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Upregulation of LncRNA FAM83H-AS1 in hepatocellular carcinoma promotes cell proliferation, migration and invasion by Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: The long non-coding RNA, FAM83H antisense RNA 1 (head to head) (FAM83H-AS1), has been reported to function as an oncogene in some types of cancer. However, the role of lncRNA FAM83H-AS1 in hepatocellular carcinoma (HCC) still remains unknown. The present work aims to explore the effect of lncRNA FAM83H-AS1 on cell proliferation and cell invasion in HCC.

PATIENTS AND METHODS: 66 pairs of HCC tissue samples and adjacent normal tissues were collected, and the expression level of lncRNA FAM83H-AS1 was detected by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) analysis. Cell Counting Kit-8 (CCK-8) assay was performed to detect cell proliferation ability, and transwell assays were applied to observe the effect of lncRNA FAM83H-AS1 on cell migration and invasion. QRT-PCR and Western blot analysis was used to determine the mRNA and protein expression.

RESULTS: In the present study, our results confirmed that lncRNA FAM83H-AS1 expression was overexpressed in HCC tissues relative to the adjacent normal tissues. Furthermore, higher lncRNA FAM83H-AS1 expression significantly associated with tumor size and vascular invasion in patients with HCC. The Kaplan-Meier methods and log rank test demonstrated that increased lncRNA FAM83H-AS1 expression associated with shorter patient overall survival compared to lower lncRNA FAM83H-AS1 expression in patients with HCC. Moreover, function assays by CCK-8 cell proliferation and transwell cell migration and invasion assays showed that the knockdown of lncRNA FAM83H-AS1 significantly inhibited cell proliferation, migration, and invasion ability in HCC. Moreover, we found that the downregulating expression of lncRNA FAM83H-AS1 inhibited Wnt/β-catenin pathway by reducing β-catenin and WNT1 expression in HCC cells.

CONCLUSIONS: Together, our results indicated that it plays an important role in HCC progression and may be a potential target for HCC treatment.

Key Words:
Gastric cancer, MicroRNA, MiR-198, Toll-like receptor 4 (TLR4).

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and represents the sixth leading cause of cancer-related death[1]. Recently, although curative improvements have made for HCC patients, the 5-year overall survival rate still remains low due to the tumor metastases and recurrence[2]. The underlying molecular mechanisms for HCC progression are not entirely clear[3]. Therefore, to explore the molecular mechanisms involved in the pathogenesis of HCC can help in the treatment of HCC.

Atkinson et al[4] have indicated that long non-coding RNAs (lncRNAs) play key roles in cancer development. To date, some lncRNAs have been found to be involved in HCC proliferation, migration, invasion, and metastasis[5]. For instance, long non-coding RNA LINC00673 promotes hepatocellular carcinoma progression and metastasis by negatively regulating miR-205[6]. Long non-coding RNA00364 represses hepatocellular carcinoma cell proliferation via modulating the p-STAT3-IFIT2 signaling axis[7]. Long noncoding RNA NNT-AS1 promotes hepatocellular carcinoma progression and metastasis through the miR-363/CDK6 axis[8]. However, investigations on the function and clinical significance of lncRNA FAM83H-AS1 remain largely limited.

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LncRNA FAM83H-AS1 is found to be associated with poor patient survival, and the knockdown of lncRNA FAM83H-AS1 impairs cell proliferation and invasion via MET/EGFR signaling in lung cancer[9]. In the present study, we confirmed that lncRNA FAM83H-AS1 expression was overexpressed in HCC and associated with worse patients' overall survival. In addition, we demonstrated that the knockdown of lncRNA FAM83H-AS1 inhibited cell proliferation, migration, and invasion ability. Therefore, our results indicated that lncRNA FAM83H-AS1 plays an important role in HCC progression and may be a potential target for HCC treatment.

Patients and Methods

Patients Tissue Samples

66 paired HCC tissue samples and adjacent normal tissue samples were obtained from patients who underwent primary surgical resection of liver cancer at the Department of Hepatobiliary Surgery, at the Affiliated Yantai Yuhuangding Hospital of Qingdao University between March 2011 and December 2014. The patients included 20 women and 46 men, aged from 35 to 78 years; the TNM stage was established according to the WHO grade[10]. All human tissue samples were immediately frozen in liquid nitrogen and stored at –80°C until RNA analyses. The present report was approved by the Research Ethics Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University (Yantai, China). Written informed consent was obtained from all patients, and the clinical data was showed in Table I.

