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Prognostic value of PUMA expression in patients with HBV-related hepatocellular carcinoma

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Abstract. – OBJECTIVE: The p53 upregulated modulator of apoptosis (PUMA) is a potent apoptosis inducer that is downexpressed in various tumor types. The aim of this study was to explore the prognostic significance of PUMA expression in patients with hepatitis B virus-related hepatocellular carcinoma (HCC).

MATERIALS AND METHODS: PUMA expressions were examined in 80 pairs of tissues to compare its expression between cancer tissues and paired noncancerous liver tissues using immunohistochemistry (IHC). Relationship between the PUMA expression level and clinicopathological characteristics and clinic outcomes was analyzed.

RESULTS: PUMA protein was all positive in paired non-tumor tissue samples. PUMA were downregulated in 61.25% (49/80) of tumor tissues compared with non-tumor tissues. PUMA levels in cancer tissues were significantly lower than non-tumor in patients with recurrence-related factors and patients at higher stage (stage II, III) ($p < 0.05$). In addition, the expressions level in tumor tissues showed a significant correlation with advanced TNM stage ($p = 0.013$) and present of recurrence-related factors ($p = 0.002$). Furthermore, Kaplan-Meier survival analysis revealed that weak PUMA expression was associated with poor 3-year disease-free survival (DFS) and overall survival (OS) in HCC patients. Finally, multivariate analyses identified that PUMA was an independent poor-prognostic predictor for DFS and OS in patients with HBV-related HCC.

CONCLUSIONS: Our results suggest that PUMA expression is a novel prognostic indicator in HBV-related HCC and may be a potential target for diagnosis and gene therapy.

Key Words:

Hepatitis B virus, Hepatocellular carcinoma, PUMA, Prognosis.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers, ranking the fifth malignancies[1]. In China, > 85% of HCCs are caused by hepatitis B virus (HBV)-related liver disease[2]. Although its postoperative 5-year survival rate is enhanced in the nearly 30 years, the tumor recurrence and metastasis are still difficult to break through[3]. Moreover, patients with recurrence-related factors, including portal vein or bile duct tumor thrombus, multiple lesions and multiple satellite lesions, are most probably to recurrent within 1 years[4,5].

The p53 upregulated modulator of apoptosis (PUMA) protein, was originally identified as a pro-apoptotic member of the BH3-only subgroup of the Bcl-2 family[6]. PUMA protein is closely related to p53-dependent and -independent apoptosis, by directly bind known anti-apoptotic members of the Bcl-2 family via its BH3 domain, results in the displacement of the proteins Bax and/or Bak, which leading to mitochondrial dysfunction and caspase activation[6,7]. In many human tumors such as cutaneous melanomas[8], colon carcinomas[9], oral squamous cell carcinoma[10], ovarian carcinoma[11], pancreatic ductal adenocarcinoma[12], and gallbladder cancer[13], PUMA was subsequently reported as a gene that is frequently dysregulated. Similarly, the dysrugulation of PUMA in HCC has also been reported[14]; however, the correlation of PUMA expression with he survival of HCC, especially with Chinese prevalent HBV-related HCC, is largely unknown.

In the present study, we quantified the expression of PUMA in tissue samples from HCC patients using immunohistochemistry. Additionally, we analyzed the relationship between PUMA ex-

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pression and the clinicopathological features of patients with HCC and evaluated its prognostic value in the postoperative survival of HCC patients.

Materials and Methods

Patients

All 80 paraffin-embedded primary HBV-related HCC samples were obtained from 2008 to 2010 at the Department of Hepatobiliary Surgery, the Affiliated Shengjing Hospital of China Medical University. Liver tissues taken from a distance of ≥ 2 cm away from the tumor, was used as paired non-tumor tissue. The diagnosis of HCC was confirmed by two pathologists. Tumor histopathological grade was defined according to the Edmondson grading system. Clinicopathological records including age, gender, tumor size, serum α-fetoprotein (AFP) level, serum HBV DNA level, recurrence-related factors (portal vein or bile duct tumor thrombus, multiple lesions and multiple satellite lesions), histologic grade and TNM stage were all recorded. Tumor staging was defined according to the 7th edition of the Union for International Cancer Control (UICC) TNM staging system. No patient received any preoperative anticancer treatments. The study protocol was approved by the ethics review board of Affiliated Shengjing Hospital of China Medical University, and written informed consent was obtained from all patients. All of the procedures were done in accordance with the Declaration of Helsinki and relevant policies in China.

