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ORIGINAL ARTICLE

Basic Study

N6-methyladenosine-modified long non-coding RNA *KIF9-AS1* promotes stemness and sorafenib resistance in hepatocellular carcinoma by upregulating SHOX2 expression

Yong Yu, Xiang-Hong Lu, Jin-Song Mu, Jiang-Yun Meng, Jiang-Shan Sun, Hai-Xu Chen, Yang Yan, Ke Meng

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) is a prevalent and aggressive tumor. Sorafenib is the first-line treatment for patients with advanced HCC, but resistance to sorafenib has become a significant challenge in this therapy. Cancer stem cells play a crucial role in sorafenib resistance in HCC. Our previous study revealed that the long non-coding RNA (lncRNA) *KIF9-AS1* is an oncogenic gene in HCC. However, the role of *KIF9-AS1* in drug resistance and cancer stemness in HCC remains unclear. Herein, we aimed to investigate the function and mechanism of the lncRNA *KIF9-AS1* in cancer stemness and drug resistance in HCC.

AIM
To describe the role of the lncRNA *KIF9-AS1* in cancer stemness and drug resistance in HCC and elucidate the underlying mechanism.

METHODS
Tumor tissue and adjacent non-cancerous tissue samples were collected from HCC patients. Sphere formation was quantified *via* a tumor sphere assay. Cell viability, proliferation, and apoptosis were evaluated *via* Cell Counting Kit-8, flow cytometry, and colony formation assays, respectively. The interactions between the lncRNA *KIF9-AS1* and its downstream targets were confirmed *via* RNA immunoprecipitation and coimmunoprecipitation. The tumorigenic role of *KIF9-AS1* was validated in a mouse model.

RESULTS
Compared with that in normal controls, the expression of the lncRNA *KIF9-AS1* was upregulated in HCC tissues. Knockdown of *KIF9-AS1* inhibited stemness and attenuated sorafenib resistance in HCC cells. Mechanistically, N6-methyladenosine modification mediated by methyltransferase-like 3/insulin-like growth factor 2 mRNA-binding protein 1 stabilized and increased the expression of *KIF9-AS1*. Additionally, *KIF9-AS1* increased the stability and expression of short stature homeobox 2 by promoting ubiquitin-specific peptidase 1-induced deubiquitination. Furthermore, depletion of *KIF9-AS1* alleviated sorafenib resistance in a xenograft mouse model of HCC.

CONCLUSION
The N6-methyladenosine-modified lncRNA *KIF9-AS1* promoted stemness and sorafenib resistance in HCC by upregulating short stature homeobox 2 expression.

Key Words: Hepatocellular carcinoma; Stemness; Sorafenib resistance; Long non-coding RNA *KIF9-AS1*; Short stature homeobox 2

Core Tip: Hepatocellular carcinoma (HCC) is a highly aggressive tumor with a poor prognosis. Our previous investigation revealed that knockdown of the long non-coding RNA *KIF9-AS1* suppressed HCC progression. However, the role of *KIF9-AS1* in cancer stemness and drug resistance in HCC remains unclear. This study revealed that N6-methyladenosine modification of *KIF9-AS1* promotes stemness and sorafenib resistance in HCC by upregulating short stature homeobox 2 expression. This finding provides new insights into the role of *KIF9-AS1* in HCC pathogenesis and highlights its potential as a biomarker and therapeutic target.

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INTRODUCTION

Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, is a highly aggressive tumor associated with a poor prognosis[1]. According to recent data, approximately 841000 cases of HCC are newly diagnosed every year globally, and the mortality rate is high[2]. Although sorafenib is the first-line treatment for advanced-stage HCC patients, resistance to sorafenib remains a major challenge in HCC therapy[3]. Cancer stem cells serve as key regulators of sorafenib resistance and tumor growth and recurrence in HCC[4]. Hence, it is crucial to explore the mechanisms of cancer cell stemness and sorafenib resistance in HCC to identify potential therapeutic targets.

Long non-coding RNAs (lncRNAs) have emerged as vital regulators of various cellular processes, and dysregulation of these lncRNAs contributes to the development of drug resistance in tumors. For example, lncRNA metastasis-associated lung adenocarcinoma transcript 1 is upregulated in HCC, where it mediates doxorubicin resistance through the microRNA-3129-5p (miR-3129-5p)/neuro-oncological ventral antigen 1 axis[5]. Moreover, Zhang *et al*[6] demonstrated that increased expression of the lncRNA small nucleolar RNA host gene 3 promoted invasion, epithelial-mesenchymal transition, and sorafenib resistance in HCC cells. Our previous study revealed that deletion of the lncRNA *KIF9-AS1* suppressed cell proliferation and migration while promoting cell death in HCC[7]. A study by Jin *et al*[8] demonstrated that *KIF9-AS1* promoted sorafenib resistance in renal cell carcinoma cells. However, research on the role of *KIF9-AS1* in drug resistance in HCC is limited. N6-methyladenosine (m6A) modification reportedly influences RNA maturation, stability, and translational regulation[9]. A previous study revealed that methyltransferase-like 3 (METTL3)-mediated m6A methylation led to a decrease in the lncRNA maternally expressed gene 3 levels, resulting in increases in the

viability, migratory ability, and invasive ability of HCC cells[10]. Furthermore, METTL3-dependent m6A methylation increased the expression of the lncRNA NIFK antisense RNA 1 (*NIFK-AS1*) in HCC, whereas depletion of *NIFK-AS1* sensitized HCC cells to sorafenib and inhibited HCC progression[11]. Another study revealed that insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) increased the expression of the lncRNA *MIR4435-2HG* in HCC cells *via* m6A methylation, which increased the stem cell properties and proliferation of HCC cells[12]. Sequence-based RNA adenosine methylation site predictor is a useful tool that is primarily used to predict m6A modification sites on RNA sequences. Notably, the presence of m6A modification sites in *KIF9-AS1* was predicted using sequence-based RNA adenosine methylation site predictor. Additionally, RM2Target predictions indicate that METTL3 and IGF2BP1 are methyltransferase recognition proteins involved in the m6A modification of *KIF9-AS1*. Recent evidence has shown that inhibition of METTL3-mediated m6A methylation reduces resistance to lenvatinib in HCC cells[13]. Shi *et al*[14] reported that increased methylation of IGF2BP1 promoted HEG1 stabilization, leading to the exacerbation of oxaliplatin resistance in HCC. These studies highlight the roles of METTL3 and IGF2BP1 as key players in drug resistance in HCC.

Several studies have focused on the downstream regulatory mechanisms of *KIF9-AS1* in tumors. Numerous studies have shown that short stature homeobox 2 (SHOX2) functions as an oncogene associated with tumor progression and metastasis in bladder cancer[15], renal cancer[16] and glioma[17]. Emerging evidence shows that SHOX2 is upregulated in HCC patients and is associated with tumor recurrence[18]. Additionally, SHOX2 has been reported to increase drug resistance in lung cancer[19]. However, the functional role of SHOX2 in cancer stemness has rarely been described in previous studies. Ubiquitin-specific peptidase 1 (USP1) reportedly promotes tumorigenesis in various cancers, including breast cancer[20] and myeloma[21], by regulating protein deubiquitination. Consistently, high USP1 expression predicts poor overall survival in HCC patients[22]. Moreover, suppression of USP1 increased the sensitivity of cancer cells to platinum[23]. USP1 inhibits the K11-linked polyubiquitination of WW domain-containing transcription regulator 1, thereby increasing the stability of WW domain-containing transcription regulator 1 in HCC[24]. SHOX2 has been predicted to be a substrate for USP1 ubiquitination *via* UbiBrowser, and the RNA-Protein Interaction Prediction tool predicted a high score for the binding of *KIF9-AS1* to USP1 (random forest classifier: 0.75; support vector machine classifier: 0.93). However, whether *KIF9-AS1* regulates the expression of SHOX2 *via* USP1 has not been determined.

Considering all the available data, we hypothesized that METTL3 stabilizes the lncRNA *KIF9-AS1* through an m6A-IGF2BP1-dependent mechanism, resulting in the upregulation of *KIF9-AS1* expression. Additionally, we hypothesized that *KIF9-AS1* enhances cancer stemness and sorafenib resistance in HCC by promoting USP1-mediated deubiquitination of SHOX2. These hypotheses suggest that targeting this pathway might be a promising therapeutic strategy for treating HCC.

MATERIALS AND METHODS

Patients and tissue samples

In this study, cancer tissues and adjacent non-cancerous tissues were obtained from 20 HCC patients for analysis. The inclusion criteria were as follows: (1) A primary diagnosis of HCC; and (2) No prior chemotherapy or radiotherapy. Patients with other types of malignant tumors were excluded. The Medical Ethics Committee of the Fifth Medical Center of the Chinese People's Liberation Army General Hospital approved this study (license KY-2024-5-75-1), and all participants provided written informed consent.

Cell culture and treatment

The HCC cell lines Huh-7 (American Type Culture Collection, United States) and Huh-7/R (Bio-129595, Biobw, China) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 μg/mL penicillin-streptomycin (Thermo Fisher Scientific, United States). The cells were then cultured at 37 °C with 5% CO_2 for 24 hours. In subsequent experiments, resistant cells (Huh-7/R) were generated *via* constant treatment with 10 μM sorafenib.

