


## RESEARCH ARTICLE

# lncRNA HAND2-AS1 overexpression inhibits cancer cell proliferation in hepatocellular carcinoma by downregulating RUNX2 expression

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**Abstract**

**Background:** The long non-coding RNA HAND2 antisense RNA 1 (HAND2-AS1) acts as a tumor suppressor in several malignancies, but its role in hepatocellular carcinoma (HCC) remains unknown. In this study, we aimed to investigate the function of HAND2-AS1 in HCC.

**Methods:** The expression levels of HAND2-AS1 and runt-related transcription factor 2 (RUNX2) were determined in patients with HCC and HCC cell lines using quantitative real-time polymerase chain reaction and western blot analyses. Cell proliferation was determined using Cell Counting Kit-8 assay, and the correlation between HAND2-AS1 and RUNX2 expression was also investigated.

**Results:** The plasma level of HAND2-AS1 was downregulated and that of RUNX2 was upregulated in patients with early-stage HCC compared with those in healthy controls. No significant differences in the plasma levels of HAND2-AS1 and RUNX2 were found among hepatitis B virus (HBV)-positive, hepatitis C virus (HCV)-positive, and HBV- and HCV-negative patients with HCC. The plasma levels of HAND2-AS1 and RUNX2 were inversely correlated in the patient groups but not in the control group. HAND2-AS1 overexpression led to the downregulation of RUNX2 expression in human HCC cells, whereas RUNX2 failed to significantly affect HAND2-AS1 expression. HAND2-AS1 overexpression inhibited and RUNX2 overexpression promoted the proliferation of HCC cells. RUNX2 overexpression attenuated the inhibitory effects of HAND2-AS1 overexpression on cancer cell proliferation.

**Conclusion:** HAND2-AS1 overexpression inhibits cancer cell proliferation in HCC by downregulating RUNX2 expression.

**KEYWORDS**

HBV, HCV, hepatocellular carcinoma, lncRNA HAND2-AS1, RUNX2

## 1 | INTRODUCTION

Despite efforts for the prevention of cancer, liver cancer remains one of the most common human malignancies and is one of the

leading causes of cancer-related death worldwide.<sup>1,2</sup> The incidence of liver cancer in the United States has been stable over the past several years; however, the mortality rate of patients with liver cancer has shown an increasing trend,<sup>3</sup> mostly because of the increased

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prevalence of cancer metastasis at the time of diagnosis, which lacks radical treatment methods.<sup>4</sup> Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections have been proven to be major causes of liver cancer<sup>5</sup>; however, the molecular mechanism underlying the pathogenesis of liver cancer remains unclear.

Runt-related transcription factor 2 (RUNX2) plays pivotal roles in osteoblast differentiation.<sup>6</sup> Increasing evidence has shown that RUNX2 also plays important roles in cancer biology, and the inhibition of RUNX2 expression is currently considered a promising therapeutic target for cancer treatment.<sup>7-9</sup> It has been reported that RUNX2 participates in cancer development through interactions with long non-coding RNAs (lncRNAs),<sup>10</sup> which are a group of non-coding RNA transcripts composed of >200 nucleotides and have essential functions in cancer biology.<sup>11</sup> The lncRNA HAND2 antisense RNA 1 (HAND2-AS1) is a tumor suppressor in endometrioid endometrial carcinoma<sup>12</sup> and osteosarcoma,<sup>13</sup> but its role in hepatocellular carcinoma (HCC) remains unknown. In the present study, we aimed to investigate the function of HAND2-AS1 in HCC.

## 2 | MATERIAL AND METHODS

### 2.1 | Human plasma and cell lines

Plasma was separated from blood extracted from 78 patients with HCC (39 males and 39 females; 30–66 years; mean age, 46.1 ± 4.9 years), and 48 healthy volunteers (23 males and 25 females; 30–67 years; mean age, 45.4 ± 4.8 years) who were admitted to Kongjiang Hospital of Yangpu District from April 2016 to May 2019. Among the 78 patients with HCC, 40 were HBV-positive (HBP), 21 were HCV-positive (HCP), and the remaining 17 were negative for both HBV and HCV (non-positive, NP). The patient inclusion criteria were as follows: 1) diagnosis via biopsy and 2) willingness of patients and their families to participate in the research. The following patients were excluded: 1) those diagnosed with multiple diseases

and 2) those treated within 3 months prior to blood extraction. All patients were in the American Joint Committee on Cancer stage I ( $n = 22$ ) or II ( $n = 56$ ), which were considered early stages of cancer. All 48 healthy volunteers underwent routine physiological examinations in Fujian Medical University Union Hospital during the same period. There were no significant differences in basic characteristics among the three patient groups and the control group (Table 1). This study was approved by the Ethics Committee of Kongjiang Hospital of Yangpu District, and all participants provided informed consent.

