

Long noncoding RNA ZFPM2-AS1 regulates ITGB1 by miR-1226-3p to promote cell proliferation and invasion in hepatocellular carcinoma

W. LIU, G.-Q. ZHANG, D.-Y. ZHU, L.-J. WANG, G.-T. LI, J.-G. XU, X.-L. JIN, Y.-M. ZHU, X.-Y. YANG

Department of Infectious Diseases, Luoyang Central Hospital Affiliated to Zhengzhou University, Luoyang, China

Abstract. – **OBJECTIVE:** Long noncoding RNA (lncRNA) is emerging as a vital regulator in various tumors. However, the biological function of ZFPM2-antisense RNA 1 (ZFPM2-AS1) in hepatocellular carcinoma (HCC) remains unclear. The present study aims to explore the function and mechanism of ZFPM2-AS1 in hepatocellular carcinoma progression.

PATIENTS AND METHODS: The ZFPM2-AS1 expression in HCC cells and tissues was measured by quantitative real-time polymerase chain reaction (qRT-PCR). Effects of ZFPM2-AS1 on tumor cell proliferation and invasion were detected by CCK8 assay or EdU assay or matrigel migration assay and Western blot. The Luciferase reporter assay, RNA pulldown assay, qRT-PCR, and Western blot were performed to explore and confirm the interaction between ZFPM2-AS1 and miR-1226-3p and integrin $\beta 1$ (ITGB1).

RESULTS: ZFPM2-AS1 was overexpressed in HCC tissues and cell lines. High levels of ZFPM2-AS1 were correlated with advanced TNM stage, distant metastasis and a poorer overall survival rate. ZFPM2-AS1 knockdown inhibited cell proliferation and invasion. ZFPM2-AS1 could directly bind to and negatively regulate miR-1226-3p expression. Moreover, ITGB1 was identified as a target gene of miR-1226-3p. ITGB1 was found to be directly negatively regulated by miR-1226-3p and indirectly upregulated by ZFPM2-AS1. Rescue assays demonstrated that ZFPM2-AS1 promotes HCC cell proliferation and invasion through modulating miR-1226/ITGB1 axis.

CONCLUSIONS: ZFPM2-AS1 promotes cell proliferation and migration by regulating miR-1226-3p/ITGB1 axis in HCC.

Key Words:

Hepatocellular carcinoma, ZFPM2-AS1, MiR-1226-3p, ITGB1, Proliferation, Invasion.

Introduction

Hepatocellular carcinoma (HCC) is a very common cancer worldwide and one of the leading causes of cancer-related death¹. Although novel therapeutic strategies have been developed, the overall survival rates for HCC patients remain low. Tumor metastasis is the main reason for the low survival rate of HCC patients. Therefore, it is urgent to investigate the molecular mechanisms underlying HCC progression, and develop a more efficient therapeutic target for treatment.

Long non-coding RNAs (lncRNAs) are transcripts with length more than 200 bp nucleotides and with little protein-coding ability². It has been reported that lncRNAs are involved in multiple cellular processes, such as cell proliferation, apoptosis, and migration³⁻⁵. Increasing studies showed that lncRNAs can function as an oncogene or a tumor suppressor in a variety of cancer, such as non-small cell lung cancer⁶, esophageal squamous cell carcinoma⁷, glioma⁸, and HCC⁹. Moreover, many studies demonstrated that lncRNAs function as competing endogenous RNAs and play an important role in the pathogenetic mechanisms of HCC. For instance, LINC00339 promotes HCC cell proliferation and migration by sponging miR-152¹⁰. Long noncoding RNA HAGLROS regulates cell proliferation, apoptosis, and autophagy in HCC by acting as a ceRNA of miR-5095¹¹. lncRNA CRNDE promotes HCC cell proliferation and migration by functioning as a ceRNA of miR-337-3p¹².

Long noncoding RNA ZFPM2-AS1 has been reported to promote proliferation and suppress apoptosis of gastric cancer cells¹³. ZFPM2-AS1

was found to promote growth, migration, and invasion of renal cell cancer cells¹⁴. However, the role of ZFPM2-AS1 in HCC, as well as the possible underlying mechanism remains unclear. Here, we found that ZFPM2-AS1 was upregulated in HCC tissues and its overexpression predicted poor prognosis. Our results showed that ZFPM2-AS1 knockdown inhibited the proliferation and migration of HCC cells by targeting miR-1226-3p/ITGB1 axis.

