tumor thrombosis, refractory ascites, or extrahepatic metastasis were excluded. In general, we performed RFA on patients with three or fewer lesions, all of which were 3 cm or smaller in diameter. However, we also performed ablation on patients with more than three lesions or lesions larger than 3 cm in diameter if the procedure could be assumed to be clinically effective without deteriorating liver function. The procedure was meticulously described elsewhere [24]. One to three days after RFA, dynamic CT with section thickness of 5 mm was performed to evaluate the efficacy of ablation. Complete ablation was defined as hypoattenuation of every lesion with a safety margin including the surrounding liver parenchyma. Patients received an additional session of RFA until the treatment was judged as complete. Follow-up consisted of monthly blood tests and monitoring of tumor markers at the outpatient clinic, and ultrasonography and dynamic CT scan were performed every 3–4 months.

Tumor recurrence was defined according to the same criteria applied to the primary HCC. Time to recurrence was defined as the interval between the first RFA treatment and the detection of recurrence. The follow-up period was defined as the interval from the date of the first RFA until diagnosis of tumor recurrence or the end of December 2007.

Viral markers

HBV serologic markers, including HBsAg, HBeAg, and antibody (HBeAb), were determined by commercially available immunoassays (Abbort Laboratories, Chicago, IL, USA). The serum level of HBV DNA was quantified by COBAS Amplicor HBV Monitor Test (Roche Diagnostic Systems, Indianapolis, IND, USA), which can quantify from 2.6–7.6 log10 copies/ml of HBV DNA in the sera. For patients treated before 2003, HBV DNA was measured using serum samples obtained before RFA and kept frozen at −70°C.

Risk factors for recurrence

The variables were selected for potential relationship to the risk of HCC development based on previous studies [9, 25–28], that is, age, sex, serum albumin concentration, total bilirubin concentration, aspartate aminotransferase (AST), alanine aminotransferase (ALT), PLT, PT, HBeAg, HBV DNA load, Child-Pugh classification (A or B), histologic fibrosis score, liver cirrhosis, inflammatory activity, alphafetoprotein (AFP), lens culinaris agglutinin A-reactive fraction of AFP (AFP-L3), and des-gamma-carboxy prothrombin (DCP). We diagnosed liver cirrhosis histologically by biopsy or clinically by the presence of portal hypertension (splenomegaly or esophageal varices), ascites, or encephalopathy. Tumor size, tumor nodule number, and tumor differentiation at the initial treatment were also included in the analysis.

The serum AFP levels were determined by fluorescence-enzyme immunoassay (STE test TOSOH II, TOSOH, Tokyo, Japan); AFP-L3 fraction by lectin-affinity electrophoresis coupled with antibody-affinity blotting (AFP Differentiation Kit L, Wako Pure Chemicals, Osaka, Japan); and DCP levels, by electrochemiluminescence immunoassay (Picolumi PIVKA-II Kit, Sankyo Junyaku, Tokyo, Japan).

Statistical analysis

Data were expressed as the mean ± standard deviation or median (range). Cumulative recurrence of HCC was analyzed using the Kaplan–Meier method. Risk factors for recurrence after RFA were evaluated using univariate and multivariate Cox' proportional hazards models. Variables that had an association ($p \leq 0.15$) with recurrence in the univariate analysis were included in a multivariate model to determine the independent contribution of each variable. We also performed subgroup analyses for the effect of viral load on recurrence stratified by liver function, tumor factors, or lamivudine status. Estimated cumulative probability was obtained with SAS PHREG procedure with baseline and covariate options. The differences with a P value of less than 0.05 were considered statistically significant. The Student's t test was used to analyze differences between groups in ages and tumor size whereas the Mann–Whitney U test was used to analyze differences in laboratory test results. The χ^2 test or Fisher's exact test was used to compare categorical data between groups. SAS version 9.1 for Windows (SAS Institute Inc., Carey, NC, USA) was used in statistical calculations.

Results

Patient characteristics

A total of 69 patients, 50 men and 19 women, with the mean age of 60.2 ± 8.6 years were enrolled. The baseline demographic characteristics of the enrolled patients are shown in Table 1. The majority had liver cirrhosis. HBeAg was negative in about three-fourth patients. Serum HBV DNA load (log10 copies/mL) was ≤4.0, 4.1–5.0, 5.1–6.0, 6.1–7.0 and ≥7.1 in 19, 14, 8, 21, and 7 patients, respectively. The total number of sessions to achieve a complete loco-regional response was 131 sessions for 69 patients, 1.9 ± 0.9 sessions per patient. The median observation period was 1.5 years after RFA and no patients were lost to follow-up. Lamivudine was being administered to four

Table 1 Baseline characteristics of patients ($N = 69$)

Variable	
Age, year[a]	60.2 ± 8.6
Male/female, N/N	50/19
Albumin, g/dL[a]	3.7 ± 0.6
Total bilirubin, mg/dL[a]	0.9 ± 0.3
AST, IU/L[a]	44.8 ± 26.1
ALT, IU/L[a]	45.7 ± 33.9
Platelet count, × 10^3/mm[3a]	134 ± 70
Prothrombin activity, %[a]	75.4 ± 13.9
HBeAg+/eAg−, N/N	16/53
HBV DNA, log copies	
–4/4.1–5/5.1–6/6.1–7/7.1–	19/14/8/21/7
Child-pugh class, A/B	54/15
Edmondson grade 1/2/3	15/37/8
Fibrosis, F 0/1/2/3/4[c]	2/1/10/13/36
CH/LC	29/40
Activity, A 0/1/2/3[c]	1/40/18/3
AFP, ng/mL[b]	17.7 (0.8–16,709)
AFP-L3, %[b]	0.5 (0.5–76.8)
DCP, mAU/mL[b]	22 (10–3,735)
Size of tumor, cm[a]	2.7 ± 1.0
–2.0/2.1–3.0/3.1–5.0/5.1–	17/32/19/1
Number of nodules[a]	1.7 ± 1.0
1/2/3/4–	42/15/7/5

[a] mean ± SD, [b]median (range)

[c] Examined in limited number of patients

AST Aspartate aminotransferase, *ATL* alanine aminotransferase, HBeAg hepatitis B e antigen, *HBV* hepatitis B virus, *CH* chronic hepatitis, *LC* Liver cirrhosis, *AFP* alpha-fetoprotein, *AFP-L3* A-reactive fraction of alpha-fetoprotein, *DCP* Des-gamma-carboxy prothrombin

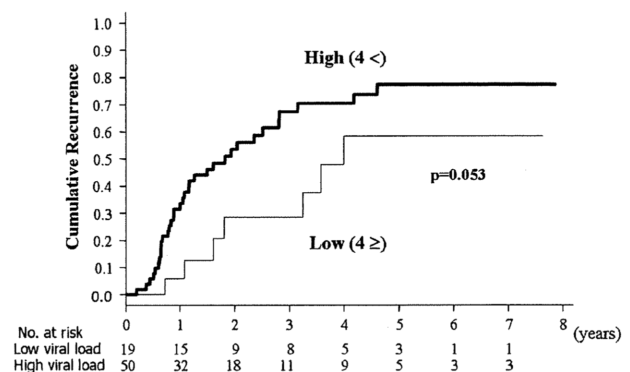

Fig. 1 Cumulative incidence of HCC recurrence grouped by serum HBV DNA load

patients at the time of RFA. Other 12 patients started receiving lamivudine during the follow-up period.

HCC recurrence

HCC recurrence was observed in 42 patients during the study period. Intrahepatic recurrence sites were distant from the site of primary nodule in 40 cases and adjacent in 2 cases. Because of the small number of latter cases, they were not distinguished in analysis. A number of 38 of 42 patients with recurrence were treated with RFA. Three patients were treated with transarterial chemoembolization because of multiple recurrences, and the irradiation therapy was chosen in one patient for portal vein tumor thrombus. A number of 15 patients died by the end of the study period.

Cumulative recurrence rates at 1, 3, and 5 years were 26.5, 57.8, and 72.8%, respectively. Although not reaching statistical significance in univariate analysis ($P = 0.053$), there was a tendency of more frequent HCC recurrence in patients with high viral load (>4.0 log10 copies/mL) (Fig. 1). In the evaluation of factors related to recurrence, 19 variables shown in Table 2 were first assessed in univariate analysis. Lower serum albumin concentration (<3.5 g/dl, $P = 0.037$), lower PLT (<150 × 10^3/mm^3, $P = 0.027$), lower PT ($P = 0.0019$), higher HBV DNA load (>4.0 log10 copies/ml, $P = 0.053$), Child-Pugh class B ($P = 0.15$), and the number of tumors (>3, $P = 0.13$) were associated with HCC recurrence at a significance level of $p \leq 0.15$. These factors were then analyzed with multivariate Cox regression analysis with stepwise variable selection, in which the number of tumors (risk ratio, 4.63; 95% CI, 1.50–14.25, $P = 0.0076$), lower PLT (risk ratio, 3.39; 95% CI, 1.52–5.78, $P = 0.0029$), and high HBV DNA load (risk ratio, 2.67; 95% CI, 1.16–6.14, $P = 0.021$) were retained as significant independent risk factors for HCC recurrence (Table 3).