The human HCC cell lines SNU-398 (ATCC<sup>®</sup> CRL-2233<sup>™</sup>) and SNU-182 (ATCC<sup>®</sup> CRL-2235<sup>™</sup>) were purchased from American Type Culture Collection (Virginia, USA). Cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

### 2.2 | Cell transfection

HAND2-AS1 and RUNX2 expression vectors (pcDNA3.1 vector) were designed and synthesized by GenePharma (Shanghai, China). Lipofectamine 3000 reagent (Thermo Fisher Scientific, USA) was used to transfect 20 nM vectors into 10<sup>5</sup> SNU-398 and SNU-182 cells. Untransfected cells were used as control cells. Transfection with empty vectors was used as the negative control. The subsequent experiments were performed 24 h after transfection. Transfection efficiencies were measured using quantitative real-time polymerase chain reaction (qRT-PCR). To achieve co-transfection, HAND2-AS1, and RUNX2 expression vectors (10 nM) were transfected simultaneously.

### 2.3 | qRT-PCR

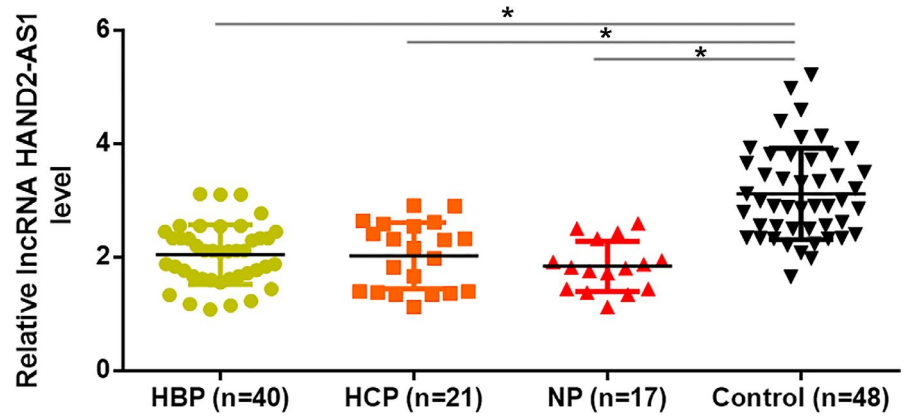
The MPure<sup>™</sup> Total RNA Extraction Kit (117022160, MP Biomedicals) was used to extract total RNA from plasma and SNU-398 and

	HBP	HCP	NP	Control	Chi-square	<i>p</i>
Cases	40	21	17	48		
Mean age (years)	46.1 ± 4.4	45.4 ± 3.9	46.4 ± 4.6	45.4 ± 4.8		
Age (year)						
≥45	22	11	10	26	0.17	0.98
<45	18	10	7	22		
Gender						
Male	19	12	8	23	0.64	0.89
Female	21	9	9	25		
AJCC stage						
I	12	6	4		0.25	0.8
II	28	15	13			

TABLE 1 Basic characteristics of the four participant groups

Note: The chi-squared test was performed to compare clinical characteristics among the participant groups.

**FIGURE 1** HAND2-AS1 expression was downregulated in patients with HCC and remained unaffected by HBV or HCV infection. Plasma HAND2-AS1 expression was significantly downregulated in the HBP, HCP, and NP patient groups compared with that in the healthy control group, and no significant differences in the plasma HAND2-AS1 levels were found among the three patient groups ( $*p < 0.05$ )



