Cancer Science
The official journal of the Japanese Cancer Association

Downregulation of HTPAP transcript variant 1 correlates with tumor metastasis and poor survival in patients with hepatocellular carcinoma

Chun Dai,[1,2] Qiong-Zhu Dong,[1,2] Ning Ren,[1] Jun-Jie Zhu,[1] Hai-Jun Zhou,[1] Hai-Jing Sun,[1] Guan Wang,[1] Xiao-Fei Zhang,[1] Yu-Hua Xue,[1] Hu-Liang Jia,[1] Qing-Hai Ye[1] and Lun-Xiu Qin[1,3]

[1]Liver Cancer Institute & Zhongshan Hospital, Institutes of Biomedical Science, Fudan University, Shanghai, China; Key Laboratory of Carcinogenesis & Cancer Invasion, Ministry of Education, China

(Received July 13, 2010/Revised December 3, 2010/Accepted December 13, 2010/Accepted manuscript online January 10, 2011)

Our previous study has identified HTPAP as a novel metastasis suppressor from chromosome 8p which is often deleted in metastatic HCC. We sought to further evaluate the expression levels of transcript variants of HTPAP (HTPAP-1, HTPAP-2 and HTPAP-3) in 67 HCC tumor tissues and 11 normal liver tissues by RT-PCR with specific TaqMan probes and primer sets, and explore their association with HCC metastasis and survival. We found that the expression levels of three HTPAP transcript variants were quite different in HCCs. Only HTPAP-1 was found to be significantly associated with HCC metastasis (P = 0.00053), overall survival (P = 0.0023) and time to recurrence (P = 0.010) of HCC. Patients with a lower expression of HTPAP-1 were inclined to accompany intrahepatic metastases and tumor thrombi (P < 0.05) and had a poor prognosis. *In vitro*, three fusion pEGFP-N1 vectors encoding HTPAP-1, HTPAP-2 and HTPAP-3 were introduced into HCC cells respectively to track HTPAPs' expressions and identify their function. We found overexpression of HTPAP-1 conferred HCC cells reduced ability of invasion without significant impact on cell proliferation, and also displayed a distinct cell location on cell membrane and in cytoplasm, which were different from two other variants. Consequently, HTPAP-1 may be the transcript of HTPAP to exhibit a suppressive role on HCC metastasis, and can be a prognostic marker for HCC. (*Cancer Sci* 2011; 102: 583–590)

HCC is one of the most frequent and aggressive human cancers worldwide, and it is the third most common cause of death from cancer.[1,2] It has an extremely poor prognosis, mainly attributed to the high frequency of intrahepatic metastatic recurrence.[3,4] Metastasis is a complex process that is regulated by multiple genes.[5] Exploration of these genes' mechanism will advance novel diagnostic, therapeutic and prognostic applications of patients with HCC.

In our previous study, HTPAP was identified as a novel metastasis suppressor from chromosome 8p which is often deleted in metastatic HCCs.[6] However, HTPAP was found to exhibit oncogenic properties in cell survival and cell transformation of breast cancer.[7] These indicate that HTPAP is a critical gene in the progress of carcinogenesis and may play distinct roles in different tissue environments.

The official full name of HTPAP is phosphatidic acid phosphatase type 2 domain containing 1B, which has three transcript variants with different 3′ ends. Transcript variant 1 (HTPAP-1; GenBank: NM_001102559.1) represents the longest transcript and encodes a set of complete functional domains. In comparison with variant 1, transcript variant 2 (HTPAP-2; GenBank: NM_032483.3) has a different exon at the 3′ terminus; and transcript variant 3 (HTPAP-3; GenBank: NM_001102560.1) uses an alternate splice site in the 3′ coding region and lacks multiple exons (Fig. 1A). Nevertheless, no study on the expression or function of HTPAP transcripts has yet been reported. Together with the central role of HTPAP in inhibiting HCC metastasis, we were promoted to explore deep into the impact of each transcript variant of HTPAP in HCC and make clear which plays the key role in inhibiting metastasis.

Metastasis suppressor genes can be broadly defined as genes that suppress the ability of metastases to form, without affecting the growth of the primary tumor.[8] Just as the name suggests, metastasis suppressor genes have been identified by their reduced or absent expression in metastatic tumor cells relative to tumorigenic, but non-metastatic cells. Moreover, according to one of the tumorigenesis and metastasis theories that genes favoring metastasis progression are initiated in the primary tumors,[9,10] it is becoming a routine strategy to compare gene expression levels in tumor samples with and without metastasis[11–13] to distinguish the metastasis related genes. Real-time PCR is a powerful tool to quantify gene expression,[14,15] especially for genes with conserve regions just like transcript variants which may possess too subtle distinctions to be accurately identified by antibodies in protein level. Our study aimed to analyze the expression of three HTPAP transcripts among 67 HCC patients with or without metastasis as well as 11 normal liver tissues by real-time PCR, and identify the real actor of metastasis suppressor within three HTPAPs *in vitro*.

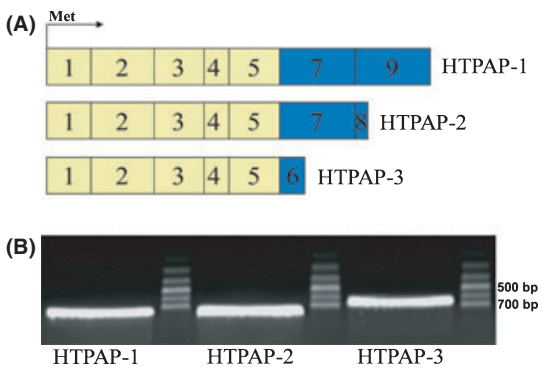

Fig. 1. Identification of expression of 3 transcripts of HTPAP. (A) Schematic showing the structure of three HTPAP isoforms. (B) Representative ethidium bromide-stained agarose gel photo showing expression of three HTPAPs amplified from MHCC97-L cDNA by PCR. HTPAP, phosphatidic acid phosphatase type 2 domain containing 1B; HTPAP-1, HTPAP transcript variant 1; HTPAP-2, HTPAP transcript variant 2; HTPAP-3, HTPAP transcript variant 3.