Patients were divided by serum HBV DNA load as low (thin line, ≤4 logcopies/mL) and high (thick line, >4 logcopies/mL). Cumulative incidence of HCC recurrence rates is shown for patients with a high viral load and a low viral load (Fig. 1). Cumulative recurrence rates at 1, 3, and 5 years were 34, 67, and 77%, respectively, in high viral load group, and 6, 28, and 58%, respectively, in the low viral load group.

Subgroup analysis

We performed subgroup analyses for the effect of HBV DNA load on recurrence stratified by liver function or tumor factors. As shown in Fig. 2, the effect of HBV DNA load on recurrence was shown in subgroups except for those with Child-Pugh class B or multinodular primary HCC. The reason for this is not clear but multicentric recurrence may play a relatively lesser role in these patients.

We also performed subgroup analyses for the effect of viral load on recurrence stratified by lamivudine status. As

Table 2 Univariate analysis of factors related to HCC recurrence

Variable	Hazard ratio (95%CI)	p value
Age, ≥60 year/< 60 (31/38)	0.944 (0.434–2.05)	0.882
Male sex/female (50/19)	0.963 (0.498–1.86)	0.912
Albumin, <3.5 g/dL/≥3.5 (17/52)	1.983 (1.041–3.78)	0.037
Total bilirubin, >1.0 mg/dL/≤1.0 (16/53)	2.00 (0.681–5.87)	0.21
AST, >40 IU/L/≤40 (33/36)	1.15 (0.621–2.14)	0.65
ALT, >40 IU/L/≤40 (32/37)	1.00 (0.539–1.86)	0.68
Platelet count, <15 × 10^3/mm^3/≥15 (47/22)	2.54 (1.11–5.78)	0.027
Prothrombin activity, <70%/≥70 (22/47)	5.55 (1.88–16.4)	0.0019
HBeAg+/eAg−(16/53)	1.11 (0.527–2.34)	0.78
HBV DNA, >4 log copies/≤4 (50/19)	2.24 (0.991–5.07)	0.053
Child-Pugh Class, B/A (15/54)	1.69 (0.820–3.50)	0.15
Edmondson grade 1–2/3 (52/8)	0.65 (0.23–1.84)	0.42
Fibrosis, F 0–3/4 (26/36)	0.834 (0.42–6.63)	0.60
CH/LC (29/40)	0.627 (0.33–1.19)	0.16
Activity, A 0–1/2–3 (41/21)	1.52 (0.80–2.90)	0.20
AFP, >400 ng/mL/≤400 (11/58)	1.04 (0.453–2.38)	0.93
AFP-L3, >10%/≤10 (14/55)	1.18 (0.564–2.47)	0.66
DCP, >100 mAU/mL/≤100 (11/58)	1.38 (0.640–2.99)	0.41
Size of tumor, ≥3.0 cm/<3.0 (26/43)	1.04 (0.564–2.02)	0.90
Number of nodules >3/1–3 (5/64)	2.17 (0.77–4.52)	0.13

AST aspartate aminotransferase, *ATL* alanine aminotransferase, *HBeAg* hepatitis B e antigen, *HBV* hepatitis B virus, *CH* chronic hepatitis, *LC* liver cirrhosis, *AFP* alpha-fetoprotein, *AFP-L3* A-reactive fraction of alpha-fetoprotein, *DCP* Des-gamma-carboxy prothrombin

Table 3 Multivariate analysis of factors related to HCC recurrence

Variable	Hazard Ratio (95%CI)	p value
Number of nodules >3	4.63 (1.50–14.25)	0.0076
Platelet count, <150 × 10^3/mm^3	3.39 (1.52–7.58)	0.0029
HBV DNA, >4 log10 copies	2.67 (1.16–6.14)	0.021

HBV Hepatitis B virus

shown in Fig. 2, the three analyses, overall, excluding the 4 patients, and excluding 16 patients (the 4 patients plus 12 who received lamivudine after RFA), showed similar results.

Comparison of patients with low and high HBV DNA loads

There was no significant difference between low HBV DNA load patients and high load patients in gender, AST, ALT, PLT, tumor size, or the number of tumors (Table 4). In low viral load patients, the age was younger, albumin concentration was higher, inflammatory activity was lower, and HBeAg seropositivity was lower, although none of these differences were statistically significant.

Discussion

Recently, Chen et al. [9] has reported that high serum HBV DNA load (> 4.0 log10 copies/ml) is a strong risk factor of HCC development, independently of HBeAg status, serum ALT levels, and the presence of cirrhosis. Consequently, it is currently recommended by most treatment guidelines for chronic hepatitis B to reduce serum HBV DNA level as the primary objective of antiviral therapy [29, 30]. The risk factors for recurrence of HBV-related HCC found significant in the current study were the number of tumors that had been treated with RFA (>3), low PLT (<150 × 10^3/mm^3), and high serum HBV DNA load (>4.0 log10 copies/ml).

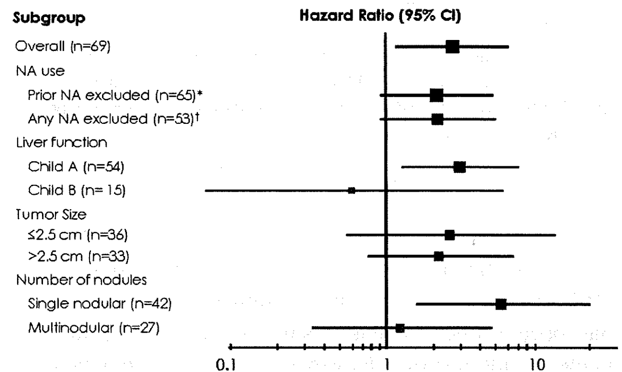

Fig. 2 Result of subgroup analysis. Hazard ratios for the risk of hepatocelullar carcinoma recurrence are shown for the patients with high viral load (HBV DNA >4 log copy/mL), as compared with the patients with low viral load (HBV DNA ≤4 log copy/mL) for various subgroups according to clinical characteristics. Those who received lamivudine before ablation were excluded (*asterisk*). Those who received lamivudine before or during follow-up period were excluded (*dagger*)

Table 4 Comparison of laboratory data between low and high HBV DNA load patients

	Low viral load $N = 19$	High viral load $N = 50$	p value
Age, year*	57.6 ± 7.0	61.2 ± 9.0	0.117
Sex, M/F	16/3	34/16	0.235
Albumin, g/dL*	4.0 ± 0.5	3.7 ± 0.6	0.0506
Total bilirubin, mg/dL*	0.85 ± 0.29	0.90 ± 0.35	0.595
AST, IU/L*	39.0 ± 23.3	47.0 ± 26.9	0.255
ALT, IU/L*	39.0 ± 22.2	48.3 ± 37.3	0.311
Platelet count, $\times 10^3/mm^3$ *	148.4 ± 9.6	128.4 ± 5.8	0.297
Prothrombin activity, %*	76.9 ± 12.8	74.8 ± 14.4	0.580
HBeAg +/eAg −	2/17	14/36	0.202
Child-Pugh Class, B/A	3/16	12/38	0.534
Edmondson grade 1–2/3	14/2	38/6	1.00
Fibrosis, F 0–3/4	8/10	18/26	1.00
CH/LC	9/10	20/30	0.597
Activity, A 0–1/2–3	15/3	26/18	0.0825
AFP, ≥400 ng/mL/<	2/17	9/41	0.715
AFP-L3, ≥10%/<	3/16	11/39	0.742
DCP, ≥100 mAU/mL/<	2/17	9/41	0.715
Size of tumor, cm*	2.8 ± 1.0	2.7 ± 0.9	0.643
Number of nodules*	1.5 ± 0.9	1.7 ± 1.1	0.493

*mean ± SD

AST aspartate aminotransferase, *ATL* alanine aminotransferase, *HBeAg* hepatitis B e antigen, *CH* chronic hepatitis, *LC* liver cirrhosis, *AFP* alpha-fetoprotein, *AFP-L3* A-reactive fraction of alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin

Kubo et al. [25] reported that high serum HBV DNA load was a risk factor of recurrence after surgical resection of HBV-related HCC. The clinical importance of this finding has been further increased since the advent of antiviral nucleot(s)ide analogs. However, the study population was small in size and limited by the indication for surgery.

HCC recurrence may be due to residual cancer cells in ablated nodule, growth of already-existing intrahepatic metastasis, or metachronous multicentric carcinogenesis. Even when recurrence site is distant from the primary site, the latter two mechanisms are not easily distinguishable. Nevertheless, the fact that PLT and HBV viral load, risk factors for primary carcinogenesis, are also risk factors for recurrence suggests that multicentric carcinogenesis contributes to a portion of HCC recurrence. While the tumor-related factors, such as tumor size or number may affect the probability of intrahepatic metastasis, PLT and HBV DNA load are unlikely to be related to residual HCC at RFA. Thus, we may safely conclude that de novo carcinogenesis, which is likely to be affected by HBV DNA load, contributes to a substantial portion of intrahepatic recurrence of HBV-related HCC after RFA.

