**Table 1.** Main characteristics of all studies included in the meta-analysis.

| First author (year) | Ref. | Population ethnicity, region | IL-28B SNP rsID, Allele | Outcome measure F(Fibrosis) A(Activity) S(Steatosis) | Patients* | | | HCV genotype | Genotype for patients rs12979860 | | Genotype for patients rs8099917 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Male | Female | Total | | CC | CT/TT | TT | TG/GG |
| Abe (2010) | [48] | Asian, Japan | rs8099917 T/G | F, A: Inuyama | 212 | 152 | 364 | 1/2 | | | 265 | 99 |
| Honda (2010) | [49] | Asian, Japan | rs8099917 T/G | F, A: Inuyama | 58 | 33 | 91 | 1 | | | 60 | 31 |
| Lotrich (2010) | [50] | Mixed (African-American/Caucasian), USA | rs12979860 C/T | F: Ishak | 101 | 32 | 133 | 1/2 | 57 | 76 | | |
| Monte (2010) | [51] | Caucasian, Spain | rs12979860 C/T | F: Scheuer | 166 | 117 | 283 | 1–4 | 129 | 154 | | |
| Thompson (2010) | [52] | Mixed (African-American/Caucasian/Asian/Hispanic), USA | rs12979860 C/T | F: METAVIR | 986 | 642 | 1628 | 1 | 538 | 1090 | | |
| Bochud (2011) | [53] | Caucasian, Switzerland | rs12979860 C/T rs8099917 T/G | F: Ishak, A: ALT S: Histological finding | 163 | 79 | 242 | 1–3 | 90 | 150 | 150 | 92 |
| Dill MT (2011) | [54] | Caucasian, Switzerland | rs12979860 C/T rs8099917 T/G | F, A: METAVIR | 30 | 79 | 109 | 1–4 | 33 | 96 | 52 | 57 |
| Fabris (2011) | [44] | Caucasian, Italy | rs12979860 C/T | F: Ishak | N.A | N.A | 434 | 1–4 | 133 | 301 | | |
| Falleti (2011) | [55] | Caucasian, Italy | rs12979860 C/T | F: Ishak | 357 | 272 | 629 | 1–4 | 205 | 424 | | |
| Kurosaki (2011) | [56] | Asian, Japan | rs8099917 T/G | F: METAVIR S: Histological finding | 250 | 246 | 496 | 1 | | | 269 | 106 |
| Lagging (2011) | [57] | Caucasian, Sweden | rs12979860 C/T rs8099917 T/G | F: Ishak S: Histological finding | 169 | 83 | 252 | 1–4 | 93 | 159 | 153 | 99 |
| Lin (2011) | [58] | Asian, Taiwan | rs12979860 C/T rs8099917 T/G | F: METAVIR | 123 | 68 | 191 | 1 | 171 | 20 | 170 | 21 |
| Lindh (2011)-1 | [59] | Mixed (Caucasian/Asian), Sweden | rs12979860 C/T rs8099917 T/G | F: Batts Ludwig | 67 | 43 | 110 | 1 | 38 | 72 | 66 | 44 |
| Lindh (2011)-2 | [60] | Caucasian, Sweden | rs12979860 C/T | F: Ishak | 204 | 137 | 341 | 2/3 | 150 | 191 | | |
| Marabita (2011) | [61] | Caucasian, Italy | rs12979860 C/T rs8099917 T/G | F: Ishak | 129 | 118 | 247 | 1–4 | 88 | 159 | 131 | 116 |
| Miyamura (2011) | [62] | Asian, Japan | rs8099917 T/G | F, A: Inuyama | 37 | 42 | 79 | 1 | | | 53 | 26 |
| Moghaddam(2011) | [63] | Caucasian, Norway | rs12979860 C/T rs8099917 T/G | F: APRI score | 166 | 115 | 281 | 3 | 129 | 152 | 201 | 80 |
| Rueda (2011) | [64] | Caucasian, Spain | rs12979860 C/T | F, A: Scheuer | 246 | 177 | 423 | 1–4 | 83 | 184 | | |
| Tillman (2011) | [35] | Mixed (African-American/Caucasian/Asian), USA | rs12979860 C/T rs8099917 T/G | S: Histological finding | 215 | 110 | 325 | 1 | 88 | 237 | 97 | 67 |
| Yu (2011) | [65] | Asian, Taiwan | rs8099917 T/G | F: Knodell and Scheuer | 264 | 218 | 482 | 2 | | | 315 | 34 |
| Asahina (2011) | [66] | Asian, Japan | rs12979860 C/T rs8099917 T/G | F: Inuyama | 28 | 60 | 88 | 1 | 54 | 34 | 54 | 34 |

**Table 1.** Cont.

| First author (year) | Ref. | Population ethnicity, region | IL-28B SNP rsID, Allele | Outcome measure F(Fibrosis) A(Activity) S(Steatosis) | Patients* | | | HCV genotype | Genotype for patients rs12979860 | | Genotype for patients rs8099917 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Male | Female | Total | | CC | CT/TT | TT | TG/GG |
| Bochud (2012) | [47] | Caucasian, Switzerland | rs12979860 C/T rs8099917 T/G | F, A: METAVIR | 870 | 657 | 1527 | 1–4 | 534 | 993 | 855 | 672 |
| Mach (2012) | [67] | Slav: Poland | rs12979860 C/T | F: Batts Ludwig | 82 | 60 | 142 | 1 | 38 | 104 | | |
| Miyashita (2012) | [68] | Asian, Japan | rs8099917 T/G | F, A: Desmet | 88 | 132 | 220 | 1/2 | | | 155 | 63 |
| Ohnishi (2012) | [69] | Asian, Japan | rs8099917 T/G | S: Histological finding | 83 | 70 | 153 | 1 | | | 116 | 37 |
| Rembeck (2012) | [70] | Caucasian, Sweden | rs12979860 C/T | F: Ishak | 199 | 140 | 339 | 2/3 | 144 | 179 | | |
| Tolmane (2012) | [71] | Caucasian, Latvia | rs12979860 C/T | F: Knodell histology activity index S: Histological finding | 84 | 58 | 142 | 1–3 | 41 | 80 | | |
| Toyoda (2012) | [72] | Asian, Japan | rs8099917 T/G | F, A: METAVIR | 139 | 133 | 272 | 1 | | | 187 | 59 |

*Patients included in the original study.
Thus, patients without information regarding IL28B polymorphism were also included.
APRI, aminotransferase platelet ratio index.
doi:10.1371/journal.pone.0091822.t001

