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PES1 enhances proliferation and tumorigenesis in hepatocellular carcinoma via the PI3K/AKT pathway

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ABSTRACT

Aim: We investigated the potential role of pescadillo ribosomal biogenesis factor 1 (PES1) in the development of hepatocellular carcinoma (HCC).

Material and methods: One hundred and thirty-four patients with hepatocellular carcinoma were chosen to evaluate the association between the expression of PES1 and survival, clinical characteristics of these patients. Western blotting, real-time PCR, immunohistochemistry, CCK-8 assay, colony formation and subcutaneous tumors in nude mice were conducted.

Key findings: We found that PES1 was commonly upregulated in HCC tissues and cells. Immunohistochemical analysis of 134 paraffin-embedded archived HCC tissues showed that the protein expression level of PES1 was positively correlated with clinical characteristics and reduced the survival time of HCC patients. Univariate and multivariate analysis revealed that PES1 expression may be an independent prognostic indicator of poorer overall survival in HCC patients. Furthermore, silencing of endogenous PES1 significantly inhibited the proliferation and tumorigenicity of SMMC 7721 and HepG2 cells in vitro as well as in vivo in nude mice. Finally, we found that PES1 affected cell proliferation by regulating the PI3K/AKT/GSK3β/cyclinD1 signaling pathway.

Significance: Our data suggest that PES1 may promote proliferation and tumorigenicity, and potentially representing a novel prognostic marker for overall survival in HCC.

Core tip: We report that pescadillo ribosomal biogenesis factor 1 (PES1) plays an oncogenic role in hepatocellular carcinoma, which was commonly upregulated in hepatocellular carcinoma tissues and cells. Immunostaining analysis found that the protein expression level of PES1 was positively correlated with clinical characteristics and reduced survival time of hepatocellular carcinoma patients. Multivariate analysis revealed that PES1 expression might be an independent prognostic indicator of survival in hepatocellular carcinoma patients. Furthermore, PES1 knockdown inhibited the proliferation and tumorigenesis in hepatocellular carcinoma cell lines. Additionally, we found that PES1 is involved in the cell proliferation by regulating the AKT/GSK3β/cyclinD1 signaling pathway.

1. Introduction

Pescadillo ribosomal biogenesis factor 1 (PES1) is expressed in eukaryotes including fungus and humans [8,10]. PES1 is upregulated in breast, ovarian and colon cancers, as well as neuroblastoma [3,16,18,25]. Moreover, PES1 knockdown inhibits proliferation and turmorigenesis in above cancer cell lines. The close relationship between PES1 and oncogenesis can be the leading reason underlying this phenomenon.

Despite these discoveries, however, little is currently known regarding the role of PES1 in hepatocellular carcinoma (HCC). Given the potential role of PES1 in DNA replication and ribosomal biogenesis, both of which are important aspects of cancer initiation and progression, we hypothesized that PES1 may display growth promoting properties and drive tumorigenesis.

Primary liver cancer is a leading cause of cancer deaths, and it remains one of the refractory cancer types [6]. HCC accounts for 90% of all primary liver cancers [22]. The five-year survival rate of HCC still lingers at a low level. Even with major clinical interventions, including surgical resection, transplantation, locoregional therapy (such as

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TACE), targeted therapy (such as tyrosine-kinase inhibitors), and immunotherapy (such as anti-PD-1 monoclonal antibody), the survival rate has not significantly improved. The treatment of HCC is fairly challenging because of its molecular heterogeneity. Recent years have witnessed some success in targeted therapies for particular mutations in HCC, such as those in vascular endothelial growth factor and platelet-derived growth factor, and these strategies have been approved for use as first or second-line therapies in HCC [2,6,7,17,20]. Conceptually, investigation of PES1 in HCC may aid in identifying new targets that may add to the growing biomarker panel that can be used for diagnosis. It is imperative to uncover more novel molecules that are beneficial to the treatment and diagnosis of HCC.

In this study, we found that PES1 is up-regulated in clinical HCC samples, as revealed by western blotting (WB), real-time PCR (RT-PCR) and immunohistochemical (IHC) staining. Up-regulation of PES1 significantly correlated with poor clinical characteristics and risk factors, including survival, largest tumor size and T classification. Moreover, we knocked down PES1 in two HCC cell lines, SMMC 7721 and HepG2 by using shRNA, and evaluated the proliferation phenotype. We further evaluated the in vivo growth of PES1-knockdown cells in a mouse xenograft model. The current data support a growth promoting function of PES1 in HCC.

