Long noncoding RNA AFAP1-AS1 indicates a poor prognosis of hepatocellular carcinoma and promotes cell proliferation and invasion via upregulation of the RhoA/Rac2 signaling

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Abstract. It has been shown that long noncoding RNAs (lncRNAs) play a critical role in the regulation of cellular processes including cancer progression and metastasis. However, the biological functions and clinical significance of lncRNA AFAP1-AS1 in hepatocellular carcinoma (HCC) remain unclear. Expression of AFAP1-AS1 was analyzed in 78 HCC tissues by real-time PCR. The effect of AFAP1-AS1 on cell proliferation was examined by MTT assay, cell apoptosis was detected by flow cytometric analysis and cell invasion was determined by Transwell assay. RhoA/Rac2 signaling and downstream factors were verified by western blotting. HCC cells infected with si-AFAP1-AS1 were injected into nude mice to investigate the effect of AFAP1-AS1 on the tumorigenesis *in vivo*. We found that increased expression of AFAP1-AS1 was significantly correlated with pathological staging (P=0.024) and lymph-vascular space invasion (LVSI) in HCC patients (P=0.007). Multivariate analyses indicated that AFAP1-AS1 represented an independent predictor for overall survival of HCC (P=0.029). Further experiments showed that knockdown of AFAP1-AS1 by si-AFAP1-AS1 decreased the proliferation and invasion *in vitro* and *in vivo*, induced cell apoptosis and blocked cell cycle in S phase via inhibition of the RhoA/Rac2 signaling. Taken together, our findings indicate that AFAP1-AS1 may promote the HCC development through upregulation of RhoA/Rac2 signaling and provide a potential therapeutic target for HCC.

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Introduction

Hepatocellular carcinoma (HCC) occurs mostly on the basis of pre-existing chronic liver disease and cirrhosis (1) and is a major health issue worldwide as the sixth most common cancer and second leading etiology of cancer-related deaths due to its poor prognosis associated with high recurrence rate and limited treatment options (2-4). Further investigations show that HCC is a genetic disease developing from a multi-step process. Gene aberrance linked to growth control, invasion and metastasis is frequent and provides molecular genetic basis of malignant transformation and tumor progression (5,6). Therefore, to find key genes related to tumorigenesis is of great importance for the diagnosis, targeted therapy, disease monitoring and clinical outcomes in HCC patients.

Long noncoding RNAs (lncRNAs) have no open reading frame and map to intronic and intergenic regions involved in regulating several biological processes such as transcription, translation, cellular differentiation, cell cycle regulation, and chromatin modification (7-9). lncRNAs (1772) have been found differentially expressed between HCC tissues and normal liver tissues (10), of which lncRNA GAS5 is downregulated in HCC indicating an independent prognostic factor for HCC patients (11), and lncRNA MEG3 functions as a growth suppressor via activation of p53 protein (12,13). Inhibition of cellular lncRNA-DREH by Hepatitis B virus X protein (HBx) promotes HCC cell proliferation *in vivo* and *in vivo* (14). In addition, overexpression of lncRNA HOTAIR and MALAT-1 may be candidate biomarkers for predicting tumor recurrence in HCC patients (15,16). Enforced expression of lncRNA HEIH facilitates HCC growth through enhancer of zeste homolog 2 (EZH2) (17) and lncRNA MVIH promotes tumor-inducing angiogenesis through inhibiting the secretion of phosphoglycerate kinase 1 (PGK1) (18). In HBV-related HCC, lncRNA HULC decreases p18 expression and boosts growth (19). Hence, lncRNAs play an important role in hepatocarcinogenesis, invasion, and metastasis.

Moreover, investigations have revealed that lncRNA AFAP1-AS1 has been implicated in tumorigenesis of various cancers. Increased expression of AFAP1-AS1 is found in Barrett esophagus, esophageal adenocarcinoma (20) and

pancreatic ductal adenocarcinoma (21). Upregulation of AFAP1-AS1 promotes cell invasion and metastasis via regulation of the actin filament integrity, suggesting a poor prognosis and survival for nasopharyngeal carcinoma (22) and lung cancer (23).

However, to our knowledge, few studies have been reported regarding the expression and functions of AFAP1-AS1 in HCC. In the present study, we showed that AFAP1-AS1 was remarkably increased in HCC tissues compared with the adjacent non-tumor tissues and served as an independent predictor for overall survival in HCC. In addition, knockdown of AFAP1-AS1 by si-AFAP1-AS1 inhibited cell growth *in vitro* and *in vivo* and cell invasion and induced cell apoptosis and cycle arrest in S phase, associated with regulating the transduction of the RhoA/Rac2 signaling, indicating that AFAP1-AS1 plays a critical role in the progression of HCC.

Materials and methods

Materials. Human HCC cell lines (SMCC7721 and HepG2) were from Institute of Biochemistry and Cell Biology (Shanghai, China). Lentivirus-mediated si-AFAP1-AS1 was purchased from Genechem Biotech Co., Ltd. (Shanghai, China). All antibodies including RhoA, Rac2, PCNA, MMP-9, CyclinD1 and Bax were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Drugs and reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD, USA); TRIzol Reagent and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA, USA); M-MLV Reverse Transcriptase was from Promega (Madison, WI, USA); SYBR Green Master Mixture was from Takara (Otsu, Japan). ECL-PLUS/kit was obtained from Beyotime (Hainan, China).