Plasmid construction and cell transfection

GenePharma (Shanghai, China) synthesized a USP1-overexpressing vector (pcDNA3.1-USP1); small hairpin RNAs (shRNAs) targeting *KIF9-AS1* (sh-KIF9-AS1), *IGF2BP1* (sh-IGF2BP1), and *METTL3* (sh-METTL3); and negative control (NC) constructs. In brief, these constructs or their corresponding NC constructs were transfected into cells with Lipo6000 reagent (Beyotime Biotechnology, China) for 48 hours.

Assessment of mRNA stability

To investigate the effect of IGF2BP1 knockdown on mRNA stability, Huh-7 and Huh-7/R cells were treated with 5 μg/mL actinomycin D for 0, 3, and 6 hours. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was subsequently conducted to analyze relative mRNA expression.

Cell Counting Kit-8 assay

Cells (1.0×10^4) were seeded into 96-well plates and exposed to sorafenib at different concentrations (0, 3, 6, 9, 12 and 15 μM). After treatment for 48 hours, 10 μL of Cell Counting Kit-8 solution was added to each well, and the plates were then maintained at 37 °C for an additional hour. The optical density of the solutions at 450 nm was measured with a scanning microplate reader (Bio-Rad, United States), and the IC_{50} values of the cells were calculated.

Sphere formation ability

After digestion and centrifugation, Huh-7 cells were washed twice with phosphate-buffered saline and plated into 24-well plates (500 cells/well) supplemented with antibiotics. The plates were then incubated at 37 °C and 5% CO_2. After 10 days, the number of spheres was counted under a microscope.

Colony formation assay

In brief, a total of 5 × 10^3 Huh-7 cells and Huh-7/R cells were plated in 6-well plates and incubated for 14 days. After fixation with glutaraldehyde, the cells were stained with 1% crystal violet solution for 15 minutes. The colonies were photographed and counted using ImageJ (NIH, United States).

Flow cytometry

To assess cell apoptosis, the cells were digested with trypsin (without EDTA). The cells were rinsed with phosphate-buffered saline and resuspended in Annexin V binding buffer. The cells were then exposed to a mixture of 5 μL of Annexin V-FITC and 5 μL of propidium iodide in the dark at room temperature for 15 minutes. Finally, cell apoptosis was measured using a FSCAN flow cytometer (BD Biosciences, United States).

m6A content

Polyadenylated mRNA was purified from total RNA using the GenElute™ mRNA Miniprep Kit (Sigma-Aldrich, Germany). The m6A level was assessed with the EpiQuik m6A RNA Methylation Quantification Kit (EpiQuik, United States). For this assay, 200 ng of extracted RNA was added to each well of a 96-well microplate and incubated with the prepared antibody solution. The optical density of each well at 450 nm was measured with a microplate reader (Bio-Tek, United States).

Methylated RNA immunoprecipitation assay

Briefly, 10 μg of mRNA was fragmented into oligonucleotides using RNA fragmentation reagents (Invitrogen) in a thermomixer. Magna ChIP Protein A/G Magnetic Beads were treated with 5 μg of m6A-specific antibody at 4 °C for 2 hours in the presence of immunoprecipitation buffer. The mixture was subsequently incubated with methylated RNA immunoprecipitation (RIP) reaction reagents at 4 °C for 1 hour. The m6A-modified RNA was then eluted and purified for further analysis *via* qRT-PCR.

RIP

Cells were subjected to an RIP assay using the Magna RIP Kit (Millipore, United States). Magnetic beads coated with 5 mg of normal antibodies against IGF2BP1 (ab313422, Abcam) or USP1 (LSC288484, LsBio) were incubated with the cell lysates at 4 °C overnight. Immunoglobulin G (IgG) was used as a NC. The beads containing the immunoprecipitated RNA-protein complex were treated with proteinase K to eliminate proteins. The RNA coprecipitated with the complex was detected *via* qRT-PCR, and the results were normalized to the input.

Coimmunoprecipitation

Cells were suspended in immunoprecipitation lysis buffer and incubated with specific antibodies against SHOX2 (#MBS6004620), ubiquitin (#MBS2118113) and USP1 (LSC288484, LsBio) at 4 °C overnight. Next, the lysate was mixed with magnetic beads conjugated with protein A/G. Finally, the antibody-protein complexes were collected for immunoblotting.

Xenograft tumor mouse model

Twelve male BALB/c nude mice (6-8 weeks, 25-30 g) were purchased from SJA Laboratory Animal Company (China) and housed in a light-controlled cage at 24 °C. The mice were allocated into two groups, the experimental group and the control group. The experimental group received subcutaneous implants of Huh-7/R cells transfected with stable *KIF9-AS1*-knockdown shRNA (5 × 10^6 cells/mouse), whereas the control group was injected with normal saline. After tumor implantation, the tumor volume was measured every 7 days. Sorafenib was administered orally (p.o.) twice a week beginning on day 7 at a dose of 30 mg/kg body weight. After 4 weeks, the tumor tissues were dissected, and the weights were recorded. This study was approved by the Institutional Animal Care Committee of the Fifth Medical Center of the Chinese People's Liberation Army General Hospital (license IACUC-2019-0025).

Immunohistochemistry

In brief, tissue samples were fixed in 10% formalin, embedded in paraffin and sectioned. The sections were subsequently treated with 3% H_2O_2 and incubated overnight at 4 °C with an anti-Ki67 antibody (ab279653; Abcam), followed by incubation with rabbit anti-mouse IgG H&L (HRP) (ab6728) at 37 °C for 30 minutes. Finally, the slides were stained with diaminobenzidine and examined with a Nikon Eclipse Ti2 confocal microscope (Nikon, Japan).

qRT-PCR

RNA extraction was performed with TRIzol reagent (Invitrogen, United States). The reverse transcription of mRNA into cDNA was conducted with the PrimeScript RT Master Mix (Takara Bio, Japan). The quantification of mRNA expression was performed on an ABI 7500 System (Applied Biosystems, United States) with TB Green® Premix Ex Taq™ II (Takara, Japan). The sequences of the PCR amplification primers used are provided in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to

Table 1 The primer sequence in quantitative reverse transcription polymerase chain reaction

Genes	Sequence (5'-3')
KIF9-AS1 F	TCCCTGCTGAAGTGACAGTG
KIF9-AS1 R	ACCCCAACCCTCTCTGTTCT
SHOX2 F	GCCTTCATGCGAGAGGAACT
SHOX2 R	CGCCTGAACCTGCTGAAATG
PD-L1 F	ACCACCACCAATTCCAAGAG
PD-L1 R	GATGGCTCCCAGAATTACCA
GAPDH F	CCAGGTGGTCTCCTCTGA
GAPDH R	GCTGTAGCCAAATCGTTGT

SHOX2: Short stature homeobox 2; PD-L1: Programmed death-ligand 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

calculate the fold change in the expression of the target genes, with *GAPDH* serving as the internal control.

Western blotting

Protein extraction was performed, and protein expression was evaluated using a BCA Protein Assay Kit (Beyotime, China). The samples were separated by 8%-10% polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Bio-Rad, United States). After blocking with 5% defatted dry milk, the membranes were incubated overnight at 4 °C with primary antibodies against CD44 (ab243894, 1/1000), CD133 (ab284389, 1/1000), epithelial cell adhesion molecule (EpCAM, ab282457, 1/1000), SHOX2 (ab55740, 1 μg/mL), USP1 (ab227551, 1/500), METTL3 (ab240595, 1/2000), IGF2BP1 (ab313422, 1/1000) and GAPDH (ab9485, 1/2500, Abcam). The samples were then incubated with secondary antibodies (ab96879 or ab6940, Abcam) at 37 °C for 1 hour. The bands were visualized and analyzed with a SuperSignal West Pico chemiluminescence system (Pierce, Inc., United States) and ImageJ software (NIH).

Statistical analysis

All the data are expressed as the means ± SDs; statistical analysis and figure generation were performed with SPSS 20.0 (IBM, United States). Statistical comparisons were performed *via* Student's *t* test. Kaplan-Meier curves were generated and log-rank tests were performed to analyze the prognostic significance of *KIF9-AS1* for HCC patients. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Expression of KIF9-AS1 is upregulated in HCC tissues compared with normal tissues

The results of this study confirmed that the expression of the lncRNA *KIF9-AS1* was elevated in HCC patients (Figure 1A). Additionally, the upregulation of *KIF9-AS1* demonstrated a notable correlation with poor overall survival in HCC patients (Figure 1B). The relationships between the clinical characteristics of HCC patients and *KIF9-AS1* expression are shown in Table 2. Notably, high *KIF9-AS1* expression in HCC patients was strongly associated with advanced tumor-node-metastasis stage and lymph node metastasis, whereas no significant relationships were found with sex, age, or tumor size. Hence, these data suggested that *KIF9-AS1* promoted HCC progression.

