

# Upregulation of hypoxia-inducible factor 1 $\alpha$ mRNA expression was associated with poor prognosis in patients with hepatocellular carcinoma

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**Background:** HIF1 $\alpha$  mRNA expression in hepatocellular carcinoma (HCC) tissues and its relationship with the prognosis in HCC patients is still unclear. We performed this study to investigate the expression of HIF1 $\alpha$  mRNA and its correlation with the prognosis in HCC patients.

**Materials and methods:** GSE14520 and Oncomine database were used to analyse the differential expression of HIF1 $\alpha$  mRNA among HCC tissues and corresponding peritumour tissues or normal liver tissues. The relationship between HIF1 $\alpha$  mRNA expression and the clinicopathological features and survival in HCC patients was analysed using the GSE14520 dataset. CCK-8 assay, wound-healing assay, transwell invasion assay, tube formation assay, and subcutaneous xenograft tumour assays using nude mice were used to confirm the function of HIF1 $\alpha$ .

**Results:** Expression of HIF1 $\alpha$  mRNA was significantly upregulated in HCC tissues ( $P < 0.05$  in all cases); this was supported by the results of the Western blotting ( $P = 0.031$ ) and IHC analyses. Our analysis of the clinicopathological features of HCC patients indicated that high HIF1 $\alpha$  mRNA expression was strongly related with TNM stage III ( $P = 0.002$ ) and BCLC stage C ( $P = 0.038$ ). Survival analysis demonstrated that HCC patients with high HIF1 $\alpha$  mRNA expression had a short overall survival (OS) ( $P = 0.048$ ), but showed no significant difference in recurrence-free survival (RFS) ( $P = 0.066$ ) compared to patients with low HIF1 $\alpha$  mRNA expression. We further demonstrated that HIF1 $\alpha$  promoted the proliferation, migration, invasion, and angiogenic ability of HCC cells, by using the stably transformed SK-Hep1 and Hep-3B cell lines showing HIF1 $\alpha$  overexpression. Finally, xenograft tumour models of nude mice showed that RNA interference-mediated HIF1 $\alpha$  silencing suppressed tumour growth and angiogenesis in HCC.

**Conclusion:** Our study suggests that the upregulation of HIF1 $\alpha$  mRNA, which is found in HCC tissues and associated with poor prognosis in HCC patients, contributed to the proliferation, migration, invasion, and angiogenic ability of HCC cells.

**Keywords:** HIF1 $\alpha$ , mRNA, prognosis, hepatocellular carcinoma

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## Introduction

Hepatocellular carcinoma (HCC) is the fifth most common tumour and the second major cause of tumour-associated deaths worldwide.<sup>1,2</sup> More than half of all HCC patients in the world have been diagnosed and treated in China.<sup>3</sup> Liver tumour resection and liver transplantation are the major treatment strategies for early HCC. However, treatment of advanced HCC mainly depends on transcatheter arterial

chemoembolisation, radiotherapy, chemotherapy, and targeted therapy with sorafenib.<sup>4–6</sup> Most HCC patients have been diagnosed at the advanced stage of the disease, especially in China, and have lost the chance of surgery and transplantation, with relatively short overall survival (OS) and recurrence-free survival (RFS).

Migration and invasion are the main causes of cancer progression and treatment failure in advanced-stage HCC patients.<sup>7</sup> Angiogenesis also plays a vital role in the development of HCC, which is a highly vascularised solid tumour.<sup>8</sup> Thus, suppression of HCC cell migration and invasion, and angiogenesis is the current trend of HCC treatment research.

Hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) was discovered by Semenza in 1992 during the analysis of the expression of the human erythropoietin gene.<sup>9</sup> HIF1 $\alpha$  is a heterodimer protein comprising an oxygen-sensitive  $\alpha$  subunit and constantly expressed  $\beta$  subunit.<sup>10,11</sup> HIF1 $\alpha$  plays a critical role in regulating cellular responses to hypoxia and the biological behaviour of tumours by affecting cell apoptosis, cell viability, vasomotor function, and energy metabolism.<sup>12–14</sup> HIF1 $\alpha$  has also been reported to promote HCC cells migration and invasion, and angiogenesis by activating the expression of downstream-related genes such as VEGF.<sup>15–19</sup> Thus, elucidating the mechanisms underlying the effects of HIF1 $\alpha$  in HCC is urgently needed for the prevention and treatment of liver cancer.

In recent years, many articles have reported that the overexpression of the HIF1 $\alpha$  protein is associated with poor OS and RFS in HCC patients.<sup>20–25</sup> However, very few studies have reported the correlation between HIF1 $\alpha$  mRNA overexpression and prognosis of liver cancers.<sup>26,27</sup> The relationship between the upregulation of HIF1 $\alpha$  mRNA expression and prognosis of HCC patients is still unclear. It is very essential to explore the HIF1 $\alpha$  mRNA expression and its prognostic value and specific function in HCC.

This study aims to explore the expression of HIF1 $\alpha$  mRNA in HCC tissues, and its relationship with the prognosis in HCC patients using GSE14520 and two datasets from the Oncomine database. We also verified the function of HIF1 $\alpha$  by constructing the stably transformed SK-Hep1 and Hep-3B cell lines showing HIF1 $\alpha$  overexpression and HIF1 $\alpha$  silencing (by RNA interference).

## Materials and methods

### Patients and ethics statement

HCC tissues and corresponding peritumour tissues were obtained from 12 patients admitted at the Shanghai Public

Health Clinical Center, Fudan University. These HCC patients were diagnosed according to histopathological analysis.<sup>28</sup> Seven patients were men and five were women; the patients were between 25 and 70 years old. Among these patients, three, four, and five patients showed TNM stage IA, IB, and II HCC, respectively. No anti-tumour treatment was administered to these patients before operation. They all signed the informed consent forms during their hospitalisation. The protocols for this study and informed consent documents were reviewed and approved by the Ethics Committee of the Shanghai Public Health Clinical Center, Fudan University. This study was conducted in accordance with the Declaration of Helsinki.

### Datasets source

The Roessler Liver and Roessler Liver 2 datasets were chosen from the Oncomine database (<https://www.oncomine.org>).<sup>29</sup> The GSE14520 dataset, which contains data for 242 HCC patients and the related information regarding their clinicopathological features, was downloaded from Gene Expression Omnibus (GEO) database in the National Center for Biotechnology Information (NCBI). The expression of *HIF1 $\alpha$*  mRNA in HCC tissues was compared with that in the corresponding peritumour tissues or normal liver tissues. The immunohistochemistry (IHC) data of the HIF1 $\alpha$  protein in the HCC tissues and normal liver tissues were downloaded from The Human Protein Atlas (<http://www.proteinatlas.org>).