Cell Lines Culture

The human HCC cell lines, including HepG2, MHCC97H (97H), Huh-7, SMCC-7721, and immortalized normal liver epithelial THLE-3 cell lines were obtained from the Cell Bank of Type Culture Collection (Chinese Academy of Sciences, Shanghai, China). Cells were maintained in

Table I. Association of lncRNA FAM83H-AS1 expression with clinicopathologic parameters in 66 HCC patients.

Clinicopathologic feathers	Total	LncRNA FAM83H-AS1 expression		*p*-value
		Lower (n = 34)	Higher (n = 32)	
Age (year)				0.148
≤ 60	46	21	25	
> 60	20	13	7	
Gender				0.709
Male	46	23	23	
Female	20	11	9	
Tumor size				0.007*
< 5 cm	36	24	12	
> 5 cm	30	10	20	
HBV infection				0.465
Yes	52	28	24	
No	14	6	8	
AFP (ng/ml)				0.852
< 400	24	12	12	
> 400	42	22	20	
Differentiation status				0.954
Higher	24	12	12	
Moderately	26	14	12	
Poor	16	8	8	
Cirrhosis				0.215
Yes	32	19	13	
No	34	15	19	
Vascular invasion				0.003*
Negative	39	26	13	
Positive	27	8	19	
TNM stage				0.485
I/II	46	25	21	
III/IV	20	9	11	

*$p < 0.05$.

Dulbecco's Modified Eagle medium (DMEM; Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37°C in a 5% CO_2 atmosphere.

Cell Transfection

Two target siRNAs and negative controls (siRNAs; Dharmacon, Lafayette, CO, USA) were transfected with a final concentration of 100 nM. Lipofectamine RNAi MAX reagent and Opti MEM medium were used for the cell interference assays according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Knockdown efficiency was measured by the qRT-PCR analysis.

Quantitative Reverse Transcription Polymerase Chain Reaction (QRT-PCR) Analysis

Total RNA was extracted from HCC tissues and cell lines using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Reverse transcription was performed by the QIAGEN One Step RT-PCR Kit (Qiagen China Co., Ltd., Shanghai, China), according to the manufacturer's protocol. The relative mRNA expression was detected using a One Step SYBR® Prime Script™ miRNA RT-PCR kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's protocol. The thermocycling conditions for qPCR were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The primers sequences were as follows: lncRNA-FAM83H-AS1-forward: 5'-TAGGAAACGAGCGAGCCC-3', lncRNAFAM83H-AS1-reverse: 5'-GCTTTGGGTCTCCCCTTCTT-3'; GAPDH-forward, 5'-GGAGTCAACGGATTTGGT-3' and GAPDH-reverse 5'-GTGATGGGATTTCCATTGAT-3'. GADPH mRNA expression fold was used as internal controls. All the researches were repeated in triplicate, and data was analyzed according to the $2^{-\Delta\Delta Ct}$ methods[11].

Cell Proliferation Assays

Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay was performed to evaluate the effects of lncNRA FAM83H-AS1 expression on cell proliferation. A total of 2000 transfected cells were seeded into each well in a 96-well plate. The cells were incubated at 37°C to indicate time, including 1, 2, 3, and 4 days. A total of 10 µl CCK-8 reagent was added into each well and cells were incubated at 37°C for an additional 2 h. The absorbance was determined at a wavelength of 450 nm by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Migration and Invasion Assays

Cell migration and invasion assay was performed using transwell chambers with a pore size of 8 µm (Corning, Corning, NY, USA) with Matrigel or without Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Following cell transfection for 48 h, 1×10^5 transfected cells with 300 µl medium without FBS were added into the upper chamber, and 500 µl medium supplemented with 10% FBS was added into the lower chamber. After cell transfection at 48 h, the cells in the lower chamber were fixed with 100% methanol for 15 min and stained with 0.5% crystal violet for 5 min at room temperature, respectively. Then, the migrated and invaded cells were counted by an inverted microscope (Olympus, Tokyo, Japan) in five randomly selected fields.