2. Materials and methods

2.1. Cell lines and cell culture

The HCC cell lines HepG2, SMMC 7721, BEL 7402, Huh7, Hep3B, SK-Hep-1, and PLC/PRF/5 were used. L02 were used as normal human liver cells. HepG2, Huh7, Hep3B, SK-Hep-1, and PLC/PRF/5 were purchased from the Library of Typical Culture of Chinese Academy of Sciences (Shanghai, China). L02 and BEL 7402 were purchased from Modern Analysis and Testing Center of Central South University (Changsha, China). SMMC 7721 cells were purchased from GeneChem Corporation (Shanghai, China). HepG2 and Hep3B were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. SMMC-7721, BEL 7402, and L02 cells were cultured in RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin. SK-Hep-1 and PLC/PRF/5 were cultured in MEM supplemented with 10% FBS and 1% penicillin/streptomycin. The above-mentioned cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Packaging of sh-PES1 lentivirus

The lentivirus vector system included the following vectors: pFu-GW-EGFP, for stable expression of a short hairpin RNA (shRNA) and a marker (EGFP fusion protein); pHelper1.0 (gag/pol element); and pHelper2.0 (VSVG element). The shRNA targeting human PES1 (#1: 5′-AGAAGATCATGTTTGGCAA-3′, #2: 5′-ATCATCAAGGAACGGTAT-3′) and the negative control (NC) shRNA (5′-TTCTCCGAACGTGTCA CGT-3′) were designed, synthesized and cloned into the pFu-GW-EGFP vector by the GeneChem Corporation (Shanghai, China). pFu-GW–shRNA-EGFP, pHelper1.0, and pHelper2.0 were mixed and transfected into 293T cells with Lipofectamine TM 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. After transfection for 48 h, the viral supernatants were collected, centrifuged, and filtered.

2.3. RNA extraction and real-time PCR

Total RNA from cells and tissues was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The 1000 ng of extracted RNA was used for cDNA synthesis (Takara, Tokyo, Japan). RT-PCR was performed with a denaturation step, followed by 40 cycles of amplification, and one cycle of both melting and cooling (Takara, Tokyo, Japan; Roche, Basel, Switzerland). Expression data were normalized to the geometric mean of the housekeeping gene β-actin and calculated as $2^{-[(\text{CT of indicated genes}) - (\text{CT of β-actin})]}$, where CT represents the threshold cycle for each transcript.

The primers used were PES1, forward: 5′-GGGCATTTATCCCCATG AACC-3′; PES1 reverse: 5′-CACCTTGTATTCACGGAACTTGT-3′; β-actin forward: 5′-CTACCTCATGAAGATCCTCACCGA-3′; and β-actin reverse: 5′-TTCTCCTTAATGTCACGCACGATT-3′.

Table 1
Clinicopathological characteristics of patient samples and expression of PES1 in hepatocellular carcinoma.

Characteristics	No. of cases (%)
Age	
< 60	82(54.7)
≥60	68(45.3)
Gender	
Male	112(83.6)
Female	22(16.4)
Etiology	
HBV	119(88.8)
HCV	3(2.2)
Alcohol	1(0.7)
Unknown	11(8.2)
Imaging cirrhosis	
Present	101(75.4)
Absent	21(15.7)
Distribution	
Unilobar	128(95.5)
Bilobar	6(4.5)
AFP level, ng/mL	
< 200	87(64.9)
≥200	47(35.1)
Method of diagnosis	
Imaging	133(99.3)
Biopsy	1(0.7)
BCLC stage	
0	12(9.0)
A	101(75.4)
B	15(11.2)
C	6(4.5)
Child-Pugh (at randomization)	
A	125(93.3)
B	8(6)
C	1(0.7)
T classification	
T1	99(73.9)
T2	9(6.7)
T3	25(18.7)
T4	1(0.7)
N classification	
N0	129(96.3)
N1	5(3.7)
Metastasis	
No	132(98.5)
Yes	2(1.5)
Vital states (at follow-up)	
Alive	56(41.8)
Dead	23(17.2)
Lost	55(41.0)
Expression of PES1	
Low expression	23(17.2)
High expression	111(82.8)

2.4. Western blotting

Cells and tissues were harvested in RIPA lysis buffer (Beyotime, Shanghai, China) and heated for 10 min at 100 °C. Protein concentrations were determined with a BCA protein assay kit (Beyotime, Shanghai, China). Equal quantities of protein were separated electrophoretically on 10% SDS/polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Billerica, MA, USA). The membranes were probed with anti-PES1 rabbit antibody