### Materials and reagents

Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin were purchased from Hyclone (Logan, USA). Foetal bovine serum (FBS) was purchased from Gibco (South America). Anti-HIF1 $\alpha$  monoclonal antibodies were purchased from CST (Boston, USA). Anti-VEGF monoclonal antibodies were purchased from RD (Minneapolis, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies, New Super ECL Assay kit, and anti- $\beta$ -actin antibodies were purchased from KeyGEN BioTECH (Nanjing, China). Transwell chambers and Matrigel were purchased from Corning Life Sciences (8- $\mu$ m pores, Tewksbury, MA, USA) and BD Biosciences (San Jose, CA, USA), respectively. The QRT-PCR kit was purchased from Takara (Shiga, Japan). The Cell Counting kit-8 (CCK-8) assay was purchased from Dojindo Molecular Technologies, Inc. The BCA Protein Assay Kit was purchased from Beyotime Biotechnology (Shanghai, China). Recombinant lentiviral expression

vectors (LV-HIF1 $\alpha$ , LV-Con, LV-ShHIF1 $\alpha$ , and LV-Scramble), and polybrene were obtained from Genechem (Shanghai, China). BALB/c nude mice were purchased from Shanghai Jiesijie Experimental Animal Co., Ltd (Shanghai, China).

## Cell culture

Human umbilical vein endothelial cells (HUVECs) and two types HCC cell lines (SK-Hep1 and Hep-3B) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and then cultured in 5% CO<sub>2</sub> at 37 °C in DMEM containing 10% FBS and 1% penicillin-streptomycin. A hypoxic culture was defined when the cells were cultured under conditions of 5% CO<sub>2</sub>, 94% N<sub>2</sub>, and 1% O<sub>2</sub> at 37 °C in DMEM with 10% FBS and 1% penicillin-streptomycin.

## In vitro transfection

LV-HIF1 $\alpha$  and LV-ShHIF1 $\alpha$  (sequence: CATCTCCATCT CCTACCCACAT) are lentiviral vectors that can express GFP, and also overexpress and silence HIF1 $\alpha$ , respectively. LV-Con and LV-Scramble (sequence: TTCTC CGAACGTGTACGT) are also lentiviral vectors that only express GFP, without specific HIF1 $\alpha$ -overexpression or -silencing (via RNA interference) effects; cells transfected with these constructs have been commonly used as controls. For lentivirus infection,  $2 \times 10^5$  cells/well were seeded into six-well culture plates for 24 h. Further, 10  $\mu$ l of LV-HIF1 $\alpha$  ( $2 \times 10^8$  TU/ml), 2.5  $\mu$ l of LV-Con ( $8 \times 10^8$  TU/ml), 5  $\mu$ l of LV-ShHIF1 $\alpha$  ( $4 \times 10^8$  TU/ml), or 2  $\mu$ l of LV-Scramble ( $1 \times 10^9$  TU/ml) and 1  $\mu$ l of polybrene (10 mg/ml) were added to 1.0 ml of DMEM containing 10% FBS without antibiotics. The old culture medium was replaced with the mixture containing the lentivirus expression vectors, polybrene, and DMEM without antibiotics. After 12 h, the cell culture medium was replaced again with 2 ml of DMEM containing 10% FBS and antibiotics. After 72 h of incubation, the fluorescence of the lentivirus-infected cells was observed using fluorescence microscopy. The infected cells can be cultured continually or collected immediately and used for QRT-PCR assay or Western blotting.

## QRT-PCR assay

Total RNA from the cultured cells was extracted using TRIzol reagent, according to the manufacturer's instructions. A QRT-PCR kit and 0.5  $\mu$ g of total RNA were used to perform the QRT-PCR analysis. All the primers used

were synthesised by Sangon Biotech. The specific primers used are listed in Table 1.

## Western blotting analysis

Cells were scraped off from the six-well culture plates and lysed using RIPA lysis buffer containing protease inhibitors and phenylmethylsulfonyl fluoride (PMSF) for 30 min on ice. The protein concentrations were quantified using the BCA Assay Kit. Equal amounts of protein (50–100  $\mu$ g) and the corresponding amounts of  $5 \times$  loading buffer were mixed, and these mixtures were subjected to 7.5% or 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis; the protein bands were then transferred onto pure nitrocellulose blotting membranes. After blocking the membranes with 5% non-fat milk at room temperature for 2 h, the membranes were incubated overnight at 4 °C with the primary antibodies (diluted at 1:1000), and then with the HRP-conjugated secondary antibodies (diluted at 1:2000) at room temperature for 1 h. The targeted proteins were visualised using a New Super ECL Assay kit and then exposed onto films for visualisation.

## Determination of cell viability by the CCK-8 assay

Cell viabilities were assessed with the Cell Counting Kit-8 assay. A total of 10000 cells/well were plated onto 96-well culture plates and then cultured for 24 h. The cells were washed thrice with sterile PBS; then, 10  $\mu$ l of CCK-8 reagent and 90  $\mu$ l of fresh medium was added to the cells. Finally, the cells were incubated for 1 h at 37 °C. The optical density (OD) of the samples was measured at 450 nm using a microplate reader. All measurements were carried out in triplicate. We calculated the cell viability index based on the following formula: (experimental optical density value/control optical density value)  $\times 100\%$ .<sup>30</sup>

## Wound healing assay

Cells were plated in six-well culture plates. When the cells grew to 80–90% confluence, the cell layers were scratched in the form of a straight line (in the middle of each well)

**Table 1** Primers used for QRT-PCR

Name	Sequences (5'-3')
GAPDH-F	CAGGAGGCATTGCTGATGAT
GAPDH-R	GAAGGCTGGGGCTCATTT
HIF-1 $\alpha$ -F	TGACAAGCCACCTGAGGAGA
HIF-1 $\alpha$ -R	ACACGCGGAGAAGAGAAGGA

**Abbreviation:** QRT-PCR, quantitative real-time polymerase chain reaction.

using a sterile white pipette tip. The cells were then rinsed thrice with fresh serum-free DMEM to wash away the cell debris; these cells were cultured for 24 h. The scratched cell layers were photographed with an inverted microscope at 0 h and 24 h. These experiments were repeated thrice.

### Transwell invasion assay

We performed the transwell assay to detect the invasive ability of the cells. First, 600  $\mu$ l of DMEM containing 20% FBS was dispensed into the lower chamber. Next, 100  $\mu$ l of Matrigel diluted with fresh serum-free DMEM (in a ratio of 1:3) was dispensed into the upper chamber and allowed to solidify for 30 min at 37 °C before the cells were plated. The cells were then plated into the upper chamber, followed by incubation for 72 h. The invasive cells were analysed by crystal violet staining; the number of invasive cells was counted under a light microscope. These experiments were repeated thrice.