Depletion of KIF9-AS1 suppresses the stemness characteristics and sorafenib resistance of HCC cells

Depletion of *KIF9-AS1* suppressed stemness characteristics and reduced sorafenib resistance in HCC cells. To evaluate the impact of *KIF9-AS1* on HCC cell stemness, we silenced *KIF9-AS1* in Huh-7 cells through transfection with sh-KIF9-AS1 (Figure 2A). Suppression of *KIF9-AS1* significantly reduced Huh-7 cell viability (Figure 2B). Furthermore, suppression of *KIF9-AS1* reduced the number of tumor spheres formed and the expression of stem cell markers, including CD44, CD133, and EpCAM, in Huh-7 cells (Figure 2C and D). Next, we assessed the impact of *KIF9-AS1* on sorafenib resistance in HCC cells. Compared with Huh-7 cells, Huh-7/R cells had a greater IC_{50} value and greater expression of *KIF9-AS1*, whereas silencing *KIF9-AS1* resulted in a reduced IC_{50} value (Figure 2E and F). Additionally, colony formation was accelerated and cell apoptosis was suppressed in Huh-7/R cells; these effects were reversed by downregulation of *KIF9-AS1* (Figure 2G and H). Together, these findings suggested that inhibition of *KIF9-AS1* suppressed stemness characteristics and attenuated sorafenib resistance in HCC cells.

METTL3 stabilizes and upregulates KIF9-AS1 expression via an m6A-IGF2BP1-dependent pathway

The importance of m6A modification in tumor stemness and drug resistance has gained widespread recognition. For example, YTHDF1 regulates the m6A modification of *NOTCH1* mRNA to increase its stability and upregulate its

Table 2 Association between long non-coding RNA *KIF9-AS1* expression and clinical features of hepatocellular carcinoma patients

Clinical features	Cases (*n* = 20)	LncRNA *KIF9-AS1*		*P* value
		High (*n* = 10)	Low (*n* = 10)	
Gender				0.3698
Male	11	4	7	
Female	9	6	3	
Age (years)				0.6499
< 65	8	5	3	
≥ 65	12	5	7	
TNM stage				0.0055[b]
Stage 1/2	11	2	9	
Stage 3/4	9	8	1	
Tumor size (cm)				0.6285
< 5	14	6	8	
≥ 5	6	4	2	
Lymph node metastasis				0.0198[a]
Positive	8	7	1	
Negative	12	3	9	

[a]$P < 0.05$.
[b]$P < 0.05$.
LncRNA: Long noncoding RNA; TNM: Tumor-node-metastasis.

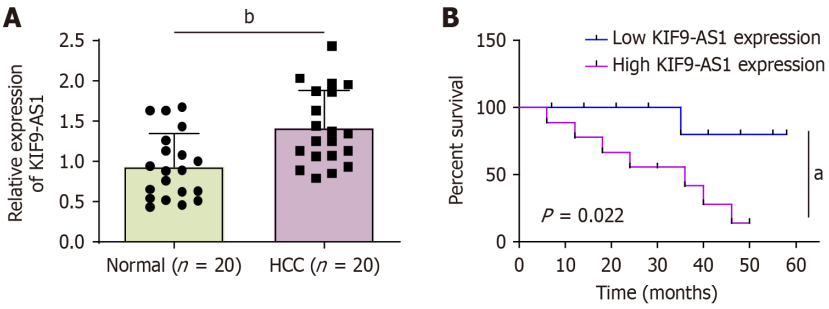
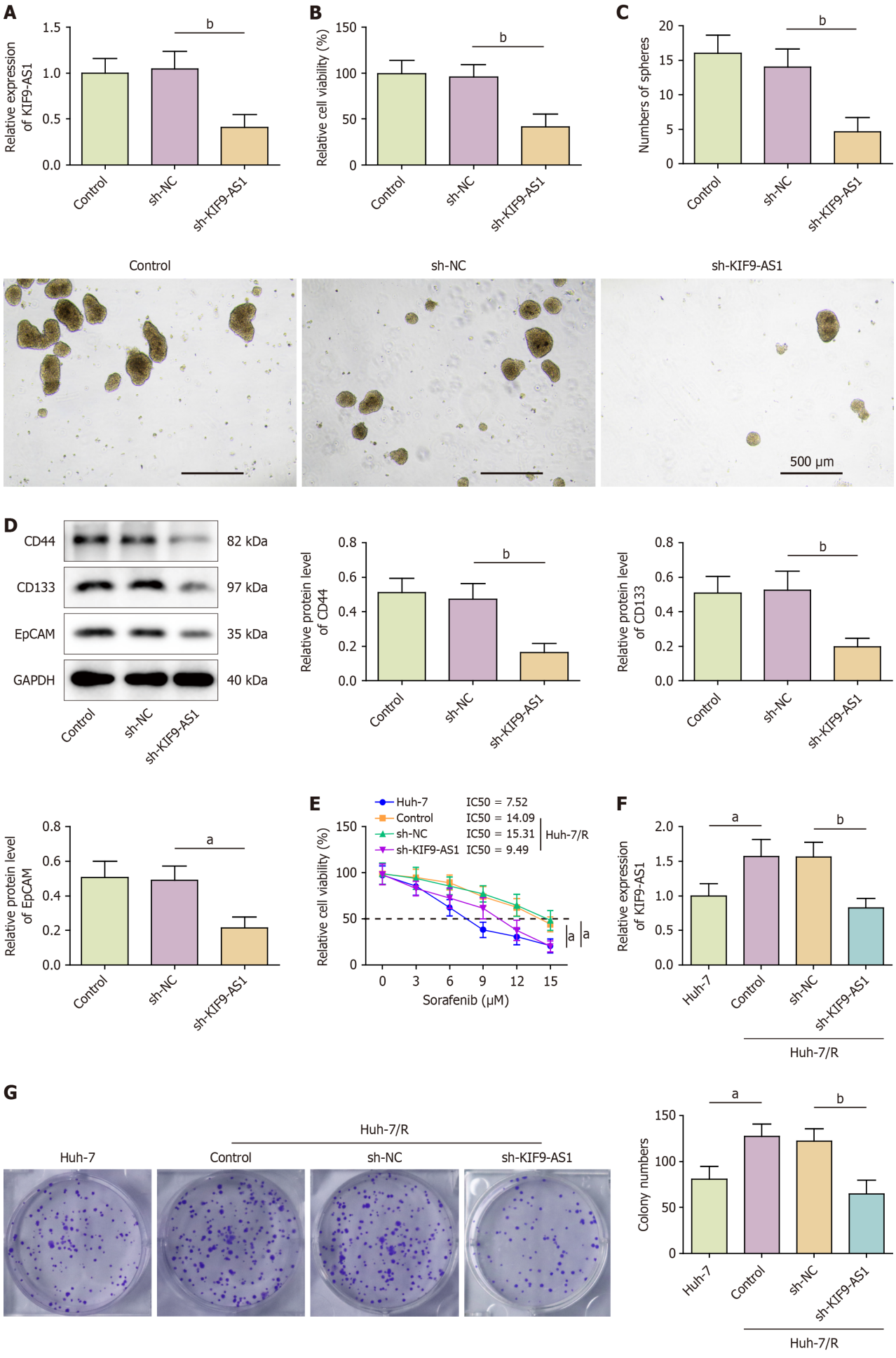


Figure 1 The long noncoding RNA *KIF9-AS1* was upregulated in hepatocellular carcinoma patients. Cancer tissues and adjacent non-cancerous tissues were collected from hepatocellular carcinoma patients. A: The expression of *KIF9-AS1* in hepatocellular carcinoma tissues and para-cancerous tissues was calculated *via* quantitative reverse transcription polymerase chain reaction; B: The overall survival rate was analyzed *via* Kaplan-Meier analysis, $n = 20$. The data are shown as the means ± SDs. [a]$P < 0.05$, [b]$P < 0.01$. HCC: Hepatocellular carcinoma.

expression, which facilitates stemness and drug resistance in HCC cells[25]. As depicted in Figure 3A, we observed an obvious increase in the total m6A content in tissue samples from HCC patients. Notably, the m6A level of *KIF9-AS1* and the protein level of METTL3 were elevated in Huh-7/R cells compared with Huh-7 cells (Figure 3B and C). Silencing *METTL3* decreased the m6A levels of *KIF9-AS1* (Figure 3D and E). The data presented in Figure 3F revealed a significant increase in IGF2BP1 expression in Huh-7/R cells compared with that in Huh-7 cells. Moreover, the anti-IGF2BP1 antibody bound significantly more *KIF9-AS1* than the IgG controls did in both Huh-7 cells and Huh-7/R cells (Figure 3G). Finally, IGF2BP1 was knocked down in both Huh-7 and Huh-7/R cells (Figure 3H). When both cell types were treated with actinomycin D (5 μg/mL), the stability of *KIF9-AS1* in Huh-7/R cells was greater than that in Huh-7 cells; however, this stability was decreased in Huh-7/R cells upon *IGF2BP1* silencing (Figure 3I). These results highlighted that METTL3 stabilized and increased *KIF9-AS1* expression through an m6A-IGF2BP1-dependent mechanism.

