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■ ORIGINAL ARTICLE ■

Overexpression of C-reactive Protein as a Poor Prognostic Marker of Resectable Hepatocellular Carcinomas

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Background: C-reactive protein (CRP) is an acute phase reactant synthesized in the liver. CRP immunoreactivity is a feature of inflammatory hepatocellular adenomas with a higher risk of malignant transformation. A high serum CRP level denotes poor prognosis in hepatocellular carcinoma (HCC) patients. This study was conducted to determine whether CRP is produced in HCC and to assess the clinicopathologic significance of CRP expression in cancer cells. **Methods:** CRP immunoreactivity was examined in treatment-naïve HCCs (n=224) using tissue microarrays and was correlated with clinicopathologic parameters. The expression of CRP mRNA and protein was also assessed in 12 HCC cases by quantitative real-time polymerase chain reaction and immunoblotting. Hep3B and SNU-449 HCC cell lines were used for the analysis of CRP mRNA regulation by interleukin 6 (IL-6). **Results:** CRP was expressed in 133 of 224 HCCs (59.4%) with a variable degree of immunoreactivity (grade 1 in 25.9%; grade 2 in 20.1%; grade 3 in 13.4%). There was an inverse relationship between grade 3 CRP immunoreactivity and cancer-specific survival (p=.0047), while no associations were found with other parameters, including recurrence-free survival. The CRP mRNA expression level was significantly higher in CRP immunopositive cases than in immunonegative cases (p<.05). CRP mRNA expression was increased in Hep3B cells, but was not detected in SNU-449 cells even after IL-6 treatment. **Conclusions:** We report the expression of CRP in HCC for the first time. CRP expression was associated with poor cancer-specific survival in patients with resectable HCC.

Key Words: Carcinoma, hepatocellular; C-reactive protein; Immunohistochemistry; Prognosis

Acute phase reactants are a group of hepatic proteins that are synthesized and released into the circulation in response to certain stresses such as infection and physical injury.[1] C-reactive protein (CRP) is a well-known, major acute phase reactant that was originally found in the sera of patients infected with *Streptococcus pneumoniae*.[2,3] CRP mRNA transcription is primarily induced by pro-inflammatory cytokines interleukin (IL)-6 and IL-1.[4-6] The blood concentration of CRP is increased in inflammatory conditions of both infectious and non-infectious etiologies such as urinary tract infection and hyperlipidemic acute pancreatitis.[7,8] A growing body of evidence indicates a solid pathobiological relationship between chronic inflammation and carcinogenesis.[9] Innate immune components such as Toll-like receptors and NOD-like receptors play a role in the regulation of inflammation and development of cancer.[10-12] Serum CRP level has been shown to be a prognostic marker in various human cancers.[13,14] Likewise, high serum CRP level is a poor prognostic factor in hepatocellular carcinoma (HCC) in relation to early recurrence.[15,16]

As hepatocytes are the primary origin of CRP synthesis, it is highly likely that neoplastic hepatocytes retain a functional capacity to synthesize CRP under the influence of pro-inflammatory stimuli. Not surprisingly, CRP expression has been rather extensively studied in hepatocellular adenomas,[17,18] and CRP immunoreactivity is a critical parameter for molecular phenotyping and defining of the inflammatory subtype of hepatocel-

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lular adenoma, which has a higher risk of malignant transformation.[19-21] Of note, based on the results of immunohistochemistry using a panel of antibodies and fluorescence *in situ* hybridization for gains of chromosomes 1, 8, and MYC in HCC arising in adenoma, Kakar *et al.*[22] recently proposed that a certain subset of hepatocellular adenomas may represent a well-differentiated version of HCCs. However, CRP expression in HCCs has not yet been studied. We postulated that the evaluation of CRP expression in HCC may provide valuable information regarding the unique biology of HCCs. This study was conducted to determine whether CRP is produced by HCC cells and to assess its clinicopathologic significance.

