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Reduced expression of CENP-E contributes to the development of hepatocellular carcinoma and is associated with adverse clinical features

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ABSTRACT

Human kinesin centromere-associated protein E (CENP-E), one of spindle checkpoint proteins, has been identified as a tumor suppressor in several types of cancer, however, its role in hepatocarcinogenesis remains unknown. Here we investigated the role of CENP-E in human hepatocellular carcinoma (HCC) employing HCC cell lines (Hep3B, SMMC7721, and QGY7701), animal models, and patient's clinical samples and data. We demonstrated that down-regulation of CENP-E by CENP-E-silencing shRNAs significantly promoted HCC proliferation/growth both *in vitro* and *in vivo*. Further studies found that CENP-E suppressed the proliferation of HCC cells by halting cell cycle progression at the G1-S phase and accelerating cell apoptosis. Analyses of HCC patient samples and clinical data revealed that CENP-E was significantly down-regulated in HCC tissues and low CENP-E expression was significantly associated with patient's adverse clinicopathological features: poor prognosis, advanced TNM stage, metastasis, and larger tumor size. Multivariate analysis indicated that CENP-E was an independent prognostic factor predicting outcomes of advanced HCC patients. Our data suggest that loss of CENP-E contributes to HCC development and is strongly associated with adverse HCC clinical pathology. Thus, CENP-E could be a novel target for new treatments and a useful prognostic biomarker for HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of malignancy in humans and ranked second worldwide in cancer mortality; the highest incidence and mortality rates being in Eastern and South-Eastern Asia [1]. Approximately 782,500 new cases of liver cancer are estimated each year [2]. The incidence of HCC in China is the fourth most common cancer with over 492,000 new cases every year, counting for approximately 55 % of total HCC new cases worldwide [3]. While the prevalence of many cancer types such as lung cancer is declining, the incidence of liver cancer is increasing in the United States, from 2.6–8.6 per 100,000 between 1975 and 2011 [2]. Adding to the burden, HCC is an aggressive cancer with a poor prognosis with an overall ratio of mortality to incidence of 0.95 [1,4]. The majority of HCC patients are diagnosed at an advanced stage when treatment options are limited and most available treatments are ineffective. Therefore there is an urgent need to improve treatment outcomes by identifying novel therapeutic targets and actionable prognosis predictors for advanced stage hepatocellular carcinoma [5].

The mitotic kinesin centromere-associated protein E (CENP-E) is a large (312 kDa) kinesin motor protein localized on the kinetochore; it accumulates in the late G2 phase of the cell cycle and plays an essential role during metaphase chromosome alignment and the mitotic checkpoint [6–10]. During mitosis, CENP-E serves to link chromosomes and the microtubules of the mitotic spindle [11–13]. CENP-E also activates the major cell cycle control mechanism to prevent chromosomal mis-segregation that can result in aneuploidy [8,9]. Reduction of CENP-E produces aneuploid progeny leading to chromosomal instability (CIN) [9,11–14]. CIN is a hallmark in most solid cancers such as hepatocellular carcinoma [15]. As a type of genomic instability, CIN, occurs frequently in many cancer cells [16,17]. Previous studies demonstrated that increased aneuploidy by inhibiting CENP-E in aged CENP-E$^{+/-}$

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mice led to the development of spontaneous spleen and lung tumors more frequently than the wild-type counterparts [14]. However, inhibition of CENP-E has also been reported to initiate tumor cell apoptosis or regression [18]. Thus, CENP-E can both suppress and promote tumors [14,17,19].

As one of spindle checkpoint proteins (SCP), CENP-E has a crucial role to play in the process of cell division, ensuring faithful chromosome segregation. Interruption of the spindle checkpoints has long been known to be involved in aneuploidy and tumorigenesis [20]. Links between centromere proteins and solid tumors such as breast cancer, ovarian cancer and HCC have also been demonstrated [19,21,22]. It is believed that studies investigating centromere proteins, such as CENP-E, in cancer tissues could uncover molecular mechanism of carcinogenesis and lead to novel cancer biomarker discoveries and enhance prognosis estimation of cancer patients [23].

The importance of CENP-E functioning as a tumor suppressor in liver cancer is supported by a few studies. It has been shown that there is a reduction in CENP-E expression in HCC tissue [24]. The authors further confirmed that CENP-E reduction in a normal liver cell line, LO2, using shRNA expressing vectors to knock down CENP-E, resulted in chromosome abnormality, aneuploidy [24]. Liu et al. recently reported that significant lower expression of CENP-E was found in human hepatoma cells compared to LO2 normal hepatic cells [25]. Despite these findings, to-date, there is no evidence linking reduced expression of CENP-E to the initiation and development of HCC. Furthermore, no study to-date has been conducted to investigate the clinical relevance of reduced CENP-E in HCC patients.

In this study, we investigated the contribution of reduced expression of CENP-E protein in promoting HCC and the clinical relevance of reduced CENP-E in HCC patients utilizing human liver cancer cell lines (Hep3B, SMMC7721, and QGY7701), animal models, and human HCC specimens with clinical data.

2. Materials and methods

2.1. Cell culture

Human liver cancer cell lines Hep3B, SMMC7721 and QGY7701 were purchased from the Typical Culture Preservation Commission cell bank (Chinese Academy of Sciences, Shanghai, China). The other HCC cell lines PCL5, Hep G2, and Huh7 were preserved in our laboratory. All these cells were cultured in DMEM (Hyclone, Logan, Utah, USA) supplemented with 10 % fetal bovine serum (FBS) (Gibco, Invitrogen, Paisley, UK), and maintained in a 37 ˚C humidified incubator in the presence of 5 % CO2. The identity of all cell lines used was confirmed by relevant authorized STR reports.

2.2. Patients and clinical tissue specimens

A total of 90 pairs of clinical liver cancer samples and matched adjacent non-tumor tissue samples, presented as tissue microarrays (TMAs) chips, containing pathological and clinical information were purchased from Shanghai Outdo Biotech Co. Ltd (Shanghai, China). The use of these materials for research purpose was approved by the Taizhou Hospital Human Ethics Committee with relevant patients' consents. Clinical data were available for all clinical samples, including age, gender, tumor size, tumor location, liver cirrhosis, metastasis, TNM stage, differentiation status, and patient survival time.

To validate the results from our clinical samples, a small set of data on CENP-E expression levels in HCC samples from a published study [24] was extracted (sTable 1). Independent datasets from Oncomine Cancer Microarray Database (https://www.concomine.org) containing data on the expression of CENP-E in HCC samples were used to analyze CENP-E expression in human HCC samples and its associations with overall survival. Extracted data were included in Supplementary Tables: sTable 2 and sTable 3.