Patients and Methods

Patients and Tissue Specimens

Fifty-two patients were enrolled in this research. All patients were pathologically diagnosed with hepatocellular carcinoma at Luoyang Central Hospital Affiliated to Zhengzhou University. Tissues were not treated with radiotherapy or chemotherapy before surgery. Tissue samples were preserved in liquid nitrogen. Study approaches were approved by the Luoyang Central Hospital Research Ethics Committee and conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and the informed consents were obtained from all the enrolled patients in accordance with the committee regulations.

Cell Culture and Transfection

HCC cell lines (HepG2, Huh7, SMMC-7721, and HCCLM3) and normal hepatic cell line H7702 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C.

To knock down lncRNA ZFPM2-AS1, small interfering RNAs (siRNAs) targeting lncRNA ZFPM2-AS1 (5'- CCTCCTGGGTTCAAGCAAT-3') and negative control siRNA (si-NC) were synthesized by GenePharma (Shanghai, China). The ZFPM2-AS1 and ITGB1 expression vector was synthesized and constructed by GenePharma and pcDNA3.1 was used as control. MiR-1226-3p mimics, negative control (NC) mimics, miR-1226-3p inhibitor and NC inhibitor were obtained from GenePharma. Cell transfections were conducted by using Lipofectamine 2000 Re-

agent (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturers' instruction.

Quantitative Real Time-PCR

Total RNAs were extracted using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) from tissues or cells. Complementary DNAs were generated using RNAs as templates through the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). qPCR was analyzed using the SYBR Green PCR Master Mix Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6 and calculated according to the 2^{-ΔΔCt} method. The primer sequences were listed as follows: lnc RNA ZFPM2-AS1 (forward: 5'-CCCAGGGAGAGTATGAGTGTA-3' and reverse: 5'-ACCAAGTTCCTCACTGGCTT-3'). miR-1226-3p (forward: 5'-GCGGCTCACCAGCCCTGTGT-3' and reverse: 5'-CAGCCACAAAAGAGCACAAT-3'); GAPDH (forward: 5'-GCTCCCTCTTTCTTTG-CAGC-3' and reverse: 5'-GGAAAGCCAGTCCCAGAAC-3'); U6 small nuclear 6 (U6) (forward: 5'-CAGCGACTCGCAACCTTTTC-3' and reverse: 5'-AAACGCTGATCCCCGAAACT-3').

CCK-8 Assay

Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) was conducted to measure cell proliferation. Specifically, a total of 2.5 × 10³ cells was cultured in 96-well plates for 0, 24, 48, 72 and 96 hr. We then applied the CCK-8 solution reagent to measure the cell viability after transfection treatments. Subsequently, the absorbance was measured at 490 nm after incubating cells with CCK-8 solution (10 µl) at 37°C for 2 h.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

The Molecular Probes EdU-Alexa Imaging Detection Kit (Life Technologies, Carlsbad, CA, USA) was used for EdU assay. Transfected cells were cultured with 10 µM EdU. After incubation for 2 hours, 4% paraformaldehyde was used to fix these cells and 1% Triton X-100 was utilized for permeabilization. Then, the Alexa-Fluor 594 reaction cocktail was introduced to stain these cells. Images were collected with a fluorescence microscope (Olympus, Tokyo, Japan).

Subcellular Fractionation Assay

The cytoplasmic and nuclear extracts were extracted from HCC cells with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA). The distribution of long noncoding RNA ZFPM2-AS1 in cytoplasm or nucleus was analyzed by qRT-PCR analysis. The expression of U6 in nucleus, GAPDH in cytoplasm was used as control.

RNA FISH Assay

ZFPM2-AS1 probes were designed and synthesized by RiboBio (Guangzhou, China). The probe signals were detected with a Fluorescent in Situ Hybridization Kit (RiboBio) according to the manufacturer's instructions. Briefly, HCC cells were fixed in 4% paraformaldehyde for 15 min. After prehybridization in PBS, the cells were hybridized at 37°C for 30 min in hybridization solution. Then, cell nuclei underwent counterstaining by utilizing DAPI (Beyotime, Guangzhou, China). A fluorescence microscope (Olympus, Japan) was applied to capture the images of the RNA FISH assay.

Matrigel Transwell Assay

For analysis of cell invasion, 2.5×10^4 cells were seeded in a transwell chamber (8- μ m pore size; EMD Millipore, Billerica, MA, USA) with a Matrigel-coated membrane in 200 μ L serum-free DMEM. A volume of 500 μ L of the medium containing 10% FBS was added to the lower chamber. The 24-well chambers were then incubated at 37°C for 24h, the cells on the lower surface of the membrane were fixed in 4% paraformaldehyde for 30 minutes and stained with 0.5% crystal violet. The transmembrane cells were counted under high-power microscope fields. Mean values were obtained from six randomly selected fields for each well. The experiment was repeated three times.

