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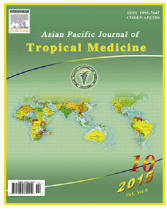

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Expression of HIP/PAP in hepatocellular carcinoma and effect of siRNA on migration and invasion in HCC cells

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ABSTRACT

Objective: To investigate the expression of HIP/PAP in hepatocellular carcinoma (HCC) patients and explore its role in migration and invasion of HCC.
Methods: The expression of HIP/PAP in HCC tissue and corresponding adjacent noncancerous tissue was assessed by IHC, RT-PCR and Western blot. The correlation between clinicopathological features and HIP/PAP expression was analyzed. The role of HIP/PAP on invasion and migration of HCC cells was observed by RNA interference, wound healing and Transwell assay.
Results: Both mRNA and protein expression of HIP/PAP was upregulated in HCC tissues compared to tumor-adjacent tissue and correlated with poor tumor differentiation, advanced tumor stage and vascular invasion. HIP/PAP expression was also upregulated in HCC cells, and silencing its expression by specific siRNA could inhibit the invasion and migration of HCC cells.
Conclusions: HIP/APA is overexpressed in HCC and contributes to the migration and invasion of HCC cells.

1. Introduction

Hepatocellular carcinoma (HCC), the most common type of primary liver carcinoma, is a leading cause of morbidity and mortality worldwide. Due to the prevalence of viral hepatitis, China has the largest HCC population in the world [1]. Despite numerous advances have been made regarding the diagnostic and therapeutic methods during the past decades, the prognosis of HCC patients is still not improved due to the high rate of recurrence and metastasis [2]. The Insidious onset of early-stage HCC makes it difficult to diagnose, and a number of HCC patients have lost opportunity of surgery at the time of diagnosis due to intrahepatic or distant metastasis [3]. The increasing researches into molecular targeting therapy have opened a new era for the treatment of HCC, and thoroughly understanding the molecular mechanisms of migration and invasion of HCC is basis of targeting therapy [2].

Human hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein (HIP/PAP), also known as Reg3A, a member of regenerating gene (*Reg*) family [4], was initially described as an acute phase secretory protein expressed during acute pancreatitis. Under physiological condition, HIP/PAP is mainly secreted by intestine and pancreas, promoting regeneration of intestinal epithelial cells and maintaining the integrity of Intestinal barrier [5]. Later, researches have shown the expression of HIP/PAP is upregulated in some digestive system carcinoma (colorectal carcinoma, HCC, pancreatic carcinoma) [4,6]. In recent years, HIP/PAP is profoundly studied as a pleiotropic molecule with mitogenic, anti-apoptotic and antimicrobial potential [7–10], the mitogenic and anti-apoptotic activity can ameliorate acute pancreatitis induced by cerulean and promote regeneration of hepatocytes after partial hepatectomy [7], and protects mice from acetaminophen induced liver injury [11], however it also can promote proliferation and inhibit apoptosis of cancer cells [12].

HIP/PAP is overexpressed in human HCC but not in normal adult liver, and its serum level is also elevated [13]. Previous studies have revealed that in HCC cells HIP/PAP can inhibit apoptosis induced by TNF-α and promote proliferation by up-regulating the expression of Cyclin A [11], suggesting its critical role in the carcinogenesis and progression of HCC. In this study, we investigated expression of HIP/PAP in HCC tissue and cells, and explore its role in migration and invasion.

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2. Materials and methods

2.1. Ethical statement

All protocols were approved by the Institutional Animal Care and Use Committee of Soochow University. All patients gave fully consent to the research.

2.2. Clinical specimens and HCC cells

A total of 75 pairs (50 males and 25 females, range 32–74 year old; median 55.6 year old) of surgically resected HCC and adjacent liver tissue were collected from Department of General Surgery, First Affiliated Hospital of Soochow University during 2013–2015. None of these patients received any radiotherapy, chemotherapy or radiofrequency ablation before surgery. HCC tissues and matched adjacent noncancerous tissues (at least 2 cm away from the surgical border) were immediately fixed in 4% paraformaldehyde (dissolved in PBS solution) or stored in −80 °C refrigerator for further research. Besides, the clinicopathological characteristics were also recorded. Staging and differentiation of the tumor was based on the TMN standard (Union for International Cancer Control) and Edmondson method respectively.