MATERIALS AND METHODS

Patients and tissue samples

A total of 224 cases of treatment-naïve HCCs (n = 224) were retrieved from the files of the Department of Pathology, Asan Medical Center, Seoul, Korea. All cases were surgically resectable (R0). Early recurrence was defined as a recurrence of the tumor within 2 years after surgery. All patients provided written informed consent, and this study was approved by the Institutional Review Board of Asan Medical Center, Seoul, Korea.

Tissue microarray and immunohistochemistry

Tissue microarrays were prepared using representative formalin-fixed, paraffin-embedded blocks of HCC cases. Two 2-mm-thick tissue cores were obtained from the donor blocks and transferred onto the recipient blocks after reviewing hematoxylin and eosin–stained slides. Four-micrometer-thick tissue microarray sections were immunostained using a rabbit polyclonal anti-CRP antibody (1:1,000, AbCam, Cambridge, UK). The sections were transferred onto 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). The signals were detected using the OptiView DAB IHC Detection Kit (Ventana Medical Systems). The immunoreactivity was evaluated by a pathologist (C.J.K.) blinded to clinical information, using a 4-tier grading system: negative, grade 0; positive in less than 10% of tumor cells, grade 1; positive in less than 50% of tumor cells, grade 2; diffusely positive, grade 3.

Cell culture

Human HCC cell lines, Hep3B and SNU-449, were used for the analysis of CRP mRNA regulation by IL-6. Hep3B cells were cultured in Dulbecco's modified Eagle medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. SNU-449 cells were cultured in RPMI 1640 medium (GE Healthcare Life Sciences, Pittsburgh, PA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. For IL-6 treatment, 5×10^5 cells were seeded into 100-mm dishes and were treated with 50 ng/mL of IL-6 (Cell Sciences, Canton, MA, USA) on the following day for 6 hours.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from liver tissues (n = 12) and cultured Hep3B and SNU-449 cells using Trizol and the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA (1 μg) was reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) analyses of CRP mRNA expression were performed using a TaqMan Gene Expression Assay (Hs00357041_m1, Applied Biosystems, Carlsbad, CA, USA) and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The human RPLPO (Large Ribosomal Protein) was used as an endogenous control for normalization.

Immunoblotting

Immunoblotting was done in 12 cases. Liver tissues were pulverized in liquid nitrogen, and total protein lysates were obtained using RIPA lysis buffer. Thirty micrograms of protein were electrophoresed in 12% sodium dodecyl sulfate–polyacrylamide gel and transferred onto polyvinylidene fluoride membranes (GE Healthcare Life Sciences). The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and incubated at 4°C overnight with mouse monoclonal primary antibodies against CRP (1:1,000, AbCam, Cambridge, MA, USA) and vinculin (1:1,000; Sigma-Aldrich, St. Louis, MO, USA), respectively. The blots were subsequently incubated at room temperature with horseradish peroxidase–conjugated secondary antibody for 1 hour (Cell Signaling Technology, Danvers, MA, USA). The signals were detected using a SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA).

Statistical analyses

The analyses of continuous variables and proportions were done using Pearson's chi-square test and Fisher exact test. The survival analysis was done using the Kaplan-Meier method. In-

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dependent variables and groups were compared using the Mann-Whitney U test. SPSS ver. 18.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS

CRP expression in HCC

The demographics of the study population are summarized in Table 1. CRP immunoreactivity was observed as diffuse cytoplasmic immunostaining. Overall, 59.4% (133/224) of HCC cases were immunopositive for CRP, with grades 1, 2, and 3 immunoreactivity in 25.9% (58/224), 20.1% (45/224), and 13.4% (30/224) of the cases, respectively (Fig. 1). When we analyzed the relationship between multiple clinicopathologic parameters and CRP immunoreactivity, we found a significant difference in cancer-specific survival between CRP-negative or non-diffuse immunopositive cases (grades 0, 1, and 2) and grade 3 immunopositive cases (Fig. 2A). Grade 3 CRP immunopositive cases showed significantly shorter survival compared to CRP-negative or non-diffuse immunopositive cases (median of 50.7 months and range of 6-58 months vs. median of 58.5 months and range of 5–88 years; p = .0047). However, no significant correlation was found between the degree of CRP immunoreactivity and other clinicopathologic parameters including recurrence-free survival (Fig. 2B).