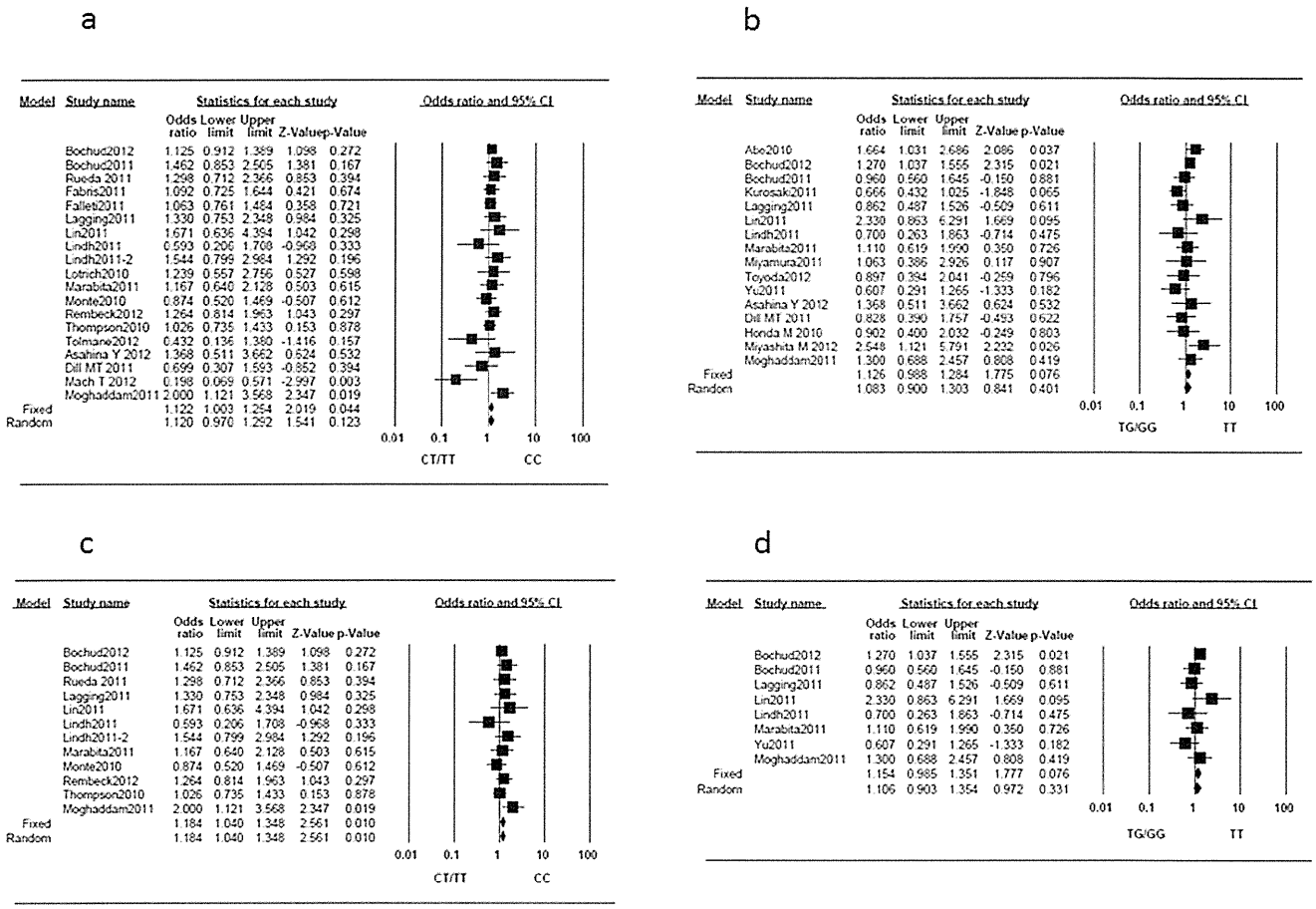


**Figure 2. Forest plot of the IL28B genotypes and the risk of severe fibrosis.** (a) rs12979860 in all patients, (b) rs8099917 in all patients, (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
doi:10.1371/journal.pone.0091822.g002

strong predictors of a sustained viral response [17–20] or spontaneous clearance of HCV [21]. The level of IL28B gene transcripts is reportedly higher in patients homozygous for the IFN responsive allele [18,19]. Therefore, in patients with the rs12979860 CC and rs8099917 TT genotype, IL28B production, which induces expression of interferon-stimulated genes, including some inflammatory cytokines, was thought to be increased. This may be the underlying cause of the higher inflammation activity and progressed fibrosis in patients with the IFN responsive allele. In analysis with the studies involving only patients without a history of IFN-based treatment, rs12979860 CC and rs8099917 TT genotypes were associated with higher possibility of having severe inflammation activity; however, the differences did not reach to the significant level. Only three studies according to rs12979860 polymorphism and two studies according to rs8099917 polymorphism were included when restricted to studies with only treatment-naïve patients, and may be underpowered to detect the effects of IL28B polymorphisms on inflammation activity. The further analyses with larger sample are needed to confirm this association. Additionally, meta-regression analysis showed that the effect of the rs12979860 polymorphism was influenced by viral genotype distribution. This result may imply a different influence of rs12979860 polymorphism on immune response according to viral genotype in treatment-naïve patients.

IL28B polymorphisms were also shown to be associated with lipid metabolism [25]. In the present study, the rs8099917 TT genotype was significantly associated with a lower possibility of severe steatosis. This association still remained statistically significant after we restricted to studies in which only treatment-naïve patients were included. The lower hepatic steatosis in patients with the IFN responsive allele could be explained by a more efficient export of lipids from hepatocytes. Higher interferon expression was shown to lead to suppression of lipoprotein lipase, which would result in decreased conversion of VLDL to LDL and subsequent higher steatosis [30–33]. The difference in IL28B expression might cause an aberration of lipid metabolism in patients with CHC. We found no significant association of rs12979860 with steatosis. And when we restricted to treatment-naïve patients, rs12979860 CC genotype was significantly associated with a higher possibility of severe steatosis. Previous studies have shown that racial differences or viral genotypes make a difference in the effects of rs12979860 and rs8099917 polymorphisms [34,35]. This may explain the discrepancy between the effect of rs12979860 and rs8099917 on hepatic steatosis. However, only four studies (962 patients) were included in the analysis of rs12979860; or when it comes to the studies with only treatment-naïve patients, only two studies (495 patients) were extracted. Thus, we should not make any definite conclusion on this matter right now. Further studies with larger sample sizes are needed to identify their exact correlation.

According to the meta-regression analysis, the effect of rs8099917 polymorphisms on steatosis became smaller with the

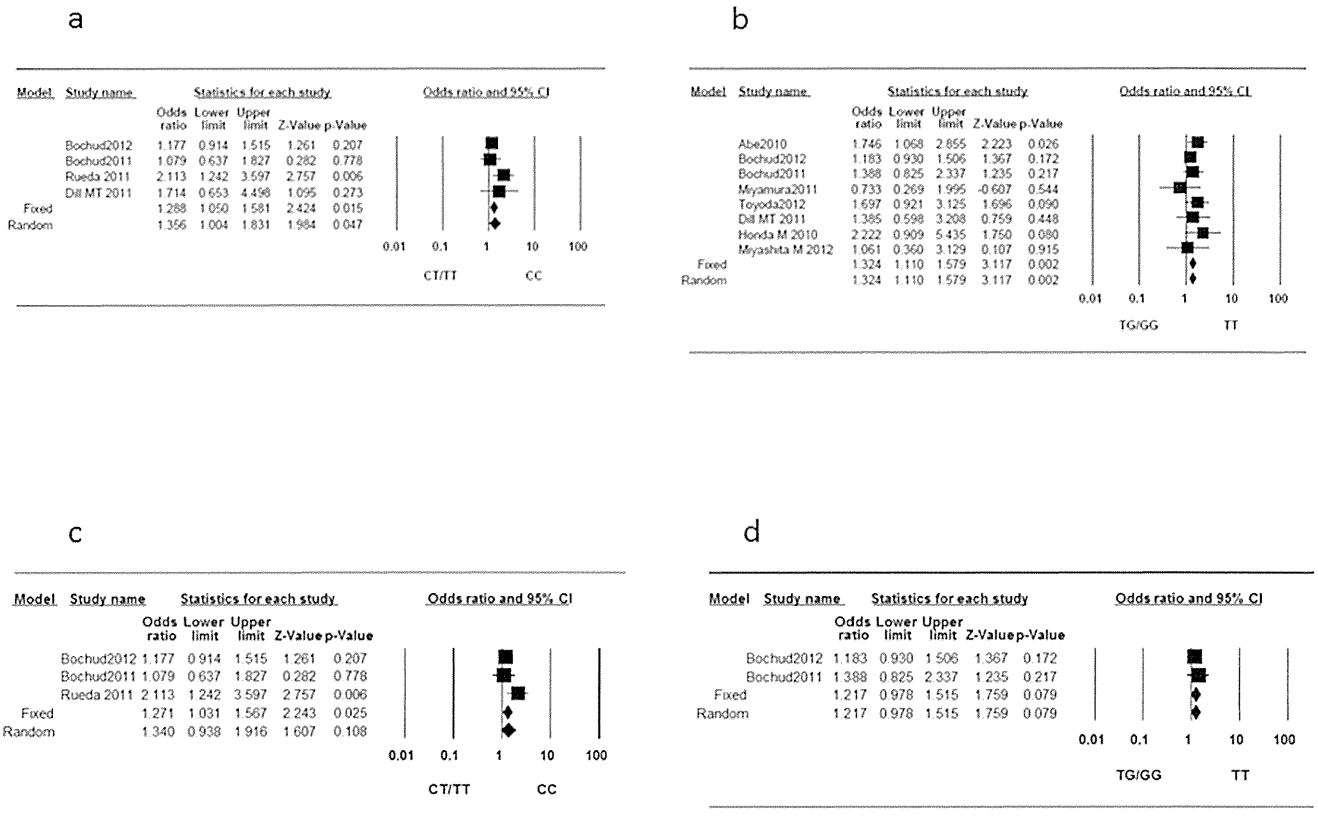


**Figure 3. Forest plot of the IL28B genotypes and the risk of severe inflammation activity.** (a) rs12979860 and (b) rs8099917. (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
doi:10.1371/journal.pone.0091822.g003

increase in the male proportion (Fig. 5), suggesting that a sexual dimorphism might be involved in the effect of rs8099917 polymorphisms on the liver fat content. Although the present study cannot explain the interaction between the polymorphism and sex, immune systems responding to IFN are reportedly controlled by estrogenic sex hormones [36,37]. Differences in IL28B expression mediated by sex hormones could be a possible mechanism for the sexual dimorphism in the effect of rs8099917 polymorphisms on liver steatosis.