Immunohistochemistry (IHC)

The subcellular localization and expression patterns of PUMA (1:100 dilution; Cell Signaling Technology®, Danvers, MA, USA) were detected by immunohistochemical methods described as follows: the 5 μm thick sections were mounted onto glass-slides. After dewaxed in xylene, the slides were rehydrated and washed with Tris-buffered saline (TBS). The endogenous peroxidase activity was quenched by incubation in a mixture of 3% hydrogen peroxide solution for 5 min. After being boiled in citrate buffer pH 6.0 for 15 min, sections were sealed and kept in 10% nonimmune goat serum in TBS (pH 7.5) at room temperature for 20 min. After that, they were incubated with primary antibody at the temperature of 4°C overnight, then with a HRP-conjugated secondary antibody (Envision™ Detection Kit; Gene Tech, Shanghai, China) at room temperature for 30 min. After washing in TBS, the slides were kept in diaminobenzidine for 5-10 min and counterstained with Mayers hematoxylin.

All the specimens were observed and photographed. Under high-power view, images of four representative fields were captured by the Leica QWin Plus v3 software (Leica Microsystems Imaging Solutions, Cambridge, UK) using identical image system settings, and integrated optical density (IOD) (pixels) was measured by NIS-Elements Br3.0. software.

HBV DNA Load Quantified By Real-Time PCR

From cell lysates, DNA was extracted and amplified by real-time PCR. The amplification was performed according to the manufacturer's protocol (Qiagen, Hilden, Germany), the manufacturer of the primers, using anRotor-Gene Q cycler: 37°C for 5 min, 94°C for 1 min, 95°C 5sec, followed by 60°C 30 sec for 40 cycles. Values under or over the detection range were recorded.

Adjuvant Therapy and Follow-up

All patients received prophylactic antibiotic therapy, liver medication and appropriate fluid therapy before resuming the diet; Patients with HBV DNA > 10^3 were given anti-viral therapy. A diagnosis of recurrence was based on the typical imaging appearance of hepatic lesions in computed tomography (CT) and/or magnetic resonance imaging (MRI) scans with elevated AFP levels. Patients with recurrence were treated with surgical resection, ablation, transhepatic arterial chemotherapy and embolization (TACE), sorafenib treatment or supportive care. The treatment decision was based on the recurrence patterns and hepatic functional reserve. Full follow-up data of all 80 patients were recorded until Sept 31, 2013.

Statistical Analysis

All statistical analyses were performed using SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL, USA). Differences between groups was analyzed using Student's *t*-test, 2 test or Wilcoxon test, as appropriate. The overall survival (OS) and disease-free survival (DFS) time was analyzed using the Kaplan-Meier method, and differences in survival were estimated by using the log-rank test. Prognostic factors were ex-

amined by univariate and multivariate analyses (Cox proportional hazards regression model). $p < 0.05$ was considered statistically significant.

Results

Baseline Characteristics

Among 80 cases of HBV patients with primary HCC, 63 were males and 17 were females, with an average age of 54.4 years (range from 43 to 69 years). Patient demographic characteristics were shown in Table I. 26 patients were confirmed to with recurrence-related factors by preoperative examination of enhanced CT, enhanced MRI or pathology, including portal vein or bile duct tumor thrombus, multiple lesions and multiple satellite lesions[4,5]. There were 37 patients with HBV DNA $>1.0 \times 10^3$ copy/mL and 43 patients with HBV DNA $< 1.0 \times 10^3$ copy/mL.

Expression of PUMA in HCC Tissues

PUMA expression levels were examined using IHC in 80 paraffin-embedded primary HBV-related HCC samples. Positive PUMA expression was observed in the cell cytoplasm, representative immunohistochemical PUMA staining images of cancer and paired no-tumor tissue samples are presented in Figure 1. Integrated optical density (IOD) of PUMA expression in tumor tissues and non-tumor tissues was 43.97 ± 32.74 and 54.16 ± 45.30, respectively. No negative cytoplasmic staining was noted in adjacent normal mucosa. In 61.25% (49/80) of patients, PUMA expression in tumor tissues were lower than non-tumor tissues, or similar with non-tumor ones, and PUMA expression in tumor tissues of the other 38.75% (31/80) of patients were higher than non-tumor tissues. Therefore, PUMA expression levels in tumor tissues were categorized as low or high relative based on this comparison result. In addition, PUMA levels in cancer tissues were significantly lower than non-tumor in patients at higher stage (stage II, III) (50.81 ± 15.47 *vs.* 75.27 ± 18.09, n = 48, Figures 2A; $p < 0.05$); and PUMA levels in cancer tissues were significantly lower than non-tumor in patients with recurrence associated risk factors (46.65 ± 10.22 *vs.* 67.75 ± 14.19, n = 26, Figures 2B; $p < 0.05$).