Human immortalized liver cell line LO2 and 4 HCC cell lines (Huh7, HepG2, Hep3B and SMMC-7721) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in complete DMEM medium with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) at 37 °C, 5% CO_2.

2.3. Immunohistochemistry assay

Paraformaldehyde-fixed and paraffin-embedded sections were used to in immunohistochemical assay (SP-IHC method). Sections were deparaffinized in xylene and rehydrated in a series of ethanol with decreasing concentration, and then undergone antigen retrieval in citrate buffer for 15 min at 95 °C. Sections were treated in 3% H_2O_2 to quench endogenous peroxidase and incubated with HIP/PAP primary rabbit polyclonal antibody (Abcam, 1:100) for overnight at 4 °C, then incubated with biotinylated IgG secondary antibody (ZYMED, USA) then horseradish peroxidase conjugated streptavidin, then color development with DAB solution. HIP/PAP primary rabbit polyclonal antibody was replaced with PBS solution for negative control.

Sections were checked by two pathologists blind to the clinical situation of all patients. The staining result for the HIP/PAP protein was semi-quantitatively assessed by Mattern method: intensity score × percentage score. The intensity score was expressed in the following four grades: 0 = none; 1 = weak; 2 = moderate; and 3 = strong. The percentage score (percentage of positively-stained cells) was expressed as the following grades: <5%, 0; 6%–25%, 1; 26%–50%, 2; 51%–75%, 3; 4 > 75%, 4. The average score of ten high magnification (×400) fields in each section became the final score. The overall score ≥1 was defined as HIP/PAP positive.

2.4. Real time polymerase chain reaction (RT-PCR)

Total RNA was extracted from HCC specimens or HCC cells by TRIZOL reagent (Invitrogen) following manufacturer's instructions. RNA concentration was determined to ensure equal amount of RNA was added in reverse transcription PCR. The cDNA was synthesized using the Revertid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada) at 37 °C for 30 min. Following inactivation at 94 °C for 2 min, Real-time PCR was performed to amplify and quantify the cDNA of each sample using SYBR® Premix Ex Taq™ II (Takara, Japan) at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s with a total of 30 cycles, and a final extension at 72 °C for 10 min. The human *β-actin* gene was used as a internal control. Quantitative RT-PCR was performed at least three times per sample with three replications each time. The relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method. The primers were synthesized by Augct Bio-tech (Beijing, China), the sequence of primers listed as follow, HIP/PAP (351 bp): Sense 5′-CCACCAGAGAGTGACTC-3′, antisense 5′-GAGTAGCTGTTACCAATG-3′; β-actin (416 bp): Sense 5′-GAGCTACGAGCTGCCTGACG-3′, Antisense 5′-CCTAGAAGCATTTGCGGTGG-3′. The PCR product was visualized on 2% agarose gel.

2.5. Western blotting analysis

Total protein of cultured cells or tissues was extracted by RIPA Lysis Buffer (Beyotime Biotechnology, Beijing, China). Protein concentration was determined by the BCA assay kit (Beyotime Biotechnology, Beijing, China). A total of 60 μg protein was separated by 12% SDS-PAGE electrophoresis and transferred to Polyvinylidene Fluoride membranes. Membranes were blocked by Tris-buffered saline–Tween 20 containing 5% skim milk and probed with HIP/PAP (Abcam, ab134309, 1:500) or β-actin (Santa Cruz, sc-7210, 1:2 000) antibody. Secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz, sc-2004) was used at a 1:10 000 dilution and detected by the Enhanced Chemiluminescence Reagent (Millipore, Billerica, MA, USA).

2.6. siRNA transfection

Specific siRNA of HIP/PAP and scramble siRNA as negative control were synthesized by Augct Bio-tech (Beijing, China). Cells were seeded in 6-well plates (2×10^5 cells per well), and transfected with 100 pmol siRNA (HIP/PAP siRNA or scramble siRNA) by Lipofectamine 2000 method (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions, and continued to culture in the incubator at 37 °C, 5% CO_2. Reduced serum medium was replaced with complete medium after 6 h. Cells were harvested after 48 h. The effect of siRNA was tested by RT-PCR and western blotting. The specific siRNA against HIP/PAP sequence (5′–3′): GUGAAGAGCAUUGGUAACAGCTAdTdT, Scramble siRNA sequence (5′–3′): UUCUCCGAACGUGUCACGUdTdT.