Statistical Analysis

All statistical analyses were performed using SPSS 19.0 (IBM SPSS, Armonk, NY, USA) and GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). The differences among groups were analyzed by a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests or a two-tailed Student's *t*-test, as appropriate. All data are expressed as the mean ± standard error of the mean. $p<0.05$ was identified as a statistically significant difference.

Results

LncRNA FAM83H-AS1 Expression is Upregulated in HCC Tissues and Cell Lines

We detected the expression of lncRNA FAM83H-AS1 in HCC tissue samples and the corresponding adjacent non-tumor tissues using qRT-PCR. We observed that lncRNA FAM83H-AS1 expression was significantly upregulated in HCC tissues compared with adjacent non-tumor tissues (Figure 1A; $p<0.05$). Furthermore, the expression of lncRNA FAM83H-AS1 in four HCC cell lines including HepG2, SMCC-7721, Huh7, 97H, and immortalized normal liver epithelial THLE-3 cells was measured. As shown in Figure 1B, lncRNA FAM83H-AS1 expres-

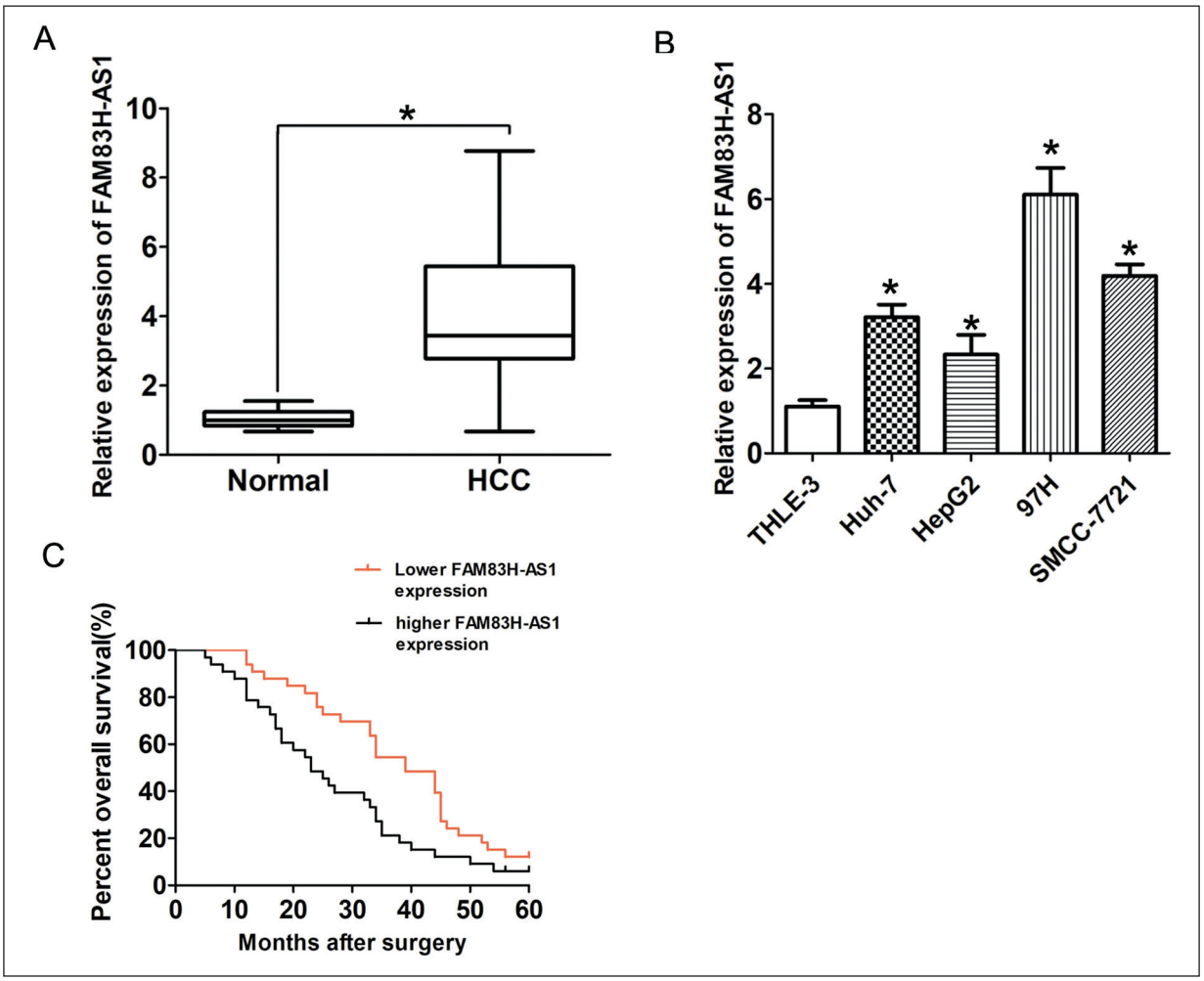

Figure 1. LncRNA FAM83H-AS1 expression is significantly upregulated in HCC tissues and cell lines. **A,** LncRNA FAM83H-AS1 expression was significantly upregulated in 66 pairs of HCC tissues when compared with corresponding adjacent non-tumor tissues using qRT-PCR. GAPDH was used as internal control. **B,** Expression level of lncRNA FAM83H-AS1 was increased in HCC cell lines compared with immortalized normal liver epithelial THLE-3 cells. *$p<0.05$. **C,** Higher lncRNA FAM83H-AS1 expression showed a shorter overall survival rate in patients with HCC compared to lower lncRNA FAM83H-AS1 expression, log rank test, $p<0.05$.

sion was also increased in HCC cell lines compared with THLE-3 ($p<0.05$). Therefore, these results indicated that lncRNA FAM83H-AS1 may perform crucial clinical value and functional effects in HCC carcinogenesis and progression.

LncRNA FAM83H-AS1 Expression Associates with Tumor Size, Vascular Invasion, and Prognosis of HCC