SNU-182 cells cultured *in vitro*. After digestion with DNase I (Thermo Fisher Scientific), the gb Reverse Transcription Kit (Generi Biotech) was used to synthesize cDNA under the following conditions: 25°C for 5 min, 52°C for 20 min, and 80°C for 10 min. All PCR reaction systems were performed using Luna® Universal One-Step RT-qPCR Kit (NEB). The PCR reaction conditions were as follows: 95°C for 1 min, followed by 40 cycles of 95°C for 10 s, and 56.6°C for 35 s. The following primer sequences were used for PCR: 5'-GTTATGAAAAACCAAGTAGCCAGGTC-3' (forward) and 5'-GTAATCTGACTCTGTCCTTGTGGAT-3' (reverse) for RUNX2 mRNA, 5'-GGGTGTTTACGTAGACCAGAACC-3' (forward) and 5'-CTTCCAAAAGCCTTCTGCCTTAG-3' (reverse) for HAND2-AS1, and 5'-GACCTCTATGCCAACACAGT-3' (forward) and 5'-AGTACTTGCGCTCAGGAGGA-3' (reverse) for human  $\beta$ -actin. This experiment was performed in triplicate, and all data were processed using the  $2^{-\Delta\Delta CT}$  method.<sup>14</sup>

## 2.4 | Cell proliferation assay

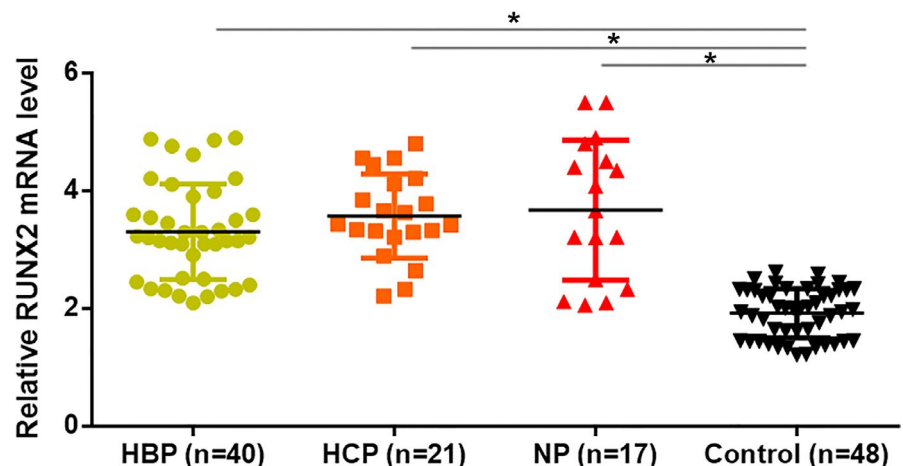
The proliferation of cancer cells was detected using the Cell Counting Kit-8 (CCK-8) assay (Beyotime Biotechnology, Jiangsu, China) for cases in which HAND2-AS1, and RUNX2 overexpression rates reached 200%. Briefly, cell suspensions ( $3 \times 10^4$  cells/ml) were prepared and transferred to a 96-well plate (100  $\mu$ l per

well). The plates were incubated at 37°C and 5% CO<sub>2</sub>, and 10  $\mu$ l of CCK-8 reagent was added every 24 h for 96 h. Cells were cultured for an additional 4 h, and the absorbance at 450 nm was measured using the Fisherbrand™ accuScan™ GO UV/Vis Microplate Spectrophotometer (Fisher Scientific). The experiment was repeated three times to obtain mean values.

## 2.5 | Western blot analysis

The ReadyPrep™ Protein Extraction Kit (Bio-Rad) was used to extract total protein from cancer cells. Protein concentration was measured using the BCA assay, and proteins were subjected to western blotting. Electrophoresis was conducted on a 10% SDS-PAGE gel. The gels were transferred to PVDF membranes, and the membranes were blocked using 5% non-fat milk at room temperature for 2 h. The samples were incubated with rabbit anti-human RUNX2 (ab23981, 1:1200, Abcam) and rabbit anti-human GAPDH (ab9485, 1:1200, Abcam) primary antibodies at 4°C overnight. The samples were then incubated with goat anti-rabbit IgG-HRP (1:1200, MBS435036, MyBioSource) secondary antibody at room temperature for 2 h. Blots were developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Grayscale images were processed using Image J version 1.46 software to normalize protein expression levels. The experiments were performed in triplicate.