Table 1. Clinical parameters and their relationship with CRP immunoreactivity

Clinical parameter	CRP grade 0, 1, 2	CRP grade 3	p-value
Gender			.019
Male	150 (67.0)	20 (8.9)	
Female	44 (19.6)	10 (4.5)	
Age (yr)			.054
< 60	123 (54.9)	22 (9.8)	
≥ 60	71 (31.7)	8 (3.6)	
Tumor size (cm)			.110
< 5	138 (61.6)	19 (8.5)	
≥ 5	56 (25.0)	11 (4.9)	
Serum AFP (ng/mL)			.125
< 400	141 (62.9)	24 (10.7)	
≥ 400	53 (23.6)	6 (2.7)	
BCLC stage			< .001
A	185 (82.6)	26 (11.6)	
B	9 (4.0)	4 (1.8)	
Etiology			.096
HBV	139 (62.1)	23 (10.3)	
HCV	20 (8.9)	1 (0.4)	
NBNC	35 (15.6)	6 (2.7)	
Fibrosis stage (Batts-Ludwig)			.454
Stage 1, 2	39 (17.4)	7 (3.1)	
Stage 3, 4	155 (69.2)	23 (10.3)	
Microvascular invasion			.983
Not identified	136 (60.7)	21 (9.4)	
Present	58 (25.9)	9 (4.0)	
Tumor number			< .001
< 3	185 (82.6)	26 (11.6)	
≥ 3	9 (4.0)	4 (1.8)	
Edmondson-Steiner grade (worst)			.814
Grade 1, 2	67 (29.9)	10 (4.5)	
Grade 3, 4	127 (56.7)	20 (8.9)	
Edmondson-Steiner grade (most)			.241
Grade 1, 2	128 (57.1)	18 (8.0)	
Grade 3, 4	66 (29.5)	12 (5.4)	
Capsular invasion			.492
Absent	156 (69.6)	25 (11.2)	
Present	38 (17.0)	5 (2.2)	
Early recurrence			.098
Absent	114 (50.9)	15 (6.7)	
Occurs	80 (35.7)	15 (6.7)	

Values are presented as number (%).
CRP, C-reactive protein; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; HCB, hepatitis C virus; NBNC, non-B, non-C hepatocellular carcinoma.

CRP mRNA expression in HCC

We further analyzed CRP protein and mRNA expression in HCCs (n = 12) to see whether CRP expression is regulated at the transcriptional level. Immunoblotting for CRP confirmed variable expression of CRP in HCC cases (Fig. 3A). CRP signals were readily detected in six cases, but not in the remaining six cases. qRT-PCR analysis demonstrated a higher CRP mRNA expression in CRP immunopositive cases than in immunonegative cases (p = .046; ΔCt median of 3.62, range of 2.8–5.98 vs ΔCt median of 8.56, range of 1.07–15) (Fig. 3B).

After confirming the relationship between CRP protein and mRNA expression, we tested if CRP mRNA transcription was dependent on pro-inflammatory mediators. The treatment of Hep3B and SNU-449 cells with IL-6 at the concentration of 50 ng/mL for 6 hours induced a 17.5-fold increase in CRP mRNA expression in Hep3B cells, but CRP mRNA expression was not detected in SNU-449 cells even after IL-6 treatment (Fig. 4).

