



# C-reactive Protein Overexpression in the Background Liver of Hepatitis B Virus–Associated Hepatocellular Carcinoma Is a Prognostic Biomarker

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**Background:** Chronic hepatitis B virus (HBV) infection is a leading cause of hepatocellular carcinoma (HCC). Peripheral blood C-reactive protein (CRP) concentration and CRP overexpression in HCC cells are proven to be prognostic markers for HCC, but the significance of CRP expression in non-neoplastic hepatocytes, which are the primary origin of CRP, has not been studied. This study was conducted to determine the clinicopathologic significance of CRP immunoreactivity in the background liver of HBV-associated HCC. **Methods:** CRP immunostaining was done on tissue microarrays of non-neoplastic liver tissues obtained from surgically resected, treatment-naïve HBV-associated HCCs (n = 156). The relationship between CRP immunoreactivity and other clinicopathologic parameters including cancer-specific survival was analyzed. CRP immunoreactivity was determined using a 4-tier grading system: grades 0, 1, 2, and 3. **Results:** CRP was positive in 139 of 156 cases (89.1%) of non-neoplastic liver in patients with HCCs: grade 1 in 83 cases (53.2%); grade 2 in 50 cases (32.1%); and grade 3 in six cases (3.8%). The patients with diffuse CRP immunoreactivity (grade 3) had decreased cancer-specific survival ( $p = .031$ ) and a tendency for shorter interval before early recurrence ( $p = .050$ ). The degree of CRP immunoreactivity correlated with serum CRP concentration ( $p < .001$ ). **Conclusions:** CRP immunoreactivity in non-neoplastic liver is a novel biomarker for poor cancer-specific survival of HBV-associated HCC and correlates with serum CRP concentration.

**Key Words:** Carcinoma, hepatocellular; C-reactive protein; Hepatitis B virus; Immunohistochemistry; Prognosis

Acute-phase reactant C-reactive protein (CRP) is primarily synthesized by hepatocytes in association with inflammation.<sup>1</sup> Pro-inflammatory cytokines interleukin (IL)-6 and IL-1 upregulate CRP transcription,<sup>2,3</sup> and chronic inflammatory conditions of infectious (e.g., bacteria, virus) and noninfectious origins (e.g., toxins, obesity) provide the microenvironment for carcinogenesis primarily by inducing instability of the cellular genome.<sup>4,5</sup> Inflammatory cellular infiltrates induce the pooling of pro-inflammatory cytokines and oxidative stress at the site of inflammation. Common molecules involved in inflammation-induced carcinogenesis include prostaglandins, nuclear factor  $\kappa$ B, and cytokines. The prognostic significance of CRP blood level has been shown in several human malignancies such as hepatocellular carcinoma (HCC).<sup>6,7</sup> CRP-based risk assessment algorithms, combined with other parameters such as absolute neutrophil count and blood albumin level, also serve as prognostic indices.<sup>8-10</sup>

HCC is a viral infection-associated cancer posing a significant health-care burden worldwide.<sup>11</sup> We have shown that CRP immunoreactivity in HCC cells is a prognostic marker of treat-

ment-naïve HCC.<sup>12</sup> However, the clinicopathologic significance of CRP expression in non-neoplastic background liver, which should be the primary origin of CRP, has not been studied. We postulated that CRP expression in non-neoplastic liver would have pathobiological meaning because the liver is primarily responsible for CRP synthesis. Chronic infection by hepatitis B virus (HBV) is a leading cause of HCC, and this study was conducted to determine the clinicopathologic significance of CRP immunoreactivity in non-neoplastic liver tissue in HBV-associated HCC cases.

## MATERIALS AND METHODS

### Patients and tissue samples

A total of 156 cases of surgically resectable (R0) HBV-associated HCCs was retrieved from the files of the Department of Pathology, Asan Medical Center, Seoul, Republic of Korea. A tumor recurrence within 2 years after surgical resection was defined as early recurrence. The patients have provided written informed con-

sent, and this study was approved by the Institutional Review Board of Asan Medical Center, Seoul, Republic of Korea (S2011-0931).

