Hepatology International (2019) 13:180–189
https://doi.org/10.1007/s12072-018-9920-8

ORIGINAL ARTICLE

Plasminogen activator inhibitor 2 (PAI2) inhibits invasive potential of hepatocellular carcinoma cells in vitro via uPA- and RB/E2F1-related mechanisms

Ye Jin[1] · Zhi-Yong Liang[2] · Wei-Xun Zhou[2] · Li Zhou[3]

Received: 10 September 2018 / Accepted: 10 December 2018 / Published online: 1 January 2019

Abstract

Background Plasminogen activator inhibitor 2 (PAI2) has been shown to be associated with invasive phenotypes and prognosis in hepatocellular carcinoma (HCC). However, its biological roles and underlying mechanisms in invasion of HCC have not been explored. The present study aimed to address the issues.

Methods First, sub-lines in that PAI2 was stably overexpressed and silenced were established based on MHCC97H and BEL7402 cell lines, respectively. Wound-healing and transwell assays were applied to evaluate cell migration and invasion. Urokinase-type plasminogen activator (uPA) activity was measured using an ELISA kit. Real-time RT-PCR and western blotting were used to show gene expression at mRNA and protein levels. E2F1 expression in human specimens was determined by tissue microarray-based immunohistochemical staining.

Results The sub-lines, MHCC97H-PAI2 and BEL7402-siPAI2, were successfully established. The two sub-lines carried much lower and higher migration and invasion powers, respectively, in contrast to the controls. In MHCC97H-PAI2 sub-line, intra-medium uPA activity was significantly decreased, while RB expression was obviously elevated, compared with the controls. The BEL7402-siPAI2 sub-line presented the opposite trend. To identify the role of RB/E2F1 pathway, we transiently overexpressed E2F1 in MHCC97H-PAI2 sub-line, and largely reversed the inhibitory effects of PAI2 on cell migration and invasion, through regulating multiple matrix metalloproteinases and epithelial–mesenchymal transition. In HCC specimens, E2F1 expression was much higher in tumor than in non-tumor tissues, and was significantly related to Edmondson–Steiner grade, overall as well as tumor-free survival.

Conclusions Our data suggest that PAI2 inhibits invasive potential of HCC cells via uPA- and RB/E2F1-related mechanisms.

Keywords Hepatocellular carcinoma · Plasminogen activator inhibitor 2 · Urokinase-type plasminogen activator · RB/E2F1 · Prognosis

Introduction

Hepatocellular carcinoma (HCC) is a highly prevalent malignant neoplasm worldwide. Currently, HCC remains to carry a poor overall prognosis, based on its high ranking in the cancer-related death [1]. Although some favorable results of curative hepatic resection for extensively selected patients have been reported [2, 3], tumor recurrence was well known as a frequent event after surgery for HCC [1]. No doubt post-resectional recurrence largely limits the long-term outcome of HCC [4]. Therefore, its underlying mechanisms are of large interest. Thus far, some recurrence-associated genes have been found, including those involved in cell invasive phenotypes, such as cell migration, motility and adhesion [5, 6], whereas some tumor biological factors, such as tumor

---

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12072-018-9920-8) contains supplementary material, which is available to authorized users.

✉ Li Zhou
lizhou02@hotmail.com

[1] Clinical Research Laboratory, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences/Peking Union Medical College, Beijing 100730, China

[2] Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences/Peking Union Medical College, Beijing 100730, China

[3] Department of General Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences/Peking Union Medical College, Beijing 100730, China

microenvironment, cancer stem cells (CSC) and epithelial–mesenchymal transition (EMT), were also recognized to play important roles in invasive/metastatic proclivity of HCC [7–9]. However, novel clues are quite expected.

It has been well acknowledged that proteolytic degradation of extracellular matrix (ECM) is an important step in tumor invasion and metastasis [10]. In the process, urokinase-type plasminogen activator (uPA) system serves as a central player, proven by many investigations [11, 12]. Among the system, plasminogen activator inhibitor 2 (PAI2) is one of two serine protease inhibitors [13]. Thus far, PAI2 was shown, by observations of different biological behaviors, to exert pro-oncogenic or anti-oncogenic function in different malignant tumors [14–18], indicating that it might have a unique molecular mechanism for an individual phenotype. In HCC, we previously found that PAI2 expression was much downregulated and was significantly associated with the absence of portal vein tumor thrombosis (PVTT), smaller tumor size and prolonged patient survival [19, 20]. These findings support the possible role of PAI2 as a negative regulator of invasion in HCC. However, its biological roles, in particular its underlying mechanisms, remain unknown. Considering part nuclear location of PAI2 in HCC cells [20], and clues that retinoblastoma (RB) was protected by PAI2 [21, 22], whether RB was involved in them was of large interest.

The present study was designed to address the issues, based on invasion-related experiments in HCC cell lines.

Materials and methods

Establishment of PAI2 stably overexpressed and silenced sub-lines, and transient overexpression of E2F1

The sub-lines in that PAI2 were stably overexpressed and silenced were established, based on two HCC cell lines with different invasive power, i.e. MHCC97H and BEL7402. The cell lines, obtained from the Cell Bank of Chinese Academy of Sciences Shanghai Institute of Cell Biology (Shanghai, China), were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS, Hyclone, Thermo Fisher Scientific Inc, Waltham, MA), 100 U/mL penicillin and 10 mg/mL streptomycin at 37 °C with 5%CO_2 at a humidified atmosphere. In the first, PAI2 cDNA was amplified by polymerase chain reaction (PCR) and then sub-cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). On the other hand, four PAI2 siRNA sequences (no. 1, sense: AAGGUAUCCCUAUUUUUGAAGCCUGUCUC, antisense: AACUUCAAAAAUAG GGAUACCCCUGUCUC; no. 2, sense: CAGAGAACAACCAGAUUGA, antisense: UCAAUCUGGUUGUUCUCUG; no. 3, sense: GCGCACACCUGUACAGAUG, antisense: CAUCUGUACAGGUGUGCGC; no. 4, sense: GCGCACACCUGUACAGAUGAUGUAC, antisense: GUACAUCAUCUGUACAGGUGUGCGC) were used. Scrambled sequences were used as the controls. Following, the duplexes were inserted into the vector pcDNA6.2 (Invitrogen); the recombinant lentiviruses were packaged using the pLent6.31 expression system for overexpression and the pLenti6.2 miR RNAi expression system for knockdown (Invitrogen). For overexpression of E2F1, the E2F1 coding sequences were first amplified and inserted into pcDNA3.1 vector (Invitrogen), according to the method previously described [23]. Then, transient transfection was performed using the Lipofectamine 3000 reagent (Invitrogen).