2.3. RNA interference and transfection

For down-regulation of human CENP-E (NM_001813), CENP-E-silencing shRNAs were designed, synthesized, and cloned into the pGPU6/GFP/Neo plasmid vector. The sh-CENP-E gene-targeted sequence used was (5′ CACCGCCACTAGAGTTGAAAGATAATCAAGAG TTATCTTTCAACTCTAGTGGCTTTTTTG), and a scrambled sequence was used as the sequence control (Shanghai GenePharma Co. Ltd, Shanghai, China). Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA) was used to transfect the recombinant plasmids into Hep3B, SMMC7721, and QGY7701 cells. After G418 selection (1 mg/ml) for 14 days, pooled G418-resistant stable cells, transfected successfully, were established. The protein level of CENP-E was determined by Western blot.

2.4. Immunohistochemistry (IHC) and scoring

IHC and scoring were conducted according to the methods published previously [26] with modifications [27]. Briefly, the TMAs were heated for 3 h at 65 ˚C, then deparaffinized and rehydrated through dimethylbenzene and graded alcohols, then briefly rinsed in tap water (several seconds). Endogenous peroxidase was blocked with 3 % hydrogen peroxide for 15 min at room temperature. The slides were rinsed in 0.01 mol/L phosphate-buffered saline solution (PBS) for 3 min with three repeat washes. Antigen retrieval was performed in 10 mM sodium citrate, pH 6.0, and placed in a microwave oven for 20 min. After 30 min of pre-incubation in 10 % normal goat serum, to prevent non-specific staining, the TMAs were incubated (2 h at room temperature) with primary antibody; anti-CENP-E in a 1:200 dilution in PBS (Santa Cruz Biotechnology, Dallas, USA). After washing the slides with PBS, 3 × 3 min, the slides were incubated with horseradish peroxidase (HRP)-labeled anti-rabbit/mouse secondary antibodies (Gene Tech, Shanghai, China) for 30 min at room temperature. The tissues were stained with DAB working solution (1:50; Gene Tech, Shanghai, China). Hematoxylin staining was performed on the TMAs for 5 min, then dehydrated through a series of graded alcohols. The degree of immunostaining of the sections was viewed and scored separately by two independent pathologists blinded to the histopathological features and patient data. The intensity of CENP-E staining was scored as negative (0), weak (1), moderate (2) and strong (3). The extent of CENP-E staining was defined as the percentage of positive stained cells: 1 ($<$ 10 %), 2 (10–50 %), 3 (51–80 %), and 4 ($>$ 80 %). The level of CENP-E expression in each sample was expressed as an overall score, ranging from 0 to 12, which was calculated by multiple the score of intensity and that of extent of staining [27]. CENP-E expression level was considered high when the final scores were $>$ 8 and low or none when the final scores were $\leq$8.

Xenograft tumors were sectioned (3 − 4 μm per section) for IHC analysis. Primary antibodies used for xenograft tumor staining were against CENP-E, ARG-1 (Arginase-1) and GPC-3(Glypican-3) at dilutions of 1:100 in PBS (Affinity Biosciences, OH, USA).

2.5. Western blotting

Protein extractions and Western blotting were performed as described previously [28]. Primary antibodies include anti-CENP-E (1:200). β-tubulin was used as a loading control to normalize the protein signal. All western blot experiments were repeated in biological triplicates. Protein bands were quantitated using an Odyssey bands scanner (S/N ODY-2792 model: 9120). The intensities of the bands were analyzed using Bandscan Software.

2.6. Cell proliferation assay

Cell proliferation assays were performed using the Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) as described previously

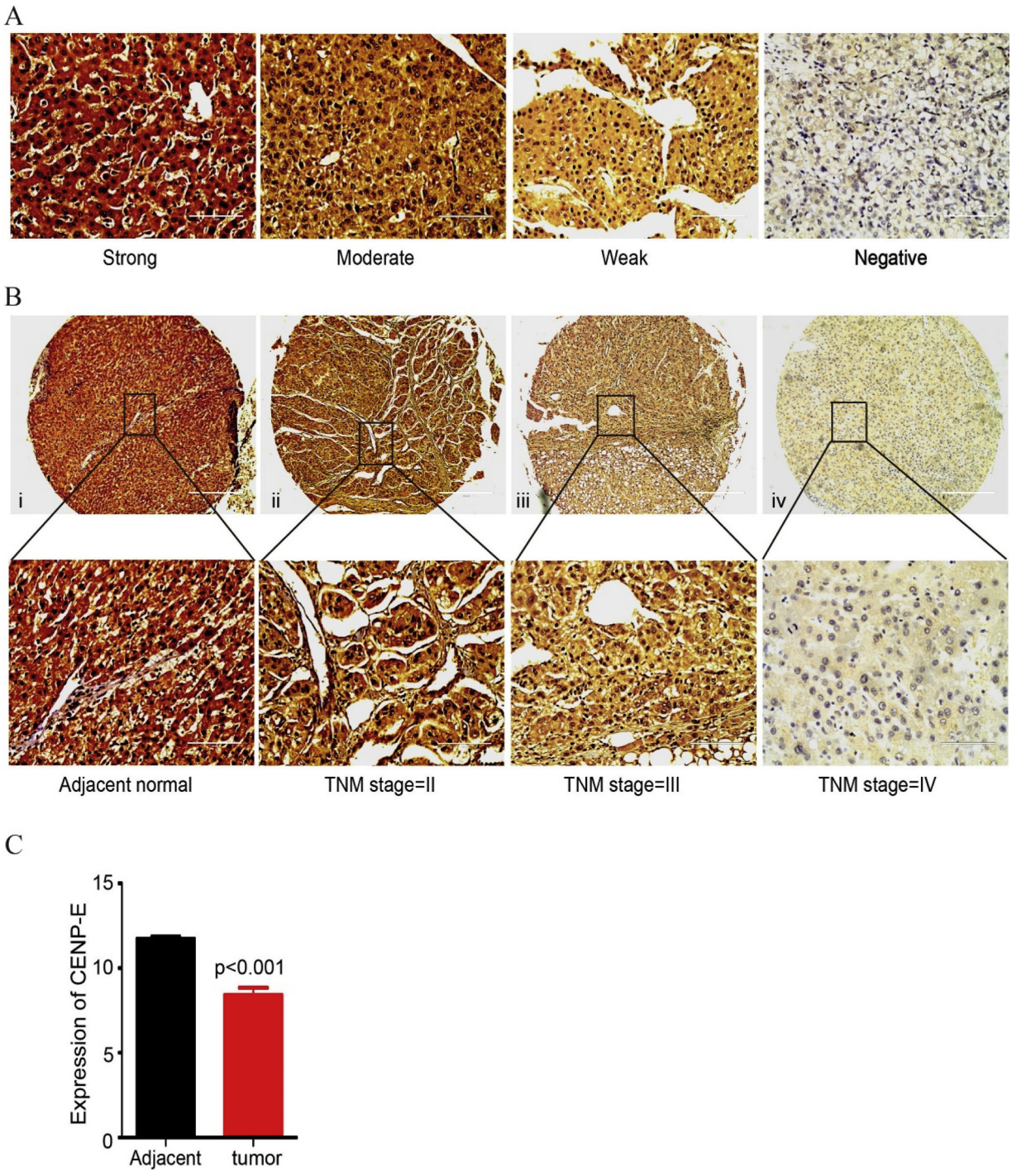

Fig. 1. CENP-E was down-regulated in HCC and correlated with clinicopathological features.
(A) Immunohistochemical analysis of CENP-E expression levels in 90 pairs of liver cancer samples and adjacent tissues. Representative images of CENP-E staining intensity: (i) strong staining in adjacent tissue; (ii)-(iv) moderate, weak, and negative staining of CENP-E in cancer tissues. 40× magnifications, scale bar 100 μm. (B) CENP-E expression level associates with liver cancer staging. (i) Strong in adjacent normal tissue; (ii) moderate in TNM stage II; (iii) weak in TNM stage III; (iv) negative in TNM stage IV. Upper graphs: 10× magnification, scale bar 400 μm; lower graphs: 40× magnification, scale bar 100 μm. (C) IHC scores of CENP-E protein expression level in paired liver cancer tissue and adjacent tissue ($P < 0.001$, Student's *t*-test).