[3]To whom correspondence should be addressed.
E-mail: qin.lunxiu@zs-hospital.sh.cn
[2]Co-first authors.

doi: 10.1111/j.1349-7006.2011.01863.x

Table 1. Associations of HTPAP-1 expression level with clinicopathological features of HCC patients

Variables	HTPAP-1		*P*-value
	Low (*n* = 33)	High (*n* = 34)	
Age (years)			
Median	52.5	55	0.912
Range	35–69	37–75	
Sex			
Male	27	27	0.803
Female	6	7	
HBsAg			
+	31	26	0.096
–	2	8	
ALT (U/L)			
≤50	26	21	0.078
>50	7	13	
AFP (ng/mL)			
≤20	9	16	0.094
>20	24	18	
Liver cirrhosis			
Yes	31	30	0.414#
No	2	4	
Tumor size (cm)			
≤5	8	10	0.562
>5	25	24	
Encapsulation of tumor			
Complete	15	19	0.393
No	18	15	
TNM Stage			
I	6	14	0.23
II	15	11	
III	6	5	
IV	6	4	
Tumor thrombus			
Yes	22	11	0.01
No	11	23	

#25% of all the cells have expected count <5, Fisher's exact test. AFP, alpha-fetoprotein; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; HTPAP-1, HTPAP Transcript variant 1; TNM, TNM classification of malignant tumors.

Table 2. Some clinic pathological characteristics of samples enrolled in this study

Clinical variable	*n* (%)		
	HCC without metastasis (*n* = 34)	HCC with metastasis (*n* = 33)	Normal (*n* = 11)
Sex			
Male	25 (73.53)	29 (87.88)	4 (36.36)
Female	9 (26.47)	4 (12.12)	7 (63.64)
Age (years)			
≤50	23 (67.65)	24 (72.72)	7 (63.64)
>50	11 (32.35)	9 (27.28)	4 (36.36)

HCC, hepatocellular carcinoma.

Materials and Methods

Patients and tissue specimens. A total of 67 patients who underwent surgical resection for HCC at the Liver Cancer Institute and Zhongshan Hospital (Fudan University, Shanghai, China) between February 2003 and February 2006 were enrolled in this study. The preoperative liver function of all patients was Child-Pugh A. Among them, 57 patients (85.1%) were positive for HBsAg and 61 patients (91.04%) had liver cirrhosis and 42 patients (62%) were positive for AFP (>20 ng/mL). Based on the International Union Against Cancer TNM classification system (6th edition),[16] 20 patients (29.8%) were in stage I, 26 patients (38.8%) were in stage II, 11 patients (16.4%) were in stage III and 10 patients (14.9%) were in stage IV. Tumor differentiation was graded by the Edmondson grading system.

Because it is difficult to ascertain whether tumors occurring after surgery are due to metastatic development or de novo tumorigenesis, we selected these 67 cases identified with clear outcomes; some accompanying metastasis at surgery and the others without metastasis and recurrence at surgery and at 3-year follow up. Accordingly, 33 patients developing distant metastasis or intrahepatic vascular tumor thrombi at surgery were considered as metastases; 34 patients with no evidence of metastasis at the time of surgery and no tumor recurrence during 3-years follow up were considered as non-metastasis cases. The HCC and nontumorous liver tissues were collected and frozen in liquid nitrogen. The detail clinicopathological information was summarized in Table 1. An additional 11 normal liver specimens were collected from patients with non-HCC liver disease as previously described[9] (Table 2). The study was approved by the Research Ethics Committee of Zhongshan Hospital, Fudan University, Shanghai. Informed consent was obtained according to the committee's regulations.

All patients were followed up every 2 months after operation until March 2009 (follow-up time ≥3 years). Serum AFP level and liver ultrasonography were detected during each visit by independent doctors without knowledge of the study. A CT scan or MRI was performed every 6 months. If metastasis or recurrence was suspected, CT or MRI was performed immediately.

RNA isolation and the real-time PCR. Total RNA was extracted from HCC and liver tissues by Trizol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and quality of the isolated RNA were measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). First-strand cDNA was synthesized using both Oligo dT Primer and Random 6 mers by PrimeScript RT Enzyme Mix I according to the manufacturer's instructions (Takara, Ostu, Shiga, Japan).

To exam the expression levels of the transcript variants of HTPAP, RT-PCR was performed with the specific TaqMan probes and primer sets. HPRT1 that was considered to be one of the reliable reference genes for q-PCR normalization in HCC specimens[17] was used as control. Commercialized probes and primer sets specific to HTPAP-2 and HPRT1 were purchased from Applied Biosystems (Foster City, CA, USA). TaqMan primers and probes of HTPAP-1 and HTPAP-2 were designed using Primer Express (Applied Biosystems). The primer

Table 3. The probes and primer sets for HTPAP transcript variants and reference gene for HPRT1 in real-time PCR

Gene symbol	Gene name	Accession number	Primer/probe†
HPRT1	Hypoxanthine phosphoribosyl-transferase I	NM_000194	Hs99999909_m1
HTPAP-1	Phosphatidic acid phosphatase type 2 domain containing 1B), transcript variant 1	NM_001102559.1	Hs1-pap
HTPAP-2	Phosphatidic acid phosphatase type 2 domain containing 1B, transcript variant 2	NM_032483.3	Hs00261167_m1
HTPAP-3	Phosphatidic acid phosphatase type 2 domain containing 1B, transcript variant 3	NM_001102560.1	Hs2-ANY

†ABI gene expression assay ID.

