two pathologists. Macrovesicular steatosis affecting at least 5% of the hepatocytes was observed in all the cases, and the patients were classified as having steatosis or steatohepatitis. In addition to steatosis, the minimum criteria for the diagnosis of steatohepatitis include the presence of lobular inflammation and either ballooning of cells or perisinusoidal/pericellular fibrosis in zone 3 of the hepatic acini.[30–32] Subjects with cirrhosis were defined as having NASH-associated cirrhosis according to a previously proposed clinicopathological classification.[32] In all cases, the severity of fibrosis was scored according to the method of Brunt.[33] The degree of steatosis was assessed on the basis of the percentage of hepatocytes containing macrovesicular fat droplets, as follows: grade 0, no steatosis; grade 1, <33% of hepatocytes containing macrovesicular fat droplets; grade 2, 33%–66% of hepatocytes containing macrovesicular fat droplets; and grade 3, >66% of hepatocytes containing macrovesicular fat droplets. The severity of fibrosis was expressed on a 4-point scale, as follows: 0 = none, 1 = perivenular and/or perisinusoidal fibrosis in zone 3, 2 = combined pericellular portal fibrosis, 3 = septal/bridging fibrosis, and 4 = cirrhosis. On the basis of this classification, the subjects were grouped into two categories, namely, those with mild fibrosis (stage 0–2) and those with advanced fibrosis (stage 3–4).

Real-time reverse transcriptase-polymerase chain reaction for measurement of CRP and interleukin-6 mRNA

Liver biopsy specimens (6–10 mg) obtained from the patients with NASH (n = 10) or hepatic steatosis (n = 10) were snap-frozen in liquid nitrogen and stored at –80°C. Total RNA was purified using an RNeasy kit (Qiagen, Hilden, Germany). The RNA was reverse-transcribed in the presence of reverse transcriptase (RT) and random hexamer primers at 23°C for 10 min, 42°C for 60 min, and 95°C for 10 min, in accordance with the manufacturer's instructions (GeneAmp RNA PCR kit from Perkin-Elmer Cetus, Norwalk, CT, USA), and then in polymerase chain reaction (PCR) master mix containing the specific primers, Hot Star *Taq* DNA polymerase, and SYBER-Green PCR buffer; all samples were measured in triplicate. To establish a negative control, the same setup was used except for the addition of RT. No PCR product was detected under these latter conditions.

The following CRP primers yielded a product of 249 bp: 5′-AGGAGGGGATAGCTCTGAGG and 5′-CCGTGCTTTGAGGGTTACAT and the following interleukin (IL)-6 primers yielded a product of 193 bp: 5′-CTCTGCAATGAGAAAGGAGA and 5′-GGTAG TCCAGGTATATCTGA. β-*Actin*, a housekeeping gene, was amplified using the primers 5′-ACGGGGTC ACCCACACTGTGC and 5′-CTAGAAGCATTTGC GGTGGACGATG. Real-time PCR quantification of CRP and IL-6 in relation to β-actin mRNA was performed using a SYBR-Green PCR assay kit and an iCycler PCR machine (Bio-Rad Laboratories, Hercules, CA, USA), as previously described.[34]

Statistical analysis

Data are expressed as means ± SD, unless indicated otherwise. Statistical analysis was conducted using SPSS 12.0 software (SPSS, Chicago, IL, USA). A t test or Wilcoxon rank sum test, as appropriate, was used for univariate comparisons between patient groups. Because the variables were often not normally distributed, group comparisons of more than two independent groups were performed by the Kruskal-Wallis test. The diagnostic performance of hs-CRP was assessed by analysis of receiver-operating characteristic (ROC) curves. The ROC curve is a plot of sensitivity versus (1—specificity) for all possible cutoff values. The most commonly used index of accuracy is the area under the ROC curve (AUROC), with values close to 1.0 indicating high diagnostic accuracy. Calculations of correlation coefficients and linear regression analysis were used to test for associations between the variables. Multivariate analysis was performed by using a binary logistic regression analysis. P values of <0.05 were considered significant.

Results

Characteristics of the patients

The histological findings in the liver biopsy specimens of the subjects with simple steatosis (n = 29) and steatohepatitis (NASH) (n = 71) are shown in Table 1. The clinical and biochemical characteristics of the NASH patients and simple steatosis patients are shown in Table 2. Marked elevation of the serum hs-CRP level was observed in the patients with NASH compared with that in those with simple steatosis, and the difference in level between the two groups was strongly significant ($P < 0.0001$) (Fig. 1A). Significant differences were observed in SFA ($P = 0.0100$), AST ($P = 0.0128$), serum ferritin concentration ($P = 0.0450$), and hyaluronic acid ($P = 0.0478$) between the patients with NASH and those with simple steatosis (Table 2). The clinical and biochemical characteristics of the patients with NASH with mild fibrosis and those with NASH with advanced fibrosis patients are shown in Table 3. Significant differences were observed in hs-CRP ($P = 0.0223$) between NASH patients with advanced fibrosis and those with mild fibrosis (Fig. 1B), and marked elevation of hyaluronic

Table 1. Histopathological findings in subjects with steatohepatitis and steatosis

	NASH (n = 71) no. (%)	Steatosis (n = 29) no. (%)
Steatosis grade		
1	43 (61%)	20 (73%)
2	24 (34%)	6 (18%)
3	4 (6%)	3 (9%)
Fibrosis stage		
0	3 (4%)	NA
1	30 (42%)	NA
2	20 (28%)	NA
3	14 (20%)	NA
4	5 (7%)	NA

NASH, nonalcoholic steatohepatitis; NA, not applicable

acid (P = 0.0010) and type IV collagen 7s domain (P = 0.0001) was also observed between these two groups (Table 3).

Hepatic fibrosis and hs-CRP levels

Since the serum levels of hs-CRP were significantly elevated in the NASH patients compared with in the simple steatosis patients, and in advanced fibrosis NASH patients compared with in mild fibrosis NASH patients, we investigated further the relationship between the severity of fibrosis and the serum level of hs-CRP in NASH patients. When serum hs-CRP concentrations were analyzed in relation to the histological stage of fibrosis in NASH patients, a stepwise increase in the

Table 2. Clinical and biochemical characteristics of patients with NASH and steatosis patients

	Steatosis patients	NASH patients	P value
Age (years)	44.4 ± 16.8	50.9 ± 13.7	0.0642
Sex (male:female)	21:8	32:39	
BMI (kg/m²)	27.2 ± 4.1	28.4 ± 5.2	0.3306
VFA (cm²)	116.7 ± 46.1	132.9 ± 50.6	0.2363
SFA (cm²)	164.9 ± 58.0	236.6 ± 107.6	0.0100
AST (U/ml)	33.3 ± 12.8	53.0 ± 35.5	0.0128
ALT (U/ml)	57.0 ± 32.3	80.6 ± 60.0	0.0808
FBS (ml/ml)	108.4 ± 16.4	123.6 ± 36.8	0.0630
IRI (μl/ml)	11.4 ± 7.8	14.1 ± 10.6	0.2776
HOMA-IR	3.10 ± 2.30	4.07 ± 3.73	0.2634
HDL cholesterol (mg/dl)	49.9 ± 14.4	47.9 ± 11.5	0.5038
LDL cholesterol (mg/dl)	115.1 ± 31.0	129.3 ± 38.5	0.1282
Triglycerides (mg/dl)	170.7 ± 82.3	172.6 ± 98.5	0.9368
Iron (ng/ml)	116.1 ± 46.9	110.1 ± 40.2	0.5874
Ferritin (ng/ml)	146.6 ± 96.1	264.2 ± 245.4	0.0450
Hyaluronic acid (ng/dl)	27.3 ± 26.2	51.2 ± 52.2	0.0478
Type IV collagen 7s domain (ng/dl)	4.21 ± 0.93	4.67 ± 1.23	0.1218

Values are means ± SD

BMI, body mass index; VFA, visceral fat area; SFA, subcutaneous fat area; AST, aspartate aminotransferase; ALT, alanine aminotransferase; FBS, fasting blood sugar; IRI, immunoreactive insulin; HOMA-IR, homeostasis model assessment of insulin resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein

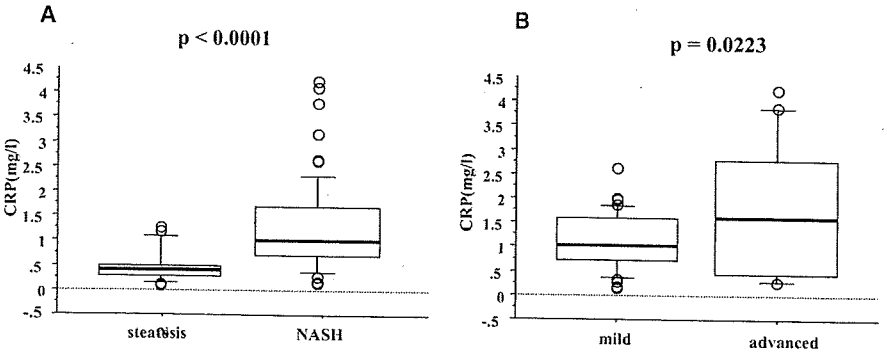

Fig. 1A,B. Box plots showing interquartile range (*box*), median (*thick line*), range (*thin lines*), and outliers (*circles*) for high-sensitivity (hs) C-reactive protein (*CRP*). **A** hs-CRP in patients with simple steatosis and in patients with nonalcoholic steatohepatitis (*NASH*). **B** hs-CRP in NASH patients with mild fibrosis and in NASH patients with advanced fibrosis

Table 3. Clinical and biochemical characteristics of patients with mild and advanced fibrosis

	Mild fibrosis	Advanced fibrosis	*P* value
Age (years)	49.4 ± 13.6	55.4 ± 13.4	0.0980
Sex (male:female)	25:28	8:11	
BMI (kg/m²)	27.9 ± 5.5	29.6 ± 4.6	0.2253
VFA (cm²)	131.3 ± 53.1	140.0 ± 38.8	0.5973
SFA (cm²)	226.2 ± 105.4	281.8 ± 109.8	0.1070
AST (U/ml)	50.2 ± 38.1	61.1 ± 25.4	0.2511
ALT (U/ml)	78.6 ± 62.5	86.7 ± 53.0	0.6112
FBS (ml/ml)	124.3 ± 38.5	121.7 ± 32.6	0.7966
IRI (μl/ml)	13.6 ± 11.5	15.5 ± 7.5	0.5545
HOMA-IR	3.91 ± 4.21	4.43 ± 2.09	0.7246
HDL cholesterol (mg/dl)	47.2 ± 12.2	50.1 ± 8.7	0.3605
LDL cholesterol (mg/dl)	128.9 ± 37.6	130.2 ± 43.0	0.9052
Triglycerides (mg/dl)	172.7 ± 99.8	172.1 ± 97.2	0.9813
Iron (ng/ml)	110.7 ± 38.8	108.6 ± 45.2	0.8538
Ferritin (ng/ml)	286.6 ± 265.0	202.4 ± 172.4	0.2280
Hyaluronic acid (ng/dl)	37.9 ± 37.5	84.3 ± 68.3	0.0010
Type IV collagen 7s domain (ng/dl)	4.32 ± 0.90	5.63 ± 1.54	0.0001