### HUVECS tube formation assay

Human HCC cells infected with the lentiviral constructs (LV- HIF1 $\alpha$  and LV-Con) were seeded into six-well plates and cultured for 24 h; then, the cell supernatants were collected as conditional medium for subsequent use. Next, 50  $\mu$ l of Matrigel was dispensed into the 96-well plates and allowed to solidify at 37 °C for 30 min, after which 100  $\mu$ l of conditional medium was added to the 96-well plates containing Matrigel. HUVECs ( $2.5 \times 10^4$  cells) were seeded onto the Matrigel, followed by incubation at 37 °C for 24 h. The formation of capillary-like structures was photographed, and the tube numbers were analysed using the Image J software. These experiments were repeated thrice.

### In vivo xenograft model assay

In order to verify the function of HIF1 $\alpha$ , we performed the in vivo xenograft model assay. Ten male BALB/c nude mice (aged four–six weeks old) were divided into two groups (LV-Scramble and LV-ShHIF1 $\alpha$  groups). Further,  $5 \times 10^6$  transformed Sk-Hep1 cells (transfected with LV-Scramble-Sk-Hep1 or LV-ShHIF1 $\alpha$ -Sk-Hep1) (in a volume of 100  $\mu$ l) were subcutaneously injected into the right flank of the nude mice after one week of adaptive feeding. The size of the xenograft tumours was measured every seven days for five weeks and calculated using the formula  $V = 0.5 \times L \times W^2$  (V, volume; L, length; W, width). All animals were sacrificed by euthanasia and the xenograft tumours were obtained for the IHC assay.

### IHC staining

Paraffin sections were deparaffinised in an oven and then hydrated. The sections were then treated with 3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase, and were then subjected to thermal repair. After cooling, the sections were blocked with 10% normal goat serum for 1 h and then incubated with the primary antibodies (diluted at 1:200) against HIF1 $\alpha$  and VEGF overnight at 4 °C. The sections were then incubated with HRP-conjugated secondary antibodies (diluted at 1:600) at room temperature for 1 h. Finally, all sections were counterstained with Diaminobenzidine and Mayer's haematoxylin.

### Statistical analysis

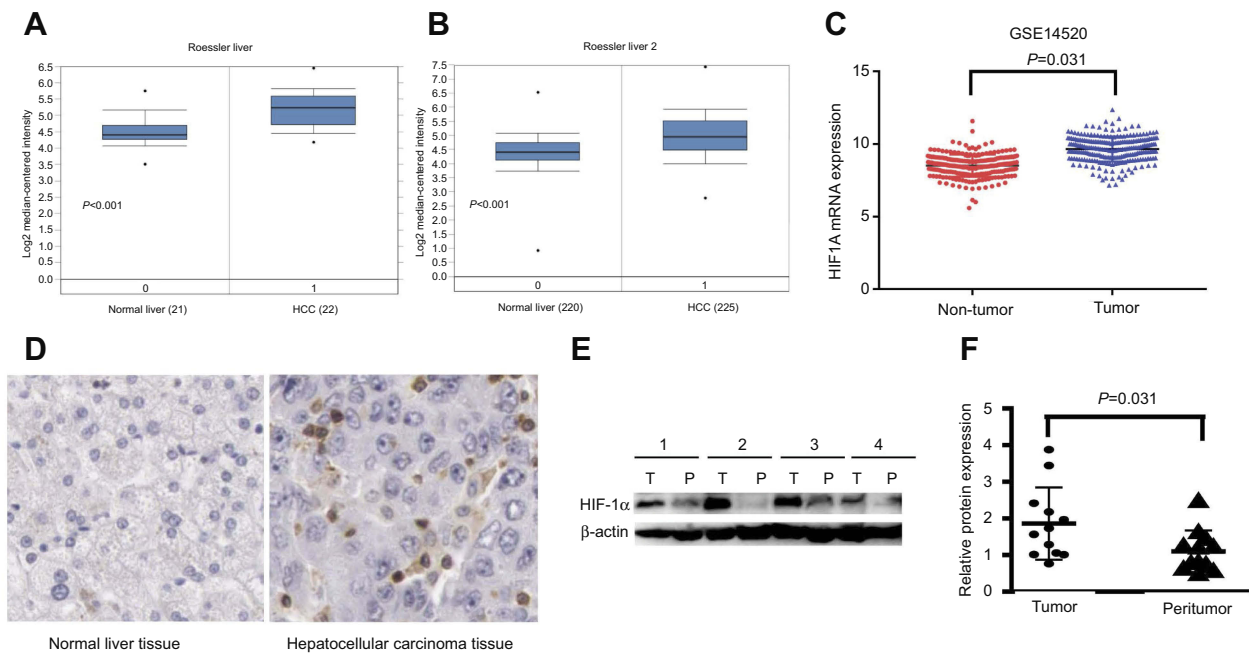
Categorical data were compared using the Chi-square test. Probabilities of OS and RFS were calculated using the Kaplan-Meier method, and the log-rank test was used to evaluate the differences between survival distributions. All experimental data were presented at the means  $\pm$  standard deviations, and the significance of data from two groups was estimated using the Student's *t* test. Statistical analyses were carried out using the IBM SPSS Statistics 21 software.  $P < 0.05$  was considered statistically significant for all the tests.

## Results

### Expression of HIF1 $\alpha$ is significantly upregulated in HCC tissues

The 'Roessler Liver' dataset, which contains data for 22 HCC tissues and 21 normal liver tissues, and the 'Roessler Liver 2' dataset, which contains data for 225 HCC tissues and 220 normal liver tissues, are from the Oncomine database. The GSE14520 dataset contains data for 242 HCC tissues and 239 peritumour tissues. Analysis of these datasets showed that the expression of HIF1 $\alpha$  mRNA was significantly upregulated in HCC tissues, compared to the case in normal liver tissues or corresponding peritumour tissues ( $P < 0.001$ , Figure 1A;  $P < 0.001$ , Figure 1B; and  $P = 0.031$ , Figure 1C). IHC results from the human protein atlas database demonstrated that a positive staining of the HIF1 $\alpha$  protein was observed in HCC tissues, whereas the normal liver tissues showed negative staining for the HIF1 $\alpha$  protein (Figure 1D). Finally, based on the Western blotting analysis, HIF1 $\alpha$  protein expression was also observed to be upregulated in HCC tissues, compared to the case for the corresponding peritumour tissues ( $P = 0.031$ , Figure 1E and F).