Clinical samples. HCC tissues and the adjacent non-tumor tissues were acquired from Shanghai First People's hospital from May 2010 to Dec 2014. Our present study was approved by Medical Ethics Committee of Shanghai Jiaotong University School of Medicine and written informed consent was received from the HCC patients or their parents before sample collection. Two pathologists decided and checked the HCC cases.

Cell culture and infection. HCC cells, placed in a humidified atmosphere containing 5% CO_2 at 37˚C, were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. When the cells reached more than 50% confluence, they were infected with lentivirus vector si-AFAP1-AS1 or negative control virus, and cultured at 37˚C and 5% CO_2. The clone infected with si-AFAP1-AS1 was defined as si-AFAP1-AS1 group, and that infected with negative control vectors was considered as s-i-NC group. si-AFAP1-AS1 forward, 5'-CCG GAACACCAATCCCAAGAGGTGACTCGAGTCACCTCT TGGGATTGGTGTTTTTTTG-3' and reverse, 5'-AATTCA AAAAAACACCAATCCCAAGAGGTGACTCGAGTCACC TCTTGGGATTGGTGTT-3'; si-NC, forward 5'-CCGGTTTC TCCGAACGTGTCACGTCTCGAGACGTGACACGTTCG GAGAATTTTTG-3' and reverse, 5'-AATTCAAAAAGTTC TCCGAACGTGTCACGTCTCGAGACGTGACACGTTCG GAGAA-3'.

Quantitative real-time PCR. To quantitatively examine the RNA expression of AFAP1-AS1 in HCC cells, real-time PCR was carried out. Total RNA of each clone was extracted with TRIzol according to the manufacturer's protocol. Reverse-transcription was performed using M-MLV and cDNA amplification was done using SYBR Green Master Mix kit. The AFAP1-AS1 gene was amplified using a specific oligonucleotide primer: sense 5'-ACTGAAGAGGAACCAGGGA CAG-3' and antisense 5'-GGGGAAACTGAAATGAATG AAG-3'. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control.

Western blot assay. HCC cells were harvested and extracted using lysis buffer (Tris-HCl, SDS, mercaptoethanol, glycerol). Cell extracts were boiled for 5 min in loading buffer and then equal amount of cell extracts were separated on 15% SDS-PAGE gels. Separated protein bands were transferred into polyvinylidene fluoride (PVDF) membranes and the membranes were blocked in 5% skim milk powder. The primary antibodies against RhoA, Rac2, PCNA, MMP-9, CyclinD1 and Bax were diluted according to the instructions of antibodies and incubated overnight at 4˚C. Horseradish peroxidase-linked secondary antibodies were added at a dilution ratio of 1:1000, and incubated at room temperature for 2 h. The membranes were washed with PBS three times and the immunoreactive bands were visualized using ECL-PLUS kit according to the kit instructions.

Cell proliferation assay. HCC cells infected with si-AFAP1-AS1 were incubated in 96-well-plates with DEME medium supplemented with 10% FBS. HCC cells were treated with 20 μl MTT dye and incubated with 150 μl of DMSO for 5 min. The color reaction was measured at 570 nm with enzyme immunoassay analyzer (Bio-Rad, Berkeley, CA, USA).

Transwell invasion assay. Transwell assay was performed by using a Transwell chamber (Qiagen, Hilden, Germany) with pore size of 8.0 μm. The Transwell chamber was coated with Matrigel. Total $1x10^6$ cells were suspended in 200 μl serum-free medium and seeded in the upper compartment of the chamber. The lower compartment was loaded with 750 μl full culture medium containing 10% FBS. After being incubated at 37˚C for 12 h, the membrane was fixed with formaldehyde, and stained with hematoxylin. Then the trans-membrane cells were counted.

Flow cytometric analysis. To detect cell apoptosis, HCC cells were trypsinized, washed with cold PBS and resuspended in binding buffer according to the instruction of the apoptosis kit. FITC-AnnexinV and PI were added to the fixed cells for 20 min in the dark, at room temperature. Then, Annexin V binding buffer was added to the mixture before the fluorescence was measured on FAC sort flow cytometer. The cell apoptosis was analyzed using Cell Quest software (Becton Dickinson, Mountain View, CA, USA). Three separate experiments were performed for each clone.

