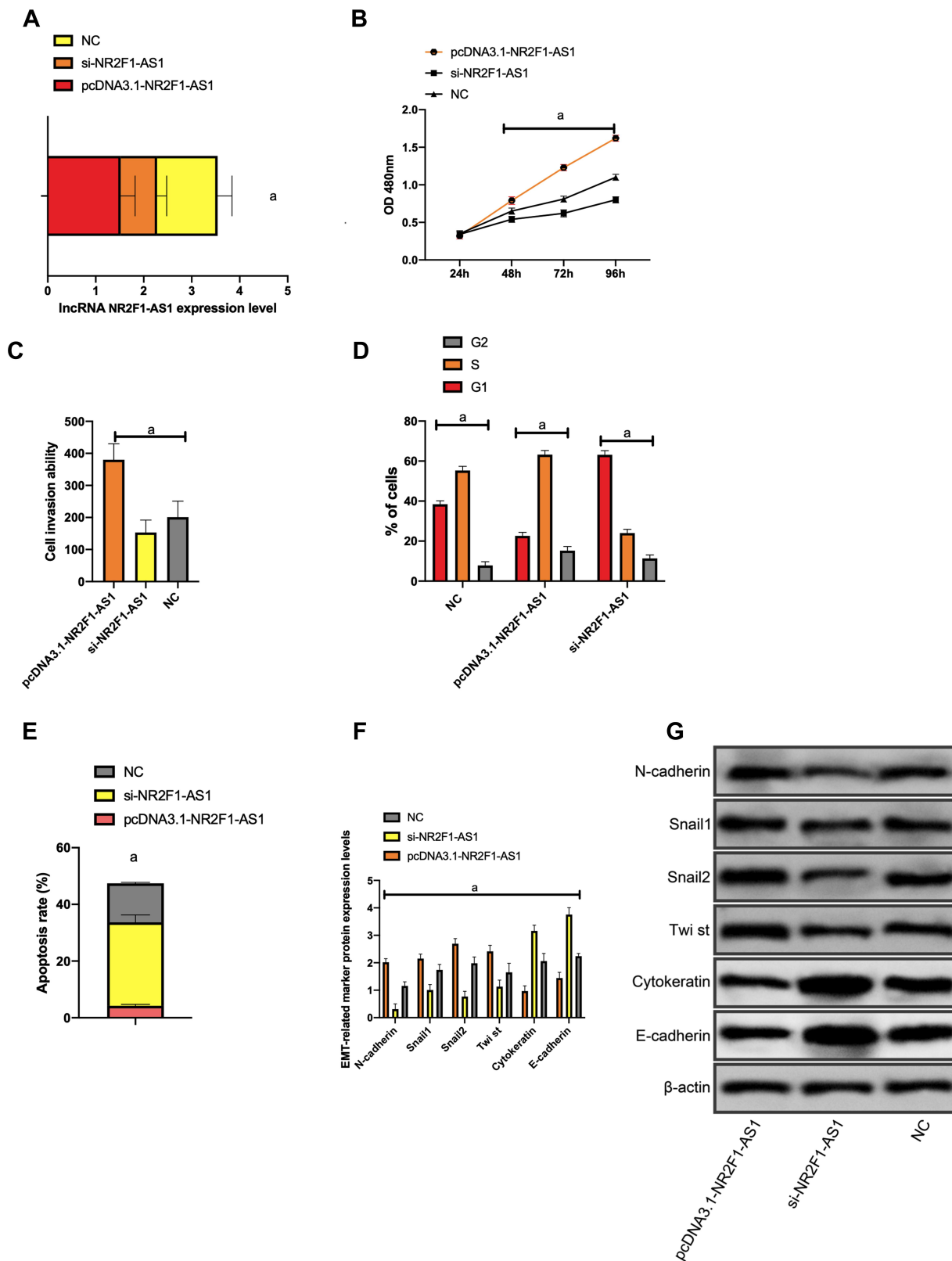


Figure 3 Effect of NR2F1-AS1 on biological function of Huh-7 cells NR2F1-AS1 expression after transfection of Huh-7 cells; **(B)** proliferation of Huh-7 cells after transfection; **(C)** invasion of Huh-7 cells after transfection; **(D)** cell cycle of Huh-7 after transfection; **(E)** apoptosis of Huh-7 after transfection; **(F)** expression of EMT-related markers after transfection; **(G)** WB figure.