KIF9-AS1 increases the stability and expression of SHOX2 through USP1-mediated deubiquitination

We further explored the downstream mechanism of *KIF9-AS1* in HCC. As shown in Figure 4A-C, the levels of USP1 and SHOX2 were increased in HCC tissues, and positive correlations were observed between the levels of *KIF9-AS1* and USP1


A
Relative expression of KIF9-AS1
1.5
1.0
0.5
0.0
b
Control
sh-NC
sh-KIF9-AS1
B
Relative cell viability (%)
150
100
50
0
b
Control
sh-NC
sh-KIF9-AS1
C
Numbers of spheres
20
15
10
5
0
b
Control
sh-NC
sh-KIF9-AS1
Control
sh-NC
sh-KIF9-AS1
500 μm
D
CD44
CD133
EpCAM
GAPDH
82 kDa
97 kDa
35 kDa
40 kDa
Control
sh-NC
sh-KIF9-AS1
Relative protein level of CD44
0.8
0.6
0.4
0.2
0.0
b
Control
sh-NC
sh-KIF9-AS1
Relative protein level of CD133
0.8
0.6
0.4
0.2
0.0
b
Control
sh-NC
sh-KIF9-AS1
Relative protein level of EpCAM
0.8
0.6
0.4
0.2
0.0
a
Control
sh-NC
sh-KIF9-AS1
E
Huh-7 IC50 = 7.52
Control IC50 = 14.09
sh-NC IC50 = 15.31
sh-KIF9-AS1 IC50 = 9.49
Huh-7/R
Relative cell viability (%)
150
100
50
0
a
a
0
3
6
9
12
15
Sorafenib (μM)
F
Relative expression of KIF9-AS1
2.0
1.5
1.0
0.5
0.0
a
b
Huh-7
Control
sh-NC
sh-KIF9-AS1
Huh-7/R
G
Huh-7/R
Huh-7
Control
sh-NC
sh-KIF9-AS1
Colony numbers
150
100
50
0
a
b
Huh-7
Control
sh-NC
sh-KIF9-AS1
Huh-7/R

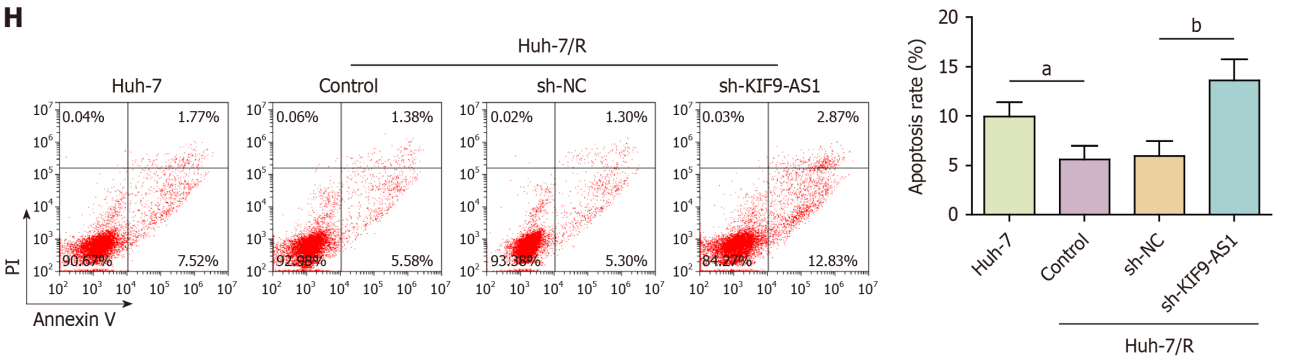


Figure 2 Depletion of the long noncoding RNA *KIF9-AS1* suppressed the stemness characteristics and sorafenib resistance of hepatocellular carcinoma cells. Huh-7 cells were transfected with sh-KIF9-AS1 or sh-NC. A: The expression of *KIF9-AS1* was measured *via* quantitative reverse transcription polymerase chain reaction; B: The viability of Huh-7 cells was assessed with a Cell Counting Kit-8 assay; C: The sphere formation ability of Huh-7 cells was examined; D: The protein levels of CD44, CD133 and epithelial cell adhesion molecule in Huh-7 cells were quantified *via* western blotting. Huh-7/R cells were transfected with sh-KIF9-AS1 or sh-NC; E: The IC_{50} values in Huh-7 and Huh-7/R cells were calculated *via* a Cell Counting Kit-8 assay; F: The expression of *KIF9-AS1* was measured *via* quantitative reverse transcription polymerase chain reaction; G and H: Cell proliferation and cell apoptosis were evaluated *via* colony formation assays and flow cytometry, respectively. The data are shown as the means ± SDs (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$. Each experiment was repeated three times. EpCAM: Epithelial cell adhesion molecule.

and between the levels of USP1 and SHOX2. Compared with those in Huh-7 cells, the expression levels of USP1 and SHOX2 were upregulated in Huh-7/R cells (Figure 4D). UbiBrowser predicted SHOX2 as a substrate for USP1 ubiquitination, and RNA-Protein Interaction Prediction predicted a high score for the binding of *KIF9-AS1* to USP1 (random forest classifier: 0.75; support vector machine classifier: 0.93). Consistent with this prediction, the RIP and coimmunoprecipitation results revealed that *KIF9-AS1* was enriched by the anti-USP1 antibody and that USP1 efficiently coimmunoprecipitated with SHOX2 in both cell lines (Figure 4E and F). Next, we knocked down *USP1* and observed an increase in the level of SHOX2 ubiquitination, leading to reduced SHOX2 protein expression (Figure 4G). Moreover, downregulation of *KIF9-AS1* led to increased SHOX2 ubiquitination and decreased SHOX2 protein expression, whereas simultaneous knockdown of *KIF9-AS1* and overexpression of USP1 repressed SHOX2 ubiquitination and increased SHOX2 protein expression (Figure 4H). *USP1* knockdown promoted degradation of the SHOX2 protein after cycloheximide treatment (Figure 5A). Overall, the results revealed that *KIF9-AS1* knockdown inhibited the protein expression of SHOX2, whereas the overexpression of USP1 increased SHOX2 protein expression, which reversed the effect of silencing *KIF9-AS1* (Figure 5B). In conclusion, *KIF9-AS1* increased the stability and expression of SHOX2 by promoting USP1-mediated deubiquitination.

KIF9-AS1 regulates the stemness and sorafenib resistance of HCC cells through SHOX2

Further investigations were conducted to explore the function of the *KIF9-AS1*/SHOX2 axis in mediating stemness and sorafenib resistance in HCC cells. Silencing of *KIF9-AS1* decreased SHOX2 protein levels and cell viability, which were reversed by SHOX2 overexpression (Figure 6A and B). Co-silencing of *KIF9-AS1* and SHOX2 overexpression resulted in increased formation of tumor spheres and expression of stemness markers (CD44, CD133, EpCAM) (Figure 6C and D). These results suggested that increased levels of SHOX2 mitigated the suppressive effects of sh-KIF9-AS1 on tumor sphere formation and stemness marker expression in Huh-7 cells. Similar trends were observed in Huh-7/R cells. Knockdown of *KIF9-AS1* significantly reduced SHOX2 expression, whereas co-transfection with OE-SHOX2 restored its expression (Figure 6E). Furthermore, SHOX2 overexpression increased the IC_{50} value and the number of colonies formed and inhibited cell apoptosis, effectively reversing the impacts of *KIF9-AS1* knockdown in Huh-7/R cells (Figure 6F-H). Thus, *KIF9-AS1* promoted stemness and sorafenib resistance in HCC cells through the regulation of SHOX2.

Knockdown of KIF9-AS1 inhibits sorafenib resistance in HCC xenograft model mice

We validated the role of *KIF9-AS1* in regulating the stemness and sorafenib resistance of HCC cells in an HCC xenograft mouse model. The mice were subcutaneously implanted with Huh-7/R cells transfected with sh-KIF9-AS1 and orally administered sorafenib. sh-KIF9-AS1 treatment resulted in significant reductions in both tumor volume and weight (Figure 7A-C). In addition, depletion of *KIF9-AS1* downregulated the expression of Ki67, SHOX2, and stemness markers (CD44, CD133, and EpCAM) in HCC model mice (Figure 7D and E). These findings suggested that the suppression of *KIF9-AS1* alleviated sorafenib resistance in HCC xenograft model mice.