After PBS washing, the fixed cells were stained with PI in the presence of RNase A for 30 min at room temperature in the

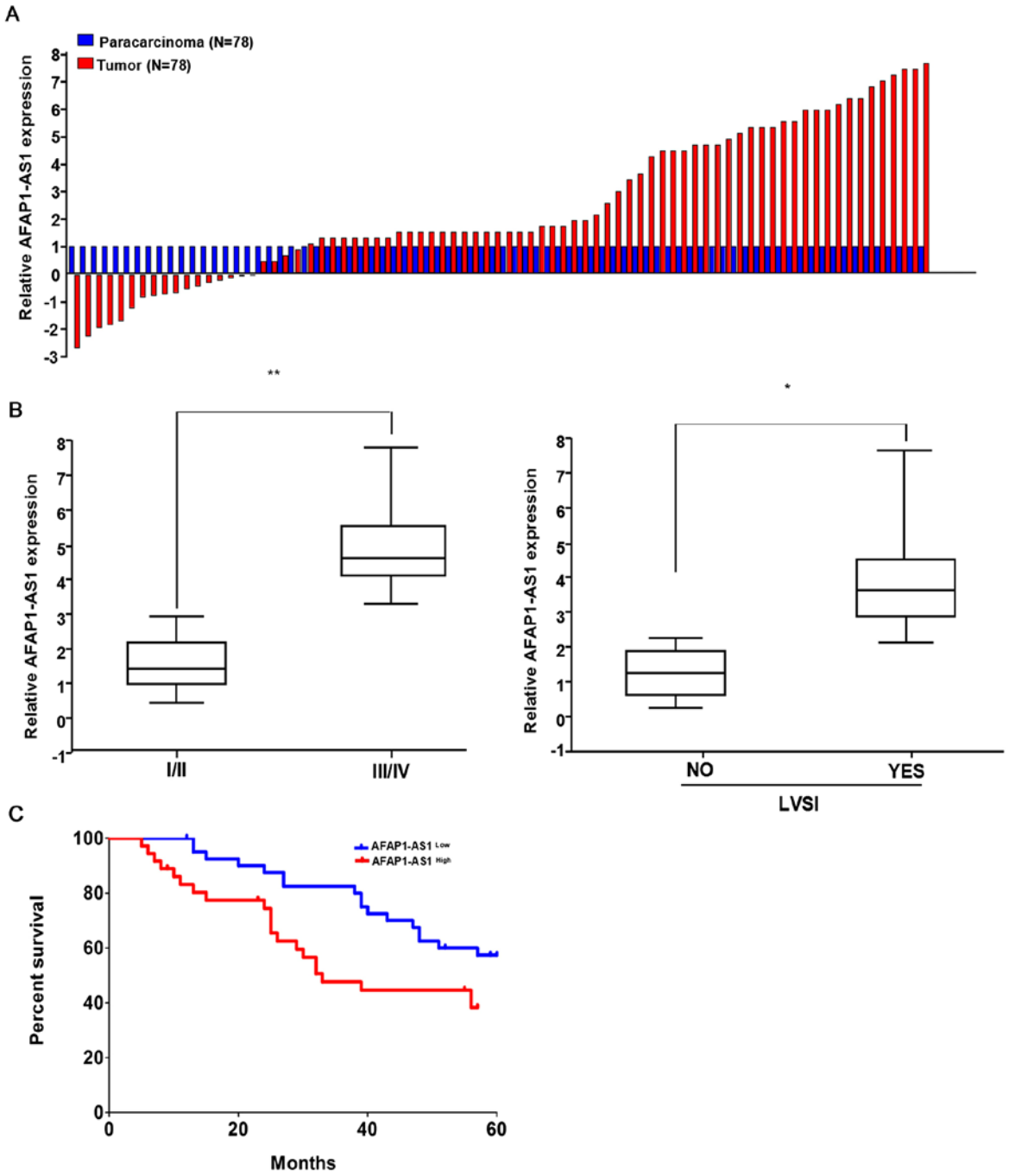

Figure 1. Expression of AFAP1-AS1 in human HCC tissues and its correlation with the clinical features and prognosis of HCC patients. (A) Relative expression of AFAP1-AS1 in HCC tissues (N=78) compared with corresponding non-tumor tissues. (B) Expression of AFAP1-AS1 was significantly higher in patients with pathological staging (III/IV) and lymph-vascular space invasion (LVSI) than in those with pathological staging (I/II) and no LVSI, respectively. (C) Kaplan-Meier analysis of survival was analyzed according to AFAP1-AS1 expression levels. *P<0.05, **P<0.01.

dark. Each sample was filtered through a 50 μm nylon filter to obtain single-cell suspension. The samples were then analyzed on FACsort flow cytometer (Becton Dickinson). ModFit3.0 software (Verity Software House, Topsham, ME, USA) was used for cell cycle analysis. Three separate experiments were performed for each clone.

In vivo tumor xenograft studies. Six-week-old female immune-deficient nude mice (BALB/c-nu) were bred at the laboratory animal facility (Institute of Chinese Academy of Sciences, Shanghai), and were housed individually in microisolator ventilated cages with free access to water and food. All experimental procedures were performed according to the regulations and internal biosafety and bioethics guidelines of Shanghai Jiaotong University and the Shanghai Municipal Science and Technology Commission. Two mice were injected subcutaneously with $1x10^6$ HCC cells in 50 μl of PBS pre-mixed with an equal volume of matrigel matrix (Becton Dickinson). Mice were monitored daily and developed a subcutaneous tumor. When the tumor size reached approximately 5 mm in length, they were surgically removed, cut into 1-2 mm^3 pieces, and reseeded individually into other mice. When tumor