According to the results of this study, we simulated the cumulative incidence of intrahepatic HCC recurrence assuming that every patient had had low HBV DNA load. The cohort could be expected to have shown cumulative recurrence rates of 11.4, 31.2, and 58.0% at 1, 3, and 5 years, respectively, instead of actual 26.5, 57.8, and 72.8%, if viral load were kept low in all patients. The estimated cumulative incidence curve indicated that the decrease in the incidence of intrahepatic recurrence would be substantial. Although yet to be confirmed in prospective studies, there is the possibility that antiviral therapy, by strongly inhibiting HBV replication, may reduce HCC recurrence down to the level shown in this simulation. Admittedly, the risk of HCC recurrence is still high even in this optimistic simulation. However, these patients are more likely to be able to undergo further treatment for recurrence because of preserved liver function, as we have previously shown [31]. Taken together antiviral therapy after RFA, when HBV DNA load is high, will produce substantially beneficial effect on the long-term prognosis of HBV-related HCC patients.

It is yet to be elucidated whether the relationship between HBV DNA load and HCC recurrence found in the present study holds outside Japan, where 85% of HBV strains are of genotype C, which is known for the high incidence rate of HCC as compared to other genotypes [32, 33]. Consequently, it can be suspected that de novo hepatocarcinogenesis after the treatment of primary HCC is also more frequent for genotype C [34]. With other HBV genotypes de novo carcinogenesis may be relatively few among overall HCC recurrence, with a smaller effect of HBV DNA load on overall recurrence.

The present study indicated that HBV viral load is associated with the rate of intrahepatic HCC recurrence after RFA, probably by influencing multicentric carcinogenesis. Since there are few practical options that can reduce HCC recurrence, it is clinically very important to evaluate prospectively whether antiviral therapy after RFA reduces HCC recurrence.

References

1. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat 2004;11:97–107
2. Shiratori Y, Shiina S, Zhang PY, Ohno E, Okudaira T, Payawal DA, Ono-Nita SK, Imamura M, Kato N, Omata M. Does dual infection by hepatitis B and C viruses play an important role in the pathogenesis of hepatocellular carcinoma in Japan? Cancer 1997;80:2060–2067
3. Lok AS, McMahon BJ. Chronic hepatitis B. Hepatology 2007;45: 507–539
4. Beasley RP. Hepatitis B virus. The major etiology of hepatocellular carcinoma. Cancer 1988;61:1942–1956

5. Lok AS. Chronic hepatitis B. N Engl J Med 2002;346:1682–1683
6. Imazeki F, Omata M, Yokosuka O, Okuda K. Integration of hepatitis B virus DNA in hepatocellular carcinoma. Cancer 1986;58:1055–1060
7. Br′echot C. Hepatitis B virus (HBV) and hepatocellular carcinoma. HBV DNA status and its implications. J Hepatol 1987;4:269–279
8. Br′echot C, Gozuacik D, Murakami Y, Paterlini-Bréchot P. Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). Semin Cancer Biol 2000;10:211–231
9. Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH, REVEAL-HBV Study Group. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 2006;295:65–73
10. Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, Tanwandee T, Tao QM, Shue K, Keene ON, Dixon JS, Gray DF, Sabbat J, Cirrhosis Asian Lamivudine Multicentre Study Group. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 2004;351:1521–1531
11. Poon RT, Fan ST, Lo CM, Ng IO, Liu CL, Lam CM, Wong J. Improving survival results after resection of hepatocellular carcinoma A prospective study of 377 patients. Ann Surg 2001;234:63–70
12. Shiina S, Teratani T, Obi S, Sato S, Tateishi R, Fujishima T, Ishikawa T, Koike Y, Yoshida H, Kawabe T, Omata M. A randomized controlled trial of radiofrequency ablation with ethanol injection for small hepatocellular carcinoma. Gastroenterology 2005;129:122–30
13. Waki K, Aikata H, Katamura Y, Kawaoka T, Takaki S, Hiramatsu A, Takahashi S, Toyota N, Ito K, Chayama K. Percutaneous radiofrequency ablation as first-line treatment for small hepatocellular carcinoma: results and prognostic factors on long-term follow up. J Gastroenterol Hepatol 2010;25:597–604
14. N'Kontchou G, Mahamoudi A, Aout M, Ganne-Carrié N, Grando V, Coderc E, Vicaut E, Trinchet JC, Sellier N, Beaugrand M, Seror O. Radiofrequency ablation of hepatocellular carcinoma: Long-term results and prognostic factors in 235 Western patients with cirrhosis. Hepatology 2009;50:1475–83
15. Zerbini A, Pilli M, Laccabue D, Pelosi G, Molinari A, Negri E, Cerioni S, Fagnoni F, Soliani P, Ferrari C, Missale G. Radiofrequency thermal ablation for hepatocellular carcinoma stimulates autologous NK-cell response. Gastroenterology 2010;138: 1931–1942
16. Kim CM, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 1991;251:317–320
17. Koike K, Tsutsumi T, Fujie H, Shintani Y, Kyoji M. Molecular mechanism of viral hepatocarcinogenesis. Oncology 2002; 62(Suppl 1):29–37
18. Tateishi R, Shiina S, Teratani T, Obi S, Sato S, Koike Y, Fujishima T, Yoshida H, Kawabe T, Omata M. Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1,000 cases. Cancer 2005;103:1201–1209
19. Huang GT, Lee PH, Tsang YM, Lai MY, Yang PM, Hu RH, Chen PJ, Kao JH, Sheu JC, Lee CZ, Chen DS. Percutaneous ethanol injection versus surgical resection for the treatment of small hepatocellular carcinoma: a prospective study. Ann Surg 2005;242:36–42
20. Chen MS, Li JQ, Zheng Y, Guo RP, Liang HH, Zhang YQ, Lin XJ, Lau WY. A prospective randomized trial comparing percutaneous local ablative therapy and partial hepatectomy for small hepatocellular carcinoma. Ann Surg 2006;243:321–328
21. Torzilli G, Minagawa M, Takayama T, Inoue K, Hui AM, Kubota K, Ohtomo K, Makuuchi M. Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. Hepatology 1999;30:889–893
22. Edmondson HA, Steiner PE. Primary carcinoma of the liver: a study of 100 cases among 4,900 necropsies. Cancer 1954;7: 462–503
23. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Sheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–1520
24. Omata M, Tateishi R, Yoshida H, Shiina S. Treatment of hepatocellular carcinoma by percutaneous tumor ablation methods: ethanol injection therapy and radiofrequency ablation. Gastroenterology 2004;127:S159–166
25. Kubo S, Hirohashi K, Tanaka H, Tsukamoto T, Shuto T, Yamamoto T, Ikebe T, Wakasa K, Nishiguchi S, Kinoshita H. Effect of viral status on recurrence after liver resection for patients with hepatitis B virus-related hepatocellular carcinoma. Cancer 2000;88:1016–1024
26. Yang HI, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, Hsiao CK, Chen PJ, Chen DS, Chen CJ, Taiwan Community-Based Cancer Screening Project Group. Hepatitis B e antigen and the risk of hepatocellular carcinoma. N Engl J Med 2002;347: 168–174
27. Sherman M. Hepatocellular carcinoma: epidemiology, risk factors, and screening. Semin Liver Dis 2005;25:143–154
28. Yuen MF, Yuan HJ, Wong DK, Yuen JC, Wong WM, Chan AO, Wong BC, Lai KC, Lai CL. Prognostic determinants for chronic hepatitis B in Asians: therapeutic implications. Gut 2005;54: 1610–1614
29. de Franchis R, Hadengue A, Lau G, Lavanchy D, Lok A, McIntyre N, Mele A, Paumgartner G, Pietrangelo A, Rodés J, Rosenberg W, Valla D, EASL Jury. EASL international consensus conference on hepatitis B. 13–14 September, 2002: Geneva, Switzerland. Consensus statement (long version). J Hepatol 2003;39:S3–25
30. ACT-HBV Asia-Pacific Steering Committee Members. Chronic hepatitis B: treatment alert. Liver Int 2006;26:47–58
31. Yoshida H, Yoshida H, Goto E, Sato T, Ohki T, Masuzaki R, Tateishi R, Goto T, Shiina S, Kawabe T, Omata M. Safety and efficacy of lamivudine after radiofrequency ablation in patients with hepatitis B virus-related hepatocellular carcinoma. Hepotol Int 2008;2:89–94
32. Sumi H, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T, Kanda T, Fukai K, Kato M, Saisho H. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. Hepatology 2003;37:19–26
33. Orito E, Mizokami M. Hepatitis B virus genotypes and hepatocellular carcinoma in Japan. Intervirology 2003;46:408–12
34. Chen JD, Liu CJ, Lee PH, Chen PJ, Lai MY, Kao JH, Chen DS. Hepatitis B genotypes correlate with tumor recurrence after curative resection of hepatocellular carcinoma. Clin Gastroenterol Hepatol 2004;2:64–71

Published OnlineFirst August 29, 2011; DOI:10.1158/1055-9965.EPI-11-0464

Cancer Epidemiology, Biomarkers & Prevention

Prediction of Hepatocellular Carcinoma Development by Plasma ADAMTS13 in Chronic Hepatitis B and C

Hitoshi Ikeda, Ryosuke Tateishi, Kenichiro Enooku, et al.