DISCUSSION

Studies have shown that tumor stem cells play crucial roles in drug resistance, cell renewal and proliferation, differentiation ability, and sphere formation ability[26,27]. Despite the widespread use of sorafenib as a first-line treatment for HCC, the emergence of sorafenib resistance remains a significant challenge in HCC treatment[28]. This study

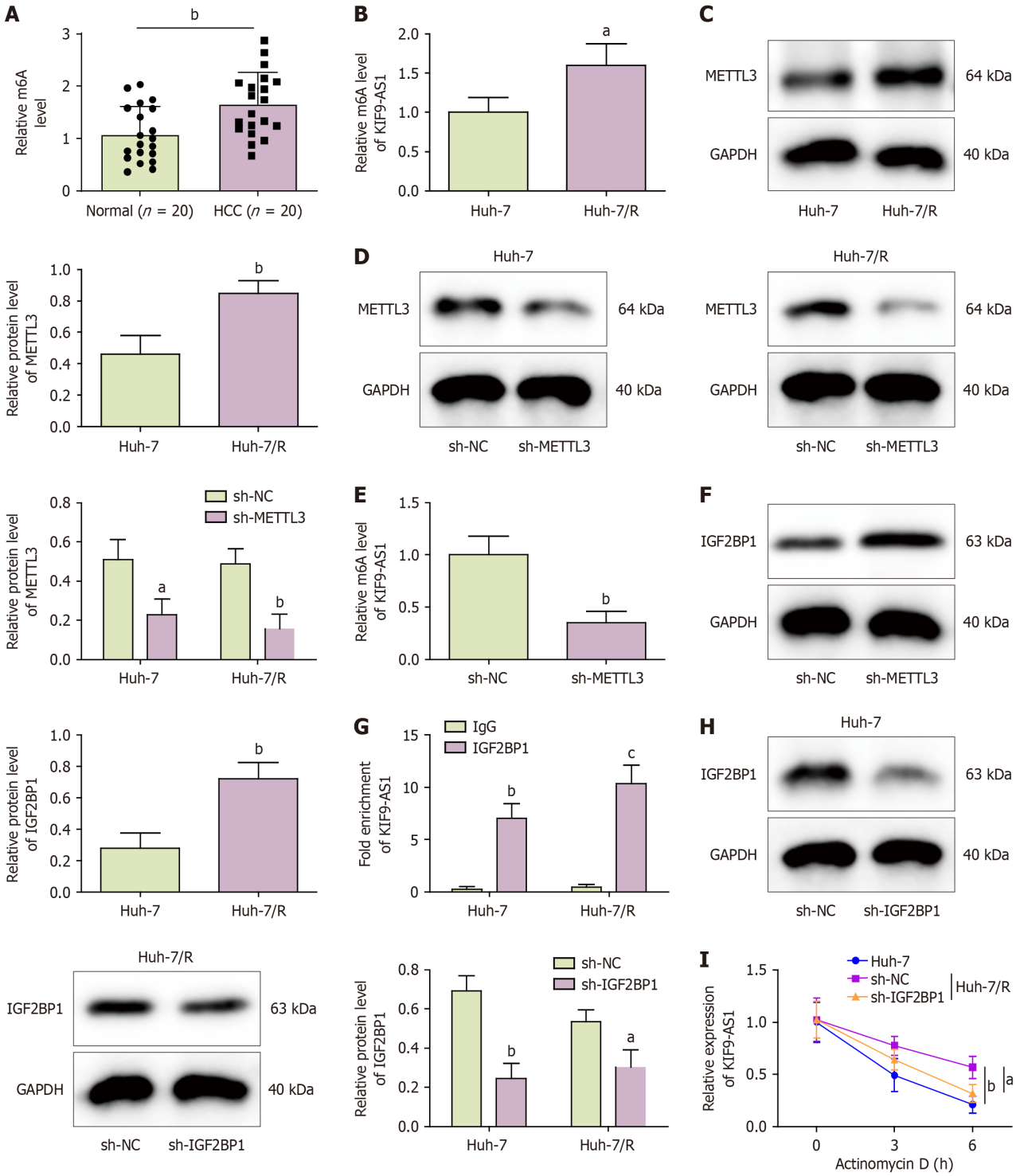


Figure 3 Methyltransferase-like 3 stabilizes and upregulates long noncoding RNA *KIF9-AS1* expression in an N6-methyladenosine-insulin-like growth factor 2 mRNA-binding protein 1-dependent manner. A: The N6-methyladenosine (m6A) content in hepatocellular carcinoma tissues and para-cancerous tissues was examined using a commercial kit (n = 20); B: m6A levels of *KIF9-AS1* in Huh-7 and Huh-7/R cells were examined *via* methylated RNA immunoprecipitation-quantitative polymerase chain reaction; C: Methyltransferase-like 3 (METTL3) protein expression in Huh-7 and Huh-7/R cells was quantified *via* western blotting; D: METTL3 protein expression in Huh-7 and Huh-7/R cells transfected with sh-METTL3 or sh-NC was quantified *via* western blotting; E: The m6A level of *KIF9-AS1* was calculated *via* methylated RNA immunoprecipitation-quantitative polymerase chain reaction; F: The insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) protein level was evaluated *via* western blotting; G: The interaction between IGF2BP1 and *KIF9-AS1* was verified *via* an RNA immunoprecipitation assay; H: The protein expression of IGF2BP1 was assessed *via* western blotting; I: After blocking the synthesis of new RNA with actinomycin D (5 μg/mL) for 0, 3, and 6 hours, the expression of *KIF9-AS1* was evaluated *via* quantitative reverse transcription polymerase chain reaction. The data are shown as the means ± SDs (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. Each experiment was repeated three times. m6A: N6-methyladenosine; METTL3: Methyltransferase-like 3; IGF2BP1: Insulin-like growth factor 2 mRNA-binding protein 1.

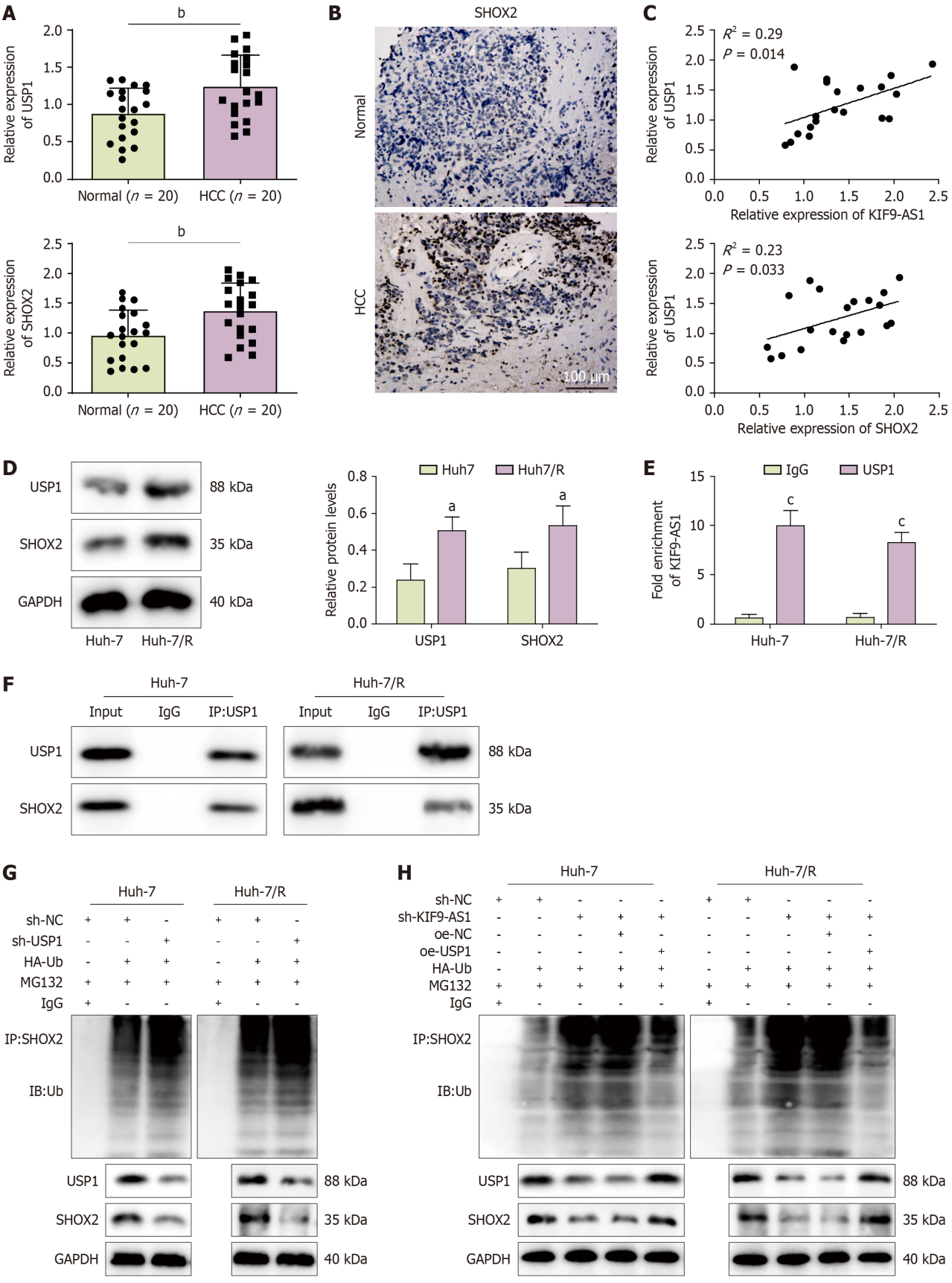


Figure 4 The long noncoding RNA *KIF9-AS1* increases the stability and expression of short stature homeobox 2 through ubiquitin-specific peptidase 1-mediated short stature homeobox 2 deubiquitination. A: The expression of ubiquitin-specific peptidase 1 (USP1) and short stature homeobox 2 (SHOX2) in hepatocellular carcinoma tissues and para-cancerous tissues was evaluated *via* quantitative reverse transcription polymerase chain reaction (n = 20); B: Image of immunohistochemical staining of SHOX2 in tumor tissues and para-cancerous tissues; C: Correlation analysis between *KIF9-AS1* and USP1 and between USP1 and SHOX2; D: The protein levels of USP1 and SHOX2 in Huh-7 and Huh-7/R cells were assessed *via* western blotting; E: The interaction

between *KIF9-AS1* and USP1 was verified *via* an RNA immunoprecipitation assay; F: The interaction between USP1 and SHOX2 was confirmed *via* a coimmunoprecipitation assay; G: The ubiquitination levels of SHOX2 in Huh-7 and Huh-7/R cells were determined *via* immunoprecipitation and western blotting; H: After cycloheximide treatment for 0, 3, 6, or 12 hours, the protein level of SHOX2 was evaluated *via* western blotting. [a]*P* < 0.05, [b]*P* < 0.01, [c]*P* < 0.001. Each experiment was repeated three times. USP1: Ubiquitin-specific peptidase 1; SHOX2: Short stature homeobox 2; HCC: Hepatocellular carcinoma; IgG: Immunoglobulin G.