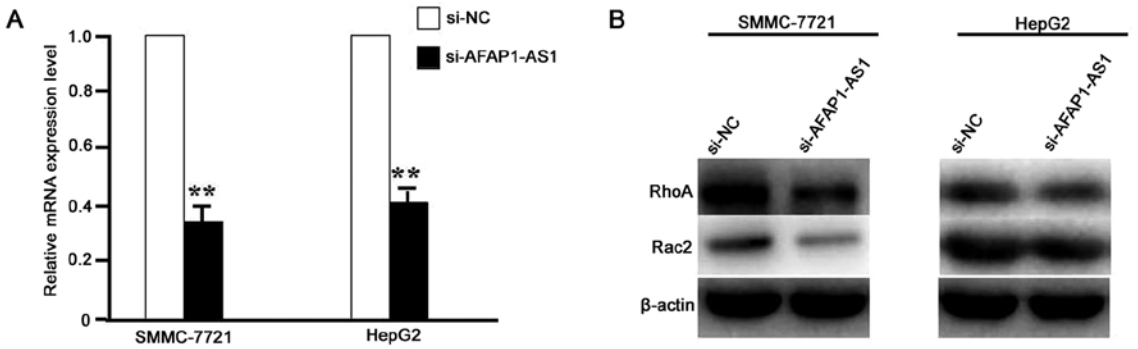

Figure 2. AFAP1-AS1 knockdown downregulates RhoA/Rac2 signaling in HCC cells. (A) Expression level of AFAP1-AS1 was detected by real-time PCR assay after HCC cell lines (SMCC-721 and HepG2) were infected with lentivirus-mediated si-AFAP1-AS1 for 24 h (**P<0.01). (B) Protein expression levels of RhoA and Rac2 were detected by western blot assay after AFAP1-AS1 knockdown.

size reached approximately 5 mm in length, the mice were randomly assigned as si-NC group (n=5) and si-AFAP1-AS1 group (n=5). In si-AFAP1-AS1 treatment group, 15 μl of lentivirus was injected into subcutaneous tumors using a multi-site injection format. Injections were repeated every other day after initial treatment. The tumor volume was measured with a caliper, using the formula volume = (length x width)^2/2.

Statistical analysis. The result of each experiment was shown as mean ± SD when applicable. Statistically significant difference in each assay was determined by SPSS version 20.0. Difference in each group was tested for significance using Kruskal-Wallis H test and ANOVA analysis of variance. P<0.05 was considered significant.

Results

Expression of AFAP1-AS1 is increased in human HCC tissues and correlates with poor prognosis. To observe the expression of AFAP1-AS1 in HCC, we examined the AFAP1-AS1 expression levels in 78 paired HCC tissues and corresponding non-tumor tissues by using qRT-PCR. The transcript levels of AFAP1-AS1 were significantly increased in 71.25% (57 of 78) cancerous tissues compared with their corresponding adjacent non-tumor tissues (P<0.01) (Fig. 1A). Then, we analyzed the correlation of AFAP1-AS1 expression level with the clinical features in HCC patients. As shown in Fig. 1B and Table I, high expression of AFAP1-AS1 was associated with pathological staging (P=0.024) and lymph-vascular space invasion (LVSI) (P=0.007). However, other clinical parameters were not found correlated with AFAP1-AS1 expression.

Kaplan-Meier analysis using the log-rank test indicated that HCC patients with high AFAP1-AS1 expression had a shorter median survival time of 33.7 months, while those with low AFAP1-AS1expression had a median survival time of 59.3 months (P=0.0378; Fig. 1C). Multivariate analysis showed that, AFAP1-AS1 expression might serve as an independent prognostic factor for overall survival (OS) in HCC patients (P=0.029, Table II).

AFAP1-AS1 knockdown downregulated the transduction of RhoA/Rac2 signaling. After HCC cell lines (SMCC-721 and HepG2) were infected with lentivirus-mediated si-AFAP1-AS1 for 24 h, the RNA expression level of AFAP1-AS1 (Fig. 2A) and protein expression levels of RhoA and Rac2 (Fig. 2B) were detected by real-time PCR and western blot assays, which indicated the decreased expression levels of AFAP1-AS1, RhoA and Rac2 in si-AFAP1-AS1 group compared with the si-NC group (P<0.01).

AFAP1-AS1 knockdown inhibits cell proliferation. To investigate the effect of AFAP1-AS1 on HCC cell proliferation, MTT

Table I. Correlation of lncRNA AFAP1-AS1 expression with clinicopathological features in HCC patients.