**Figure 1** HIF1 $\alpha$  expression in HCC tissues and normal liver tissues or corresponding peritumour tissues.

**Notes:** (A) HIF1 $\alpha$  mRNA expression from the "Roessler Liver" dataset in the Oncomine database. (B) HIF1 $\alpha$  mRNA expression from the "Roessler Liver 2" dataset in the Oncomine database. (C) HIF1 $\alpha$  mRNA expression from the GSE14520 dataset. (D) HIF1 $\alpha$  protein expression assayed by IHC from the human protein atlas database. (E) HIF1 $\alpha$  protein expression in 4 pairs of HCC tissues and the corresponding peritumour tissues assayed by Western blotting. (F) The relative fold change of the HIF-1 $\alpha$ / $\beta$ -actin expression (obtained by Western blotting analysis) in 12 pairs of HCC tissues and the corresponding peritumour tissues was calculated using the ImageJ software.

**Abbreviations:** HCC, hepatocellular carcinoma; IHC, immunohistochemistry.

## Clinicopathological characteristics of patients and their relationship with the HIF1 $\alpha$ mRNA level in HCC

Clinicopathological characteristics of all HCC patients from the GSE14520 dataset are fully described in Table 2. This dataset comprises data for 242 HCC patients, with complete survival information. Our study included 193 HCC patients younger than 60 years old. 210 HCC patients were men. With regards to the HBV status, the numbers of HCC patients described as chronic carriers (CCs), active viral replication-chronic carriers (AVR-CCs), and as those with unavailable HBV status were 162, 57, and 6, respectively. The number of HCC patients with an ALT level of more than 50 U/L was 100. The number of HCC patients with an AFP level of more than 300 ng/ml was 97. The number of HCC patients with multinodular tumours was 51. The number of HCC patients with a main tumour size of more than 5 cm was 88. The number of cirrhosis patients was 223. The numbers of patients with TNM stage I, II, and III HCC were 97, 78, and 59, respectively, and with BCLC stage A, B, and C were 153, 24, and 27, respectively. Based on the median HIF1 $\alpha$  mRNA expression levels, the patients were divided into the high expression and low expression groups. HCC patients

with an HIF1 $\alpha$  mRNA expression  $\geq$  the median HIF1 $\alpha$  mRNA expression level were considered as those with high HIF1 $\alpha$  expression, while the others were considered as those with low HIF1 $\alpha$  expression. There was no significant difference between the high HIF1 $\alpha$  mRNA expression group and low HIF1 $\alpha$  mRNA expression group when comparing age ( $\geq 60$  versus  $< 60$ ), gender (male versus female), HBV status (CC and AVR-CC versus NA), ALT ( $> 50$  U/L versus  $\leq 50$  U/L), AFP ( $> 300$  ng/mL versus  $\leq 300$  ng/mL), multinodular tumours (Yes versus No), main tumour size ( $> 5$  cm versus  $\leq 5$  cm), and cirrhosis (Yes versus No). However, HCC patients with high HIF1 $\alpha$  mRNA expression had a higher tendency to be associated with TNM stage III and BCLC stage C of the disease than those with low HIF1 $\alpha$  mRNA expression ( $P = 0.002$  and  $P = 0.038$ , respectively; Table 2). Thus, the upregulation of HIF1 $\alpha$  mRNA expression was found to be correlated with TNM stage III and BCLC stage C of liver cancer.

## Overexpression of HIF1 $\alpha$ mRNA contributes to poor prognosis in HCC patients

Kaplan-Meier analysis of data from the GSE14520 cohort demonstrated that HCC patients with high

**Table 2** Correlations between HIF1 $\alpha$  mRNA expression and the clinic-pathologic variables of HCC patients

Clini-pathologic Features	Case(N)	HIF1 $\alpha$ mRNA Expression		P
		Low	High	
Age				0.150
≥60	49	29	20	
<60	193	92	101	
Gender				0.448
Male	210	107	103	
Female	32	14	18	
HBV viral status				0.193
CC	161	79	82	
AVR-CC	57	32	25	0.446
NA	6	5	1	0.249
ALT(>/≤50 U/L)				1.000
High	100	50	50	
Low	142	71	71	
AFP(>/≤300 ng/ml)				0.179
High	97	43	54	
Low	141	75	66	
Multinodular				0.083
Yes	51	20	31	
No	191	101	90	
Main tumor size (>/≤5 cm)				0.098
Large	88	38	50	
Small	154	83	70	
Cirrhosis				0.811
Yes	223	111	112	
No	19	10	9	
TNM stage				0.282
I	97	54	43	
II	78	46	32	0.105
III	59	16	34	0.002
BCLC stage				0.075
A	153	85	68	
B	24	12	12	0.838
C	27	9	18	0.038

**Abbreviations:** HCC, hepatocellular carcinoma; NA, not available; AVR-CC, active viral replication chronic carrier; CC, chronic carrier; BCLC, Barcelona Clinic Liver Cancer; TNM, tumor node metastasis; HBV, hepatitis B virus; AFP, alpha fetoprotein; ALT, alanine aminotransferase.

HIF1 $\alpha$  mRNA expression showed poor OS, compared to those with low HIF-1 $\alpha$  mRNA expression (HR=1.503, 95% CI=1.001–2.245,  $P=0.048$ , Figure 2A). There was no significant difference between the RFS of patients

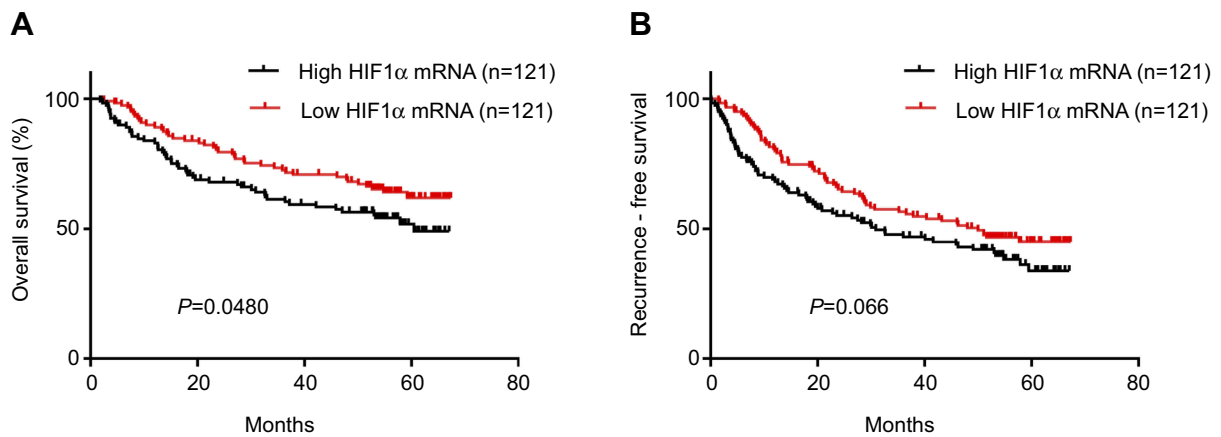
showing high and low HIF1 $\alpha$  mRNA expression; however, with regards to the RFS, there was an obvious survival advantage for HCC patients with low HIF1 $\alpha$  mRNA expression (HR=1.374, 95% CI=0.976–1.922,  $P=0.066$ , Figure 2B).