Data are expressed as means ± SD

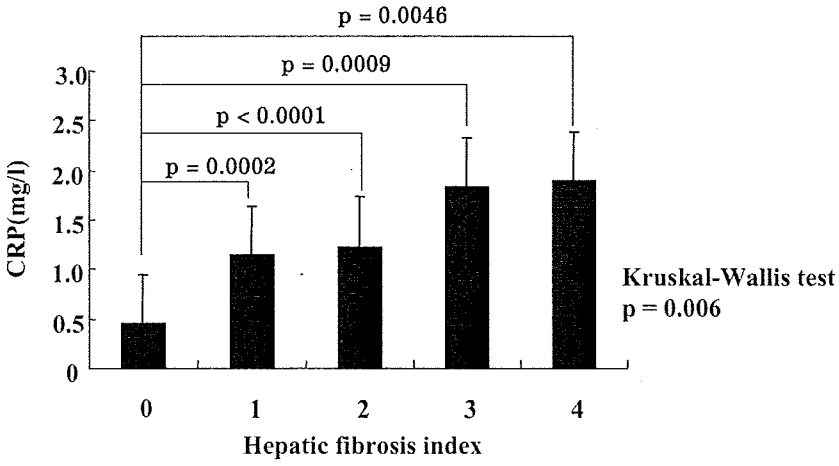

Fig. 2. Comparison between serum hs-CRP levels at various stages of fibrosis. A steady stepwise increase in serum hs-CRP was observed with increasing severity of hepatic fibrosis ($P = 0.006$). The Wilcoxon rank sum test was performed to evaluate the differences between pairs of groups

serum CRP level with increasing severity of hepatic fibrosis ($P = 0.0060$ by Kruskal-Wallis test) was observed (Fig. 2). There were significant differences between F0 and F1, F0 and F2, F0 and F3, and F0 and F4 ($P = 0.0002$, $P < 0.0001$, $P = 0.0009$, and $P = 0.0046$, respectively).

Relation between hs-CRP and grade of hepatic steatosis or grade of necroinflammation

Analysis of the serum hs-CRP concentrations in relation to the histological grade of steatosis or grade of necroinflammation showed no relation between serum hs-CRP levels and either the steatosis or necroinflammation grade (Fig. 3).

Multiple regression analysis for demographic factors in NASH patients

We performed a multiple logistic regression analysis by using the SFA, AST, hyaluronic acid, ferritin, and hs-CRP values, which were significantly elevated in NASH patients compared with in those with simple steatosis according to the univariate analysis. Serum hs-CRP was still significantly higher in the NASH patients compared with in the simple steatosis patients by the multiple logistic regression analysis (Table 4). Multiple logistic regression analyses were then performed to determine whether the observed differences in the serum hs-CRP between the NASH patients and the simple steatosis patients were independent of the effect of age, sex, BMI, VFA, SFA, presence of diabetes, insulin resis-

tance, and hyperlipidemia, and to quantify the extent of these differences by the odds ratio (OR). The results of the multivariate analysis revealed marked elevation of the serum hs-CRP concentration in NASH patients compared with in the simple steatosis patients, even after adjustment for age, sex, presence of diabetes, HOMA-IR, serum triglyceride, HDL and LDL cholesterol levels, BMI, VFA, and SFA [OR, 0.004; 95% confidence interval (CI), 8.178×10^{-5} to 0.494; $P = 0.0048$] (Table 5). The AUROC curve for distinguishing between NASH and steatosis using hs-CRP levels was 0.833 (Fig. 4).

Table 4. Multiple logistic regression analysis of factors associated with NASH compared with simple steatosis

Factor	Odds ratio	95% CI	*P* value
SFA (cm^2)	0.988	0.976–1.001	0.0609
AST (U/ml)	0.989	0.959–1.021	0.5041
Hyaluronic acid (ng/dl)	0.982	0.953–1.012	0.2491
Ferritin (ng/ml)	0.997	0.991–1.003	0.3998
CRP (mg/l)	0.097	0.012–0.753	0.0256

Intercept = –35.373; R^2 for entire model = 0.378
CI, confidence interval; CRP, C-reactive protein

Multiple regression analysis for factors associated with the fibrosis grade in subjects with NASH (mild or advanced fibrosis)

We performed a multiple logistic regression analysis by using hs-CRP, type IV collagen 7s domain, and hyaluronic acid, which were the significantly elevated in NASH with advanced fibrosis compared with in NASH with mild fibrosis according to the univariate analysis. The serum hs-CRP and type IV collagen 7s domain values were still significant in the NASH patients with advanced fibrosis compared with in those with mild fibrosis by the multiple logistic regression analysis (Table 6). Multiple logistic regression analyses were then performed to determine whether the observed differences in serum hs-CRP between the NASH patients with advanced fibrosis and those with mild fibrosis were independent of the effect of age, sex, BMI, VFA, SFA, presence of diabetes, insulin resistance, and hyperlipidemia, and to quantify the extent of these differences by the OR. The results revealed that even after adjusting for the effect of age, sex, presence of diabetes, HOMA-IR, triglyceride, HDL cholesterol, LDL cholesterol, BMI, VFA, and SFA, the serum hs-CRP

Table 5. Multiple logistic regression analysis of factors associated with NASH compared with simple steatosis

Factor	Odds ratio	95% CI	*P* value
Age (year)	0.943	0.864–1.028	0.1807
Sex	1.725	0.169–17.585	0.6454
CRP (mg/l)	0.004	8.178×10^{-5} to 0.185	0.0048
BMI (kg/m^2)	1.544	1.043–2.287	0.0301
Diabetes	3.054	0.388–24.021	0.2887
VFA (cm^2)	0.987	0.965–1.010	0.2814
SFA (cm^2)	0.982	0.961–1.004	0.1071
HOMA-IR	1.062	0.662–1.704	0.8031
HDL cholesterol (mg/dl)	1.045	0.952–1.148	0.3498
Trigtyceride (mg/dl)	1.012	1.000–1.025	0.0490
LDL cholesterol (mg/dl)	0.988	0.966–1.010	0.2798

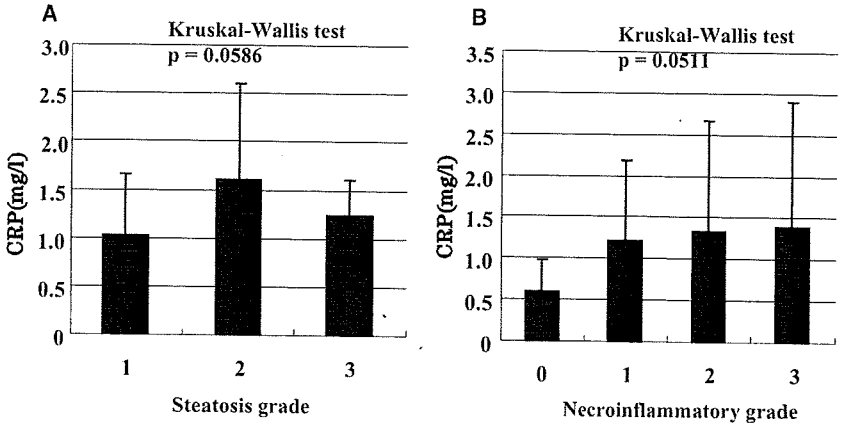

Fig. 3. **A** Comparison of the hs-CRP levels at various grades of steatosis. There were no significant differences among the three groups ($P = 0.0586$) **B** Comparison of hs-CRP levels at various grades of necroinflammation. There were no significant differences among the four groups ($P = 0.0511$)

concentrations were more markedly elevated in the advanced fibrosis group (OR, 0.155; 95% CI, 0.026–0.905; $P = 0.0384$) compared with in the mild fibrosis group (Table 7).

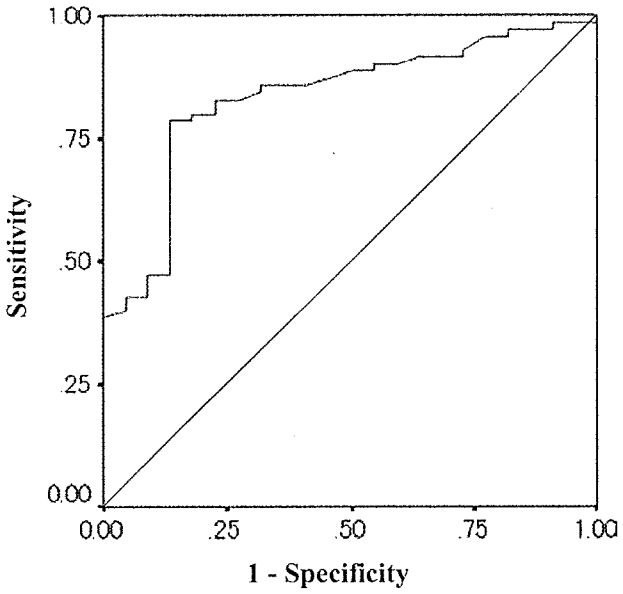

Fig. 4. Receiver-operating characteristics (*ROC*) curve for differentiating steatosis and NASH using hs-CRP (area under curve = 0.833)

mRNA expression of CRP and IL-6 in the liver

The expression of CRP mRNA and IL-6 mRNA in the liver biopsy specimens of NASH patients ($n = 10$) and steatosis patients ($n = 10$) was compared by quantitative RT-PCR. As shown in Fig. 5, the expression of CRP mRNA transcripts was markedly elevated in the liver of NASH patients compared with in the liver of simple steatosis patients ($P = 0.0258$). On the other hand, while the expression of IL-6 mRNA transcripts tended to be increased in the liver of NASH patients compared with in the liver of simple steatosis patients, the difference was not statistically significant.

Discussion

The results of this study demonstrated markedly elevated serum concentrations of hs-CRP in NASH patients compared with in patients with simple steatosis, and in NASH patients with advanced fibrosis compared in NASH patients with mild fibrosis, even after adjustment for age, sex, presence of diabetes, BMI, VFA, SFA, insulin resistance, and hyperlipidemia.