### Tissue microarray and immunohistochemistry

Tissue microarrays (TMAs) were generated using paraffin blocks of formalin-fixed liver tissues. The hematoxylin and eosin-stained slides were reviewed to select the regions for TMA construction, and 2-mm-thick tissue cores were obtained in duplicates from the donor blocks and transferred onto the recipient blocks. From each TMA, 4- $\mu$ m-thick sections were obtained, and subsequent immunohistochemistry was done. For CRP immunohistochemical staining, a rabbit polyclonal anti-CRP antibody (Abcam, Cambridge, UK) was used at 1:1,000 dilution. The sections were placed on silanized slides, and heat-induced epitope retrieval was performed by treating the slides with Cell Conditioning 1 buffer for 32 minutes in a BenchMark XT automatic immunostainer (Ventana Medical Systems, Tucson, AZ, USA). An OptiView DAB IHC Detection Kit (Ventana Medical Systems) was used for the detection of signal. Evaluation of the immunoreactivity was done by a pathologist (C.J.K.), who was blinded to clinical information, using a 4-tier grading system: negative as grade 0; positive in less than 10% of tumor cells as grade 1; positive in less than 50% of tumor cells as grade 2; and diffusely positive in more than 50% of tumor cells as grade 3.

### Statistical analysis

For statistical evaluation of the significance of the clinicopathologic parameters, the analyses of noncontinuous categorical variables were done using linear by linear association (chi-square test for trend). We also used logistic regression analysis for multivariate analysis of statistically significant variables in univariate analysis. The relationship between serum CRP level and liver tissue CRP immunoreactivity was analyzed using Spearman correlation analysis and displayed using dot plot. The Kaplan-Meier method and Cox regression analysis were used in the survival analysis. SPSS ver. 18.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

## RESULTS

### CRP immunoreactivity in liver tissues

CRP expression was observed as cytoplasmic immunoreactivity. Immunoreactive CRP was found in 139 of 156 cases (89.1%), and 17 cases (10.9%) were negative. Among CRP-positive cases (n = 139), more than half (n = 83; 59.7%) showed grade 1 immu-

noreactivity, followed by the cases with grade 2 (n = 50, 36.0%) and grade 3 (n = 6, 4.3%) immunoreactivity (Fig. 1). The patient demographics and the relationship between CRP immunoreactivity and other clinicopathologic parameters are summarized in Tables 1 and 2. Among the clinicopathologic parameters analyzed, tumor size and serum CRP concentration significantly varied according to CRP immunoreactivity ( $p < .001$  and  $p < .005$ , respectively). Serum CRP concentration (median, 0.135 mg/dL; range, 0.03 to 6.95 mg/dL) showed a positive linear correlation with CRP immunoreactivity ( $p < .001$ ;  $\rho = 0.513$ ) (Fig. 2). The number of tumors ( $p = .058$ ), serum  $\alpha$ -fetoprotein level ( $p = .056$ ), and Edmondson-Steiner histologic grade ( $p = .055$ ) also tended to be different depending on the grade of CRP immunoreactivity.

