Biochemical and Biophysical Research Communications 436 (2013) 711–718

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization of the oncogenic function of centromere protein F in hepatocellular carcinoma

Yongdong Dai [a,1], Lulu Liu [a,1], Tingting Zeng [a], Ying-Hui Zhu [a], Jiangchao Li [d], Leilei Chen [b], Yan Li [a], Yun-Fei Yuan [a], Stephanie Ma [b,c,*], Xin-Yuan Guan [a,b,c,*]

[a] State Key Laboratory of Oncology in Southern China, Sun Yat-Sen University Cancer Center, Guangzhou, China
[b] Department of Clinical Oncology, The University of Hong Kong, Pokfulam, Hong Kong, China
[c] State Key Laboratory for Liver Research, The University of Hong Kong, Pokfulam, Hong Kong, China
[d] Vascular Biology Research Institute, Guangdong Pharmaceutical University, Guangzhou, China

ARTICLE INFO

Article history:
Received 6 June 2013
Available online 17 June 2013

Keywords:
HCC
CENPF
Prognosis factor
G2/M transition

ABSTRACT

Centromere protein F (CENPF) is an essential nuclear protein associated with the centromere-kinetochore complex and plays a critical role in chromosome segregation during mitosis. Up-regulation of CENPF expression has previously been detected in several solid tumors. In this study, we aim to study the expression and functional role of CENPF in hepatocellular carcinoma (HCC). We found CENPF was frequently overexpressed in HCC as compared with non-tumor tissue. Up-regulated CENPF expression in HCC was positively correlated with serum AFP, venous invasion, advanced differentiation stage and a shorter overall survival. Cox regression analysis found that overexpression of CENPF was an independent prognosis factor in HCC. Functional studies found that silencing CENPF could decrease the ability of the cells to proliferate, form colonies and induce tumor formation in nude mice. Silencing CENPF also resulted in the cell cycle arrest at G2/M checkpoint by down-regulating cell cycle proteins cdc2 and cyclin B1. Our data suggest that CENPF is frequently overexpressed in HCC and plays a critical role in driving HCC tumorigenesis.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common and the third most deadly cancer in the world [1]. Although progress has been made in recent decades to improve detection and treatment of HCC, the 5-year survival rate for the disease remains poor due to late presentation and the high incidence of recurrence and metastasis [2,3]. Thus, there is a need to identify better markers to detect the disease at an earlier stage, as well as to develop new therapeutic regimens that can better target HCC. Amplification of the long arm of chromosome 1 (1q) is one of the most frequent genetic alterations in HCC [4,5]. Several candidate oncogenes at 1q have been reported to be associated with the development and progression of HCC, including CHD1L [6,7], JTB and SHC1 [5]. In the present study, another candidate oncogene, centromere protein F (*CENPF*) at 1q41, was characterized.

CENPF is a member of the centromere protein family of kinetochore proteins [8]. When bound together with nuclear proteins like CENP-E, cytoplasmic dynein, MAD1, MAD2, Bub1 and BubR1, CENPF acts as a subunit of the protein complex which is responsible for kinetochore assembly, microtubule attachment, microtubule dynamics and spindle checkpoint signaling during mitosis [9]. *CENPF* is expressed in a cell cycle-dependent manner. At S phase, only low levels of CENPF can be detected in cell nucleus. At early G2 phase, CENPF level increases, and at late G2 phase, a dynamic spatial and temporal distribution of CENPF appears at the nuclear envelope [10]. Following nuclear envelope breakdown, CENPF becomes soluble in the mitotic cytoplasm while a subset remains bound at the outer kinetochore region until metaphase–anaphase transition [11]. During telophase, CENPF accumulates to the intraceullar bridge between the daughter cells [10], and then rapidly proteolyzed by farnesylation and ubiquitylation at the end of mitosis [12].

Abbreviations: CENPF, centromere protein F; HCC, hepatocellular carcinoma; CHD1L, chromodomain helicase DNA binding protein 1-like; JTB, jumping translocation breakpoint; SHC1, Src homology 2 domain containing transforming protein 1; MAD1, mitotic arrest deficient-like 1; MAD2, mitotic arrest deficient-like 2; BUB1, mitotic checkpoint serine/threonine kinase; TMA, tissue microarray; IHC, immunohistochemistry; DAB, Diaminobenzidine; qRT-PCR, quantitative real time polymerase chain reaction; siRNAs, small interfering RNAs; HE, hematoxylin and eosin.

* Corresponding authors. Address: Department of Clinical Oncology, Li Ka Shing Faculty of Medicine, University of Hong Kong, Room 56, 10/F, Laboratory Block, 21 Sassoon Road, Pok Fu Lam, Hong Kong, China. Fax: +86 852 2218 5244 (S. Ma), Room 605, State Key Laboratory of Oncology in Southern China, Sun Yat-Sen University Cancer Center, 651 Dongfeng Road East, Guangzhou 510060, China. Fax: +86 852 2819 9629 (X.-Y. Guan).

E-mail addresses: stefma@hku.hk (S. Ma), xyguan@hkucc.hku.hk (X.-Y. Guan).

[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.06.021

Overexpression of CENPF has previously been reported to be associated with non-Hodgkin's lymphoma [13], pancreatic ductal carcinoma [14], Wilm's tumor [15], neuroblastoma [16], breast cancer [17], colorectal gastrointestinal stromal tumors [18] and nasopharyngeal carcinoma [19]. CENPF overexpression has been suggested to serve as a poor prognostic factor in breast cancer and colorectal gastrointestinal stromal tumors [17,18]. Recently, CENPF was also found to be frequently amplified in HCC [20]. However, the oncogenic role of CENPF and its clinicopathological significance in HCC has not been explored to date. In the present study, immunohistochemistry was applied to investigate the expression pattern of CENPF in a large cohort of HCC specimens, as well as the clinical significances of CENPF overexpression in HCC. Oncogenic function of CENPF was characterized by various functional assays.