We then assessed the association of lncRNA FAM83H-AS1 expression with clinicopathological factors in patients with HCC. The lncRNA FAM83H-AS1 expression was divided into higher and lower expression groups according to the mean expression of lncRNA FAM83H-AS1 in HCC tissues. As shown in Table I, we observed that higher lncRNA FAM83H-AS1 expression was significantly associated with tumor size ($p=0.007$) and vascular invasion ($p=0.003$) in HCC patients. However, no correlation was found between lncRNA FAM83H-AS1 expression and other clinicopathological feathers, including age, gender, differentiation, HBV infection, and so on ($p>0.05$, Table I). The survival plots by Kaplan-Meier methods showed that higher lncRNA FAM83H-AS1 in patients with HCC exhibited a shorter overall survival rate compared to lower lncRNA FAM83H-AS1 in patients with HCC (Figure 1C, log-rank test, $p<0.05$).

LncRNA FAM83H-AS1 Knockdown Inhibits Cell Proliferation, Migration and Invasion in HCC

Cell proliferation, migration, and invasion assays were performed to assess the effects of lncRNA FAM83H-AS1 on the biological function of HCC. As demonstrated in Figure 2A-2B, lncRNA FAM83H-AS1 was knocked down by two si-FAM83H-AS1 oligos. We found that the si-FAM83H-AS1-2 showed higher silencing efficiency for FAM83H-AS1 in 97H and SMCC-7721 cells. Through the CCK8 cell proliferation assays, we showed that lncRNA FAM83H-AS1 silencing inhibited the HCC 97H and SMCC-7721 cellular proliferation ability, compared with cells transfected with si-NC (Figure 2C-2D). Furthermore, we detected the effects of lncRNA FAM83H-AS1 silencing on cell migration and invasion of HCC. We observed that lncRNA FAM83H-AS1 silencing also significantly inhibited the 97H and SMCC-7721 cellular migration and invasion ability compared with cells transfected with si-NC (Figure 3A-3D). Thus, these above results indicate that the abnormal expression of lncRNA FAM83H-AS1 may be capable of regulating HCC proliferation, migration, and invasion.