The rs738409 genotype within the patatin-like phospholipase domain containing 3 locus was also reported to be associated with hepatic steatosis in patients with CHC [38–40]. Notably, previous meta-analysis evaluating the effect of patatin-like phospholipase domain containing 3 polymorphisms on steatosis also reported a

**Table 2.** Meta-regression analysis between each continuous variable among the studies (only treatment- naïve patients were included) and the effect (log odds ratio) of IL28B polymorphisms on inflammation activity.

| Variables | Slope* | Standard error | P-value |
|---|---|---|---|
| **Proportion of patients with genotype 1 or 4 virus, per 1% increase** | | | |
| **rs12979860** | **2.992** | **1.497** | **0.046** |
| **Proportion of male patients, per 1% increase** | | | |
| **rs12979860** | **−2.963** | **5.802** | **0.610** |
| **Proportion of Caucasian patients, per 1% increase** | | | |
| **rs12979860†** | – | – | – |
| **Proportion of African-American patients, per 1% increase** | | | |
| **rs12979860†** | – | – | – |
| **Proportion of Asian patients, per 1% increase** | | | |
| **rs12979860†** | – | – | – |

*Positive (negative) slope values indicate that the proportions of patients with the rs12979860 CC genotype with severe inflammation activity are increasing (decreasing) as the values of each contentious variable (proportions of genotype 1 or 4 virus, male, or each race) is increasing.
†We could not perform meta-regression analyses for these outcomes because only caucasian patients were included in all 3 studies included in this analysis.
doi:10.1371/journal.pone.0091822.t002

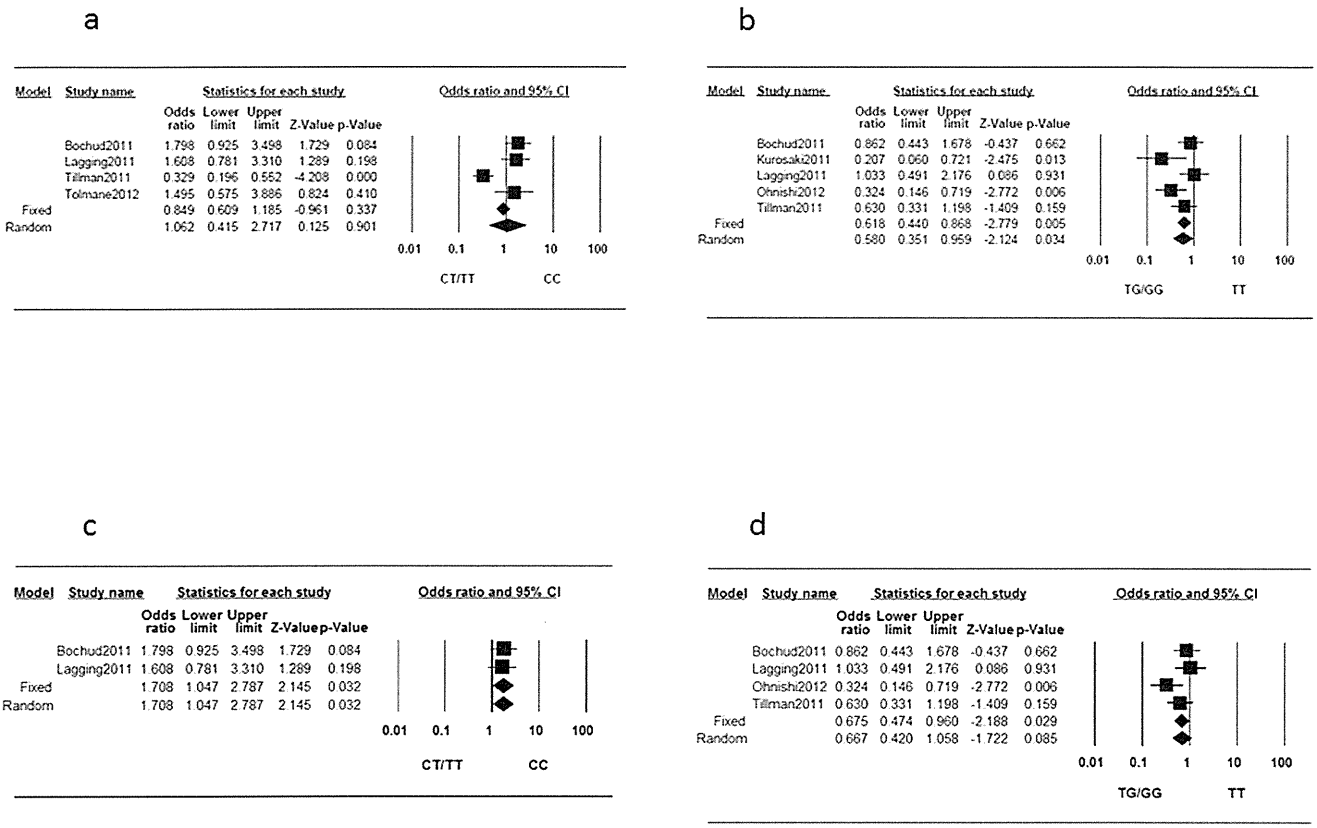


**Figure 4. Forest plot of the IL28B genotypes and the risk of hepatic steatosis.** (a) rs12979860 and (b) rs8099917. (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
doi:10.1371/journal.pone.0091822.g004

**Table 3.** Meta-regression analysis between each continuous variable among the studies and the effect (log odds ratio) of IL28B polymorphisms on steatosis.

| Variables | Slope* | Standard error | P-value |
|---|---|---|---|
| Proportion of patients with genotype 1 or 4 virus, per 1% increase | | | |
| rs12979860 | −4.947 | 1.086 | <0.001 |
| rs8099917 | −2.704 | 1.277 | 0.034 |
| Proportion of male patients, per 1% increase | | | |
| rs12979860 | −2.899 | 16.577 | 0.861 |
| rs8099917 | 6.225 | 2.530 | 0.014 |
| Proportion of Caucasian patients, per 1% increase | | | |
| rs12979860 | 7.361 | 1.569 | <0.001 |
| rs8099917 | 1.168 | 0.422 | 0.006 |
| Proportion of African-American patients, per 1% increase | | | |
| rs12979860 | −8.996 | 1.918 | <0.001 |
| rs8099917 | 0.142 | 2.147 | 0.947 |
| Proportion of Asian patients, per 1% increase | | | |
| rs12979860† | – | – | – |
| rs8099917 | −1.049 | 0.398 | 0.008 |

*Positive (negative) slope values indicate that the proportions of patients with the rs12979860 CC or rs8099917 TT genotypes with severe steatosis are increasing (decreasing) as the values of each contentious variable (proportions of genotype 1 or 4 virus, male, or each race) is increasing.
†We could not perform a meta-regression analysis for this outcome because only one patient was included in the corresponding studies.
doi:10.1371/journal.pone.0091822.t003

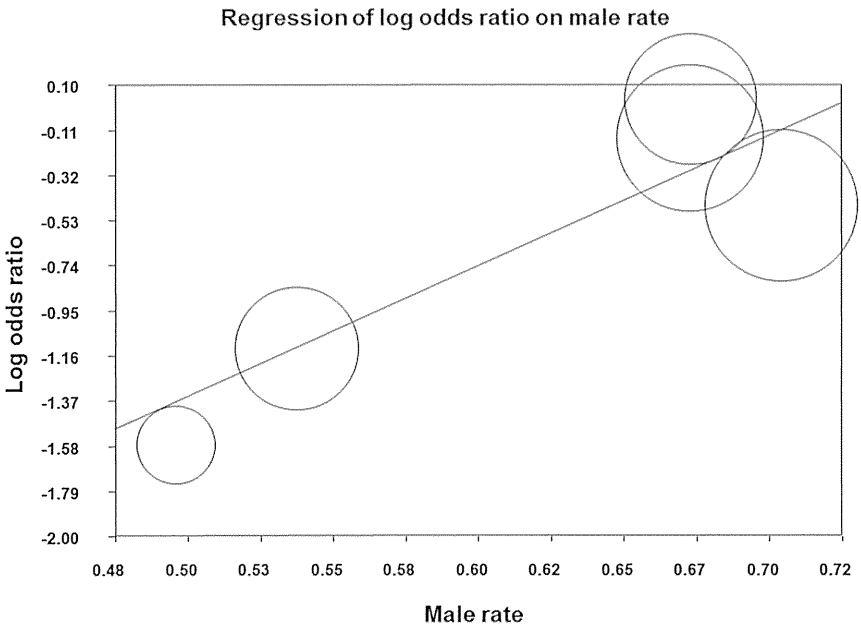


**Figure 5. Meta-regression plot for log odds ratios in rates of patients with severe hepatic steatosis by proportion of males (%) in rs8099917.**
doi:10.1371/journal.pone.0091822.g005

negative correlation between the male proportion and the effect of rs738409 on the liver fat content in nonalcoholic fatty liver disease [41]. Interestingly, the meta-regression analysis in the present study showed that the effect of the IL28B (rs12979860 and rs8099917) polymorphisms on steatosis was also influenced by racial and viral genotype distributions.