Western Blot Analysis

Total proteins were prepared using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Guangzhou, China). Proteins were quantified by bicinchoninic acid assay (BCA) assay. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride (PDVF) membranes (Bio-Rad, Hercules, CA, USA). After being blocked using 5% non-fat milk for 1 h at room temperature, the primary rabbit anti-human antibodies against ITGB1 (Abcam, Cambridge, MA,

USA) and GAPDH (Abcam, Cambridge, MA, USA) were supplemented overnight at 4°C. Then, the members were washed with tris-buffered saline Tween 20 (TBST) for three times and probed with horse radish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge, MA, USA) for 1 h at 37°C. The bands were visualized using a Chemic DocXRS system (Bio-Rad, Hercules, CA, USA).

Luciferase Reporter Assay

For Luciferase reporter assay, the predicted sequences of ZFPM2-AS1 or ITGB1 were inserted into pmirGLO vector (Promega, Madison, WI, USA). Afterward, these vectors were co-transfected with miR-1226-3p mimics and Ctrl mimics into Huh7 and HepG2 cells. 48 hours later, the relative Luciferase activity was measured using the Dual-Luciferase Assay System.

RNA Pull-Down Assay

RNA pull-down assays were performed to examine whether miR-1226-3p was combined with long noncoding RNA ZFPM2-AS1. Biotin-labeled wild-type miR-1226-3p (bio-wt-miR-1226-3p), mutant miR-1226-3p (bio-mut-miR-1226-3p) or negative control (bio-NC) was obtained from Guangzhou RiboBio Co., Ltd (Guangzhou, China). Pulldown assay was carried out using Dynabeads™ M-280 Streptavidin (Invitrogen, CA, USA) according to the manufacturer's protocol. Precipitated RNAs were analyzed using qRT-PCR.

Statistical Analysis

All data of multiple experiments are presented as the mean $\pm$ standard deviation. SPSS 19.0 software (SPSS, IBM, Armonk, NY, USA) was adopted for statistical analysis. The Kaplan-Meier analysis and log-rank test were used to calculate the overall survival. Statistical differences between the two groups were analyzed using the Student's *t*-test, and one-way ANOVA for more groups followed by the Post-Hoc test (Least Significant Difference). The value of $p < 0.05$ was considered to be significant.

Results

Long Noncoding RNA ZFPM2-AS1 Was Upregulated in HCC

To assess the expression of ZFPM2-AS1 in HCC, we analyzed ZFPM2-AS1 expression by qRT-PCR. First, we explored the ZFPM2-AS1

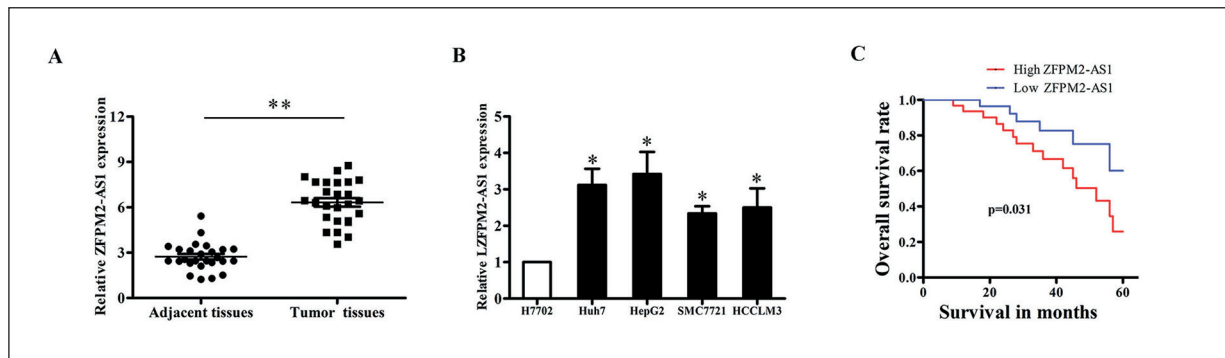


Figure 1. ZFPM2-AS1 was upregulated in HCC. **A**, Increased expression of ZFPM2-AS1 in HCC tissues compared with normal tissues. **B**, ZFPM2-AS1 was overexpressed in HCC cell lines. **C**, Kaplan-Meier analysis was employed to estimate the relation between ZFPM2-AS1 expression and overall survival of HCC patients. (* $p < 0.05$, ** $p < 0.01$).