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

After RNA extraction using TRIzol reagents (Invitrogen) and reverse transcription with a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA), qRT-PCR was performed with All-in-One qRT-PCR Mix (GeneCopoeia, Rockville, MD, USA) on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The used primers were as follows: PAI2, 5′-AACCCCAGGCAGTAGACTTC-3′ (forward), 5′-ACAGCATTCACCAGGACCAT-3′ (reverse); RB, 5′-ACCCAGAAGCCATTGAAATC-3′ (forward), 5′-TCTGGGTGCTCAG ACAGAAG-3′ (reverse); E2F1, 5′-CATCCCAGGAGGTCACTTCTG-3′ (forward), 5′-GAC AACAGCGG TTCTTGCTC-3′ (reverse); MMP9, 5′-CTTTGGACACGCACGAC-3′ (forward), 5′-CCACCTGGTTCAACTCACT-3′ (reverse); MMP14, 5′-TGGACTGTCAGGAATGAGGAT-3′ (forward), 5′-CATAGGTGGGGTTTTTGGGT-3′ (reverse); MMP15, 5′-GCTGCTCCTGGTGCTTCT-3′ (forward), 5′-CTGAGGCAGGTAGCCATAAAG-3′ (reverse); GAPDH, 5′-CATCTTCTTTTGCGTCGCCA-3′ (forward), 5′-TTAAAAGCAGCCCTGGTGACC-3′ (reverse). GAPDH was served as a housekeeping gene. All reactions were repeated in triplicates.

Western blotting

Extracted total proteins (80 μg/lane), after protein concentration measurement by a BCA protein assay kit (Pierce), were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking by 5% non-fat dry milk, the membranes were incubated overnight at 4 °C with primary antibodies for PAI2, RB, E2F1, MMP9, MMP14, MMP15, E-cadherin, *N*-cadherin, Vimentin, GAPDH and β-actin (Abcam, Cambridge, UK). The subsequent incubations with horseradish peroxidase-conjugated secondary antibodies were performed at room temperature. Protein bands were detected by enhanced chemiluminescence reagents (Merck, Darmstadt, Germany). GAPDH or β-actin was used as the loading control. All blots were repeated three times.

Wound-healing assay

Cells were seeded in six-well plates. When 80% confluence was reached, cell layers were scratched using 200-μL pipette tips. After a culture of 24 h, the wound closure was observed by microscopy. For cells with E2F1 overexpression, the experiments were started 72 h after plasmid transfection. All assays were repeated in triplicates.

Transwell invasion analysis

Transwell inserts (pore size: 8.0 μm, Corning, Chelmsford, ST, USA) and extracellular matrix (ECM) gel (Sigma-Aldrich, St. Louis, MO, USA), that was coated under surface of the membrane, were used. In brief, 500 μL of medium with 20% FBS was added to the lower chamber. Cells (5×10^4/well) were re-suspended in serum-free media and seeded to the upper chamber. After an incubation of 24 h at 37 °C, cells on the upper surface of the membrane were removed. Invaded cells were fixed with methanol and stained with crystal violet. Finally, the cells were counted in five random fields at a magnification of × 200. The results were derived from three independent experiments.

Cell proliferation assay

Cell proliferation was determined using the Cell Counting Kit-8 (CCK8, Dojindo, Kumamoto, Japan). Cells were seeded at 4.0×10^3 cells/well in 96-well microplates. After cultures of 0 h, 24 h, 48 h and 72 h, fresh DMEM and 10 μL/well CCK-8 solution were replaced. Then, the plates were incubated at 37 °C for 2 h. Finally, the absorbance at 450 nm was measured.