In the present study, we included studies that did not report the associations between IL28B genotypes and background liver diseases as study outcomes, but provided raw data that allowed us to calculate the OR for each outcome, which may have minimized potential publication bias. In fact, no publication bias was observed in the present study. The Human Genome Epidemiology Network highlighted the necessity of meta-analysis before evidence for a particular association can be regarded as strong [42]. The impact of IL28B genotypes on the disease progression found in the present meta-analysis may provide clinically important information in the follow-up of patients with CHC. The effect of IL28B polymorphisms on hepatocarcinogenesis, which is also crucial information in the HCC screening of patients with CHC, remains controversial [43–47]. Further analysis with larger sample sizes may be needed to elucidate the exact effect of IL28B polymorphisms on hepatocarcinogenesis.

A potential limitation of this study is inter-study variability in the outcome measure and the definition of "severe" among studies, where some discrepancies among studies exist. The studies without a pathological diagnosis, using laboratory data as surrogates, were also included. These studies may have diminished the accuracy of our research results concerning liver disease severity.

In conclusion, the present study highlighted the impact of IL28B polymorphisms on liver fibrosis, inflammation activity, and steatosis in patients with CHC. Disease progression appeared to be promoted in patients with rs12979860 CC or rs8099917 TT genotypes. The current findings may provide clinically important information in the follow-up of patients with CHC.

## Supporting Information

**Checklist S1 PRISMA 2009 Checklist.**
(DOC)

## Acknowledgments

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/IWcYpT.

## Author Contributions

Conceived and designed the experiments: MS RT NK. Performed the experiments: MS MK RT. Analyzed the data: MS RT. Contributed reagents/materials/analysis tools: MS. Wrote the paper: MS RT HY. Critical revision of manuscript: NF MT KK.

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Hepatology Research

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***Original Article***

# Slight elevation of high-sensitivity C-reactive protein to predict recurrence and survival in patients with early stage hepatitis C-related hepatocellular carcinoma

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*Aim:* Hepatocellular carcinoma (HCC) is associated with chronic inflammation derived from various origins. We investigated whether high-sensitivity C-reactive protein (hsCRP) could predict recurrence and survival after curative treatment for early stage hepatitis C virus-related HCC (C-HCC).

*Methods:* We enrolled 387 patients with three or fewer C-HCC nodules, none of which exceeded 3 cm, and of Child–Pugh class A or B who underwent radiofrequency ablation. We divided the patients into high and low hsCRP groups based on the optimal cut-off value for recurrence using a split-sample method and maximally selected rank statistics. Differences in recurrence and survival rates were evaluated by the Kaplan–Meier method and the log–rank test. Hazard ratios of hsCRP were adjusted with confounding factors using a multiple Cox regression model. We also assessed the correlations between hsCRP levels and clinical parameters.

*Results:* The optimal hsCRP cut-off value was 0.08 mg/dL. The cumulative recurrence rates after 5 years in the high and low hsCRP groups were 90.0% and 82.2%, respectively ($P = 0.028$), and the corresponding survival rates were 50.9% and 71.8%, respectively ($P < 0.001$). Higher hsCRP was an independent predictor for recurrence (adjusted hazard ratio [aHR], 1.32; 95% confidence interval [CI], 1.03–1.67; $P = 0.026$) and survival (aHR, 1.59; 95% CI, 1.14–2.22; $P = 0.007$). hsCRP was correlated with central obesity as well as tumor burden and liver dysfunction.

*Conclusion:* Slight elevation of the hsCRP level, even within the normal range, can predict recurrence and survival after curative treatment among patients with early stage C-HCC.

**Key words:** curative treatment, hepatocellular carcinoma, high-sensitivity C-reactive protein, prognosis, recurrence

## INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most frequently diagnosed cancer and the third most frequent cause of cancer-related death.[1] Chronic hepatitis C virus (HCV) infection is the predominant cause of HCC in Japan and other countries. These patients are often under surveillance for HCC because of frequent hepatocarcinogenesis; as a result, HCC is usually detected when the tumors are small and treatment is more likely to succeed.[2] Nevertheless, recurrence eventually occurs in most patients, which can lead to a long-term poor prognosis.[3] Several methods have been used to predict recurrence or survival among patients with HCC, including biomarkers,[4,5] but these may become less useful as HCC is more frequently diagnosed at earlier stages.

C-Reactive protein (CRP) is one of the major acute-phase proteins and is a marker of systemic inflammation. CRP has been reported to be associated with a wide range of diseases, including atherosclerosis, diabetes mellitus and various cancers.[6–8] Notably, high-sensitivity

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CRP (hsCRP) can be used to detect low-grade inflammation, and slight elevation in the hsCRP level is therefore a useful indicator of future cardiovascular disease development even within the normal range of the conventional CRP tests.[8] The use of hsCRP as a prognostic predictor also has been evaluated in patients with a variety of malignancies.[9,10] Indeed, there was a report on the relationship between elevated hsCRP and survival of patients with HCC. However, that study included patients with advanced stage HCC, such as those with tumors of more than 5 cm in diameter, multiple nodules and portal venous tumor invasion, who were treated by various methods, including resection, ablation and transarterial chemoembolization. To clarify what represents elevated hsCRP and whether hsCRP may predict survival and recurrence in HCC patients, it would be ideal to enroll patients with early HCC treated with single-modality therapies.

The aim of this study was to assess whether the hsCRP level at the time of diagnosis of HCC could predict recurrence and long-term outcomes in patients with HCV-related HCC, classified as stage 0 or A according to the Barcelona Clinic Liver Cancer staging system (BCLC).[11]

## METHODS

THIS RETROSPECTIVE STUDY was conducted according to the ethical guidelines for epidemiological research of the Japanese Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare. The study design was included in a comprehensive protocol from the Department of Gastroenterology, the University of Tokyo Hospital, and approved by The University of Tokyo Medical Research Center Ethics Committee (approval no. 2058).

### Patients

Of the 750 patients undergoing percutaneous radiofrequency ablation (RFA) for naïve HCC in our hospital between January 2004 and December 2009, there were 413 patients with BCLC stage 0 or A HCC. To clarify recurrence patterns, we excluded two patients in whom RFA was only intended to reduce tumor burden because it had been judged that curative treatment, either before or after RFA, was not feasible. Serum hsCRP levels and/or other variables were unavailable for 24 patients. Thus, we reviewed the remaining 387 (94.2%) patients retrospectively.

### Laboratory data

All laboratory values recorded in this study were evaluated within 3 days prior to RFA. Because we postponed the treatment when a patient had clinical evidence of an infection at the time of admission, such as pneumonia or spontaneous bacterial peritonitis, and when the hsCRP value had substantially increased compared to the value at the last outpatient clinic prior to the admission, we could exclude patients with elevated hsCRP related to overt infection. Slightly increased hsCRP levels may reflect inapparent infection related to cirrhosis. However, such elevations of hsCRP may be useful in terms of long-term mortality prediction. Therefore, we included these patients in this study.

### Anthropometric parameters

A previous report showed that obesity influenced serum inflammatory markers in chronic hepatitis C.[12] Thus, we assessed the following anthropometric parameters in patients with HCC: body weight, body mass index (BMI; the weight in kilograms divided by the square of the height in meters), waist circumference and fat tissue area. The fat tissue area and waist circumference were measured in each patient by analyzing a computed tomography (CT) image at the level of the umbilicus using Slim Vision software (KGT, Tokyo, Japan).[13] The subcutaneous fat area (SFA) was defined as the sum of the extraperitoneal fat areas between the skin and muscle on the CT image, which showed attenuation ranging from −150 to −50 Hounsfield units. The visceral fat area (VFA) was defined as the sum of the intraperitoneal fat areas showing the same attenuation.