Note: ^aP<0.001.

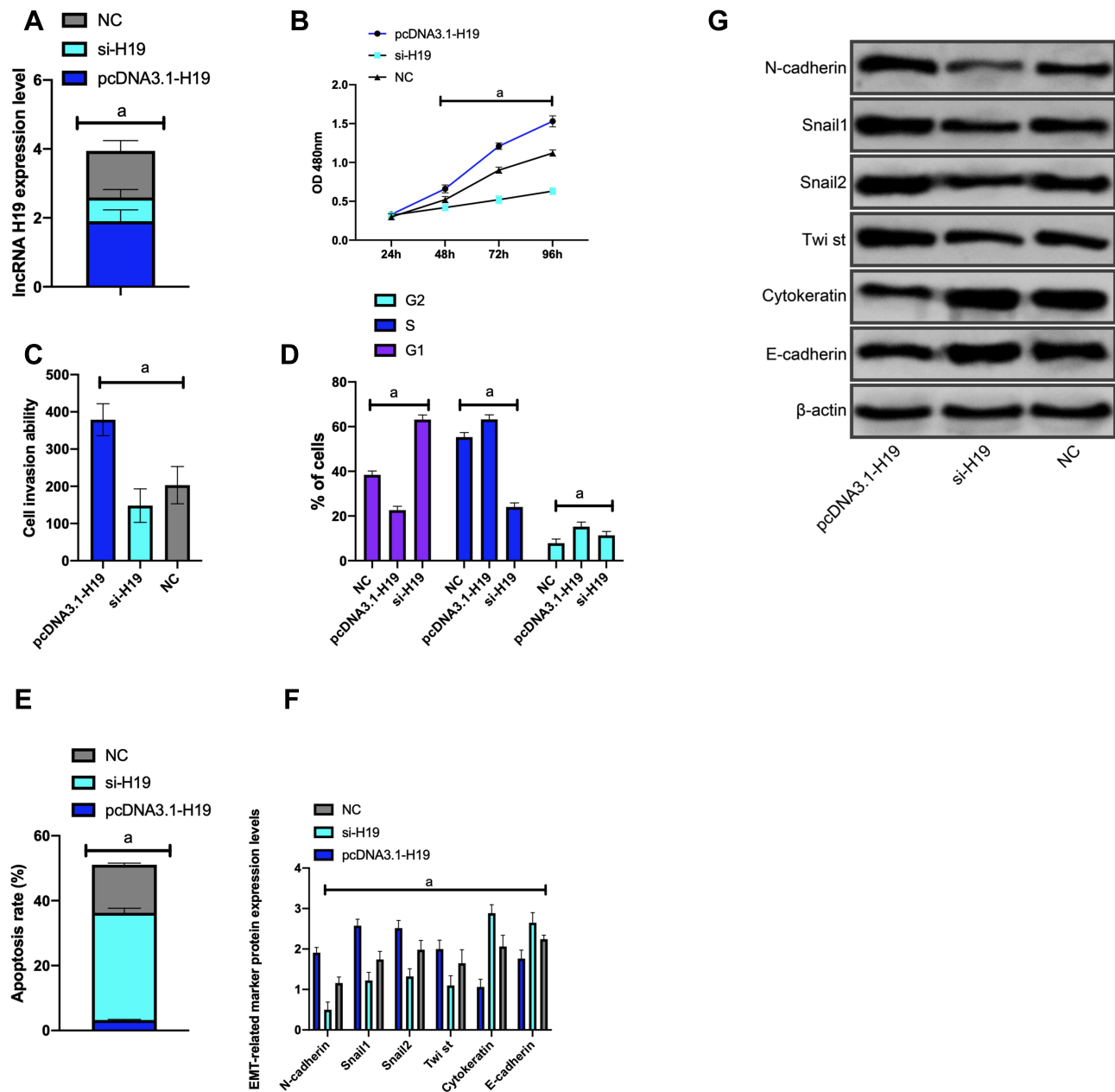


Figure 4 Effect of H19 on biological function of Huh-7 cells (A) H19 expression after transfection of Huh-7 cells; (B) proliferation of Huh-7 cells after transfection; (C) invasion of Huh-7 cells after transfection; (D) cell cycle of Huh-7 after transfection; (E) Huh-7 Apoptosis after transfection; (F) expression of EMT-related markers after transfection; (G) WB figure.