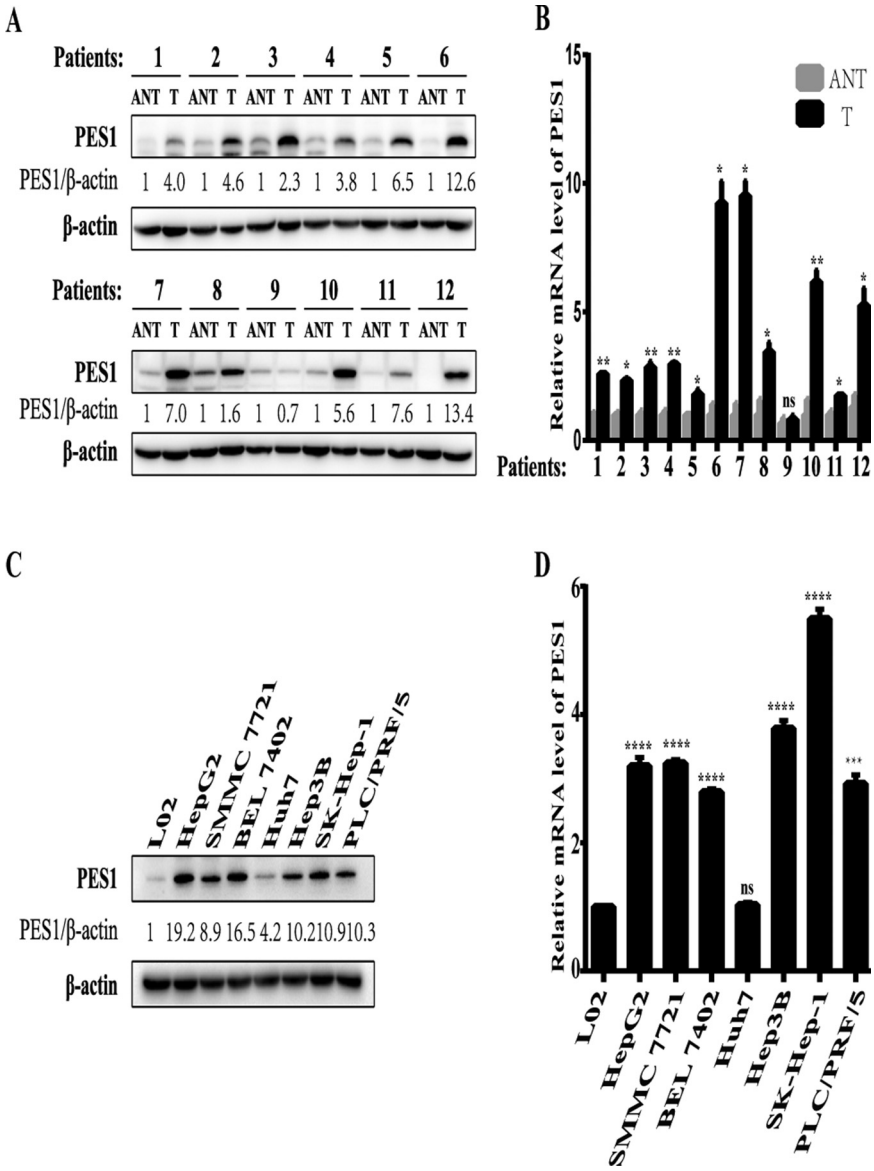


Fig. 1. PES1 is upregulated in HCC. A. WB analysis of PES1 in HCC tissues (T) and matched adjacent non-cancerous tissues (ANT). Levels of PES1 quantified by optical density and normalized to β-actin levels in the same samples are shown below the WB results relative to levels in ANT. B. RT-PCR analysis of PES1 in 12 paired T and their ANT. The average PES1 mRNA expression was normalized to the expression of β-actin. C. PES1 protein expression was detected in L02 and HCC cell lines through WB analysis. Levels of PES1 quantified on the basis of optical density and normalized to β-actin levels in the same samples are shown below the WB results relative to levels in L02 cells. D. PES1 mRNA expression was detected in L02 and HCC cell lines by using RT-PCR analysis. Three independent experiments were conducted in each assay. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(Proteintech, Chicago, USA), and antibodies to phosphor-(p-)PI3K, PI3K, p-AKT, AKT, and cyclinD1. The expression of each protein was determined with secondary antibodies (Zhongshan Golden Bridge, Beijing, China) and High-sig ECL Western Blotting Substrate (Tanon, Shanghai, China) according to the manufacturers' suggested protocols. The membranes were stripped and reprobed with anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA) as a loading control.

2.5. Immunohistochemistry

A total of 134 paraffin-embedded HCC samples from patients diagnosed at the First Affiliated Hospital of Anhui Medical University between 2014 and 2016 were used. Clinicopathological classification and staging were determined according to the criteria of the American Joint Committee on Cancer [4]. Patient consent and approval from the Institutional Research Ethics Committee were obtained for the use of these clinical materials for research purposes before the experiments were performed. The clinicopathological features of the patients are summarized in Table 1.

To study PES1 protein expression in HCC tissues, we performed IHC. In short, paraffin-embedded specimens were cut into 5 μm sections. The sections were deparaffinized and microwaved for antigenic retrieval. Then the sections were treated with an endogenous peroxidase blocker, then, incubated with goat serum to block nonspecific binding, and then with anti-PES1 overnight at 4 °C.

After samples were washed in PBS three times, a secondary antibody was used for further incubation. Diaminobenzidene (Zhongshan Golden Bridge, Beijing, China) was used as a chromogen for color development. Then the slides were counterstained with hematoxylin, and dehydrated. Finally, the tissue sections were observed at 100×, 200×, and 400× magnification, and images were taken from six random fields of view. Quantitative analysis of IHC was performed with ImageJ and IHC Profiler software [23].

2.6. Cell proliferation assays

The proliferation of HepG2 and SMMC 7721 cells was determined with CCK-8 assays (Biosharp, Hefei, China). These two cell lines were seeded in four 96-well plates. One of the plates was measured after growing for 4 h, and the other three plates maintained for 24 h, 48 h, and 72 h at 37 °C, respectively. Then 100 μL fresh medium containing CCK-8 (10 μL) was put into each well and incubated at 37 °C for 2 h. The absorbance was measured at 450 nm.

Table 2
Correlation between PES1 expression and clinicopathological characteristics of hepatocellular carcinoma patients.