Cancer Epidemiol Biomarkers Prev 2011;20:2204-2211. Published OnlineFirst August 29, 2011.

Updated Version	Access the most recent version of this article at: doi:10.1158/1055-9965.EPI-11-0464

Cited Articles	This article cites 44 articles, 6 of which you can access for free at: http://cebp.aacrjournals.org/content/20/10/2204.full.html#ref-list-1

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Downloaded from cebp.aacrjournals.org on February 21, 2012
Copyright © 2011 American Association for Cancer Research

Published OnlineFirst August 29, 2011; DOI:10.1158/1055-9965.EPI-11-0464

Cancer Epidemiology, Biomarkers & Prevention

Research Article

Prediction of Hepatocellular Carcinoma Development by Plasma ADAMTS13 in Chronic Hepatitis B and C

Hitoshi Ikeda[1,2], Ryosuke Tateishi[2], Kenichiro Enooku[1,2], Haruhiko Yoshida[2], Hayato Nakagawa[1,2], Ryota Masuzaki[2], Yuji Kondo[2], Tadashi Goto[2], Shuichiro Shiina[2], Yukio Kume[1], Tomoaki Tomiya[2], Yukiko Inoue[2], Takako Nishikawa[2], Natsuko Ohtomo[2], Yasushi Tanoue[2], Tomoko Ono[3], Kazuhiko Koike[2], and Yutaka Yatomi[1]

Abstract

Background: Chronic liver injury evokes a wound healing response, promoting fibrosis and finally hepatocellular carcinoma (HCC), in which hepatic stellate cells play an important role. Although a blood marker of hepatic stellate cells is not known, those cells importantly contribute to the regulation of plasma a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) activity, a defect of which causes thrombotic thrombocytopenic purpura.

Methods: Plasma ADAMTS13 was evaluated in chronic hepatitis B or C patients with or without HCC.

Results: Plasma ADAMTS13 activity significantly correlated with serum aspartate aminotransferase and alanine aminotransferase, liver stiffness value, and aspartate aminotransferase-to-platelet ratio index, irrespective of the presence of HCC, suggesting that it may reflect hepatocellular damage and subsequent wound healing and fibrosis as a result of hepatic stellate cell action. During the three-year follow-up period for patients without HCC, it developed in 10 among 81 patients. Plasma ADAMTS13 activity was significantly higher in patients with HCC development than in those without and was a significant risk for HCC development by univariate and multivariate analyses. Furthermore, during the one-year follow-up period for patients with HCC treated with radiofrequency ablation, HCC recurred in 55 among 107 patients. Plasma ADAMTS13 activity or antigen level was significantly higher in patients with HCC recurrence than in those without and was retained as a significant risk for HCC recurrence by multivariate analysis.

Conclusions: Higher plasma ADAMTS13 activity and antigen level was a risk of HCC development in chronic liver disease.

Impact: Plasma ADAMTS13 as a potential marker of hepatic stellate cells may be useful in the prediction of hepatocarcinogenesis. *Cancer Epidemiol Biomarkers Prev; 20(10); 2204–11. ©2011 AACR.*

Introduction

It is well known that chronic wound healing generally provides a microenvironment that gives rise to cancer (1). Indeed, chronic injury in the liver evokes a perpetuating wound healing response, promoting the development of fibrosis and finally hepatocellular carcinoma (HCC; ref. 2). Among the cells in the liver, hepatic stellate cells are known as a main effector of wound healing and fibrosis following liver injury of any etiology (3), however, a useful blood marker to reflect the activity of those cells has not been found yet in the clinical setting.

Authors' Affiliations: Departments of [1]Clinical Laboratory Medicine and [2]Gastroenterology, Graduate School of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo; and [3]Mitsubishi Chemical Medicine Corporation, Ohwadashinden, Yachiyo-shi Chiba, Japan

Corresponding Author: Hitoshi Ikeda, Department of Clinical Laboratory Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Phone: 81-3-3815-5411; Fax: 81-3-5689-0495; E-mail: ikeda-1im@h.u-tokyo.ac.jp

doi: 10.1158/1055-9965.EPI-11-0464

©2011 American Association for Cancer Research.

In this context, we have focused on a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13), a defect of which increases unusually large multimers of von Willebrand factor in the plasma, causes platelet thrombosis under high sheer stress, and results finally in thrombotic thrombocytopenic purpura (4–6). With regard to the site of production, *ADAMTS13* mRNA expression was shown exclusively in the liver (7–9) and then both *ADAMTS13* mRNA expression and ADAMTS13 activity were determined primarily in hepatic stellate cells among the liver cells in mice (10). ADAMTS13 expression was also detected in hepatic stellate cells in human and thereby ADAMTS13 is reportedly produced in those cells (11). To elucidate a regulatory mechanism of plasma ADAMTS13 activity, we previously determined that selective hepatic stellate cell damage caused by dimethylnitrosamine in rats leads to decreased plasma ADAMTS13 activity (12). On the other hand, plasma ADAMTS13 activity was upregulated during the process of liver fibrosis due to cholestasis caused by bile duct ligation and steatohepatitis induced by a choline-deficient L-amino acid–defined diet in rats, in which hepatic stellate cells actively proliferate (13).

Downloaded from cebp.aacrjournals.org on February 21, 2012
Copyright © 2011 American Association for Cancer Research

Published OnlineFirst August 29, 2011; DOI:10.1158/1055-9965.EPI-11-0464

These results indicate that hepatic stellate cells play an important role in the regulation of plasma ADAMTS13 activity, although other sources of ADAMTS13 were reported (14–16).

On the basis of these previous findings, we wondered whether plasma ADAMTS13 could be a blood marker of hepatic stellate cells. To examine this, plasma ADAMTS13 was evaluated in patients with chronic hepatitis B or C, in whom chronic wound healing and fibrosis are observed with a high risk of HCC development (17), in which hepatic stellate cells play an important role (3). In this study, we have found that plasma ADAMTS13 was increased in relation with serum levels of aspartate aminotransferase (AST) or alanine aminotransferase (ALT), and the markers of liver fibrosis and that higher plasma ADAMTS13 was more frequently found in patients who later developed HCC.

Patients and Methods

Patients

Eighty-one patients with chronic hepatitis B and C, who visited the Department of Gastroenterology, the University of Tokyo Hospital, Tokyo, Japan, between April and August in 2007, were first enrolled. Chronic hepatitis B was defined as hepatitis B surface antigen (HBsAg) positivity, and chronic hepatitis C was defined as serum anti-hepatitis C virus antibody (HCVAb) positivity and a detectable HCV RNA level, having persistent liver damage for more than 6 months. Patients with HCC at the time of enrollment or with past history of HCC were excluded from this analysis.

Next, between July and September in 2009, 107 consecutive patients with chronic hepatitis B and C with HCC who were scheduled to undergo radiofrequency ablation (RFA) for HCC were enrolled.

All the studies were carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and were approved by the Institutional Research Ethics Committee of the Faculty of Medicine of the University of Tokyo. Informed consent from the patients was obtained for the use of the samples in this study.

Measurement of ADAMTS13 activity

ADAMTS13 enzymatic activity was measured manually using a chromogenic ELISA kit, ADAMTS13-act-ELISA (Kainos Inc./Technoclone GmbH), which captures products cleaved by ADAMTS13 using a sandwich method, and expressed as percentage of healthy control. The very high correlation of the values measured by classical VWF multimer assay and this novel chromogenic ADAMTS13-act-ELISA was reported previously (18).

Measurement of ADAMTS13 antigen level

ADAMTS13 antigen level was measured by a latex photometric immunoassay, in which suspended polystyrene latex particles coated with polyclonal antibody F(ab') 2 fragment against ADAMTS13 were employed. Antisera against ADAMTS13 were obtained by immunization with pCAG-ADAMTS13 plasmid DNA (donated by Dr. Soejima from The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) using electroporation. Latex agglutination was analyzed using LPIA-A700 (Mitsubishi Chemical Medience Co.), a fully automated quantitative latex photometric immunoassay instrument. ADAMTS13 antigen level in sample of each patient was expressed as the percentage of that in pooled normal human plasma.