2. Materials and methods

2.1. Clinical samples and cell lines

Forty-one pairs of freshly frozen tissue samples containing HCC and adjacent non-tumor counterparts were obtained from the Department of Hepatobiliary Tumor, Sun Yat-Sen University Cancer Center (Guangzhou, China). Patient's consent and approval from the Institute's Research Ethics Committee was obtained before clinical material collection for research purposes. HCC cell lines QGY7703, BEL7402, SMMC7721, QSG7701, HepG2, PLC8024, Huh7, H2P and H2M, and immortalized normal human liver cell lines MIHA and LO2 have been described in previous studies [7,21,22].

2.2. Tissue microarray (TMA) construction and immunohistochemistry (IHC)

A total of 142 pairs of paraffin-embedded HCC samples were used for construction of the TMA. Samples were histologically and clinical diagnosed between 2003 and 2010 at the Sun Yat-Sen University Cancer Center. TMA blocks construction and IHC were performed as previously described [23]. The degree of immunostaining was assessed and scored independently by two investigators, blinded from the clinical parameters, according to both intensity and extent of staining. Only cells stained in the nucleus were taken into account. The extent of immunopositive cells was categorized as follow: 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%). The staining intensity was categorized by relative intensity as follow: 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). An overall score taking into account both parameters were then given: low CENPF expression with sum of both scores (extent and intensity) <3 or high CENPF expression with sum of both scores (extent and intensity) $\geqslant 3$.

2.3. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured cells or frozen tissues using TRIZOL Reagent (Invitrogen, Carlsbad, CA). Reverse

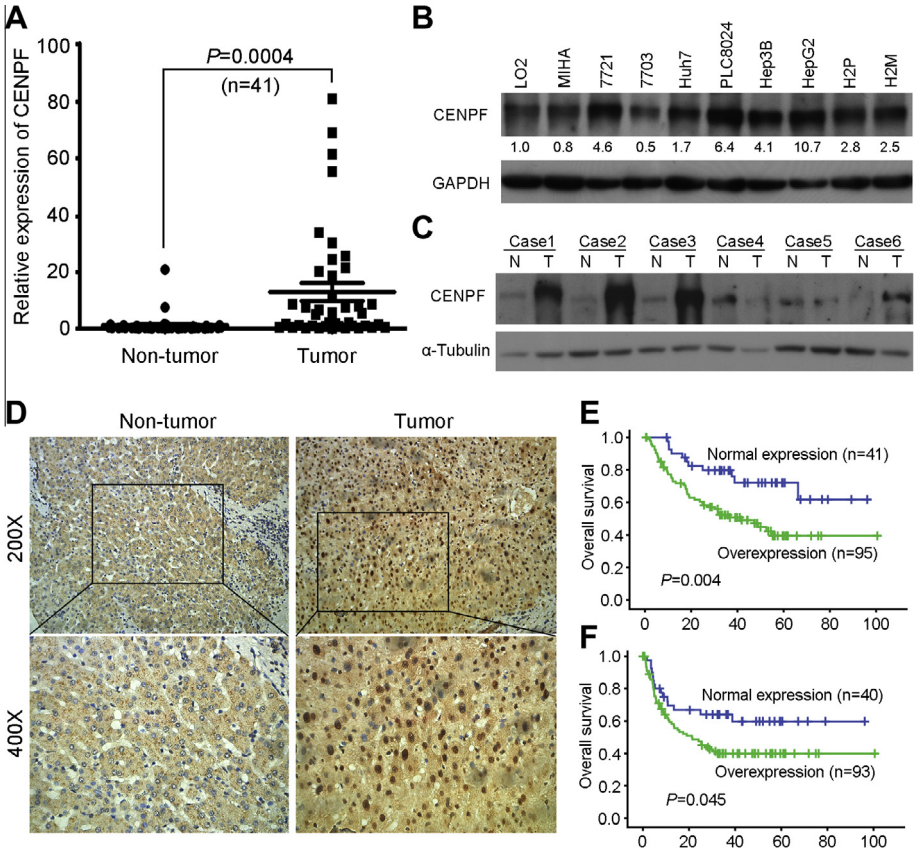

Fig. 1. Overexpression CENPF was determined in HCC and serve as an independent prognosis factor for cancer patients. (A) mRNA expression of CENPF in 41 matched non-tumor and primary HCC cases as detected by qRT-PCR. Average relative expression in tumor tissues (12.63 ± 3.10) was markedly higher than as compared with adjacent non-tumor tissues (1.00 ± 0.51; $P = 0.0004$). 18S was amplified as an internal control. (B) Protein expression of CENPF in a panel of liver cell lines as detected by Western blot. LO2 and MIHA are immortalized, normal liver cell lines. 7721, 7703, Huh7, PLC8024, Hep3B, HepG2, H2P and H2M are HCC cell lines. Numeric number represents the intensities of the bands of CENPF related to GAPDH. (C) Protein expression of CENPF in 6 pairs of non-tumor and HCC clinical samples as detected by Western blot. CENPF was up-regulated in 4 of the 6 samples examined. α-Tubulin was used as a loading control. (D) Representative IHC images of CENPF staining on the TMA. Only nuclear CENPF staining was regarded as positive signals. (E) Probability of survival of all patients with HCC: low CENPF expression, $n = 41$; high CENPF expression, $n = 95$ ($P = 0.004$). (F) Recurrence-free survival analysis of CENPF expression in tumor tissues ($P = 0.045$).

Table 1
Clinicopathological correlation of CENPF expression in HCC.