LncRNA FAM83H-AS1 Knockdown Inhibits Wnt/β-Catenin Pathway in HCC

To explore whether lncRNA FAM83H-AS1 silencing affects the Wnt/β-catenin pathway in

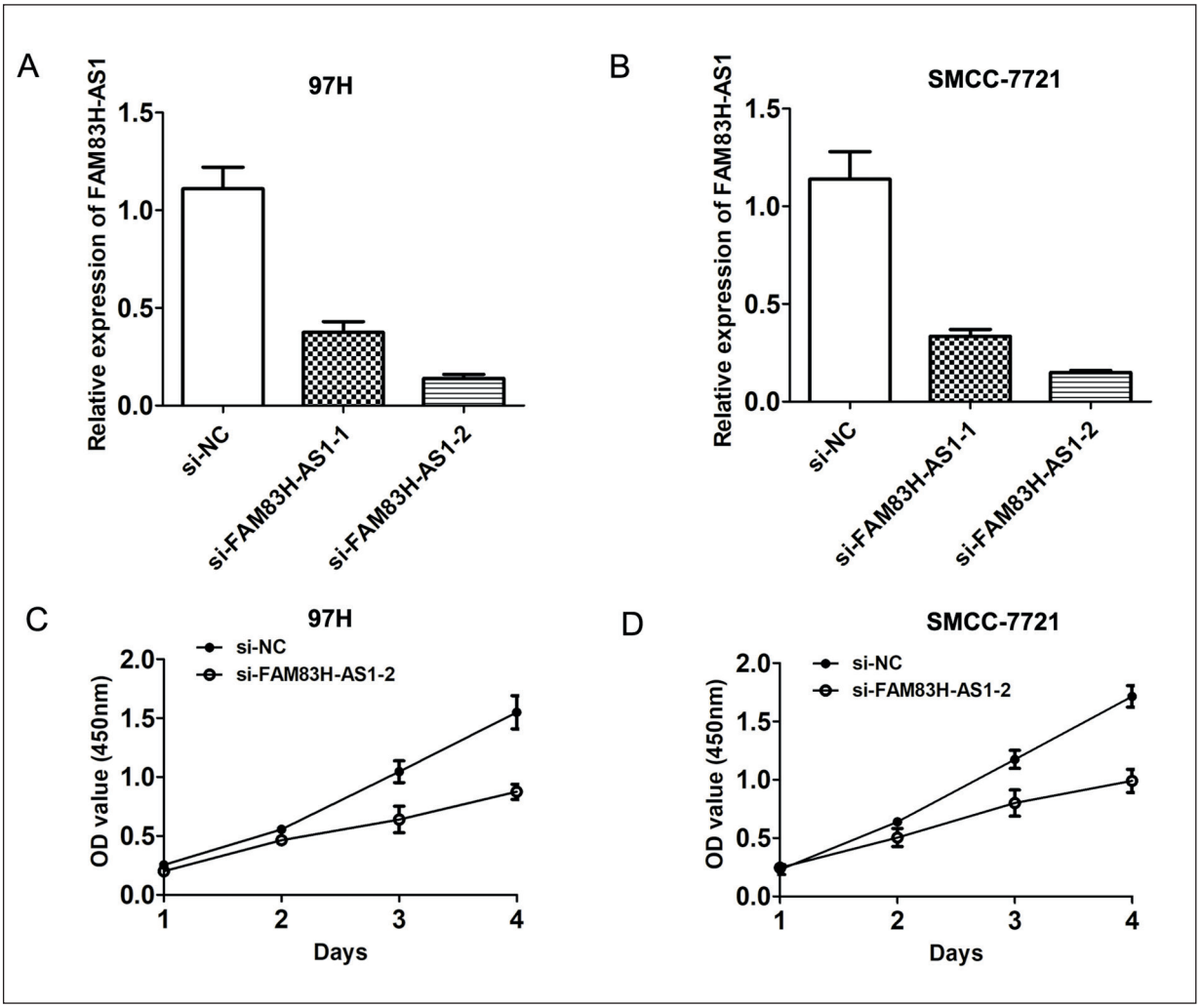

Figure 2. LncRNA FAM83H-AS1 knockdown significantly inhibits HCC cell proliferation ability. **A-B,** Expression was detected using qRT-PCR after 97H, and SMCC-7721 cells were transfected with si-FAM83H-AS1-2 or si-NC. GAPDH was used as internal control. **C-D,** Cell proliferation ability was detected using CCK8 assay after 97H, and SMCC-7721 cells were transfected with si-FAM83H-AS1-2 or si-NC. *$p<0.05$.