Metabolic syndrome, characterized by a core set of disorders that include abdominal obesity, dyslipidemia, hypertension, and hyperglycemia, has been shown to be an important predictor of type 2 diabetes and cardiovascular disease.[35–37] Recently, a growing body of evi-

Table 6. Multiple logistic regression analysis of factors associated with mild fibrosis compared with advanced fibrosis

Factor	Odds ratio	95% CI	*P* value
Hyaluronic acid (ng/dl)	0.987	0.971–1.002	0.0912
Type IV collagen 7s domain (ng/dl)	0.451	0.232–0.879	0.0193
CRP (mg/l)	0.488	0.256–0.927	0.0283

Intercept = −34.162; R^2 for entire model = 0.286

Table 7. Multiple logistic regression analysis of factors associated with mild fibrosis compared with advanced fibrosis

Factor	Odds ratio	95% CI	*P* value
Age (year)	0.885	0.775–1.011	0.0710
Sex	0.329	0.018–5.843	0.4486
CRP (mg/l)	0.155	0.026–0.905	0.0384
BMI (kg/m^2)	1.063	0.742–1.522	0.7399
Diabetes	0.006	8.161×10^{-5} to 0.484	0.0222
VFA (cm^2)	1.011	0.985–1.037	0.4112
SFA (cm^2)	0.986	0.966–1.005	0.1509
HOMA-IR	1.042	0.797–1.362	0.7624
HDL cholesterol (mg/dl)	0.925	0.808–1.059	0.2593
Trigryceride (mg/dl)	1.000	0.991–1.009	0.9509
LDL cholesterol (mg/dl)	1.003	0.979–1.028	0.7893

Intercept = −28.604; R^2 for entire model = 0.452

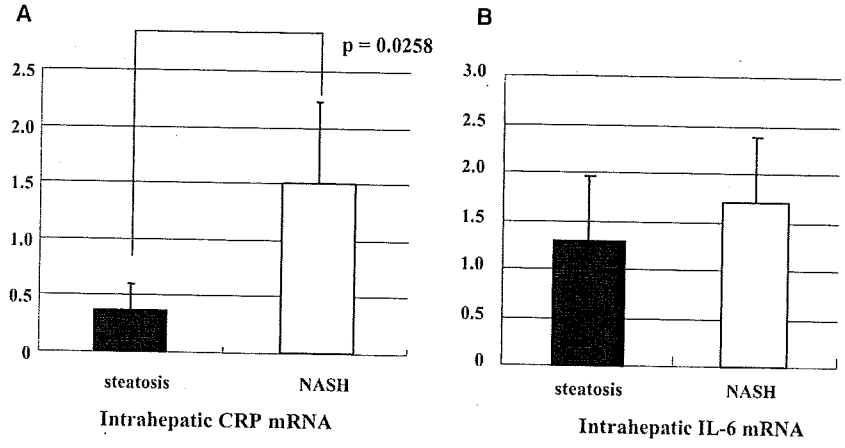

Fig. 5. **A** Significant difference in the intrahepatic mRNA expression of CRP between NASH patients and simple steatosis patients ($P = 0.0288$). **B** The intrahepatic mRNA expression level of interleukin-6 (*IL-6*) tended to be increased in NASH patients in comparison with in the simple steatosis patients; however, the association was not statistically significant

dence has been collected to support the notion that NAFLD may be a feature of metabolic syndrome. Conceivably, NAFLD, which can progress to cirrhosis, can be considered the most common form of chronic liver disease in obese patients. Because there is no proven treatment for NAFLD, the identification of important risk factors for disease progression can be expected to provide valuable information in regard to both risk stratification and development of risk-reduction strategies.[38]

CRP is a major acute-phase protein and a marker of systemic inflammation. Recently, elevated serum hs-CRP was reported to be a strong predictor of future cardiovascular events.[8-10] It has been suggested that insulin resistance syndrome may be associated with increased serum hs-CRP levels and visceral obesity;[11,12] therefore hs-CRP is a very important parameter to consider in cases of metabolic syndrome.[8,9,21,39] Recently, some reports have also suggested that hs-CRP may be an independent risk factor for disease progression in cases of NAFLD.[16,18,40] However, reliable interpretation of the results of these studies is limited because they did not use a gold standard measure, such as liver biopsy, for the evaluation of NAFLD. Although serum markers of liver damage, including ALT and AST, may be reasonable noninvasive surrogate markers in epidemiological studies,[41,42] some nondifferential misclassification of NAFLD based on transaminase levels is likely, and the repeatability of elevated ALT is poor.[43] Thus, there are no established noninvasive methods for the evaluation of patients with NASH. Haukeland et al.,[44] who investigated several inflammation markers, including serum hs-CRP, in 45 biopsy-proven NAFLD patients,[44] were able to demonstrate differences in the levels of inflammation markers such as CC-chemokine ligand 2/monocyte chemoattractant protein-1 between NASH patients and simple steatosis patients. Regrettably, however, they could not demonstrate any significant differences in serum hs-CRP levels between the NASH patients and the simple steatosis patients.

We were therefore prompted to investigate potential interrelationships between serum hs-CRP and the progression to NASH of cases of NAFLD. We excluded patients with a history of treatment with aspirin or statins, because these agents may reduce vascular risk among patients with elevated serum hs-CRP levels,[21-23] as well as patients treated with pioglitazone and metformin, which also may influence NASH.[24,25] Our results demonstrated that serum hs-CRP can be used to reliably distinguish patients with NASH from those with simple steatosis even after adjustment for age, sex, presence of diabetes, BMI, VFA, SFA, insulin resistance, and serum levels of HDL cholesterol, triglyceride, and LDL cholesterol, all of which may be associated with elevation of the serum hs-CRP level. Furthermore, we found that intrahepatic CRP mRNA expression was significantly higher in NASH patients than in those with simple steatosis. Further detailed study revealed that serum hs-CRP could significantly distinguish between cases of NASH with mild fibrosis and those with advanced fibrosis, even after adjustment for age, sex, presence of diabetes, BMI, VFA, SFA, insulin resistance, and serum levels of HDL cholesterol, triglyceride, and LDL cholesterol.

In this study, we confirmed that serum hs-CRP levels are higher in patients with NASH than in those with the more benign form of NAFLD, namely, simple steatosis. Also, higher serum hs-CRP levels were associated with a higher grade of hepatic fibrosis in the NASH patients. Thus, in this cohort, we showed that the baseline serum hs-CRP level was a strong clinical feature of not only disease progression to NASH but also the severity of liver fibrosis in NASH patients.

While the reasons for the higher serum hs-CRP levels observed in NASH patients, especially those with advanced fibrosis, are still not clear, several mechanisms

have been proposed. One proposed mechanism is elevation of circulating levels of IL-6, which also induces the production of CRP.[45] However, Haukeland et al.[44] reported that both patients with simple steatosis and those with NASH had significantly raised serum levels of IL-6 compared with healthy controls, and they found no significant difference between the two groups. Therefore, to investigate this possible mechanism further, we determined intrahepatic IL-6 mRNA and CRP mRNA expression. The results revealed that while CRP mRNA expression in the liver was significantly elevated in the NASH patients compared with in the simple steatosis patients, intrahepatic IL-6 mRNA expression was only slightly increased in the NASH patients, but was not significantly different from the expression in the simple steatosis patients. Further study is therefore required to investigate the possible involvement of this mechanism.

Measurement of serum hs-CRP is likely to be useful for targeted therapies in the primary prevention of cardiovascular disease. Similarly, hs-CRP is also useful for targeted therapy against fibrosis in NASH patients, because in our study, the serum hs-CRP level was also found to be significantly elevated in the NASH patients with advanced hepatic fibrosis compared with in those with mild fibrosis. At the same time, weight reduction and exercise, the first-line therapies stressed by ATP-III (Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults) for management of metabolic syndrome, may also reduce the serum hs-CRP levels.[46] Furthermore, a recent report has suggested that rosiglitazone directly reduces serum hs-CRP levels, an intriguing observation, since this PPARγ inhibitor has already been used in the therapy of NASH patients.[24]

A scoring system combining such noninvasive markers with age and other clinical features may permit the reliable differentiation of NASH from simple steatosis, and hence the selection of patients with a higher probability of significant pathology on liver biopsy.

Finally, our result is highly significant clinically, because hs-CRP determination is now available as a routine clinical laboratory test.

In conclusion, this is the first report to demonstrate a consistent and profound elevation of serum hs-CRP in NASH patients compared with in patients with simple steatosis, and also marked elevation of serum CRP in NASH patients with severe fibrosis compared with mild fibrosis, based on results of liver biopsy, the gold standard for evaluation of NASH. These results were also supported by the results of measurements of intrahepatic CRP mRNA expression. Serum hs-CRP measurement is noninvasive and appears to hold promise for identifying patients with NASH from simple steatosis patients among subjects with NAFLD, and also as a clinical feature for determining the severity of liver fibrosis in NASH patients. Further studies must be conducted to explore the prognostic value of serum hs-CRP to determine the long-term outcome of patients with NAFLD.

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Type IV collagen 7s domain is an independent clinical marker of the severity of fibrosis in patients with nonalcoholic steatohepatitis before the cirrhotic stage

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Background. The changes in nonalcoholic fatty liver disease range over a wide spectrum, extending from simple steatosis to nonalcoholic steatohepatitis (NASH). We investigated the clinical usefulness of the type IV collagen 7s domain and hyaluronic acid for predicting the severity of fibrosis before progression to the cirrhotic stage in NASH patients. ***Methods.*** The type IV collagen 7s domain and hyaluronic acid were measured in 72 patients with histologically verified NASH. ***Results.*** In a univariate analysis, marked elevation of hyaluronic acid and the type IV collagen 7s domain was observed in the NASH patients with advanced fibrosis compared with those with mild fibrosis ($P = 0.0028$, $P = 0.0006$, respectively). For detection of NASH with advanced fibrosis, the area under the receiver-operating characteristic curves for type IV collagen 7s domain and hyaluronic acid were 0.767 and 0.754, respectively. However, multiple regression analysis revealed that the type IV collagen 7s domain, but not hyaluronic acid, was significantly elevated in patients with advanced fibrosis even after adjustment for age, sex, platelet count, prothrombin time, aspartate aminotransferase/alanine aminotransferase ratio, body mass index, and presence of underlying type 2 diabetes mellitus, all of which have previously been reported as useful predictors of advanced fibrosis in patients with NASH ($P = 0.0127$, $P = 0.2804$, respectively). ***Conclusions.*** This is the first report to demonstrate a consistent and profound elevation of the type IV collagen 7s domain in NASH patients with advanced fibrosis (before progression to the stage of cirrhosis) compared with those with mild fibrosis.