### Diagnosis of HCC

Hepatocellular carcinoma was diagnosed using dynamic CT, considering hyperattenuation in the arterial phase with washout in the late phase as a definite sign of the disease.[14] The diagnosis of HCC was based on typical findings on CT, including hyperattenuation in the arterial phase and hypoattenuation in the equilibrium phase.[15,16] Most nodules (88.4%) were also confirmed histopathologically with ultrasound-guided biopsies. The pathological grade was determined based on the Edmondson–Steiner criteria.[17]

### Biopsy of non-tumorous liver

Ultrasound-guided biopsy of the non-tumorous liver was performed in 315 patients (81.4%), excluding patients with a risk of hemorrhage such as those with

severe thrombocytopenia. The background liver was pathologically graded using the METAVIR system.[18]

## Treatment and follow up

The inclusion criteria for percutaneous ablation were as follows: total bilirubin concentration of less than 3.0 mg/dL, platelet count of $50 \times 10^3/mm^3$ or more, and prothrombin activity of 50% or more. Patients with portal vein tumor thrombosis, massive refractory ascites or extrahepatic metastasis were excluded. Generally, we performed percutaneous ablation in patients with three or fewer lesions, all of which were 3.0 cm or less in diameter. However, we also performed ablation in patients with more than three lesions or lesions of more than 3.0 cm if the procedure was considered to be beneficial clinically. This procedure has been described elsewhere.[19] After several sessions of percutaneous ablation, dynamic CT was performed with a section thickness of 0.5 cm to evaluate treatment efficacy. Complete ablation was defined as hypoattenuation of the whole lesion along with the surrounding liver parenchyma. We usually attempt to ablate an area larger than the size of the tumors with consideration of possible underestimation of the tumor boundary by imaging modalities. Patients received additional sessions of ablation until complete ablation was confirmed for each HCC nodule. The follow up comprised monthly blood tests and monitoring of tumor markers at the outpatient clinic, with ultrasonography and dynamic CT scans performed every 4 months. Tumor recurrence was diagnosed according to the same criteria as the initial HCC. Chest CT or bone scintigraphy was performed if extrahepatic recurrence was suspected. RFA was used for the treatment of recurrence if the patient still met the indication criteria. If multiple recurrences were not treatable by RFA, we typically performed transcatheter arterial chemoembolization.

## Analysis of recurrence and survival

Recurrence time was defined as the interval between the first ablation and the detection of HCC recurrence or the last examination before 31 December 2012, whichever came first. Survival analysis was performed on a patient basis. Survival time was defined as the interval between the first treatment and death or the last visit to the outpatient clinic prior to 31 December 2012.

## Statistical analyses

Quantitative variables are expressed as medians and interquartile ranges (IQR) unless otherwise indicated. Numbers and percentages are used for qualitative variables.

To identify the optimal hsCRP cut-off value, we randomly split the data into two sets: a training set (193 patients) and the validation set (194 patients). In the training set, we estimated the optimal cut-off value of hsCRP for predicting recurrence after curative treatment using maximally selected rank statistics, as described by Lausen and Schumacher.[20] This cut-off value is optimal for separation into high and low levels of prognosis. Statistically significant differences in prognosis using this cut-off value were examined using an adjustment for *P*-values, taking into account the arising multiple test situation. We then validated the cut-off value by the log–rank test in the validation set. We classified patients into high and low hsCRP groups according to the cut-off value and compared their baseline characteristics using Student's *t*-test or the Mann–Whitney *U*-test for continuous data and the $\chi^2$-test for categorical data.

Correlations between hsCRP and the following clinical parameters were estimated using Spearman's rank correlation coefficient: age, body height, body weight, BMI, waist circumference, SFA, VFA, platelet count, aspartate aminotransferase (AST) level, alanine aminotransferase (ALT) level, total bilirubin, albumin, tumor factors (including the size and number of nodules), and HCC-specific biomarkers α-fetoprotein (AFP) and des-γ-carboxyprothrombin.

Cumulative recurrence and survival curves were plotted using the Kaplan–Meier method, and differences were assessed using the log–rank test. We investigated predictors of recurrence and survival using the Cox proportional hazard regression model. Stepwise variable selection with the Akaike information criterion (AIC) was used to find the best model in the multivariate analysis. The Cox proportional hazards assumption was checked using the smoothed plots of Schoenfeld residuals. Moreover, the hazard ratio (HR) of hsCRP for survival was estimated as a continuous number using a restricted cubic spline with three knots after adjusting for other significant predictors.[21]

Regarding long-term survival, subgroup analyses using the Cox proportional hazards model were used to estimate the HR of higher versus lower hsCRP, with two-tailed *P*-values, for explanatory variables. The explanatory variables used for HR estimation were as follows: age, sex, serum albumin concentration, BMI, ALT level, platelet counts, clinical cirrhosis, AFP concentration and tumor stage.

Statistical analyses were performed using the "R" software (ver. 2.13.0; www.r-project.org), with the

"survival", "maxstat" and "rms" packages. All tests were two-sided and $P < 0.05$ was considered to indicate statistical significance.

## RESULTS

### Optimal cut-off value of hsCRP

THE DISTRIBUTION OF hsCRP values is shown in Figure 1(a). The median hsCRP was 0.05 mg/dL (IQR, 0.02–0.11). In the training set, maximally selected rank statistics showed that the optimal cut-off value of hsCRP to predict HCC recurrence was 0.08 mg/dL (Fig. 1b, Fig. S1A,B). This cut-off value was confirmed in the validation set (Fig. S1C,D). Thus, we classified patients with a hsCRP of 0.08 mg/dL or less as the low hsCRP group and the remaining patients as the high hsCRP group.

### Patients

Baseline characteristics of the patients are shown in Table 1. Of the 387 patients, 139 (35.9%) were classified into the high hsCRP group, and they were more likely to be heavier in weight, especially with central obesity, and to have worse liver function (Table 1). Diabetes was also more prevalent among patients in the high hsCRP group. Tumor size in the high hsCRP group tended to be larger.

### Correlation between hsCRP and clinical parameters

Correlations between hsCRP and clinical parameters are shown in Table 2. Serum hsCRP was correlated not only with liver dysfunction and tumor size but also with high weight in HCC patients. Serum hsCRP had a positive correlation with VFA but not with SFA.

### Recurrence rate and long-term survival

The median observation period was 4.4 years (IQR, 3.1–6.0). In the low hsCRP group, the cumulative recurrence rates at 1, 3 and 5 years were 27.1%, 68.8% and 82.2%, respectively, while in the high hsCRP group, the rates were 39.1%, 79.3% and 90.0%, respectively (Fig. 2a). The recurrence rate curves for the two groups differed significantly (log–rank test, $P = 0.0028$).

The overall survival rates at 1, 3 and 5 years were 98.4%, 87.9% and 71.8% in the low hsCRP group, respectively, and 94.2%, 73.1% and 50.9% in the high hsCRP group, respectively (Fig. 2b). The survival curves for the two groups differed significantly (log–rank test, $P < 0.001$).