Note: ^aP<0.001.

pcDNA3.1-H19 transfection, and up-regulated after si-H19 transfection (P<0.001). pcDNA3.1-H19 and miR-140-5p-mimics were co-transfected into Huh-7 cells. Functional analysis manifested that after miR-140-5p-mimics transfection, the proliferation and invasion of Huh-7 cells decreased markedly, while the apoptosis increased (all P<0.01). Transfecting pcDNA3.1-H19/miR-140-5p-mimics could partially reverse the pro-apoptosis effect of pcDNA3.1-H19 on Huh-7 cells.

These results indicated that miR-140-5p was the functional target of H19 in Huh-7 cells (Figure 6).

Effects of lncRNA NR2F1-AS1 and lncRNA H19 Gene on DNA Synthesis Phase (S Phase) of Huh-7 Cells

We monitored the cell cycle changes of Huh-7 cells transfected with pcDNA3.1-NR2F1-AS1/miR-363-mimics and pcDNA3.1-H19/miR-140-5p-mimics.

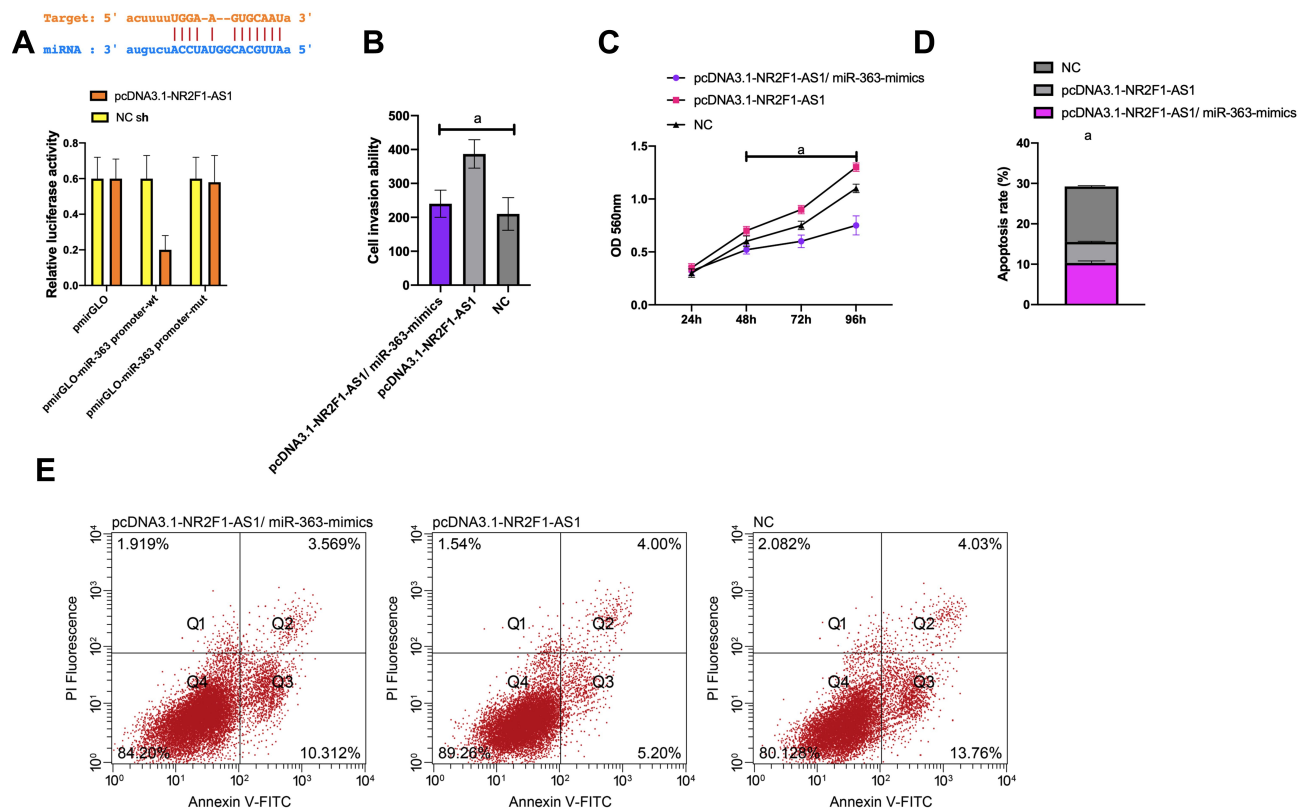


Figure 5 Dual-luciferase activity assay. **(A)** There is a binding site between NR2F1-AS1 and miR-363 through relative luciferase activity-dual luciferase report assay; **(B)** invasion of Huh-7 cells after transfection; **(C)** cell proliferation; **(D)** apoptosis; **(E)** Flow cytometry. **Note:** ^aP<0.001.