[27]. A total of 5×10^3 cells (Hep3B, SMMC7721, and QGY7701) were seeded into 24-well plates and cultured for 3 days. Then 20 μl CCK-8 solution was added. After incubated at 37 °C for 4 h, absorbance at 450 nm was measured using a microplate reader (Bio-Rad). Each group was plated in three duplicate wells. Each experiment was repeated in biological triplicates.

2.7. Cell cycle analysis

Approximately 1×10^6 cells were trypsinized, washed twice with PBS, and fixed in ice-cold 70 % ethanol for 1 h. The samples were then centrifuged to remove the ethanol and incubated with 100 μl RNaseA (keyGEN BioTECH, Nanjing, China) for 30 min at 37 °C. Cellular DNA was stained with propidium iodide (PI). Cell cycle distributions were determined using a flow cytometer, BD FACSCalibur system, and data

were analyzed using CellQuest software, as descripted previously [27].

2.8. Cell apoptosis assay

Approximately 1×10^6 cells were harvested and stained with AnnexinV-APC and PI according to the manufacturer's protocol (keyGEN BioTECH). Annexin V-APC/PI binding was analyzed by flow cytometry using a BD FACSCalibur system, and data were analyzed using CellQuest software.

2.9. Cell migration assay

Cell migration activity was measured using Boyden transwell chambers (8-μm pore, Corning star, Cambridge, USA). Cells in serum-free medium (30×10^4 cells/200 μl) were added to the upper chambers of transwell plates. Then 10 % FBS-containing medium was added to the lower chambers as a chemoattractant. After incubation for 4.5 h at 37 °C, cells that had migrated and attached to the lower surface of the membrane were fixed with methanol and stained with 0.1 % crystal violet. For quantification, cells were counted under a microscope in five randomly selected fields (original magnification, 200×).

2.10. Wound closure assays

Wound closure assays were performed in 6-well plates (3×10^5 cells/well). A scratch was made down the center of each well using a plastic tip (100 μl size). Along the scratch line, the cells were washed away and replaced with serum-free medium. Three randomly chosen identical locations were imaged at 0 and 24 h under light microscopy (200×) for each replicate. Results are expressed as the distance between the edges of individual wounds at 24 h and compared with the t = 0 time point.

2.11. Colony forming assays

Cells in logarithmic growth phase were digested with 0.25 % trypsin and cells were suspended in DMEM containing 10 % FBS. Cell suspensions from each group were diluted to 1000 cells per well (6-well plates) and cultured at 37 °C in a humidified incubator in the presence of 5 % CO2 for 2 weeks. When clones became visible to the naked eye, Then the medium was removed, and 4 % paraformaldehyde was added to the 6-well plates for 5 min to fix the cell clones. The 4 % paraformaldehyde was washed off then 0.1 % crystal violet staining was overlayed on the cells for 15 min. The plate was rinsed with water to remove the excess dye and plates dried at room temperature. Numbers of cell colony were counted for statistical analysis.

2.12. In vivo Xenograft experiments

BALB/c nude mice aged 5–6 weeks old were purchased from the Experimental Animal Centre of Southern Medical University (Guangzhou, China) and maintained under standard pathogen-free conditions. 200×10^4 HCC cells CENP-E down-regulating or scramble controls were injected subcutaneously into the left or right flanks of nude mice (n = 3 per group). Tumors were measured with calipers to estimate volume from day 7 to day 20 after injection. The mice were sacrificed 20 days later and xenograft tumors were photographed and collected for western blotting and IHC analyses. All experimental procedures were performed according to the regulation of animal usage for scientific research of Southern Medical University.

2.13. Statistical analysis

All statistical analyses were carried out with SPSS 23.0 software (IBM Corp., Armonk, NY). Statistical significance was tested by Student's *t*-test or chi-square test as appropriate. All tests were two-sided. $P < 0.05$ was considered statistically significant. Analysis of patient survival was performed using Kaplan–Meier analysis and Cox regression analysis (SPSS).

3. Results

3.1. Reduced expression of CENP-E in human HCC tissues

Clinical samples from the cohort of 90 HCC patients, 74 males and 16 females with a mean age of 43 years ranging from 28 to 76 years, were used for this study. CENP-E expression in the tissue samples was analyzed in 90 pairs of HCC and adjacent non-cancer tissues by immunohistochemistry (IHC). As shown in Fig. 1A and B, positive immunostaining were mainly located in both nucleus and cytoplasm of the para-neoplastic cells. We found that CENP-E protein was detected abundantly in non-malignant tissues adjacent to HCC tissues. Our quantification results demonstrated that CENP-E was strongly expressed in adjacent non-malignant tissues, 86/90 (95.6 %) samples of adjacent tissues had a score of 12 (maximum score). In contrast, the expression of CENP-E in human HCC tissues was relatively weak, with 47/90 (52.2 %) of HCC samples having a score of ≤8 representing low CENP-E expression. In 12/90 (13.3 %) HCC samples, CENP-E was not detectable with a score of 0. Overall, the IHC results clearly showed that the expression level of CENP-E in human HCC tissues was significantly lower than in adjacent non-malignant tissues (8.43 ± 0.41 *vs* 11.76 ± 0.10, $P < 0.0001$; Fig. 1C).

3.2. Association between CENP-E expression and clinicopathological features of HCC

The patient cohort was divided into a low CENP-E expression group (n = 47) and a high CENP-E expression group (n = 43) based on the results of IHC staining. Correlations between the HCC clinicopathological features and CENP-E expression are summarized in Table 1. Low expression of CENP-E was positively associated with tumor size ($P < 0.05$), metastasis ($P < 0.05$), and TNM stage ($P < 0.05$). Lower CENP-E intensity was associated with higher tumor stage (Fig. 1B). However, CENP-E expression did not appear to be

Table 1
Association of CENP-E expression with clinicopathological characteristics of HCC patients.