**FIGURE 2** Runt-related transcription factor 2 mRNA expression was upregulated in patients with HCC and was unaffected by HBV or HCV infection. Plasma RUNX2 mRNA expression was significantly upregulated in the HBP, HCP, and NP patient groups compared with that in the healthy control group, and no significant differences in plasma RUNX2 mRNA levels were found among the three patient groups ( $*p < 0.05$ )



	Cases	HAND2-AS1		Chi-square	<i>p</i>	RUNX2		Chi-square	<i>p</i>
		High	Low			High	Low		
Gender									
Male	39	18	21	0.46	0.50	17	22	1.28	0.26
Female	39	21	18			22	17		
Age (years)									
≥45	41	22	19	0.46	0.50	20	21	0.05	0.82
<45	37	17	20			19	18		
AJCC stage									
I	22	10	12	0.25	0.61	9	13	1.01	0.31
II	56	29	27			30	26		

**TABLE 2** Correlation between HAND2-AS1 and RUNX2 expression levels and clinical characteristics of patients with HCC

Note: The chi-squared test was performed to analyze the correlation between HAND2-AS1 and RUNX2 expression levels and clinical characteristics of patients with HCC.

## 2.6 | Statistical analysis

GraphPad Prism 6 software was used for statistical analyses. Data were expressed as the mean ± standard deviation. Correlation analysis was performed using the Pearson correlation coefficient. Comparisons between two groups were performed using Student's *t* test, and comparisons among multiple groups were performed using analysis of variance followed by Tukey's test. Receiver operating characteristic (ROC) curve analysis was performed on the assumption that all patients with HCC were true positive cases, and all healthy controls were true negative cases. The chi-squared test was performed to compare clinical characteristics among the participant groups. The correlations between the HAND2-AS1 and RUNX2 expression levels, and clinical characteristics of patients were also analyzed using the chi-squared test.  $p < 0.05$  was used to indicate statistical significance.

## 3 | RESULTS

### 3.1 | HAND2-AS1 expression was downregulated in patients with HCC and was not affected by HBV or HCV infection

The plasma levels of HAND2-AS1 were determined using qRT-PCR. Compared with that in healthy controls, the plasma level of HAND2-AS1 was significantly downregulated in the HBP (0.57-fold,  $p = 0.022$ ), HCP (0.57-fold,  $p = 0.031$ ) and NP (0.42-fold,  $p = 0.020$ ) patient groups. In addition, there were no significant differences in the plasma levels of HAND2-AS1 among the HBP, HCP, and NP patient groups (Figure 1).

### 3.2 | RUNX2 mRNA expression was upregulated in patients with HCC and was not affected by HBV or HCV infection

The qRT-PCR results showed that plasma levels of RUNX2 mRNA were significantly upregulated in the HBP (1.71-fold,  $p = 0.017$ ),