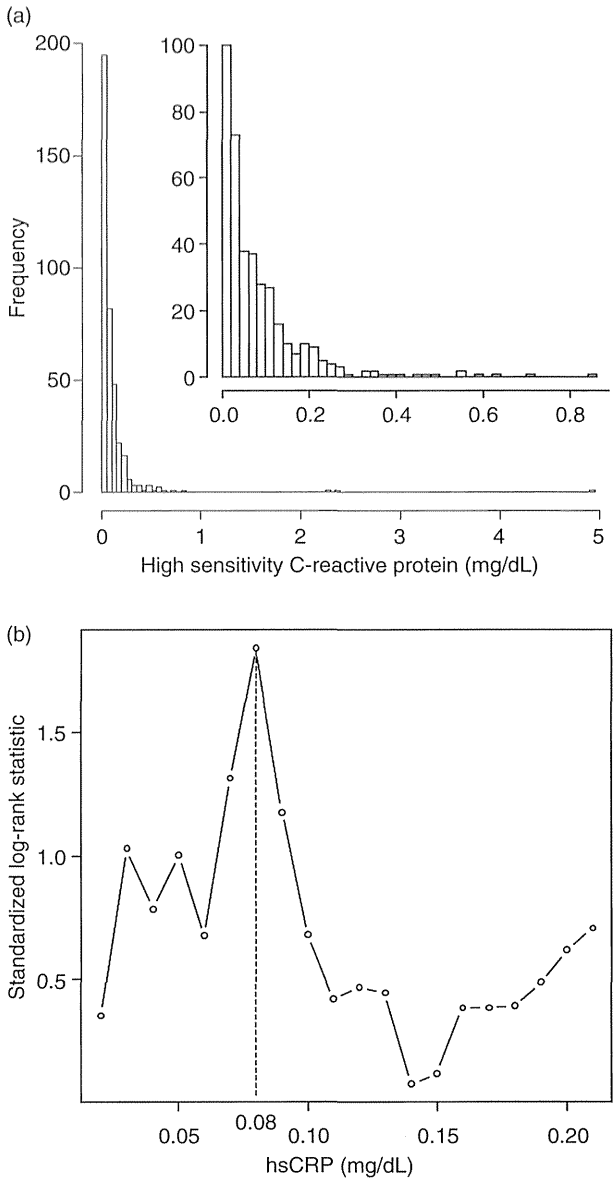


**Figure 1** Distribution and the optimal cut-off value of serum high-sensitivity C-reactive protein (hsCRP) concentrations. (a) The distribution of serum hsCRP concentrations is shown. The median serum hsCRP concentration was 0.05 mg/dL (interquartile range, 0.02–0.11). The upper right histogram shows the frequency of patients whose hsCRP levels ranged within 1.0 mg/dL. (b) Maximally selected log–rank statistics performed for hsCRP to determine an optimal cut-off value for separation of the two groups with different recurrence distribution. In the training set, the estimated cut-off point was 0.08 mg/dL with an M statistic of 1.85 and a corresponding corrected *P*-value of less than 0.001.

**Table 1** Baseline characteristics

| Characteristics | Overall ($n = 387$) | Low hsCRP ($n = 248$) | High hsCRP ($n = 139$) | $P$ value |
|---|---|---|---|---|
| Sex, male, $n$ (%) | 210 (54.3) | 126 (50.8) | 84 (60.4) | 0.086 |
| Age† | 70.1 ± 8.0 | 70.7 ± 7.8 | 69.1 ± 8.4 | 0.062 |
| Body weight (kg) | 57.0 (50.1–64.0) | 56.0 (49.1–62.8) | 58.4 (52.0–66.3) | 0.009 |
| BMI (kg/m$^2$) | 22.8 (20.8–24.8) | 22.4 (20.6–24.4) | 23.6 (21.1–26.0) | 0.005 |
| Waist circumference (cm) | 81.0 (75.5–88.1) | 80.3 (75.1–86.8) | 83.8 (77.0–89.0) | 0.003 |
| Visceral fat area (cm$^2$) | 65.7 (36.4–96.0) | 61.1 (35.5–90.9) | 73.8 (38.9–106.4) | 0.028 |
| Subcutaneous fat area (cm$^2$) | 111.1 (68.4–154.9) | 110.1 (66.6–150.2) | 117.6 (73.1–163.8) | 0.31 |
| Diabetes | | | | 0.014 |
| Yes, $n$ (%) | 81 (20.9) | 42 (16.9) | 39 (28.1) | |
| Atherosclerosing disease | | | | 0.35 |
| Yes, $n$ (%) | 189 (48.8) | 126 (50.8) | 63 (45.3) | |
| Alcohol consumption, $n$ (%) | | | | 0.68 |
| >80 g per day | 41 (10.6) | 28 (11.3) | 13 (9.4) | |
| Clinical cirrhosis, $n$ (%) | 321 (82.9) | 197 (79.4) | 124 (89.2) | 0.021 |
| Fibrotic stage, $n$ (%)‡ | | | | 0.38 |
| F0–2 | 58 (18.4) | 42 (20.0) | 16 (15.2) | |
| F3–4 | 257 (81.6) | 168 (80.0) | 89 (84.8) | |
| Inflammatory activity in non-cancerous tissue‡ | | | | 0.38 |
| 0 | 0 (0) | 0 (0) | 0 (0) | |
| 1 | 195 (61.9) | 132 (62.9) | 63 (60.0) | |
| 2 | 117 (37.1) | 75 (35.7) | 42 (40.0) | |
| 3 | 3 (1.0) | 3 (1.4) | 0 (0) | |
| hsCRP (mg/dL) | 0.05 (0.02–0.11) | 0.03 (0.02–0.05) | 0.14 (0.11–0.22) | |
| Platelet count (×10$^3$/mm$^3$) | 101 (76–136) | 101 (78–137) | 100 (76–132) | 0.82 |
| AST (IU/L) | 58 (45–78) | 56 (42–76) | 61 (50–80) | 0.026 |
| ALT (IU/L) | 51 (34–77) | 50 (33–77) | 54 (36–75) | 0.43 |
| Total bilirubin (mg/dL) | 0.8 (0.6–1.1) | 0.8 (0.6–1.0) | 0.9 (0.7–1.3) | 0.004 |
| Albumin (g/dL) | 3.6 (3.3–3.9) | 3.7 (3.4–4.0) | 3.4 (3.1–3.8) | <0.001 |
| Tumor size (cm) | 2.0 (1.7–2.5) | 2.0 (1.6–2.4) | 2.1 (1.7–2.5) | 0.06 |
| No. of nodules | | | | 0.95 |
| Solitary, $n$ (%) | 250 (64.6) | 161 (64.9) | 89 (64.0) | |
| 2 or 3 nodules, $n$ (%) | 137 (35.4) | 87 (35.1) | 50 (36.0) | |
| Tumor stage, $n$ (%) | | | | 0.11 |
| Solitary nodule <2 cm | 115 (29.7) | 81 (32.7) | 34 (24.5) | |
| Others | 272 (70.3) | 167 (67.3) | 105 (75.5) | |
| Serum AFP (ng/mL) | 21.6 (8.2–74.9) | 24.5 (8.1–81.9) | 17.1 (9.0–53.7) | 0.33 |
| Serum DCP (mAU/mL)§ | 21.0 (15.0–42.0) | 21.0 (15.0–40.5) | 21.0 (15.0–47.0) | 0.49 |

†Expressed as mean ± standard deviation.
‡Biopsies were available in 315 patients (81.4%). Background liver was pathologically graded based on the METAVIR system.
§Serum DCP level could not be measured in two patients because they were taking warfarin.
AFP, α-fetoprotein; ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; DCP, des-γ-carboxyprothrombin; hsCRP, high-sensitivity C-reactive protein.

## Risk factors for survival and recurrence

Univariate analysis showed that a high hsCRP level was a significant predictor of overall survival (HR, 1.95; 95% confidence interval [CI], 1.43–2.66; $P < 0.001$). The risk of recurrence after RFA increased in accordance with high hsCRP (HR, 1.43; 95% CI, 1.13–1.82; $P = 0.003$). Other risk factors for recurrence and survival are shown in Table 3.

Stepwise multivariate analysis using the AIC established the best model for survival and recurrence (Table 3). Patients with higher hsCRP levels were at a significantly higher risk for both survival (HR, 1.59; 95% CI, 1.14–2.22; $P = 0.007$) and recurrence (HR, 1.32; 95% CI, 1.03–1.67; $P = 0.026$). After adjusting for other risk factors, we found that the estimated log-transformed HR of survival in relation to hsCRP peaked

**Table 2** Spearman's rank correlation coefficients and *P*-values between hsCRP and clinical parameters

| Characteristics | Spearman's rho | *P* |
|---|---|---|
| Age | −0.055 | 0.28 |
| Body weight (kg) | 0.199 | <0.001 |
| BMI (kg/m$^2$) | 0.181 | <0.001 |
| Waist circumference (cm) | 0.200 | <0.001 |
| Visceral fat area (cm$^2$) | 0.176 | <0.001 |
| Subcutaneous fat area (cm$^2$) | 0.064 | 0.21 |
| Platelet count (×10$^3$/mm$^3$) | 0.044 | 0.39 |
| AST (IU/L) | 0.091 | 0.074 |
| ALT (IU/L) | −0.002 | 0.96 |
| Total bilirubin (mg/dL) | 0.120 | 0.019 |
| Albumin (g/dL) | −0.285 | <0.001 |
| Tumor size (cm) | 0.124 | 0.015 |
| No. of tumors | 0.091 | 0.072 |
| Serum AFP (ng/mL) | −0.086 | 0.091 |
| Serum DCP (mAU/mL)† | 0.050 | 0.32 |

†Serum DCP level could not be measured in two patients because they were taking warfarin.
AFP, α-fetoprotein; ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; DCP, des-γ-carboxyprothrombin; hsCRP, high-sensitivity C-reactive protein.

at approximately 0.16 mg/dL hsCRP and then plateaued (Fig. 3). This estimated HR was significant after adjustment ($P = 0.0019$).