Clinicopathological feature	Number	*R*	CENPF expression		*P*-value
			Low	High	
Gender					
Male	125		38	87	
Female	9	0.016	3	6	1.000
Age					
<60 years	105		28	77	
⩾60 years	26	−0.169	12	14	0.061
HBsAg					
Negative	20		11	9	
Positive	113	0.220	30	83	**0.017**
Serum AFP					
⩽400 ng/ml	76		34	42	
>400 ng/ml	54	0.359	6	48	**0.000**
Tumor size					
⩽5 cm	48		12	36	
>5 cm	84	−0.087	28	56	0.334
Cirrhosis					
Absent	22		9	13	
Present	108	0.099	31	77	0.312
Encapsulation					
Absent	50		19	31	
Present	80	0.124	21	59	0.176
Microsatellite					
Absent	100		31	69	
Present	35	0.023	10	25	0.834
Venous invasion					
Absent	112		38	74	
Present	23	0.171	3	20	**0.050**
Adjacent organ invasion					
Absent	99		32	67	
Present	31	0.091	7	24	0.373
Recurrence					
No recurrence	66		25	41	
Recurrence	67	0.169	15	52	0.060
Metastasis					
No metastasis	115		32	82	
Metastasis	17	−0.122	8	10	0.175
Tumor grade[a]					
Stage I–II	81		30	51	
Stage III–IV	53	0.194	10	43	**0.033**

Statistical significance ($P < 0.05$) is shown in bold.
[a] AJCC.

transcription of total RNA (2 μg) was carried out using the reverse transcription (RT)-PCR kit (Roche, Basel, Switzerland), according to manufacturer's instructions. For qRT-PCR, cDNA was amplified using the SYBR Green PCR Kit (Roche) on an ABI PRISM 7900 sequence detection system, with the following CENPF primers (sense 5′-TACTGAGTTTGAGCCAGAGGGACT-3′ and antisense 5′-CATGGTTGTTCTTCGCAGGATAT-3′). 18S rRNA was amplified as an internal control. To ensure reproducibility of results, qRT-PCR was performed in triplicates.

2.4. Western blot analysis and antibodies

Twenty-four microgram of protein was separated by SDS–PAGE and subsequently blotted onto a polyvinylidene fluoride (PVDF) membrane. Transferred membranes were blocked in 4% bovine serum albumin in phosphate buffered saline (4% BSA in PBS) and then incubated with primary antibodies overnight. Primary antibodies against cyclin D1 (DCS6), p53 (7F5), CDK4 (DCS156), cyclin B1 (V152), cdc2 and α-tublin were purchased from Cell Signaling Technology (Beverly, MA). Anti-CENPF (ab90) was purchased from Abcam (Cambridge, MA). Following primary antibody incubation, membranes were rinsed and incubated with HRP-conjugated secondary antibody and developed using a standard ECL system with signal captured on an X-ray film. Intensities of bands in cell lines were analyzed by the Macintosh densitometry program ImageJ (NIH, Bethesda, MD).

2.5. RNA interference

CENPF expression was repressed in PLC8024 and SMMC7721 cells using double-stranded siRNAs (siRNA1: 5′-GGAGAAUCAAAGAUUGAUGGA-3′ and 5′-UCCAUCAAUCUUUGAUUCUCC-3′; siRNA2: 5′-GAGGGACUUCCAGAAGUUGUA-3′ and 5′-UACAACUUCUGGAAGUCCCUC-3′). Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Scramble siRNA was used as a negative control. For *in vivo* studies, stable shCENPF cells were established by shRNA plasmid purchased from Genecopoeia (Rockville, MD).

Table 2
Univariate and multivariate analyses.

Clinicopathological feature	Univariate analysis			Multivariate analysis		
	HR[b]	95%CI[c]	*P*	HR[b]	95%CI[c]	*P*
Gender Male vs. female	0.735	0.294–1.836	0.509			
Age ⩽60 vs. >60	0.695	0.352–1.372	0.294			
HBsAg (−) vs. (+)	1.855	0.798–4.311	0.151			
Encapsulation (absent vs. present)	1.194	0.697–2.046	0.518			
Cirrhosis (absent vs. present)	1.828	0.831–4.021	0.134			
Tumor grade[a] (I–II vs. III–IV)	1.438	0.869–2.379	0.157			
AFP (ng/ml) (⩽400 vs. >400)	2.604	1.559–4.348	**0.000**	1.653	0.956–2.858	0.072
Microsatellite (absent vs. present)	1.857	1.092–3.157	**0.022**	1.664	0.971–2.853	0.064
Tumor size (⩾5 cm vs. <5 cm)	2.284	1.271–4.105	**0.006**	1.635	0.858–3.118	0.135
Tumor thrombus (absent vs. present)	4.450	2.469–8.022	**0.000**	2.614	1.384–4.939	**0.003**
Recurrent (No vs. Yes)	3.393	2.015–5.716	**0.000**	2.342	1.316–4.169	**0.004**
Metastasis (No vs. Yes)	2.493	1.386–4.482	**0.002**	1.259	0.633–2.502	0.511
CENPF overexpression	2.532	1.313–4.883	**0.006**	2.161	1.070–4.363	**0.032**

Statistical significance ($P < 0.05$) is shown in bold.
[a] AJCC.
[b] Hazard ratio.
[c] Confidence interval.

2.6. In vivo tumorigenicity assay

The study protocol was approved by and performed in accordance with the Committee of the Use of Live Animals in Teaching and Research at the Sun Yat-Sen University. For CENPF transplantation studies, lentiviral-delivered shRNA CENPF and shRNA control SMMC7721 cells were injected subcutaneously into the flank of nude mice in complete medium at a concentration of 4×10^6. Tumor size was measured every week for 4 weeks and calculated using the formula $V = 0.5 \times L \times W^2$. Cryosections (4 μm thick) were stained with hematoxylin and eosin (HE) and used forIHC.