2.7. Wound healing assay

Cells were cultured and transfected with HIP/PAP siRNA or scramble siRNA, then seeded in 6-well plates and allowed to grow into cell monolayer overnight. A wound was created by a yellow sterile pipette tip on the surface of plates, and the suspended cells were gently cleared with PBS. Cells in the plates were cultured in serum-free medium. Images of wound were taken under the phase-contrast microscope at 0, 24 and 48 h. The distance was measured by Image Pro-Plus 6.0 software (Media

Cybernetics, USA). For each experiment, 5 visual fields and 2 repeated wells were measured with three replications.

2.8. Migration and invasion assay

24-well transwell (8-μm pore size; Millipore) was used to perform migration assay and Matrigel invasion assay. Matrigel-coated chamber was prepared following manufacturer's instructions. For migration assay, 24 h after transfection, the concentration of cells was adjusted to 2.5×10^5/mL by DMEM fetal bovine serum-free medium, 100 μL cell suspension was loaded into the top chamber of transwell insert with non-coated membrane. For invasion assay, 100 μL cell suspension was loaded into the upper Matrigel-coated chamber instead. In both assays, 600 μL DMEM with 10% fetal bovine serum was added into the bottom chamber. Cells were then allowed to migrate or invade for 48 h at 37 °C. The cells that migrated or invaded into the bottom chamber were fixed in paraformaldehyde and permeabilized in methanol, then stained with crystal violet dye. Images were taken and penetrated cells were counted under a light microscope. The average cell number of four random fields was the final result.

2.9. Statistical analysis

Data was expressed as mean ± SD. Difference analysis was performed by SPSS 18.0. Difference was considered statistically significant when $P < 0.05$.

3. Results

3.1. HIP/PAP expression in HCC tissues

To observe the expression of HIP/PAP in HCC tissues, HIP/PAP expression at mRNA and protein level was assessed in

Table 1
Correlation of HIP/PAP expression with clinicopathological characteristics of 75 HCC cases.

Clinical parameters	Cases (*n*)	Expression level		*P* value
		Positive (*n* = 51)	Negative (*n* = 24)	
Age (years)				
<65 years	50	35	15	0.610
≥65 years	25	16	9	
Gender				
Male	50	34	16	1.000
Female	25	17	8	
Tumor size (cm)				
<5 cm	47	35	12	0.134
≥5 cm	28	16	12	
Tumor number				
Solitary	43	28	15	0.621
Multiple	32	23	9	
Edmondson				
I+II	30	12	18	0.001*
III+IV	45	39	6	
TNM stage				
I–II	49	28	21	0.008*
III–IV	26	23	3	
Capsular infiltration				
Present	44	31	13	0.622
Absent	31	20	11	
Vascular invasion				
Present	40	22	18	0.013*
Absent	35	29	6	
AFP				
<400 ng/mL	28	19	9	1.000
≥400 ng/mL	47	32	15	
HBsAg				
Positive	62	43	19	0.745
Negative	13	8	5	

*$P < 0.05$.