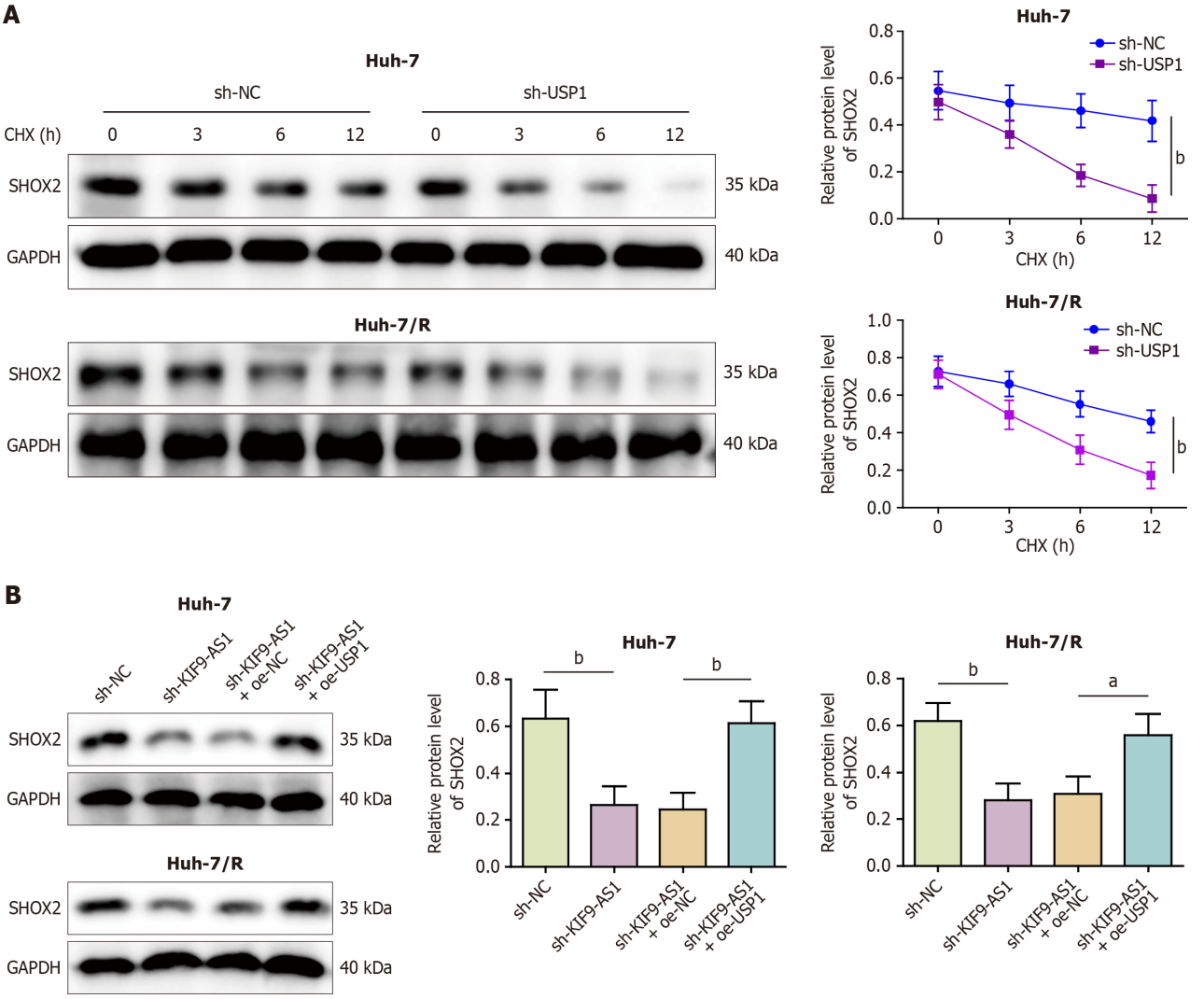
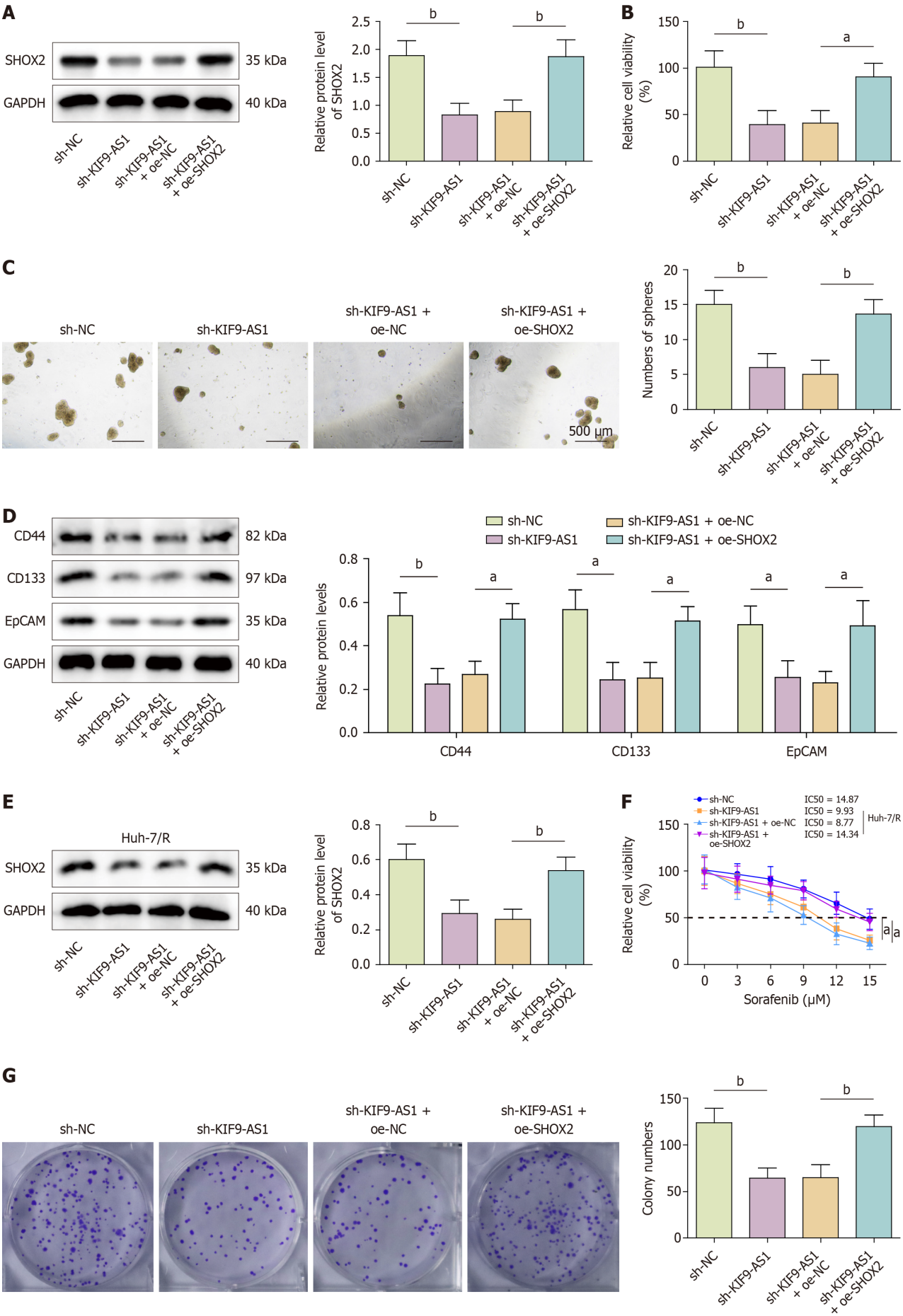


Figure 5 The long noncoding RNA *KIF9-AS1* enhances the stability and expression of short stature homeobox 2 through ubiquitin-specific peptidase 1-mediated short stature homeobox 2 deubiquitination. A: The ubiquitination levels of short stature homeobox 2 in Huh-7 and Huh-7/R cells were determined *via* immunoprecipitation and western blotting; B: Western blotting was conducted to analyze the short stature homeobox 2 protein. [a]*P* < 0.05, [b]*P* < 0.01. Each experiment was repeated three times. USP1: Ubiquitin-specific peptidase 1; SHOX2: Short stature homeobox 2; CHX: Cyclohexamide.

demonstrated that the m6A-modified lncRNA *KIF9-AS1* promoted stemness and sorafenib resistance in HCC by upregulating SHOX2 expression.