Measurement of liver stiffness

Liver stiffness was measured by transient elastography (FibroScan 502; EchoSens) as described previously (19–21). Briefly, the measurements were done in the right lobe of the liver through the intercostal spaces, with the patient lying in the dorsal decubitus position, and were considered valid only when at least 10 acquisitions were successful, with a success rate of at least 60% and the ratio of interquartile range to the median value was larger than 30%. Liver stiffness value was expressed in kilopascals (kPa).

Patient follow-up and diagnosis of HCC

Patients without HCC were followed up at the outpatient clinic with monthly blood tests, including tumor markers and ultrasonography every 4 to 6 months. Contrast-enhanced computed tomography (CT) was done when serum alpha-fetoprotein (AFP) levels and/or plasma des-gamma-carboxy prothrombin (DCP) levels showed an abnormal rise and/or tumors were detected as possible HCC on ultrasonography. The diagnosis of HCC was based on typical findings on CT, that is, hyperattenuation in the arterial phase and hypoattenuation in the equilibrium phase (22–24).

The end points consisted of the interval between the first measurement of plasma ADAMTS13 activity and the detection of HCC development, death without HCC development, or the last examination until 30 July 2010, whichever came first. Death without HCC development was treated as censored data.

Radiofrequency ablation, patient follow-up, and analysis of HCC recurrence

The detailed procedure of RFA was meticulously described elsewhere (25). The indication criteria for RFA consisted of total bilirubin concentration less than 3.0 mg/dL and platelet count more than $5 \times 10^4/\mu L$. Patients with portal vein tumor thrombosis, massive refractory ascites, or extrahepatic metastasis were excluded. In general, RFA was done on patients with 3 or fewer lesions, each less than 3.0 cm in diameter. However, RFA was also done on patients who did not meet these criteria when complete ablation could be anticipated in all tumors without deteriorating liver function. After RFA, dynamic CT was done to evaluate treatment efficacy. Complete ablation was defined as hypoattenuation of the whole lesion together with the surrounding liver parenchyma as a safety margin.

Downloaded from cebp.aacrjournals.org on February 21, 2012
Copyright © 2011 American Association for Cancer Research

Published OnlineFirst August 29, 2011; DOI:10.1158/1055-9965.EPI-11-0464

Patients received additional RFA until complete ablation was confirmed for each HCC nodule.

The follow-up consisted of monthly blood tests and monitoring of tumor markers at the outpatient clinic, with ultrasonography and dynamic CT scan done every 4 months. HCC recurrence was diagnosed on the basis of the criteria as described earlier.

The end points consisted of the interval between the first ablation and the detection of HCC recurrence, death without recurrence, or the last examination until 30 September 2010, whichever came first. Death without recurrence was treated as censored data.

Statistical analysis

Comparisons between groups were made using Student's t test or χ^2 test. The correlation between 2 groups, in which the data points were distribution free, was analyzed using Spearman's rank correlation coefficient (ρs). The cumulative incidence of HCC was estimated using the Kaplan–Meier method. In the analysis of risk factors for hepatocarcinogenesis, we tested the following variables obtained at the time of entry in univariate and multivariate Cox proportional hazard regression analyses: age, sex, positivity for HBsAg and HCVAb, albumin, total bilirubin, AST, ALT, prothrombin time, platelet counts, liver stiffness value, APRI, AFP, DCP, and either plasma ADAMTS13 activity or antigen level. Multichotomous categorical variables were represented by corresponding binary dummy variables. Factors that had a $P < 0.2$ in univariate analysis were subsequently included in a multivariate Cox proportional hazard regression model, with stepwise selection of variables based on the Akaike information criterion (AIC). Data processing and analysis were done by using the S-plus Ver. 7 (TIBCO Software Inc.).

Results

Characteristics of the patients without HCC and correlation between plasma ADAMTS13 activity and clinical variables

The characteristics of the patients, who were first enrolled for the measurement of plasma ADAMTS13 activity, are summarized in Table 1. There were 21 patients with chronic hepatitis B and 60 patients with chronic hepatitis C. All the patients were outpatients without HCC at the time of enrollment and past history of HCC.

Plasma ADAMTS13 activity in these patients was 114.0 ± 45.4% (mean ± SD) of control, ranged from 28.0% to 221.5%, as shown in Table 1. Relationships between plasma ADAMTS13 activity and clinical variables are shown in Table 2. The significant correlations were determined between plasma ADAMTS13 activity and serum AST and ALT levels ($P < 0.001$). On the other hand, the significant correlations were also determined between plasma ADAMTS13 activity and the variables predicting the stage of liver fibrosis, liver stiffness value ($P < 0.001$), and aspartate aminotransferase-to-platelet ratio index (APRI; $P = 0.027$). Of note is the finding that plasma ADAMTS13 activity significantly correlated with serum AFP level ($P < 0.001$).

HCC development and risk analysis

Next, a potential link between plasma ADAMTS13 activity and HCC was examined. During the mean follow-up period of 35.4 months, one patient had been lost to follow-up evaluation and one patient died before HCC was identified. By the end of the follow-up, HCC developed in 10 patients, among whom 2 patients died of HCC. The cumulative incidence rates of HCC at

Table 1. Characteristics of patients without HCC or with HCC

Variables	Patients without HCC	Patients with HCC
Age (y)	63 ± 12 (23–85)	68.9 ± 8.5 (43–86)
Man/Woman	49/32	68/39
HBV/HCV	21/60	15/92
Albumin (g/dL)	4.1 ± 0.4 (3.1–4.9)	3.7 ± 0.6 (2.0–5.1)
AST (U/L)	48 ± 35 (3–270)	61.4 ± 39.2 (16–289)
ALT (U/L)	53 ± 66 (11–542)	54.1 ± 38.6 (11–276)
Platelet count (× 10^4/μL)	15.2 ± 6.3 (3.4–30.8)	10.8 ± 4.7 (3.4–25.2)
Prothrombin time (%)	87.7 ± 11.6 (49.2–100.0)	98.3 ± 5.2 (73.0–100.0)
Plasma ADAMTS13 activity (%)	114.0 ± 45.4 (28.0–221.5)	125.0 ± 32.4 (62.0–223.0)
Plasma ADAMTS13 antigen level (%)	Not measured	128.6 ± 39.6 (48.9–258.3)
Liver stiffness (kPa)	11.4 ± 9.2 (3.1–48.0)	28.5 ± 17.9 (6.1–75.0)
APRI	1.07 ± 1.00 (0.08–5.92)	1.89 ± 1.44 (0.22–7.81)
AFP (ng/mL)	12.6 ± 38.2 (1–319)	99.4 ± 361.2 (1–3,399)
DCP (mAu/mL)	18.1 ± 17.6 (10–165)	70.7 ± 194.3 (8–1,462)
Maximum size of HCC (mm)	Not available	17.8 ± 6.0 (6.0–33.0)

NOTE: Values are expressed as the mean ± SD (range).

Downloaded from cebp.aacrjournals.org on February 21, 2012
Copyright © 2011 American Association for Cancer Research

Published OnlineFirst August 29, 2011; DOI:10.1158/1055-9965.EPI-11-0464

Table 2. Relation between plasma ADAMTS13 activity and clinical variables in patients without HCC or with HCC

Variables	Patients without HCC		Patients with HCC	
	ρs[a]	P	ρs[a]	P
Age	−0.067	0.554	−0.030	0.760
AST (U/L)	0.360	<0.001	0.531	<0.001
ALT (U/L)	0.426	<0.001	0.519	<0.001
Albumin (g/dL)	−0.114	0.309	−0.146	0.133
Platelet count (× 10^4/μL)	−0.091	0.418	−0.129	0.185
Prothrombin time (%)	−0.343	<0.005	−0.029	0.764
Liver stiffness (kPa)	0.379	<0.001	0.216	0.026
APRI	0.245	0.027	0.403	<0.001
AFP (ng/mL)	0.465	<0.001	0.554	<0.001
DCP (mAu/mL)	0.135	0.230	−0.281	0.003
Size of HCC (mm)[b]	Not available		−0.075	0.571

[a]Spearman's rank correlation coefficient.
[b]Analyzed in patients with single nodule of HCC.