Characteristics	No of cases	Expression of CENP-E (case, %)		*P* Value
		Low	High	
Age				
≥61	25	13 (52.0 %)	12 (48.0 %)	0.979
< 61	65	34 (52.3 %)	31 (47.7 %)	
Gender				
Male	74	41 (55.4 %)	33 (44.6 %)	0.194
Female	16	6 (37.5 %)	10 (62.5 %)	
Tumor Size (cm)				
> 5	50	31 (62.0 %)	19 (38.0 %)	0.038*
≤5	40	16 (40.0 %)	24 (60.0 %)	
Liver cirrhosis				
Yes	17	7 (41.2 %)	10 (58.8 %)	0.311
No	73	40 (54.8 %)	33 (45.2 %)	
Metastasis				
Negative	83	40 (48.2 %)	43 (51.8 %)	0.013*
Positive	7	7 (100 %)	0 (0 %)	
TNM stage				
I + II	52	21 (40.4 %)	31 (59.6 %)	0.009**
III + IV	38	26 (68.4 %)	12 (31.6 %)	
Differentiation				
Well/moderate	65	32 (49.2 %)	33 (50.8 %)	0.54
Poor	25	15 (60.0 %)	10 (40.0 %)	

* $p < 0.05$.
** $p < 0.01$.

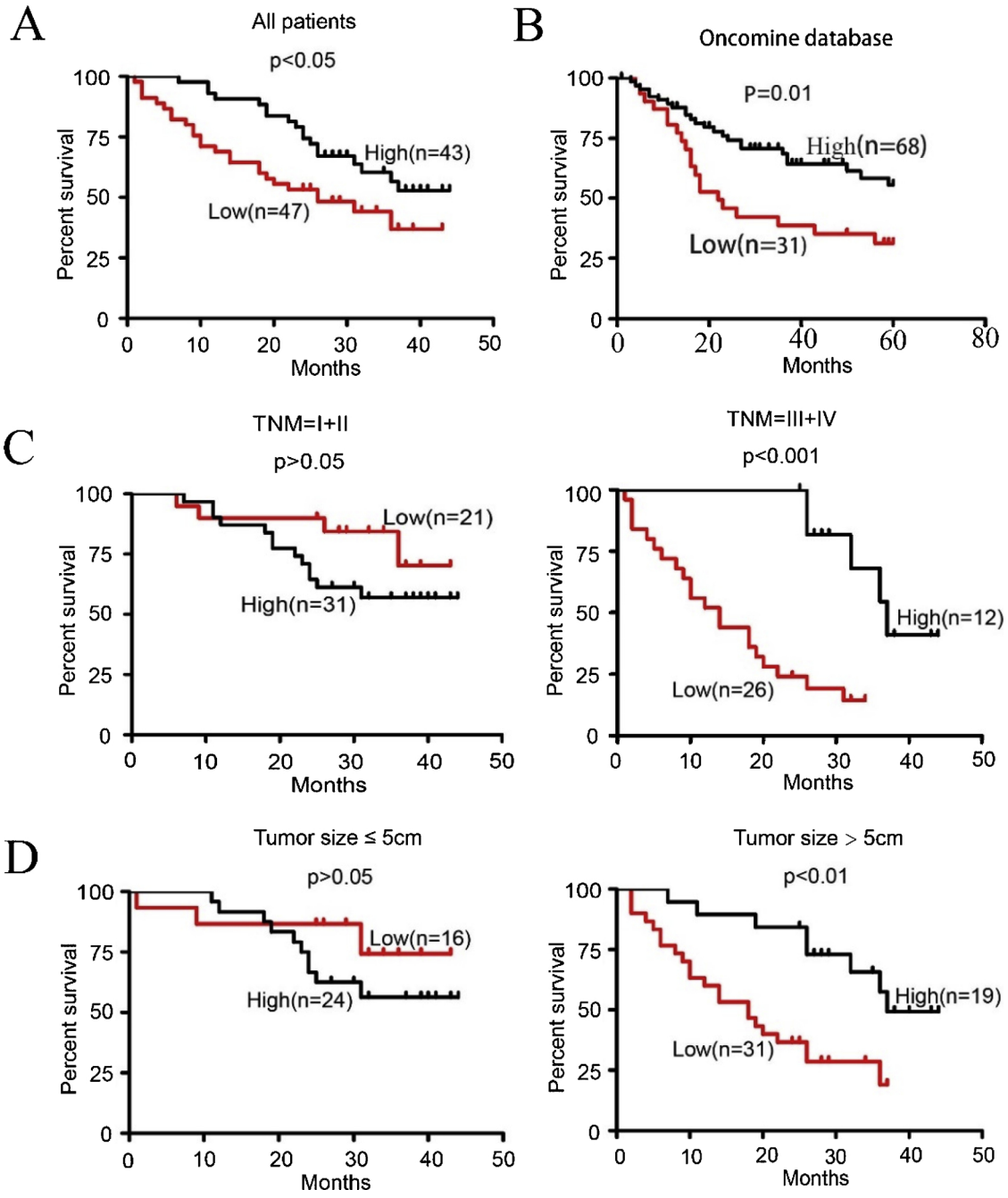

Fig. 2. CENP-E expression is correlated with patient survival with stratification.
(A) Kaplan–Meier analysis of overall survival based on CENP-E expression in 90 patients. HCC patients with low CENP-E expression had a higher risk of death ($P < 0.05$). (B) Kaplan-Meier survival analysis comparing the survival time of HCC patients with low or high CENP-E expression at different TNM stages (left: $P > 0.05$, right: $P < 0.001$) and tumor sizes (left: $P > 0.05$, right: $P < 0.01$).

associated with age, gender, liver cirrhosis and differentiation. These results provided a strong indication that the loss of CENP-E is involved in the pathogenesis of HCC.

3.3. Correlation between CENP-E expression and patient survival

Kaplan-Meier analysis revealed that HCC patients with low CENP-E expression had a higher risk of death (HR 2.023, 95 %CI 1.107–3.696; $P < 0.05$; Fig. 2A). Furthermore, stratification of patients by TNM stage and tumor size revealed that low CENP-E expression only correlated with poor prognosis in specific subgroups of HCC patients: TNM stage = III/IV ($P < 0.001$) (Fig. 2B), and tumor size > 5 cm ($P < 0.01$) (Fig. 2C). Univariate Cox regression analysis demonstrated that CENP-E expression and other parameters including tumor size, metastasis, differentiation, and TNM stage were significantly associated with overall survival of HCC patients. However, age, sex, and liver cirrhosis had no prognostic significance in this population (Table 2). Although multivariate analysis showed that CENP-E expression was not independently prognostic for all HCC patients (Table 2), CENP-E expression was identified as an independent prognostic factor for patients with TNM stage III + IV (HR: 7.092, 95 %CI: 2.080–24.186, $P < 0.01$; Table 3), and HCC patients with tumor size > 5 cm (HR: 4.042, 95 %CI: 1.588–10.285, $P < 0.01$; Table 4).

Table 2
Univariate and multivariate analysis of potential prognostic factors in HCC patients (HR: hazard ratio and CI confidence interval).