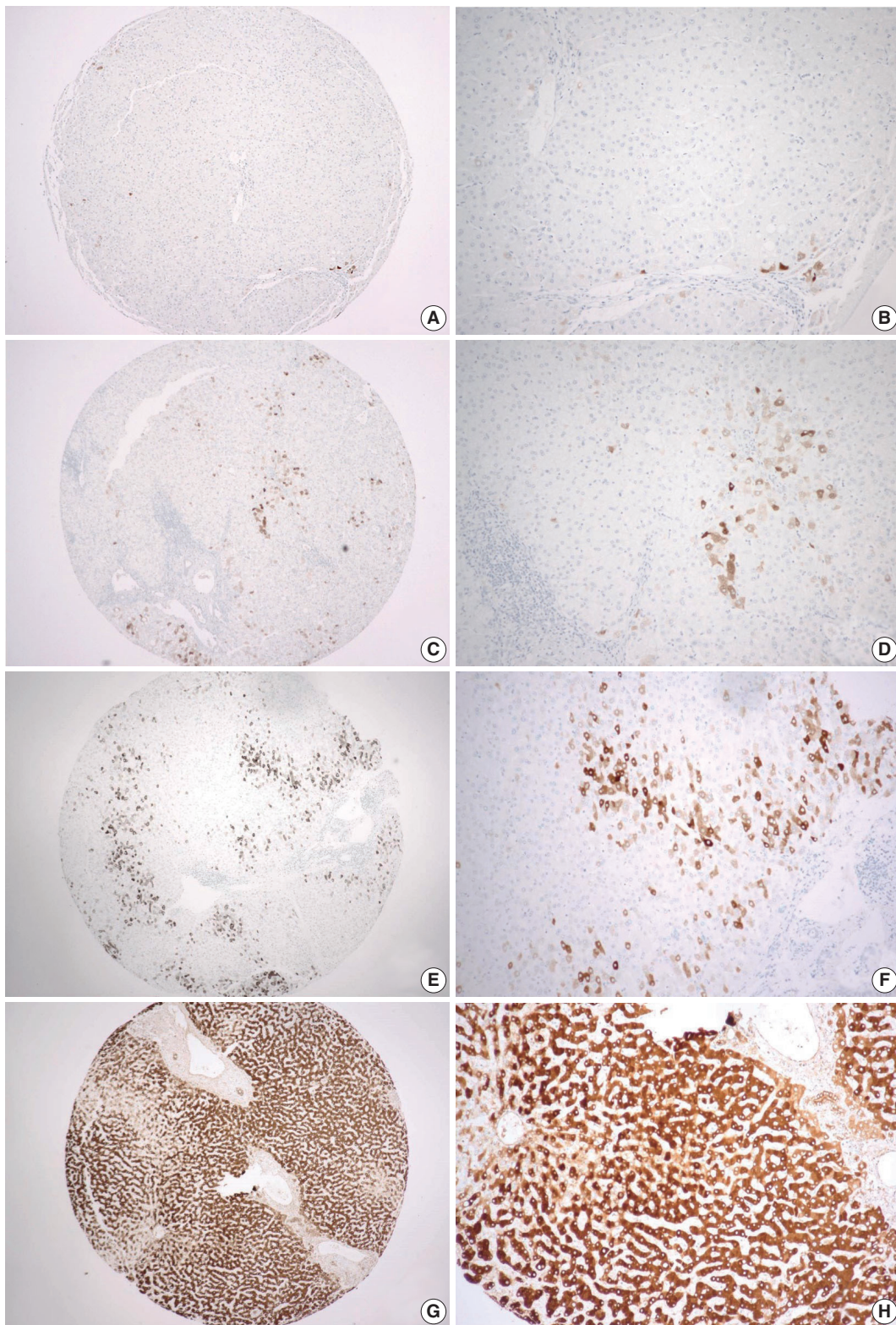
Of note, CRP immunoreactivity grade 3 was exclusively associated with the presence of satellite nodules ( $p < .005$ ). Furthermore, the patients with CRP immunoreactivity grade 3 showed significantly shorter cancer-specific survival (median, 89.3 months; range, 5 to 104 months) compared with CRP-negative or nondiffuse immunopositive cases (median, 60.7 months; range, 8 to 100 months;  $p < .05$ ) (Fig. 3A). Early recurrence occurred in 63 cases (40.4%). Recurrence-free survival tended to be different between strong and diffuse immunopositive cases (grade 3) and CRP-negative or nondiffuse immunopositive cases (grades 0, 1, and 2). The CRP grade 3 group tended to have shorter time before recurrence (median, 37.8 months; range, 2 to 100 months) than CRP-negative or nondiffuse immunopositive cases did (median, 67.4 months; range, 2 to 103 months;  $p = .050$ ) (Fig. 3B).

Multiple logistic analysis showed serum CRP level greater than 1 mg/dL to be independently correlated with high CRP grade ( $p = .017$ ; odds ratio, 12.229; 95% confidence interval, 1.573 to 95.087), whereas tumor size greater than 5 cm and satellite nodule presence are not (Table 3). Kaplan-Meier survival curves demonstrated shorter survival period in satellite nodule-present group (median, 34.0 months; range, 17 to 64 months;  $p < .001$ ) (Fig. 3C) and vascular invasion-present group (mean, 77.4 months; range, 67 to 88 months;  $p = .001$ ) (Fig. 3D). Cox regression analysis results suggest that the presence of satellite nodule is an independent factor affecting cancer-specific survival (Table 4). Vascular invasion and high-grade CRP immunoreactivity might have independent influence on survival rate; however, statistical significance was not observed.