2.7. Cell proliferation assay

Proliferation rates were determined using the CCK-8 Kit (Tojindo, Shanghai, China) according to manufacturer's instruction. For foci formation assays, cell growth rates were determined by colorimetric assay using crystal violet (Sigma–Aldrich, St Louis, MO), a cytochemical stain that binds to chromatin.

2.8. Cell cycle analysis

Cells were fixed in 70% ethanol and stained with propidium iodide and DNA content was analyzed by Cytomics FC 500 (Beckman Coulter, Brea, CA). Results were analyzed by Multicycle AV software for Windows (Phoenix Flow Systems, San Diego, CA).

2.9. Statistical analysis

All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL). Independent Student's *t* test was used to analyze the results expressed as mean ± SD. χ^2 test or Fisher's exact test was used to analyze the association of CENPF protein expression and clinicopathological parameters. Kaplan–Meier plots and log-rank tests were used for survival analysis. Univariate and multivariate survival analyses were performed using the Cox proportional hazards regression model. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. CENPF is frequently overexpressed in HCC

To analyze the expression levels of CENPF in HCC, qRT-PCR was performed to compare the CENPF mRNA expression between tumor and their paired adjacent non-tumor tissues in 41 primary HCC samples. Results indicated that the average fold change of CENPF mRNA in HCC was significantly higher than that in non-tumor tissues (12.63 vs. 1.00, $P = 0.0004$, independent Student's *t* test, Fig. 1A). Western blot analysis was subsequently applied to determine protein expression levels of CENPF in 10 liver cell lines, including two immortalized normal liver cell lines (LO2 and MIHA), and eight HCC cell lines (SMMC7721, QGY7703, Huh7, PLC8024, Hep3B, HepG2, H2P and H2M); as well as in 6 pairs of fresh frozen non-tumor and HCC tissue clinical samples. CENPF expression was found to be relatively overexpresssed in 6 of 8 HCC cell lines

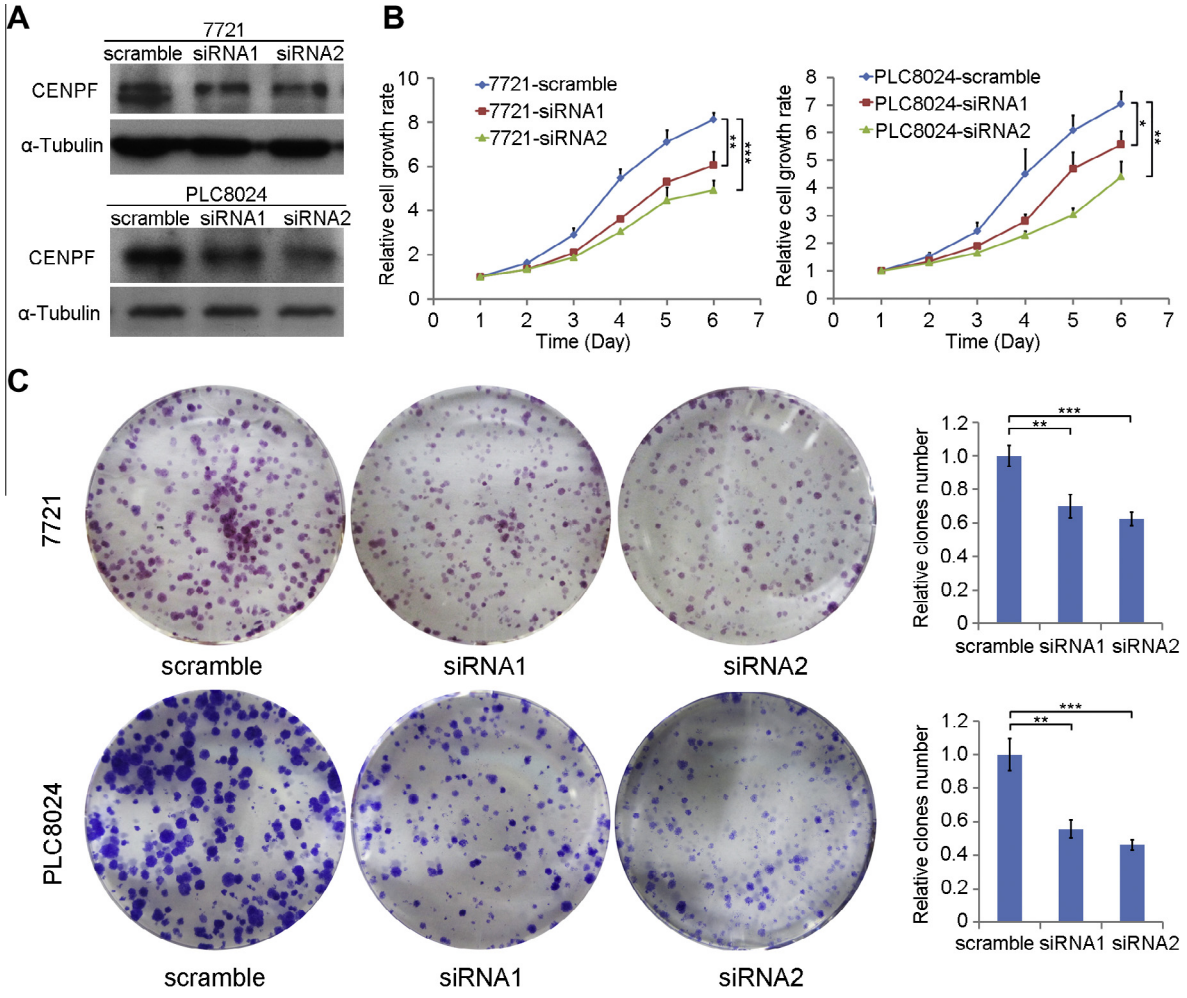

Fig. 2. *In vitro* functional studies in HCC cells with or without CENPF repressed. (A) Efficiency of siRNA knockdown was verified by Western blot. (B) XTT assay. 7721-scramble and 7721-siCENPF (left) and PLC8024-scramble and PLC8024-siCENPF (right). Cells with CENPF suppressed had a decreased ability to grow than as compared with control cells. (C) Foci formation assay. 7721-scramble and 7721-siCENPF (top) and PLC8024-scramble and PLC8024-siCENPF (bottom). Cells with CENPF suppressed had a decreased ability to induce colony formation than as compared with control cells. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Values expressed as mean ± SD of 3 independent experiments.