1, 2, and 3 years estimated by the Kaplan–Meier method were 4.9%, 9.1%, and 11.1%, respectively, as shown in Figure 1A. In these patients who developed HCC,

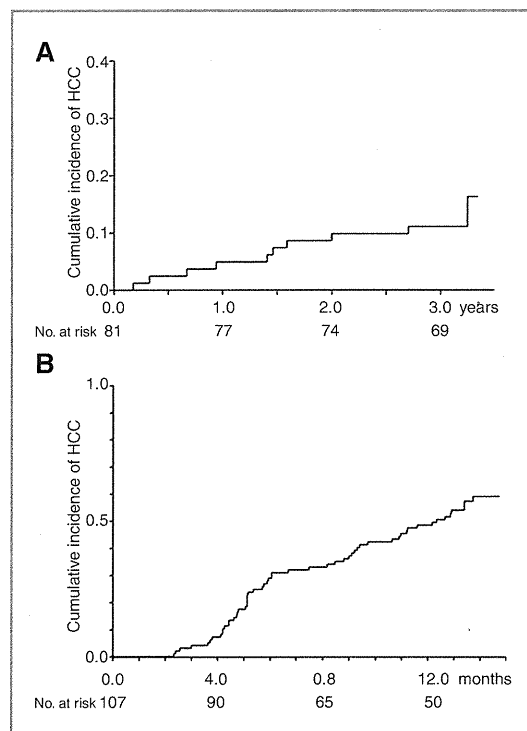

Figure 1. Cumulative incidence of HCC development (A) and recurrence (B).

plasma ADAMTS13 activity was significantly higher than that in patients who did not develop HCC ($P < 0.001$), as depicted in Table 3; plasma ADAMTS13 activity was 161.9 ± 33.8% in patients who developed HCC and 108.8 ± 42.2% in patients who did not develop HCC. Liver stiffness value was also significantly higher in patients with HCC development, and serum albumin level and prothrombin time (%) were significantly lower in those patients. Then, univariate analyses showed that the higher plasma ADAMTS13 activity was a risk for HCC development ($P < 0.001$; Table 4). Other significant risk factors for HCC included lower albumin level, higher ALT level, lower prothrombin time (%), and higher liver stiffness value. Next, stepwise variable selection with AIC was used to find the best model in multivariate analysis (Table 4), which revealed that the higher plasma ADAMTS13 activity ($P = 0.03$) and the higher liver stiffness value ($P = 0.03$) were the significant risk factors for HCC. These results suggest that plasma ADAMTS13 activity may predict HCC development in patients with chronic hepatitis B or C.

Then, the relation between plasma ADAMTS13 activity and HCC development was analyzed separately in patients with chronic hepatitis B and with chronic hepatitis C. In patients with chronic hepatitis B ($n = 20$), plasma ADAMTS13 activity was significantly higher in patients who developed HCC than that in patients who did not develop HCC ($P < 0.005$); plasma ADAMTS13 activity was 158.9 ± 36.7% in patients who developed HCC and 95.3 ± 35.0% in patients who did not develop HCC. Then, univariate analyses showed that the higher plasma ADAMTS13 activity was a risk for HCC development ($P < 0.001$), and further multivariate analysis revealed that the higher plasma ADAMTS13 activity was a significant risk factor for HCC ($P = 0.03$) in these patients. In patients

Downloaded from cebp.aacrjournals.org on February 21, 2012
Copyright © 2011 American Association for Cancer Research

Published OnlineFirst August 29, 2011; DOI:10.1158/1055-9965.EPI-11-0464

Table 3. Characteristics of patients according to HCC development and recurrence

Variables	Development (−)	Development (+)	P	Recurrence (−)	Recurrence (+)	P
Age (y)	63.0 ± 12.4	61.4 ± 12.4	0.695	70.2 ± 7.5	68.3 ± 9.2	0.267
Man/Woman	40/29	6/4	0.82	23/19	36/19	0.39
HBV/HCV	16/53	4/6	0.45	7/35	7/48	0.80
Albumin (g/dL)	4.1 ± 0.3	3.8 ± 0.6	0.024	3.8 ± 0.5	3.6 ± 0.6	0.296
AST (IU/L)	46.9 ± 36.6	55.1 ± 23.0	0.494	61.0 ± 46.5	60.5 ± 33.3	0.951
ALT (IU/L)	52.0 ± 68.8	63.2 ± 46.9	0.620	56.9 ± 46.7	49.7 ± 29.5	0.359
Platelet count (× 10^4/μL)	15.7 ± 6.5	12.2 ± 3.9	0.097	10.9 ± 5.5	10.5 ± 4.3	0.667
Prothrombin time (%)	89.5 ± 10.5	74.7 ± 11.6	<0.001	98.9 ± 3.5	97.5 ± 6.5	0.188
Plasma ADAMTS13 activity (%)	108.8 ± 42.2	161.9 ± 33.8	<0.001	116.8 ± 28.5	130.0 ± 30.8	0.039
Plasma ADAMTS13 antigen (%)	Not measured	Not measured		118.9 ± 35.4	134.3 ± 36.1	0.037
Liver stiffness (kPa)	9.2 ± 5.7	22.6 ± 13.7	<0.001	23.4 ± 15.0	30.6 ± 18.4	0.053
APRI	1.03 ± 1.03	1.35 ± 0.80	0.35	1.57 ± 0.81	1.47 ± 0.77	0.517
AFP (ng/mL)	10.7 ± 38.1	27.4 ± 41.4	0.203	131.5 ± 546.0	81.2 ± 158.1	0.521
DCP (mAu/mL)	17.9 ± 18.8	19.7 ± 8.2	0.766	114.0 ± 297.6	41.7 ± 64.3	0.082

NOTE: Values are expressed as the mean ± SD (range).

with chronic hepatitis C ($n = 59$), plasma ADAMTS13 activity was significantly higher in patients who develop HCC than that in patients who did not develop HCC ($P < 0.01$); plasma ADAMTS13 activity was 163.9 ± 35.2% in patients who developed HCC and 112.9 ± 43.6% in patients who did not develop HCC. Then, univariate analyses showed that the higher plasma ADAMTS13 activity was a risk for HCC development ($P < 0.001$), and multivariate analysis revealed that the higher plasma ADAMTS13 activity was a significant risk factor for HCC ($P = 0.02$) in these patients.

Characteristics of the patients with HCC and correlation between plasma ADAMTS13 activity or antigen level and clinical variables

To further examine a potential link between plasma ADAMTS13 and HCC, plasma ADAMTS13 activity and antigen level were measured in 107 patients with HCC. Their characteristics are summarized in Table 1. There were 15 patients with chronic hepatitis B and 92 patients with chronic hepatitis C.

Plasma ADAMTS13 activity in these patients was 124.9% ± 32.3% (mean ± SD) of control, ranged from 62.0% to 223.0%, and plasma ADAMTS13 antigen level, 128.3% ± 39.3% (mean ± SD) of control, ranged from 48.9% to 258.3%, respectively (Table 1). Of note, the strong correlation between plasma ADAMTS13 activity and plasma ADAMTS13 antigen level was observed (Spearman's rank; $\rho s = 0.803$, $P < 0.00001$, $n = 107$). Relationships between plasma ADAMTS13 activity and clinical variables are shown in Table 2. Same as in patients without HCC, the significant correlations were determined between plasma ADAMTS13 activity and serum AST and ALT levels ($P < 0.001$), liver stiffness value ($P = 0.026$), APRI ($P < 0.001$), and serum AFP level ($P < 0.001$). Of note, there was no significant correlation between plasma ADAMTS13 activity and maximum tumor size in patients with single nodule, suggesting that plasma ADAMTS13 activity is not a tumor marker of HCC.

Table 4. Risk factors for HCC development–univariate and multivariate analyses

Variable	HR (95% CI)	P
Univariate analysis		
ADAMTS13 (per 10% increase)	1.29 (1.11–1.50)	<0.001
Age (per 1 year increase)	0.990 (0.943–1.04)	0.68
Sex (male vs. female)	1.07 (0.563–2.02)	0.84
Hepatitis virus (HCV vs. HBV)	0.718 (0.380–1.36)	0.31
Albumin (per 1 g/dL increase)	0.208 (0.0477–0.905)	0.04
AST >40 U/L	1.73 (0.881–3.41)	0.11
ALT >40 U/L	2.06 (1.05–4.07)	0.04
PLT <15 × 10^4/μL	1.97 (0.908–4.29)	0.09
Prothrombin time (%; per 10% increase)	0.490 (0.324–0.743)	<0.001
Liver stiffness (per 10% increase)	1.16 (1.07–1.26)	<0.001
APRI (per 10% increase)	1.05 (0.983–1.13)	0.14
AFP >20 ng/mL	1.71 (0.785–3.71)	0.18
DCP >40 mAU/mL[a]	NA	
Multivariate analysis		
ADAMTS13 (per 10% increase)	1.20 (1.02–1.40)	0.03
Liver stiffness (per 10% increase)	1.12 (1.01–1.23)	0.03

[a]Not accessed as only DCP was more than 40 mAU/mL in only 1 patient.