Differentially expressed lncRNAs in HCC are associated with prognosis and sensitivity to therapy[29]. For example, the lncRNA *AC007639.1* increased drug resistance in HCC cells and accelerated tumor growth in HCC mouse models [30]. Additionally, increased levels of the lncRNA nuclear paraspeckle assembly transcript 1 are correlated with poor prognosis in HCC patients, and a molecular mechanism study revealed that nuclear paraspeckle assembly transcript 1 decreases sorafenib resistance in HCC by targeting the miR-149-5p/AKT serine/threonine kinase 1 axis[31]. Our previous study revealed that *KIF9-AS1* is upregulated in HCC tumor tissues and that deletion of this lncRNA suppressed cell proliferation and migration but accelerated cell death in HCC cells[7]. In the present study, we confirmed that *KIF9-AS1* expression was increased in HCC patient tissues and that high expression of *KIF9-AS1* predicted poor clinical outcomes in patients. These results suggested the oncogenic potential of *KIF9-AS1* in the development of HCC. To date, the regulatory role of *KIF9-AS1* in drug resistance in tumors has rarely been discussed. Our previous study revealed that the upregulation of *KIF9-AS1* accelerated cell proliferation, suppressed cell apoptosis, and enhanced sorafenib resistance in renal cell carcinoma cells[8]. Consistent with these findings, the present study revealed that knocking down *KIF9-AS1*


A
SHOX2
GAPDH
35 kDa
40 kDa
sh-NC
sh-KIF9-AS1
sh-KIF9-AS1 + oe-NC
sh-KIF9-AS1 + oe-SHOX2
Relative protein level of SHOX2
B
Relative cell viability (%)
C
sh-NC
sh-KIF9-AS1
sh-KIF9-AS1 + oe-NC
sh-KIF9-AS1 + oe-SHOX2
500 μm
Numbers of spheres
D
CD44
CD133
EpCAM
82 kDa
97 kDa
35 kDa
40 kDa
Relative protein levels
E
Huh-7/R
F
IC50 = 14.87
IC50 = 9.93
IC50 = 8.77
IC50 = 14.34
Sorafenib (μM)
G
Colony numbers

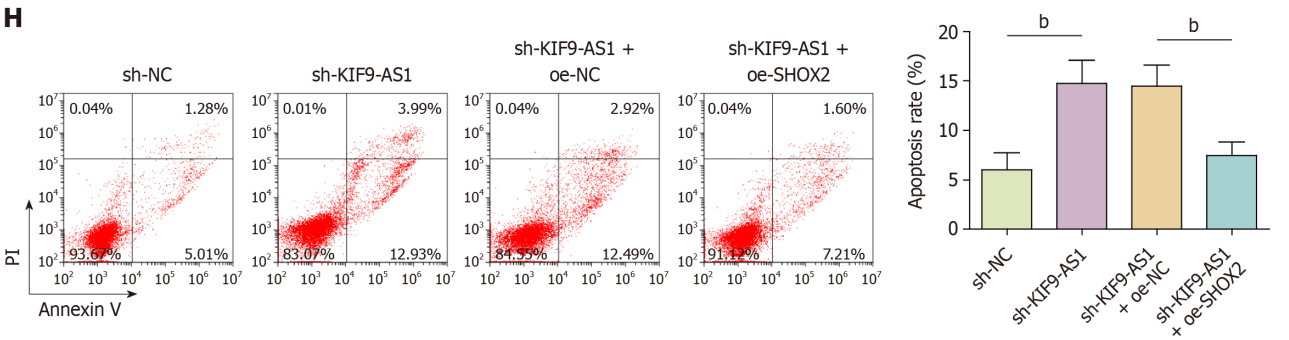


Figure 6 The long noncoding RNA *KIF9-AS1* increases the stemness and sorafenib resistance of hepatocellular carcinoma cells through short stature homeobox 2. Huh-7 cells were transfected with sh-NC, sh-KIF9-AS1, sh-KIF9-AS1 + OE-NC or sh-KIF9-AS1 + OE-short stature homeobox 2. A: Short stature homeobox 2 (SHOX2) protein expression was quantified *via* western blotting; B: Cell viability was assessed *via* a Cell Counting Kit-8 assay; C: The sphere formation ability of Huh-7 cells was examined; D: The protein levels of the stemness markers CD44, CD133 and epithelial cell adhesion molecule in Huh-7 cells were evaluated *via* western blotting. Huh-7/R cells were transfected with sh-NC, sh-KIF9-AS1, sh-KIF9-AS1 + OE-NC or sh-KIF9-AS1 + OE-SHOX2; E: SHOX2 expression was measured *via* western blotting; F: The IC_{50} values were determined *via* a Cell Counting Kit-8 assay; G: A colony formation assay was performed to evaluate cell proliferation; H: A flow cytometry assay was conducted to detect cell apoptosis. $^{a}P < 0.05$, $^{b}P < 0.01$. Each experiment was repeated three times. SHOX2: Short stature homeobox 2; EpCAM: Epithelial cell adhesion molecule.

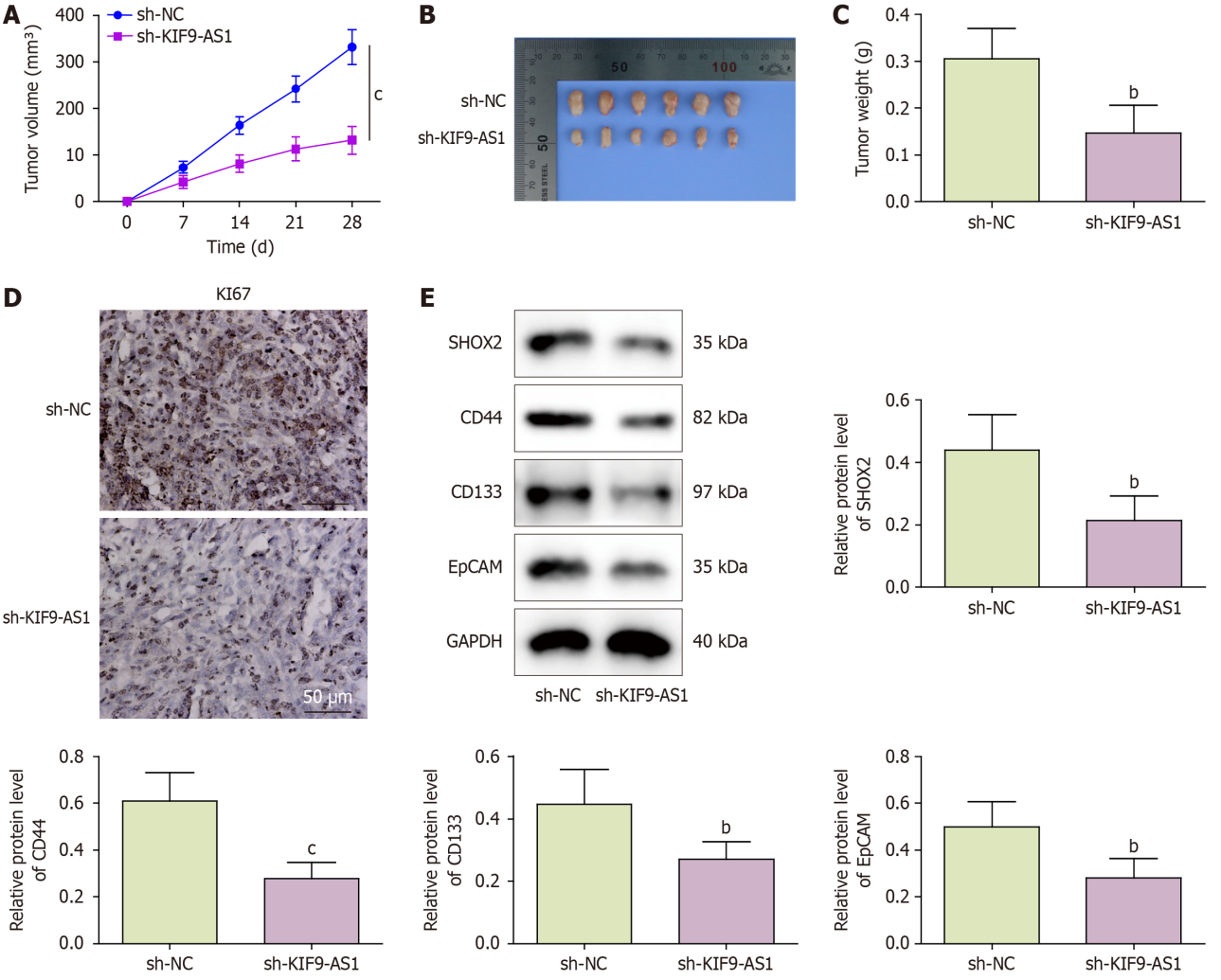


Figure 7 Knockdown of the long noncoding RNA *KIF9-AS1* inhibited sorafenib resistance in hepatocellular carcinoma xenograft model mice. Huh-7/R cells transfected with sh-KIF9-AS1 or sh-NC were subcutaneously transplanted into BALB/c nude mice to establish a mouse model of xenograft tumor growth. Sorafenib was administered orally twice a week at a dosage of 30 mg/kg from days 7-14 and 21-28. A-C: The tumor volume and weight of the mice were recorded; D: Images of immunohistochemical staining for Ki67 in tumor tissues; E: Short stature homeobox 2, CD44, CD133 and epithelial cell adhesion molecule expression levels were assessed *via* western blotting. $n = 6$. $^{b}P < 0.01$, $^{c}P < 0.001$. EpCAM: Epithelial cell adhesion molecule.

alleviated resistance to sorafenib, suppressed cell proliferation and promoted cell death in HCC cells. As previously reported, lncRNAs can be targeted to combat chemoresistance mediated by cancer stem cells, suggesting that stemness and therapeutic resistance are linked by lncRNAs[32]. However, the functional role of *KIF9-AS1* in stemness in HCC has not been thoroughly elucidated. Notably, our study demonstrated for the first time that *KIF9-AS1* promoted the expression of stemness marker genes in sorafenib-resistant HCC cells, which is consistent with previous studies. For example, the lncRNA DPPA2 upstream binding RNA increased cancer stemness marker expression and amplified chemoresistance in HCC through the E2F transcription factor 1/cellular inhibitor of PP2A axis[33]. These results suggest the potential of *KIF9-AS1* as a promising therapeutic target for HCC in the future.