## DISCUSSION

The primary findings of this study are as follows: (1) CRP expression is relatively common in the background liver tissues





**Fig. 1.** Cytoplasmic C-reactive protein immunoreactivity in non-neoplastic hepatocytes in treatment-naive, surgically resected HBV-associated hepatocellular carcinoma cases. Immunoreactivity was determined using a 4-tier grading system: A–B, grade 0; C–D, grade 1; E–F, grade 2; and G–H, grade 3.

**Table 1.** Clinical parameters and their relationship with CRP immunoreactivity

Clinical parameter	CRP grade 0	CRP grade 1	CRP grade 2	CRP grade 3	p-value
Sex					.590
Male	10 (6.4)	63 (40.4)	37 (23.7)	4 (2.6)	
Female	7 (4.5)	20 (12.8)	13 (8.3)	2 (1.3)	
Age (yr)					.764
<60	14 (9.0)	60 (38.5)	36 (23.1)	5 (3.2)	
≥60	3 (1.9)	23 (14.7)	14 (9.0)	1 (0.6)	
Tumor size (cm)					.000
<5	17 (10.9)	67 (42.9)	31 (19.9)	3 (1.9)	
≥5	0	16 (10.3)	19 (12.2)	3 (1.9)	
Serum AFP (ng/mL)					.056
<400	40 (25.6)	29 (18.6)	26 (16.7)	16 (10.3)	
≥400	24 (15.4)	11 (7.1)	5 (3.2)	5 (3.2)	
Serum CRP (mg/dL)					.002
<1	12 (7.7)	43 (27.6)	28 (17.9)	2 (1.3)	
≥1	0	3 (1.9)	5 (3.2)	3 (1.9)	
BCLC stage					.497
A	17 (10.9)	78 (50)	46 (29.5)	6 (3.8)	
B	0	5 (3.2)	4 (2.6)	0	
Fibrosis stage (Batts–Ludwig)					.773
Stage 1, 2	3 (1.9)	13 (8.3)	7 (4.5)	1 (0.6)	
Stage 3, 4	14 (9.0)	70 (44.9)	43 (27.6)	5 (3.2)	
Microvascular invasion					.890
Not identified	14 (9.0)	57 (36.5)	33 (21.1)	4 (2.6)	
Present	3 (1.9)	26 (16.7)	17 (10.9)	2 (1.3)	
Tumor number					.058
Less than 3	17 (10.9)	80 (51.3)	43 (27.6)	6 (3.8)	
3 or more	0	3 (1.9)	7 (4.5)	0	
Satellite nodule					.099
Absent	17 (10.9)	78 (50)	48 (30.8)	4 (2.6)	
Present	0	5 (3.2)	2 (1.3)	2 (1.3)	
Edmondson–Steiner grade (worst)					.169
Grade 1, 2	7 (4.5)	29 (18.6)	14 (9.0)	1 (0.6)	
Grade 3, 4	10 (6.4)	54 (34.6)	36 (23.1)	5 (3.2)	
Edmondson–Steiner grade (most)					.055
Grade 1, 2	14 (9.0)	57 (36.5)	30 (19.2)	3 (1.9)	
Grade 3, 4	3 (1.9)	26 (16.7)	20 (12.8)	3 (1.9)	
Capsular invasion					.292
Absent	17 (10.9)	65 (41.7)	40 (25.6)	5 (3.2)	
Present	0	18 (11.5)	10 (6.4)	1 (0.6)	
Early recurrence					.415
Absent	38 (24.4)	27 (17.3)	18 (11.5)	10 (6.4)	
Present	26 (16.7)	13 (8.3)	13 (8.3)	11 (7.1)	

Values are presented as number (%).