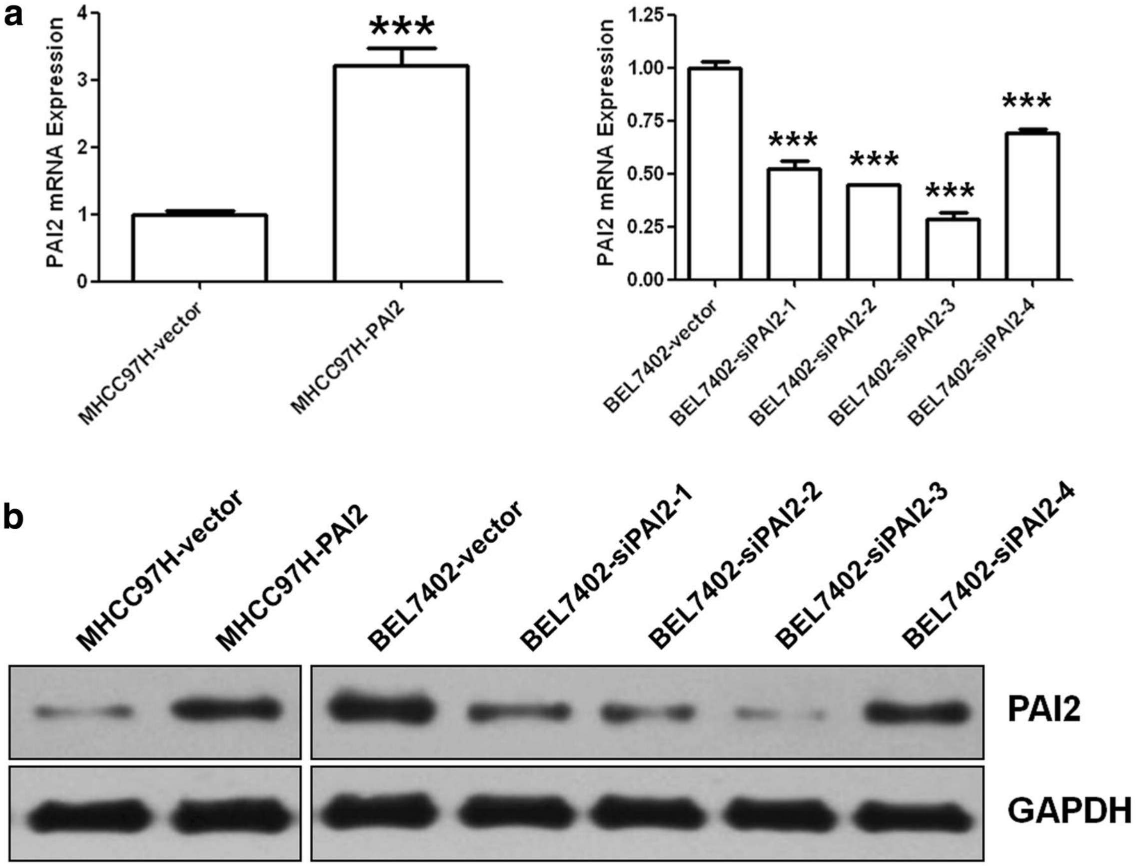

Fig. 1 Establishment of PAI2 stably overexpressed and silenced sub-lines. **a** Significantly enhanced and decreased PAI2 mRNA expression in MHCC97H-PAI2 sub-line and four BEL7402-siPAI2 sub-lines; **b** significantly enhanced and decreased PAI2 protein expression in MHCC97H-PAI2 sub-line and four BEL7402-siPAI2 sub-lines. ***$p < 0.001$

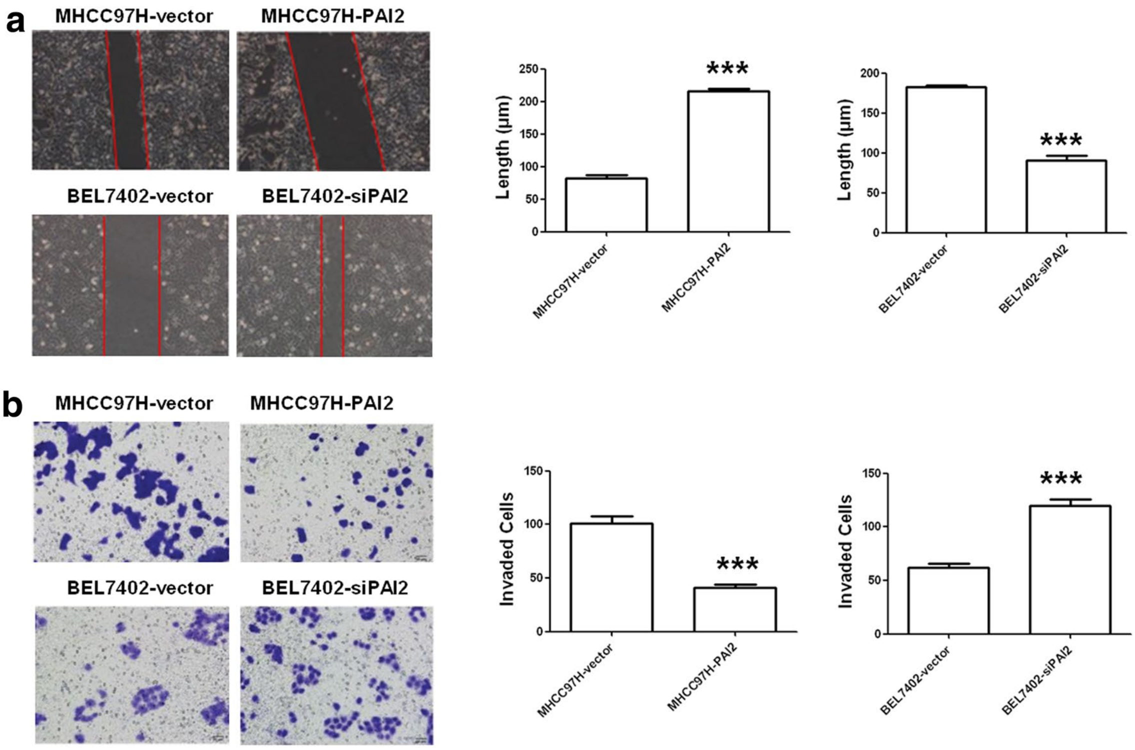

Fig. 2 Changes of migration and invasion in PAI2 stably overexpressed and silenced sub-lines. **a** Wound-healing assays in MHCC97H-PAI2 and BEL7402-siPAI2 sub-lines; **b** transwell invasion analyses in MHCC97H-PAI2 and BEL7402-siPAI2 sub-lines. ***$p < 0.001$

Measurement of uPA activity in the cell medium

uPA activity was measured by a uPA Activity Assay Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, a standard curve was first generated. Then, the mixture of cell medium and assay buffer was incubated with chromogenic substrate. The optical density (OD) value of each well was measured using a standard microplate reader (405 nm). The two measured OD values of the same well were averaged to form the final one. The relative uPA activity was calculated based on the standard curve.

Patients, tissue microarray construction and immunohistochemistry

The study group consisted of 149 curatively resected patients with HCC. The inclusion criteria were as follows: (1), histologically confirmed diagnosis; (2), no neoadjuvant therapy; (3), with matched tumor and adjacent non-tumor samples; (4), with complete follow-up data. There were 125 (83.9%) male and 24 (16.1%) female. Ages ranged 26–76 years (median 50 years). Seventy-four patients (49.7%) had tumor size larger than 5 cm. The follow-up terms were 42–2077 days (median 445 days).

Tissue microarray (TMA) construction was in accordance to the previous report [24].

The rabbit anti-human E2F1 monoclonal antibody (Abcam) and a two-step staining kit (EnVision™ + kit, Dako, Denmark) were adopted for immunohistochemical staining. In brief, 4-µm-thick slides were mounted, deparaffinized and rehydrated, followed by autoclave antigen retrieval and blockage of endogenous peroxidase with 3% hydrogen peroxide. Slides were then incubated with the primary antibody (1:50) overnight at 4 °C. A horseradish peroxidase (HRP)-labeled secondary antibody was subsequently used. Diaminobenzidine was applied as a chromogen. Finally, slides were counterstained with hematoxylin. Pre-immune rabbit serum at the same dilution functioned as the negative control.