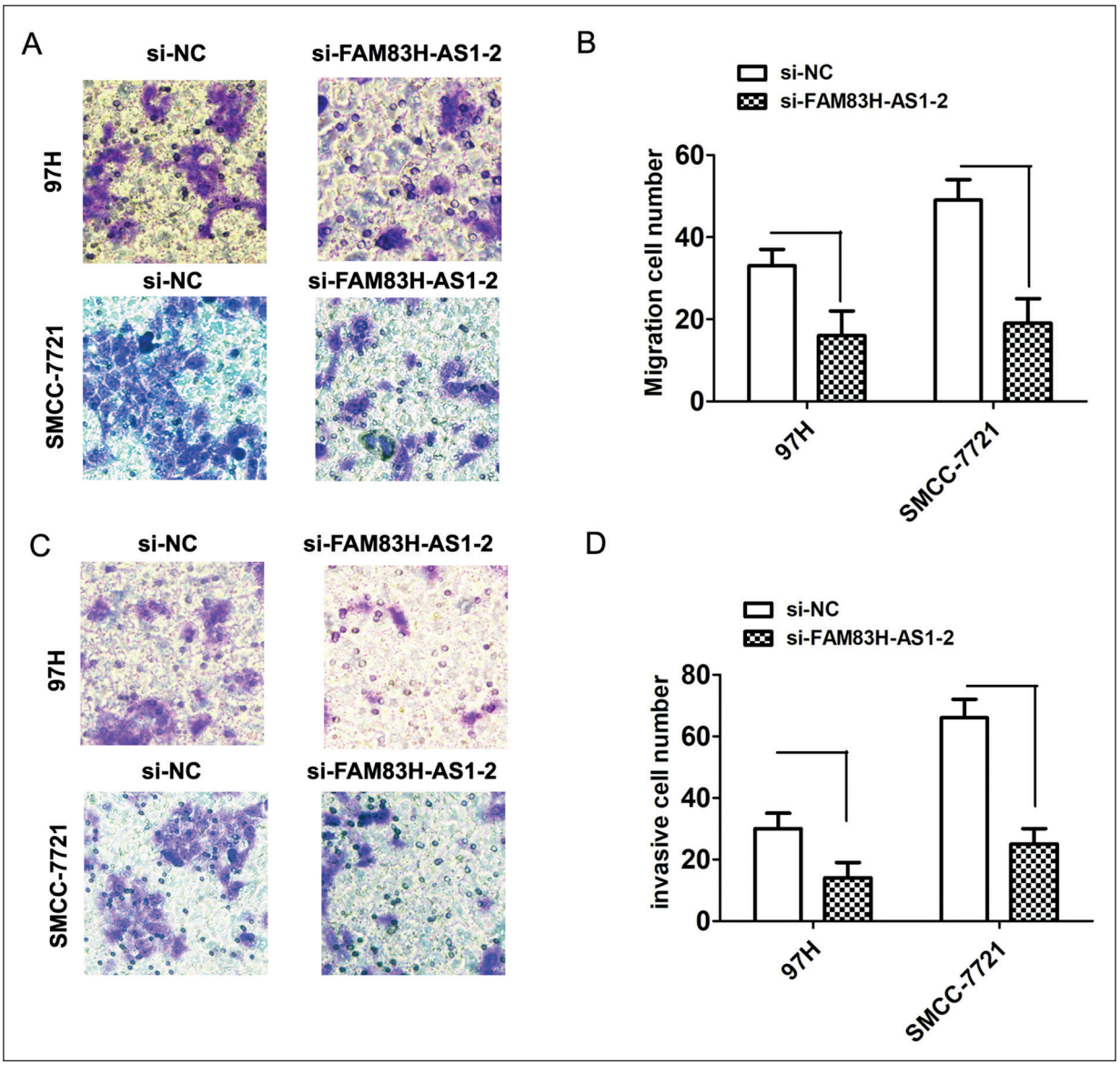

Figure 3. LncRNA FAM83H-AS1 knockdown significantly inhibits HCC cell migration and invasion ability. **A-B,** Cell migration ability was detected using transwell migration assay after 97H, and SMCC-7721 cells were transfected with si-FAM83H-AS1-2 or si-NC. **C-D,** Cell invasion ability was detected using transwell invasion assay after 97H, and SMCC-7721 cells were transfected with si-FAM83H-AS1-2 or si-NC. *$p<0.05$. **A-C,** Magnification 200×.