After pcDNA3.1-NR2F1-AS1 and pcDNA3.1-H19 transfection, S-phase cells, EMT-related epithelial phenotypic protein level, and apoptosis-related protein expression decreased, while apoptosis-inhibiting protein expression increased.

After pcDNA3.1-NR2F1-AS1/miR-140-5p-mimics and pcDNA3.1-H19/miR-140-5p-mimics transfection, the number of S-phase cells and the level of EMT-related epithelial phenotypic proteins increased, the expression of pro-apoptotic protein was obviously up-regulated and that of anti-apoptotic protein was obviously down-regulated (P<0.05) (Figure 7).

Discussion

HCC, as an aggressive malignancy with high recurrence rate, is always unsatisfactory in clinical treatment.¹⁶ It is widely verified that lncRNA takes part in tumorigenesis and regulation, so improving the research on “lncRNA regulates chemotherapy resistance of tumor cells and its influence on biological function of related tumor cells” is a vital purpose of the continuous development of bioinformatics prediction tools.^{17,18}

lncRNA H19 is located on human chromosome 11p15, and H19 is up-regulated and has a strong carcinogenic function in cancer progression.^{19–21} Comparing the differences of lncRNA NR2F1-AS1 and lncRNA H19 between HCC and healthy people, we found that both of them were highly expressed in HCC patients’ serum and cancer tissues. The lncRNA NR2F1-AS1 up-regulated the carcinogenesis of FOXA1 in osteosarcoma by sponging miR-483-3p.²² In the cell transfection experiment, we silenced and over-expressed the NR2F1-AS1 and H19 levels in Huh-7 cells, and observed cell biological function. It was found that the invasion of HCC after inhibiting NR2F1-AS1 or H19 was obviously inhibited. This suggested that NR2F1-AS1 or H19 could influence the proliferation and apoptosis of Huh-7 cells, which could predict HCC progression. lncRNA mainly plays various roles in cancer by colliding with microRNA (miRNA). It was found that lncRNA AGAP2-AS1 might become a key inhibitor in HCC by competing with miR-574. This potential lncRNA-miRNA-regulatory target provided a novel therapeutic strategy for HCC.²³ The lncRNA NR2F1-AS1 was highly expressed in esophageal squamous cell carcinoma tissues and cells. In addition, it also promoted