expression in 52 pairs of HCC and paired normal liver tissues. The results showed that ZFPM2-AS1 was highly expressed in HCC (Figure 1A). Then, we detected the ZFPM2-AS1 expression in HCC cell lines and the hepatocyte cell line. We found that ZFPM2-AS1 was significantly upregulated in HCC cells (Figure 1B). Furthermore, the relationship between ZFPM2-AS1 expression and clinicopathological variables was analyzed. The expression level of ZFPM2-AS1 was positively correlated with tumor size, distant metastasis and TNM stage (Table I). Kaplan-Meier analysis showed that HCC patients with higher ZFPM2-AS1 level had a lower overall survival rate (Figure 1C).

ZFPM2-AS1 Knockdown Inhibited HCC Cell Proliferation and Invasion

To investigate its biological functions, ZFPM2-AS1 was knocked down in HepG2 and Huh7 cells using si-ZFPM2-AS1 (Figure 2A). The CCK-8 assay showed that cell proliferation was inhibited by knockdown of ZFPM2-AS1 (Figure 2B). The EdU assay demonstrated that ZFPM2-AS1 knockdown inhibited cell proliferation (Figure 2C). Moreover, the Matrigel transwell assay showed that ZFPM2-AS1 knockdown remarkably suppressed the HCC cells invasion (Figure 2D). These results indicated that ZFPM2-AS1 promoted the proliferation and invasion of HCC cells.

Table I. Correlation between lncRNA ZFPM2-AS1 expression with clinicopathological features of HCC patients.

Features	ZFPM2-AS1 expression		p-value
	Low (n = 24)	High (n = 28)	
Age			0.085
≤ 50	8	16	
> 50	16	12	
Gender			0.493
Male	15	20	
Female	9	8	
Tumor size			0.0146*
≤ 5 cm	10	21	
> 5 cm	14	7	
Distance metastasis			0.0061**
Yes	11	23	
No	13	5	
TNM stage			0.0054**
I-II	17	9	
III-IV	7	19	

Low/high by the sample median. Pearson χ^2 -test. $p < 0.05$ was considered. Statistically significant. * $p < 0.05$; ** $p < 0.001$.