### Establishment of the SK-Hep1 and Hep-3B cell lines stably transfected with the HIF1 $\alpha$ gene (HIF1 $\alpha$ overexpression)

In order to further explore the function of HIF1 $\alpha$  in HCC, we constructed the stably transformed SK-Hep1 and Hep-3B cell lines with HIF1 $\alpha$  overexpression using lentiviral vectors. Our results showed that HIF1 $\alpha$  was significantly upregulated at both the mRNA and protein levels in SK-Hep1 and Hep-3B cells from the LV-HIF1 $\alpha$  group, compared to the case for those from the LV-Con group in (Figure 3A–C). Additionally, we found that VEGF, a protein present downstream of HIF1 $\alpha$ , was also upregulated in cells from the LV-HIF1 $\alpha$  group (Figure 3B and D). Thus, we successfully constructed the stably transformed SK-Hep1 and Hep-3B cell lines with HIF1 $\alpha$  overexpression.

### Overexpression of HIF-1 $\alpha$ promotes the proliferation, migration, invasion, and angiogenic ability of SK-Hep1 and Hep-3B cells

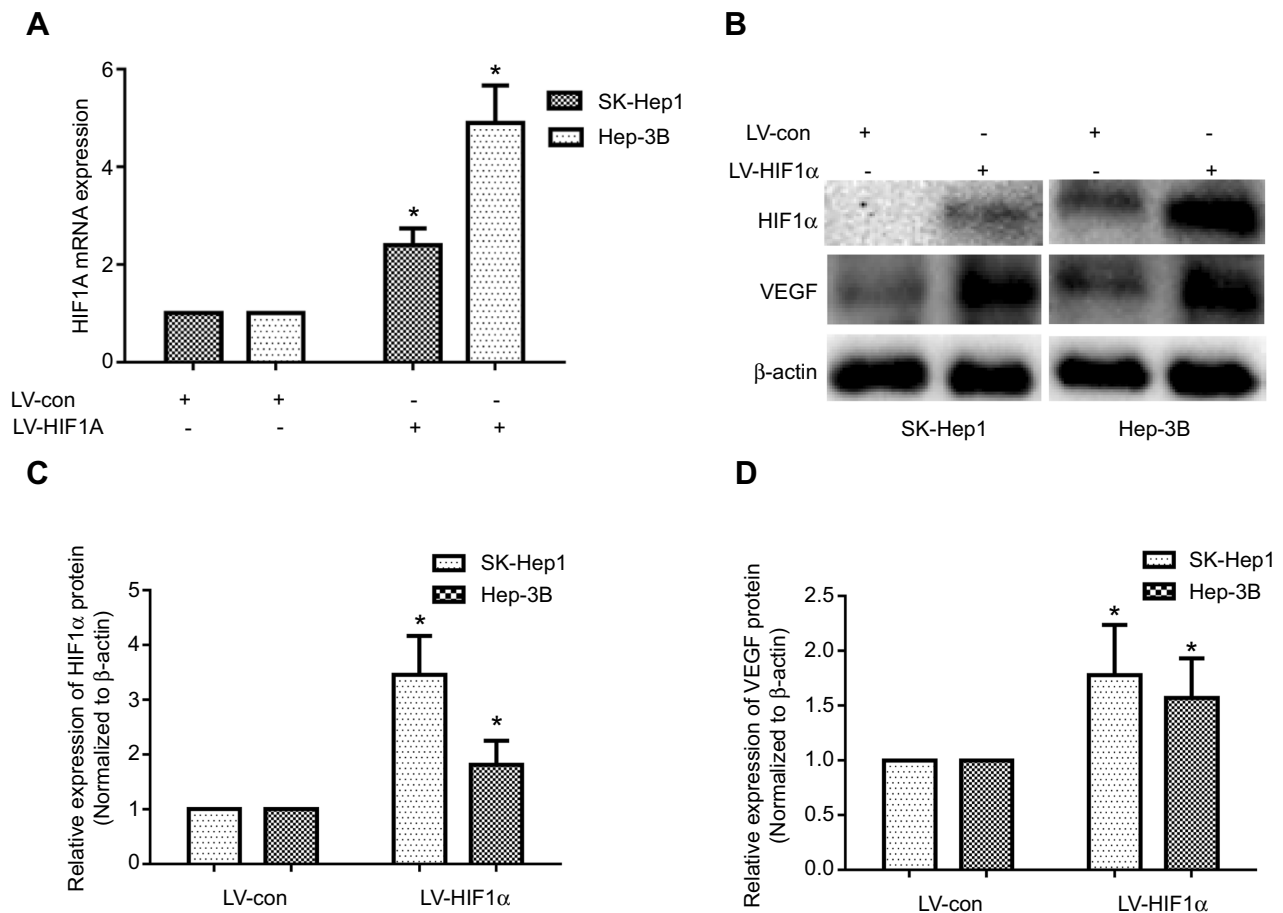
We performed the CCK-8 assay to assess the effect of HIF1 $\alpha$  overexpression on the proliferation of SK-Hep1 and Hep-3B cells. Our results showed that the viability of SK-Hep1 and Hep-3B cells from the LV-HIF1 $\alpha$  group increased significantly, compared to the case for those from the LV-Con group (Figure 4A). To identify the effects of HIF1 $\alpha$  overexpression on the migration and invasion abilities of SK-Hep1 and Hep-3B cells, we performed the wound healing assay and transwell invasion assay. The results indicated that HIF1 $\alpha$  overexpression significantly promoted the migration (Figure 4B and C) and invasion (Figure 4D and E) of SK-Hep1 and Hep-3B cells. Finally, our results also demonstrated that the SK-Hep1 and Hep-3B cell culture supernatants from the LV-HIF1 $\alpha$  group significantly promoted the angiogenesis of HUVECs, compared to those from the LV-Con group (Figure 4F and G).