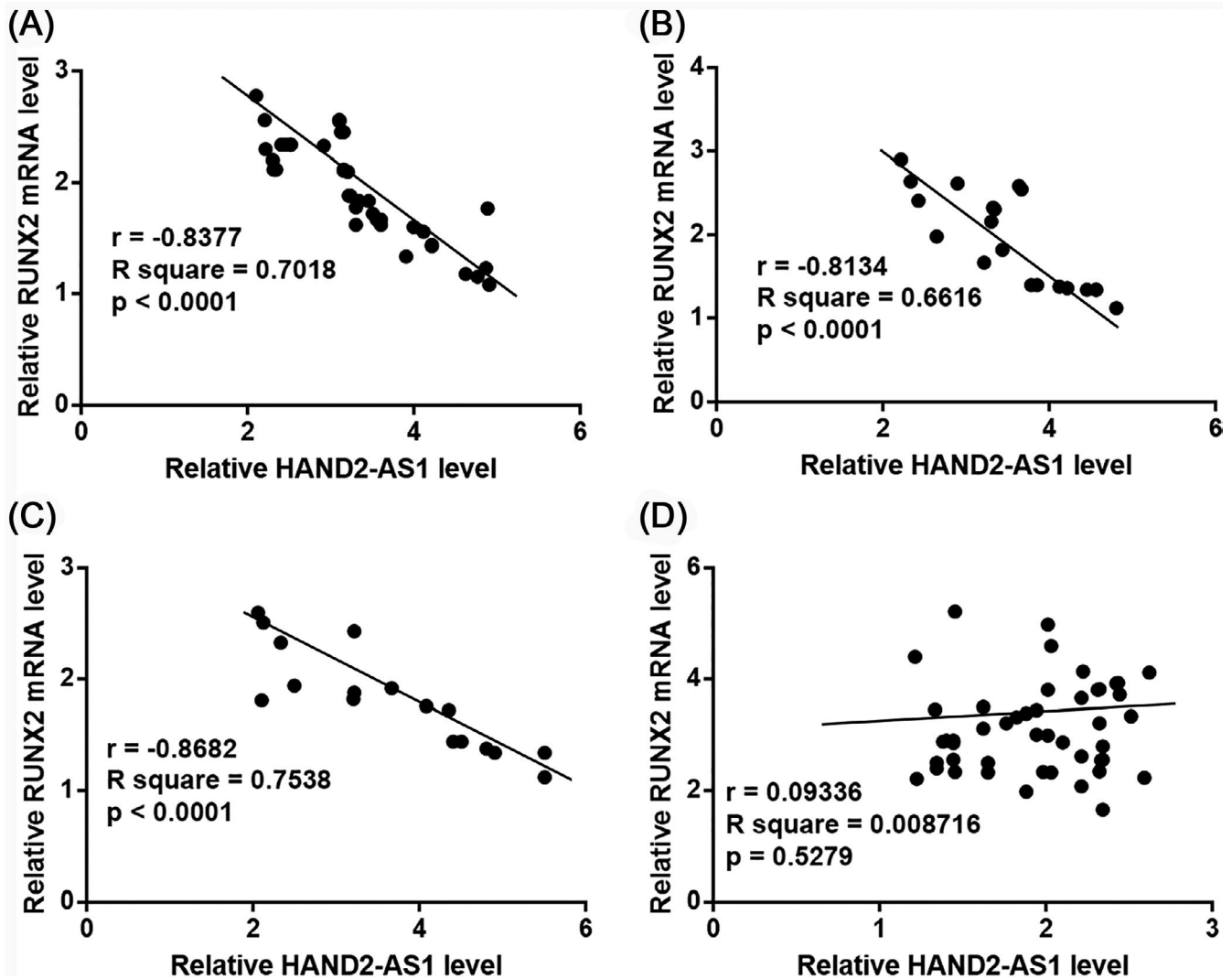
HCP (1.85-fold,  $p = 0.012$ ), and NP (1.88-fold,  $p = 0.011$ ) patient groups compared with those in the healthy controls. There were no significant differences in the plasma levels of RUNX2 mRNA among the three patient groups (Figure 2). In addition, sex, age, and clinical stage had no significant effects on HAND2-AS1 expression (Table 1).

### 3.3 | Plasma RUNX2 mRNA and HAND2-AS1 expression levels were inversely correlated in the patient groups but not in the control group

Patients were grouped into high- and low-expression groups ( $n = 39$ ) according to the median levels of HAND2-AS1 and RUNX2. Chi-square tests showed that sex, age, and clinical stage had no significant effects on HAND2-AS1 and RUNX2 expression (Table 2). The correlation between plasma RUNX2 mRNA and HAND2-AS1 expression in different groups was analyzed using the Pearson correlation coefficient. A significantly inverse correlation between plasma RUNX2 mRNA and HAND2-AS1 expression was found in the HBP (Figure 3A,  $p < 0.0001$ ), HCP (Figure 3B,  $p < 0.0001$ ), and NP (Figure 3C,  $p < 0.0001$ ) patient groups, and the correlation between plasma RUNX2 mRNA and HAND2-AS1 expression was not strong in the control group (Figure 3D,  $p = 0.5279$ ). In addition, sex, age, and clinical stage did not show significant effects on RUNX2 expression.

### 3.4 | Downregulation of plasma HAND2-AS1 expression assisted HCC diagnosis

Receiver operating characteristic curve analysis was performed under the assumption that all patients with HCC were true positive cases, and all healthy controls were true negative cases. As shown in Figure 4, the area under the curve was 0.8802, with a standard error of 0.03007 and 95% confident interval of 0.8212–0.9391 ( $p < 0.0001$ ).



**FIGURE 3** Plasma RUNX2 mRNA expression and HAND2-AS1 expression were inversely correlated in the patient groups but not in the control group. A significant inverse correlation between plasma RUNX2 mRNA and HAND2-AS1 expression was found in the HBP (A), HCP (B), and NP (C) groups but not in the control group (D)

### 3.5 | HAND2-AS1 is likely an upstream inhibitor of RUNX2 in HCC cells

Compared with the control (C) and negative control (NC) groups, HAND2-AS1 overexpression significantly downregulated RUNX2 mRNA expression in SNU-398 and SNU-182 cells, as shown by qRT-PCR results (Figure 5A,  $p < 0.05$ ). In addition, western blot results showed that HAND2-AS1 overexpression led to downregulated RUNX2 protein expression (Figure 5B,  $p < 0.05$ ). In contrast, RUNX2 overexpression did not significantly alter the expression of HAND2-AS1.