doi: 10.1111/j.1349-7006.2011.01863.x

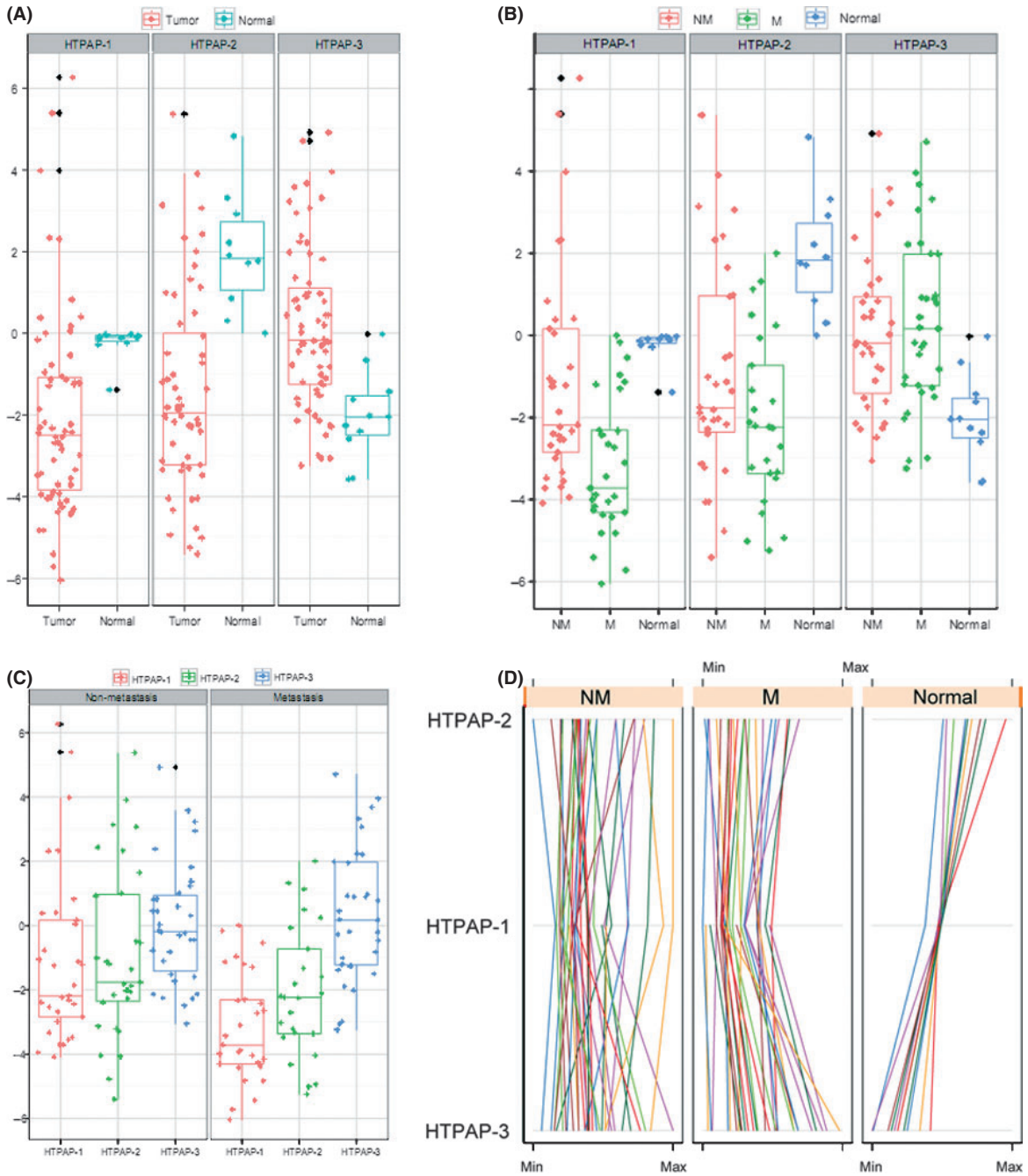

Fig. 2. Expression levels of HTPAP transcript variants in hepatocellular carcinoma (HCC) tissues and normal liver tissues. (A) Expression of varied transcripts in tumor and normal liver tissues. Expression level of HTPAP-1 was lower in tumor than in normal tissue ($P = 0.001413$); HTPAP-2 had the same tendency as HTPAP-1 ($P = 0.0002140$); HTPAP-3 was on the contrary ($P = 0.0003228$). (B) Expression of varied transcripts of HTPAP in HCC without metastasis (NM), HCC with metastasis (M) and normal liver specimens (Normal). (C) Expression of varied transcripts of HTPAP between HCC with and without metastasis. (D) Expression tendency of HTPAP transcript variants in HCC with and without metastasis and in normal liver tissues, along with the expression tendency of individual patient by slop ratio of every curve. Values are given as – CT. Boxes represent the lower and upper quartiles with medians. HTPAP, phosphatidic acid phosphatase type 2 domain containing 1B; HTPAP-1, HTPAP transcript variant 1; HTPAP-2, HTPAP transcript variant 2; HTPAP-3, HTPAP transcript variant 3.

sequences for HTPAP-1 were 5′-CGCACATGTGACTACAAG-CAT-3′ (forward primer) and 5′-GCCGATAGCAGACATAGG-CAAAT-3′ (reverse primer) with a MGB probe sequence VIC-CTGGCAAGATGTACTAGTTG-MGB; the primer sequences for HTPAP-3 were 5′-TCTGAGCCTTGACTATTTGGGTAAA-TATTAC-3′ (forward primer) and 5′-GTACAAGATTGCAG-GATCTAGGCAAA-3′ (reverse primer) with a MGB probe sequence VIC-ACCTTCATGAGATTTGC-MGB; their essential gene-specific data were shown in Table 3. The transcripts were amplified with the TaqMan One-Step RT-PCR Master Mix Reagent and ABI Prism 7900HT sequence detection system (Applied Biosystems). The expression levels of tested genes were quantified in relation to the expression of HPRT1 with the use of sequence detector software and the relative quantification

method (Applied Biosystems). The relative mRNA levels of HTPAP transcript variants were determined with the – ΔCT method.[15]

Construction of recombinant plasmids. Three full-length complementary DNA (cDNA) coding human HTPAP sequence lacking stop codons were amplified from MHCC97L cells cDNAs by PCR (Fig. 1B) and cloned into pEGFP-N1 vector (clontech, Palo Alto, CA, USA) respectively to construct pEGFP-N1-HTPAP-1, pEGFP-N1-HTPAP-2 and pEGFP-N1-HTPAP-3, carboxy-terminally tagged with green fluorescence protein. The primers of HTPAP-1 were 5′-ccgctcgagatggggaaggcggcggcggc-3′ (forward), 5′-cggggtaccgtaatatcaaaacaataag-3′ (reverse); the primers of HTPAP-2 were 5′-ccgctcgagatggggaaggcggcggcggc-3′ (forward), 5′-cgggtaccgtccatttaaagggtcctt-3′ (reverse); the primers of HTPAP-3 were 5′-ccgctcgagatggggaaggcggcggcggc-3′ (forward), 5′-cggggtaccgtgctttctaggttaaatt-3′ (reverse). The recombinant plasmids were extracted and identified by gene sequencing.

Cell culture and transfection. The human hepatoma cell lines of HepG2, MHCC97L, MHCC97H and HCCLM3 were used in this study. MHCC97L, MHCC97H and HCCLM3 were established from the same parent human HCC cell line of MHCC97 in the authors' institution, but with increasing metastatic potential.[18,19] HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured and maintained in DMEM (Gibco BRL, Grand Island, NY, USA), high glucose medium containing 10% FBS (Hyclone, Logan, UT, USA), penicillin (100 units/mL), and streptomycin (0.1 mg/mL) and incubated at 37°C in a humidified incubator under an atmosphere of 5% CO_2.