Characteristics	PES1		*p* value
	Low, no. cases	High, no. cases	
Age			
< 55	12	61	0.059
≥55	11	50	
Gender			
Male	16	96	0.046
Female	7	15	
Etiology			
HBV	21	98	0.113
HCV	0	3	
Alcohol	1	0	
Unknown	1	10	
Imaging cirrhosis			
Present	19	82	0.305
Absent	2	19	
Distribution			
Unilobar	23	105	0.254
Bilobar	0	6	
AFP level, ng/mL			
< 200	17	70	0.321
≥200	6	41	
BCLC stage			
0	4	8	0.306
A	17	84	
B	2	13	
C	0	6	
Child-Pugh (at randomization)			
A	20	105	0.265
B	3	5	
C	0	1	
T classification			
T1	21	78	0.201
T2	1	8	
T3	1	24	
T4	0	1	
N classification			
N0	22	107	0.864
N1	1	4	
Metastasis			
No	23	109	0.517
Yes	0	2	
Vital states (at follow-up)			
Alive	11	45	0.022
Dead	0	23	

2.7. Colony formation assays

For colony formation assays, approximately 200 infected cells were seeded into six-well plates and cultured for 2 weeks at 37 °C. After removal of the medium and washing with PBS, the cells were fixed in 4% paraformaldehyde for 20 min and then stained with Giemsa stain (Solarbio, Beijing, China). Only positive colonies (> 50 cells/colony) in the plates were counted and compared.

2.8. Nude mouse tumor growth and IHC analysis

Nude mice (BALB/c) were purchased from SLAC Laboratory Animal (Shanghai, China). The experiment was approved by the Institute Animal Ethical and Welfare Committee. The mice were acclimated to 12 h light-dark cycle with 50% humidity and with free access to food and water for 2 weeks prior to experimentation. Cell stably expressing SMMC7721/control shRNA, and SMMC7721 shRNA were resuspended at 5×10^6 cells/mL, and a 0.1 mL aliquot of cell suspension was injected subcutaneously into the right flank of the nude mice ($n = 10$/group). Tumor size was measured every 2 days. Tumor volumes were determined by external measurements and calculated according to the following equation: $V = [L \times W^2] \times \pi/6$ (V = volume, L = length and W = width). Thirty-three days later, the mice were sacrificed, and transplanted tumors were removed for assessment. All protocols in our animal experiments were in accordance with the Animal Ethical and Welfare Committee.

IHC analysis was conducted as described above. Briefly, after being embedded in paraffin for 48 h, xenografts were cut into 5 μm sections and incubated with the anti-PES1, anti-Ki67 (Abcam, Cambridge, MA, USA) primary antibodies, then visualized with corresponding secondary antibodies. After staining and sealing of samples, photographs were taken at the magnifications of 100×, 200×, and 400×.

2.9. Statistical analysis

SPSS 23.0 (IBM, Armonk, NY, USA) and GraphPad Prism 7.0 software (La Jolla, CA, USA) were used for analysis. Chi-square tests were used to analyze the correlation between PES1 expression and the clinicopathological characteristics. Spearman's rank correlation coefficient was used to evaluate the bivariate correlations between the variables. Survival curves were plotted with the Kaplan-Meier method and compared through the log-rank test. The significance of various variables for survival was analyzed with the Cox proportional hazards model in univariate and multivariate analysis. Student's *t*-tests or nonparametric tests were applied to analyze the differences between two groups. Differences among groups were calculated by one-way analysis of variance (ANOVA) with repeated measures. All data are shown as mean ± SEM from at least three independent experiments. *p*-values < 0.05 were considered significant.

3. Results

3.1. PES1 is up-regulated in HCC tissues and cell lines

To investigate the crucial role of PES1 in HCC, we first detected the expression patterns of PES1 in HCC tissues and cell lines. WB analysis revealed that PES1 protein levels were significantly up-regulated in the 12 tested liver tumor tissues (T) compared with the matched adjacent noncancerous tissues (ANT) (Fig. 1A). RT-PCR analysis verified that PES1 mRNA expression was indeed up-regulated in T compared with ANT samples (Fig. 1B). Furthermore, to examine the expression of PES1 in cell lines, we analyzed seven HCC cell lines and L02. WB and RT-PCR analyses revealed that PES1 protein and mRNA were both markedly overexpressed in the six HCC cell lines tested compared with L02 (Fig. 1C and 1D). These results were strongly indicative of upregulation of PES1 in human liver cancer.

3.2. High PES1 expression in HCC tissues correlates with poor patient survival

To explore the frequency of PES1 upregulation in HCC, we examined its expression by using IHC in 134 paraffin-embedded, archived HCC tissues (Table 1). These samples were stained with antibody to PES1 and scored with a standard method (summarized in Table 2). Compared with the ANT tissues, in which PES1 was undetectable or was detected at low levels, PES1 was overexpressed in the HCC specimens. As shown in Fig. 2A, PES1 expression was observed at high levels in the tumor cells from 111 samples (111/134, 82.8%), and PES1 was predominantly localized to the nucleus. Kaplan-Meier survival curves demonstrated that the overall survival of patients with high expression of PES1 was significantly shorter than that of patients with low PES1 expression (Fig. 2B, $p < 0.05$). Collectively, these results indicate that overexpression of PES1 in HCC patients correlates with poor survival.