Factors	Case No	Univariate analysis		Multivariate analysis	
		HR (95 %C I)	*P* value	HR (95 %CI)	*P* value
Age (≥61/ < 61)	25/65	0.908 (0.46–1.794)	0.782		
Gender (male/female)	74/16	2.253(0.885–5.737)	0.089		
Tumor size (> 5/≤5 cm)	50/40	2.211(1.175–4.161)	0.014*		
Liver cirrhosis (negative/positive)	73/17	0.615(0.259–1.459)	0.270		
Metastasis(negative/positive)	83/7	0.205(0.089-0.470)	0.000**	0.338(0.140-0.811)	0.015*
TNM stage (I + II/III + IV)	52/38	0.345(0.189-0.628)	0.001**	0.412(0.217-0.780)	0.007**
Differentiation (well or moderate/poor)	65/25	0.488(0.267-0.893)	0.020*		
CENP-E expression (low /high)	47/43	2.023(1.107–3.696)	0.022*		0.115

* p < 0.05.
** p < 0.01.

Table 3
Multivariate analysis of potential prognostic factors in HCC patients in TNM stage III/IV (n = 38) (HR: hazard ratio and CI confidence interval).

Factors	Case No	Multivariate analysis	
		HR (95 %CI)	*P* value
Age (≥61/ < 61)	29/9		0.918
Gender (male/female)	32/6	7.563 (1.014–56.429)	0.048
Tumor size (> 5/≤5 cm)	35/3		0.288
Liver cirrhosis (negative/positive)	34/4		0.742
Metastasis (negative/positive)	31/7		0.056
Differentiation (well or moderate/poor)	23/15		0.621
CENP-E expression (low /high)	26/12	7.092(2.080–24.186)	0.002**

** p < 0.01.

Table 4
Multivariate analysis of potential prognostic factors in HCC patients in Tumor size > 5 cm (n = 50) (HR: hazard ratio and CI confidence interval).

Factors	Case No	Multivariate analysis	
		HR (95 %CI)	*P* value
Age (≥61/ < 61)	8/42		0.822
Gender (male/female)	41/9	3.649 (1.089–12.228)	0.036*
TNM Stage (I + II/III + IV)	15/35	0.352 (0.135-0.916)	0.032*
Liver cirrhosis (negative/positive)	41/9		0.604
Metastasis (negative/positive)	44/6		0.057
Differentiation (well or moderate/poor)	33/17		0.476
CENPE expression (low /high)	31/19	4.042 (1.588–10.285)	0.003**

* p < 0.05.
** p < 0.01.

3.4. Validation of findings from clinical samples and clinical data

Data on the expression of CENP-E in HCC samples and its clinical relevance are very limited. We were unable to find an identical cohort to validate our findings. However, there was one dataset (Lizuka Liver 2, n = 60) from Oncomine reporting that the CENP-E mRNA expression in HCC samples was reduced with a similar trend to the CENP-E protein level we observed in our clinical samples. In a published study [24], it was also found that both mRNA and protein expression levels of CENP-E were significantly reduced in HCC tissues (n = 21) compared to the adjacent tissues (n = 8). These two sets of data provided additional evidence to support our observation that CENP-E expression was reduced in human HCC tissues. As shown in Fig. 2B, Kaplan–Meier analysis of overall survival of two sets of Oncomine data (n = 99) with both CENP-E expression and survival revealed that low expression of CENP-E, as measured by CENP-E DNA copy number, was associated with poor survival (P = 0.01), which is entirely consistent with our findings.

3.5. CENP-E suppresses HCC cell proliferation in vitro

Different expression of CENP-E was observed in the six HCC cell lines used in this study (Fig. 3A). Cell lines with minimum CENP-E expression were avoided for CENP-E knockdown experiments. Therefore, Hep3B, SMMC7721, and QGY7701 cell lines were chosen for the study. We further investigated the biological function of CENP-E by down-regulating the expression of CENP-E in Hep3B, SMMC7721, and QGY7701 cells. CENP-E knockdown was accomplished by transfecting cells with recombinant interfering plasmid (pGPU6-GFP-Neo /sh-CENP-E) to establish stable cell lines. Down-regulation of CENP-E protein in Hep3B, SMMC7721, and QGY7701 cells was confirmed by western blot analysis. As shown in Fig. 3A, CENP-E protein level in sh-CENP-E cells was reduced by approximately one-fold compared with the scramble control in HCC cells. The effect of CENP-E on cell proliferation *in vitro* was assessed using the CCK-8 assay and demonstrated that reduction of CENP-E significantly increased the proliferation of Hep3B, SMMC7721, and QGY7701 cells (P < 0.005; Fig. 3B). In addition, the colony formation capability of CENP-E low-expression HCC cell lines was significantly enhanced (P < 0.001; Fig. 3C). Cell cycle analysis, by flow cytometry, showed that CENP-E low-expressing cell lines exhibited a significant decrease in the percentage of cells in the G1/G0 peak (P < 0.01), and a concomitant increase in the S phase compared with the control lines (P < 0.01) (Fig. 3D). The Hep3B, SMMC7721, and QGY7701 cells with CENP-E knockdown showed decreased apoptosis compared to the control transfected cell lines (P < 0.05; Fig. 3E). These results supported that loss of CENP-E expression increased cell cycle proliferation, increasing cycle transition from G1-S phase and reduced apoptosis in HCC cells.

3.6. CENP-E suppresses HCC cell migration and invasion in vitro

Boyden chamber migration assays showed that down-regulation of CENP-E promoted the migration of Hep3B, SMMC7721 and QGY7701 cells toward the bottom chamber (P < 0.001; Fig. 4 A). Consistent with the Boyden chamber results, monolayer wound healing assays exhibited similar phenomena, the loss of CENP-E increased the migratory potential of the cells (P < 0.05) (Fig. 4B). These results demonstrated down-regulation of CENP-E enhanced migration and wound healing.