Recent evidence indicates that m6A modification can mediate RNA metabolism and gene expression, influencing the malignant phenotypes and drug resistance of HCC[34]. A study by Wu *et al*[10] demonstrated that m6A modification increased lncRNA *NIFK-AS1* expression in HCC, thus promoting resistance to sorafenib and tumor development. Another study revealed that METTL14 modulates m6A modification, stabilization and upregulation of the lncRNA MIR155 host gene, thereby facilitating immune escape in HCC cells[35]. Bioinformatics analyses have predicted m6A modification sites in *KIF9-AS1*. However, the role of m6A-modified *KIF9-AS1* in HCC has not been clarified. Our findings provide evidence that m6A modification of *KIF9-AS1* is significantly enhanced in sorafenib-resistant HCC cells. METTL3 and IGF2BP1 are considered regulators of m6A methylation; the RM2Target database also predicts that these proteins regulate the m6A modification of *KIF9-AS1*. However, regulation of the METTL3/IGF2BP1 axis by m6A-modified *KIF9-AS1* has not been reported in HCC. A previous study revealed that METTL3 mediated m6A modification and upregulation of the expression of the lncRNA *LINC00958*, leading to lipogenesis in HCC and tumor progression[36]. Liu *et al*[37] reported that METTL3, an m6A writer, facilitates m6A modification and the upregulation of the expression of the lncRNA glucosylceramidase beta pseudogene 1, increasing the migratory and invasive capabilities of HCC cells. Our findings revealed that METTL3 expression was elevated in HCC cells and that silencing *METTL3* suppressed m6A modification of *KIF9-AS1*. Cai *et al*[38] reported that RNA binding motif protein 15 promoted the proliferation and invasion of HCC cells through m6A methylation of YES1 *via* an IGF2BP1-dependent mechanism. This study also revealed that silencing *IGF2BP1* decreased the stability of *KIF9-AS1* in HCC cells. Our study confirmed that METTL3 stabilized and promoted *KIF9-AS1* expression in HCC cells in an m6A-IGF2BP1-dependent manner. These results indicate that the METTL3/IGF2BP1 axis regulates m6A modification and *KIF9-AS1* expression in HCC.

Finally, we explored the downstream mechanism of *KIF9-AS1* in HCC. As demonstrated in a study by Yang *et al*[18], the upregulation of SHOX2 was positively correlated with tumor recurrence and advanced tumor-node-metastasis stage, while silencing *SHOX2* obviously suppressed the proliferative and invasive abilities of HCC cells. Consistently, our findings revealed that SHOX2 overexpression enhanced the stemness and sorafenib resistance of HCC cells. According to the bioinformatics analysis, USP1 is predicted to interact with SHOX2 and *KIF9-AS1*. The ubiquitination function of USP1 plays an essential regulatory role in various types of cancers. Reportedly, an increase in USP1 can lead to the deubiquitination of tumor-related proteins, thereby increasing protein stability and function in tumor cells[39]. Another study demonstrated that USP1 suppression decreased tumor stemness and sensitized HCC cells to doxycycline by increasing the ubiquitylation of proliferating cell nuclear antigen[40]. Accordingly, we observed that USP1 suppression induced an increase in SHOX2 ubiquitination, leading to the downregulation of SHOX2 expression in HCC cells. Moreover, we found that *KIF9-AS1* increased SHOX2 stability and expression by promoting deubiquitination *via* USP1. The functional mechanism of SHOX2 has been implicated in different cancers. For example, SHOX2 facilitates the oncogenic behavior of prostate cancer cells by blocking the Hippo-Yes-associated protein pathway through the activation of nephronophthisis type 4 transcription[41]. Teng *et al*[42] reported that SHOX2 accelerated breast cancer metastasis by activating WASF3 transcription in cooperation with signal transducer and activator of transcription 3 (STAT3)[42]. Notably, Yes-associated protein expression was found to be associated with vascular invasion, stemness and epithelial-mesenchymal transition in HCC cells[43]. Furthermore, glycochenodeoxycholic acid-induced activation of the STAT3 signaling pathway enhances chemoresistance and stemness in HCC[44]. Another study revealed that inhibition of the NANOG/STAT3 pathway increased sensitivity to sorafenib treatment in HCC[45]. Therefore, we speculate that SHOX2 might exert its functional effects on stemness and sorafenib resistance in HCC by mediating downstream pathways or proteins.

This study still has limitations. First, we plan to collect more clinical samples to analyze the use of *KIF9-AS1* as a diagnostic biomarker for HCC in future work. Second, this study revealed that m6A modification led to the differential expression of *KIF9-AS1* in HCC; however, other epigenetic mechanisms, such as transcription factor expression and histone acetylation, may also be involved. Third, our findings revealed that *KIF9-AS1* regulated SHOX2 expression through USP1-mediated ubiquitination. Further investigations are needed to clarify whether *KIF9-AS1* influences SHOX2 expression through other factors or epigenetic modifications. Overall, our study also indicates the potential clinical application of *KIF9-AS1*. For example, this study might provide a theoretical foundation for the use of *KIF9-AS1* as a diagnostic marker for HCC. Furthermore, *KIF9-AS1* might be delivered to target sites through various delivery systems for HCC treatment.

CONCLUSION

In conclusion, the present study demonstrated that the m6A-modified lncRNA *KIF9-AS1* promoted stemness and sorafenib resistance in HCC through USP1-mediated deubiquitination of SHOX2 (Figure 8). Our work might offer a promising new clinical target for treating HCC.

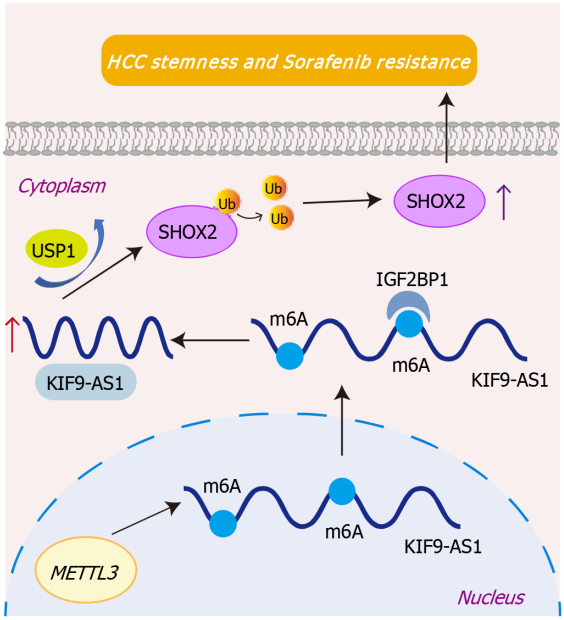


Figure 8 The mechanism by which the m6A-modified long noncoding RNA *KIF9-AS1* promotes stemness and sorafenib resistance in hepatocellular carcinoma through ubiquitin-specific peptidase 1-mediated deubiquitination of short stature homeobox 2. HCC: Hepatocellular carcinoma; USP1: Ubiquitin-specific peptidase 1; SHOX2: Short-stature homeobox 2; METTL3: Methyltransferase-like 3; IGF2BP1: Insulin-like growth factor 2 mRNA binding protein 1; m6A: N6-methyladenosine; Ub: Ubiquitin.

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FOOTNOTES

Author contributions: Yu Y and Lu XH jointly wrote the manuscript; Yu Y drafting the initial version; Lu XH making substantial revisions and additions to ensure accuracy and consistency; Yu Y and Lu XH made equal contributions in study design, data analysis, and manuscript preparation, and they contributed equally to this work as co-first authors. Yu Y, Mu JS, Yan Y, and Meng K designed and coordinated the study; Lu XH, Meng JY, Sun JS, and Chen HX performed the experiments and acquired and analyzed the data; Mu JS, Yan Y, and Meng K revised the manuscript; and all authors approved the final version of the article. Yan Y and Meng K designed, financed and coordinated the study and contributed equally to this work as co-corresponding authors.

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