(including SMMC7721, PLC8024, Hep3B, HepG2, H2P and H2M), than as compared to the two immortalized normal liver cell lines (Fig. 1B). Similarly, CENPF expression was also found to be overexpressed in 4 of 6 HCC samples as compared to their matched non-tumor tissue (Cases 1–3 and 6, Fig. 1C).

To further validate this observation, we expanded our analysis using a larger cohort of clinical specimens sampled on a tissue microarray ($n = 142$). IHC staining showed that CENPF expression was found mostly located in the nucleus (Fig. 1D). Informative CENPF expression was detected in 136 of the 142 cases. Other non-informative samples included lost samples, or samples with too few tumor cells, and thus were not used in data compilation. CENPF overexpression was observed in 92 of 136 (67.6%) of tumor tissues, compared with adjacent non-tumor tissue.

3.2. Overexpression of CENPF is associated with poor prognosis

Using data obtained from our IHC analysis, we further performed correlative analysis (χ^2 test) of CENPF expression in HCC with various patient's clinicopathological features. Overexpression of CENPF was found to be closely associated with HBsAg ($P = 0.017$), AFP ($P = 0.000$), tumor venous invasion ($P = 0.050$) and differentiation stage ($P = 0.033$) (Table 1). However, no significant association was found between CENPF expression and other clinicopathological characteristics like gender, age, tumor size, cirrhosis, microsatellite formation, adjacent organ invasion, recurrence and metastasis (Table 1). Kaplan–Meier analysis found both the trend of overall survival and recurrence-free survival for HCC patients expressing low or high levels of CENPF to be significantly diverse ($P = 0.004$ and $P = 0.045$, respectively; log-rank test, Fig. 1E and F), suggesting that high CENPF expression would lead to a worse overall prognosis for HCC patients.

3.3. Overexpression of CENPF protein is an independent prognostic factor of HCC

By univariate Cox regression analyses, AFP, microsatellite formation, tumor size, tumor thrombus, recurrence, metastasis and CENPF expression were all found to be closely associated with overall survival (Table 2). Further, multivariate Cox proportional hazard regression analysis found tumor thrombus, recurrence and overexpression of CENPF to be independent prognostic factors for the overall survival of HCC patients (Table 2).

3.4. CENPF knockdown inhibited tumorigenic ability of HCC cells

Kim and colleagues previously reported that overexpression of *CENPF* in NIH-3T3 cells promoted cell clonogenic and invasive

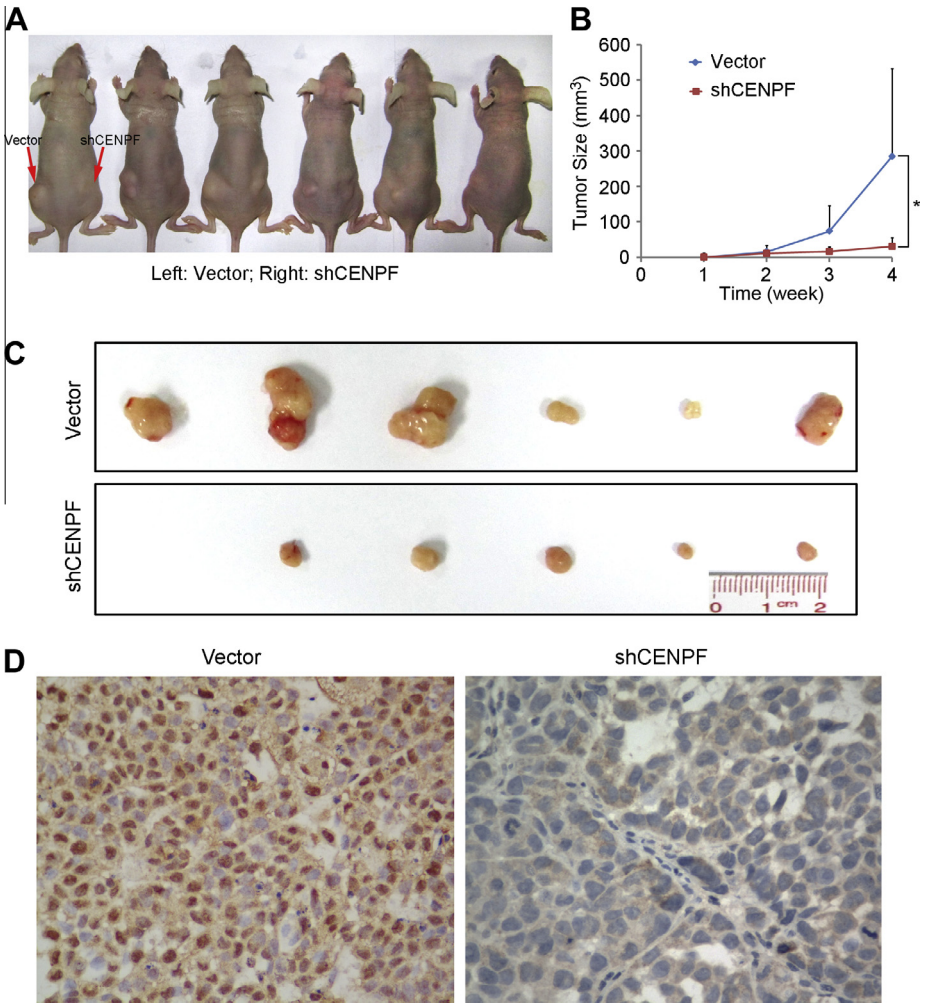

Fig. 3. *In vivo* tumorigenicity assay. (A) Nude mice injected with 7721 vector control cells (left dorsal flank) or 7721-shCENPF cells (right dorsal flank). (B) Tumor size of xenografts 4 weeks post injection. Average tumor volume of 7721-shCENPF xenografts was smaller than 7721-vector. Values expressed as mean ± SD of volumes of six mice. *Represents $P < 0.05$ (independent Student's t test). (C) Images of tumors harvested from the sacrificed animals. (D) IHC staining of CENPF in the harvested xenografts. 7721-vector (left) and 7721-shCENPF (right).