HCC, we performed Western blot to detect the β-catenin and WNT1 expression.

We detected that lncRNA FAM83H-AS1 silencing also significantly inhibited the protein expression β-catenin and WNT1 in 97H and SMCC-7721 cells compared with cells transfected with si-NC (Figure 4A-4B). Thus, these above results indicate that the downregulating expression of lncRNA FAM83H-AS1 inhibited Wnt/β-catenin pathway by reducing β-catenin and WNT1 expression in HCC cells.

Discussion

The deregulation of lncRNAs expression in human tumor sample tissues compared to normal tissues is a common event may be important for tumorigenesis and development[5,12]. Thus, to explore the clinical role and biological function of lncRNAs may be applied to identify the biomarker for clinical diagnosis and prognosis or target for tumor therapy in various types of cancer[13,14]. Yang et al[15] reported that lncRNA FAM83H-AS1

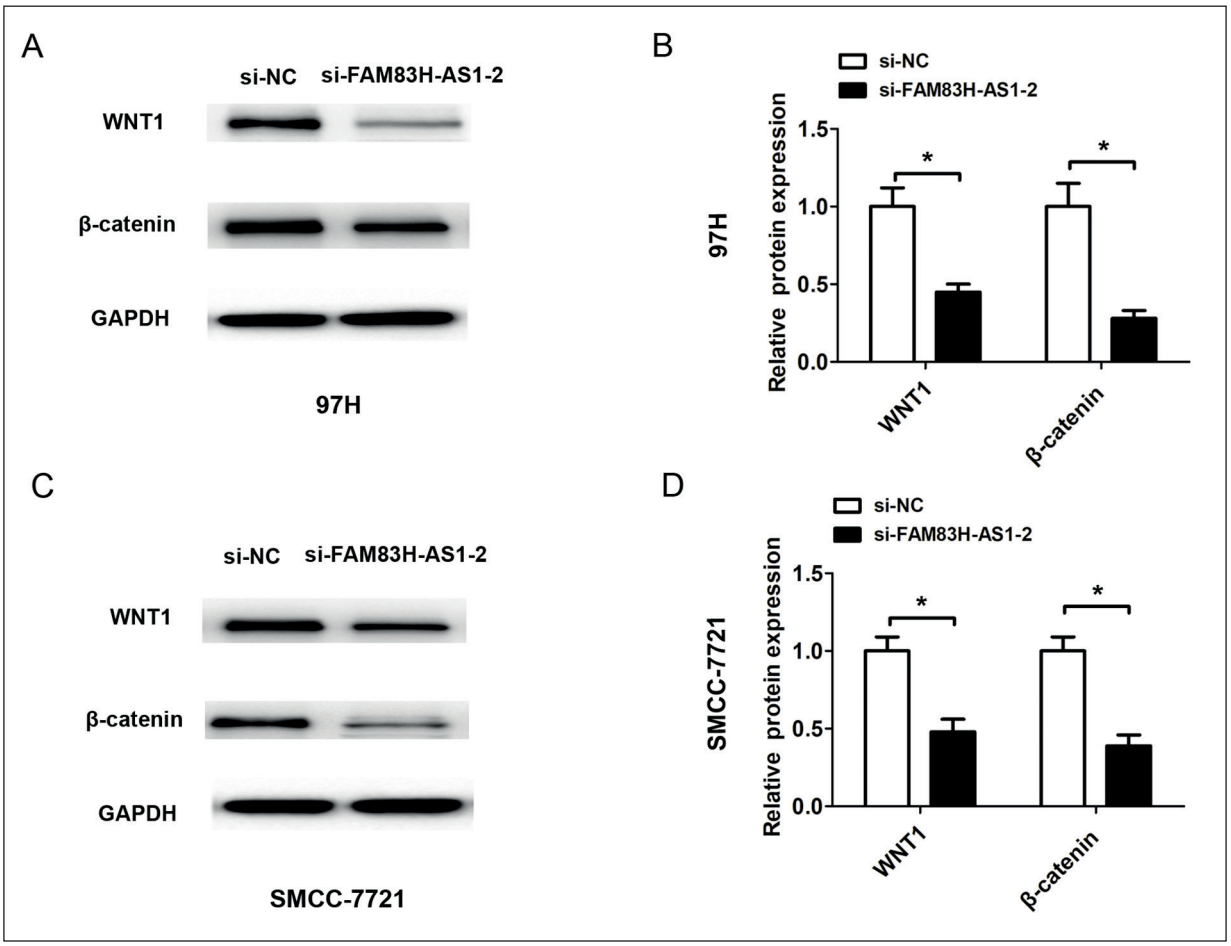

Figure 4. LncRNA FAM83H-AS1 knockdown inhibits Wnt/β-catenin pathway in HCC. **A-B,** β-catenin and WNT1 expression was detected by Western blot analysis in 97H cells were transfected with si-FAM83H-AS1-2 or si-NC. **C-D,** β-catenin and WNT1 expression was detected by Western blot analysis in SMCC-7721 cells were transfected with si-FAM83H-AS1-2 or si-NC. *$p<0.05$.

was identified as a novel prognostic marker in luminal subtype breast cancer. Overexpression of FAM83H-AS1 indicates poor patient survival and the knockdown impairs cell proliferation and invasion via MET/EGFR signaling in lung cancer[9]. Aberrant expression of lncRNA FAM83H-AS1 is associated with the prognosis of colorectal carcinoma and promotes cell proliferation by targeting the Notch signaling pathway[16]. Bi et al[17] showed that long noncoding RNA FAM83H-AS1 exerts an oncogenic role in glioma by epigenetically silencing CDKN1A (p21). However, the clinical role and functional effects of lncRNA FAM83H-AS1 in HCC still remains unknown.