3.3. Upregulation of PES1 is associated with advanced clinicopathological features of HCC

We analyzed the association between PES1 and the

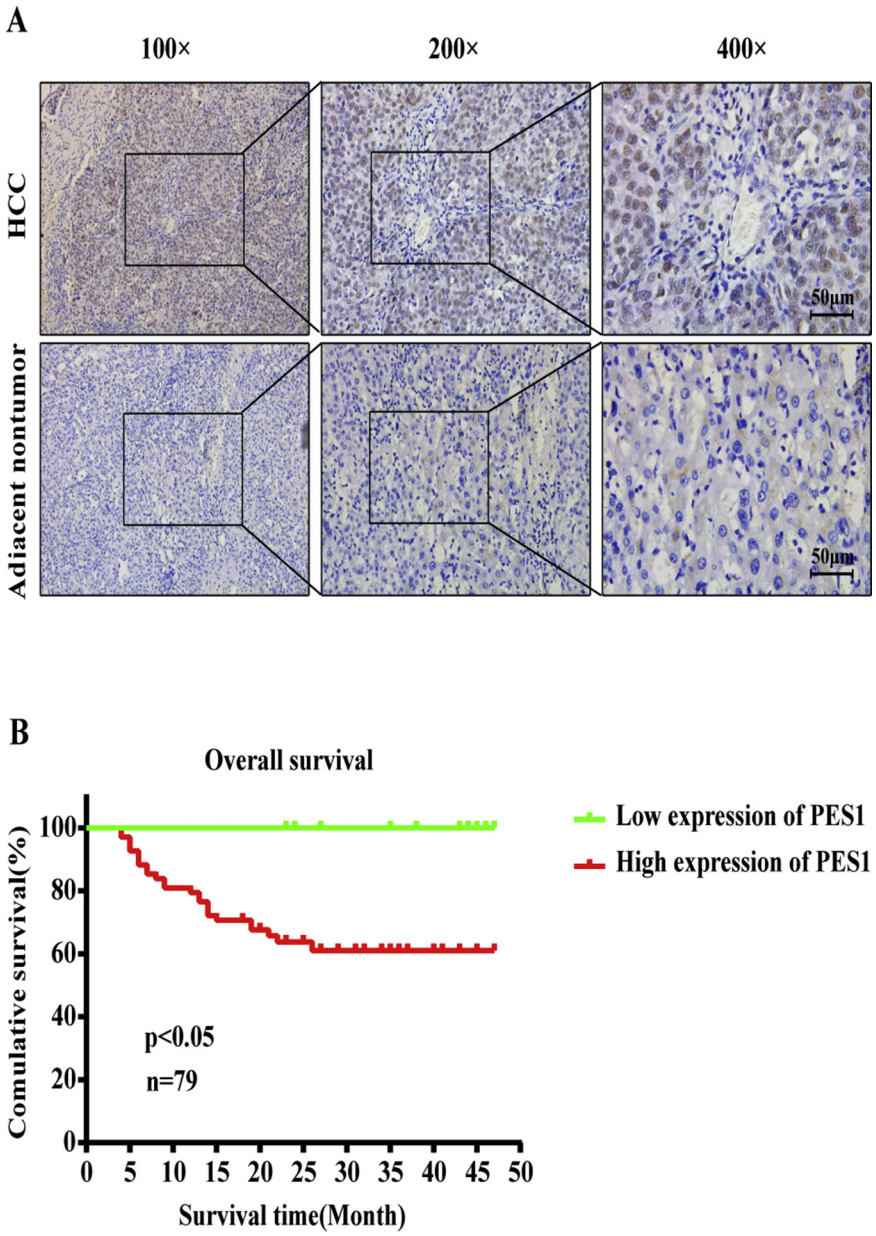


Fig. 2. High PES1 expression in HCC tissues correlates with poor patient survival. A. Expression levels of PES1 in HCC tissues compared with their paired adjacent noncancerous tissues as determined through IHC staining. B. Overall survival curves for all 79 patients with HCC stratified by high and low expression of PES1.

clinicopathological features of HCC. As shown in Table 2, there were associations between PES1 expression and gender ($p = 0.046$), vital states ($p = 0.022$). However, there was no association between PES1 expression and age ($p = 0.059$), etiology ($p = 0.113$), imaging cirrhosis ($p = 0.305$), tumor distribution ($p = 0.254$), AFP level (p = 0.254), BCLC stage ($p = 0.306$), Child-Pugh score ($p = 0.265$), T classification ($p = 0.201$), N classification ($p = 0.864$), or metastasis ($p = 0.517$).

As shown in Table 3, Spearman analysis of the correlation between PES1 expression and clinicopathological features manifested that the expression of PES1 was significantly correlated with the largest tumor size ($p = 0.046$) and vital states ($p < 0.001$). Furthermore, the relative risk associated with PES1 in the prognosis of HCC was analyzed. Cox-regression analysis was used to determine whether PES1 might be a risk factor. According to Table 4, high expression of PES1 was associated with a significantly increased risk of death in HCC patients ($p < 0.001$) compared with patients with low PES1 expression, on the basis of univariate Cox regression analyses (Table 4). Multivariate Cox regression analysis illustrated that PES1 was a factor predictive of poor survival when PES1 expression ($p < 0.001$), largest tumor size ($p = 0.044$) and T classification ($p = 0.034$) were included (Table 4). These results demonstrated a significant correlation between the PES1 expression with and HCC prognosis.