Two senior pathologists who had no prior information for follow-up data (Z.Y.L. and W.X.Z.) evaluated the staining, using *H*-score [25], a staining evaluation method that

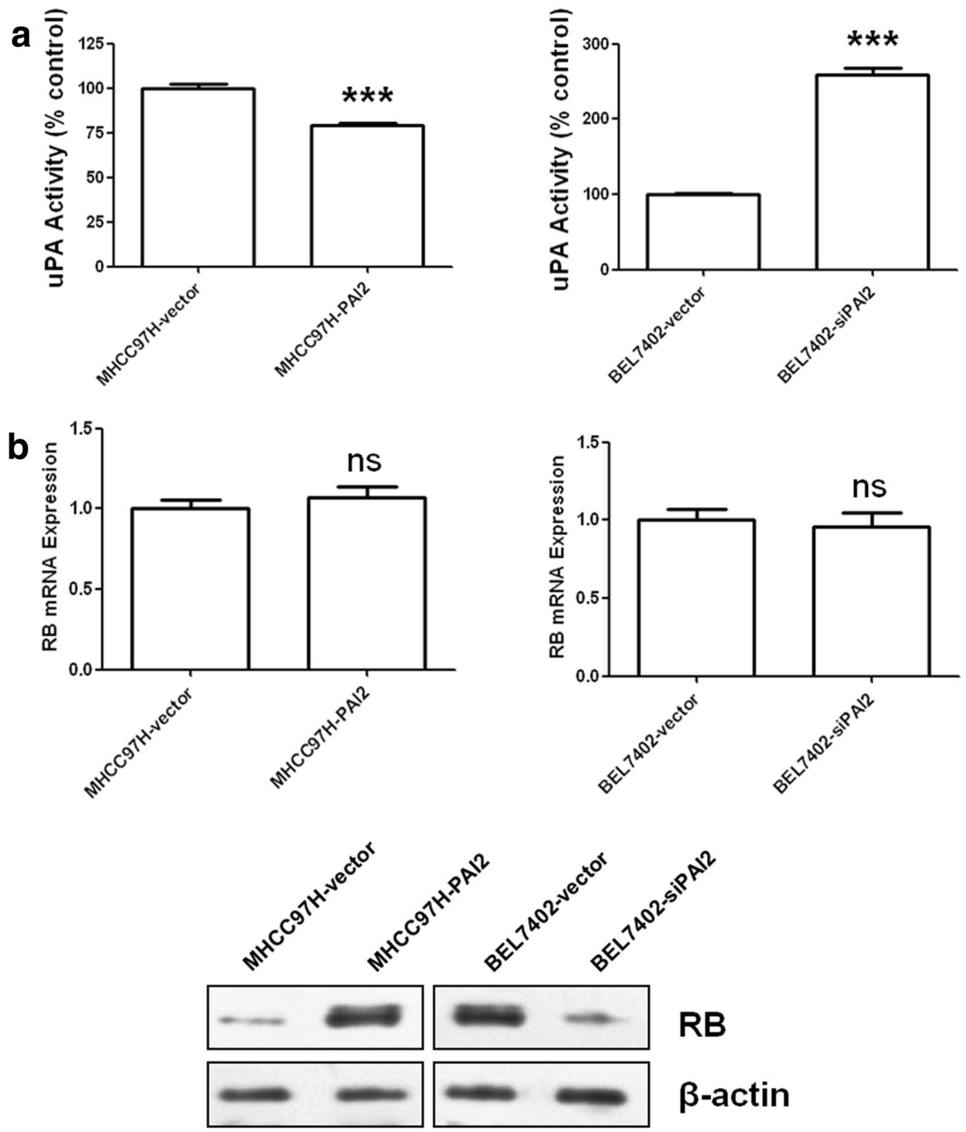

Fig. 3 Mechanistic explorations for the impacts of PAI2 stably overexpression and silence on cell migration and invasion. **a** Cell medium uPA activity measurements; **b** RB mRNA and protein expression detected by real-time RT-PCR and western blotting. *ns* not significant; ***$p < 0.001$

was widely used [26, 27]. The median *H*-score in tumor tissues was selected as the cut-off value.

Statistical analyses

The comparison of continuous variables was performed using the student *t* test or Mann–Whitney *U*-test. The survival curves were got by the Kaplan–Meier method and compared by the log-rank test. Multivariate Cox regression (proportional hazard model) was adopted to identify independent prognostic indicators. Statistical software package SPSS11.5 (SPSS Inc, Chicago, IL, USA) was used for all the analyses. A *p* value less than 0.05 was defined as statistically significant.

Results

Successful establishment of PAI2 stably overexpressed and silenced sub-lines

As shown in Fig. S1, the highly invasive HCC cell line, MHCC97H, had much lower PAI2 expression than the lowly invasive one, BEL7402. Therefore, sub-lines in that PAI2 were stably overexpressed and silenced were established on the basis of MHCC97H and BEL7402, respectively. Real-time RT-PCR and western blotting found significantly increased and decreased PAI2 mRNA and protein expression in MHCC97H- and BEL7402-based sub-lines (Fig. 1), suggesting successful establishment. In four PAI2 silenced sub-lines, the lowest PAI2 expression was present in BEL7402-siPAI2-3 (Fig. 1). Thus, it was subjected to further experiments.