Table I. Relationship between PUMA expression and clinicopathologic parameters of HBV-related HCC patients.

Characteristics	Number of case	PUMA expression		*p* value[b]
		High (n = 31)	Low (n = 49)	
Age (years)	80	54.4 ± 6.40	55.1 ± 7.13	0.549
Gender				0.665
Male	63	22	41	
Female	17	7	10	
Tumor size				0.587
< 5 cm	28	9	19	
≥ 5 cm	52	20	32	
HBV-DNA				0.486
$< 10^3$	43	14	29	
$\geq 10^3$	37	15	22	
AFP level				0.096
< 400 ng/mL	19	8	11	
≥ 400 ng/mL	61	21	40	
TNM stage[a]				0.013
I	32	12	20	
II, III	48	31	17	
Histologic grade				0.160
Well/moderately	20	9	11	
Poorly/others	60	20	40	
Recurrence-related factors				0.002
Absent	54	43	11	
Present	26	6	20	

[a]Tumor stage was obtained according to the TNM criteria. [b]All statistical tests were 2-sided. Significance level: $p < 0.05$.

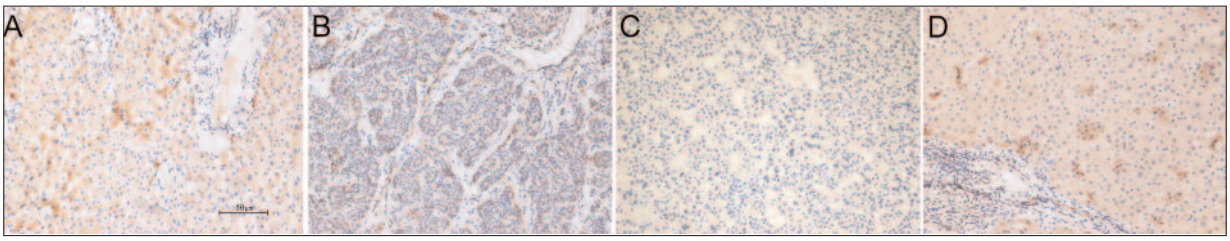

Figure 1. Representative sections of immunhistochemical staining for PUMA protein in malignant and paired non-tumor tissue samples from patients with hepatocellular carcinoma. PUMA was expressed in cytoplasm (brown staining). Malignant hepatocellular tissue samples showing: ***A,*** High level of PUMA in the cytoplasm. ***B,*** Low level of PUMA in the cytoplasm. ***C,*** No PUMA staining. ***D,*** Normal hepatocellular tissue sample showing high level of PUMA staining (200 ×).

Correlations Between PUMA Expression And Clinicopathological Characteristics

According to the results of IHC, 49 out of 80 patients were classified as low PUMA group ($tumor_{IOD} \leq non\text{-}tumor_{IOD}$) and 31 patients were classified as high PUMA group ($tumor_{IOD} > non\text{-}tumor_{IOD}$). PUMA expression in tumor tissues was significantly associated with advanced TNM stage ($p = 0.013$) and present of recurrence-related factors ($p = 0.002$). However, no significant associations were observed between PUMA expression and age, gender, tumor size, HBV DNA load, AFP level, or histologic grade.

Association of PUMA Expression with Prognosis in Patients with HBV-Related HCC

The median survival time for the 80 cases of HBV-related HCC studied was 31.60 ± 1.68 months, ranging from 12 to 49 months. The 3-year DFS and OS of patients with weak PUMA expression was significantly poorer than those with strong PUMA expression (log-rank test: $p = 0.008$ and $p = 0.000$, Figure 3). Univariate and multivariate analyses were carried out using a Cox proportional hazard model to compare the effect of PUMA expression and other clinicopathological parameters on the prognosis of patients with HBV-related HCC. Univariate Cox regression analysis showed that PUMA expression, TNM stage, and recurrence associated risk factors were significantly related with DFS and OS (Tables II and III). Multivariate Cox regression analysis indicated that PUMA expression was an independent predictor for DFS and OS in addition to recurrence-related factors (Tables II and III), which suggested that PUMA expression might be useful for predicting the prognosis of HBV-related HCC patients.