Key words: NASH, type IV collagen 7s domain, liver fibrosis, biomarker

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver injury in many countries around the world.[1,2] NAFLD represents a spectrum of conditions that are histologically characterized by macrovesicular hepatic steatosis, and the diagnosis is made in patients who have not consumed alcohol in amounts sufficient to be considered to be harmful to the liver. The histological changes range over a wide spectrum, extending from simple steatosis, which is generally nonprogressive, to nonalcoholic steatohepatitis (NASH), liver cirrhosis, and liver failure, and sometimes even hepatocellular carcinoma.[3,4] Liver biopsy is recommended as the gold standard method for the diagnosis as well as staging of fibrosis in patients with NASH.[1,4,5] This procedure, however, is invasive and associated with a risk of complications.[6,7] Several clinical studies have attempted to identify serum markers that might be correlated with the degree of fibrosis in these patients. To date, many clinical variables have been proposed as predictors of severe fibrosis in patients with NAFLD, including old age, underlying type 2 diabetes mellitus (DM), obesity, serum transaminase levels, and platelet count.[8–10] However, NASH patients in the cirrhotic stage, which is generally recognized as cryptogenic cirrhosis, were included in all of these studies. Cryptogenic cirrhosis, which is of unknown origin and is diagnosed by a process of elimination of a history of excessive alcohol consumption and other evidence of liver diseases, is mainly caused by NASH. Clinically, one of the most important the goals of therapy in NASH patients is the prevention of progression to the stage of cirrhosis. Therefore, it is important to make the diagnosis of NASH at least by the stage of advanced fibrosis, before progression to the cirrhotic stage.

Assessment of the degree of liver fibrosis is important in the evaluation of the prognosis of NAFLD patients. One noninvasive approach that could be employed is

the measurement of substances that regulate fibrosis or participate in the generation of the liver extracellular matrix. The most suitable candidate substances include hyaluronic acid, which is a high-molecular-weight glycosaminoglycan and an essential component of the extracellular matrix in virtually every tissue in the body,[11] and type IV collagen. Recently, the type IV collagen 7s domain (7s collagen), which is involved in connective tissue metabolism, has been identified as a biochemical marker for assessing fibrogenesis and the severity of fibrosis in patients with cirrhosis.[12–14] The serum levels of 7s collagen and hyaluronic acid have been reported to be significantly different between NASH patients with severe fibrosis and those with mild to moderate fibrosis in a few recent studies.[8–10,15] However in these studies, the percentage of patients with liver cirrhosis ranged from 8%–21%. Thus, the clinical usefulness of measurement of the serum levels of 7s collagen and hyaluronic acid before progression to the cirrhotic stage in NASH patients has not yet been investigated.

We, therefore, investigated the potential clinical usefulness of the serum levels of the 7s collagen and hyaluronic acid to predict the severity of fibrosis in patients with NASH before their progression to the cirrhotic stage.

Patients and methods

Patients

We prospectively evaluated 72 NASH patients who underwent liver biopsy at Yokohama City University Hospital between April 2004 and October 2006. The study was conducted with the approval of the Ethics Committee of Yokohama City University Hospital.

A detailed history was obtained from all 72 patients, and each was given a thorough physical examination. The exclusion criteria from the study included patients with cryptogenic cirrhosis, simple steatosis, history of hepatic disease such as chronic hepatitis C or concurrent active hepatitis B (serum positive for hepatitis B surface antigen), autoimmune hepatitis, primary biliary cirrhosis (PBC), sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's disease, or hepatic injury caused by substance abuse, or current or past history of consumption of more than 20 g of alcohol daily. None of the patients had any clinical evidence of hepatic decompensation, such as hepatic encephalopathy, ascites, variceal bleeding, or elevation of the serum bilirubin level to more than twice the upper limit of normal.

Clinical and laboratory evaluation

The weight and height of the patients were measured with a calibrated scale after they had removed their shoes and any heavy items of clothing. Venous blood samples were obtained after the patients had fasted overnight (12 h) for measurement of the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, insulin, 7s collagen, and hyaluronic acid. 7s collagen was measured with a type IV collagen 7s radioimmunoassay kit (Mitsubishi Chemical Group, Tokyo, Japan), and serum hyaluronic acid was measured by latex agglutination immunoassay (Mitsubishi Chemical Group). The serum insulin levels were measured by radioimmunoassay, while the other laboratory biochemical parameters were measured in a conventional automated analyzer.

Insulin resistance was calculated by the modified homeostasis model assessment of insulin resistance (HOMA-IR), using the following formula: HOMA-IR = fasting insulin (μU/ml) × plasma glucose (mg/dl)/405. HOMA-IR was originally reported by Matthews and has since been modified.[16,17] This index has been shown to be well-correlated with the results of the euglycemic–hyperinsulinemic clamp method to determine the levels of insulin resistance in type 2 DM patients. HOMA-IR has been reported to be a suitable method for evaluating the presence of insulin resistance in patients with type 2 diabetes only when the fasting glucose levels are <170 mg/dl.[18] Ono et al.[18] reported that the results of HOMA-IR were not significantly correlated with insulin resistance in subjects with fasting glucose levels >200 mg/dl. Therefore, type 2 DM patients with poor glycemic control (fasting plasma glucose >170 mg/dl) were excluded from the HOMA-IR measurement.

Determination of the visceral and subcutaneous fat areas

The abdominal fat distribution in the study subjects was determined by computed tomography (CT), conducted with the subjects in supine position in accordance with a previously described procedure.[19] The subcutaneous fat area and intra-abdominal visceral fat area were measured at the level of the umbilicus in terms of the CT number, using a standardized method. In brief, a region of interest was defined in the subcutaneous fat layer by tracing its contour on each scan, and the attenuation range for fat tissue in this area was measured in terms of the CT number (in Hounsfield units).

Pathology

Liver biopsies were obtained with an 18-gauge needle biopsy apparatus (Pro-Mag, Medical Device Technolo-

gies, Gainesville, FL, USA) with a minimum of five portal tracts and a minimum length of 15 mm. The number of biopsy specimen fragments was one or two. The liver biopsy specimens were stained with hematoxylin-eosin, reticulin, and Masson trichrome stains, and the histopathological findings were scored by two pathologists. Macrovesicular steatosis affecting at least 5% of the hepatocytes was observed in all patients, who were classified as having steatosis or steatohepatitis depending on the additional findings, as follows. In addition to steatosis, the minimum criteria for the diagnosis of steatohepatitis included the presence of lobular inflammation and either ballooning of the cells or perisinusoidal/pericellular fibrosis in zone 3 of the hepatic acini.[20–22] In all cases, the severity of fibrosis was scored according to the method of Brunt.[5] The degree of steatosis was assessed based on the percentage of hepatocytes containing macrovesicular fat droplets, as follows: grade 0, no steatosis; grade 1, <33% of hepatocytes containing macrovesicular fat droplets; grade 2, 33%–66% hepatocytes containing macrovesicular fat droplets; and grade 3, >66% hepatocytes containing macrovesicular fat droplets. The severity of fibrosis was expressed on a 4 point scale, as follows: 0 = none; 1 = perivenular and/or perisinusoidal fibrosis in zone 3; 2 = combined pericellular portal fibrosis; 3 = septal/bridging fibrosis; and 4 = cirrhosis. On the basis of this classification, the subjects were grouped into two categories, namely, those with mild fibrosis (stages 0–2) and those with advanced fibrosis, before progression to the stage of cirrhosis (stage 3).

Statistical analysis

Data are expressed as means ± SD, unless indicated otherwise. Statistical analysis was conducted using SPSS 12.0 (SPSS, Chicago, IL, USA). For univariate comparisons between the patient groups, the *t* test or Mann-Whitney's *U* test was used, as appropriate. Because variables were often not normally distributed, comparisons of more than two independent groups were performed by the Kruskal-Wallis test. The diagnostic performance of 7s collagen and hyaluronic acid were assessed by analysis of receiver-operating characteristic (ROC) curves. The ROC curve is a plot of sensitivity versus (1 – specificity) for all possible cutoff values. The most commonly used index of accuracy is the area under the ROC curve, with values close to 1.0 indicating a high diagnostic accuracy. A patient was assessed as being positive or negative according to whether the noninvasive marker value was greater than, less than, or equal to a given cutoff value. Determination of correlation coefficients and linear regression analysis were conducted to test for associations between the variables. *P* values of <0.05 were considered to be significant.

Table 1. Histopathological findings in subjects with nonalcoholic steatohepatitis

	Nonalcoholic steatohepatitis (n = 72) No. (%)
Steatosis grade	
1	43 (59.7)
2	25 (34.7)
3	4 (5.6)
Fibrosis Stage	
0	6 (6.9)
1	27 (37.5)
2	21 (29.2)
3	18 (25)

Results

Characteristics of the patients

The histological findings in the liver biopsy specimens of the NASH patients with mild fibrosis (stage 0–2) (n = 54) and those with advanced fibrosis, before progression to the stage of cirrhosis (stage 3; n = 18) are shown in Table 1. The clinical and biochemical characteristics of the NASH patients with mild fibrosis and advanced fibrosis are shown in Table 2. Serum levels of hyaluronic acid and 7s collagen were markedly elevated in the NASH patients with advanced fibrosis compared with those with mild fibrosis; these differences between the two groups were strongly significant (P = 0.0028, P = 0.0006, respectively).

Hepatic fibrosis and serum levels of hyaluronic acid and 7s collagen

Since the serum levels of hyaluronic acid and 7s collagen were significantly elevated in NASH patients with advanced fibrosis compared with those with mild fibrosis, we further investigated the relationship between the histological severity of fibrosis and the serum levels of hyaluronic acid and 7s collagen in the NASH patients. The results of this analysis revealed stepwise increases in the serum levels of both hyaluronic acid and 7s collagen with increasing histological severity of hepatic fibrosis (P = 0.0142, P = 0.0059, respectively, by Kruskal-Wallis test) (Figs. 1 and 2). For detection of NASH with advanced fibrosis, the area under the curves for 7s collagen and hyaluronic acid were 0.767 and 0.754, respectively, by ROC analysis (Figs. 3 and 4). The best cutoff values to detect NASH with advanced fibrosis (stage 3) were also assessed using the ROC analysis, and sensitivity, specificity, positive predictive value, and negative predictive value were calculated (Table 3).