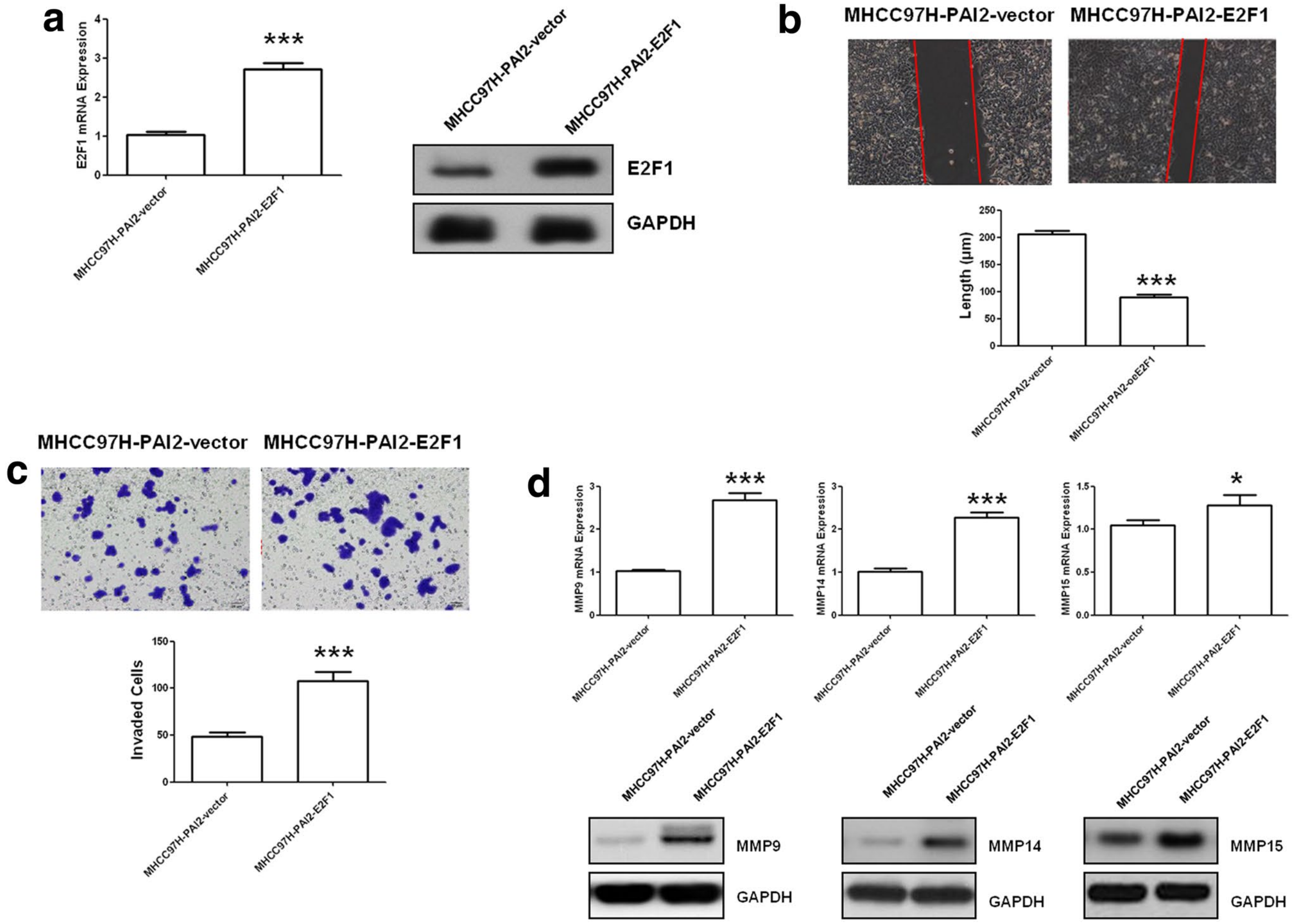

Fig. 4 Impacts of E2F1 transient overexpression on cell migration and invasion in PAI2 stably overexpressed sub-line. **a** Significantly enhanced E2F1 mRNA and protein expression after E2F1 transient overexpression in MHCC97H-PAI2 sub-line; **b** changes of migration after E2F1 transient overexpression in MHCC97H-PAI2 sub-line; **c** changes of invasion after E2F1 transient overexpression in MHCC97H-PAI2 sub-line; **d** significantly enhanced mRNA and protein expression of MMP9, MMP14 and MMP15 after E2F1 transient overexpression in MHCC97H-PAI2 sub-line. $*p<0.05$, $***p<0.001$

PAI2 inhibits migration and invasion of HCC cells

The wound-healing assay that reflects cell migration showed that MHCC97H-PAI2 cells migrated remarkably slower than its vector control, while faster migration was observed in BEL7402-siPAI2 sub-line compared with the control (Fig. 2a). The less and more invaded MHCC97H-PAI2 and BEL7402-siPAI2 cells, in contrast to controls, were also revealed in the transwell invasion analyses (Fig. 2b). These results indicated the inhibitory role of PAI2 for migration and invasion of HCC cells. However, there seemed to be a slight impact of PAI2 on cell proliferation, but not statistically significant (Fig. S2, $p>0.05$).

PAI2 inhibits uPA activity and increases RB protein expression in HCC cells

For the underlying mechanisms of PAI2-mediated inhibition of invasive potential of HCC cells, we first measured uPA activity in established sub-lines. It was shown that uPA activity was markedly declined and elevated in MHCC97H-PAI2 and BEL7402-siPAI2 cells, respectively, compared with controls (Fig. 3a). To explore the other possible mechanism, RB expression at mRNA and protein levels was detected. It could be clearly found that MHCC97H-PAI2 and BEL7402-siPAI2 cells had higher and lower RB protein expression, but not its mRNA level, than controls, respectively (Fig. 3b). These data suggested that PAI2 might exert its inhibitory role through multiple mechanisms.

