Research Article

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PEBP1 downregulation is associated to poor prognosis in HCC related to hepatitis B infection

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Background & aims: Phosphatidylethanolamine-binding protein 1 (PEBP1, also RKIP) plays a pivotal role in cancer by regulating multiple cellular signaling processes and suppressing metastasis in animal models. We examined whether PEBP1 expression in hepatocellular carcinoma (HCC) correlated with the risk of recurrence and survival after resection.

Methods: A randomly selected cohort of 240 Chinese HCC patients, predominantly hepatitis B related, formed the basis of the study. PEBP1 expression levels were evaluated by immunohistochemistry and real-time reverse-transcriptase PCR. Survival analysis was performed by univariate and multivariate analyses. The results were further validated in an independent series of 403 patients. The relevance of PEBP1 to phospho-ERK was determined by Western blot analysis on clinical samples and hepatoma cell lines.

Results: PEBP1, prevalently down-regulated in HCC, was significantly associated with tumor invasive characteristics (such as vascular invasion, lack of encapsulation, poor differentiation and large size). Both PEBP1 protein and mRNA levels were independent predictors for tumor recurrence (hazard ratio (HR) = 1.877, $p = 0.001$; HR = 2.633, $p = 0.001$; respectively), and patient survival (HR = 1.796, $p = 0.004$; HR = 1.730, $p = 0.044$; respectively). The prognostic value of PEBP1 was then confirmed in the validation cohort. In addition, Western blot suggested that loss of PEBP1 led to hyperactivity of MAPK signaling.

Conclusions: Down-regulation of PEBP1 in HCC indicated aggressive tumor behaviors and predicted a worse clinical outcome, which may be a useful biomarker to identify the patients at high risk of post-operative recurrence.

Keywords: Phosphatidylethanolamine-binding protein 1 (PEBP1); Prognosis; Hepatocellular carcinoma.

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide. The majority of patients, who are usually diagnosed at an advanced stage, still lack effective treatment and have an extremely dismal prognosis. Even in well-selected patients, who underwent potentially curative treatments such as resection, the recurrence rate can be as high as 50% at 3 years [1]. Therefore, there is an urgent need to explore novel therapeutic approaches, especially for the adjuvant setting of resection, as well as to better stratify patients, and at an earlier stage, according to their risk of recurrence and survival.

Data about molecular pathogenesis, signal transduction pathways, and potential therapeutic targets in HCC have been accumulating, providing new encouraging treatment options [2]. Importantly, the role of aberrant Ras/Raf/MEK/ERK signaling in HCC progression has been increasingly recognized [3–4]. More recently, sorafenib, a multi-kinase inhibitor, targeting Raf kinase and VEGFR, has proved to be effective in prolonging the survival of patients with advanced HCC [5], thus heralding a new era of molecular targeting therapy and reinforcing the utility of blocking Ras/MAPK signals in the treatment of HCC. However, given the complexity of HCC, unless we develop successful strategies or patient selection, it is unlikely that a single agent will have meaningful impact [6].

PEBP1, also known as Raf kinase inhibitory protein (RKIP), was originally identified as an endogenous inhibitor of Raf and it negatively regulates the Raf/MEK/ERK-signaling cascade [7]. It has been well-established that PEBP1 suppresses the metastatic spread of tumor cells, moreover, the down-regulated expression of PEBP1 is observed in a number of human cancers [8]. Subsequent studies revealed that PEBP1 can also inhibit G protein-coupled receptor (GPCR) kinase and NFκB-signaling pathways [9], two other major signaling cascades in HCC initiation and progression [10,11]. As a modulator of key signaling pathways, in addition to its pivotal role in regulating cell differentiation, cycle and migration, evidence has also suggested that PEBP1 potentiates the apoptosis of tumor cell induced by chemotherapy or radiotherapy [12,13]. What is more, an *in vitro* study demonstrated that PEBP1 over-expression can sensitize tumor cells to TRAIL and allow tumor cells to be eliminated by host cytotoxic lymphocytes [14]. Thus, the attenuation of PEBP1 in tumor cells may also represent an underlying molecular mechanism of tumor progression. Taken together, we hypothesize that PEBP1 may

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serve as a useful marker for prognosis in HCC patients and may also be a potential therapeutic target in HCC.

However, as for its clinical utility, the data are still conflicting. Although it was reported that PEBP1 was an independent prognostic factor in prostate [15], colorectal [16–17], and gastric cancers [18], independent observations also showed no correlation between PEBP1 expression and the clinical course in melanoma [19]. As such, PEBP1's role in human HCC warrants a cancer-specific exploration. However, to our knowledge, few studies have described the clinical significance of PEBP1 in HCC; some limited data were obtained from cell lines and a small number of tumor samples, showing that PEBP1 was down-regulated in HCC and that the loss of PEBP1 could promote proliferation and migration of hepatoma cells [20,21].

Based on the considerations mentioned above, we evaluated tumor PEBP1 expression in a large, randomly selected HCC cohort and validated in an independent cohort. We demonstrated that the down-regulation of PEBP1 in HCC significantly correlated with tumor aggressiveness, and PEBP1 was an independent prognosticator for recurrence and poor survival.