The day before transfection, cells were plated in 6-well plate in growth medium without antibiotics at a density of 80%. Then 2 μg of three pEGFP-HTPAPs were transfected into HepG2 cells with Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells with pEGFP-N1 were as a negative control. Cells transfected with pEGFP-N1-HTPAPs were also directly examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Western blot analysis. Cells were lysed with RIPA buffer (Pierce Biotechnology, Rockford, IL, USA) containing protease inhibitors (Pierce Biotechnology). Four HCC patients were obtained randomly from the whole cohort to assess the protein expression of three HTPAPs in patients. Beta-actin (Kangchen, Beijing, China) levels were used to normalize loading. Primary antibody of HTPAP were purchased from Lifespan (Lifespan Biosciences, Seattle, WA, USA), which was raised against synthetic peptide of N-Terminus, a conserved region of three transcript variants and, thus can be applied to detect the expression of all three HTPAPs.

Cellular proliferation assay. Three pEGFP-N1-HTPAPs were transfected into HepG2 cells individually with a rapid protocol for 96-well transfections without pre-plating cells (Lipofectamine 2000; Invitrogen Corporation). Cells were seeded at a density of 5×10^3 cells per well in 96-well plates in transfection. The cellular proliferation of transfected cells was measured via CyQUANT Cell Proliferation Assay kit (Invitrogen Corporation). Each assay was repeated three times.

***In vitro* invasion Matrigel assays.** The invasive ability of HCC cells was determined using Matrigel (BD Pharmingen, San Diego, CA, USA) coated 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8-um pore (Costar, New York, NY, USA). The bottom chamber was filled with DMEM containing 10% FBS as a chemoattractant. The transfected cells (5×10^4) were seeded on the top chamber and incubated at 37°C in 5% CO2 humidified air for 24 h. The cells that migrated to the underside of the membrane were stained with Giemsa (Sigma, St. Louis, MO, USA) and counted with a microscope (Olympus).

Statistical analysis. Analyses were performed on all included patients. The endpoints included the TTR and OS. TTR was defined as the time between the surgery and the first report of intrahepatic or distant recurrence (excluding patients who died before recurrence from causes unrelated to liver cancer). TTR was recorded as the date of death or the last follow up for patients who had not experienced a recurrence at the time of death or last follow up, respectively. OS was defined as the interval between the dates of surgery and death. Diagnosis of recurrence was based on typical imaging appearance and/or an elevated AFP level.[20] The Pearson chi-squared test or Fisher exact test were used to compare qualitative variables; and Student's *t*-test was used to analyze the quantitative variables. Kaplan–Meier analysis was used to determine the OS. Median value was used to determine the cutoff parameter dividing high and low expression of HTPAP-1. Statistical analyses were performed with SPSS 15.0 for Windows (SPSS Software, Chicago, IL, USA). A P-value < 0.05 was considered statistically significant.

Results

The expression levels of HTPAP transcript variants detected by real time PCR. The expression levels of three HTPAP variants were detected in 67 HCC tissues and 11 normal liver specimens. As shown in Figure 2, both the expression levels of HTPAP-1 and HTPAP-2 in HCC tissues were much lower than that of normal liver tissues; However, HTPAP-3 was much higher in HCC tissues in comparison with normal liver

Table 4. Association of the expression levels of HTPAP transcript variants with HCC metastasis

Variables	Median of relative expression		*P*-value
	Non-metastasis (n = 34)	Metastasis (n = 33)	
HTPAP-1	−2.21699	−3.927996	0.00053
HTPAP-2	−1.85179825	−3.231766	0.085
HTPAP-3	−0.1983425	0.169273	0.6426

HCC, hepatocellular carcinoma; HTPAP, phosphatidic acid phosphatase type 2 domain containing 1B; HTPAP-1, HTPAP transcript variant 1; HTPAP-2, HTPAP transcript variant 2; HTPAP-3, HTPAP transcript variant 3.

Table 5. Univariate and multivariate analysis of factors associated with overall survival

Clinical variable	Hazard ratio (95% CI)	*P*-value
Univariate analysis		
Tumor size, cm (≤5 *vs* >5)	3.612 (1.322–10.247)	<0.001
Tumor thrombus (yes *versus* no)	3.077 (1.756–5.824)	0.004
HTPAP-1 level (low *versus* high)	0.452 (0.207–0.976)	<0.001
Age, years (<51 *vs* ≥51)	1.000 (1.000–1.000)	0.560
Sex (male *versus* female)	2.831 (0.822–6.470)	0.230
HBsAg (positive *versus* negative)	2.015 (0.784–2.593)	0.312
Liver cirrhosis (yes *versus* no)	2.148 (0.984–9.485)	0.047
Tumor encapsulation (none *versus* complete)	2.413 (1.376–4.523)	0.265
Multivariate analysis		
HTPAP-1 level	0.441 (0.176–0.894)	0.048
Tumor size	1.384 (0.300–8.759)	0.031
Tumor thrombus	2.844 (1.684–4.91)	0.080

HTPAP-1, HTPAP transcript variant 1.

doi: 10.1111/j.1349-7006.2011.01863.x

tissues ($P < 0.05$). Moreover, the HTPAP-1 level in the metastatic HCCs was markedly lower than that of the non-metastasis ones ($P = 0.00053$), which indicated the expression of HTPAP-1 was inversely correlated with HCC metastasis. However, no significant difference in HTPAP-2 and HTPAP-3 levels was found between the metastatic and non-metastatic HCCs (Table 4).

Association of HTPAP-1 expression level with the clinicopathologic features of HCC patients. The association of HTPAP-1 expression level with the clinicopathological features including patients' age, gender, HBsAg positivity, preoperative serum ALT level, serum AFP level, liver cirrhosis, tumor size, tumor encapsulation, vascular invasion and TNM stage etc. was assessed (Table 1). The expression level of HTPAP-1 was found to be significantly correlated with tumor thrombus of HCC; patients with a lower HTPAP-1 level tended to have tumor thrombus in portal vein ($P = 0.01$).

Association of HTPAP-1 expression level with HCC prognosis. Clinicopathological features showing significance by univariate analysis were adopted as covariates when multivariate Cox proportional hazards analysis were performed. HTPAP-1 was independent risk factor for OS (RR = 0.441, 95% CI 0.176–0.894, $P = 0.048$). Tumor size was also independent risk factor for OS (RR = 1.384, $P = 0.031$) (Table 5).