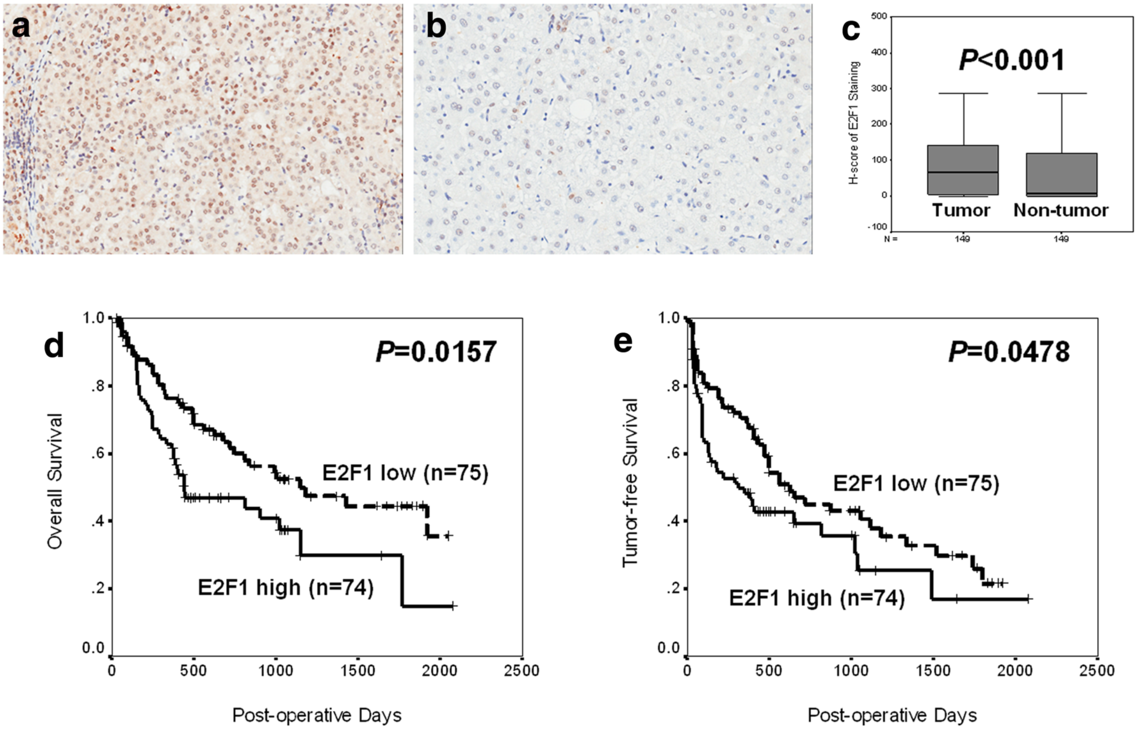

Fig. 5 E2F1 expression in hepatocellular carcinoma. **a** High E2F1 expression in tumor tissue (original magnification × 200); **b** low E2F1 expression in non-tumor tissue (original magnification × 200); **c** comparison of *H*-scores of E2F1 between tumor and non-tumor tissues (Mann–Whitney *U*-test; $p < 0.001$). **d** Overall survival of patients with high and low tumoral E2F1 expression (Log-rank test; $p = 0.0157$), **e** tumor-free survival of patients with high and low tumoral E2F1 expression (Log-rank test; $p = 0.0478$)

Transient overexpression of E2F1 reverses PAI2-mediated inhibition of migration and invasion of HCC cells

To confirm the RB-related mechanism of PAI2, we transiently overexpressed E2F1 in MHCC97H-PAI2 cells. After overexpression (Fig. 4a), cells carried faster migration and more intensive invasion than control cells (Fig. 4b, c), thus reversing the PAI2-mediated inhibition of cell migration and invasion. For relative mechanisms, MMP9, MMP14 and MMP15 expressions at mRNA and protein levels were elevated (Fig. 4d). In addition, E-cadherin was downregulated, while *N*-cadherin and Vimentin were upregulated, indicating the induction of EMT (Fig. S3).

E2F1 is overexpressed in HCC and predicts poor prognosis

Immunohistochemical staining E2F1 was highly expressed in tumor tissues, in contrast to non-tumor ones (Fig. 5a–c, $p < 0.001$). The E2F1 H-score was significantly higher in Edmondson–Steiner grade III–IV tumors than in grade I–II ones (Fig. S4, $p = 0.006$).

Using the median tumoral *H*-score (67.5) as the cut-off value, high E2F1 expression in tumor tissues was significantly associated with poor overall and tumor-free survival (Fig. 5d, e; Table 1, $p = 0.0157$ and 0.0478, respectively). In multivariate Cox regression test, E2F1 expression was very close to have statistical significance (Table 2, $p = 0.050$ and 0.084).

Discussion

Tissue invasion/metastasis has been well known as one of cancer hallmarks [28]. During the complex process, proteolytic degradation of ECM, a key modulator of the hallmarks [29], plays a crucial role [10]. Among factors involved in ECM proteolysis, uPA, binding with its receptor, effectively converts plasminogen to active plasmin, thus promoting the proteolytic cascade [11, 12]. As one of two specific inhibitors of uPA activity, PAI2 was thought to block the cascade, thus being a tumor suppressor [13]. However, PAI2 was found to prevent plasmin generation and its

Table 1 Univariate analysis for overall and tumor-free survival of HCC after curative resection

Variables	*n*	Overall survival		Tumor-free survival	
		Median	*p* value	Median	*p* value
Age			0.8257		0.8835
> 60 years	31	713		403	
≤ 60 years	118	992		558	
Gender			0.3622		0.9398
Male	125	992		496	
Female	24	496		347	
HBsAg			0.3353		0.3054
Positive	122	806		465	
Negative	26	1147		1023	
Tumor size			0.0721		0.0628
> 5 cm	74	620		372	
≤ 5 cm	75	1018		620	
Tumor number			0.0631		0.3531
Solitary	114	992		496	
Multiple	34	389		403	
VI			**0.0014**		0.1748
Present	81	434		405	
Absent	66	1922		645	
TNM stage			**< 0.0001**		**< 0.0001**
I–II	94	1178		815	
III–IV$_A$	55	364		127	
Edmondson–Steiner grade			0.9130		0.6687
I–II	81	837		465	
III–IV	68	903		620	
AFP			0.3146		0.1157
High (> 20 ng/mL)	92	682		403	
Normal	45	1005		867	
E2F1			**0.0157**		**0.0478**
High	74	435		310	
Low	75	1147		620	

HCC hepatocellular carcinoma, *HBsAg* hepatitis B surface antigen, *VI* vascular invasion, *TNM* Tumor node metastasis, *AFP* α-fetoprotein, *E2F1* E2F transcription factor 1

Bold numbers indicate the *p* values less than 0.05, which are defined as statistically significant

metastasis-suppressive effects in brain metastasis from lung and breast cancers [16], although its anti-invasive/metastatic and anti-chemoresistant activities were also revealed in other malignancies [14, 15, 17, 18]. Therefore, its biological roles and molecular mechanisms are complicated, especially for different phenotypes in different cancer types. In HCC, the current data focused on downregulated expression of PAI2 and its prognostic significance [19, 20], but its biological roles and molecular mechanisms remain unclear.

In the present study, we showed that PAI2 inhibits migration and invasion, but not proliferation, of HCC cells, on the basis of established PAI2 stably overexpressed and silenced MHCC97H-PAI2 and BEL7402-siPAI2 sub-lines. The results provide a mechanistic explanation for the previously found negative association between PAI2 expression and PVTT, an important marker of invasion/metastasis in HCC [20]. Because PAI2 is a specific inhibitor of uPA activity, an uPA-related mechanism was first considered. The findings that uPA activity was markedly declined or elevated in MHCC97H-PAI2 or BEL7402-siPAI2 cells, in contrast to controls, prove the inhibitory effect of PAI2 on uPA activity, that was previously found [30, 31]. Therefore, direct inhibition of uPA activity seems to be one of relative molecular mechanisms. Clinically, high uPA activity was shown to be closely related to unfavorable phenotypes and recurrence in HCC [32]. We here preliminarily suggest a novel therapeutic strategy.