The effects of high hsCRP on the risk of HCC survival were also evaluated in subgroup analyses to assess whether high hsCRP was a significant risk factor over strata (Fig. 4). Indeed, a higher hsCRP level was found to be a significant risk factor for survival excluding the subgroups of patients with non-liver cirrhosis and serum albumin levels of more than 4.0 g/dL. A higher hsCRP level was an especially strong predictor for survival in patients with HCC with a single nodule of less than 2 cm (HR, 2.88; 95% CI, 1.46–5.66; $P < 0.001$).

## DISCUSSION

IN THIS STUDY, we identified an optimal hsCRP cut-off value of 0.08 mg/dL to predict recurrence after curative treatment for early stage HCC. This value was much lower than the conventional cut-off value of 0.3 mg/dL and was below the range detected by conventional CRP assays. Using the hsCRP assay enabled us to predict early recurrence and poor prognosis after curative treatment for early/very early stage HCC.

What pathological mechanism(s) may underlie this association between hsCRP and prognosis? We speculated that visceral fat was the dominant factor associated with elevations in hsCRP level compared with inflammation of non-tumorous liver tissue, considering that neither AST, ALT nor the grade of liver inflammation were associated with higher hsCRP levels. As shown in Table 2, there were weak correlations between hsCRP and anthropometric parameters related to central obesity, including waist circumference and VFA. Accordingly, visceral adipose accumulation may link higher hsCRP with a poor prognosis, because obesity-induced dysregulation of adipokines can directly accelerate HCC

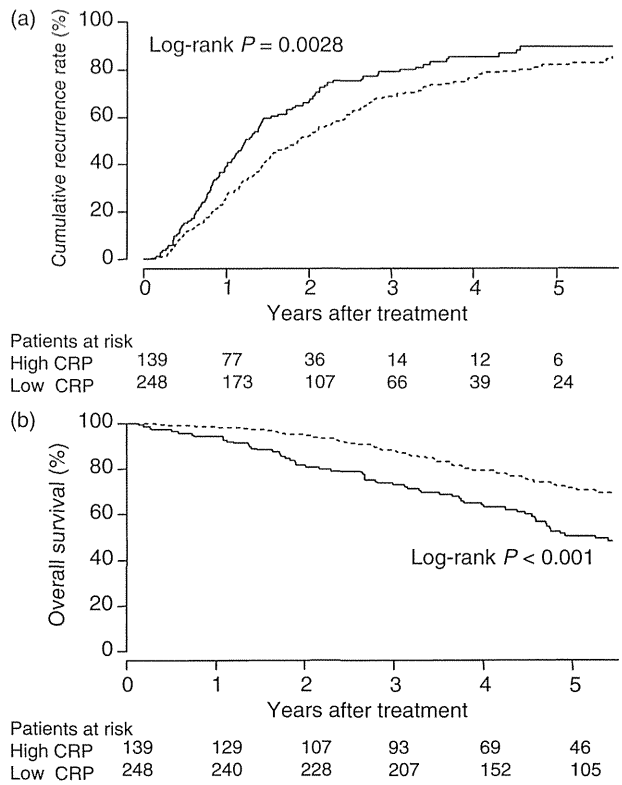


**Figure 2** Cumulative recurrence rate and overall survival stratified by high-sensitivity C-reactive protein (hsCRP) level. (a) The cumulative recurrence rate is shown. In the low hsCRP group, the cumulative recurrence rates at 1, 3 and 5 years were 27.1%, 68.8% and 82.2%, respectively, while in the high hsCRP group, the rates were 39.1%, 79.3% and 90.0%, respectively. There was a significant difference between the two cumulative rates (log-rank test, $P = 0.0028$). (b) The overall survival is shown. In the low hsCRP group, the overall survival rates at 1, 3 and 5 years were 98.4%, 87.9% and 71.8%, respectively, while in the high hsCRP group, the rates were 94.2%, 73.1% and 50.9%, respectively. The survival curves of the two groups were significantly different (log-rank test, $P < 0.001$). —, High hsCRP; - - -, low hsCRP.

**Table 3** Risk factors for recurrence and survival

| Variables | Survival | | | | | | Recurrence | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Univariate | | | Multivariate | | | Univariate | | | Multivariate | | |
| | HR | 95% CI | *P* | HR | 95% CI | *P* | HR | 95% CI | *P* | HR | 95% CI | *P* |
| High hsCRP | 1.95 | 1.43–2.66 | <0.001 | 1.59 | 1.14–2.22 | 0.007 | 1.43 | 1.13–1.82 | 0.003 | 1.32 | 1.03–1.67 | 0.026 |
| Sex, female | 1.05 | 0.77–1.43 | 0.78 | | | | 0.86 | 0.68–1.08 | 0.20 | | | |
| Age per 1 year | 1.03 | 1.01–1.05 | 0.005 | 1.04 | 1.02–1.06 | <0.001 | 1.01 | 0.99–1.02 | 0.49 | | | |
| BMI per 1 kg/m$^2$ | 0.97 | 0.92–1.01 | 0.14 | | | | 0.98 | 0.95–1.02 | 0.28 | | | |
| VFA per 10 cm$^2$ | 0.98 | 0.94–1.01 | 0.22 | | | | 1.00 | 0.97–1.02 | 0.71 | | | |
| Presence of diabetes | 1.07 | 0.73–1.57 | 0.72 | | | | 1.03 | 0.78–1.37 | 0.83 | | | |
| Presence of atherosclerosis | 1.03 | 0.76–1.40 | 0.86 | | | | 1.09 | 0.87–1.37 | 0.44 | | | |
| Alcohol consumption >80 g/day | 1.08 | 0.65–1.78 | 0.78 | | | | 0.98 | 0.66–1.45 | 0.92 | | | |
| Serum albumin, per 1 g/dL | 0.33 | 0.23–0.48 | <0.001 | 0.47 | 0.32–0.70 | <0.001 | 0.61 | 0.48–0.78 | <0.001 | 0.61 | 0.47–0.78 | <0.001 |
| Serum total bilirubin, per 1 mg/dL | 2.28 | 1.64–3.17 | <0.001 | 1.76 | 1.22–2.56 | 0.003 | 1.30 | 0.99–1.72 | 0.063 | | | |
| Platelet count, per $10 \times 10^3$/mm$^3$ | 0.96 | 0.93–1.00 | 0.033 | | | | 0.98 | 0.96–1.00 | 0.11 | | | |
| Prothrombin time, per 1% | 0.99 | 0.98–1.01 | 0.26 | | | | 1.00 | 0.99–1.01 | 0.79 | | | |
| Tumor size per 1 cm | 1.61 | 1.19–2.20 | 0.002 | 1.56 | 1.14–2.14 | 0.006 | 1.45 | 1.16–1.82 | 0.001 | 1.44 | 1.15–1.81 | 0.002 |
| No. of nodules per 1 nodule | 1.12 | 0.91–1.38 | 0.29 | | | | 1.31 | 1.11–1.53 | 0.001 | 1.34 | 1.14–1.58 | <0.001 |
| Serum AFP ≥100 ng/dL | 1.41 | 0.98–2.01 | 0.061 | 1.50 | 1.04–2.19 | 0.033 | 1.32 | 0.99–1.75 | 0.055 | 1.39 | 1.04–1.86 | 0.024 |
| Serum DCP ≥100 mAU/mL† | 1.61 | 1.00–2.61 | 0.051 | | | | 1.44 | 0.97–2.13 | 0.067 | 1.59 | 1.07–2.37 | 0.021 |

†Serum DCP level could not be measured in two patients because they were taking warfarin.
AFP, α-fetoprotein; BMI, body mass index; CI, confidence interval; DCP, des-γ-carboxyprothrombin; HR, hazard ratio; hsCRP, high-sensitivity C-reactive protein; VFA, visceral fat area.