The median follow-up time for 67 HCC patients tested was 50.2 months, with a range of 36–73 months. Their mean OS time was 46.2 months (95% CI, 39.1–53.4 months), and the mean TTR was 37.8 months (95% CI, 30.1–45.5 months). The OS and TTR time for HCC patients with low HTPAP-1 levels were 22.6 months (95% CI, 14.3–30.9 months) and 13.0 months (95% CI, 6.1–20.0 months), respectively, which were statistically shorter than those with high HTPAP-1 (53.7 months (95% CI, 45.9–61.3 months), $P = 0.0023$; and 46.4 months (95% CI, 37.8–54.9 months), $P = 0.010$) (Fig. 3). It showed that HTPAP-1 level was significantly correlated with survival and tumor recurrence of HCC patients.

The expression levels of HTPAP isoforms in HCC cell lines and patients by Western blot. HCC cell lines of MHCC97-L, MHCC97-H and HCCLM3 have identical genetic background and stepwise increasing metastatic potentials. As shown in Figure 4A, HTPAP-1 was downregulated in MHCC97-H and HCCLM3 cell lines with high metastatic potential compared with MHCC97-L of low metastatic potential in protein level, which was consistent with the expression in mRNA level (Fig. 4B). However, neither HTPAP-2 nor HTPAP-3 displayed a similar tendency on both expressional levels.

To clarify the protein expression of three HTPAPs in HCC patients, three HTPAPs were examined in four samples of HCC tissues by Western blot. On the film blotted by HTPAP antibody probing the N-terminus epitope of three isoforms' conserved region (Fig. 5A), three bands of sizes between 55

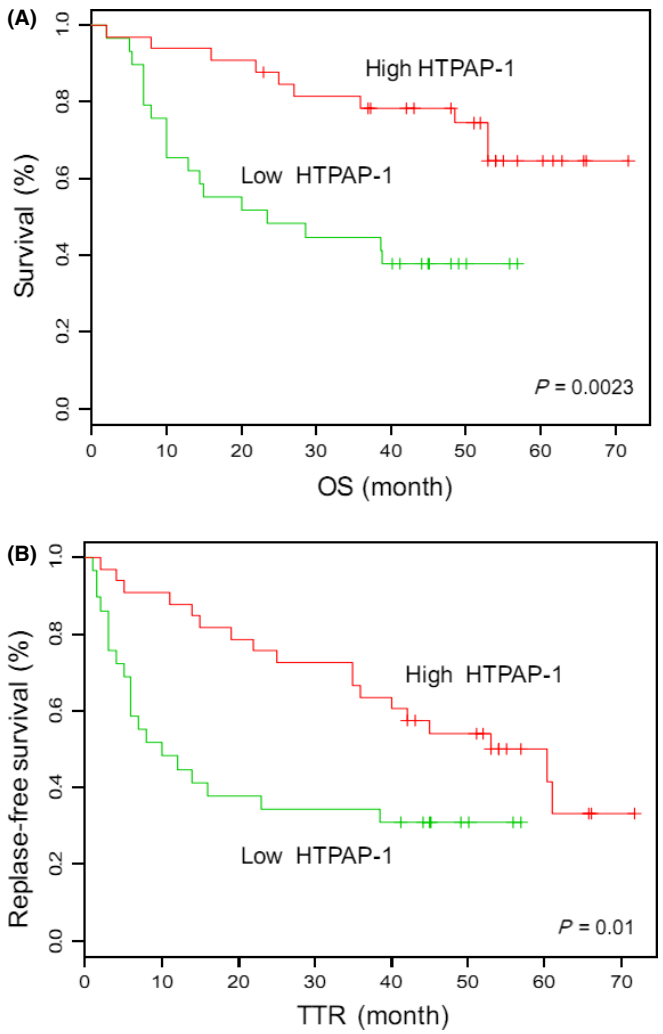

Fig. 3. The association HTPAP-1 expression level with cumulative overall survival (OS) and time to recurrence (TTR) curves of hepatocellular carcinoma (HCC) patients. High expression of HTPAP-1 in HCC was associated with both (A) prolonged survival and (B) reduced probability of tumor recurrence. HTPAP-1, HTPAP transcript variant 1.

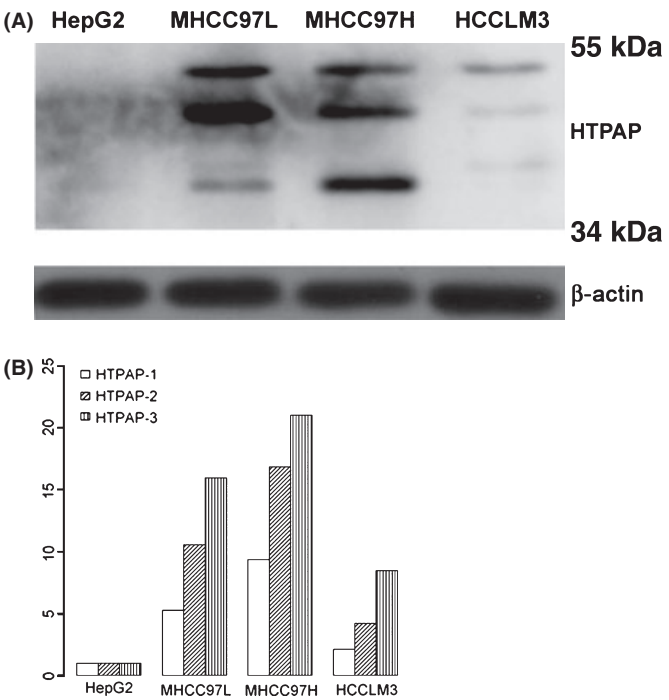

Fig. 4. The expression of HTPAPs in hepatocellular carcinoma cell lines with different metastatic potential. (A) HTPAPs expressions detected by western blot. According to the size of each isoform, the upper bands were HTPAP-1, the middle were HTPAP-2 and the lower were HTPAP-3. (B) HTPAPs expression estimated by real-time PCR. HTPAP, phosphatidic acid phosphatase type 2 domain containing 1B; HTPAP-1, HTPAP transcript variant 1; HTPAP-2, HTPAP transcript variant 2; HTPAP-3, HTPAP transcript variant 3.