### 3.6 | HAND2-AS1 overexpression potentially inhibits HCC cell proliferation by downregulating RUNX2 expression

The CCK-8 assay results showed that HAND2-AS1 overexpression significantly inhibited and RUNX2 overexpression significantly

promoted the proliferation of SNU-398 (Figure 6A) and SNU-182 (Figure 6B) cells ( $p < 0.05$ ) compared with that in the C and NC groups. In addition, compared with cells transfected with HAND2-AS1 expression vectors alone, those transfected with both HAND2-AS1 and RUNX2 expression vectors showed significantly enhanced cell proliferation. Consistent with these cell proliferation data, co-transfection of HAND2-AS1 and RUNX2 led to the upregulation of HAND2-AS1 expression and downregulation of HAND2-AS1 mRNA expression.

## 4 | DISCUSSION

Although lncRNA HAND2-AS1 has been characterized as a tumor suppressor in endometrioid endometrial carcinoma<sup>12</sup> and osteosarcoma,<sup>13</sup> its involvement in other human diseases remains poorly understood. A key finding of the present study is that HAND2-AS1 also acts as a tumor suppressor in HCC, and its effects in HCC are likely achieved via the downregulation of RUNX2 expression.

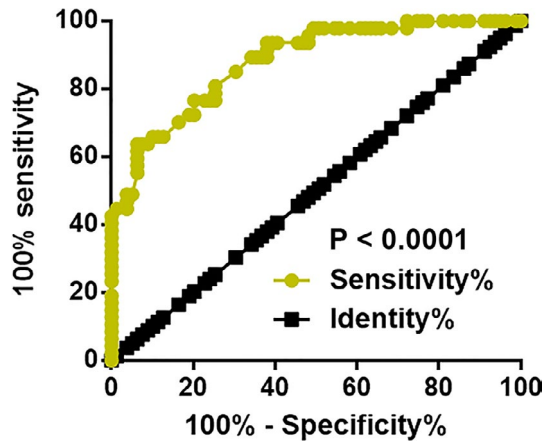


FIGURE 4 Receiver operating characteristic curve analysis of the diagnostic value of plasma HAND2-AS1 for early-stage HCC

Runx-related transcription factor 2 expression is upregulated in HCC, and its overexpression promoted the migration and invasion of HCC cells.<sup>15</sup> Consistent with the findings of this previous study, our

results also indicated increased RUNX2 expression in patients with HCC compared with that in healthy controls. However, our study also showed that RUNX2 overexpression promoted HCC cell proliferation. Our findings imply an additional function of RUNX2 in HCC. HBV and HCV infections are known to be major causes of HCC.<sup>5</sup> However, the interaction of RUNX2 with HBV and HCV remains to be clarified. In the present study, we showed that RUNX2 expression was not significantly affected by HBV and HCV infections. Therefore, RUNX2 participates in HCC through HBV- and HCV-independent pathways.

HAND2-AS1 inhibits cancer cell invasion in endometrioid endometrial carcinoma.<sup>12</sup> However, in the present study, its overexpression did not significantly affect the migration and invasion of HCC cells. In contrast, HAND2-AS1 overexpression led to the inhibition of HCC cell proliferation. Therefore, HAND2-AS1 plays different roles in various types of cancers. Kang et al. previously reported that HAND2-AS1 inhibited osteosarcoma progression by reducing energy metabolism,<sup>13</sup> which provides energy for cancer cell proliferation.<sup>16</sup> This finding suggests the involvement of HAND2-AS1 in cancer development.