CRP, C-reactive protein; AFP,  $\alpha$ -fetoprotein; BCLC, Barcelona Clinic Liver Cancer.

in HBV-associated HCC cases (89.1%); and (2) there is an inverse relationship between diffuse and strong CRP immunoreactivity and cancer-specific patient survival ( $p = .031$ ). Notable variables significantly associated with CRP immunoreactivity also included tumor size, presence of satellite nodules, and serum CRP con-

centration.

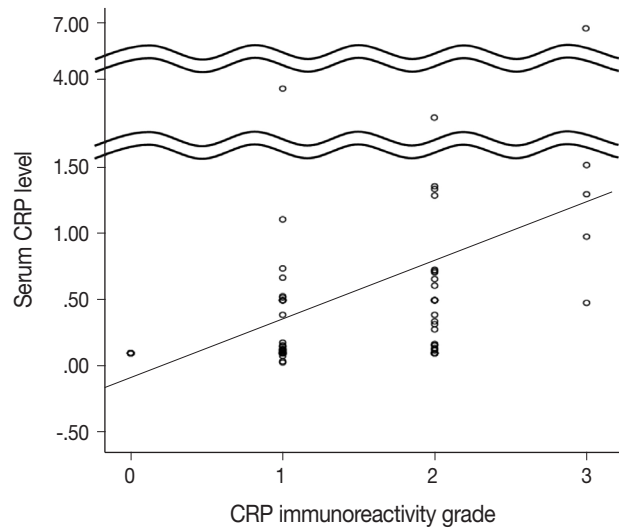
Hepatocellular carcinogenesis represents a classic model of viral etiology, and HCC is among the major causes of cancer-related mortalities.<sup>13</sup> The prevalence of hepatitis virus infection is relatively higher in East Asian countries. Chronic HBV infection

**Table 2.** Clinical parameters and their relationship with CRP immunoreactivity

Clinical parameters	CRP grade 0, 1, 2	CRP grade 3	p-value
Sex			.719
Male	110 (70.5)	4 (2.6)	
Female	40 (25.6)	2 (1.3)	
Age (yr)			.586
<60	110 (70.5)	5 (3.2)	
≥60	40 (25.6)	1 (0.6)	
Tumor size (cm)			.137
<5	115 (73.7)	3 (1.9)	
≥5	35 (22.4)	3 (1.9)	
Serum AFP (ng/mL)			.585
<400	95 (60.9)	16 (10.3)	
≥400	40 (25.6)	5 (3.2)	
Serum CRP (mg/dL)			.000
<1	83 (53.2)	2 (1.3)	
≥1	8 (5.1)	3 (1.9)	
BCLC stage			.538
A	141 (90.4)	6 (3.8)	
B	9 (5.8)	0	
Fibrosis stage (Batts–Ludwig)			.929
Stage 1, 2	23 (14.7)	1 (0.6)	
Stage 3, 4	127 (81.4)	5 (3.2)	
Microvascular invasion			.890
Not identified	104 (66.7)	4 (2.6)	
Present	46 (29.5)	2 (1.3)	
Tumor number			.515
Less than 3	140 (89.7)	6 (3.8)	
3 or more	10 (6.4)	0	
Satellite nodule			.003
Absent	143 (91.7)	4 (2.6)	
Present	7 (4.5)	2 (1.3)	
Edmondson–Steiner grade (worst)			.395
Grade 1, 2	50 (32.1)	1 (0.6)	
Grade 3, 4	100 (64.1)	5 (3.2)	
Edmondson–Steiner grade (most)			.379
Grade 1, 2	101 (64.7)	3 (1.9)	
Grade 3, 4	49 (31.4)	3 (1.9)	
Capsular invasion			.570
Absent	122 (78.2)	5 (3.2)	
Present	28 (17.9)	1 (0.6)	
Early recurrence			.182
Absent	91 (58.3)	2 (1.3)	
Present	59 (37.8)	4 (2.6)	

Values are presented as number (%). CRP, C-reactive protein; AFP,  $\alpha$ -fetoprotein; BCLC, Barcelona Clinic Liver cancer.

is more common than infection by hepatitis C virus (HCV) is, and more than half of HCCs are associated with HBV infection worldwide.<sup>14</sup> Hence, we focused our analysis of the clinicopathologic significance of CRP immunoreactivity on non-neoplastic