In view of our previous finding that PAI2 expression partly located in nuclei of HCC cells [20], and literatures concerning PAI2 protection of RB [21, 22], another RB-related mechanism was speculated. We first revealed remarkably elevated and declined RB protein level, not mRNA one, in MHCC97H-PAI2 and BEL7402-siPAI2 cells, respectively, indicating PAI2 protection of RB protein, rather than transcriptional regulation. According to the interaction between RB and E2F1, this might decrease the release of the latter. It was already known that RB loss and E2Fs were proto-oncogenic in HCC [33, 34], and E2F1 was involved in migration and invasion of HCC cells [35, 36]. Our transient overexpression of E2F1 largely rescued the invasion inhibitory role of PAI2 and upregulating MMP9, MMP14 and MMP15, three cancer invasion promoters as direct targets

Table 2 Multivariate analysis for overall and tumor-free survival of HCC after curative resection

Variables	Overall survival			Tumor-free survival		
	HR	95% CI	*p* value	HR	95% CI	*p* value
TNM stage	2.039	1.191–3.491	**0.009**	2.557	1.659–3.941	**< 0.001**
VI	1.607	0.914–2.826	0.100			
E2F1	1.601	0.999–2.565	0.050	1.465	0.950–2.258	0.084

HCC hepatocellular carcinoma, *HR* hazard ratio, *CI* confidence interval, *TNM* Tumor node metastasis, *VI* vascular invasion, *E2F1* E2F transcription factor 1

Bold numbers indicate the *p* values less than 0.05, which are defined as statistically significant of E2F1 [37], in HCC. Also, EMT was induced, same as reported [35]. Therefore, the PAI2–RB–E2F1 pathway might be a novel mechanism of PAI2 inhibition for HCC invasion. Subsequent results that E2F1 expression largely reversed the inhibitory effects of PAI2 on HCC cell migration and invasion provided further confirmative evidence.

Some papers investigated expression and prognostic significance of E2F1 in HCC [38, 39]. However, these ones were limited by smaller sample sizes, especially for prognostic analysis [39]. In the present study enrolling more specimens, E2F1 expression in tumor tissues was much higher than that in non-tumor ones. Furthermore, tumoral high E2F1 expression was positively associated with histological grade, poor overall and tumor-free survival. Although being close to multivariate significance, E2F1 remains to be a promising predictor of poor survival and recurrence, basically supporting our in vitro experiments and the work by Chen et al. [39].

However, this study has some limitations. First, the results have not been confirmed in vivo. Second, only one PAI2 stably overexpressed or silenced sub-line was used, respectively. Finally, imperfect multivariate prognostic analysis might be attributed, as least in part, to sample size that was increased but not sufficient. These will be improved in further studies.

Conclusions

Taken together, our data indicated that PAI2 inhibits invasive potential of HCC cells via uPA- and RB/E2F1-related mechanisms.

Acknowledgements This study was supported by the National Natural Science Foundation of China (81301845).

Compliance with ethical standards

Conflict of interest Ye Jin, Zhi-Yong Liang, Wei-Xun Zhou and Li Zhou declare that they have no conflict of interest.

Ethical standards All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Institutional and National) and with the Helsinki Declaration of 1975, as revised in 2008. This study was approved by the institutional ethics committee and informed consent was obtained from all patients.

References

1. Forner A, Reig M, Bruix J. Hepatocellular carcinoma. Lancet. 2018;391:1301–1314.
2. Yip VS, Poon RT, Chok KS, Chan AC, Dai WC, Tsang SH, et al. Comparison of survival outcomes between right posterior sectionectomy and right hepatectomy for hepatocellular carcinoma in cirrhotic liver: a single-centre experience. World J Surg. 2015;39:2764–2770.
3. Takayasu K, Arii S, Sakamoto M, Matsuyama Y, Kudo M, Kaneko S, et al. Impact of resection and ablation for single hypovascular hepatocellular carcinoma ≤ 2 cm analysed with propensity score weighting. Liver Int. 2018;38:484–493.
4. Lee SC, Tan HT, Chung MC. Prognostic biomarkers for prediction of recurrence of hepatocellular carcinoma: current status and future prospects. World J Gastroenterol. 2014;20:3112–3124.
5. Woo HG, Park ES, Cheon JH, Kim JH, Lee JS, Park BJ, et al. Gene expression-based recurrence prediction of hepatitis B virus-related human hepatocellular carcinoma. Clin Cancer Res. 2008;14:2056–2064.
6. Xieraili M, Yasen M, Mogushi K, Obulhasim G, Mayinuer A, Aihara A, et al. Villin 1 is a predictive factor for the recurrence of high serum alpha-fetoprotein-associated hepatocellular carcinoma after hepatectomy. Cancer Sci. 2012;103:1493–1501.
7. Yang JD, Nakamura I, Roberts LR. The tumor microenvironment in hepatocellular carcinoma: current status and therapeutic targets. Semin Cancer Biol. 2011;21:35–43.
8. Chiba T, Iwama A, Yokosuka O. Cancer stem cells in hepatocellular carcinoma: therapeutic implications based on stem cell biology. Hepatol Res. 2016;46:50–57.
9. Giannelli G, Koudelkova P, Dituri F, Mikulits W. Role of epithelial to mesenchymal transition in hepatocellular carcinoma. J Hepatol. 2016;65:798–808.
10. Rabbani SA, Mazar AP. Evaluating distant metastases in breast cancer: from biology to outcomes. Cancer Metastasis Rev. 2007;26:663–674.
11. Duffy MJ. The urokinase plasminogen activator system: role in malignancy. Curr Pharm Des. 2004;10:39–49.
12. Pillay V, Dass CR, Choong PF. The urokinase plasminogen activator receptor as a gene therapy target for cancer. Trends Biotechnol. 2007;25:33–39.
13. Croucher DR, Saunders DN, Lobov S, Ranson M. Revisiting the biological roles of PAI2 (SERPINB2) in cancer. Nat Rev Cancer. 2008;8:535–545.
14. Shimizu T, Sato K, Suzuki T, Tachibana K, Takeda K. Induction of plasminogen activator inhibitor-2 is associated with suppression of invasive activity in TPA-mediated differentiation of human prostate cancer cells. Biochem Biophys Res Commun. 2003;309:267–271.
15. Kopitz C, Gerg M, Gansbacher B, Krüger A. Plasminogen activator inhibitor-2, but not cystatin C, inhibits the prometastatic activity of tissue inhibitor of metalloproteinases-1 in the liver. Hum Gene Ther. 2008;19:1039–1049.
16. Valiente M, Obenauf AC, Jin X, Chen Q, Zhang XH, Lee DJ, et al. Serpins promote cancer cell survival and vascular co-option in brain metastasis. Cell. 2014;156:1002–1106.
17. Schroder WA, Major LD, Le TT, Gardner J, Sweet MJ, Janciauskiene S, et al. Tumor cell-expressed SerpinB2 is present on microparticles and inhibits metastasis. Cancer Med. 2014;3:500–513.
18. Huang Z, Li H, Huang Q, Chen D, Han J, Wang L, et al. SERPINB2 down-regulation contributes to chemoresistance in head and neck cancer. Mol Carcinog. 2014;53:777–786.
19. Zhou L, Hayashi Y, Itoh T, Wang W, Rui J, Itoh H. Expression of urokinase-type plasminogen activator, urokinase-type plasminogen activator receptor, and plasminogen activator inhibitor-1 and -2 in hepatocellular carcinoma. Pathol Int. 2000;50:392–397.
20. Zhou L, Jin Y, Cui QC, Jin KM, Zhou WX, Xing BC. Low expression of PAI-2 as a novel marker of portal vein tumor thrombosis and poor prognosis in hepatocellular carcinoma. World J Surg. 2013;37:608–613.