DISCUSSION

The primary findings of this study are (1) CRP expression is relatively common in HCCs, with variable immunoreactivity in nearly 60% of the cases tested, (2) there is an inverse relationship between diffuse and strong CRP immunoreactivity and cancer-specific patient survival (p = .0047), (3) CRP-positive cases in immunoblotting showed significantly higher CRP mRNA expression, suggesting that increased CRP production is a consequence of transcriptional activation of CRP, and (4) CRP mRNA transcription is induced by IL-6 in Hep3B cells, but not in SNU-449 cells, suggesting that CRP expression marks distinct mo-

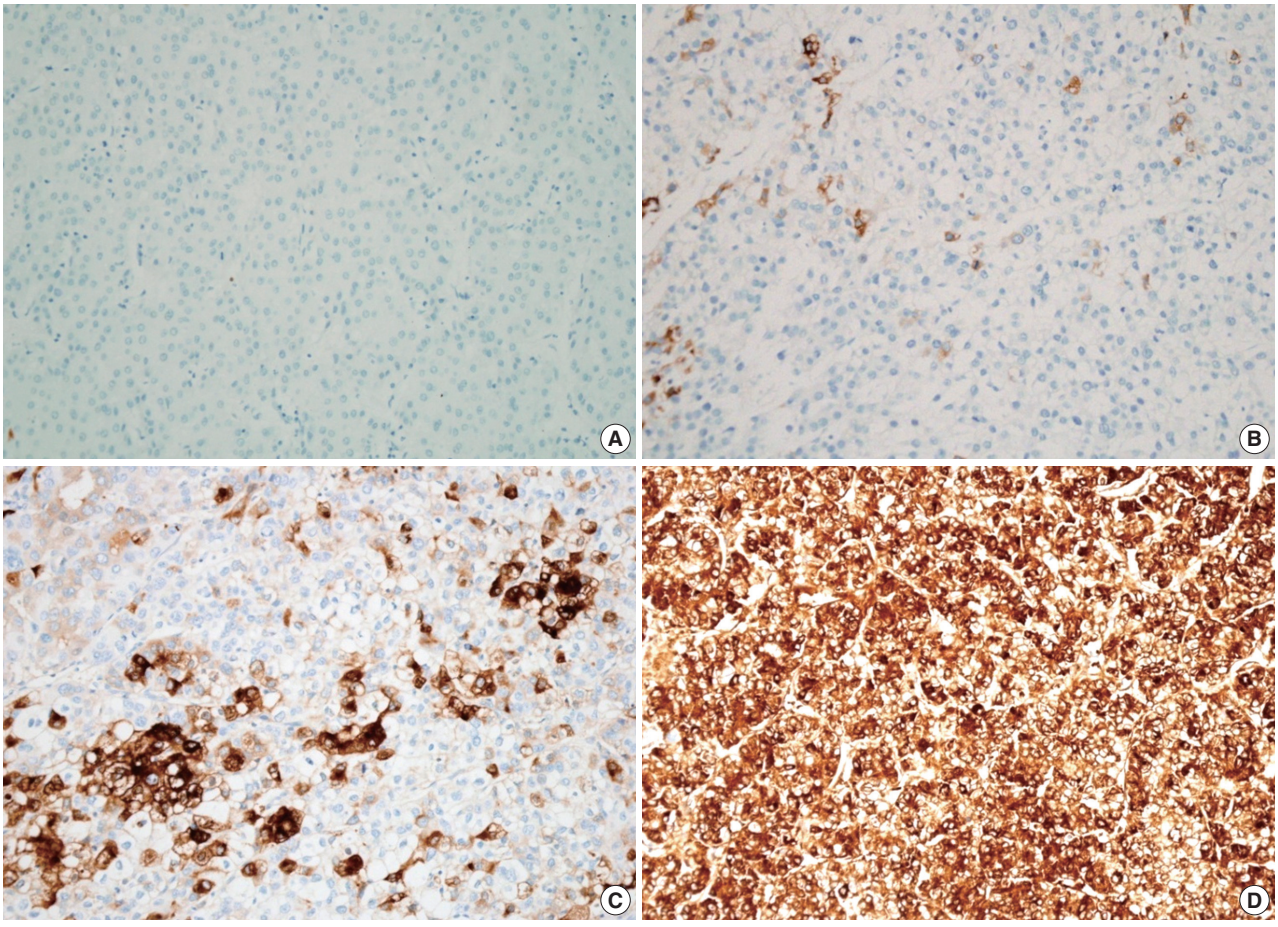

Fig. 1. Cytoplasmic C-reactive protein (CRP) immunoreactivity in hepatocellular carcinoma cases. Immunoreactivity is analyzed using a 4-tier grading system: grade 0 (A), grade 1 (B), grade 2 (C), and grade 3 (D).