Downloaded from cebp.aacrjournals.org on February 21, 2012
Copyright © 2011 American Association for Cancer Research

Published OnlineFirst August 29, 2011; DOI:10.1158/1055-9965.EPI-11-0464

HCC recurrence and risk analysis

During the follow-up period of 12 months, 1 patient died without HCC. Two patients who developed extrahepatic recurrence and 3 patients who developed recurrence at a site adjacent to the treated site were excluded from the analysis. Four patients who were treated with IFN were not analyzed because IFN is known to reduce the risk of HCC development in chronic hepatitis B and C (26, 27). By the end of the follow-up, HCC recurrence was determined in 55 patients. The cumulative recurrence rates of HCC by the Kaplan–Meier method are shown in Figure 1B. The characteristics of patients with or without HCC recurrence are shown in Table 3. Among the various parameters, plasma ADAMTS13 activity ($P = 0.039$) and antigen level ($P = 0.037$) were significantly higher in patients with HCC recurrence than those in patients without HCC recurrence (Table 3). No significant differences were determined in other parameters between patients with and without HCC recurrence. Although there was no significant risk factor for HCC recurrence in univariate analyses (Table 5), plasma ADAMTS13 activity was retained as a significant risk factor of HCC recurrence ($P = 0.028$) in the multivariate Cox proportional hazard model, as shown in Table 5. When plasma ADAMTS13 antigen level was analyzed instead of plasma ADAMTS13 activity level, plasma ADAMTS13 antigen level was also a significant risk factor of HCC recurrence ($P = 0.007$) in multivariate analysis. These results suggest that plasma ADAMTS13 activity may predict HCC recurrence in patients with chronic hepatitis B or C.

Table 5. Risk factors for HCC recurrence–univariate and multivariate analyses

Variable	HR (95%CI)	P
Univariate analysis		
ADAMTS13 activity (per 10% increase)	1.106 (0.997–1.228)	0.052
Age (per 1 year increase)	0.982 (0.953–1.013)	0.25
Sex (male vs. female)	1.22 (0.70–2.13)	0.49
Hepatitis virus (HCV vs. HBV)	1.10 (0.50–2.44)	0.81
Albumin (per 1g/dL increase)	0.723 (0.432–1.210)	0.22
AST > 40 IU/L	1.35 (0.75–2.42)	0.31
ALT > 40 IU/L	1.00 (0.58–1.71)	0.99
PLT < 15 × 10^4/μL	1.25 (0.65–2.43)	0.51
Prothrombin Activity (per 10% increase)	0.88 (0.42–1.07)	0.20
Liver stiffness (per 10% increase)	1.12 (0.98–1.28)	0.11
APRI (per 10% increase)	1.00 (0.973–1.04)	0.81
AFP > 20 ng/mL	1.24 (0.73–2.11)	0.42
DCP > 40 mAU/mL	1.17 (0.64–2.14)	0.62
Multivariate analysis		
ADAMTS13 activity (per 10% increase)	1.14 (1.01–1.29)	0.028

The relation between plasma ADAMTS13 activity and HCC recurrence was also analyzed separately in patients with chronic hepatitis B and with chronic hepatitis C. In patients with chronic hepatitis B ($n = 14$), plasma ADAMTS13 activity or antigen level was not different between patients with (105.0 ± 34.0% or 97.3 ± 24.7%) and without HCC recurrence (104.0 ± 16.3% or 98.9 ± 14.4%), possibly because the number of patients analyzed was small. On the other hand, in patients with chronic hepatitis C ($n = 83$), plasma ADAMTS13 activity or antigen level was significantly higher in patients with HCC recurrence (133.2 ± 28.9% or 139.7 ± 34.4%) than that in patients without HCC recurrence (119.4 ± 29.8% or 122.9 ± 37.1; $P = 0.037$ or $P = 0.036$). Multivariate analysis revealed that the higher plasma ADAMTS13 activity or antigen level was a significant risk factor for HCC ($P = 0.024$ or $P = 0.005$) in these patients.

Discussion

In the current study, plasma ADAMTS13 activity or antigen level significantly correlated with serum AST and ALT levels and also the variables predicting the stage of liver fibrosis, liver stiffness value, and APRI in patients with chronic hepatitis B or C, irrespective of the presence of HCC. Serum levels of AST and ALT reflect hepatocellular damage, and higher hepatocellular damage generally induces a higher wound healing response. Thus, our current findings may be in line with our speculation that plasma ADAMTS13 activity or antigen level reflects the activity of hepatic stellate cells as a main effector of wound healing and fibrosis in the liver.

Major finding of this study is that the higher plasma ADAMTS13 activity or antigen level was a significant risk factor for HCC development. With regard to HCC development among patients with chronic hepatitis B or C without the past history of HCC, plasma ADAMTS13 activity was higher in the patients who developed HCC than in those who did not develop HCC. Among the various clinical parameters, univariate analysis revealed that the higher plasma ADAMTS13 activity was a significant risk factor for HCC development. Then, multivariate analysis showed that the higher plasma ADAMTS13 activity was a significantly predicting factor for hepatocarcinogenesis, independent of other significant risk factors for HCC development, including the variables predicting the stage of liver fibrosis. This potential link between plasma ADAMTS13 activity and HCC development was further observed in the analysis of HCC recurrence: the patients who had HCC recurrence during the 1-year follow-up period had also significantly higher plasma ADAMTS13 activity or antigen level than those who did not have HCC recurrence. Then, only plasma ADAMTS13 activity or antigen level was retained in the multivariate Cox proportional hazard model as a significant risk factor of recurrence.

Downloaded from cebp.aacrjournals.org on February 21, 2012
Copyright © 2011 American Association for Cancer Research

Published OnlineFirst August 29, 2011; DOI:10.1158/1055-9965.EPI-11-0464

Then, we wondered how HCC development might be predictable by the activity or antigen level of plasma ADAMTS13, whose source is mainly hepatic stellate cells, as a key player of liver fibrosis. To explain this, the notion that advanced liver fibrosis is the strong risk factor for HCC development (17) may be important. Furthermore, the recent evidence suggests a potential direct link between hepatic stellate cells and HCC (3), as follows.

It is well known that HCC usually develops in the liver already suffering from chronic liver disease (2). In particular, HCV-related cirrhosis is associated with an extremely high risk of HCC development, with a reported annual incidence ranging between 3% and 8% (28–30). Thus, advanced liver fibrosis is one of the strongest risk factors for HCC development. In fact, the higher liver stiffness value is reportedly a strong risk for HCC development (21). In this study, a significant correlation was observed between plasma ADAMTS13 activity or antigen level and the variables predicting the stage of liver fibrosis such as liver stiffness value. Thus, we have first speculated that plasma ADAMTS13 activity is retained as a risk factor for HCC development by univariate analysis because plasma ADAMTS13 activity may reflect liver fibrosis. However, the higher plasma ADAMTS13 activity was a significant risk factor for HCC development, independent of liver stiffness value by multivariate analysis. Furthermore, in the analysis of HCC recurrence, plasma ADAMTS13 activity or antigen level was retained as a significant risk for HCC development, but not liver stiffness value, by multivariate analysis. The current finding that plasma ADAMTS13 activity or antigen level significantly correlated with serum AST and ALT levels may explain this. Of note, it was previously shown that the higher serum ALT is associated with the higher rate of incidence of HCC development (31) and HCC recurrence after the surgical treatment (32) in HCV-related cirrhosis, suggesting that more hepatocellular damage increases a risk for HCC development in the liver of the same stage of fibrosis. Because plasma ADAMTS13 activity or antigen level reflect hepatocellular damage and subsequent wound healing as well as liver fibrosis stage, plasma ADAMTS13 activity, or antigen level may act distinctly from liver stiffness value in the risk analysis of HCC development.

Alternatively, the prediction of HCC development by plasma ADAMTS13 activity or antigen level may be explained by a potential direct link between hepatic stellate cells and HCC, which has been recently reported (3). This concept is suggested based on the findings that hepatic stellate cells express the stem cell marker of CD133 (33) and both hedgehog (34, 35) and Wnt signaling (36) are found in hepatic stellate cells, two pathways implicated in stem cell differentiation and cancer (37). Furthermore, the direct promotion of tumorigenicity of HCC by hepatic stellate cells has been reported (38).

In human studies, the alteration of plasma ADAMTS13 activity in chronic liver disease has already been reported (39–44). In patients with liver cirrhosis, plasma ADAMTS13 activity was shown to be decreased (39) in relation to the severity of cirrhosis (44), although the wide range of values were detected compared with normal controls (43). In contrast, Lisman and colleagues showed that plasma ADAMTS13 activity in patients with liver cirrhosis was highly variable and not significantly different from that in normal controls (42). In line with the latter report, plasma ADAMTS13 activity in chronic hepatitis B and C was variable in the current study. We speculate that these distinct results of plasma ADAMTS13 activity in chronic liver disease may be caused by the characteristics of the patients enrolled in the analysis. The patients with reduced plasma ADAMTS13 activity in the previous reports (39, 43, 44) might have minimal hepatitis activity, that is, minimal wound healing response. Highly variable activity of plasma ADAMTS13 in liver cirrhosis (42) might also be explained by the variable hepatitis activity in those patients. This issue should be further clarified.

In conclusion, the higher plasma ADAMTS13 activity or antigen level was a significantly independent risk factor for HCC development in chronic hepatitis B or C, suggesting that plasma ADAMTS13 activity and antigen level may be useful in the prediction of hepatocarcinogenesis in chronic liver disease. It should be further evaluated whether plasma ADAMTS13 activity and antigen level could be useful as a predictor of HCC development with a larger sample size and also with other etiology of underlying chronic liver disease such as NASH.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by Kurozumi Medical Foundation Fund.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 18, 2011; revised August 19, 2011; accepted August 20, 2011; published OnlineFirst August 29, 2011.