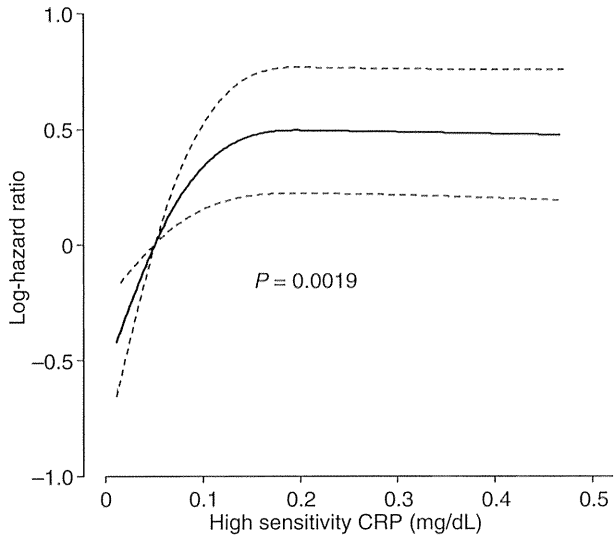


**Figure 3** Log-hazard ratio of survival related to serum high-sensitivity C-reactive protein (hsCRP) concentration. Serum hsCRP concentration was fixed at 0.05 mg/dL. The estimated log-transformed hazard ratio of survival in relation to hsCRP peaked at approximately 0.16 mg/dL and then plateaued.

development by enhancing cancer cell proliferation, inhibiting apoptosis and inducing migration which are liver steatosis- and inflammation-independent mechanisms.[22,23] However, this present study could not address the causality. Further study is required to elucidate the association between hsCRP, adipokines and the outcomes of HCC after curative treatment.

Based on previous reports,[24–26] interleukin (IL)-6 could partially explain the relationship of elevated hsCRP with higher recurrence rate and poorer survival in HCC patients. IL-6, the principal regulator of CRP production, is a multifunctional cytokine largely responsible for the hepatic response to infections or systemic inflammation. In fact, we previously reported that a higher serum IL-6 level correlated with future HCC development in patients with chronic hepatitis C.[27] IL-6 is also a key molecule linking obesity with hepatocarcinogenesis.[28] Moreover, promising molecular innovations support these results. Hoshida *et al.* reported a transcriptomic signature in liver tissue

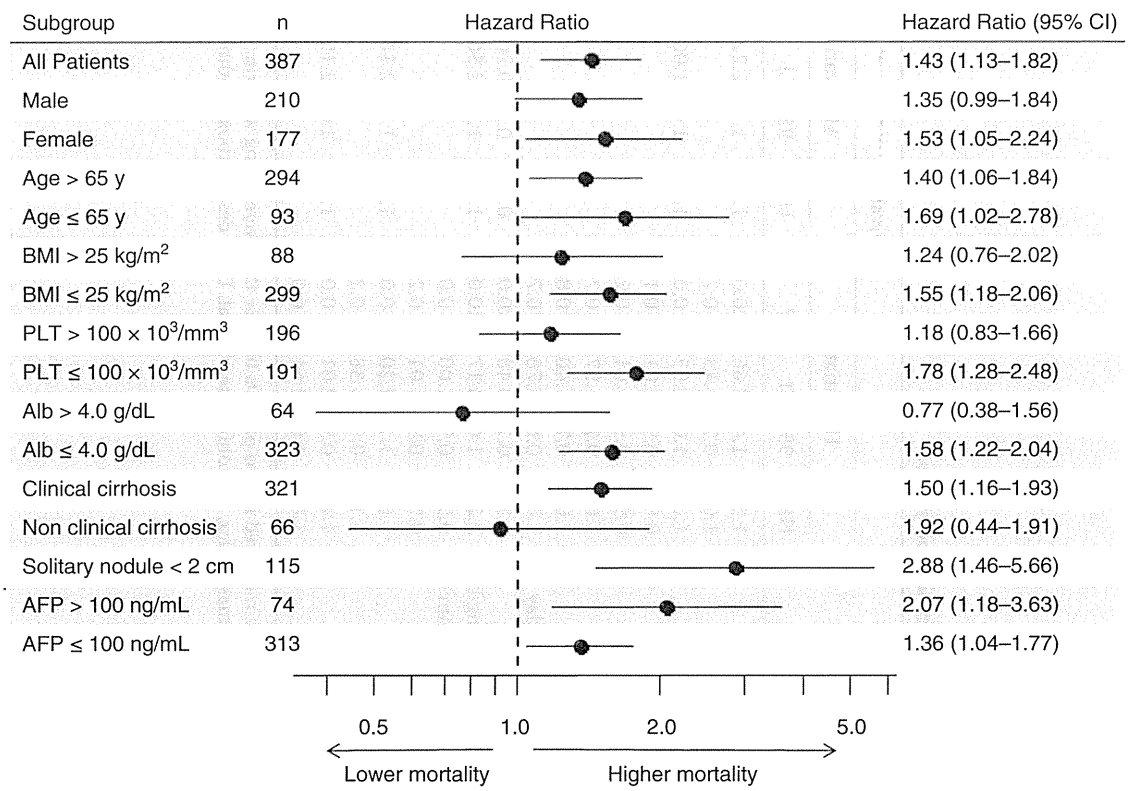


**Figure 4** Subgroup analyses stratified by risk factor. A higher high-sensitivity C-reactive protein (hsCRP) concentration was a significant risk factor for mortality over nearly all strata. A higher hsCRP level was also an independent risk factor for survival in patients with a single nodule of less than 2 cm. AFP, α-fetoprotein; Alb, albumin; BMI, body mass index; CI, confidence interval; PLT, platelets.

adjacent to HCC that could predict survival and late recurrence after HCC resection.[29] In this study, the poor prognosis signature involved genes associated with inflammation, including those related to interferon signaling and activation of nuclear factor-κB. Intriguingly, the downstream targets of IL-6 were strongly associated with the poor-prognosis signature in non-tumorous liver tissue. Thus, higher CRP may independently indicate carcinogenic potential in the underlying liver. Additionally, the transcription factor signal transducer and activator of transcription-3, which mediates the effects of IL-6, was found to be activated in most HCC and seems to be associated with more aggressive tumors. This may indicate that higher hsCRP levels reflect the HCC malignancy grade.[30]

Exploration of serum biomarkers to predict survival, progression and treatment efficacy among HCC patients in various stages is an ever-improving field.[5,31,32] Several reports have shown associations of conventional CRP with recurrence rate and long-term survival in HCC patients who were treated by surgical resection,[33] non-surgical procedures[34] and liver transplantation.[35] In these studies, 1.0 mg/dL was used as the cut-off value. However, only three patients with early HCC (0.8%) showed a hsCRP of more than 1.0 mg/dL in this study (Fig. 1a). Thus, there is a need for an optimal cut-off value in early/very early stage HCC patients.[36,37] On the basis of a statistically robust method, this study set a useful hsCRP cut-off value to identify patients at risk for rapid progression to death despite curative treatment among patients with early HCC, who have intrinsically better prognosis.[38] Additionally, measuring hsCRP is practical in real-world clinical settings because it is readily determinable, simple, widely available and inexpensive.

This study has several limitations. First, due to its retrospective nature, hsCRP was not measured in some patients, which might have caused selection bias. However, the proportion of these patients (5.9%) was small. Second, elevation of hsCRP may reflect non-specific or undetectable infection, especially that related to cirrhosis. Thus, hsCRP may be a surrogate marker for poor liver function. However, hsCRP remained significant in terms of recurrence and prognosis prediction after adjusting for indicators of liver functional reserve. Third, we did not validate the determined optimal cut-off value of hsCRP in an external cohort. However, we could validate the cut-off value using a split-sample method.

Although we analyzed only patients with C-HCC in order to restrict the background conditions of patients, whether hsCRP is an independent predictor of recurrence and survival in HCC patients with hepatitis B virus or non-alcoholic steatohepatitis is an interesting issue. Further study is required to clarify the predictability of slightly elevated hsCRP in patients with HCC of other etiologies.

In conclusion, slightly elevated hsCRP, even though below the range detected by conventional CRP assays, can identify patients with early stage C-HCC at risk for recurrence and death.

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## SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

**Figure S1** Cumulative recurrence rate and survival stratified by high-sensitivity C-reactive protein (hsCRP) in the training set and the validation set, respectively. (A) Cumulative recurrence rate in the training set. In the low hsCRP group, the cumulative recurrence rates at 1, 3 and 5 years were 27.7%, 68.0% and 83.0%, respectively; while in the high hsCRP group, the rates were 38.3%, 81.4% and 88.0%, respectively. There was a significant difference between the two cumulative rates (log–rank test, $P = 0.049$). (B) Overall survival in the training set. In the low hsCRP group, the overall survival rates at 1, 3 and 5 years were 97.5%, 84.6% and 68.2%, respectively,