3.4. PES1 modulates proliferation of HCC

To explore the role of PES1 in HCC, we knocked down PES1 with lentivirus-mediated shRNA in SMMC 7721 and HepG2 cell lines. The protein and mRNA expression levels of PES1 were significantly decreased by shRNA1 and shRNA2 in both SMMC 7721 and HepG2 cells (Fig. 3A–3B). CCK8 assays indicated that, in both SMMC 7721 and HepG2 cells, the cell numbers were substantially decreased by knockdown of PES1 (Fig. 3C–3D). Likewise, colony formation assays showed that PES1 knockdown in both SMMC 7721 and HepG2 cells dramatically impaired colony formation (Fig. 3E–3F). As exhibited in the graph, PES1 is indispensable for the proliferation of HCC.

Table 3
Spearman analysis of correlation between PES1 and clinicopathological features.

Variables	PES1 expression level	
	Spearman correlation	*p* value
Age	0.061	0.486
Gender	−0.085	0.327
Etiology	−0.056	0.524
Imaging cirrhosis	0.096	0.294
Distribution	0.107	0.217
Largest tumor size	0.172	0.046
AFP level, ng/mL	0.025	0.786
Pathological differentiation	0.018	0.84
BCLC stage	0.141	0.105
Child-Pugh (at randomization)	−0.06	0.49
T classification	0.108	0.214
N classification	0.056	0.519
Metastasis	0.095	0.274
Vital states (at follow-up)	0.419	< 0.001

3.5. PES1 regulates HCC tumorigenesis

To validate the biological function of PES1 in tumorigenesis, we performed a xenograft formation experiment in BALB/C nude mice by using SMMC 7721 cells with/without stable PES1 knockdown. As shown in Fig. 4A, PES1 knockdown cells (transfected with shRNA2), compared with the negative control (NC) group, had a weak ability to form tumors in nude mice. In addition, the xenograft tumor growth curves measured on the basis of tumor volume and tumor weight were suggestive (Fig. 4B–4C). WB analysis favored that the expression of PES1 was dramatically decreased in the tumors formed by PES1 knockdown cells rather than control cells (Fig. 4D). The expression of PES1 and Ki67 protein was lower in tumors formed by PES1 knockdown cells (Fig. 4E). These results together indicate that, PES1 may play a crucial role in the tumorigenicity of HCC cells.

3.6. The PI3K/AKT/GSK3β/cyclin D1 signaling pathway is regulated by PES1

As exhibited in Fig. 4F–4G, the knockdown of PES1 resulted in down-regulation of phosphorylated-PTEN(p-PTEN), phosphorylated-PI3K(p-PI3K), p-AKT, p-GSK-3β, and cyclinD1 in both SMMC 7721 and HepG2 cells. Thus, PES1 appears to modulate the proliferation and tumorigenesis of HCC, via regulating the crucial proteins in the PI3K/AKT/GSK3β/cyclinD1 signaling pathway.

4. Discussion

In this study, we first demonstrated that PES1 plays a tumor promoting role in HCC. First, we showed that PES1 was commonly upregulated in HCC tissues and cells. Second, IHC analysis indicated that the protein expression of PES1 was positively correlated with clinical characteristics and shorter survival time in HCC patients. Multivariate analysis revealed that PES1 expression might be an independent prognostic indicator of survival in HCC patients. Third, our data indicated that PES1 knockdown inhibited proliferation and colony formation in HCC cells in vitro and tumor growth in vivo. Fourth, PES1 was found to be involved in cell proliferation through regulation of the PI3K/AKT/GSK3β/cyclinD1 signaling pathway.

These results corroborate the idea of the ideas of Fan Ping et al., who suggested that PES1 promotes cell proliferation in HCC [5]. They demonstrated that bromodomain-containing protein 4 (BRD4) acts as upstream transcriptional component of PES1, while this study focused on the downstream pathway. Moreover, they manifested that high expression of PES1 related to poor prognosis in HCC patients according to the ONCOMINE microarray gene expression database, while the current study collected 134 HCC specimens and their clinical baseline characteristics that confirm the association between expression of PES1 and prognosis of PES1 patients.

Previous studies have reported that PES1 is involved in important biological processes, such as embryonic development, ribosome biogenesis, cell cycle regulation, DNA replication, and chromosome stability [9,11,12,14]. Recently, Long Cheng et al. reported that PES1 is overexpressed in breast cancer and promotes breast cancer [3]. Elisa Lázaro-Ibáñez et al. demonstrated that PES1 is higher in microvesicles from PC-3 and LNCaP prostate cancer cells than in benign PNT2 prostate cells [13]. Jun Li et al. reported that down-regulation of pescadillo inhibits proliferation and tumorigenicity of breast cancer cells through regulation of AKT/GSK3β/cyclinD1 signaling pathway [15]. Masato Nakaguro et al. showed that PES1 is a marker of neuroblastoma outcome and is associated with neuroblastoma differentiation [18]. In the present study, we found that the expression of PES1 was upregulated dramatically in HCC, thereby triggering the development of HCC.