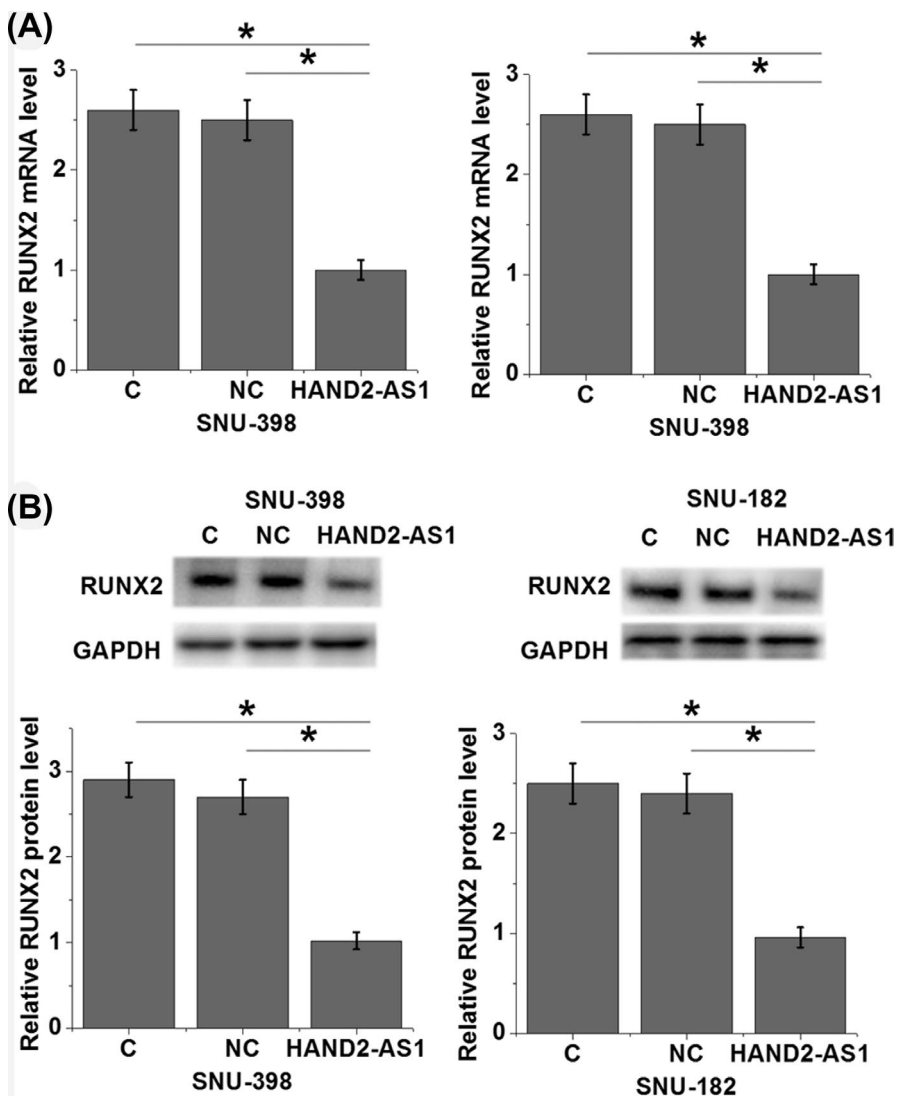
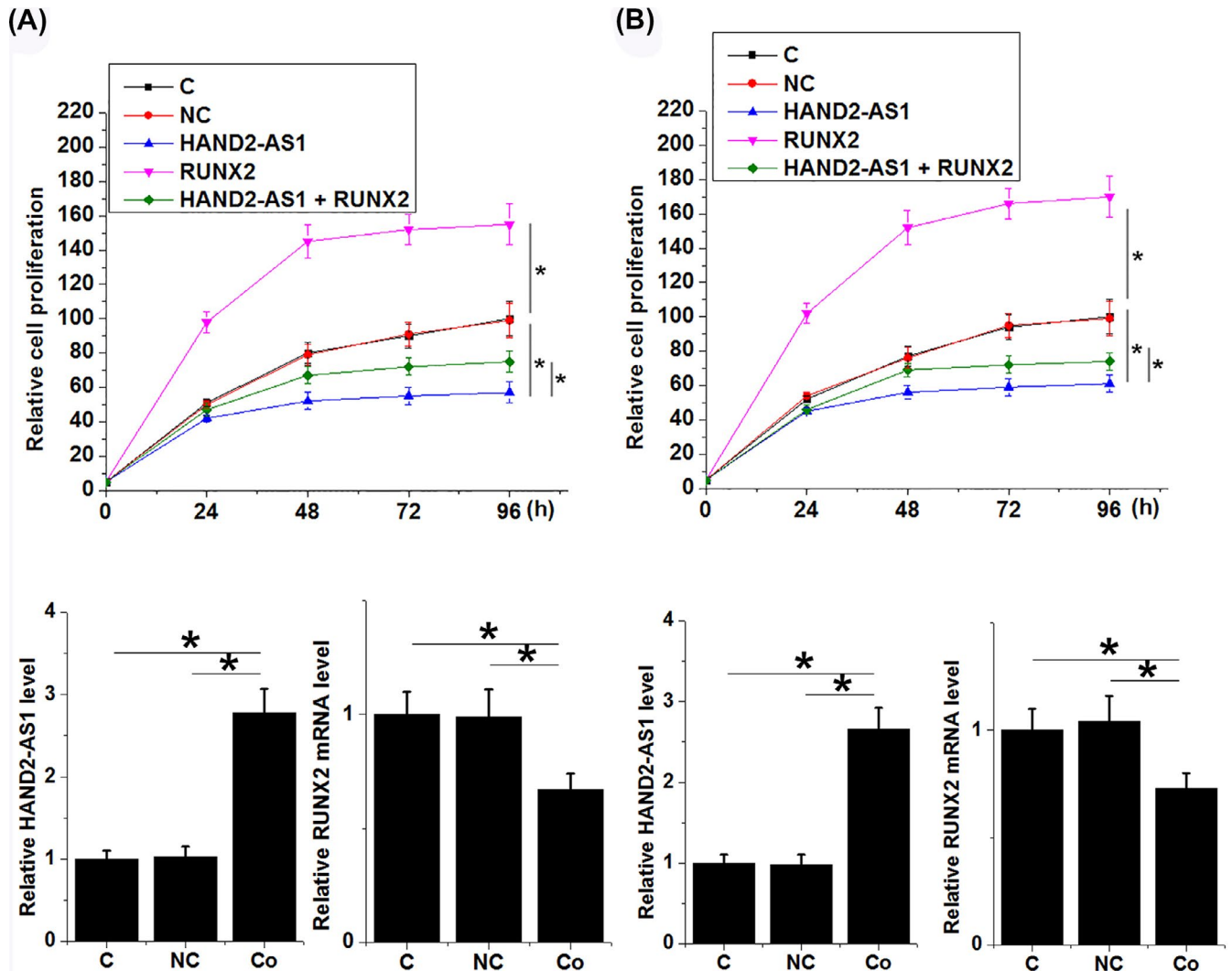