Table 2. Clinical and biochemical characteristics of patients with mild and advanced fibrosis

	Standard value	Mild fibrosis[a]	Advanced fibrosis[b]	*P* value
Age (years)		49.5 ± 13.4	55.6 ± 12.2	0.1028
BMI (kg/m²)		27.8 ± 5.3	29.6 ± 3.5	0.2096
VFA (cm²)		128.9 ± 53.4	148.1 ± 36.5	0.2575
SFA (cm²)		222.8 ± 108.5	276.1 ± 104.1	0.1386
AST (U/ml)	(14–32)	50.4 ± 36.8	61.9 ± 27.1	0.2492
ALT (U/ml)	(11–45)	79.8 ± 60.8	90.9 ± 56.0	0.5102
AST/ALT		0.70 ± 0.22	0.77 ± 0.23	0.3132
FBS (mg/dl)	(87–115)	123.5 ± 36.9	124.8 ± 34.6	0.9064
IRI (μl/ml)	(3.0–15.0)	13.2 ± 11.0	15.7 ± 7.7	0.4436
HOMA-IR		3.85 ± 4.00	4.52 ± 2.15	0.5623
Platelet count (×10⁴/μl)	(18–39)	24.5 ± 6.72	22.2 ± 8.10	0.2490
Albumin (g/dl)	(4.1–5.1)	4.56 ± 0.30	4.38 ± 0.36	0.0523
Prothrombin time (INR)	(0.87–1.15)	1.02 ± 0.06	1.06 ± 0.08	0.0509
Hyaluronic acid (ng/dl)	(≤50)	36.6 ± 36.3	77.3 ± 66.8	0.0028
Type IV collagen 7s (ng/dl)	(≤6)	4.29 ± 0.88	5.46 ± 1.58	0.0006

Data are expressed as means ± SD
BMI, body mass index; VFA, visceral fat area; SFA, subcutaneous fat area; AST, aspartate aminotransferase; ALT, alanine aminotransferase; FBS, fasting blood sugar; IRI, immunoreactive insulin; HOMA-IR, homeostasis model assessment of insulin resistance; INR, International Normalized Ratio
[a] Stages 0–2
[b] Stage 3

Table 3. Diagnostic accuracy of markers of NASH with advanced fibrosis

	Cutoff Value	Se (%)	Sp (%)	PPV (%)	NPV (%)
Type IV collagen 7s domain	4.25 (ng/dl)	88.9	59.7	42.4	94.2
Hyaluronic acid	32.5 (ng/dl)	77.8	65.8	42.7	87.8

NASH, nonalcoholic steatohepatitis; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value

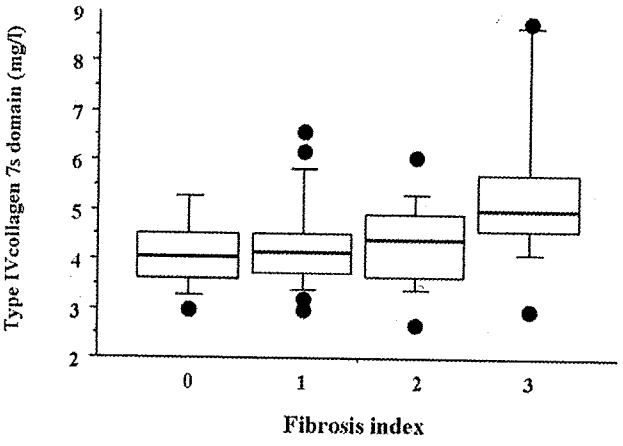

Fig. 1. Box plots showing the interquartile range (*box*), median (*thick line*), range (*thin lines*), and outliers (*circles*) of the serum levels of the type IV collagen 7s domain (7s collagen). A steady stepwise increase in the serum level of 7s collagen was observed with increasing severity of hepatic fibrosis ($P = 0.0142$)

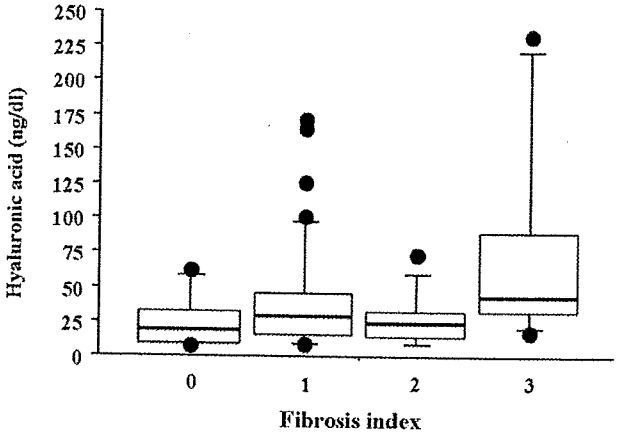

Fig. 2. Box plots showing the interquartile range (*box*), median (*thick line*), range (*thin lines*), and outliers (*circles*) of the serum levels of hyaluronic acid. A steady stepwise increase of the serum level of hyaluronic acid was observed with increasing severity of hepatic fibrosis ($P = 0.0006$)

Influence of demographic factors on serum levels of hyaluronic acid and 7s collagen

To determine if the observed differences in the serum levels of hyaluronic acid and 7s collagen between NASH patients with advanced and those with mild hepatic fibrosis were independent of other previously reported clinical variables, including age, sex, platelet count, prothrombin time (PT), AST/ALT ratio, body mass index (BMI), and presence of underlying type 2 DM, the ex-

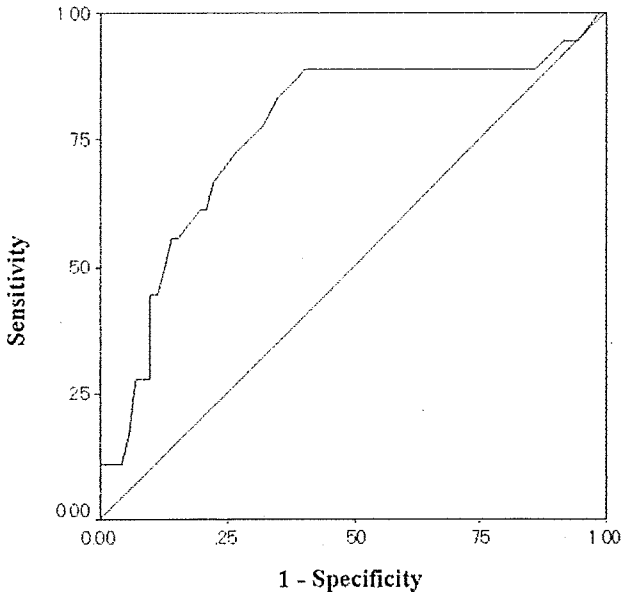

Fig. 3. Receiver-operating characteristic (ROC) curves for differentiating nonalcoholic steatohepatitis (NASH) with mild fibrosis from NASH with advanced fibrosis (stage 3) using 7s collagen

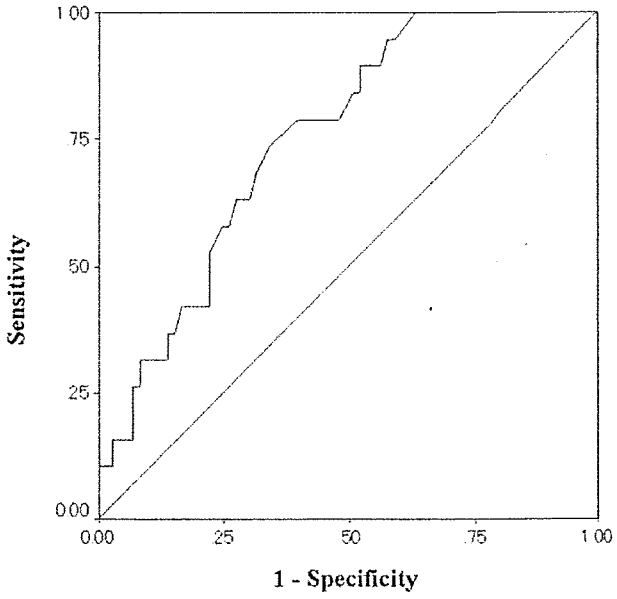

Fig. 4. ROC curves for differentiating NASH with mild fibrosis from NASH with advanced fibrosis (stage 3) using hyaluronic acid

Table 4. Multiple logistic regression analysis of factors associated with advanced fibrosis before progression to the cirrhotic stage

Factor	Odds ratio	95% CI	*P* value
Age (years)	0.936	0.851–1.031	0.1786
Sex	0.730	0.084–6.351	0.7752
Type IV collagen 7s domain (mg/l)	0.283	0.105–0.764	0.0127
Type 2 DM	0.093	0.009–0.548	0.0114
Prothrombin time	0.084	4.290×10^{-8}–2.032×10^{5}	0.7501
Platelet count	1.133	0.958–1.340	0.1456
AST/ALT	2.361	0.006–966.472	0.7795
BMI	0.877	0.700–1.100	0.2558

Intercept = –30.862; $R^2 = 0.401$ (for entire model)
CI, confidence interval; DM, diabetes mellitus

tent of these differences was quantified by multiple logistic regression analyses.

The results of the multivariate analysis indicated that the serum level of 7s collagen and the presence of underlying type 2 DM were significantly and independently associated with the presence of advanced hepatic fibrosis in the NASH patients (Table 4). The odds ratio of elevated serum levels of 7s collagen for advanced hepatic fibrosis was 0.283 (95% confidence interval, 0.105–0.764; $P = 0.0127$). On the other hand, the serum level of hyaluronic acid was not an independent predictor of advanced hepatic fibrosis in the NASH patients (Table 5). These results indicate that the serum level of 7s collagen is an independent predictor of the severity of hepatic fibrosis, before progression to the stage of cirrhosis in patients with NASH.

Discussion

In the present study, we demonstrated that the serum concentrations of 7s collagen were markedly elevated in NASH patients with advanced hepatic fibrosis compared with patients with mild hepatic fibrosis, even after adjustment for the age, sex, platelet count, PT, AST/ALT ratio, BMI, and presence of underlying type 2 DM, all of which have previously been reported as useful predictors of advanced fibrosis in patients of NASH. To our surprise, however, the serum level of hyaluronic acid was found by multivariate analysis to not be an independent predictor of the severity of hepatic fibrosis after adjustment for the aforementioned factors, even though strong significance by univariate analysis and a satisfactorily high area under the ROC curve for distin-

Table 5. Multiple logistic regression analysis of factors associated with advanced before progression to the cirrhotic stage

Factor	Odds ratio	95% CI	*P* value
Age (years)	0.984	0.891–1.088	0.7575
Sex	0.431	0.054–3.412	0.4250
Hyaluronic acid (ng/l)	0.987	0.964–1.011	0.2804
Type 2 DM	0.080	0.012–0.515	0.0079
Prothrombin time	0.007	6.121×10^{-8}–823.372	0.4056
Platelet count	1.032	0.892–1.195	0.6697
AST/ALT	0.136	0.002–9.211	0.3534
BMI	0.953	0.791–1.147	0.6075

Intercept = –31.113; $R^2 = 0.269$ (for entire model)

guishing between mild hepatic fibrosis and advanced hepatic fibrosis had been found.