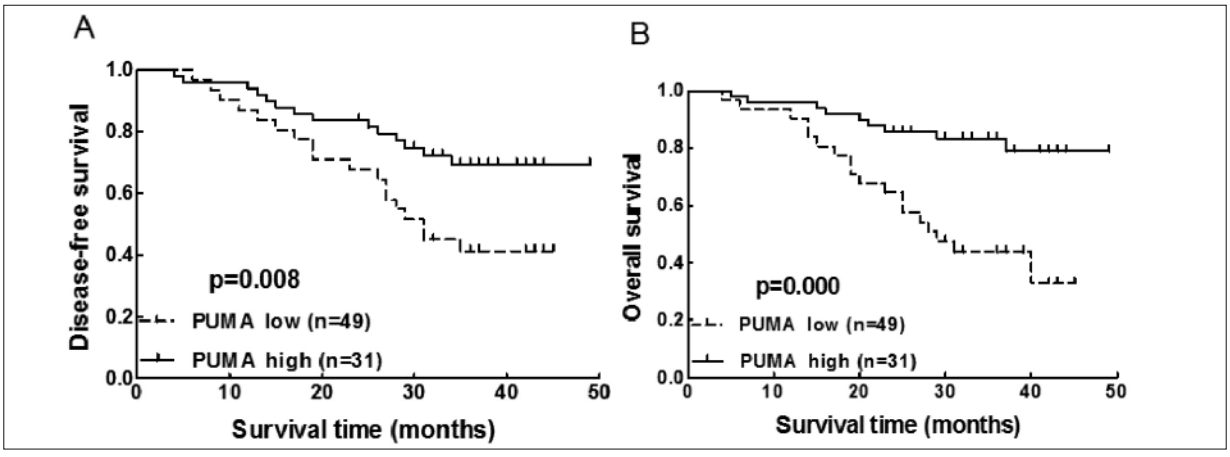

Figure 2. PUMA expression in tumor tissues in HCC patients at higher grade (stage II, III) and patients with recurrence-related factors was lower than non-tumor tissues. ***A,*** PUMA levels in cancer tissues were significantly lower than non-tumor in patients at higher stage (stage II, III) (50.81 ± 15.47 *vs*. 75.27 ± 18.09, n = 48; $p < 0.05$). ***B,*** The expression of PUMA in cancer tissues were significantly lower than non-tumor tissues in patients with recurrence-related factors (46.65 ± 10.22 *vs*. 67.75 ± 14.19, n = 26; $p < 0.05$).

Table II. Univariate and multivariate analysis of clinicopathological factors for DFS in HBV-related HCC.

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p^a	HR (95% CI)	p^a
Age (≥ 50/< 50)	0.979 (0.896-1.069)	0.633		
Gender (Male/Female)	1.083 (0.140-8.352)	0.939		
Tumor size (≥ 5/< 5)	2.621 (0.804-8.538)	0.110		
HBV-DNA (≥ 10^3/<10^3)	1.081 (0.352-3.320)	0.891		
AFP level (ng/mL) (≥ 400/< 400)	0.999 (0.997-1.000)	0.087		
TNM stage (III+II/I)	3.619 (2.990-13.234)	0.025		
Histologic grade (poor, others/well, mod)	0.863 (0.264-2.813)	0.806		
Recurrence-related factors (Present/Absent)	5.473 (1.486-20.152)	0.011	2.417 (1.167-10.039)	0.036
PUMA (high/low)	0.408 (0.203-0.821)	0.012	0.288 (0.128-0.644)	0.002

HR: Hazard ratio; CI: confidence interval. [a]All statistical tests were 2-sided. Significance level: $p < 0.05$.

Discussion

Previous studies have demonstrated the dysregulation of apoptotic regulatory protein PUMA in tumors[14-17]. The available evidence suggests that that multiple mechanisms in tumorigenesis (mutation or deletion of p53, complete deletion of the long arm of chromosome 19, overexpression of anti-apoptotic Bcl-2 family proteins) induce the deficiency of PUMA expression in cancer cells[6]. The upstream promoter sequence of PUMA gene contains p53-binding sites and transcriptional regulation of PUMA include p53-dependent pathway. p53 (tetramers) bind directly with its target site to promote PUMA transcription and protein expression under apoptotic stimuli. In HBV-related HCC, while the activated IKKα/β promotes p53 protein decrease by activation of MDM2[18]; it can also promote PUMA. Phosphorylated PUMA keep the binding properties, but it is easily depredated by proteasome[19], which reduces PUMA expression. In this study, we used IHC to analyzed PUMA protein expression in primary HBV-related HCC tissue and paired non-tumor tissue samples. Although our results showed that PUMA expressions in tumor tissues were not statistically different from those in non-tumor tissues, we found that in 61.25% (49/80) of HCC patients, the PUMA expression in tumor tissues were lower than, or similar with non-tumor tissues, which was consistent with Ahn et al[14] findings.