Variables	Cases no.	AFAP1-AS1 Low 21	AFAP1-AS1 High 57	χ^2	P-value
Age (years)					
<60	51	14	37		
≥60	27	7	20	0.021	0.896
Gender					
Male	58	16	42		
Female	19	5	14	0.011	0.915
Liver cirrhosis					
No	49	15	34		
Yes	29	6	23	0.900	0.343
Pathological staging					
I-II	32	13	19		
III-IV	46	8	38	5.111	**0.024**
Tumor size (cm)					
<5	45	15	30		
≥5	33	6	27	2.193	0.139
TNM staging					
T1+ T2	41	10	31		
T3+ T4	37	11	26	0.278	0.598
Lymph-vascular space invasion (LVSI)					
No	26	12	14		
Yes	52	9	43	7.237	**0.007**

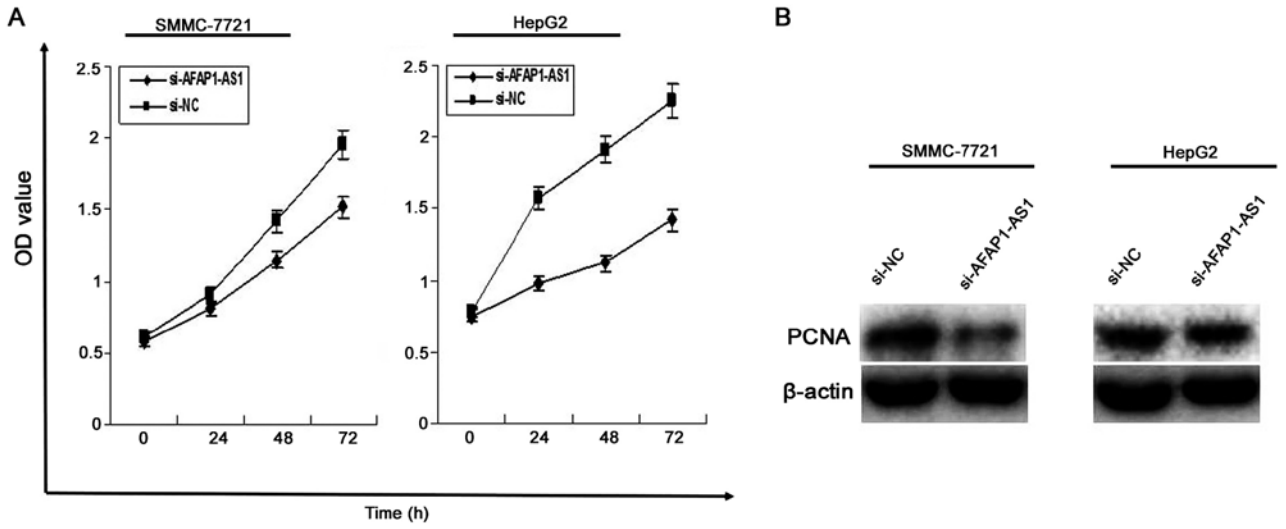

Figure 3. Effects of AFAP1-AS1 knockdown on proliferation of HCC cells. (A) MTT assay was performed to detect the proliferation of SMMC-7721 and HepG2 cells. (B) Protein expression levels of PCNA were detected by western blot assay after AFAP1-AS1 knockdown.

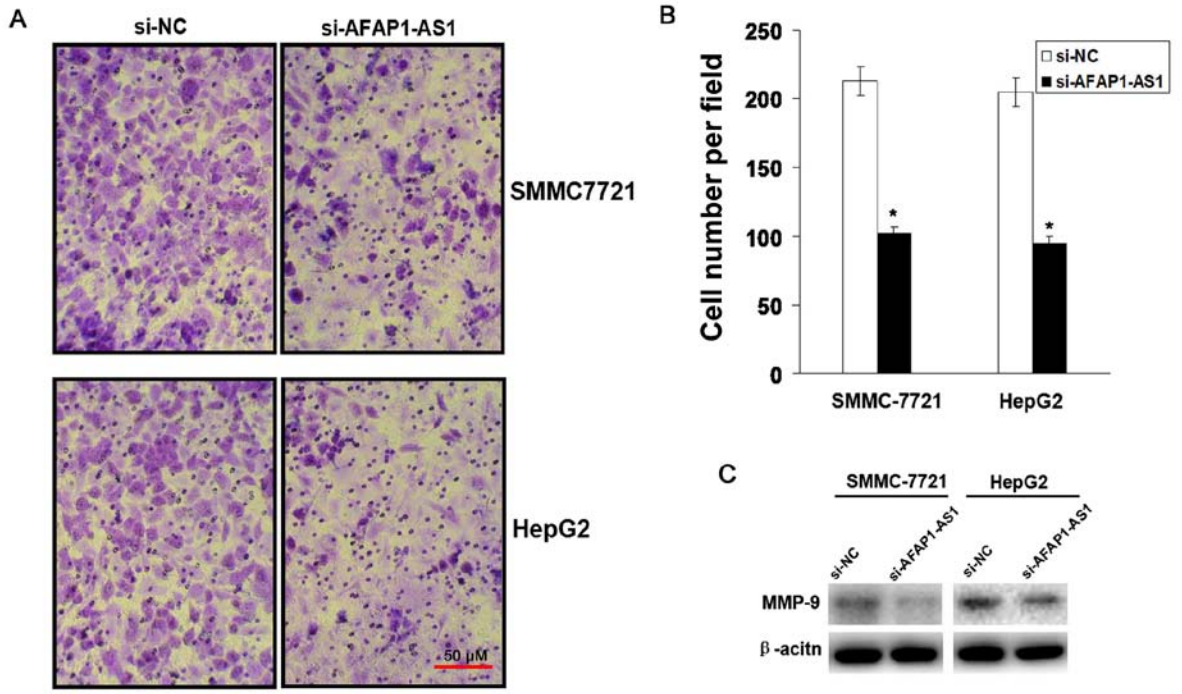

Figure 4. AFAP1-AS1 knockdown influences invasion of HCC cells. (A) Transwell assay was performed to determine the invasive activity of HCC cells after AFAP1-AS1 knockdown. (B) Cell number per field is indicated as means ± SD (*P<0.05). (C) Protein expression levels of MMP-9 were detected by western blot assay after AFAP1-AS1 knockdown.