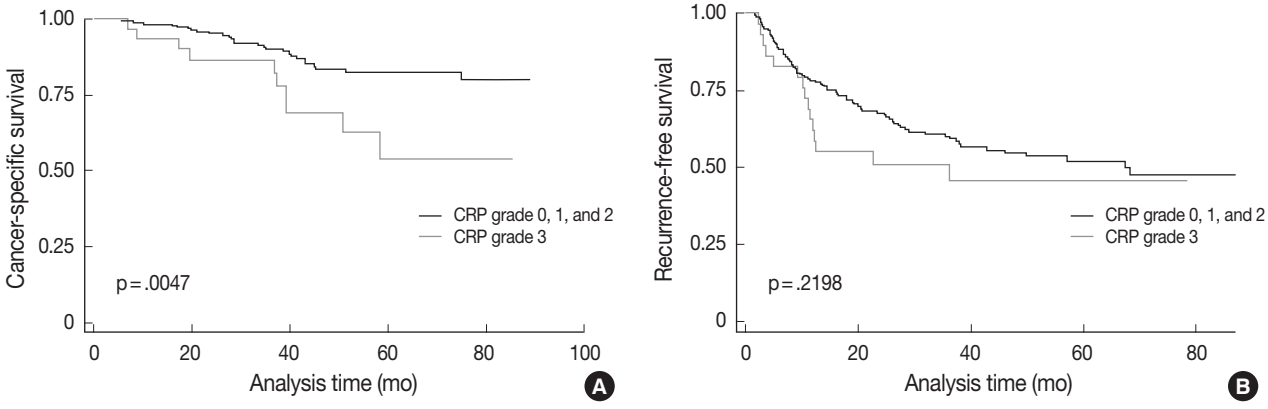

Fig. 2. Clinical significance of C-reactive protein (CRP) immunoreactivity. (A) There is a significant difference in cancer-specific survival between patients with CRP grade 3 hepatocellular carcinomas (HCCs) and those with CRP grade 0, 1, and 2 HCCs. (B) There is no difference in recurrence-free survival between the two groups.

lecular phenotypes among HCCs.

Hepatocellular carcinogenesis represents a classic model of viral etiology associated with chronic inflammation.[23] The elevation of inflammatory biomarkers such as CRP, IL-6, C-peptide, and adiponectin is associated with a higher risk of HCC.[24] Several investigations have addressed the clinical significance of

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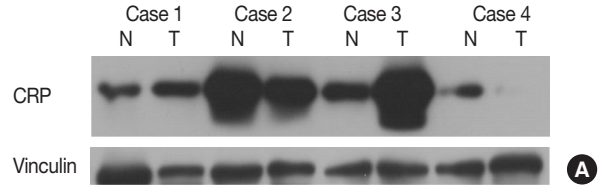