**Figure 2** Kaplan-Meier survival curves of HCC patients based on different HIF1 $\alpha$  mRNA expression levels.

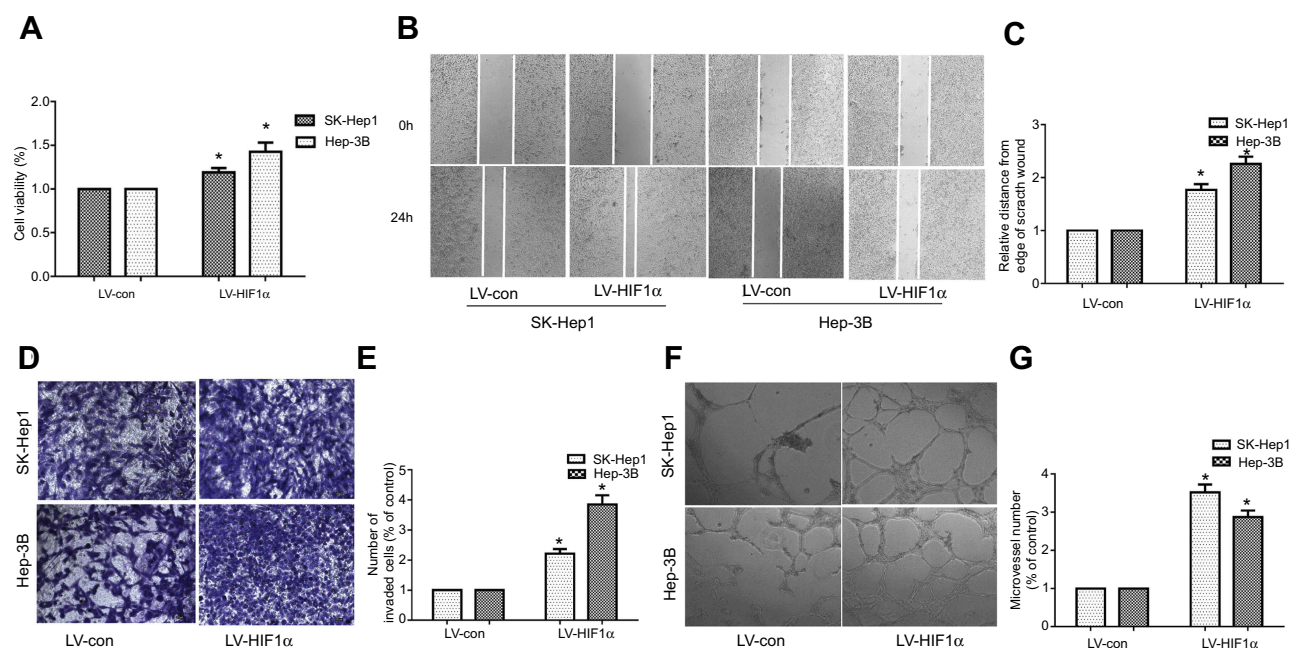
**Notes:** (A) The survival curves showed that HCC patients with high HIF1 $\alpha$  mRNA expression had a poor OS, compared to those with low HIF1 $\alpha$  mRNA expression ( $P=0.048$ ). (B) The survival curves showed that there was no significant difference between the RFS of HCC patients with high HIF-1 $\alpha$  mRNA expression and those with low HIF1 $\alpha$  mRNA expression ( $P=0.066$ ).

**Abbreviations:** HCC, hepatocellular carcinoma; OS, overall survival; RFS, recurrence free survival.



**Figure 3** Establishment of the stably transformed SK-Hep1 and Hep-3B cell lines with the HIF1 $\alpha$  gene (HIF1 $\alpha$  overexpression).

**Notes:** (A) HIF1 $\alpha$  mRNA expression levels in SK-Hep1 and Hep-3B cell lines stably transfected with HIF1 $\alpha$  gene. (B) Expression levels of the HIF1 $\alpha$  and VEGF proteins in SK-Hep1 and Hep-3B cell lines stably transfected with the HIF1 $\alpha$  gene. (C, D) Relative expression levels of the HIF1 $\alpha$  and VEGF proteins in SK-Hep1 and Hep-3B cell lines stably transfected with the HIF1 $\alpha$  gene were quantified using the ImageJ software. Data were represented using the GraphPad Prism 5.02 software. \* $P<0.05$ , compared to the control group.



**Figure 4** HIF1 $\alpha$  overexpression promotes the proliferation, migration, invasion, and angiogenic ability of SK-Hep1 and Hep-3B cells.

**Notes:** (A) Cell proliferation was assessed by the CCK-8 assay. (B) Cell migration was assessed by the wound healing test. (C) The histogram represents the migration distance relative to the control 24 h after scratching the cell layer. (D) Cell invasion was examined by the transwell invasion test. (E) The histogram represents the average number of invasive cells relative to the control. (F) HCC cell-induced angiogenesis of HUVECs was studied by the tube formation test. (G) The representative histogram of the area covered by the tube network was quantified using the Image-Pro Plus software. Data were represented with the GraphPad Prism 5.02 software. \* $P < 0.05$ , compared to the control group.

**Abbreviations:** HCC, hepatocellular carcinoma; HUVECs, Human umbilical vein endothelial cells.

## Establishment of the stably transformed SK-Hep1 and Hep-3B cell lines with HIF1 $\alpha$ silencing using RNA interference, and the inhibitory effects of HIF1 $\alpha$ silencing on HCC cell proliferation