NAFLD, which can progress to cirrhosis, may be the most common form of chronic liver disease in obese patients. As there is no proven treatment available yet for NAFLD, identification of the important risk factors for disease progression in these patients could be valuable for both risk stratification and the development of risk-reduction strategies for these patients.[23] Liver biopsy is considered the gold standard for the diagnosis and staging of NASH.[24,25] Discrimination between NASH and simple steatosis is difficult by imaging examinations alone,[26,27] and there are currently no established noninvasive methods for the evaluation of patients with NASH.

Determination of the severity of liver fibrosis is important in the evaluation of the prognosis of patients with NASH. Some of the noninvasive approaches developed to assess the severity of the histological changes in other liver diseases include assessment of clinical symptoms, routine laboratory tests, and radiographic imaging.[13,26–32] The usefulness of markers of liver fibrosis, such as the serum levels of 7s collagen and hyaluronic acid, has been reported for patients with chronic viral hepatitis.[13,28,29] On the other hand, only a few studies have investigated the clinical usefulness of serum levels of 7s collagen[15] and hyaluronic acid[8–10] to predict the severity of fibrosis in patients with NAFLD. In this context, NASH patients who have progressed to the cirrhotic stage are considered to have end-stage liver disease, which can be easily diagnosed by imaging examinations, such as ultrasonography, CT, or magnetic resonance imaging. It is not uncommon for patients to present with the complications of previously unrecognized cirrhosis despite being under long-standing medical care, because they often do not manifest the classic physical changes associated with cirrhosis. Clinically, the diagnosis of NASH in the stage of cirrhosis is considered to be too late; the diagnosis should be made at least at the stage of advanced fibrosis before progression to the stage of cirrhosis. All previous clinical studies conducted to investigate the predictive factors of advanced fibrosis in patients with NASH have included cases of cryptogenic cirrhosis; therefore, studies that exclude patients with cryptogenic cirrhosis have been needed. We, therefore, investigated the potential clinical usefulness of measurement of serum levels of hyaluronic acid and 7s collagen to detect NASH patients with advanced fibrosis, before progression to the stage of cirrhosis.

In this study, our results demonstrated that measurement of the serum levels of 7s collagen is very useful for predicting the severity of hepatic fibrosis in patients with NASH. On the other hand, the results of our multivariate analysis revealed that the serum level of hyaluronic acid is not an independent predictor of the severity of hepatic fibrosis in patients with NASH, even though the results of our univariate analysis revealed strong significance of this parameter. Therefore, monitoring of the serum level of 7s collagen is more useful for the development of targeted therapy against fibrosis in NASH patients than monitoring of hyaluronic acid.

A scoring system combining such noninvasive markers with age and other clinical features may permit reliable differentiation of NASH patients with advanced fibrosis from those with mild fibrosis, and hence, the selection of patients with a higher probability of significant pathology on liver biopsy.

In conclusion, this is the first report to demonstrate a consistent and profound elevation of the serum levels of 7s collagen in NASH patients with advanced fibrosis (before progression to the stage of cirrhosis) compared with patients with mild fibrosis, as determined by liver biopsy, the gold standard for evaluation of the severity of liver pathology in patients with NASH. Thus, measurement of the serum levels of 7s collagen is a noninvasive, clinically useful method for predicting the severity of liver fibrosis in patients with NASH. Further studies must be conducted to explore the prognostic value of the serum levels of 7s collagen to determine the long-term outcome of patients with NAFLD.

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FOXP3$^+$ Regulatory T Cells Affect the Development and Progression of Hepatocarcinogenesis

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Abstract

Purpose: Tumor-infiltrating lymphocytes represent the host immune response to cancer. CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells (Tregs) suppress the immune reaction. The aim of the present study was to investigate the clinicopathologic significance and roles of Tregs and CD8$^+$ T cells during hepatocarcinogenesis.

Experimental Design: We examined the infiltration of FOXP3$^+$ Tregs and CD8$^+$ T cells in the tumor stroma and nontumorous liver parenchyma using 323 hepatic nodules including precursor lesions, early hepatocellular carcinoma (HCC), and advanced HCC, along with 39 intrahepatic cholangiocarcinomas and 59 metastatic liver adenocarcinomas. We did immunohistochemical comparative studies.

Results: The prevalence of Tregs was significantly higher in HCC than in the nontumorous liver ($P < 0.001$). The patient group with a high prevalence of Tregs infiltrating HCC showed a significantly lower survival rate ($P = 0.007$). Multivariate analysis revealed that the prevalence of Tregs infiltrating HCC was an independent prognostic factor. The prevalence of Tregs increased in a stepwise manner ($P < 0.001$) and that of CD8$^+$ T cells decreased during the progression of hepatocarcinogenesis ($P < 0.001$). Regardless of the presence of hepatitis virus infection or histopathologic evidence of hepatitis, the prevalence of Tregs was significantly increased in nontumorous liver bearing primary hepatic tumors.

Conclusions: Tregs play a role in controlling the immune response to HCC during the progression of hepatocarcinogenesis. It has been suggested that primary hepatic cancers develop in liver that is immunosuppressed by a marked infiltration of Tregs. A high prevalence of Tregs infiltrating HCC is thought to be an unfavorable prognostic indicator.

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, representing the third most common cause of mortality among deaths from cancer (1). Even with remarkable advances in diagnostic and therapeutic techniques, the incidence of HCC is still on the increase. Hepatitis virus B (HBV) and hepatitis virus C (HCV) are known to be major risk factors, and chronic infection with these viruses is responsible for ~80% of HCCs in humans (2). Most of the HCCs occur in damaged liver (chronic hepatitis or liver cirrhosis), even if the liver is not infected with HBV or HCV (3). HCC is also characterized by an obvious multistage process of tumor progression (4–7), from a regenerative nodule to adenomatous hyperplasia (AH), and thereafter to atypical adenomatous hyperplasia (AAH), early HCC (defined as *in situ* or microinvasive cancer), and advanced HCC. It is important to detect cancers at an early stage, including their precursor lesions, and to assess their risk in order to provide appropriate treatment and reduce cancer-related mortality.

Previous studies have investigated the changes in morphology, genetics, and molecular biology of epithelial cells during tumorigenesis. Recently, many studies have suggested that the tumor microenvironment also plays an important role in the establishment and progression of tumors. Lymphocytes contribute to the tumor microenvironment through immunity and inflammation. CD8$^+$ CTLs can directly kill target cells by releasing granules including membrane-lytic materials such as perforin and granzymes in acquired immune responses, thereby playing a central role in antitumor immunity. Indeed, a high frequency of CD8$^+$ T cells infiltrating cancer tissue can be a favorable prognostic indicator in ovarian cancer (8) and colorectal cancer (9). In HCC, extremely marked infiltration of T cells including predominant CD8$^+$ T cells has been shown to be closely associated with a low recurrence rate and good prognosis (10). On the other hand, another study using a mouse model has

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shown that marked infiltration of $CD8^+$ T cells exacerbates liver damage, thus accelerating the development of HCC (11).

In contrast to $CD8^+$ CTL, which generally exert a suppressive influence on tumor growth, regulatory T cells (Tregs) are thought to have a positive effect on tumor growth through suppression of antitumor immune cells. $CD4^+CD25^+$ Tregs are a minor but functionally unique population of T cells, which maintain immune homeostasis in immune tolerance and the control of autoimmunity. Tregs can inhibit immune responses mediated by $CD4^+CD25^-$ and $CD8^+$ T cells *in vitro* by a contact-dependent and cytokine-independent mechanism (12–14), although more recent reports suggest that the immune suppression mechanisms of Tregs *in vivo* are more complex (15, 16). Forkhead or winged helix family of transcription factor P3 (FOXP3) is critical for the development and function of Tregs in mice and humans (16, 17), and is still the only marker for evaluating real Tregs that have a suppressive function. In murine models, it has been described that Tregs inhibit the antitumor immune response (15, 18–20). Involvement of $CD4^+CD25^+$ Tregs in human cancer has been observed in peripheral blood and tumor tissues from patients with several types of cancer (21–25). A few groups have reported that Tregs are increased in peripheral blood and among tumor-infiltrating lymphocytes of patients with HCC (26–28), although these were not large-scale studies and did not estimate the clinicopathologic significance of Tregs infiltrating HCC, including their prognostic value. Early studies detected Tregs not as $FOXP3^+$ T cells but as $CD4^+CD25^+$ T cells, although recent studies have revealed that $CD4^+CD25^+$ T cells consist of Tregs and activated effector T cells, the latter being increased in inflammatory lesions (29). Furthermore, no previous study has investigated host immune responses in multistage hepatocarcinogenesis.

In the present study, we first investigated the clinicopathologic values of both $FOXP3^+$ Tregs and $CD8^+$ T cells infiltrating the tumor stroma of HCC, and then examined the prevalence of $FOXP3^+$ Tregs and $CD8^+$ T cells during multistage hepatocarcinogenesis. Precursor lesions of HCC are small nodular lesions that can be detected and evaluated only by microscopic analysis, making it difficult to extract living immune cells from them and to analyze their immunophenotypes and immune functions. Therefore, we selected an immunohistochemical comparative approach for evaluating host immune responses in these HCC precursor lesions. This approach was used in the other experiments as well. We also investigated whether Tregs are involved in the development of HCC, and compared the host immune responses by measuring and comparing the infiltration of Tregs and $CD8^+$ T cells between HCC and primary hepatic adenocarcinoma, intrahepatic cholangiocarcinoma (ICC) as well as between primary and metastatic liver tumors. We compared the prevalence of Tregs in nontumorous liver parenchyma among patients with and without primary hepatic tumors, and those with and without hepatitis viral infection. The results showed that the prevalence of Tregs increases during the progression of established cancers as well as that of their precursor lesions. Furthermore, the prevalence of Tregs was significantly correlated with patient survival, independent of other prognostic factors.

Materials and Methods

Patients and samples. This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan. Clinical and pathologic data and the specimens used for immunohistochemical analysis were obtained through a detailed retrospective review of the medical records of 218 patients with 323 hepatic nodules of HCC or its precursor lesions who had undergone initial surgical resection between 1992 and 2000 at the National Cancer Center Hospital, Tokyo, Japan. None of the nodules had been treated previously with techniques such as radiofrequency ablation, percutaneous ethanol injection therapy, or transcatheter arterial embolization or injection, and none of the patients with nodules had received systemic chemotherapy. Sixty-five patients had hepatic cancers that had been treated by surgical resection, radiofrequency ablation, percutaneous ethanol injection therapy, or transcatheter arterial embolization or injection; the current nodules were also located in different lobes, as well as distant from, the previous cancers. In another six patients, curative resection was not done. The remaining 147 patients were studied in order to evaluate the clinicopathologic correlation of the prevalence of $FOXP3^+$ Tregs and $CD8^+$ T cells with specific variables. Tumors were classified according to the WHO classification (30) and the International Union against Cancer tumor-node-metastasis (TNM) classification (31). If patients had multiple nodules in the liver, we selected the nodule showing the most advanced histologic grade for our study. If a tumor had different grades of histology, the grade of the tumor was regarded as the most advanced one among them. Nontumorous liver was classified histopathologically into four categories: non-chronic hepatitis (NCH), chronic hepatitis (CH), chronic hepatitis with cirrhotic change (pre-cirrhotic stage; PC), and liver cirrhosis (LC), which corresponded to 0, 1-3, 4-5, and 6 of the fibrosis stages of the modified histological activity index system (32). There were 5 patients with HBV infection and 15 patients without HBV or HCV infection in NCH, which included liver with fatty changes and/or slight inflammatory infiltrates in the portal area. All patients had complete medical records and had been followed by the tumor registries for survival and outcome. Follow-up was available in all cases and ranged from 0.5 to 169.1 months (mean, 52.8 months). The latest survival data were collected on April 30, 2006. The overall survival rate at 5 years and the disease-free survival rate were 39.5% and 18.4%, respectively. The clinicopathologic features of the patients are summarized in Table 1.