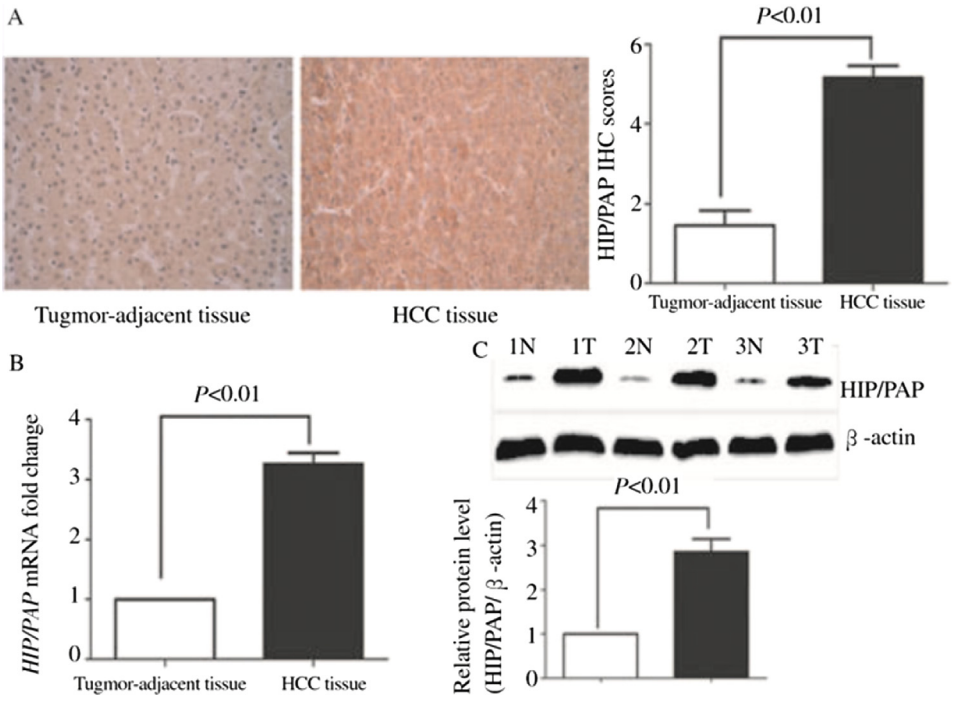

Figure 1. mRNA and protein expression of HIP/PAP in HCC tissues.
(A) Representative IHC staining and IHC scores of HIP/PAP expression in human HCC tissues and tumor-adjacent liver tissues, Original magnification: ×400; (B) Comparison of mRNA expression of *HIP/PAP* between HCC tissues and tumor-adjacent liver tissues; (C) Expression of HIP/PAP protein in HCC tissues and adjacent noncancerous tissues (N: adjacent noncancerous tissue, T: HCC tissue), Representative western blotting film ($n = 3$) and relative level of expression.

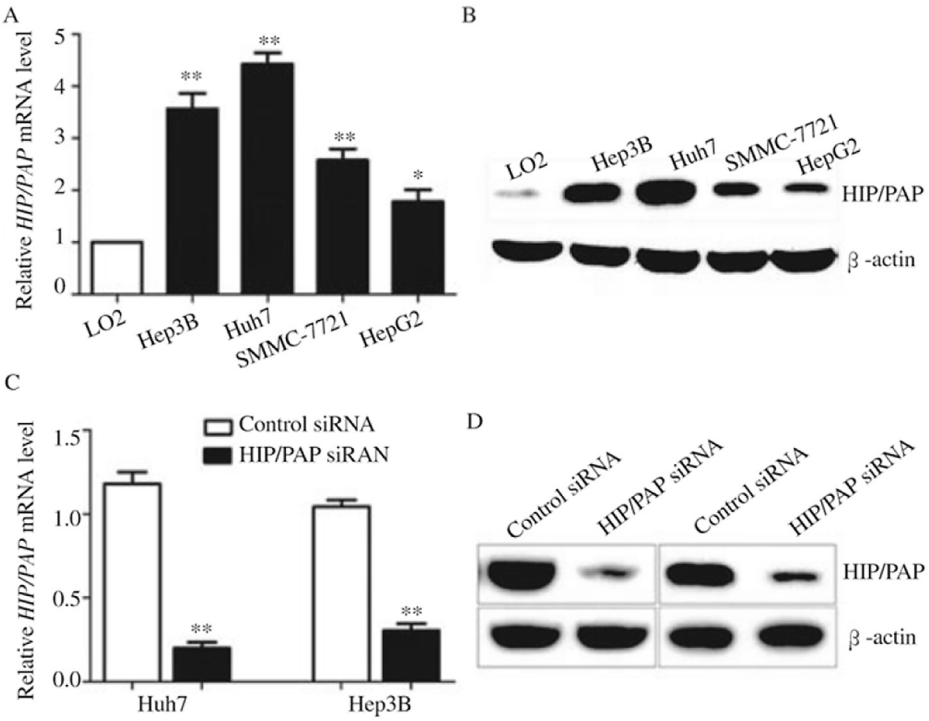

Figure 2. HIP/PAP expression in HCC cell lines and downregulation of HIP/PAP in Hep3B and Huh7 cells by specific siRNA.
(A) Real-time PCR analysis of HIP/PAP expression in different HCC cell lines; (B) Western blotting analysis of HIP/PAP expression in different HCC cell lines. Expression of HIP/PAP is remarkably downregulated in Hep3B and Huh7 cells by specific siRNA reflected by *HIP/PAP* mRNA quantification by real-time PCR (C) and HIP/PAP protein analysis by Western blotting (D). (**$P < 0.001$, *$P < 0.05$).