assay was used to evaluate cell proliferative activity, indicating that cell proliferation activity of HCC cells was significantly reduced in si-AFAP1-AS1 group compared to those in si-NC group (P<0.01, Fig. 3A). In addition, the protein expression level of PCNA examined by western blotting (Fig. 3B) assay, was decreased in si-AFAP1-AS1 group compared to the si-NC group (P<0.01).

AFAP1-AS1 knockdown inhibits cell invasion. To observe the effect of AFAP1-AS1 on cell invasive potential in HCC cells, Transwell assay was performed. We found that the invasive potential of HCC cells was lower in si-AFAP1-AS1 group compared to those in si-NC group (P<0.01, Fig. 4A and B). The protein expression level of MMP-9 examined by western blot (Fig. 4C) assay was downregulated in si-AFAP1-AS1 group compared to the si-NC group.

AFAP1-AS1 knockdown induces cell apoptosis and cycle arrest. To evaluate the effect of AFAP1-AS1 on cell apoptosis and cycle distribution in HCC cells, flow cytometric analysis was performed. We found that the apoptotic indexes of HCC cells were elevated in si-AFAP1-AS1 group compared to those in NC group (P<0.01, Fig. 5A and B). The number of HCC cells was significantly increased in S phase in si-AFAP1-AS1