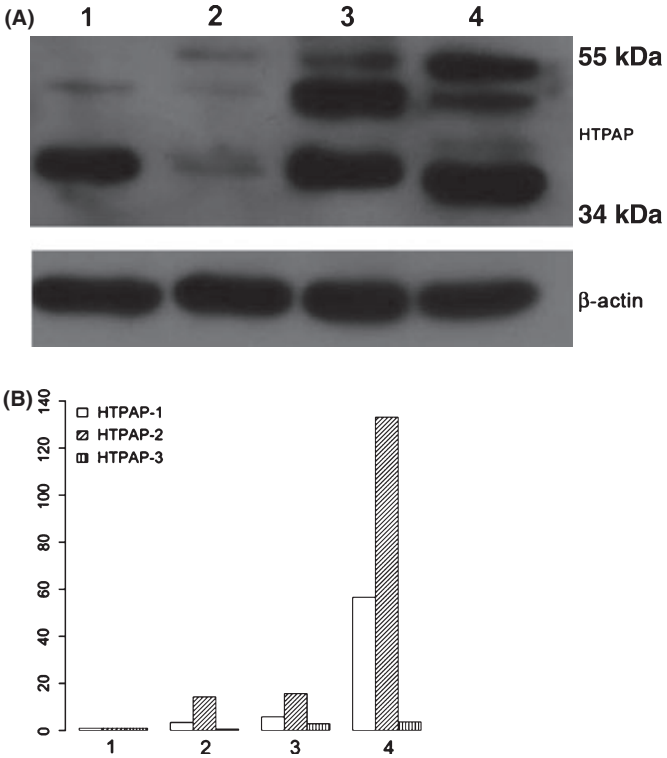

Fig. 5. The expression of HTPAPs in hepatocellular carcinoma patients. (A) HTPAPs expressions detected by western blot in four patients. According to the size of each isoform, the upper bands were HTPAP-1, the middle were HTPAP-2 and the lower were HTPAP-3. (B) HTPAPs expression of four patients estimated by real-time PCR. HTPAP, phosphatidic acid phosphatase type 2 domain containing 1B; HTPAP-1, HTPAP transcript variant 1; HTPAP-2, HTPAP transcript variant 2; HTPAP-3, HTPAP transcript variant 3.

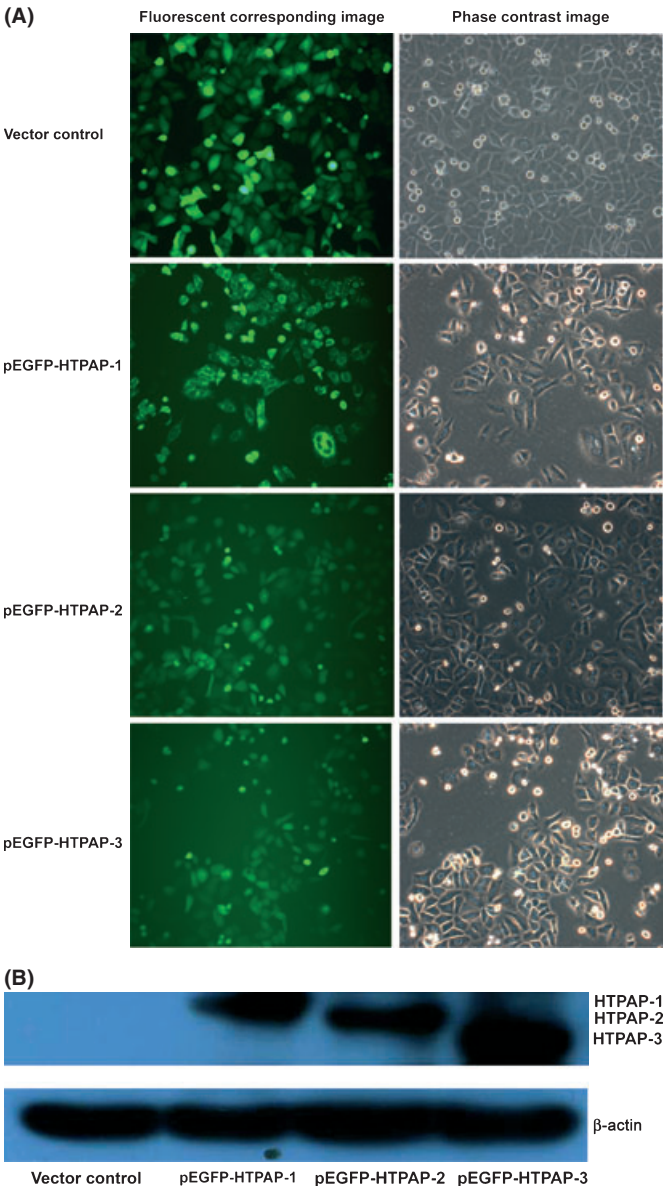

Fig. 6. Overexpression of HTPAPs in hepatocellular carcinoma cell line. (A) Expression of HTPAPs observed by fluorescence microscopy. Phase-contrast microscopy and fluorescent detection of GFP protein in pEGFP-N1 vector control and HTPAPs-GFP transfected HepG2 cells. (B) 3 HTPAP fusion isoforms detected by Western blot from HepG2 cells overexpressed with 3 HTPAPs respectively. HTPAP, phosphatidic acid phosphatase type 2 domain containing 1B.

and 34 kDa were matched to the three transcripts of HTPAP, which displayed little difference from the real-time PCR data (Fig. 5B).

Generation of three HTPAPs overexpressing cells. To clarify three HTPAPs function on HCC metastasis, three fusion GFP vector encoding three HTPAPs were introduced into HepG2. GFP was detected by fluorescence microscopy (Fig. 6A). GFP was diffusely located in the nucleus and cytoplasm of HepG2 with vector pEGFP-N1 control. Similar results were obtained in HepG2 with pEGFP-HTPAP-2 and HepG2 with pEGFP-HTPAP-3, whereas GFP was present on the membrane and in the cytoplasm, forming the adhesion macula of HepG2 with pEGFP-HTPAP-1. Overexpressions of three HTPAPs were confirmed by Western blot. As shown in Figure 6B, three HTPAP isoforms were substantially upregulated in corresponding transfected HepG2 cells. These cells overexpressing HTPAPs were used for further study.

Effect of three HTPAPs on the proliferation and invasion of HCC cells *in vitro*. Cells transfected with three HTPAPs were used to determine the role of each HTPAP on HCC cell proliferation and invasion. As shown in Figure 7, compared with cells transfected pEGFP-N1 vector, proliferation ability of HCC cells was slightly inhibited by HTPAP-1 transfection but without statistical significance ($P = 0.096$); proliferation abilities of HCC cells with either HTPAP-2 or HTPAP-3 did not vary too much.