Interestingly, the data in Table 2 indicated male dominance in all subjects ($p < 0.05$). Sex hormones metabolic abnormalities in the liver have been widely reported to give rise to the sex disparity in HCC, androgens have a tumor promoting function, whereas estrogen has a protective role [19]. Estrogen receptor α (ERα) is widely acknowledged to be abundant in the liver, and it plays a key role in cell proliferation and tumorigenesis. Moreover, the absence of ERα leads to HCC [1,21]. Recently, some researchers have suggested that the degradation of ERα ultimately leads to the progression of HCC [24]. However, others have proposed that PES1 causes breast cancer to develop by enhancing the levels of ERα and decreasing levels of ERβ [3]. Hence, we speculate that the growth promoting role of PES1 in hepatocellular carcinoma may be ascribed to the regulation of ERα and ERβ.

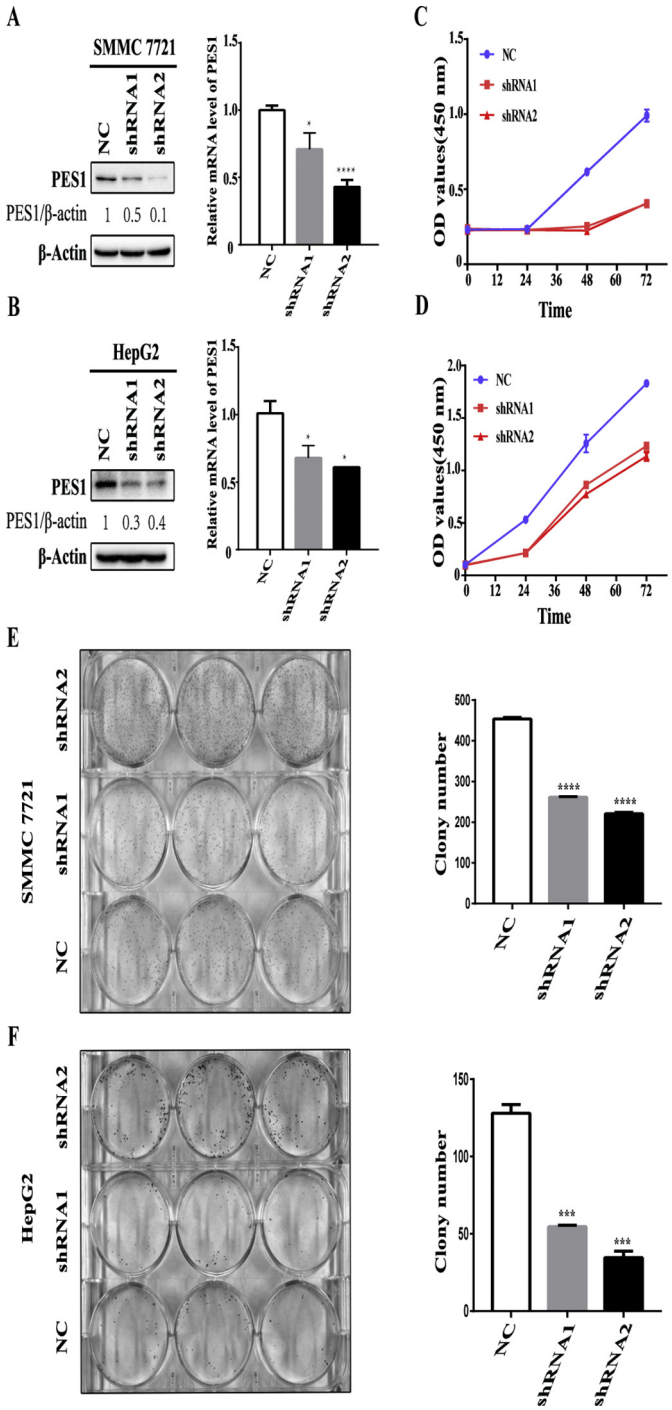
These conclusions are based on the responses of only 134 patients who met operation criteria, and our results thus might not reflect the patients without indications for operation. Because of the incorrect contact information or the changes in contact information, we were unable to determine the survival status of 55 patients. We are now confirming our results in more patients and mice. Moreover, PES1 is not a kinase and thus cannot activate PI3K/AKT/GSK3β/cyclinD1 signaling pathway directly. We are now exerting a tremendous fascination on its direct mechanism.

5. Conclusions

In summary, PES1 has a novel role in regulating proliferation and tumorigenesis in HCC and thus may be a potential therapeutic target for

Table 4
Univariate and multivariate analysis of various prognosis parameters in patients with liver cancer Cox-regression analysis.