3.7. CENP-E suppresses tumor growth in vivo

To investigate whether CENP-E affects tumor growth *in vivo*, HCC cells were subcutaneously implanted into BALB/c nude mice after modulation of CENP-E expression in the tumor cells. Twenty days later, the animals were sacrificed and the tumors were harvested. Down-

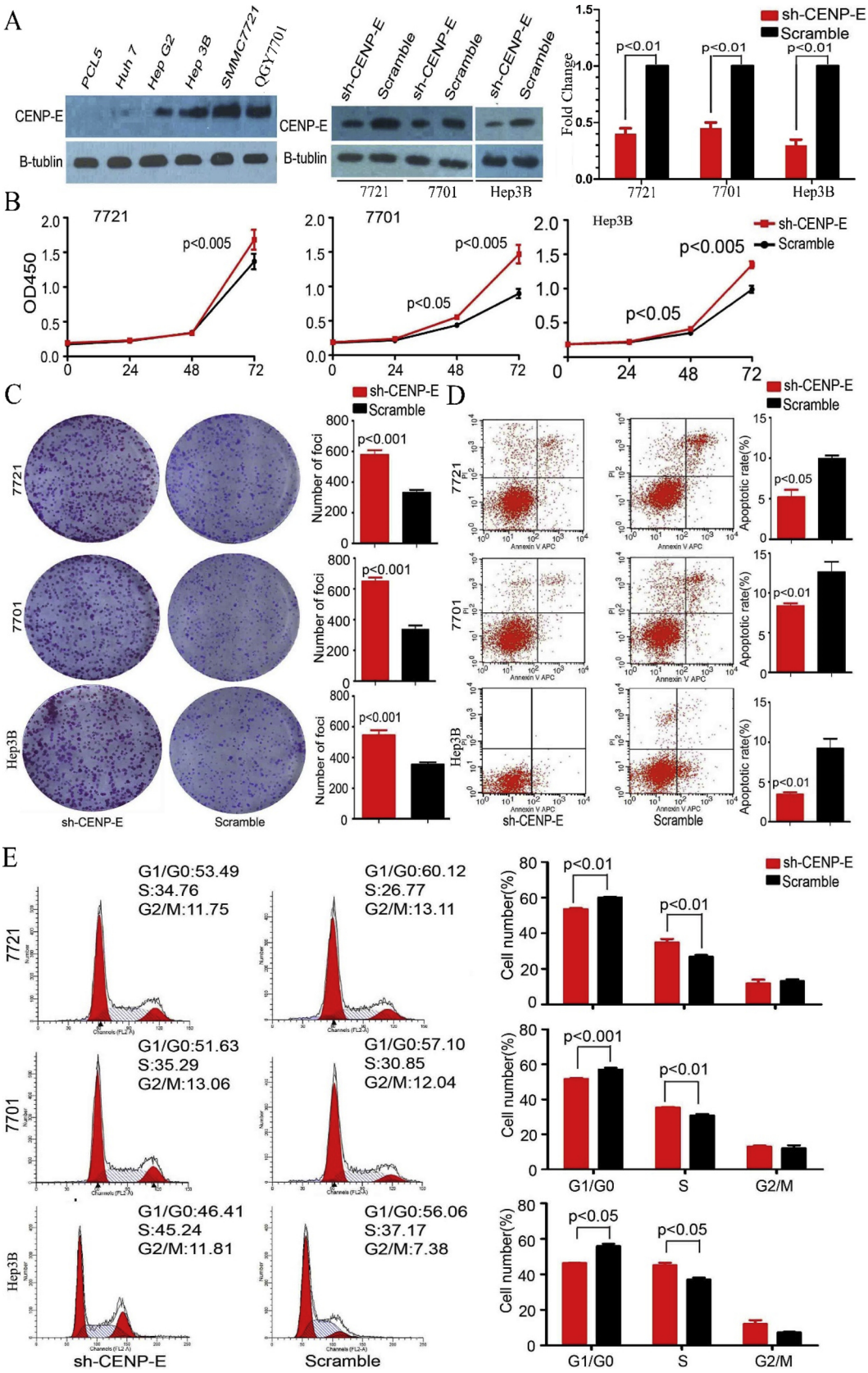

Fig. 3. Down-regulation of CENP-E expression increased HCC proliferation and colony formation *in vitro*.
(A) Western blot analysis of CENP-E in six HCC cell lines. Down-regulation of CENP-E expression in Hep3B, SMMC7721, and QGY7701 cells was confirmed by western blotting. (sh-CENP-E as the gene-targeted silencing sequence, scramble as the irrelevant sequence control). (B) Down-regulation of CENP-E increased HCC cell proliferation *in vitro* ($P < 0.005$). (C) Down-regulation of CENP-E expression increased the colony formation capacity of Hep3B, SMMC7721, and QGY7701 cells ($P < 0.001$). (D) Flow-cytometry analysis of the cell cycle progression showed a decrease in the percentage of cells in G0/G1 phase($P < 0.01$), and increase in the S phase of CENP-E-low-expressing HCC cells compared to that of control Hep3B, SMMC7721, and QGY7701 cell lines ($P < 0.01$). (E)Down-regulation of CENP-E decreased apoptosis in HCC cells ($P < 0.05$). Student's *t*-test was performed to calculate statistical significance.