In the Matrigel assays, the migrated cell number of HepG2 cell with HTPAP-1 (71 ± 4.38), was much lower than that of the negative control cells (135 ± 8.26, $P = 0.044$), which suggests that HTPAP-1 could significantly suppress the invasion of HCC cells. No significant difference was found in HepG2 cells with HTPAP-2 (107 ± 11.49, $P = 0.113$) and HepG2 cells with HTPAP-3 (120 ± 10.75, $P = 0.235$) (Fig. 8), related to the negative control.

Discussion

Metastasis suppressor genes encode proteins that prevent or reduce the development of metastases *in vivo,* without simultaneously affecting primary tumor growth.[21] Understanding the biology of metastasis suppressors provides valuable mechanistic insights that may translate to therapeutic opportunities.

doi: 10.1111/j.1349-7006.2011.01863.x

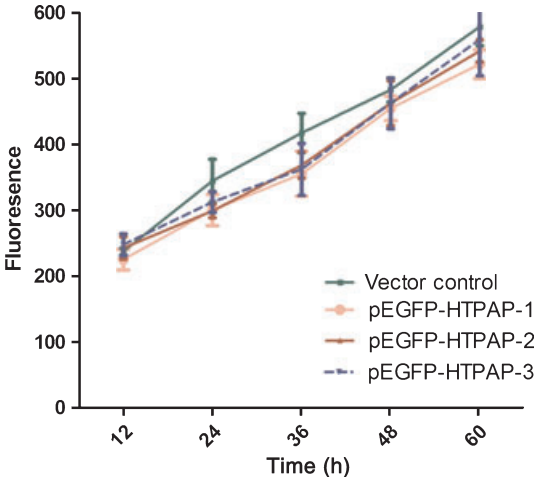

Fig. 7. The effect of HTPAPs on proliferation of HepG2 cells. No significant alterations in the proliferation abilities were found in HepG2 cells transfected with three HTPAPs genes respectively and the vector control. HTPAP, phosphatidic acid phosphatase type 2 domain containing 1B.

Nevertheless, until recently, relatively few metastasis suppressor genes had been characterized.[22]

Our previous studies' data suggest that HTPAP gene plays an important role in the process of HCC metastasis.[6,11,23] In this study, we compared the expression levels of three transcript variants of HTPAP in HCC samples by real-time PCR and found HTPAP-1 in tumor tissues was markedly reduced relative to that in normal liver tissues, and in metastatic HCC patients HTPAP-1 was largely downregulated relative to that in non-metastasis which was consistent with the expressions of HTPAP-1 in HCC cell lines with different metastatic potentials. The expression profiles of three HTPAPs indicated HTPAP-1 was the major effector among HTPAPs in the progression of metastasis. Our *in vitro* invasion data confirmed the function of HTPAP-1 on suppressing HCC metastatic.

Metastasis is the process by which tumor cells disseminate from the primary tumor, migrate through the basement membrane, survive in the circulatory system, invade into a secondary site, and start to proliferate.[24,25] However, a detailed understanding of which steps in metastasis are affected by these genes has yet to be elucidated.[8] Our present data manifested that the expression level of HTPAP-1 inversely correlated with tumor thrombus. Patients with reduced HTPAP-1 were prone to develop tumor thrombus and vice versa. It implied that HTPAP-1 might exert its function by restricting vascular metastases.

In patients with HCC and resectable tumors, the presence of tumor thrombus can indicate a poor prognosis.[26,27] To understand the prognostic value of HTPAP-1 in HCC, we analyzed the association between HTPAP-1 level and survival. We found patients with reduced HTPAP-1 had significantly short survival period and short time to recurrence. It manifested that downregulated HTPAP-1 may be a potentially life-threatening factor for HCC, and hence, it can be facilitated as a useful prognostic indicator for HCC patients.

Alternative splicing is a complex and highly regulated process. At least 15% of all disease-causing single nucleotide base changes affect splicing. Alternative splicing is thought to occur in more than 70% of all genes and may be regulated differentially during development and in a tissue-specific manner.[28,29] In our study, we found that all three variants of HTPAPs were expressed in HCC and their expressional levels varied greatly. Their different expression profiles revealed that three transcripts had independent functions, which were also supported by immunofluorescence data for distinct localizations of HTPAPs. Our data manifested poor correlations between mRNA level and protein level of HTPAPs in HCC samples. There are presumably several reasons for the discrepancy. First, there are many complicated and varied post-transcriptional mechanisms involved in turning mRNA into protein; second, proteins may differ substantially in their *in vivo* half lives; and the last, the available antibody just probing the N-terminus epitope, a conserved region of three transcript variants of HTPAP was used and three transcript variants were identified according to their sizes, which would contribute to a large amount of noise in experiments to limit our ability to get a clear picture.[30–32]

To sum up, our data suggested that HTPAP transcript variant 1 functioned as the real effector of metastasis suppressor in HCC, and had prognostic value in HCC. HTPAP-1 could predict patients' recurrence and survival after hepatectomy, and provide advice to patients and guidance for assessment and treatment of HCC patients. Moreover, with the new conception of metastasis suppressor drugs emerging, HTPAP-1 may become an

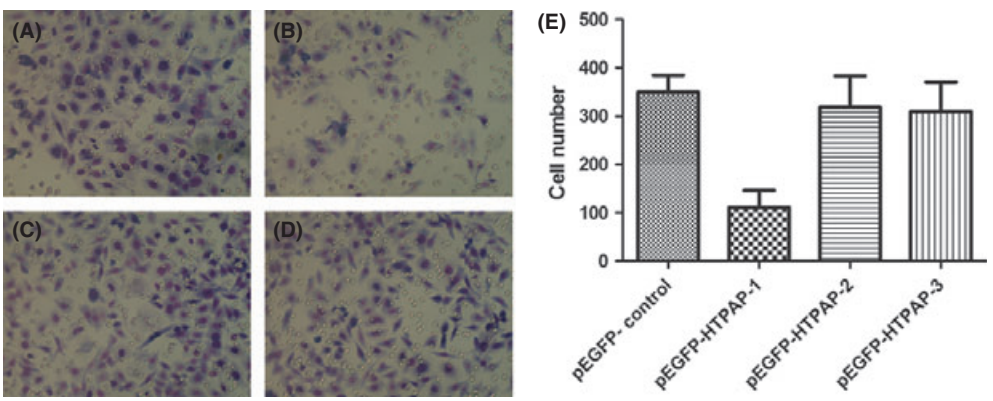

Fig. 8. The effect of HTPAPs on invasive abilities of HepG2 Cells. The data indicated the invasion ability of HepG2 cells transfected with (B) HTPAP-1 was reduced at large scale, compared with (A) HepG2 cell with vector control, (C,D) while neither of other two HTPAPs displayed any obvious alterations. The changes of invasion abilities of three HTPAPs were also exhibited in bar graphs (bottom) and (E) values are shown as cell numbers across membrane.

anti-metastasis drug aiming at its targets in the progress of metastasis, and deserve our expectation.