It is well documented that apoptosis acts as a barrier against oncogenesis, tumor formation and progression are to some extent attributed to deregulated apoptosis [20]. As a critical proapoptotic mediator[7], PUMA is involved in a large number of cancer related physiological and pathological processes by mediating p53-dependent or p53-independent apoptotic and death stimuli[21,22]. For example, PUMA ablation in Bim-deficient mice exacerbated hyperplasia of lymphatic or-

Table III. Univariate and multivariate analysis of clinicopathological factors for OS in HBV-related HCC.

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p^a	HR (95% CI)	p^a
Age (≥ 50/< 50)	0.960 (0.862-1.070)	0.464		
Gender (Male/Female)	1.884 (0.234-5.157)	0.552		
Tumor size (≥ 5/< 5)	2.621 (0.804-8.538)	0.110		
HBV-DNA (≥ 10^3/<103)	1.273 (0.341-4.749)	0.720		
AFP level (ng/mL) (≥ 400/< 400)	0.998 (0.996-1.000)	0.112		
TNM stage(III+II/I)	8.730 (1.087-19.446)	0.042		
Histologic grade (poor, others/well, mod)	1.779 (0.368-8.591)	0.473		
Recurrence-related factors (Present/Absent)	12.625 (1.563-27.005)	0.017	6.098 (1.626-9.372)	0.025
PUMA (high/low)	0.217 (0.083-0.564)	0.002	0.359 (0.121-0.914)	0.005

HR: Hazard ratio; CI: confidence interval. [a]All statistical tests were 2-sided. Significance level: $p < 0.05$.

gans, and promoted spontaneous malignancies[23], suppression of PUMA accelerates the E¼-Myc-induced lymphoma formation by blocking p53-dependent apoptosis[24]; and exogenous PUMA expression in human melanoma cell lines results in significant apoptotic cell death[8]. All these indicated that PUMA suppresses tumorigenesis. In this case, its not surprisingly that in our cohorts mojority of patients showed lower PUMA expression in cancer tissues than non-tumor tissues. Additionally, PUMA protein levels in tumor tissues in HCC patients at higher grade (stage II, III) and patients with recurrence-related factors (including portal vein or bile duct tumor thrombus, multiple lesions and multiple satellite lesions) were lower than non-tumor tissues. These results suggested that PUMA could play a important role in HCC progression and dissemination.

In recent studies, PUMA expression was found to be associated with malignant tumor phenotype. For example, in early stage ovarian carcinoma, the expression of PUMA was found to be positive associated with the positivity of pro-apoptotic Bax protein and the FIGO-stage of the cancer tissues[11]. The expression level of PUMA significantly higher in low-grade tumors (TNM stages I and II), compared with higher grade (stage III) tumors, and a higher PUMA expression was identified in pancreatic ductal adenocarcinoma patients with higher apoptosis index[12]. During the development and progression of cancer, in order to protecting themselves from various apoptotic stimuli, cancer cells activate varies of apoptosis-resistant mechanisms, such as overexpress anti-apoptotic Bcl-2 family members and downexpress pro-apoptotic Bcl-2 family members including PUMA to evade apoptosis. Our findings revealed that lower PUMA expression in tumor tissues was significantly associated with advanced TNM stage and present of recurrence-related factors. It could be that such selected HCC cells might have resistant mechanisms against PUMA-dependent apoptosis.

Conclusions

In this study, we investigated the association between PUMA and the clinical outcome of HBV-related HCC. The univariate analysis suggested that PUMA deregulation may possibly be used to predict both disease-free survival (DFS) and overall survival (OS) in HBV-related HCC patients. By univariate analysis, we also found that recurrence-related factors are closely associate with shorter DFS. Additionally, TNM stage and recurrence-related factors are related to shorter OS. To our knowledge, this is the first report indicating that PUMA expression is closely related to the outcomes of patients with HBV-related HCC after tumor resection, PUMA may serve as a prognostic marker for the survival of HBV-related HCC patients.

Finally, the relatively small sample size in our study might induce a limited statistical power, our results need to be tested in larger cohort from multicenter of China, including cases with HCC of differing etiology. Therefore, a larger well-designed study and functional evaluation are warranted to confirm these findings.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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