abilities [20]. To further examine the function of *CENPF* in HCC, we designed and synthesized two specific siRNAs to repress *CENPF* in HCC cell lines with high levels of endogenous CENPF expression. Efficiency of siRNA silencing was validated by Western blot analysis. Both siRNA sequences could effectively repress expression of CENPF in SMMC7721 and PLC8024 (Fig. 2A). Interestingly, CENPF presents two protein bands in 7721-scramble cells. This may be explained that CENPF protein can be post-translational modified by phosphorylation in multiple amino acid residues, such as Ser106, thr144, thr154, thr253, etc. [24,25]. However, after silencing CENPF in 7721, the total protein especially the unmodified protein form was decreased. To compare the cell growth and proliferation abilities of HCC cells with or without CENPF repressed, *in vitro* functional studies were performed. Relative cell growth rates in cells with CENPF suppressed were significantly retarded as compared with control cells ($P < 0.05$, Student's t test, Fig. 2B). Knockdown of CENPF could also significantly reduce the abilities of HCC cells to form colonies, as evident in the foci formation assay ($P < 0.01$, Student's t test, Fig. 2C).

To further confirm this observation, we extended our studies using an *in vivo* mouse model. SMMC7721 cells with or without CENPF stably repressed (shCENPF-7721 or Vec-7721) were injected subcutaneously into the dorsal flanks of athymic nude mice. Tumor size were measured weekly for 4 weeks. Results found cells with CENPF repressed to have a significantly retarded ability to initiate tumor formation than as compared with control cells (Fig. 3A and B). Four weeks following cell injection, all mice were sacrificed with xenografts harvested (Fig. 3C). IHC was performed to examine the expression of CENPF in the xenograft tumors. CENPF expression in the tumors formed with 7711-shCENPF cells was substantially weaker than in tumors formed with Vec-7721 control cells (Fig. 3D).

3.5. CENPF knockdown blocked cell cycle at G2/M transition

Since CENPF is a kinetochore proteinand is expressed in a cell cycle-dependent manner, we further examined whether the functional role of CENPF in HCC is regulated at the cell cycle level. Cell cycle analysis by flow cytometry found that knockdown of CENPF in HCC was able to arrest cells at G2/M transition (Fig. 4A). Subsequent analysis for cell cycle related protein expression levels by Western blot found that G2/S checkpoint promoting factors cyclin B1 and cdc2 were significantly down-regulated. Cyclin D1 and CDK4 expression levels were not affected (Fig. 4B).

4. Discussion

The pathogenesis of HCC is a long process involves multiple genetic and epigenetic changes including the amplification of

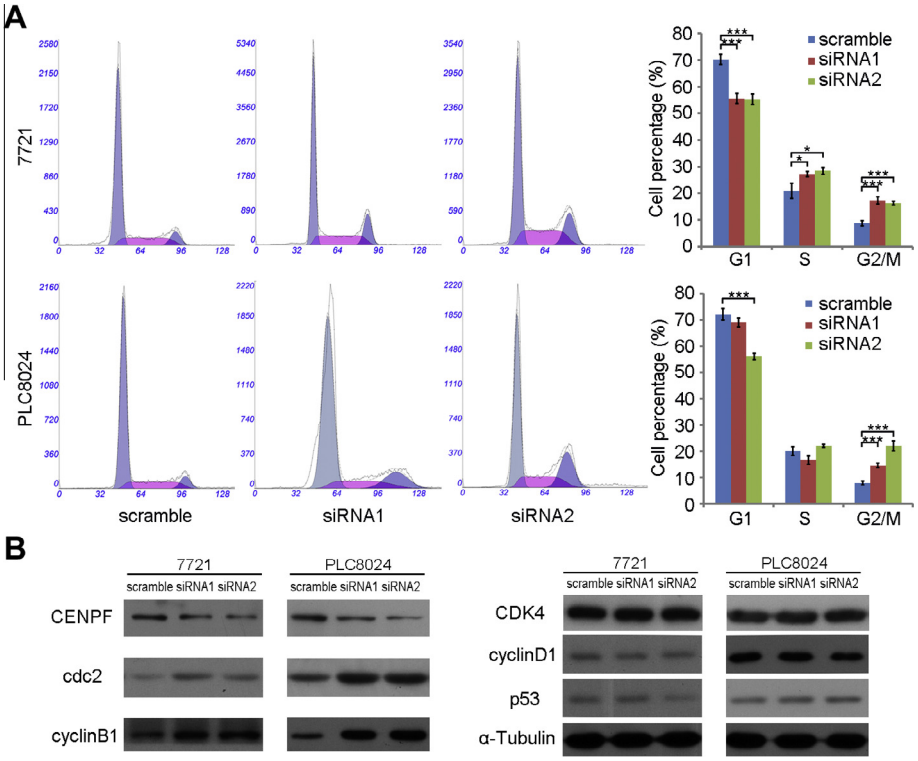

Fig. 4. CENPF knockdown blocked cell cycle at G2/M transition. (A) Representative images of cell cycle analysis by flow cytometry. A significantly greater number of cells were held at the G2/M phase in HCC cells with CENPF repressed as compared with control cells (upper, 7721; lower, PLC8024; $^{*}P < 0.05$; $^{***}P < 0.001$). Values expressed as mean ± SD of 3 independent experiments. (B) Expression of CENPF, cdc2, cyclin B1, CDK4, cyclin D1 and p53 as detected by Western blot. There is an accumulation of cdc2 and cyclin B1 following knockdown of CENPF, further indicative of arrest at G2/M.