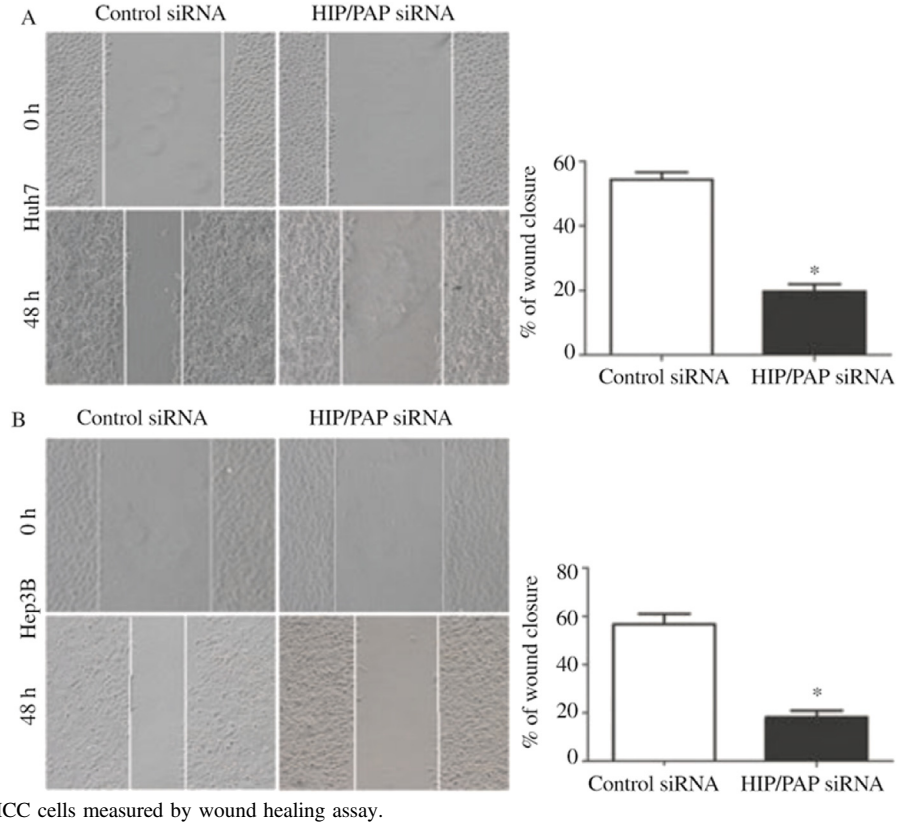

Figure 3. Motility of HCC cells measured by wound healing assay.
Wound healing assays revealed that the migration of Huh7 cells (A) and Hep3B cells (B) was inhibited by HIP/PAP silencing, original magnification ×100 (*$P < 0.01$).

HCC and matched adjacent noncancerous tissue by qRT-PCR, immunohistochemistry and western blot. Immunohistochemical staining (Figure 1A) of HIP/PAP could be detected in both cytoplasm and intercellular space. As shown in Figure 1B and C, both HIP/PAP mRNA and protein expression elevated significantly in HCC tissue compared with matched adjacent noncancerous tissues.

3.2. HIP/PAP expression correlates with poor clinicopathological features

Correlation analysis (Table 1) showed that positive HIP/PAP expression in HCC tissue was significantly associated with poor differentiation, advanced tumor stage and vascular invasion, however HIP/PAP expression was not associated with gender, age, serum AFP or tumor number and size. These results demonstrated that elevated HIP/PAP expression correlated with aggressive behaviors of HCC.