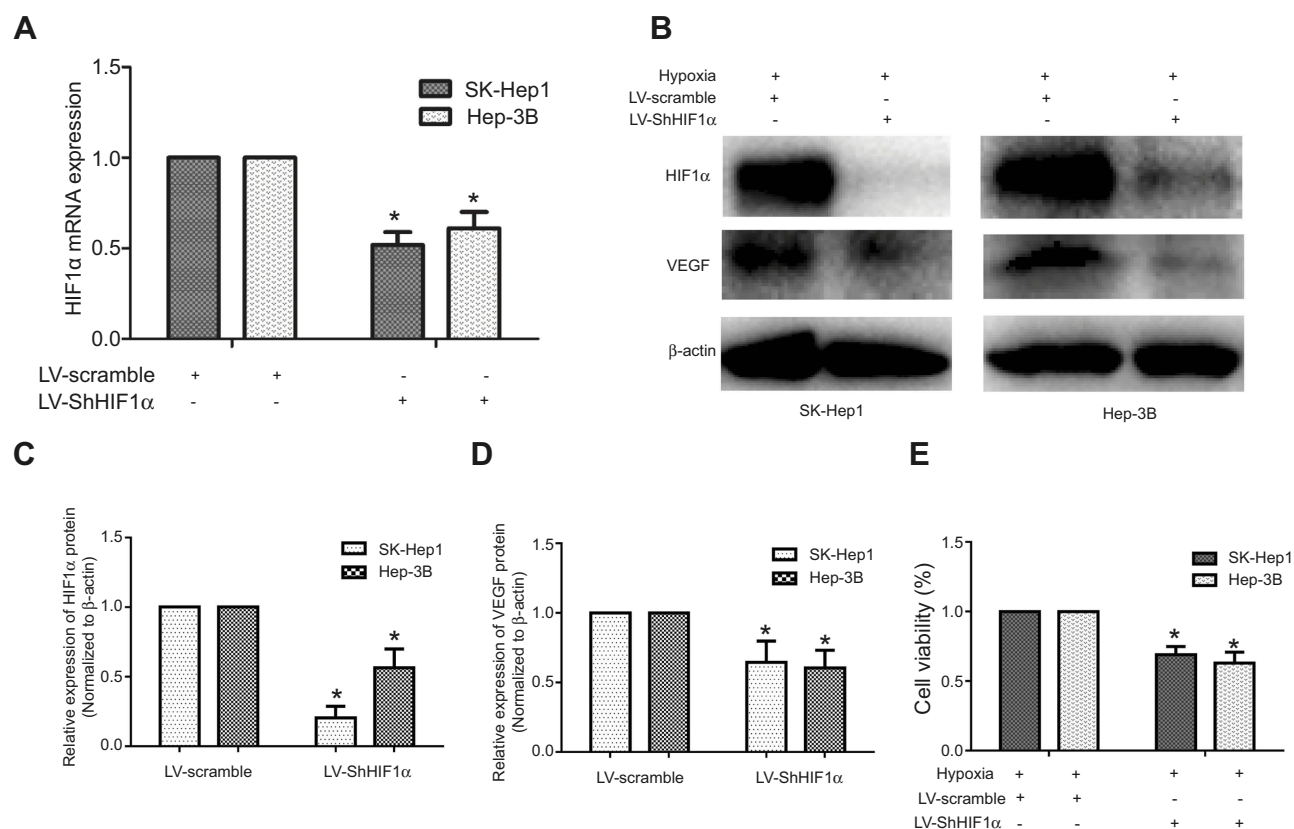
We constructed the stably transformed SK-Hep1 and Hep-3B cell lines by means of RNA interference-mediated HIF1 $\alpha$  gene silencing to verify the effects of HIF1 $\alpha$  on HCC cell proliferation, given the low expression of HIF1 $\alpha$  in Hep-3B cells under conditions of normoxia. Our results showed that HIF1 $\alpha$  was significantly downregulated at both the mRNA and protein levels in SK-Hep1 and Hep-3B cells from the LV-ShHIF1 $\alpha$  group, compared to the case for those from the LV-Scramble group (Figure 5A–C). Additionally, we also found that the expression of VEGF, a protein present downstream of HIF1 $\alpha$ , was downregulated in cells from the LV-ShHIF1 $\alpha$  group (Figure 5B and D). Thus, we successfully constructed the stably transformed SK-Hep1 and Hep-3B cell lines showing HIF1 $\alpha$  silencing (by RNA interference). We performed the CCK-8 assay to examine the effects of HIF1 $\alpha$  silencing on the proliferation of SK-Hep1 and Hep-3B cells. Our results indicated that the

viability of SK-Hep1 and Hep-3B cells from the LV-ShHIF1 $\alpha$  group decreased significantly, compared to the case for those from the LV-Scramble group (Figure 5E).

## RNA interference-mediated HIF1 $\alpha$ silencing suppressed the growth of subcutaneous xenograft tumours in nude mice

In order to further illustrate the function of HIF1 $\alpha$ , we constructed xenograft models using BALB/c nude mice. Stably transformed Sk-Hep1 cells (LV-Scramble-Sk-Hep1 or LV-ShHIF1 $\alpha$ -Sk-Hep1) were injected subcutaneously into the right flank of the nude mice. The size of the xenograft tumours was measured every seven days for five weeks. The results indicated that the xenograft tumours from mice injected with the LV-Scramble-transfected cells were significantly larger than those from mice injected with the LV-ShHIF1 $\alpha$ -transfected cells (Figure 6A and B). The IHC results demonstrated that staining of the HIF1 $\alpha$  and VEGF proteins in xenograft tumours from mice injected with the LV-Scramble-transfected cells was significantly stronger than in those from mice injected with the LV-ShHIF1 $\alpha$ -transfected cells (Figure 6C).





**Figure 5** Establishment of the stably transformed SK-Hep1 and Hep-3B cell lines with HIF1α silencing (using RNA interference), and the inhibitory effects of HIF1α silencing on HCC cell proliferation.

**Notes:** (A) HIF1α mRNA expression levels in SK-Hep1 and Hep-3B cell lines stably transfected with the HIF1α-specific interfering RNA. (B) Expression levels of the HIF1α and VEGF proteins in the SK-Hep1 and Hep-3B cell lines stably transfected with the HIF1α-specific interfering RNA. (C, D) Relative expression levels of the HIF1α and VEGF proteins in the SK-Hep1 and Hep-3B cell lines stably transfected with the HIF1α-specific interfering RNA were quantified using the ImageJ software. (E) The proliferation of the SK-Hep1 and Hep-3B cells stably transfected with the HIF1α-specific interfering RNA was assessed by the CCK-8 assay. Data were represented using the GraphPad Prism 5.02 software. \* $P < 0.05$ , compared to the control group.