chromosome 1q [4]. In the present study, we characterized another candidate oncogene *CENPF* at 1q41. Overexpression of CENPF was frequently detected in HCC cases, which was significantly associated with patient's HBsAg level ($P = 0.017$), serum AFP level ($P = 0.000$), tumor venous invasion ($P = 0.050$) and differentiation stage ($P = 0.033$). This data suggests that *CENPF* might be an oncogene playing critical role in the development and progression of HCC.

CENPF had previously been reported to act as a nuclear matrix component and to be required for kinetochore-microtubule interaction and spindle checkpoint during mitosis [25,26]. In cancer cells, Cao and colleagues found that CENPF potential target chemicals (Zoledronic acid and FTI-277) that could significantly enhance the chemotherapeutic sensitivity of NPC cell lines to cisplatin [19]. Moreover, forced expression of CENPF in NIH-3T3 promoted the abilities of the cells to invade [20]. In the present work, the functional role of CENPF was investigated by both *in vitro* and *in vivo* assays after the silencing of CENPF in HCC cell lines. Our results showed that knockdown CENPF expression in HCC cells could significantly inhibit cell growth and colony formation. Result of tumorigenicity assay in nude mice showed that ability of cells to initiate tumors *in vivo* was drastically reduced in HCC cells with CENPF silenced. Further analysis demonstrated that silencing CENPF arrested cells at the G2/M transition, concomitant with an accumulation of mature promoting factor (MPF) of G2 to M phase, cyclin B1/CDC2 complex.

Furthermore, an important finding of our present study is that overexpression of CENPF could be used as an independent prognostic factor for HCC patients. This finding is consistent with results of previous reports in other tumor types, where overexpression of CENPF had been associated with a poor prognosis in breast cancer, colorectal gastrointestinal stromal tumors and NPC patients [17–19]. Therefore, CENPF appears to play an important role in pathogenesis of various human solid tumors including HCC.

In summary, we report for the first time, that overexpression of CENPF in HCC is closely associated with tumor venous invasion and cell differentiation. CENPF overexpression may serve as a powerful prognostic marker for HCC and also a novel therapeutic target for the disease.

Author responsibility

Y.D. and L.L., initiated and designed the study, performed experiments and interpreted results, drafted the manuscript; T.Z., Y.Z., J.L., L.C. and Y.L., performed experiments and interpreted results; Y.F.Y., collected clinical samples and relevant clinical information; S.M., X.Y.G., initiated and designed the study, and supervised the project.

Funding

This work was supported by grants from the National Basic Research Program of China (2012CB967001) and Major National Science and Technology Project of China (2008ZX10002-022).

References

[1] H.B. El-Serag, K.L. Rudolph, Hepatocellular carcinoma: epidemiology and molecular carcinogenesis, Gastroenterology 132 (2007) 2557–2576.
[2] Y. Yang, H. Nagano, H. Ota, O. Morimoto, M. Nakamura, H. Wada, T. Noda, B. Damdinsuren, S. Marubashi, A. Miyamoto, Y. Takeda, K. Dono, K. Umeshita, S. Nakamori, K. Wakasa, M. Sakon, M. Monden, Patterns and clinicopathologic features of extrahepatic recurrence of hepatocellular carcinoma after curative resection, Surgery 141 (2007) 196–202.
[3] Z.Y. Tang, S.L. Ye, Y.K. Liu, L.X. Qin, H.C. Sun, Q.H. Ye, L. Wang, J. Zhou, S.J. Qiu, Y. Li, X.N. Ji, H. Liu, J.L. Xia, Z.Q. Wu, J. Fan, Z.C. Ma, X.D. Zhou, Z.Y. Lin, K.D. Liu, A decade's studies on metastasis of hepatocellular carcinoma, J. Cancer Res. Clin. Oncol. 130 (2004) 187–196.
[4] X.Y. Guan, Y. Fang, J.S. Sham, D.L. Kwong, Y. Zhang, Q. Liang, H. Li, H. Zhou, J.M. Trent, Recurrent chromosome alterations in hepatocellular carcinoma detected