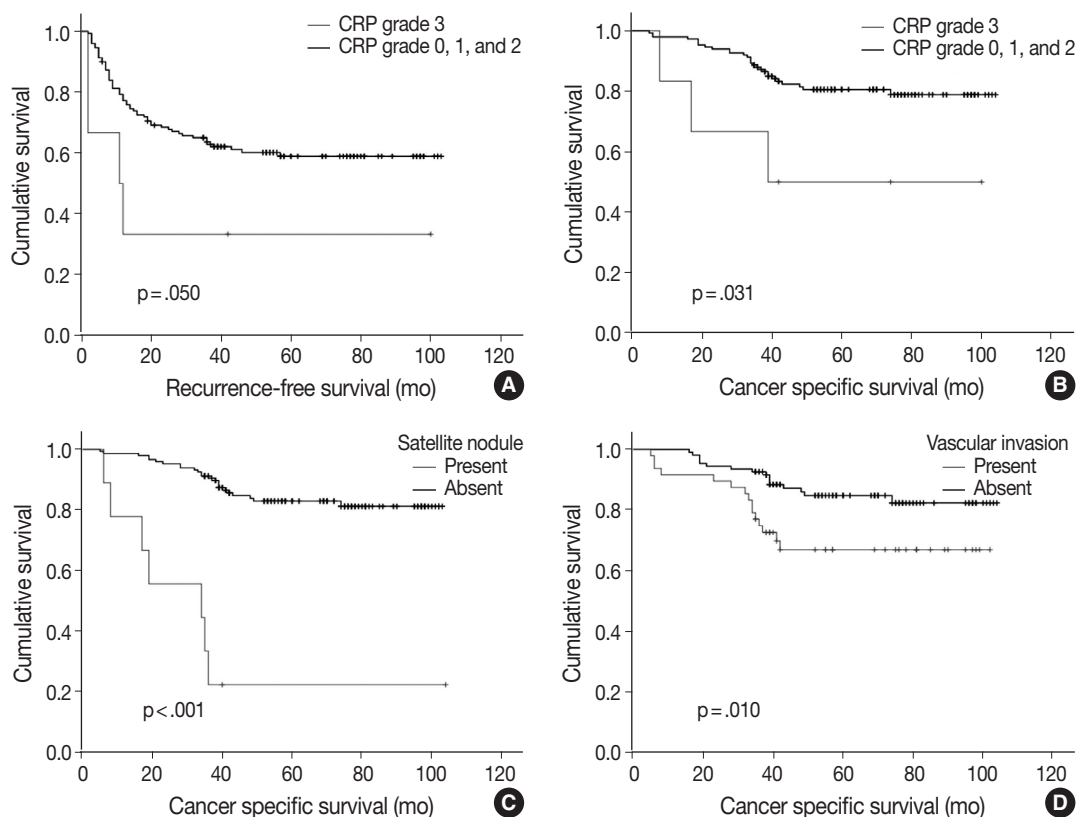


**Fig. 2.** Positive correlation between serum C-reactive protein (CRP) level and CRP immunoreactivity grades of non-neoplastic hepatocytes ( $p < .001$ ;  $\rho = 0.513$ ).

hepatocytes of HBV infection–associated HCCs. HBV infection increases the carcinogenic potential of hepatocytes, either by inducing chronic inflammation or direct integration into the host cell genome.<sup>11–15</sup> The latter mechanism would explain HBV-associated HCCs in the absence of chronic hepatitis in the background liver. Chimeric integration of HBV genome into several host genes such as cyclin E1 and myeloid/lymphoid or mixed-lineage leukemia 4 has been documented by sequencing studies. The detection and sequencing of high-throughput viral integration done by Zhao *et al.*<sup>16</sup> revealed 4,225 HBV integration events in neoplastic and non-neoplastic liver tissues from 426 patients. Among HCCs with grade 3 hepatocytic CRP immunoreactivity in this study ( $n = 6$ ), three cases were with mixed macronodular and micronodular cirrhosis, two cases were with chronic hepatitis, and one case was without significant background inflammation or cirrhosis.