**Abbreviation:** HCC, hepatocellular carcinoma.

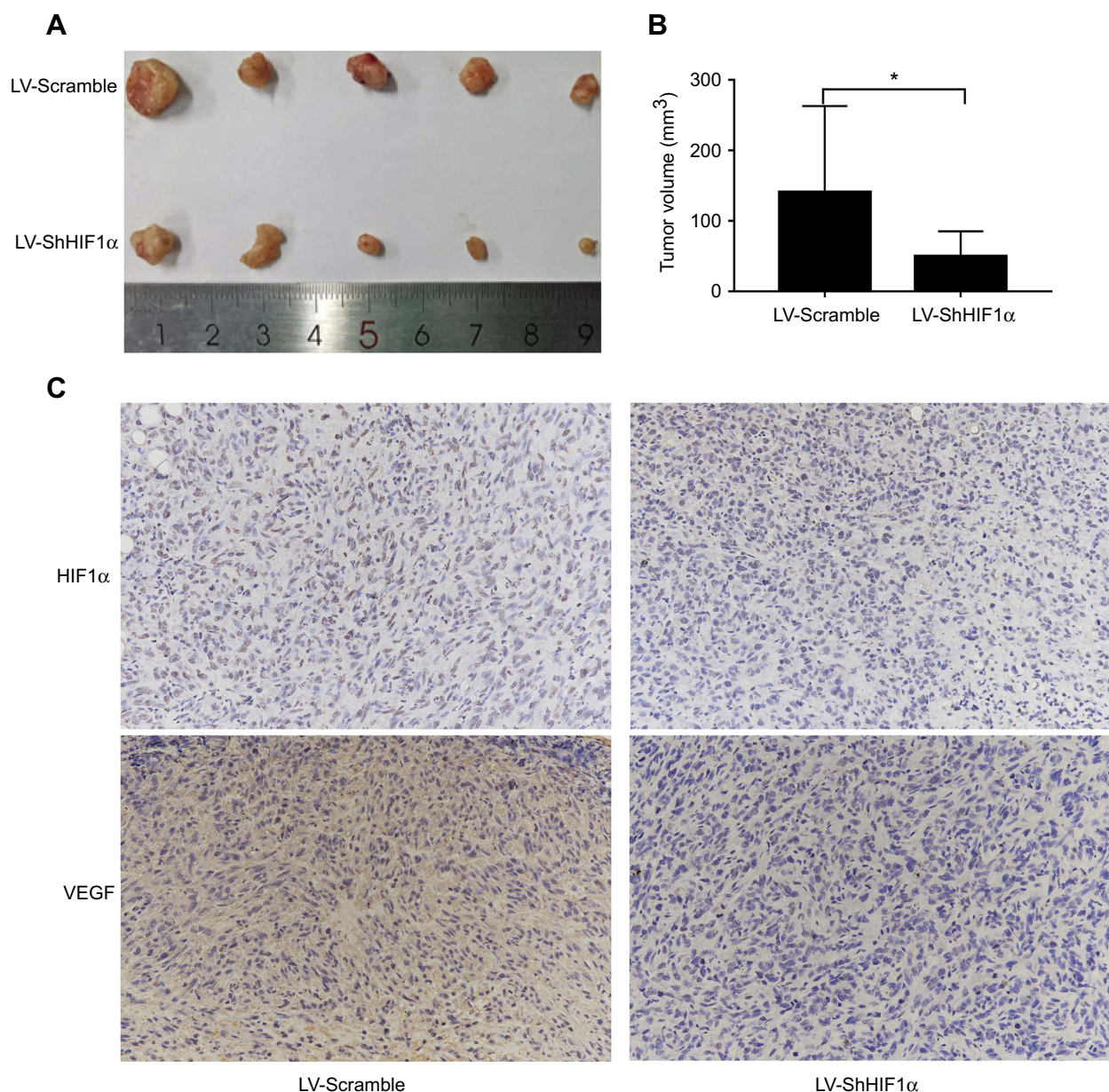
## Discussion

HCC is a malignant cancer with high morbidity and mortality. Although there are a large number of treatment strategies for HCC, the therapeutic effects are still not satisfactory. Thus, there is an urgent need to explore the new mechanisms underlying the invasion and metastasis of HCC and look for new predictive markers and targets for therapeutic interventions to improve the outcome of HCC treatment strategies.

HIF1α is a heterodimeric transcription factor containing the oxygen-sensitive α subunit and the constitutively expressed β subunit. Recent studies have demonstrated that the overexpression of HIF1α at the protein level is always linked with poor prognosis. Nevertheless, there are very few studies examining the correlation between the overexpression of HIF1α mRNA and its prognostic value in HCC patients. Hence, it is essential to clarify the effects of the upregulation of HIF1α mRNA expression on the

pathological features and prognostic value in HCC patients, and the specific function of HIF1α in human HCC cell lines.

Our study outcomes showed that HIF1α mRNA expression was significantly upregulated in HCC tissues, compared to the case in normal liver tissues or corresponding peritumour tissues; these results were further supported by those of the Western blotting and IHC analyses. Results of the survival analysis demonstrated that HCC patients with high HIF1α mRNA expression showed a shorter OS, but not RFS, than HCC patients with low HIF1α mRNA expression. Additionally, we found that in HCC patients, high HIF1α mRNA expression was associated with TNM stage III and BCLC stage C of the disease, compared to the case for low HIF1α mRNA expression. In order to explore the function of HIF1α in detail, we successfully constructed the stably transformed SK-Hep1 and Hep-3B cell lines with HIF1α overexpression and HIF1α inhibition (by means of RNA



**Figure 6** HIF1 $\alpha$  silencing using RNA interference suppressed the growth of xenograft tumours in nude mice.

**Notes:** (A) Images of subcutaneous xenograft tumours harvested from the sacrificed nude mice. (B) Comparison of the size of xenograft tumours from mice five weeks after they were subcutaneously injected with SK-HepI cells from the LV-Scramble and LV-ShHIF1 $\alpha$  groups. Values were expressed as the means  $\pm$  SDs of the tumour volumes of five mice. (C) IHC staining for the HIF1 $\alpha$  and VEGF proteins in xenograft tumours from mice injected with cells from the LV-Scramble and LV-ShHIF1 $\alpha$  groups ( $\times 200$ ). Data were represented using the GraphPad Prism 5.02. \* $P < 0.05$ , compared to the control group.

**Abbreviation:** IHC, immunohistochemistry.

interference to silence HIF1 $\alpha$  expression) using lentiviral vectors. Results indicated that HIF1 $\alpha$  overexpression significantly promoted the proliferation, migration, invasion, and angiogenic ability of HCC cells, and HIF1 $\alpha$  inhibition significantly suppressed the proliferation of HCC cells. Finally, the results of the xenograft tumour assay using nude mice showed that HIF1 $\alpha$  silencing suppressed the growth of xenograft tumours and the expression of the VEGF protein.

Considering the poor prognostic value of HIF1 $\alpha$  in HCC patients, and that it promotes the proliferation, migration, invasion, and angiogenic ability of HCC cells, many researchers have been encouraged to deplete HIF1 $\alpha$  mRNA from HCC patients to decrease the activity of tumour cells, with the aim of controlling the growth of HCC tumours. In addition, we may need to consider both the HCC patients' pathological conditions and their HIF1 $\alpha$  mRNA levels when

formulating treatment strategies. HCC patients with high HIF1 $\alpha$  mRNA levels may need more aggressive treatments after surgery. However, our study showed that there was no significant difference in the RFS of HCC patients showing high and low HIF1 $\alpha$  mRNA expression. The reason for this may be the lack of a sufficient sample size. Hence, additional prospective investigations with larger sample sizes are still needed to confirm the exact prognostic value of HIF1 $\alpha$  mRNA expression in HCC patients.

Our study has a few limitations. Firstly, in our research, the analysis of HIF1 $\alpha$  overexpression and its prognostic value is restricted to the mRNA level, which may not necessarily reflect the function of HIF1 $\alpha$  when it is activated. Secondly, our survival analysis mainly was only based on the GEO database; no other related survival data were considered. Thirdly, our results need to be verified in other HCC cell lines, such as the Huh7, bel7404, and SMMC-7721 cell lines.

## Conclusion

Our study demonstrated that the upregulation of HIF1 $\alpha$  mRNA expression was found in HCC tissues; it was associated with unfavourable OS and linked with TNM stage III and BCLC stage C in HCC patients. We also showed that HIF1 $\alpha$  promoted the proliferation, migration, invasion, and angiogenic ability of HCC cells.

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## Disclosure

The authors report no conflicts of interest in this work.

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