3.3. HIP/PAP expression in HCC cells and effect of siRNA on HIP/PAP expression in Huh7 and Hep3B cells

The result showed *HIP/PAP* mRNA (Figure 2A) and protein (Figure 2A) level were increased remarkably in HCC cells, HIP/PAP expression was significantly higher in Hep3B and Huh7 cells which possessed higher invasive behavior. Specific HIP/PAP siRNA and scramble were transfected into Huh7 and Hep3B cells, effect of RNA interfering was evaluated by RT-PCR and western blot. As shown in Figure 2C and D, HIP/PAP-siRNA significantly down-regulated HIP/PAP mRNA and protein expression in Huh7 and Hep3B cells, and could be used for subsequent experiments.

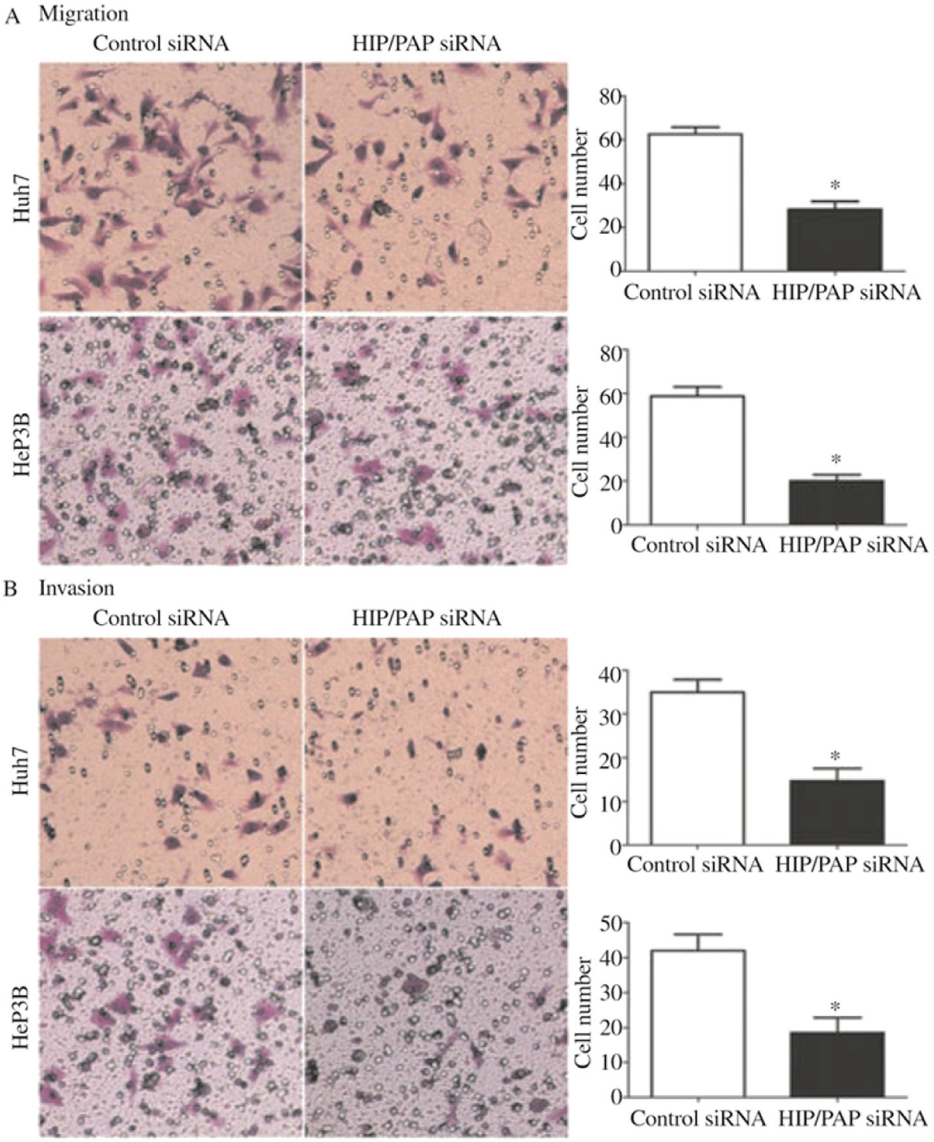

Figure 4. Migration and invasion of HCC cells measured by Transwell assay.
Migrated and invaded cells were stained with crystal violet and imaged by microscopy, original magnification ×100. Representative images showed HIP/PAP knockdown by specific siRNA significantly inhibits migration (A) and invasion (B) of Hep3B and Huh7 cells (*$P < 0.01$).