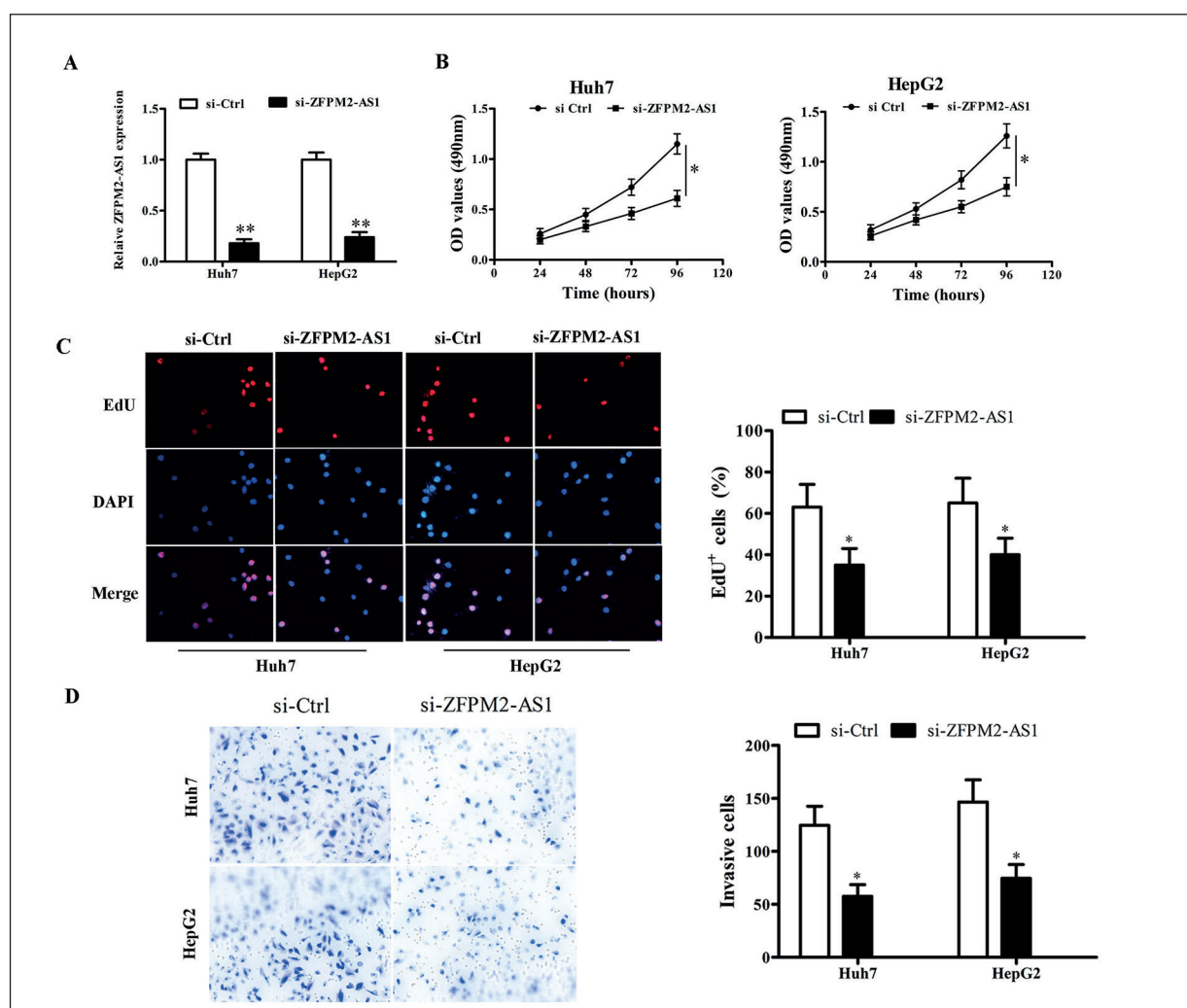


Figure 2. ZFPM2-AS1 knockdown suppressed HCC proliferation, invasion. **A**, ZFPM2-AS1 expression was decreased in Huh7 and HepG2 cells transfected with si-ZFPM2-AS1. **B**, **C**, CCK8 assay and EdU assay indicated that ZFPM2-AS1 knockdown inhibited HCC cell proliferation (magnification 200 $\times$). **D**, Transwell assay indicated that ZFPM2-AS1 knockdown suppressed HCC cell invasion (magnification 100 $\times$). Data are expressed as mean $\pm$ standard deviation. (* p <0.05, ** p <0.01).

ZFPM2-AS1 Served as a Sponge for miR-1226-3p in HCC

To investigate the regulatory mechanism of ZFPM2-AS1, we examined intracellular location of ZFPM2-AS1. Subcellular fractionation and RNA FISH assays indicated that ZFPM2-AS1 mainly localized in the cytoplasm (Figure 3A and 3B). Bioinformatics analysis showed that miR-1226-3p has binding sites of ZFPM2-AS1 (Figure 3C). Dual-Luciferase reporter assay showed that the Luciferase activity of ZFPM2-AS1-WT was markedly reduced by miR-1226-3p mimics (Figure 3D). Furthermore, qRT-PCR assay showed that miR-1226-3p expression was remarkably suppressed in ZFPM2-AS1 overexpressed HCC

cells (Figure 3E). MiR-1226-3p expression was upregulated in HCC cells transfected with siZFPM2-AS1 (Figure 3F). Moreover, RNA pulldown assay showed that biotin-labeled miR-1226-3p precipitated lncRNA ZFPM2-AS1 in HCC cells (Figure 3G). Taken together, these findings revealed that lncRNA ZFPM2-AS1 was a sponge for miR-1226-3p in HCC.

ZFPM2-AS1 Promoted HCC Proliferation and Invasion through Regulating miR-1226-3p/ITGB1

Using TargetScan software (<http://www.targetscan.org/cgibin/targetscan>), we discovered that miR-1226-3p could potentially bind to the 3'-