FIGURE 5 HAND2-AS1 is likely an upstream inhibitor of RUNX2 in HCC cells. HAND2-AS1 overexpression significantly downregulated RUNX2 expression in both SNU-398 and SNU-182 cells at the mRNA (A) and protein (B) levels ( $*p < 0.05$ )





**FIGURE 6** HAND2-AS1 overexpression potentially inhibits HCC cell proliferation by downregulating RUNX2 expression. HAND2-AS1 overexpression significantly inhibited, whereas RUNX2 overexpression significantly promoted the proliferation of SNU-398 (A) and SNU-182 (B) cells. RUNX2 overexpression partially compensated the adverse effects of HAND2-AS1 overexpression on cancer cell proliferation. Consistent with the cell proliferation data, co-transfection of HAND2-AS1, and RUNX2 led to upregulation of HAND2-AS1 and downregulation of HAND2-AS1 mRNA expression ( $*p < 0.05$ )

Runt-related transcription factor 2 functions through interactions with multiple signaling molecules, including lncRNAs.<sup>17,18</sup> Herein, we showed that HAND2-AS1 is a negative upstream regulator of RUNX2 in HCC cell proliferation. Therefore, HAND2-AS1 overexpression serves as a potential therapeutic target for HCC via downregulation of RUNX2 expression. However, the interactions between HAND2-AS1 and RUNX2 are likely indirect as there was a lack of significant correlation between their expression levels in the healthy controls.

One limitation of the present study was that the RUNX2 protein levels in plasma were not measured; however, future studies should include this investigation. In addition, although we performed diagnostic analyses, we did not obtain a robust combination of sensitivity and specificity owing to the small sample size.

We will include more patients in future studies to further test the diagnostic values.

In conclusion, lncRNA HAND2-AS1 acts as a tumor suppressor in HCC and inhibits HCC cell proliferation by downregulating RUNX2 expression.

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None.

#### CONFLICT OF INTEREST

There are no competing interests.

#### DATA AVAILABILITY STATEMENT

My manuscript has no associated data.

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