3.4. Effect of HIP/PAP knockdown on migration and invasion of Huh7 and Hep3B cells

Wound healing assay (Figure 3) demonstrated that HIP/PAP knockdown could inhibit the motility of Huh7 and Hep3B cells. Transwell assay also revealed HIP/PAP knockdown by siRNA in the highly invasive Huh7 and Hep3B cells could significantly inhibited the migratory (Figure 4A) and invasive (Figure 4B) behavior compared to control group.

4. Discussion

Tumor is a major cause of death worldwide, and HCC is one of the worst due to its rapid growth, early metastasis and relapse. The poor prognosis of HCC patients is mainly due to metastasis. Understanding the molecular mechanism of HCC carcinogenesis, progression and metastasis is top priority for advancing the therapeutic pattern. Growing studies indicated that cancer-related inflammation (tumor inflammatory microenvironment) plays pro-tumoral role, and tumor in a self-sufficient way promotes the expression of inflammatory mediators associated with proliferation and survival of malignant cells, angiogenesis, and metastasis [14–16]. The fact that most HCC cases having either HBV, HCV infection or alcoholic liver disease indicates that hepatic inflammation and fibrosis is the major risk factor of HCC carcinogenesis and progression [17], however the mechanistic basis of the association between inflammation and cancer are not completely understood, Inflammation-fibrosis-cancer axis is a recently-proposed pattern to explain the mechanism regarding how hepatic inflammation contributes to cancer. Chronic hepatocyte damage leads to the continuous release of inflammatory mediators (such as TGF-β, TNF-α, IL-6, CXCL1, CXCL2) which augment hepatocyte damage and promote fibrosis and cirrhosis, stimulating regeneration and malignant transformation of hepatocytes [16,18]. Nuclear factor-κB (NF-κB) is the pivotal transcription factor in the inflammation-fibrosis-cancer axis and can act as signal transducer for a number of membranous receptors, studies have demonstrated inflammatory mediators which compose inflammatory microenvironment are mainly released by activation of NF-κB pathway. Toll like receptors are the major upstream receptors of NF-κB. HIP/PAP is an important molecular component of the tumor inflammatory microenvironment, and can be secreted through Toll like receptors – NF-κB pathway [19].

HIP/PAP has a variety of biologic activities, and commonly expressed in digestive system malignancies. Similar to other oncogenes, the expression of HIP/PAP is high in embryonic stage of liver development, but restrained in normal adult liver. The elevated expression of HIP/PAP in cirrhotic patients before HCC development suggests that HIP/PAP is not just a marker of HCC, but also a promoter in the carcinogenesis of HCC, however the detailed molecular mechanisms are still unknown. Previous researches have confirmed that HIP/PAP exerts proliferative potential by up-regulating Cyclin A [8,10], yet its role in HCC migration and invasion remains unknown.

In this study we have shown HIP/PAP is over-expressed in HCC tissues, positive HIP/PAP expression was strongly associated with poor tumor differentiation, advanced tumor stage, vascular invasion. These clinical data suggested that HIP/PAP contributes to malignant progression of HCC. However, long term follow-up is required to confirm the prognostic role of HIP/PAP in HCC patients.

Moreover, we determined the functional significance of HIP/PAP knockdown by specific siRNA in HCC cell lines. The HIP/PAP expression is low in normal hepatocytes, and increases greatly in HCC cells. The expression of HIP/PAP is even higher in Huh7 and Hep3B cells which are highly invasive. We chose Huh7 and Hep3B cells with high invasiveness for further RNA interference assay, and as expected HIP/PAP knockdown by specific siRNA significantly inhibited the migration and invasion of HCC cells.

In conclusion, this study demonstrated HIP/PAP is upregulated in HCC and an indicator of aggressive behaviors of HCC cells, a key factor participating in the progression of HCC by promoting invasion and migration, yet the prognostic role of HIP/PAP and detailed molecular pathways involved in the pathogenesis should be further explored.

Conflict of interest statement

We declare that we have no interest of conflict.

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