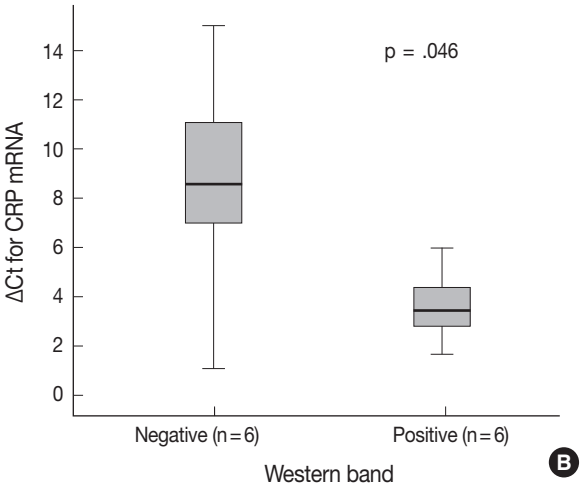
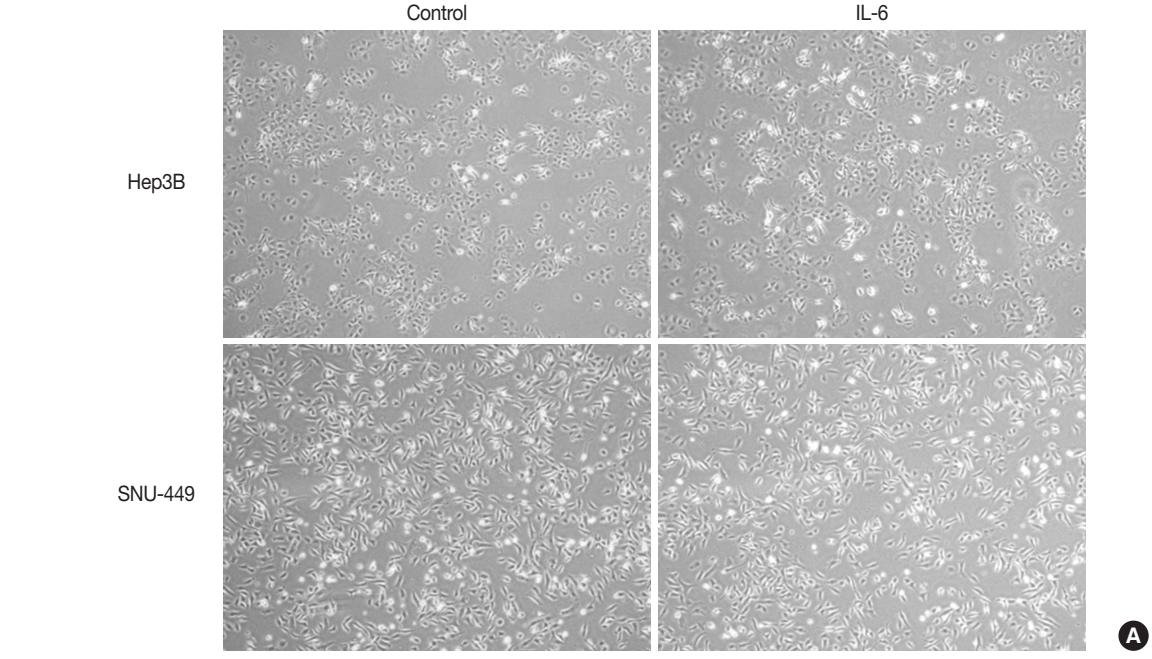


Fig. 3. Correlation between C-reactive protein (CRP) mRNA and protein expression in hepatocellular carcinomas (HCCs). (A) Immunoblotting for CRP protein shows variable expression in HCCs (N, non-neoplastic liver; T, HCC). CRP protein expression is not found in the tumor of case 4, while CRP bands are readily detectable in both non-neoplastic and HCC samples of cases 1, 2, and 3. (B) Quantitative real-time polymerase chain reaction results are shown in box plots of ΔCt for CRP mRNA expression (Ct_CRP–Ct_RPLPO).