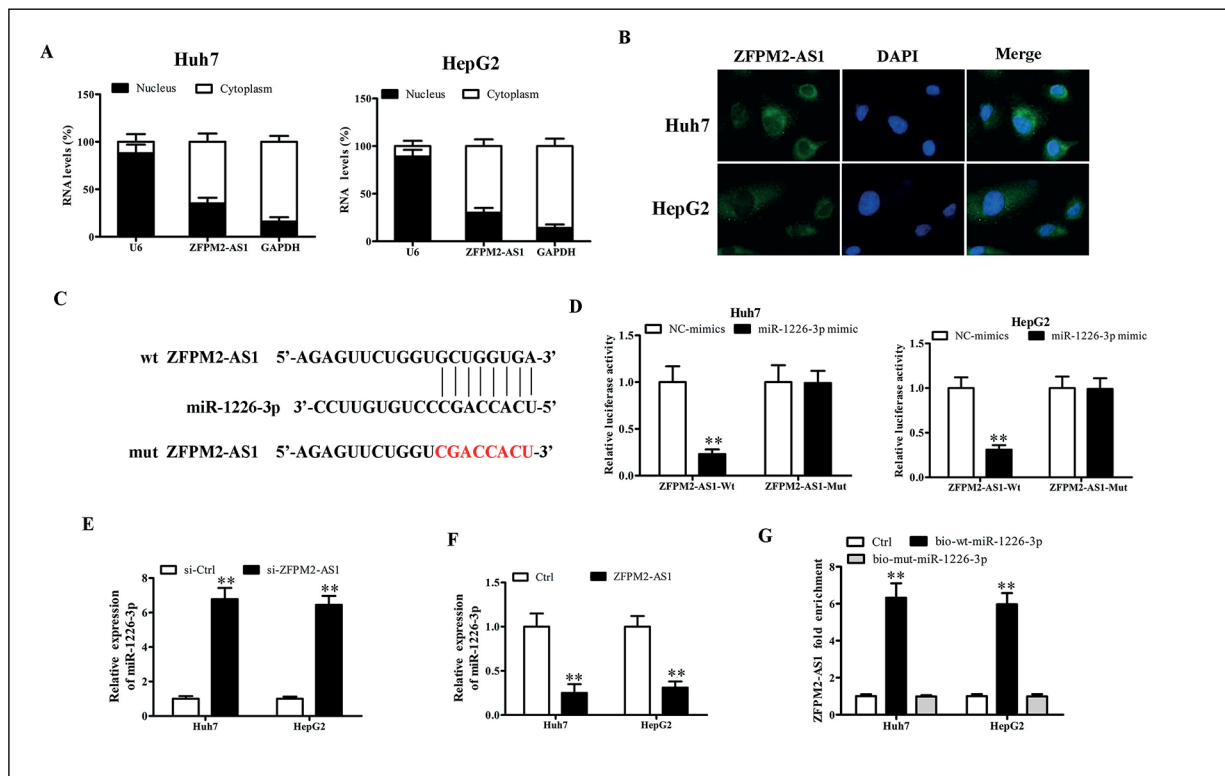


Figure 3. ZFPM2-AS1 was a sponge for miR-1226-3p in HCC. **A, B**, Subcellular fractionation and FISH staining confirmed the expression of ZFPM2-AS1 in cytoplasm (magnification 400×). **C**, The result of LncBase Predicted v.2 predicted that miR-1226-3p had a binding site for ZFPM2-AS1. **D**, Luciferase reporter assay indicated that ZFPM2-AS1 could bind with miR-1226-3p. **E**, miR-1226-3p expression was increased after ZFPM2-AS1 knockdown. **F**, ZFPM2-AS1 overexpression inhibited miR-1226-3p expression. **G**, RNA pulldown assay showed that biotin-labeled wt-miR-1226-3p precipitated ZFPM2-AS1 in HCC cell lysates. Data are expressed as mean±standard deviation. (** $p < 0.01$).

UTR of ITGB1 mRNA (Figure 4A). Luciferase reporter assay indicated that the activity of wt-ITGB1 reporter was decreased by miR-1226-3p mimics (Figure 4B). Moreover, miR-1226-3p mimics significantly inhibited ITGB1 expression in Huh7 and HepG2 (Figure 4C and 4D). To determine whether ZFPM2-AS1 could promote ITGB1 expression by sponging miR-1226-3p, we performed Luciferase reporter assay. We found that the Luciferase reporter activity of wt-ITGB1 was inhibited by si-ZFPM2-AS1 (Figure 4E). Furthermore, ZFPM2-AS1 knockdown inhibited the expression of ITGB1 in HCC cells (Figure 4F). Thus, ITGB1 expression was upregulated by ZFPM2-AS1 through sponging miR-1226-3p in HCC.

We further investigate the biological effect of the ZFPM2-AS1-miR-1226-3p-ITGB1 axis in HCC cells. We found that ITGB1 expression was upregulated in HCC tissues (Figure 4G). We restored the expression of ITGB1 in HCC cells

(Figure 4H). The CCK8 and EdU assays results showed that ITGB1 restoration rescued the abilities of proliferation in ZFPM2-AS1-knockdown HCC cells (Figure 4I, 4J). Transwell assay results demonstrated that ITGB1 reversed the effect of ZFPM2-AS1 on HCC cells invasion (Figure 4K). Taken together, these results demonstrated that ZFPM2-AS1 regulates HCC progression through miR-1226-3p/ITGB1 axis.