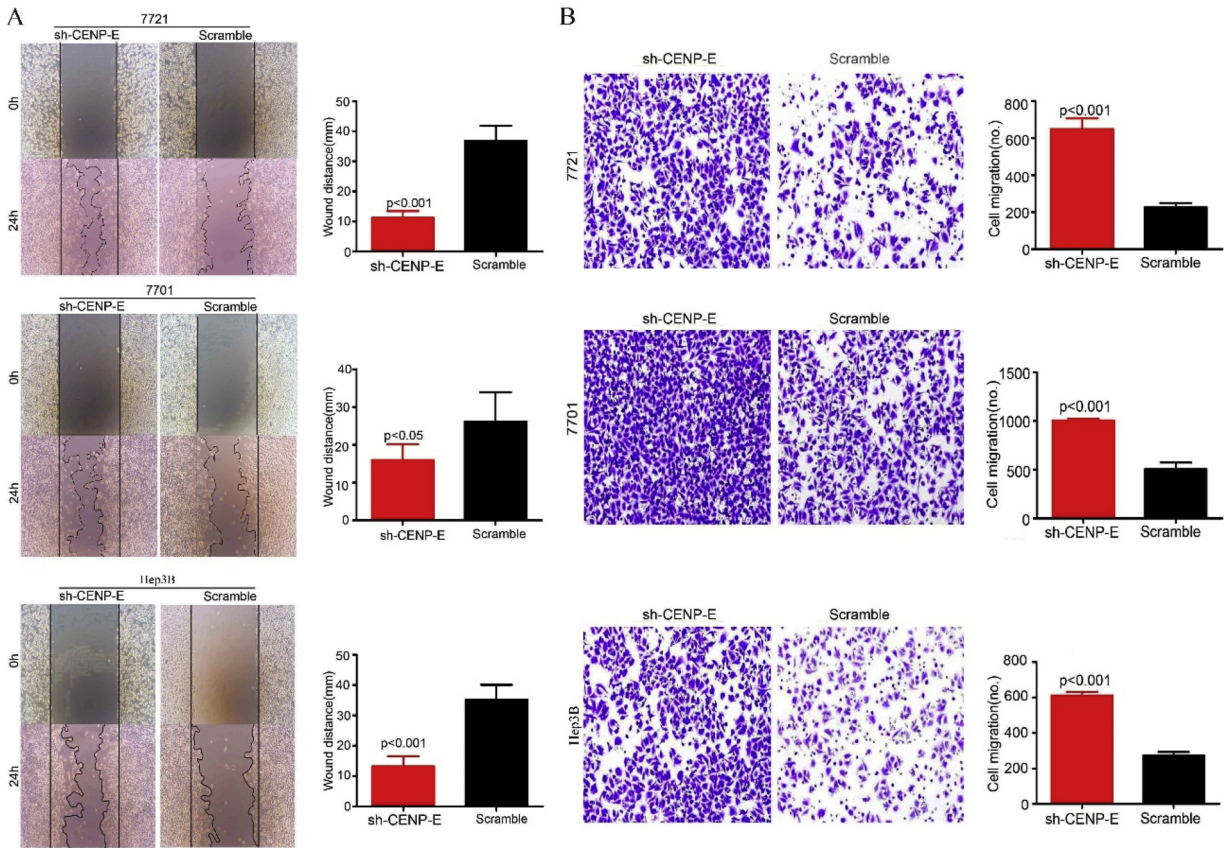

Fig. 4. Down-regulation of CENP-E expression promoted HCC migration *in vitro*.
(A) Down-regulation of CENP-E increased the ability of Hep3B, SMMC7721,and QGY7701cells to migrate toward chemoattractant in transwell chamber assays ($P < 0.001$, Student's *t*-test); (B) Wound healing assays showed a significant increase of HCC migration by down-regulation of CENP-E in all 3 cell lines($P < 0.001$ or $P < 0.05$, Student's *t*-test).

regulation of CENP-E in SMMC7721 cells significantly promoted tumor growth; the size of CENP-E knockdown SMMC7721 tumors was > 2-fold greater than that of the control SMMC7721 cells ($P < 0.05$; Fig. 5A). Western blotting analysis of tumor tissues confirmed the down-regulation of CENP-E in tumor cells (Fig. 5B). These results strongly suggest that CENP-E functions as a tumor suppressor to inhibit HCC growth *in vivo*. IHC ARG-1 staining demonstrated no difference in expression between tumors and controls ($P > 0.05$), but GPC-3 expression was significantly higher in CENP-E-low-expressing tumors *versus* control tumors ($P < 0.05$) (Fig. 5C).

4. Discussion

In this study, we demonstrated that down regulation of CENP-E in HCC cells significantly promoted HCC proliferation/growth *in vitro* and *in vivo*. This is the first piece of evidence connecting reduced expression of CENP-E with the pathogenesis of HCC, strongly suggesting that CENP-E is a HCC suppressor, which plays a role in hepatocarcinogenesis. Our analysis of 90 HCC clinical samples revealed a strong association between low CENP-E expression in HCC tissues and adverse clinicopathological features of HCC, in particular, poor prognosis, advanced TNM stage (stage III/IV), metastasis and larger tumor size (> 5 cm). Furthermore, we also found, in multivariate analysis, that CENP-E is an independent prognostic factor predicting disease outcome of advanced HCC patients. These results support that loss of CENP-E expression in liver tissue contributes to the progression of HCC, making CENP-E a novel target for new HCC therapy and a useful prognostic biomarker for HCC patients.

Previous studies have shown that CENP-E can both promote and suppress tumors depending on the context [14,17,19,29,30]. Weaver et al. proposed that the rate of chromosome missegregation or CIN, resulting from CENP-E suppression, determines on whether the effect is promotive or suppressive. low rates of CIN are tumor promoting, higher rates of CIN leads to tumor suppression [17,29]. However, our data from both *in vitro* and *in vivo* studies overwhelmingly supported that CENP-E in HCC cells/tissues functions as a tumor suppressor only. The two-ways effect of promoting and/or suppressing tumorigenesis by CENP-E has not been demonstrated in the present study.

In agreement with our data, Liu and colleagues reported that CENP-E expression, as determined by Western blot and qPCR, was significantly downregulated in human HCC as compared with adjacent non-tumor tissues. CENP-E levels in the human HCC-derived cell line HepG2 cells was also diminished to about half of that shown in LO2 cells. [24]. In a recent study, low expression of CENP-E in HepG2 cells was observed when the cells were under stress [25]. These studies confirmed that the level of CENP-E protein was reduced, not increased, in the HCC tissues, which suggested that CENP-E may have a suppressive role in some stages of human hepatocarcinogenesis. An independent set of data (n = 60) from Oncomine also confirmed that CENP-E expression as measured by mRNA level was reduced in human HCC samples (sTable 2). However, no further study has been conducted to define the role of CENP-E in the pathogenesis of HCC. In our study, we demonstrated for the first time that low expression of CENP-E in HCC tissues was strongly associated with a number of adverse clinical features. Furthermore, suppression of CENP-E in HCC cells led to significant promotion of tumor growth. Collectively, our data strongly suggested that the CENP-E protein functions as a tumor suppressor in HCC, and increasing CENP-E in the tumor potentially results in HCC suppression. Our findings are supported by previous studies that show aneuploidy drives tumorigenesis to promote tumor progression. An