We also investigated 39 patients with ICC and 59 patients with metastatic liver tumors from primary colorectal cancer who had undergone initial surgical resection between 1991 and 2005 at the National Cancer Center Hospital. The patients with ICC or metastatic liver cancer without hepatitis viral infection were randomly selected and those with hepatitis viral infection were all the patients we had. The patients with ICC comprised 22 males and 17 females, and their median age at surgery was 63 years (range, 44-85 years). HBV and HCV infection were detected in four and five patients, respectively. Their livers were diagnosed histopathologically as CH in eight patients and as PC in one patient. NCH were found in the liver of 30 patients without any HBV or HCV infection. Tumor diameters ranged from 15 to 140 mm (mean, 64.6 $\pm$ 30.6 mm). There were 8 patients at stage I, 9 patients at stage II, 3 patients at stage IIIa, 7 patients at stage IIIb, and 12 patients at stage IIIc according to the International Union against Cancer staging classification (31). ICCs were classified histopathologically as well-differentiated adenocarcinoma in 7 cases, moderately differentiated adenocarcinoma in 27, and poorly differentiated adenocarcinoma in 5 according to the WHO classification (30). The patients with liver metastasis from colorectal cancer comprised 37 males and 22 females, and their median age at surgery was 62 years (range, 34-81 years). HBV and HCV virus infection were detected in 8 and 21 patients, respectively, and their livers were diagnosed histopathologically as CH in 18 and as NCH in 11. The other 30 patients had not been infected with HBV or HCV and their nontumorous liver showed no inflammatory or fatty changes. Therefore, the nontumorous liver tissue from these patients was defined as "healthy liver." Thirty-three patients had a solitary tumor and 26 had multiple tumors. Tumor diameters ranged from 12 to 150 mm

Table 1. Clinicopathologic features of the patients

Variables	Results
Characteristics of the patients with HCC (218 cases)	
Age, y (median, range)	62, 17-84
Gender (male/female)	170/48
Virus infection [HBV/HCV/HBV+HCV/(−)]	57/117/10/34
Nontumor liver (NCH/CH/PC/LC)	20/101/35/62
Tumor nodules (AH/AAH/early HCC/WD HCC/MD HCC/PD HCC)	11/9/68/58/123/54
Clinicopathologic findings of the patients with HCC (147 cases)	
Age, y (median, range)	62, 17-83
Gender (male/female)	113/34
Virus infection [HBV/HCV/HBV+HCV/(−)]	47/79/9/12
Nontumor liver (NCH/CH/PC/LC)	17/71/23/36
Child-Pugh classification (A/B/C)	136/11/0
TNM stage (I/II/III/IV)	57/53/37/0
Histologic grade (early HCC/WD HCC/MD HCC/PD HCC)	17/15/77/38
AFP, ng/mL (median, range)	27.1, 1-27,170
VP (presence/absence)	57/90
IM (presence/absence)	33/114
Tumor size, mm (median, range)	35, 6-185

Abbreviations: MD, moderately differentiated; PD, poorly differentiated; WD, well differentiated.

(mean, 42.3 ± 28.2 mm). Histopathologically, the tumors were well-differentiated adenocarcinoma in 5 cases, moderately differentiated adenocarcinoma in 53 cases, and poorly differentiated adenocarcinoma in 1 case.

Immunohistochemical analysis. Immunohistochemistry was done on the formalin-fixed, paraffin-embedded tissue sections as described previously (33). We reacted 4-μm-thick sections of representative blocks with monoclonal antibodies against the following: CD4 (1F6; 1:50), CD8 (4B11; 1:50), and perforin (5B11; 1:50) from Novocastra Laboratories, Ltd. (Newcastle upon Tyne, United Kingdom), and FOXP3 (clone 42; ref. 25). Briefly, the sections were deparaffinized and rehydrated. After blocking of endogenous peroxidase with methanol containing 0.3% H_2O_2, the sections were autoclaved at 121°C for 10 min in citrate buffer (10 mmol/L sodium citrate; pH 6.0) for antigen retrieval. After blocking with normal goat serum, the sections were reacted overnight with appropriately diluted primary antibodies. The sections were then reacted sequentially with biotin-conjugated anti-mouse IgG antibodies (Vector Laboratories, Burlingame, CA) and Vectastain Elite ABC reagent (Vector Laboratories). For staining CD4 and CD8, a CSA system (DAKO, Glöstrup, Denmark) and EnVision$^+$ Polymer system (DAKO) were used, respectively, instead of the avidin-biotin complex system. Diaminobenzidine was used as the chromogen, and the nuclei were counterstained with hematoxylin.

Serial sections were prepared from each paraffin block. The first section was stained with H&E and the second, third, and fourth sections were subjected to immunohistochemistry to detect the CD8, CD4, and FOXP3 antigens. CD8$^+$, CD4$^+$, or FOXP3$^+$ lymphocytes were counted in the corresponding visual fields. Quantitative evaluation of lymphocytes was done by analyzing at least three different high-power fields (×40 objective and ×10 eyepiece). The proportion of FOXP3$^+$ lymphocytes among CD4$^+$ lymphocytes and that of CD8$^+$ lymphocytes among total T cells, together with the sum of CD4$^+$ and CD8$^+$ lymphocytes, were calculated for each field and the averages were compared.

Statistical analysis. Values were expressed as mean ± SD. Statistical analyses were done with StatView-J 5.0 software (Abacus Concepts, Berkeley, CA). Associations among the variables were assessed by the χ^2 test, Student's t test, Mann-Whitney U test, and Kruskal-Wallis test. If there was evidence of non-normality, the Mann-Whitney U test or the Kruskal-Wallis test was used to test the difference in medians among the groups. Survival rates were calculated by the Kaplan-Meier method. Differences between survival curves were analyzed by the log-rank test. To assess the correlation between survival time and multiple clinicopathologic variables, multivariate analyses were done by the Cox proportional hazards regression model. Differences were considered significant at $P < 0.05$.

Results

Increased populations of FOXP3$^+$ Tregs among CD4$^+$ T cells in tumor stroma of HCC. In order to assess the infiltration of Tregs in the stroma of HCC (n = 235) and nontumorous liver (n = 248), we evaluated both the absolute numbers of FOXP3$^+$ Tregs and the prevalence of FOXP3$^+$ Tregs among CD4$^+$ T cells. The absolute number of FOXP3$^+$ Tregs that had infiltrated HCC was significantly higher than that of Tregs in nontumorous liver from patients with HCC or healthy liver tissue (versus healthy controls, $P < 0.001$; versus NCH, $P < 0.001$; versus CH, $P = 0.002$; versus PC, $P = 0.023$; versus LC, $P < 0.001$; Fig. 1A). The prevalence of tumor-infiltrating FOXP3$^+$ Tregs among CD4$^+$ T cells in HCC was also significantly higher (versus healthy controls, $P < 0.001$; versus NCH, $P < 0.001$; versus CH, $P < 0.001$; versus PC, $P < 0.001$; versus LC, $P < 0.001$; Fig. 1B). Among advanced HCCs, the prevalence of FOXP3$^+$ Tregs was significantly higher in less differentiated HCCs (Kruskal-Wallis test, $P < 0.001$; Fig. 1B). No significant difference in the infiltration of Tregs was found among CH, PC, and LC. The prevalence of Tregs in NCH was lower than that in CH ($P = 0.021$), PC, and LC, but was significantly higher than that in healthy controls ($P < 0.001$; Fig. 1B).

The absolute number of CD8$^+$ T cells was increased in CH, PC, and LC, and was significantly higher than that in HCC ($P < 0.001$; Fig. 1C). The prevalence of CD8$^+$ T cells in HCC was significantly lower than that in any type of damaged and nontumorous liver from patients with HCC (versus NCH, $P = 0.025$; versus CH, $P < 0.001$; versus PC, $P = 0.015$; versus LC, $P < 0.001$; Fig. 1D). In advanced HCCs, the prevalence of CD8$^+$ T cells was significantly lower in less differentiated HCC (Kruskal-Wallis test, $P = 0.034$; Fig. 1D). CD8$^+$ T cells

were increased slightly in NCH and viral hepatitis including CH, PC, and LC compared with healthy controls. These results suggested that an immunoreaction had also occurred in nontumorous liver bearing HCC without viral hepatitis.

Clinicopathologic features of HCC and the prevalence of tumor-infiltrating Tregs and CD8⁺ T cells. We analyzed the correlation between clinicopathologic features of HCC and the prevalence of tumor-infiltrating Tregs or that of CD8⁺ T cells in HCC (Table 2A and B). Patients with HCC were divided into two groups either by the median value for the prevalence of tumor-infiltrating Tregs (29.0%) or by CD8⁺ T cells (51.5%). The high Treg group (n = 73) showed a significant correlation with high histologic grade (P = 0.021) and tended to show a lower number of infiltrating CD8⁺ T cells in HCC (P = 0.064) among the various clinicopathologic characteristics (Table 2A).