Patients and methods

Patient selection and tissue microarray construction

Informed consent was obtained and the study was approved by the Zhong Shan Hospital Research Ethics Committee. The inclusion and exclusion criteria have been reported elsewhere [22–24]. Briefly: (a) distinctive pathologic diagnosis, (b) without pre-operative anti-cancer treatment and distant metastases, (c) curative liver resection, (d) with a complete clinico-pathologic and follow-up data. According to the criteria, a total of 2523 eligible cases were identified from the database of our institute between 2002 and 2006. Using computer-generated random numbers via SPSS software, 240 patients were selected from this cohort for tissue microarrays construction, as described previously [22].

Follow-up procedures and post-operative treatments according to a uniform guideline were described previously [22–23,25–26]. The patients were followed up every 2–4 months after discharge and were monitored prospectively by serum α-fetoprotein (AFP), abdomen ultrasonography, and chest X-ray. For patients with test results suggestive of recurrence or metastasis, computed tomography and/or magnetic resonance imaging were used. The time of surgery was used to calculate time to event. Time to recurrence (TTR) or overall survival (OS) was censored at last follow-up (March 31, 2009) for the patients without recurrence or death.

The clinico-pathologic characteristics of the cohort are described in Table 1, which were also described previously [22]. Most patients (91.25%, 219/240) had hepatitis B virus background, one patient had hepatitis C virus, and four patients had both. Almost all the patients (239/240) were in Child-Pugh A classification. The Barcelona Clinic Liver Cancer (BCLC) staging system was applied to classify the disease stage [27]. Tumor differentiation was graded by the Edmondson–Steiner grading system.

Tissue microarrays were produced as described previously [22]. Each case had triplicate of 1 mm cores in 3-aminopropyltriethoxysilane-coated slides, along with different controls (spleen, lymph node, artery, and glioma).

Cells lines

Five human hepatoma cell lines were used, including HepG2, Hep3B (American Type Culture Collection), MHCC97L, MHCC97H, and HCCLM3. The latter 3 HCC cell lines were established in our institute, from the same parental cell line MHCC97, with stepwise pulmonary metastatic potential (MHCC97L < MHCC97H < HCCLM3) which had been reported elsewhere [28–30].

Cell lines were all maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C with 5% CO_2.

Real-time reverse-transcriptase PCR

The frozen specimens stored at −80 °C were subjected to RNA extraction, and real-time, reverse-transcriptase polymerase chain reaction (RT-PCR)-based expression analysis for PEBP1. The primer sequence of PEBP1 was as follows: forward: 5′-ATAGACCCACCAGCATTTCGT-3′ and reverse: 5′-GTAAACCAGCCAGACATAGCG-3′. The thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Total RNA was extracted from 240 tumors and 120 randomly selected peritumoral tissues. After the determination of the purity and the integrity, RNA samples from 225 tumors and 104 peritumoral tissues with sufficient quality and quantity were finally to be considered for experiments to analyze the gene expression of PEBP1. As a result, there were 104 matched pairs of tumor and peritumoral tissues with PEBP1 gene expression profiles.

For data analysis, the $2^{-\Delta\Delta Ct}$ method was used. The value of $2^{-\Delta\Delta Ct}$ indicated the fold change in gene expression normalized to two housekeeping genes (TBP and HPRT) and calibrated to the expression of the normal liver tissue pool ($n = 10$), as previously described [31].

Table 1. Clinicopathologic characteristics of two cohorts of HCC patients.

Characteristic		training set (n = 240)	validation set (n = 403)	*p* value
Age (years)	≤52	124	203	NS
	>52	116	200	
Gender	male	204	353	NS
	female	36	50	
γ-GT (units/L)	≤54	104	164	NS
	>54	136	239	
AFP (ng/ml)	≤20	82	142	NS
	>20	158	261	
Virus infection	HBV	219	327	0.001*
	HCV	1	5	
	HBV+HCV	4	5	
	None	16	66	
Liver cirrhosis	no	28	74	0.025
	yes	212	329	
Tumor differentiation	I-II	135	263	0.023
	III-IV	105	140	
Tumor size (cm)	≤5	117	233	0.026
	>5	123	170	
Tumor number	single	184	323	NS
	mutliple	56	80	
Encapsulation	complete	119	235	0.031
	none	121	168	
Vascular invasion	no	131	300	<0.001
	yes	109	103	
	(macroscopic/ microscopic)	(84/25)	(23/80)	<0.001
BCLC stage	A	98	318	
	B	58	62	
	C	84	23	

Note: The χ^2 test was used for comparison between groups. Abbreviations: AFP, α-fetoprotein; γ-GT, γ-glutamyl transferase.
*Fisher's exact test.

Immunohistochemistry and quantitative analysis

Immunohistochemistry was carried out as previously described [22,26]. Briefly, after microwave antigen retrieval, slides were preincubated with primary antibodies against PEBP1 (Agent) overnight, followed by incubation with secondary antibodies, and treated with horseradish peroxidase-conjugated streptavidin. Reaction products were visualized with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.

Quantification of PEBP1 expression level was evaluated by a computer-assisted image system [22,32–33]. Briefly, three images of representative fields were captured by Leica QWin Plus v3 software, under a CCD camera connected to a microscope (Leica Microsystems Imaging Solutions Ltd.) at a magnification