by comparative genomic hybridization, Genes Chromosomes Cancer 29 (2000) 110–116.
[5] N. Wong, A. Chan, S.W. Lee, E. Lam, K.F. To, P.B. Lai, X.N. Li, C.T. Liew, P.J. Johnson, Positional mapping for amplified DNA sequences on 1q21-q22 in hepatocellular carcinoma indicates candidate genes over-expression, J. Hepatol. 38 (2003) 298–306.
[6] N.F. Ma, L. Hu, J.M. Fung, D. Xie, B.J. Zheng, L. Chen, D.J. Tang, L. Fu, Z. Wu, M. Chen, Y. Fang, X.Y. Guan, Isolation and characterization of a novel oncogene, amplified in liver cancer 1, within a commonly amplified region at 1q21 in hepatocellular carcinoma, Hepatology 47 (2008) 503–510.
[7] L. Chen, T.H. Chan, Y.F. Yuan, L. Hu, J. Huang, S. Ma, J. Wang, S.S. Dong, K.H. Tang, D. Xie, Y. Li, X.Y. Guan, CHD1L promotes hepatocellular carcinoma progression and metastasis in mice and is associated with these processes in human patients, J. Clin. Invest. 120 (2010) 1178–1191.
[8] A. Varis, A.L. Salmela, M.J. Kallio, Cenp-F (mitosin) is more than a mitotic marker, Chromosoma 115 (2006) 288–295.
[9] V.L. Johnson, M.I. Scott, S.V. Holt, D. Hussein, S.S. Taylor, Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression, J. Cell Sci. 117 (2004) 1577–1589.
[10] H. Liao, R.J. Winkfein, G. Mack, J.B. Rattner, T.J. Yen, CENP-F is a protein of the nuclear matrix that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis, J. Cell Biol. 130 (1995) 507–518.
[11] J.B. Rattner, A. Rao, M.J. Fritzler, D.W. Valencia, T.J. Yen, CENP-F is a.ca 400 kDa kinetochore protein that exhibits a cell-cycle dependent localization, Cell Motil. Cytoskeleton 26 (1993) 214–226.
[12] D. Hussein, S.S. Taylor, Farnesylation of Cenp-F is required for G2/M progression and degradation after mitosis, J. Cell Sci. 115 (2002) 3403–3414.
[13] M. Erlanson, C.A. Casiano, E.M. Tan, J. Lindh, G. Roos, G. Landberg, Immunohistochemical analysis of the proliferation associated nuclear antigen CENP-F in non-Hodgkin's lymphoma, Mod. Pathol. 12 (1999) 69–74.
[14] R. Grutzmann, C. Pilarsky, O. Ammerpohl, J. Luttges, A. Bohme, B. Sipos, M. Foerder, I. Alldinger, B. Jahnke, H.K. Schackert, H. Kalthoff, B. Kremer, G. Kloppel, H.D. Saeger, Gene expression profiling of microdissected pancreatic ductal carcinomas using high-density DNA microarrays, Neoplasia 6 (2004) 611–622.
[15] B. Zirn, O. Hartmann, B. Samans, M. Krause, S. Wittmann, F. Mertens, N. Graf, M. Eilers, M. Gessler, Expression profiling of Wilms tumors reveals new candidate genes for different clinical parameters, Int. J. Cancer 118 (2006) 1954–1962.
[16] D. Albino, P. Scaruffi, S. Moretti, S. Coco, M. Truini, C. Di Cristofano, A. Cavazzana, S. Stigliani, S. Bonassi, G.P. Tonini, Identification of low intratumoral gene expression heterogeneity in neuroblastic tumors by genome-wide expression analysis and game theory, Cancer 113 (2008) 1412–1422.
[17] A. Brendle, A. Brandt, R. Johansson, K. Enquist, G. Hallmans, K. Hemminki, P. Lenner, A. Forsti, Single nucleotide polymorphisms in chromosomal instability genes and risk and clinical outcome of breast cancer: a Swedish prospective case-control study, Eur. J. Cancer 45 (2009) 435–442.
[18] W.B. Chen, X.B. Cheng, W. Ding, Y.J. Wang, D. Chen, J.H. Wang, R.S. Fei, Centromere protein F and survivin are associated with high risk and a poor prognosis in colorectal gastrointestinal stromal tumours, J. Clin. Pathol. 64 (2011) 751–755.
[19] J.Y. Cao, L. Liu, S.P. Chen, X. Zhang, Y.J. Mi, Z.G. Liu, M.Z. Li, H. Zhang, C.N. Qian, J.Y. Shao, L.W. Fu, Y.F. Xia, M.S. Zeng, Prognostic significance and therapeutic implications of centromere protein F expression in human nasopharyngeal carcinoma, Mol. Cancer 9 (2010) 237.
[20] H.E. Kim, D.G. Kim, K.J. Lee, J.G. Son, M.Y. Song, Y.M. Park, J.J. Kim, S.W. Cho, S.G. Chi, H.S. Cheong, H.D. Shin, S.W. Lee, J.K. Lee, Frequent amplification of CENPF, GMNN and CDK13 genes in hepatocellular carcinomas, PLoS One 7 (2012) e43223.
[21] M. Liu, L. Chen, T.H. Chan, J. Wang, Y. Li, T.T. Zeng, Y.F. Yuan, X.Y. Guan, Serum and glucocorticoid kinase 3 at 8q13.1 promotes cell proliferation and survival in hepatocellular carcinoma, Hepatology 55 (2012) 1754–1765.
[22] Y. Li, L. Chen, T.H. Chan, M. Liu, K.L. Kong, J.L. Qiu, Y.F. Yuan, X.Y. Guan, SPOCK1 is regulated by CHD1L and blocks apoptosis and promotes HCC cell invasiveness and metastasis in mice, Gastroenterology 144 (2013) 179–191. e174.
[23] D. Xie, J.S. Sham, W.F. Zeng, H.L. Lin, L.H. Che, H.X. Wu, J.M. Wen, Y. Fang, L. Hu, X.Y. Guan, Heterogeneous expression and association of beta-catenin, p16 and c-myc in multistage colorectal tumorigenesis and progression detected by tissue microarray, Int. J. Cancer 107 (2003) 896–902.
[24] J.V. Olsen, M. Vermeulen, A. Santamaria, C. Kumar, M.L. Miller, L.J. Jensen, F. Gnad, J. Cox, T.S. Jensen, E.A. Nigg, S. Brunak, M. Mann, Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis, Sci. Signal. 3 (2010) ra3.
[25] S.V. Holt, M.A. Vergnolle, D. Hussein, M.J. Wozniak, V.J. Allan, S.S. Taylor, Silencing Cenp-F weakens centromeric cohesion, prevents chromosome alignment and activates the spindle checkpoint, J. Cell Sci. 118 (2005) 4889–4900.
[26] J. Feng, H. Huang, T.J. Yen, CENP-F is a novel microtubule-binding protein that is essential for kinetochore attachments and affects the duration of the mitotic checkpoint delay, Chromosoma 115 (2006) 320–329.