Discussion

In the present study, we identified lncRNA ZFPM2-AS1 as an oncogene involved in HCC progression. We found that ZFPM2-AS1 was overexpressed in HCC tissues and positively correlated with TNM stage, distant metastasis and poor prognosis. Furthermore, we revealed that ZFPM2-AS1 knockdown suppressed the proliferation and invasion of HCC cells. Mechanistically,

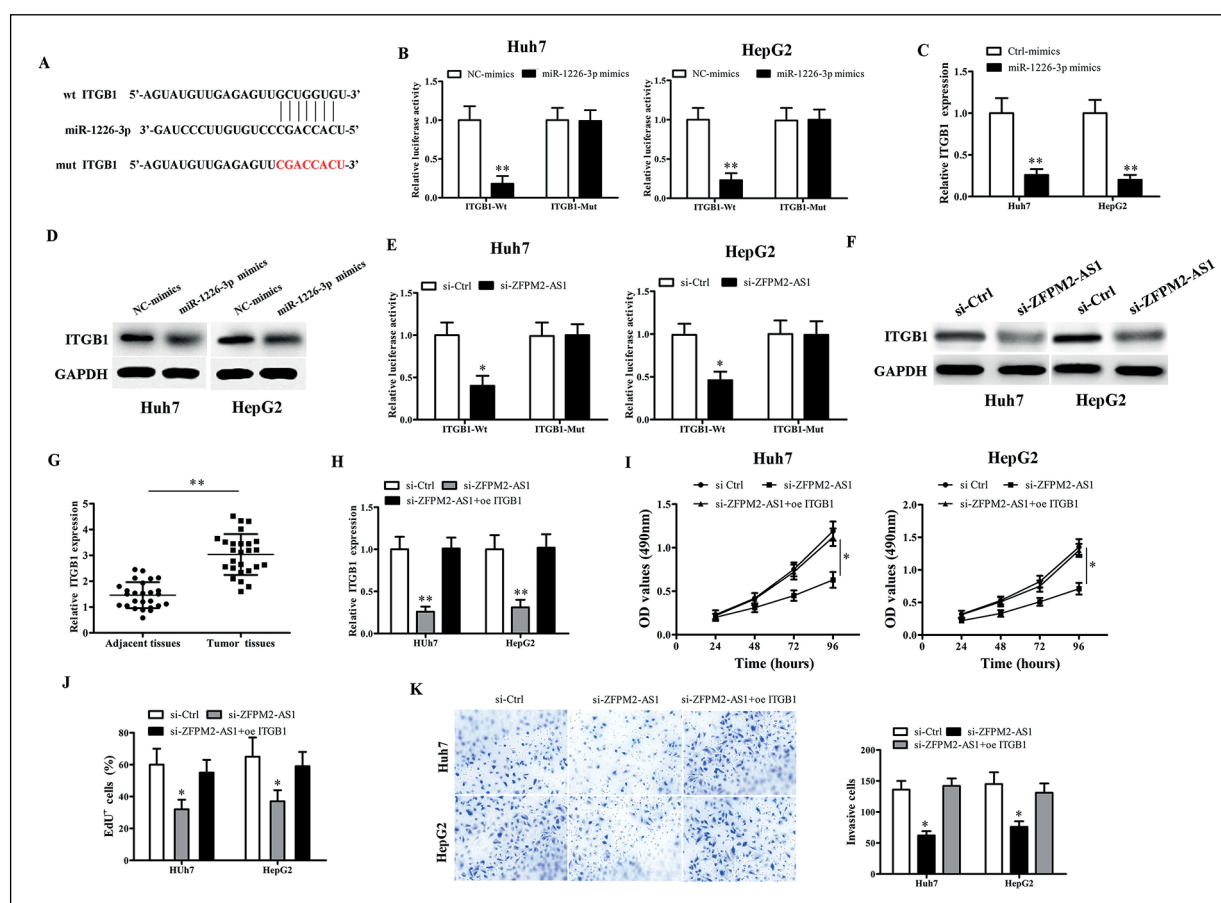


Figure 4. ZFPM2-AS1/miR-1226-3p/ITGB1 axis regulated HCC cells proliferation and migration. **A**, Target Scan was used to predict that ITGB1 had a binding site for miR-1226-3p. **B**, Luciferase reporter assay verified that ITGB1 was a target gene of miR-1226-3p. **C**, **D**, ITGB1 expression was inhibited by miR-1226-3p mimics. **E**, Luciferase reporter assay showed that ZFPM2-AS1 knockdown suppressed the activity of wt-ITGB1 reporter. **F**, Western blot showed that ZFPM2-AS1 knockdown inhibited the expression of ITGB1 in HCC cells. **G**, qRT-PCR showed that ITGB1 expression was upregulated in HCC tissues. HCC cells were transfected with si-ZFPM2-AS1, si-ZFPM2-AS1+ITGB1 overexpression vector (si-ZFPM2-AS1+oeITGB1) or the control si RNA (si-Ctrl). **H**, Relative expression of ITGB1 in Huh7 and HepG2 cells was determined by qRT-PCR. **I**, **J**, Cell proliferation was measured by CCK8 and EdU assays. **K**, Cell invasion was assessed by Matrigel transwell assay (magnification 100×). Data are expressed as mean±standard deviation. (* $p < 0.05$, ** $p < 0.01$).

we showed that ZFPM2-AS1 acts as a ceRNA for miR-1226-3p and upregulates ITGB1 expression.

ZFPM2-AS1 is located on chromosome 8q23, the studies on ZFPM2-AS1 functions are limited. Present report showed that ZFPM2-AS1 promotes progression of gastric cancer by regulating MIF/p53 axis¹³. And ZFPM2-AS1 promotes tumorigenesis of renal cell cancer by targeting miR-137¹⁴. In the present study, we found that ZFPM2-AS1 expression was significantly upregulated in HCC tissues. ZFPM2-AS1 expression was positively correlated with tumor size, TNM and distant metastasis. In addition, patients with high expression of ZFPM2-AS1 displayed poor overall survival rate, suggesting that ZFPM2-AS1 might

be a potential diagnostic biomarker and prognostic indicator for HCC. The *in vitro* experiments demonstrated that knockdown of ZFPM2-AS1 remarkably inhibits HCC cell proliferation and invasion, suggesting the oncogenic role of ZFPM2-AS1 in HCC.