21. Darnell GA, Antalis TM, Johnstone RW, Stringer BW, Ogbourne SM, Harrich D, et al. Inhibition of retinoblastoma protein degradation by interaction with the serpin plasminogen activator inhibitor 2 via a novel consensus motif. Mol Cell Biol. 2003;23:6520–6532.
22. Tonnetti L, Netzel-Arnett S, Darnell GA, Hayes T, Buzza MS, Anglin IE, et al. SerpinB2 protection of retinoblastoma protein from calpain enhances tumor cell survival. Cancer Res. 2008;68:5648–5657.
23. Luo W, Li G, Yi Z, Nie Q, Zhang X. E2F1-miR-20a-5p/20b-5p auto-regulatory feedback loop involved in myoblast proliferation and differentiation. Sci Rep. 2016;6:27904.
24. Lin SB, Zhou L, Liang ZY, Zhou WX, Jin Y. Expression of GRK2 and IGF1R in hepatocellular carcinoma: clinicopathological and prognostic significance. J Clin Pathol. 2017;70:754–759.
25. McClelland RA, Finlay P, Walker KJ, Nicholson D, Robertson JF, Blamey RW, et al. Automated quantitation of immunocytochemically localized estrogen receptors in human breast cancer. Cancer Res. 1990;50:3545–3450.
26. Righi L, Papotti MG, Ceppi P, Billè A, Bacillo E, Molinaro L, et al. Thymidylate synthase but not excision repair cross-complementation group 1 tumor expression predicts outcome in patients with malignant pleural mesothelioma treated with pemetrexed-based chemotherapy. J Clin Oncol. 2010;28:1534–1539.
27. Ortolan E, Arisio R, Morone S, Bovino P, Lo-Buono N, Nacci G, et al. Functional role and prognostic significance of CD157 in ovarian carcinoma. J Natl Cancer Inst. 2010;102:1160–1177.
28. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–674.
29. Pickup MW, Mouw JK, Weaver VM. The extracellular matrix modulates the hallmarks of cancer. EMBO Rep. 2014;15:1243–1253.
30. Al-Ejeh F, Croucher D, Ranson M. Kinetic analysis of plasminogen activator inhibitor type-2: urokinase complex formation and subsequent internalisation by carcinoma cell lines. Exp Cell Res. 2004;297:259–271.
31. Croucher D, Saunders DN, Ranson M. The urokinase/PAI-2 complex: a new high affinity ligand for the endocytosis receptor low density lipoprotein receptor-related protein. J Biol Chem. 2006;281:10206–10213.
32. Itoh T, Hayashi Y, Kanamaru T, Morita Y, Suzuki S, Wang W, et al. Clinical significance of urokinase-type plasminogen activator activity in hepatocellular carcinoma. J Gastroenterol Hepatol. 2000;15:422–430.
33. Mayhew CN, Carter SL, Fox SR, Sexton CR, Reed CA, Srinivasan SV, et al. RB loss abrogates cell cycle control and genome integrity to promote liver tumorigenesis. Gastroenterology. 2007;133:976–984.
34. Huntington JT, Tang X, Kent LN, Schmidt CR, Leone G. The spectrum of E2F in liver disease–mediated regulation in biology and cancer. J Cell Physiol. 2016;231:1438–1449.
35. Wang Y, Qin X, Guo T, Liu P, Wu P, Liu Z. Up-regulation of CDK16 by multiple mechanisms in hepatocellular carcinoma promotes tumor progression. J Exp Clin Cancer Res. 2017;36:97.
36. Chang L, Xi L, Liu Y, Liu R, Wu Z, Jian Z. SIRT5 promotes cell proliferation and invasion in hepatocellular carcinoma by targeting E2F1. Mol Med Rep. 2018;17:342–349.
37. Johnson JL, Pillai S, Pernazza D, Sebti SM, Lawrence NJ, Chellappan SP. Regulation of matrix metalloproteinase genes by E2F transcription factors: Rb-Raf-1 interaction as a novel target for metastatic disease. Cancer Res. 2012;72:516–526.
38. Palaiologou M, Koskinas J, Karanikolas M, Fatourou E, Tiniakos DG. E2F-1 is overexpressed and pro-apoptotic in human hepatocellular carcinoma. Virchows Arch. 2012;460:439–446.
39. Chen YL, Uen YH, Li CF, Horng KC, Chen LR, Wu WR, et al. The E2F transcription factor 1 transactives stathmin 1 in hepatocellular carcinoma. Ann Surg Oncol. 2013;20:4041–4054.