In the study, we showed that lncRNA FAM83H-AS1 expression was overexpressed in HCC tissues relative to adjacent normal tissues and significantly associated with tumor size and vascular invasion of HCC. Furthermore, we detected the prognostic value of lncRNA FAM83H-AS1 in HCC. The Kaplan-Meier method demonstrated that increased lncRNA FAM83H-AS1 expression was associated with worse patient overall survival. Thus, these results indicated that lncRNA FAM83H-AS1 may act as a prognostic marker of HCC prognosis.

Next, we performed cell proliferation, migration, and invasion assay to detect the effects of lncRNA FAM83H-AS1 expression on biological functions. Through CCK-8 cell proliferation assays, we demonstrated that lncRNA FAM83H-AS1 knockdown inhibited the HCC 97H and SMCC-7721 cellular proliferation ability, compared with cells transfected with NC. Furthermore, we also confirmed that knockdown of lncRNA FAM83H-AS1 suppressed the HCC 97H and SMCC-7721 cellular migration and invasion ability compared with cells transfected with NC.

These results indicated that the abnormal expression of lncRNA FAM83H-AS1 may be capable of regulating HCC proliferation, migration, and invasion.

To explore whether lncRNA FAM83H-AS1 silencing affects the Wnt/β-catenin pathway in HCC, we performed Western blot to detect the β-catenin and WNT1 expression.

We observed that lncRNA FAM83H-AS1 silencing also significantly inhibited the protein expression β-catenin and WNT1 in HCC, which indicated that the downregulating expression of lncRNA FAM83H-AS1 inhibited the Wnt/β-catenin pathway by reducing β-catenin and WNT1 expression in HCC cells.

Conclusions

LncRNA FAM83H-AS1 expression was higher in HCC tissues and cell lines, and was associated with tumor prognosis of HCC. Besides, the knockdown of lncRNA FAM83H-AS1 suppressed HCC cell proliferation, migration, and invasion ability. Thus, these results implied that lncRNA FAM83H-AS1 plays an important role in HCC progression and may be a potential target for HCC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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