Prognostic significance of the prevalence of Tregs and CD8⁺ T cells in HCC. Overall and disease-free survival were analyzed in these patients. Of the 147 patients with HCC who underwent hepatic resection, 88 (59.9%) died. The overall 5-year survival and disease-free survival rates were 39.5% and 18.4%, respectively. The low-Treg group showed significantly better overall survival than the high-Treg group (log-rank test, P = 0.007; Fig. 1E). Mean overall survival was 60.3 (±33.8) months for the low-Treg group and 45.1 (±38.7) months for the high-Treg group. The low-Treg group also showed significantly better disease-free survival than the high-Treg group (log-rank test, P = 0.015; Fig. 1F). Mean disease-free survival was 36.2 (±31.7) months for the low-Treg group and 27.3 (±32.9) months for the high-Treg group. The 15 clinicopathologic factors listed in Table 3A and B were examined for their association with overall and disease-free survival after initial resection of the tumor. Univariate analysis of overall survival revealed that the following variables had a negative influence: Child-Pugh classification (B), TNM stage (III and IV), high serum α-fetoprotein (AFP; >27.1 IU/mL), presence of portal vein invasion (VP), presence of histologic intrahepatic metastatic foci (IM), and high prevalence of tumor-infiltrating Tregs (Table 3A). In multivariate Cox proportional hazard analysis for clinicopathologic variables and prevalence of tumor-infiltrating Tregs, the

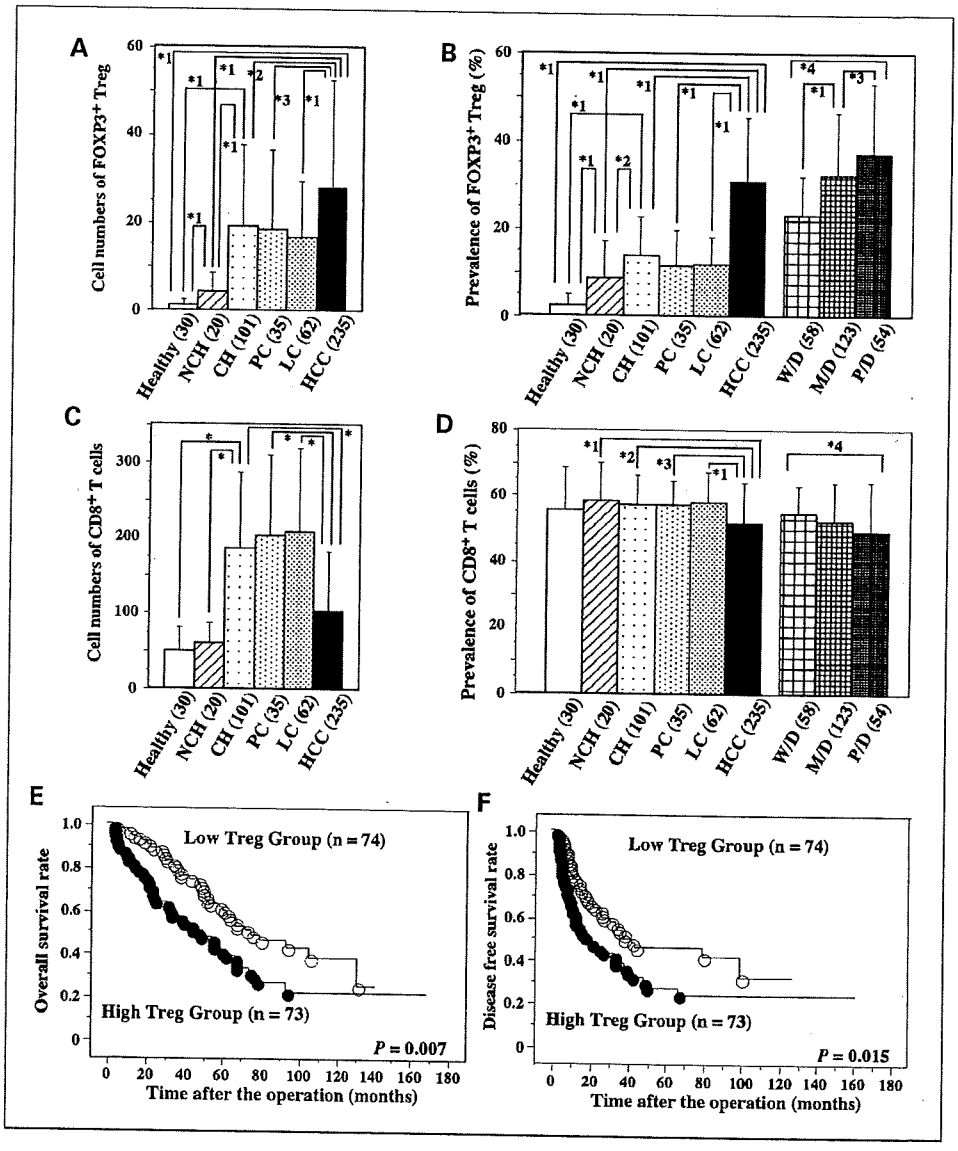

Fig. 1. Increased population of FOXP3⁺ Tregs and decreased population of CD8⁺ T cells in tumor stroma of HCC. *A* and *B*, absolute number of Tregs (*A*) and prevalence of Tregs (*B*) in HCC and nontumorous liver. Right column, the contents of HCC according to histologic grade (*B*). Number of cases tested in parentheses: *A*, *1, P < 0.001; *2, P = 0.002; *3, P = 0.023. *B*, *1, P < 0.001; *2, P = 0.021; *3: P = 0.044; *4, P < 0.001 (Kruskal-Wallis test); thin bars, SD. *C* and *D*, absolute number of CD8⁺ Tcells (*C*) and prevalence of CD8⁺ Tcells (*D*) in HCC and nontumorous liver. Right column, the contents of HCC according to histologic grade (*D*). Number of cases tested in parentheses: thin bars, SD. *C*, *, P < 0.001. *D*, *1, P = 0.025; *2, P < 0.001; *3, P = 0.015; *4, P < 0.001 (Kruskal-Wallis test). *E* and *F*, Kaplan-Meier survival curves of 147 patients with HCC. Overall survival curve (*E*) and disease-free survival curve (*F*) are shown. The prognosis was significantly worse in the high Treg prevalence group (*solid dots*, n = 73) than in the low Treg prevalence group [*white dots*, n = 74; log-rank test, P = 0.007 (*E*) and P = 0.015 (*F*)].

hazard ratio for poor prognosis was 1.640 for patients in the high-Treg group compared with patients in the low-Treg group ($P = 0.040$; Table 3A). Worse Child-Pugh classification and the presence of VP were also independent factors for overall patient survival. Univariate analysis for disease-free survival revealed that six variables negatively affected the survival rate and all of them were the same with the six variables of overall survival (Table 3B). In multivariate analysis for disease-free survival, two variables—the presence of IM and the high prevalence of Tregs infiltrating HCC—were significant factors. The hazard ratio for poor prognosis was 1.706 for patients in the high-Treg group compared with patients in the low-Treg group ($P = 0.024$; Table 3B). There was no significant difference in the overall survival rate or disease-free survival rate between the low and high $CD8^+$ T cell groups. These results indicated that the prevalence of tumor-infiltrating Tregs was an independent prognostic factor in patients with HCC, whereas the prevalence of tumor-infiltrating $CD8^+$ T cells was not.

Increased populations of Tregs among $CD4^+$ T cells in tumor stroma correspond to progression during multistage hepatocarcinogenesis. It was suggested that Tregs play important roles in the progression of HCC. Therefore, the prevalence of Tregs among $CD4^+$ T cells in the precursor lesions, AH ($n = 11$; Fig. 2E-H) and AAH ($n = 9$), and early HCC ($n = 68$; Fig. 2I-L), was analyzed during tumorigenesis of HCC. As shown in Fig. 3A, the prevalence of Tregs increased significantly in a stepwise manner during the progression of hepatocarcinogenesis (Kruskal-Wallis test, $P < 0.001$; viral hepatitis containing CH, PC, and LC versus precursor lesions containing AH and AAH, $P = 0.038$; precursor lesions versus early HCC, $P = 0.121$; early HCC versus advanced HCC, $P < 0.001$). These findings suggest that the prevalence of Tregs is closely correlated with the progression of multistage hepatocarcinogenesis. In contrast, the prevalence of $CD8^+$ T cells showed a clear, but not drastic, decrease during the progression of hepatocarcinogenesis (Kruskal-Wallis test, $P < 0.001$; Fig. 3B).

Table 2. Correlation between clinicopathologic findings and the prevalence of Tregs and $CD8^+$ T cells infiltrating HCC

(A) Correlation between clinicopathologic findings and the prevalence of Tregs infiltrating HCC

Variables	Prevalence of Tregs among $CD4^+$ T cells		
	High Treg	Low Treg	*P*
Age, y (mean ± SD)	62.6 ± 8.92	61.4 ± 10.4	0.444*
Gender (male/female)	59/14	54/20	0.259†
Viral infection			
HBV and/or HCV/(−)	66/7	69/5	0.531†
HBV(+)/(−)	27/7	29/5	0.525†
HCV(+)/(−)	45/7	43/5	0.640†
Nontumor liver (NCH/CH/PC/LC)	7/41/9/16	10/30/14/20	0.289†
Child-Pugh score (A/B/C)	68/5/0	68/6/0	0.772†
TNM stage (I/II/III/IV)	28/22/23/0	29/31/14/0	0.155†
Tumor size, mm (median, range)	40, 9-185	30, 6-150	0.113‡
Histologic grade (early HCC/WD HCC/MD HCC/PD HCC)	7/3/38/25	10/12/39/13	**0.021**†
AFP, ng/mL (median, range)	24.1 (1.8-27,170)	28.3 (1.0-25,000)	0.681‡
VP (presence/absence)	33/40	24/50	0.112†
IM (presence/absence)	20/53	13/61	0.152†
Number of $CD8^+$ T cells infiltrating tumor (median, range)	75 (12-405)	91 (9-435)	0.064‡

(B) Correlation between clinicopathologic findings and the prevalence of $CD8^+$ T cells infiltrating HCC

Variables	Prevalence of $CD8^+$ T cells in total T cells		
	High $CD8^+$ T cells	Low $CD8^+$ T cells	*P*
Age, y (mean ± SD)	63.6 ± 9.08	60.5 ± 10.0	0.051*
Gender (male/female)	51/23	62/12	**0.045**†
Viral infection			
HBV and/or HCV/(−)	69/4	66/8	0.238†
HBV(+)/(−)	30/4	26/8	0.203†
HCV(+)/(−)	42/4	46/8	0.348†
Nontumor liver (NCH/CH/PC/LC)	11/35/12/15	6/36/11/21	0.471†
Child-Pugh score (A/B/C)	67/6/0	69/5/0	0.736†
TNM stage (I/II/III/IV)	28/29/16/0	29/24/21/0	0.560†
Tumor size, mm (median, range)	40, 6-185	31, 10-150	0.075‡
Histologic grade (early HCC/WD HCC/MD HCC/PD HCC)	9/8/39/17	8/7/38/21	0.907†
AFP, ng/mL (median, range)	21.5 (1.0-27,170)	36.3 (1.8-17,430)	0.105‡
VP (presence/absence)	31/42	26/48	0.362†
IM (presence/absence)	18/55	15/59	0.524†

Abbreviations: MD, moderately differentiated; PD, poorly differentiated; WD, well differentiated.
*Student's *t* test.
†χ^2 test or Fisher exact test.
‡Mann-Whitney *U* test.