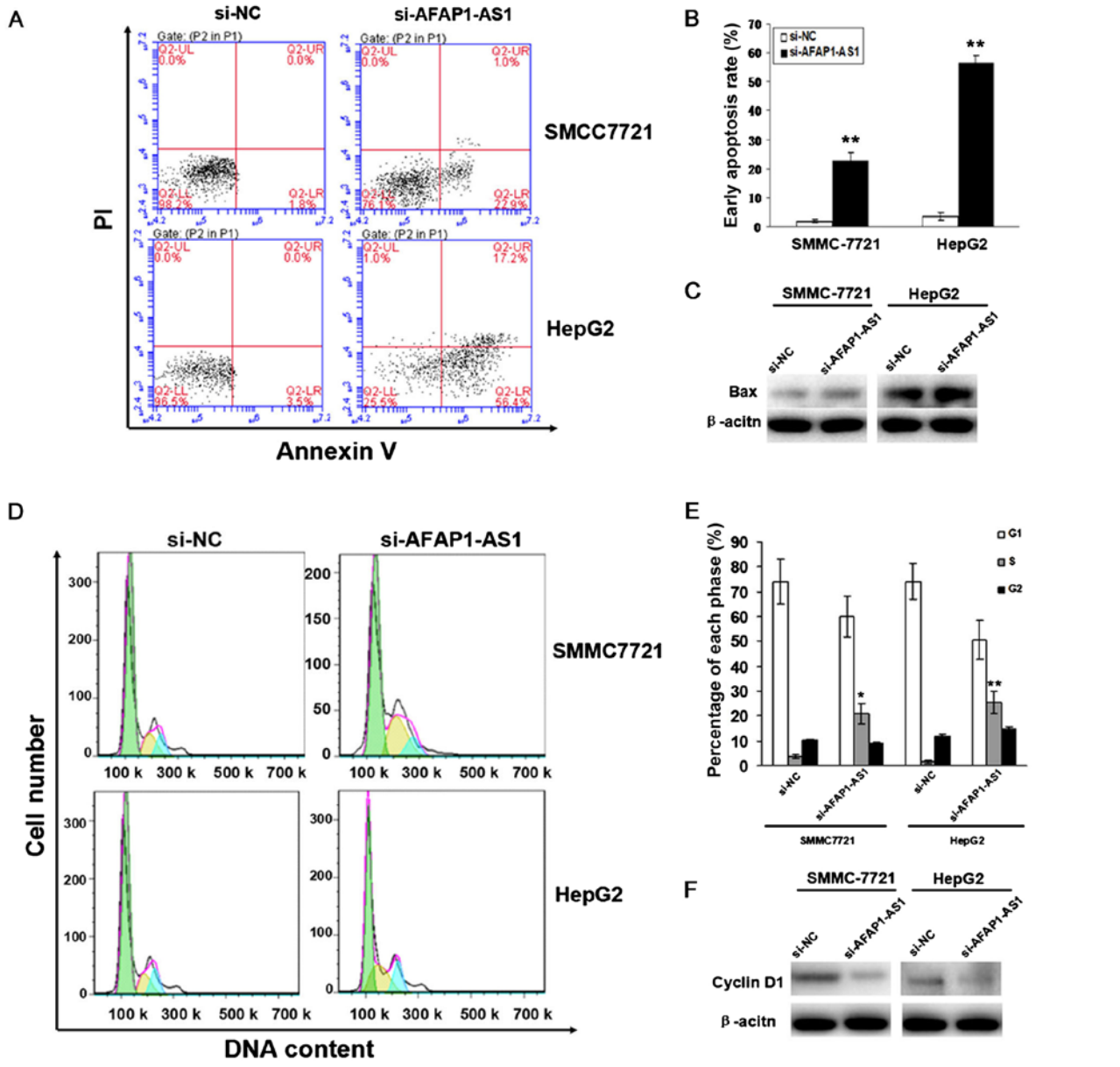

Figure 5. Effects of AFAP1-AS1 knockdown on apoptosis and cycle distribution of HCC cells. (A) The apoptotic rates of HCC cells were detected by flow cytometry after infection. (B) Early apoptotic rates are represented as means of means ± SD (**P<0.01). (C) Protein expression levels of Bax were detected by western blot assay after AFAP1-AS1 knockdown. (D) Cell cycle was analyzed by flow cytometry after infection. (E) Percentage of each phase was indicated as means ± SD (*P<0.05, **P<0.01). (F) Protein expression levels of cyclin D1 were determined by western blot assay after AFAP1-AS1 knockdown.

group compared to those in the si-NC group, and cell cycle was arrested in S phase (P<0.05, P<0.01, Fig. 5D and E). The protein expression levels of Bax examined by western blot assay were upregulated while those of and cyclinD1 were downregulated in si-AFAP1-AS1 group compared to the si-NC group (Fig. 5C and F).

AFAP1-AS1 knockdown inhibits xenograft tumor growth in vivo. Xenograft tumor models were established to assess the tumor growth *in vivo*. During the whole tumor growth period, the tumor growth activity was measured. The tumors grew substantially slowly in si-AFAP1-AS1 group compared to the si-NC group (Fig. 6A and B). When the tumors were harvested, the average weight of the tumors in si-AFAP1-AS1 group was significantly smaller than that in si-NC group (P<0.05, Fig. 6B).

Discussion

Molecular targeting therapy is of particular significance for treatment of malignancies because of the lack of effective systemic therapies and options. Tremendous evidence shows that lncRNAs over 200 nucleotides (nt) in length are emerging as important regulatory molecules at the transcriptional and post-transcriptional levels, and play essential roles in a variety of cancer development and progression and provide potential therapeutic biomarkers for cancer diagnosis and prognosis such as H19, HOTAIR, MALAT1, MEG3, and XIST (24-26). The combination of lncRNAs SOX2OT, PTPRG-AS1, ANRASSF1, ANRIL and RP11-397D12.4, AC007403.1, ERICH1-AS1 may be helpful for early detection and evaluation of prognosis in breast cancer (27) and non-small cell lung cancer (NSCLC) (28). To confirm the expression and clinical