	Univariate analysis			Multivariate analysis		
	p value	Hazard ratio	95% Confidence interval	*p* value	Hazard ratio	95% Confidence interval
PES1	< 0.001	4.42	2.103–9.292	0.001	4.094	1.838–9.121
Largest tumor size	< 0.001	1.215	1.097–1.345	0.044	1.117	1.003–1.244
T classification	< 0.001	2.168	1.419–3.312	0.034	1.677	1.039–2.707



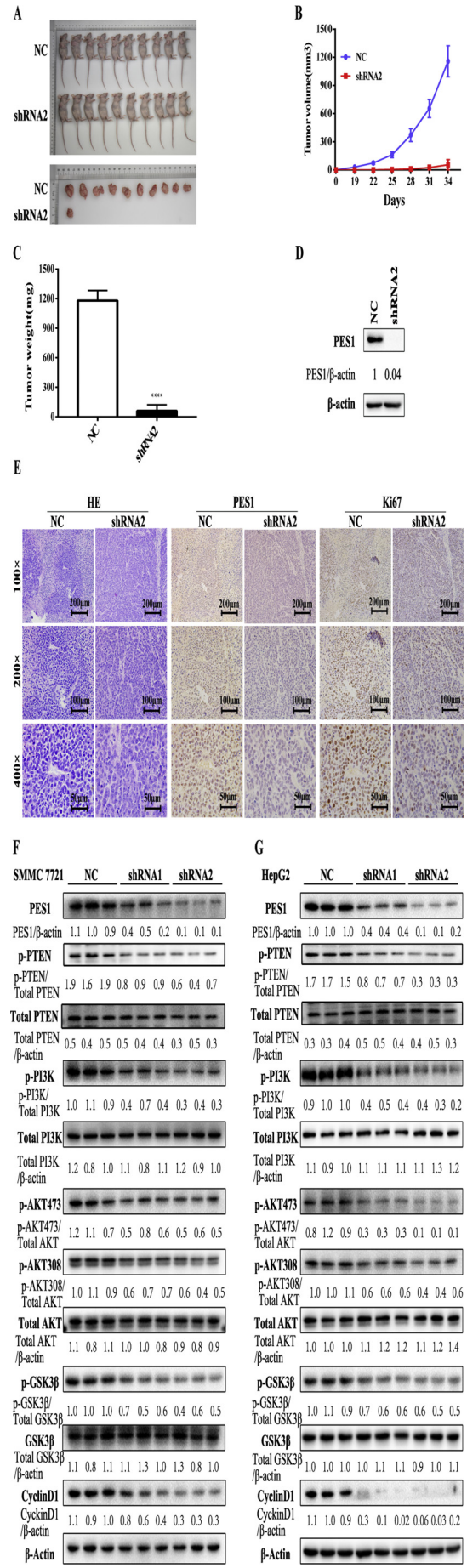


Fig. 3. PES1 modulates proliferation of HCC. A. WB and RT-PCR analysis of SMMC 7721 cells transfected with negative control (NC) shRNA, and PES1 shRNA1 and shRNA2. Levels of PES1 quantified on the basis of optical density and normalized to β-actin levels in the same samples are shown below the WB results relative to levels in NC cells. B. WB and RT-PCR analysis of HepG2 cells transfected with NC shRNA, PES1 shRNA1, and shRNA2. Levels of PES1 quantified on the basis of optical density and normalized to β-actin levels in the same samples are shown below the WB results relative to levels in NC cells. C. CCK8 assays revealing that PES1 knock-down inhibits the proliferation of SMMC 7721 cells. D. CCK8 assays revealing that PES1 knock-down inhibits the proliferation of HepG2 cells. E.–F. Downregulation of PES1 reduced the colony number in colony formation assays. Results are representative of at least three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(caption on next page)

Fig. 4. PES1 knock-down affects HCC tumorigenesis by regulating the PI3K/AKT/GSK3β/cyclin D1 signaling pathway. A. Subcutaneous tumors in nude mice and excised tumors after 34 days, which were formed by injection of SMMC 7721 cells infected with NC shRNA and PES1 shRNA2 lentivirus ($n = 10$ in each group). B. Tumor volumes were measured every 3 days. C. Average tumor weights of the xenografts. D. WB analysis of PES1 expression in excised xenograft tumors. Levels of PES1 quantified on the basis of optical density and normalized to β-actin levels in the same samples are shown below the WB results relative to levels in NC cells in treated xenograft tumors. E. Representative images of sections sliced from the indicated tumors and stained with hematoxylin and eosin, anti-PES1 and anti-Ki67. F.–G. WB analysis of PES1, p-PTEN, total PTEN, p-PI3K, total PI3K, p-AKT, total AKT, p-GSK-3β, total GSK-3β, cyclin D1 in SMMC 7721 and HepG2 cells. Levels of PES1, p-PI3K, total PI3K, p-AKT, total AKT, p-GSK-3β, total GSK-3β, and cyclin D1 quantified on the basis of optical density and normalized to β-actin levels in the same samples are shown below the WB results relative to average levels in NC cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

HCC. Moreover, the level of PES1 is predictive of the survival time of HCC patients.

Author contributions

Wang J performed the majority of experiments, analyzed the data and wrote the paper; Sun J performed the molecular investigations and the treatment of animals; Zhang N and Yang RJ participated equally in the molecular investigations and the treatment of animals; Li HX and Zhang YJ participated in the collection of clinical data; Chen KY and Kong DR designed and coordinated the research, as well as the critical revision of the manuscript.

Conflict-of-interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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