We previously reported significant correlation between CRP immunoreactivity in HCC cells and patient survival. We looked at the relationship between HCC and corresponding non-neoplastic background liver in this study (data not shown) and found a significant correlation between the two, which indicated that both neoplastic and non-neoplastic hepatocytes are under a similar pro-inflammatory microenvironment in terms of CRP expression.<sup>12</sup> Serum CRP has been consistently shown to be a key component of inflammation-based prognostication of HCC.<sup>17–19</sup> The molecular mechanisms involved in the prognostic significance of CRP in HCC remain uncertain. However, a recent experimental investigation on CRP knockdown HepG2 cell proteome sug-





**Fig. 3.** Relationships between C-reactive protein (CRP) immunoreactivity of hepatocytes and patient outcomes. (A) There is a significant difference in cancer-specific survival between patients with CRP grade 3 immunoreactivity and those with CRP grade 0, 1, or 2 immunoreactivity ( $p = .031$ ). (B) There was a marginal difference in early recurrence of cancer between the 2 groups ( $p = .050$ ). (C, D) The presence or absence of satellite nodule (C) and vascular invasion (D) shows significant difference in cancer-specific survival period ( $p < .001$  and  $p = .001$ , respectively).

**Table 3.** Factors affecting CRP immunoreactivity by multiple logistic regression analysis

Clinical parameters	OR	95% CI	p-value
Serum CRP	12.229	1.573–95.087	.017
Tumor size			.496
Satellite nodule			.282

CRP, C-reactive protein; OR, odds ratio; CI, confidence interval.

gested that CRP plays a role in promoting the migration, invasion, and metastasis of HCCs via activation of MEK/ERK and phosphoinositide 3-kinase/AKT signaling pathways. Notably, cathepsin D was among the proteins the expression of which was decreased in CRP knockdown cells.<sup>20</sup> Other studies have also shown promotion of cell proliferation and antiapoptotic effects in different cell types such as renal tubular epithelial cells and myeloma cells.<sup>21,22</sup>

Although it is difficult to determine the relative contribution of HCC cells and non-neoplastic hepatocytes to serum CRP level, significant correlation between hepatocytic CRP immunoreactivity and blood CRP concentration supports the clinicopathologic significance of CRP as a surrogate prognostic marker of HBV-

**Table 4.** Cox regression analysis of parameters affecting cancer-specific survival in patients with HBV-associated hepatocellular carcinoma

Clinical parameters	HR (95% CI)	p-value
Satellite nodule	7.457 (3.004–18.514)	< .001
Vascular invasion		.077
CRP immunoreactivity		.063

HBV, hepatitis B virus; HR, hazard ratio; CI, confidence interval; CRP, C-reactive protein.

associated HCC. Of note, Hu *et al.*<sup>23</sup> proposed that HCC cells are not primarily responsive to serum hepatocyte growth factor, IL-6, and CRP based on the fact that the levels of these proteins go up after resection of HCCs, but this does not rule out the possibility of the significant contribution of HCC cells in affecting blood CRP level.

Regarding the association with tumor size, serum CRP level and tumor size were shown to be a marker of efficacy of transcatheter arterial chemoembolization (TACE) in HCCs.<sup>24</sup> TACE reduced blood CRP level in HCC patients.<sup>25</sup> Liu *et al.*<sup>26</sup> reported

an association between preoperative serum CRP level and HCC tumor size and also found significantly decreased overall survival and recurrence-free survival in HCC patients with elevated serum CRP compared with those with normal serum CRP level. Another independent study by Hu *et al.*<sup>23</sup> demonstrated a relationship between tumor size and preoperative serum CRP level in HCC patients. A significant association with the presence of satellite nodule suggests the possibility of CRP expression and tumor growth characteristics. Kim *et al.*<sup>27</sup> reported that CRP is an independent predictor of tumor recurrence in HCC cases with portal vein invasion.

The findings in this study indicate that CRP immunoreactivity in non-neoplastic hepatocytes is a novel prognostic biomarker of HBV-associated HCCs and is associated with certain tumor growth characteristics. The use of immunohistochemistry has merit in that the methodology is easily applicable to routine diagnostic pathology services. Further studies are needed to determine the significance of hepatocytic CRP immunoreactivity in cases with HCV-associated HCCs or nonvirally induced HCCs.

### Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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