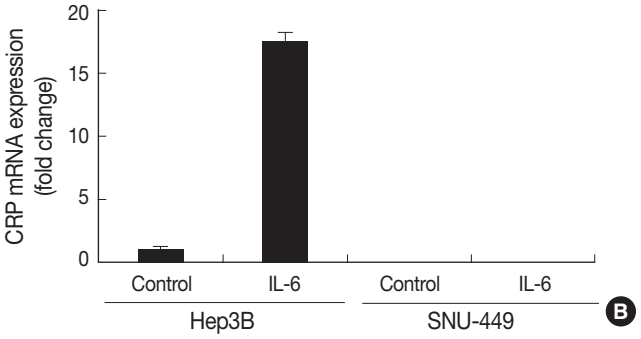


Fig. 4. Induction of C-reactive protein (CRP) mRNA in Hep3B and SNU-449 hepatocellular carcinoma cell lines after interleukin 6 (IL-6) treatment. (A) There are no significant changes in cellular morphology in either cell line after IL-6 treatment. (B) There is a 17.5-fold increase in CRP mRNA expression in IL-6–treated Hep3B cells (50 ng/mL for 6 hours), while CRP mRNA expression is not detected in SNU-449 cells. The y-axis represents fold-changes in CRP mRNA expression following IL-6 treatment.

serum CRP. Serum CRP has been consistently shown to be a key component of inflammation-based prognostication of HCC. Mori *et al.*[25] proposed that a preoperative scoring system based on the preoperative serum concentration of CRP and alpha-fetoprotein has a prognostic value in patients with HCC after hepatectomy. Although the contribution of CRP produced by tumor cells to the elevation of serum CRP cannot be directly assessed, it is very probable that CRP of tumor origin is released

into the systemic circulation. Although we expected certain differences in clinicopathologic characteristics between CRP immunopositive and immunonegative HCCs, a clear difference was found only in the cancer-specific survival of strong CRP immunopositive cases.

One of the major potential drives for CRP overexpression is IL-6, and a role of IL-6 in hepatocellular carcinogenesis has been strongly suggested. When compared to healthy controls, cirrhotic patients and HCC patients had serum IL-6 concentrations that were 4-fold and 25-fold higher, respectively.[26] IL-6 signaling mediated via IL-6 receptors activates STAT3, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase pathways. IL-6–mediated STAT3 activation is known to be a biological link between chronic inflammation and carcinogenesis,[27] and IL-6 confers anti-apoptotic effects to cells. Blocking STAT3 activation using STAT3 siRNA or small molecular STAT3 inhibitor LLL 12 has been shown to abrogate the anti-apoptotic effects of IL-6 against doxorubicin-induced apoptosis in SNU-449 HCC cells, which express a higher level of endogenous IL-6 compared to Hep3B cells.[28] We thought that CRP would be induced in both Hep3B and SNU-449 cells by IL-6 treatment. However, CRP mRNA expression after IL-6 treatment was significantly different between SNU-449 cells and Hep3B cells. Therefore, the observations in HCC tissue samples and the cell lines *in vitro* indicate that the degree of CRP expression involves several mechanisms, which need further elucidation.

A major drawback of this study is that serum CRP or IL-6 levels could not be determined due to a lack of blood samples. Future analyses of the relationship between the CRP-positive HCC phenotype and systemic inflammatory profile will provide a more comprehensive understanding of the biology of HCC. We also could not determine the underlying biochemical mechanisms involved in the differential IL-6-induced responses between Hep3B and SNU-449 cells in terms of induction of CRP mRNA expression. Considering the complexities of IL-6 signaling and CRP transcription, more *in vitro* studies are necessary.

In summary, we report the expression of CRP in HCC for the first time, and provide evidence to support the significance of CRP in HCC based on the clear difference in cancer-specific survival between CRP-positive and -negative cases. Overall findings strongly suggest that CRP is a marker for future molecular phenotyping of HCC.

Conflicts of Interest

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

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