Increasing studies have confirmed that lncRNAs play crucial roles in cancers by functioning as ceRNAs to sponge miRNA and regulate gene expression^{15,16}. For example, LINC01296 promoted progression and metastasis of HCC cells by sponging miR-122-5P¹⁷. SNHG14 served as a ceRNA by sponging miR-340 to promote NSCLC progression¹⁸. In this study, we investigated the mechanism by which ZFPM2-AS1

exerts oncogenic functions in HCC. Through subcellular fractionation assay and RNA FISH assay, we determined that ZFPM2-AS1 mainly localized in cytoplasmic localization. Notably, it has been reported that lncRNAs, miRNAs, and mRNAs in the ceRNA regulatory network mainly interact with each other in the cytoplasm¹⁹. We supposed that ZFPM2-AS1 acted as ceRNAs to sponge miRNA and exerted its oncogenic functions. We found that ZFPM2-AS1 interacted with miR-1226-3p and negatively regulated its expression.

MiR-1226-3p has been reported to promote sorafenib sensitivity in HCC²⁰. Many studies have found that miR-1226 may be associated with tamoxifen resistance in breast cancer and miR-1226 interacts with the MUC1 mRNA 3'UTR and downregulates MUC1 protein levels^{21,22}. According to ceRNA hypothesis, mRNA was upregulated due to the binding of lncRNA and miRNA. Therefore, we investigated the mRNA that could interact with miR-1226-3p in HCC cells. ITGB1 plays an important function in cancer progression^{23,24}. UCA1 promoted nasopharyngeal carcinoma cell proliferation by up-regulating ITGB1 through suppressing miR-124-3p²⁵.

In our study, we found that ITGB1 was a direct target of miR-1226-3p. We also indicated that ITGB1 expression was inhibited by miR-1226-3p and upregulated by ZFPM2-AS1. Besides, we showed that ITGB1 expression was increased in HCC tissues. In addition, rescue assays indicated that ITGB1 upregulation significantly rescued the effects caused by ZFPM2-AS1 knockdown on cell proliferation and invasion.

Conclusions

In summary, our study revealed that ZFPM2-AS1 acted as an oncogene to promote HCC cell progression through miR-1226-3p/ITGB1 axis. We revealed a novel mechanism involved in HCC progression, which provides promising therapeutical targets for HCC prevention and treatment. Lacking *in vivo* data is a deficiency of our present study. We will investigate this novel molecular pathway in animal model in the future study.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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