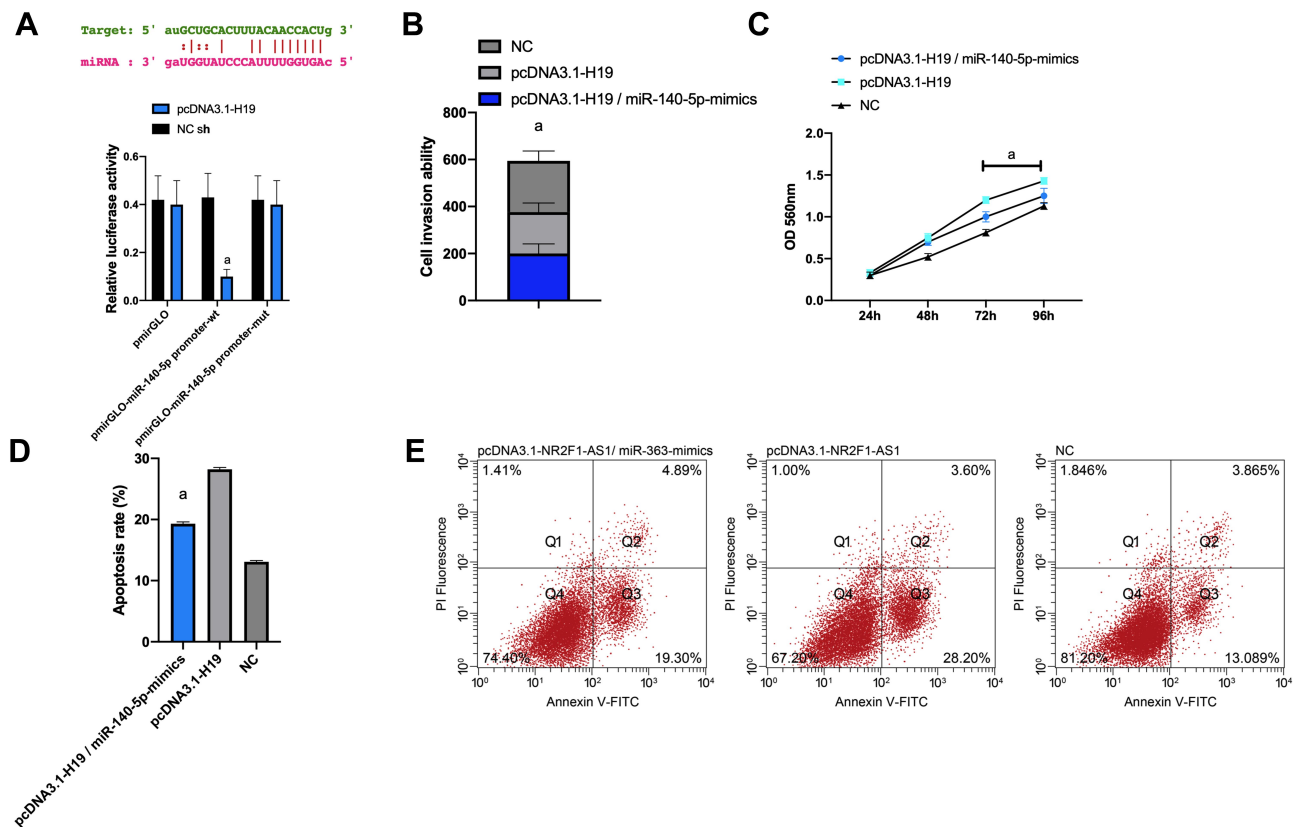


Figure 6 Dual-luciferase activity assay. (A) There is a binding site between H19 and miR-140-5p through the relative luciferase activity-dual luciferase report assay; (B) invasion of Huh-7 cells after transfection; (C) cell proliferation; (D) apoptosis; (E) Flow cytometry.

Note: ^aP<0.001.

the proliferation and metastasis of esophageal squamous cell carcinoma cells by regulating EMT.²⁴

At last, we predicted that miR-363 and NR2F1-AS1 had complementary binding sequences, and miR-140-5p and H19 had complementary binding sequences by TargetsCan6.2, and then analyzed the EMT process and cell cycle of H19 cells regulated by miRNA targeted by lncRNA NR2F1-AS1 and lncRNA H19. Many studies suggest that lncRNA can regulate G1/S-phase transition of cell cycle.²⁵ While we found that transfecting pcDNA3.1-NR2F1-AS1/miR-363-mimics and pcDNA3.1-H19/miR-140-5p-mimics could partially reverse EMT induction by pcDNA3.1-NR2F1-AS1 or H19, and restore the malignant proliferation of Huh-7 cells. The long non-coding RNA NR2F1-AS1 promoted the proliferation and migration of thyroid cancer cells by regulating miRNA-338-3p/CCND1 axis, but inhibited their apoptosis.²⁶ lncRNA H19 mediated cisplatin resistance and migration of ovarian cancer cells during EMT.²⁷ miR-675-3p derived from lncRNA H19 boosted epithelial-mesenchymal transition and stem

cell growth of human pancreatic cancer cells.²⁸

Combined with the results, we believe that knocking down lncRNA NR2F1-AS1 and lncRNA H19 can reduce the proliferation and metastasis of Huh-7 cells. What is more, over-expressing miR-363/miR-140-5p can suppress HCC progression promoted by lncRNA NR2F1-AS1/lncRNA H19.

In this research, we explored the ability of lncRNA NR2F1-AS1 and lncRNA H19 genes to interfere with the protein encoded by target gene mRNA. And we confirmed their function in HCC and influence on the biological function of Huh-7. Functional tests were conducted to explore the mechanism of regulating the behavior of Huh-7 cells by targeting miRNA with H19 and NR2F1-AS1. However, we still have some limitations. For one thing, we have not further explored the lncRNA-miRNA-mRNA network, for another, no related promotion studies have been made on other related signal pathways. Hence, we hope to screen and analyze more lncRNA and mRNA which are ceRNA in HCC in the future research, so as to provide more basis for the experiment.