Acknowledgments

This work was supported by China National Key Projects for Infectious Disease (2008ZX10002-021), the ''973'' State Key Basic Research Program of China (2009CB521701), the Program of Shanghai Chief Scientist (08XD14008), China National Natural Science Foundation (30872946,30600589), Shanghai Rising Star of Young Scientist Project (07QA14010).

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

AFP	alpha-fetoprotein
ALT	alanine aminotransferase
CT	computed tomography
HCC	hepatocellular carcinoma
HPRT1	hypoxanthine phosphoribosyl-transferase I
HTPAP	phosphatidic acid phosphatase type 2 domain containing 1B
HTPAP-1	HTPAP transcript variant 1
HTPAP-2	HTPAP transcript variant 2
HTPAP-3	HTPAP transcript variant 3
MRI	magnetic resonance imaging
OS	overall survival
TTR	time to recurrence

References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
2 Tang ZY, Ye SL, Liu YK *et al.* A decade's studies on metastasis of hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2004; **130**: 187–96.
3 Sherman M. Recurrence of Hepatocellular Carcinoma. *N Engl J Med* 2008; **359**: 2045–7.
4 Bruix J, Llovet JM. Major achievements in hepato cellular carcinoma. *Lancet* 2009; **373**: 614–6.
5 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
6 Wu X, Jia HL, Wang YF *et al.* HTPAP gene on chromosome 8p is a candidate metastasis suppressor for human hepatocellular carcinoma. *Oncogene* 2006; **25**: 1832–40.
7 Bernard-Pierrot I, Gruel N, Stransky N *et al.* Characterization of the recurrent 8p11-12 amplicon identifies PPAPDC1B, a phosphatase protein, as a new therapeutic target in breast cancer. *Cancer Res* 2008; **68**: 7165–75.
8 Steeg PS. Metastasis suppressors alter the signal transduction of cancer cells. *Nat Rev Cancer* 2003; **3**: 55–63.
9 Ye QH, Qin LX, Forgues M *et al.* Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *Nat Med* 2003; **9**: 416–23.
10 Budhu A, Forgues M, Ye QH *et al.* Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. *Cancer Cell* 2006; **10**: 99–111.
11 Qin LX, Tang XY, Sham JST *et al.* The association of chromosome 8p deletion and tumor metastasis in human hepatocellular carcinoma. *Cancer Res* 1999; **59**: 5662–5.
12 Khan J, Wei JS, Ringner M *et al.* Classification and diagnostic prediction of cancers using gene expression profiling and artificial neural networks. *Nat Med* 2001; **7**: 673–9.
13 Budhu A, Jia HL, Forgues M *et al.* Identification of metastasis-related microRNAs in hepatocellular carcinoma. *Hepatology* 2008; **47**: 897–907.
14 Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol* 2002; **30**: 503–12.
15 Schmitten TD, Livak KJ. Analyzing real-time PCR data by the comparative C method. *Nat Protoc* 2008; **3**: 1101–8.
16 Wittekind C, Compton CC, Greene FL, Sobin LH. TNM residual tumor classification revisited. *Cancer* 2002; **94**: 2511–6.
17 Fu LY, Jia HL, Dong QZ *et al.* Suitable reference genes for real-time PCR in human HBV-related hepatocellular carcinoma with different clinical prognoses. *BMC Cancer* 2009; **9**: 49.
18 Li Y, Tian B, Yang J *et al.* Stepwise metastatic human hepatocellular carcinoma cell model system with multiple metastatic potentials established through consecutive in vivo selection and studies on metastatic characteristics. *J Cancer Res Clin Oncol* 2004; **130**: 460–8.
19 Tian J, Tang ZY, Ye SL *et al.* New human hepatocellular carcinoma (HCC) cell line with highly metastatic potential (MHCC97) and its expressions of the factors associated with metastasis. *Br J Cancer* 1999; **81**: 814–21.
20 Llovet JM, Di Bisceglie AM, Bruix J *et al.* Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* 2008; **100**: 698–711.
21 Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell* 2006; **127**: 679–95.
22 Smith SC, Theodorescu D. Learning therapeutic lessons from metastasis suppressor proteins. *Nat Rev Cancer* 2009; **9**: 253–64.
23 Qin LX, Tang ZY, Ye SL *et al.* Chromosome 8p deletion is associated with metastasis of human hepatocellular carcinoma when high and low metastatic models are compared. *J Cancer Res Clin Oncol* 2001; **127**: 482–8.
24 Fidler IL. Timeline – The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 2003; **3**: 453–8.
25 Chambers AF. The metastatic process: basic research and clinical implications. *Oncol Res* 1999; **11**: 161–8.
26 Tsai TJ, Chau GY, Lui WY *et al.* Clinical significance of microscopic tumor venous invasion in patients with resectable hepatocellular carcinoma. *Surgery* 2000; **127**: 603–8.
27 Ikai I, Yamaoka Y, Yamamoto Y *et al.* Surgical intervention for patients with stage IV-A hepatocellular carcinoma without lymph node metastasis – Proposal as a standard therapy. *Ann Surg* 1998; **227**: 433–9.
28 Gopalakrishnan S, Emburgh BO, Shan JX *et al.* A novel DNMT3B splice variant expressed in tumor and pluripotent cells modultes genomic DNA methylation patterns and displays altered DNA bind. *Mol Cancer Res* 2009; **7**: 1622–34.
29 Black DL. Mechanisms of alternative pre-messenger RNA splicing. *Annu Rev Biochem* 2003; **72**: 291–336.
30 Baldi P, Long AD. A Bayesian framework for the analysis of microarray expression data: regularized t-test and statistical inferences of gene changes. *Bioinformatics* 2001; **17**: 509–19.
31 Szallasi Z. Genetic network analysis in light of massively parallel biological data acquisition. *Pac Symp Biocomput* 1999; **4**: 5–16.
32 Dov G, Christopher C, Kenneth W *et al.* Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol* 2003; **4**: 117.

doi: 10.1111/j.1349-7006.2011.01863.x