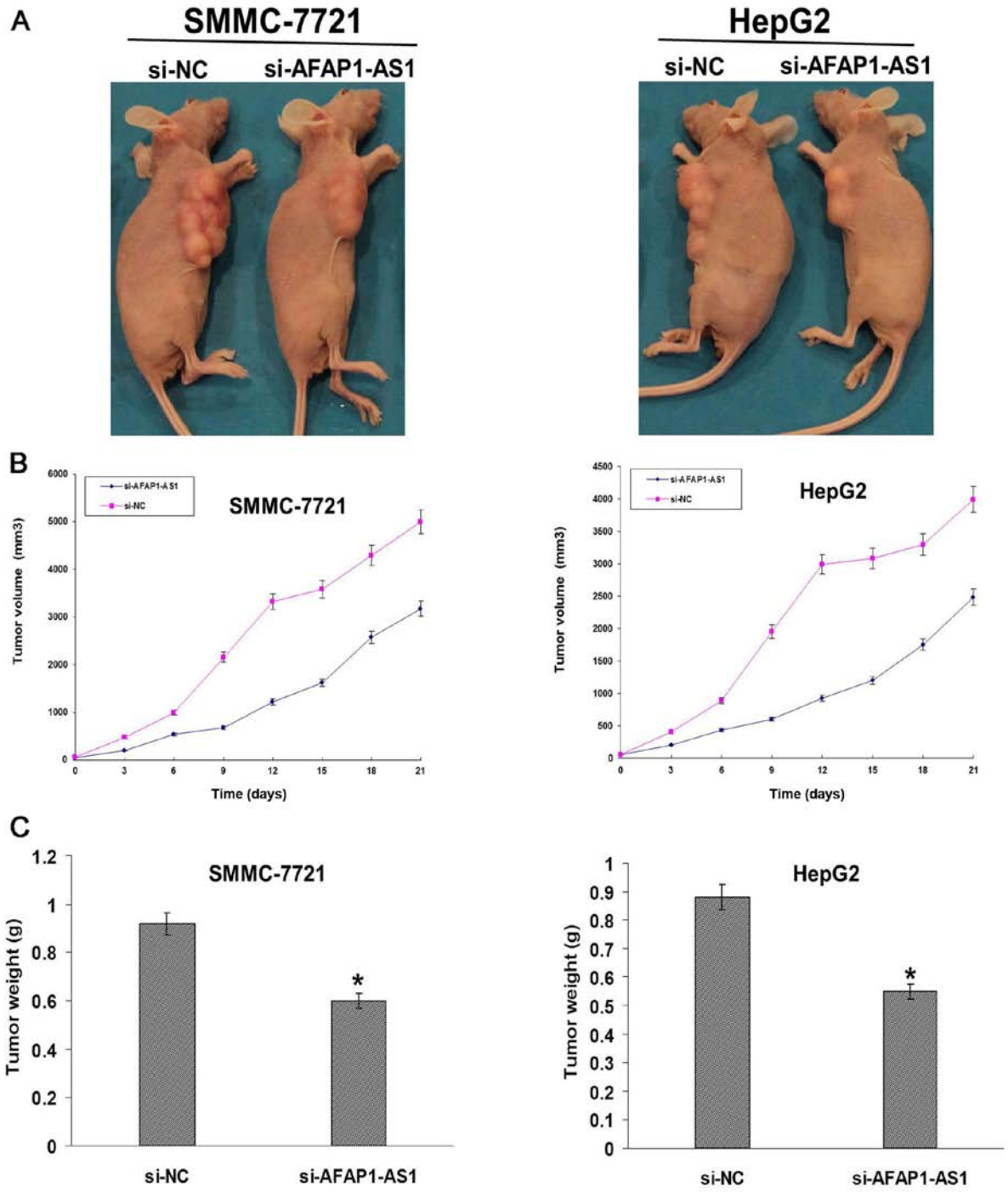

Figure 6. Effects of AFAP1-AS1 knockdown on xenograft tumor growth *in vivo*. (A) Subcutaneous tumors of nude mice in each group after sacrifice. (B) HCC xenograft tumors treated with si-AFAP1-AS1 grew significantly slowly compared to those in si-NC group. (C) The average weight of the tumors in si-AFAP1-AS1 group was significantly lower than those in si-NC group ($^{*}P<0.05$).

significance of AFAP1-AS1 in HCC, in the present study, we found that AFAP1-AS1 was highly expressed in HCC tissues and was correlated with the LVSI in HCC patients. Multivariate analysis showed that AFAP1-AS1 might serve as an independent prognostic factor for overall survival in HCC patients.

Many studies have confirmed that lncRNAs are involved in cell proliferation, angiogenesis, invasion and metastasis in various types of cancers (29-32). LncRNA Hh maintains the mammosphere-formation efficiency (MFE) and self-renewal capacity of cancer stem cells in Twist-positive breast cancer (29), and HOTAIR induces androgen-independent androgen receptor (AR) activation, drives the AR-mediated transcriptional program and facilitates castration-resistant prostate cancer progression (30). Silencing of lncRNA MALAT1 or HOXA-AS2 inhibits epithelial-mesenchymal transition and malignant transformation by inducing G1 arrest and promoting apoptosis in gastric cancer (31,32). Depletion of lncRNA ANRIL leads to cell cycle arrest at the G2/M phase in NSCLC and cervical cancer (33). LncRNA ODRUL increases doxorubicin-resis-

Table II. Summary of univariate and multivariate Cox regression analysis of overall survival duration.

Parameter	Univariate P	Multivariate analysis		
		P	HR	95% CI
Age (≥60 vs. <60 years)	0.137	NA		
Gender (Male vs. Female)	0.269	NA		
Liver cirrhosis (Positive vs. Negative)	0.371	NA		
Pathological stage (I/II vs. III/IV)	0.217	NA		
Tumor size (≥5 vs. <5 cm)	0.016	NS	1.175	0.914-1.939
TNM classification (T1/T2 vs. T3/T4)	0.067	NA		
LVSI (Positive vs. Negative)	0.017	NS	2.013	1.237-2.514
AFAP1-AS1 expression (High vs. Low)	0.0012	0.029	1.471	0.987-2.626

NA, not anayzed; NS, not significant.

tance molecule via ABCB1 gene in osteosarcoma cells (34). To demonstrate the function of AFAP1-AS1 in HCC, we found that knockdown of AFAP1-AS1 by si-AFAP1-AS1 decreased the proliferation and invasion *in vitro* and *in vivo*, induced cell apoptosis and blocked cell cycle in S phase.

RhoA activation has been confirmed to regulate many molecular events including cell proliferation, differentiation, inflammation response and angiogenesis (35). Activation of RhoA contributes to a poor prognosis and mediates cell migration in HCC (36-38). However, inhibition of RhoA by miR-200b/200c/429 counteracts the metastatic capacity of HCC cells (39). Rac2 is frequently mutated and have a high transcript level in HCC (40,41). However, the relationship between AFAP1-AS1 expression and RhoA/Rac2 signaling is not comprehensively understood. Our present studies showed that knockdown of AFAP1-AS1 decreased the expression of RhoA and Rac2 in HCC cells, suggesting that AFAP1-AS1 might promote the HCC progression via upregulation of RhoA/Rac2 signaling.

In conclusion, our findings indicate that AFAP1-AS1 may promote the HCC progression and invasion through upregulation of RhoA/Rac2 signaling. Our studies may provide a novel and potential therapeutic target for treatment of HCC.

Acknowledgements

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