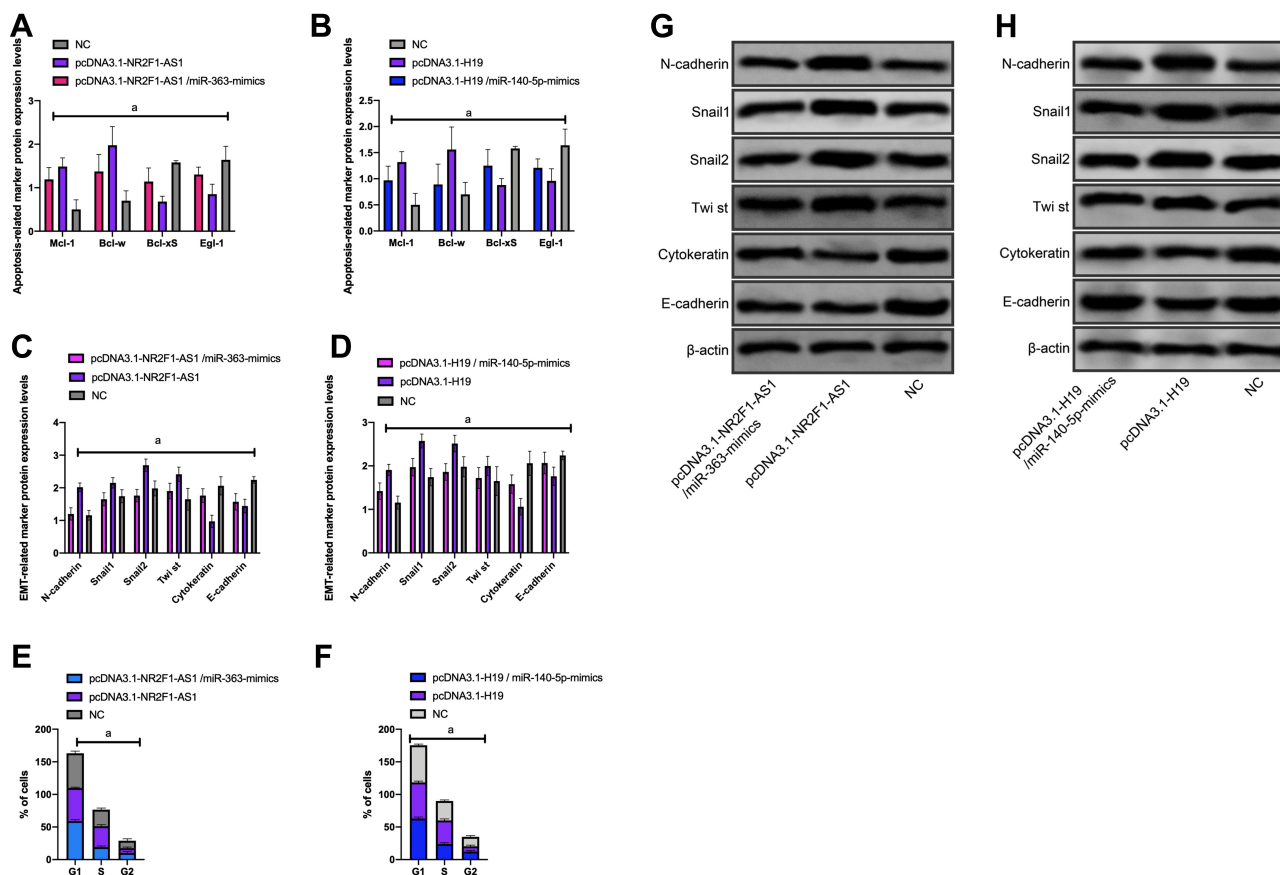


Figure 7 Effect of cell cycle and apoptosis-related proteins in HUH-7 cells. (A) Expression of apoptotic protein after pcDNA3.1-NR2F1-AS1/miR-363-mimics transfection; (B) expression of apoptotic protein; (C) expression of EMT protein; (D) expression of EMT protein; (E) cell cycle; (F) cell cycle; (G) WB diagram; (H) WB diagram; ³P<0.001.

Conclusion

To summarize, lncRNA NR2F1-AS1 and lncRNA H19 genes are abnormally high in HCC. Furthermore, the two can effectively inhibit the EMT process of Huh-7 cells and promote apoptosis.

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Disclosure

The authors report no conflicts of interest in this work.

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