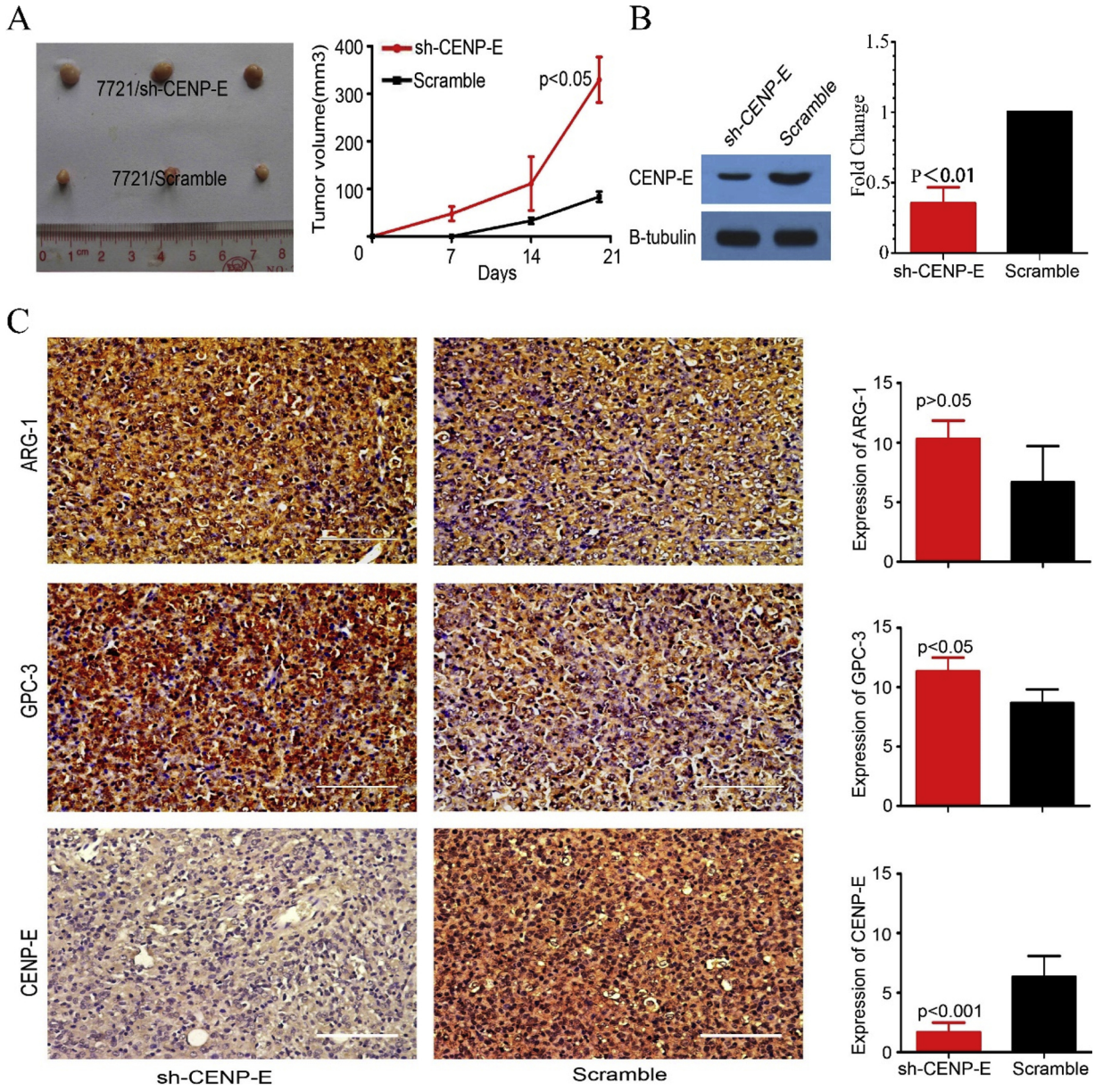

Fig. 5. Down regulation of CENP-E in HCC cells promoted tumor growth of *in vivo*.
(A) Down-regulation of CENP-E expression in human HCC derived cell line SMMC7721 cells promoted the growth of HCC *in vivo* ($P < 0.05$). (B) Tumors of HCC *in vivo* were collected for western blotting. (C) Tumor sections were subjected to IHC staining, showing the increased expression of GPC-3 but no difference of ARG-1 expression in CENP-E-low-expressing tumors *versus* control tumors (ARG-1:$P > 0.05$, GPC-3:$P < 0.05$). 40× magnifications, scale bar 100 μm. Statistical significance was tested by Student's *t*-test.

increase in chromosomal aneuploidy, resulting from suppression of CENP-E, promotes tumors in some contexts [14,17,29,31]. When CENP-E expression was inhibited, the chromosomes were unable to separate procedurally, resulting in an increase in aneuploidy and CIN, a hallmark of cancer [19]. Specifically, in liver cells, reduced CENP-E expression has been shown to interfere with the separation of chromosome [24]. Consistent results were also obtained from the study by Putkey and colleagues who demonstrated that selective deletion of CENP-E led to aberrant mitoses marked by chromosome missegregation during tissue regeneration, confirming the essential role of CENP-E in the maintenance of chromosomal stability [12].

In our laboratory studies and in the clinical sample cohort different levels of CENP-E suppression were recorded. The overall inhibition of CENP-E expression in the HCC tissues was about 30 % (8.43/11.76, Fig. 1C) while the suppression of CENP-E in animal xenograft tumors was nearly 90 % (Fig. 5C). In all experiments, only the tumor-promoting effects of reduced CENP-E were observed, regardless of how low the level of CENP-E protein was expressed in the tissues. Therefore we were unable to observe any tumor-suppression induced by higher rates of CIN, possibly resulting from significant suppression of CENP-E in xenograft tumors, as reported in murine models by other researchers [17,29]. These data suggest that the effects of CENP-E may be cell type and species specific. The frequency of aneuploidy and polyploidy in hepatocytes was noted to be variable between species [32].

Deregulation of signaling pathways during the G1 phase of the cell cycle represents a major driving force in the initiation and development of cancer [33]. Interestingly, our *in vitro* studies revealed that down-regulation of CENP-E significantly decreased the percentage of cells in G1/G0 phase and increased the percentage of cells in S phase, suggesting that CENP-E regulates the cell cycle transition from G1-S phase in HCC. We also observed that down-regulation of CENP-E significantly decreased cell apoptosis, again suggestive of an apoptotic role for

CENP-E a major goal for anticancer therapy [34].

A highlight of this study was the finding that down-regulation of CENP-E in HCC promoted tumor cell proliferation. This observation was confirmed in the *in vitro* experiments, and well substantiated by the *in vivo* results. In the *in vivo* animal study, subcutaneous injection of down-regulated CENP-E expressing HCC cells into nude mice resulted in significantly larger (> 2 folds) tumor growth compared to the control mice. The findings from the *in vitro* and *in vivo* animal studies were further supported by the results from human HCC clinical samples and data, where low expression of CENP-E in HCC tissues was associated with adverse clinical features (including larger tumor size and metastasis) and poor HCC patient survival. Multivariate analysis revealed that CENP-E could be an independent and useful prognostic biomarker predicting outcomes for HCC patients in the advanced TNM stage III/IV or tumor size > 5 cm groups. Interestingly, the expression of CENP-E correlated well with the expression of GPC-3 (Fig. 5C), a specific clinical diagnostic biomarker for HCC [35], adding support for CENP-E as a potential prognostic biomarker, another translational implication of CENP-E.

Our work has provided original evidence to strongly support that reduced expression of CENP-E protein in HCC cells promotes HCC growth *in vitro* and *in vivo*, highlighting the relevance of CENP-E and its role in promoting tumorigenesis in the liver. However, whether reduced/depleted expression of CENP-E in normal liver cells would initiate and drive the formation of spontaneous HCC *in vivo* is not known. Furthermore, it would be interesting to explore if upregulation of CENP-E in HCC cells does lead to inhibition of tumor growth. Thus, further work is warranted to elucidate the role of CENP-E in the initiation of HCC and as new therapeutic target.

In conclusion, our data from *in vitro* studies, *in vivo* animal models, and human clinical sample analysis, suggest that CENP-E is a tumor suppressor and reduced level of CENP-E in HCC contributes to tumor growth. Mechanistically, reduced expression of CENP-E promotes the growth of HCC through acceleration of cell cycle transition from the G1 to S phase and lead to suppression of apoptosis in HCC cells. Low expression of CENP-E in the HCC tissue could serve as an independent prognostic indicator for the poor outcome of advanced HCC patients. Overall, our work not only provides important evidence supporting a tumor-suppressive role for CENP-E in HCC and possible underlying mechanisms providing important insights into the hepatocarcinogenesis, but also suggests CENP-E expression as a potential therapeutic target and prognostic biomarker for HCC patients.

Data availability

The data [3 supplementary tables] used to support the findings of this study are included within the supplementary information file-Suppl.zip submitted.

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Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Supplementary materials

The submitted compressed file (Suppl.zip) contains the following supplementary tables: **sTable 1.** Expression of CENP-E mRNA and Protein in HCC Samples; **sTable 2.** CENP-E mRNA Expression in HCC Samples (n = 60); and **sTable 3.** Association between CENP-E Expression and Overall Survival

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