References

1. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 1986;315:1650–9.
2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907–17.
3. Friedman SL. Mechanisms of hepatic fibrogenesis. Gastroenterology 2008;134:1655–69.
4. Furlan M, Robles R, Solenthaler M, Wassmer M, Sandoz P, Lammle B. Deficient activity of von Willebrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. Blood 1997;89:3097–103.
5. Kinoshita S, Yoshioka A, Park YD, Ishizashi H, Konno M, Funato M, et al. Upshaw-Schulman syndrome revisited: a concept of

Downloaded from cebp.aacrjournals.org on February 21, 2012
Copyright © 2011 American Association for Cancer Research

Published OnlineFirst August 29, 2011; DOI:10.1158/1055-9965.EPI-11-0464

congenital thrombotic thrombocytopenic purpura. Int J Hematol 2001;74:101–8.
6. Sasahara Y, Kumaki S, Ohashi Y, Minegishi M, Kano H, Bessho F, et al. Deficient activity of von Willebrand factor-cleaving protease in patients with Upshaw-Schulman syndrome. Int J Hematol 2001;74:109–14.
7. Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. J Biol Chem 2001;276:41059–63.
8. Soejima K, Mimura N, Hirashima M, Maeda H, Hamamoto T, Nakagaki T, et al. A novel human metalloprotease synthesized in the liver and secreted into the blood: possibly, the von Willebrand factor-cleaving protease? J Biochem (Tokyo) 2001;130:475–80.
9. Cal S, Obaya AJ, Llamazares M, Garabaya C, Quesada V, Lopez-Otin C. Cloning, expression analysis, and structural characterization of seven novel human ADAMTSs, a family of metalloproteinases with disintegrin and thrombospondin-1 domains. Gene 2002;283:49–62.
10. Zhou W, Inada M, Lee TP, Benten D, Lyubsky S, Bouhassira EE, et al. ADAMTS13 is expressed in hepatic stellate cells. Lab Invest 2005;85: 780–8.
11. Uemura M, Tatsumi K, Matsumoto M, Fujimoto M, Matsuyama T, Ishikawa M, et al. Localization of ADAMTS13 to the stellate cells of human liver. Blood 2005;106:922–4.
12. Kume Y, Ikeda H, Inoue M, Tejima K, Tomiya T, Nishikawa T, et al. Hepatic stellate cell damage may lead to decreased plasma ADAMTS13 activity in rats. FEBS Lett 2007;581:1631–4.
13. Watanabe N, Ikeda H, Kume Y, Satoh Y, Kaneko M, Takai D, et al. Increased production of ADAMTS13 in hepatic stellate cells contributes to enhanced plasma ADAMTS13 activity in rat models of cholestasis and steatohepatitis. Thromb Haemost 2009;102:389–96.
14. Liu L, Choi H, Bernardo A, Bergeron AL, Nolasco L, Ruan C, et al. Platelet-derived VWF-cleaving metalloprotease ADAMTS-13. J Thromb Haemost 2005;3:2536–44.
15. Turner N, Nolasco L, Tao Z, Dong JF, Moake J. Human endothelial cells synthesize and release ADAMTS-13. J Thromb Haemost 2006;4: 1396–404.
16. Manea M, Kristoffersson A, Schneppenheim R, Saleem MA, Mathieson PW, Morgelin M, et al. Podocytes express ADAMTS13 in normal renal cortex and in patients with thrombotic thrombocytopenic purpura. Br J Haematol 2007;138:651–62.
17. El-Serag HB, Marrero JA, Rudolph L, Reddy KR. Diagnosis and treatment of hepatocellular carcinoma. Gastroenterology 2008;134: 1752–63.
18. Kato S, Matsumoto M, Matsuyama T, Isonishi A, Hiura H, Fujimura Y. Novel monoclonal antibody-based enzyme immunoassay for determining plasma levels of ADAMTS13 activity. Transfusion 2006;46: 1444–52.
19. Castera L, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology 2005;128:343–50.
20. Ziol M, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, et al. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. Hepatology 2005;41:48–54.
21. Masuzaki R, Tateishi R, Yoshida H, Goto E, Sato T, Ohki T, et al. Prospective risk assessment for hepatocellular carcinoma development in patients with chronic hepatitis C by transient elastography. Hepatology 2009;49:1954–61.
22. Torzilli G, Minagawa M, Takayama T, Inoue K, Hui AM, Kubota K, et al. Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. Hepatology 1999;30:889–93.
23. Teratani T, Yoshida H, Shiina S, Obi S, Sato S, Koike Y, et al. A novel display of reconstruction computed tomography for the detection of small hepatocellular carcinoma. Liver Int 2004;24:619–24.
24. Fujishima T, Yoshida H, Obi S, Shiina S, Kanda M, Tateishi R, et al. Analysis of factors influencing hepatocellular carcinoma detection: efficient use of computed tomography during arterial portography and during hepatic arteriography. J Gastroenterol 2005;40:266–73.
25. Omata M, Tateishi R, Yoshida H, Shiina S. Treatment of hepatocellular carcinoma by percutaneous tumor ablation methods: Ethanol injection therapy and radiofrequency ablation. Gastroenterology 2004;127: S159–66.
26. Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Fukuda M, et al. Interferon decreases hepatocellular carcinogenesis in patients with cirrhosis caused by the hepatitis B virus: a pilot study. Cancer 1998;82: 827–35.
27. Kasahara A, Hayashi N, Mochizuki K, Takayanagi M, Yoshioka K, Kakumu S, et al. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. Hepatology 1998;27:1394–402.
28. Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of hepatocarcinogenesis by interferon therapy. Ann Intern Med 1999;131:174–81.
29. Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med 1993;328:1797–801.
30. Ikeda K, Saitoh S, Koida I, Arase Y, Tsubota A, Chayama K, et al. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. Hepatology 1993;18:47–53.
31. Tarao K, Rino Y, Ohkawa S, Shimizu A, Tamai S, Miyakawa K, et al. Association between high serum alanine aminotransferase levels and more rapid development and higher rate of incidence of hepatocellular carcinoma in patients with hepatitis C virus-associated cirrhosis. Cancer 1999;86:589–95.
32. Tarao K, Takemiya S, Tamai S, Sugimasa Y, Ohkawa S, Akaike M, et al. Relationship between the recurrence of hepatocellular carcinoma (HCC) and serum alanine aminotransferase levels in hepatectomized patients with hepatitis C virus-associated cirrhosis and HCC. Cancer 1997;79:688–94.
33. Kordes C, Sawitza I, Muller-Marbach A, Ale-Agha N, Keitel V, Klonowski-Stumpe H, et al. CD133+ hepatic stellate cells are progenitor cells. Biochem Biophys Res Commun 2007;352:410–7.
34. Sicklick JK, Li YX, Choi SS, Qi Y, Chen W, Bustamante M, et al. Role for hedgehog signaling in hepatic stellate cell activation and viability. Lab Invest 2005;85:1368–80.
35. Yang L, Wang Y, Mao H, Fleig S, Omenetti A, Brown KD, et al. Sonic hedgehog is an autocrine viability factor for myofibroblastic hepatic stellate cells. J Hepatol 2008;48:98–106.
36. Myung SJ, Yoon JH, Gwak GY, Kim W, Lee JH, Kim KM, et al. Wnt signaling enhances the activation and survival of human hepatic stellate cells. FEBS Lett 2007;581:2954–8.
37. Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. Nature 2001;411:349–54.
38. Amann T, Bataille F, Spruss T, Muhlbauer M, Gabele E, Scholmerich J, et al. Activated hepatic stellate cells promote tumorigenicity of hepatocellular carcinoma. Cancer Sci 2009;100:646–53.
39. Mannucci PM, Canciani MT, Forza I, Lussana F, Lattuada A, Rossi E. Changes in health and disease of the metalloprotease that cleaves von Willebrand factor. Blood 2001;98:2730–5.
40. Kavakli K, Canciani MT, Mannucci PM. Plasma levels of the von Willebrand factor-cleaving protease in physiological and pathological conditions in children. Pediatr Hematol Oncol 2002;19:467–73.
41. Uemura M, Matsuyama T, Ishikawa M, Fujimoto M, Kojima H, Sakurai S, et al. Decreased activity of plasma ADAMTS13 may contribute to the development of liver disturbance and multiorgan failure in patients with alcoholic hepatitis. Alcohol Clin Exp Res 2005;29:264S–71S.
42. Lisman T, Bongers TN, Adelmeijer J, Janssen HL, de Maat MP, de Groot PG, et al. Elevated levels of von Willebrand factor in cirrhosis support platelet adhesion despite reduced functional capacity. Hepatology 2006;44:53–61.
43. Feys HB, Canciani MT, Peyvandi F, Deckmyn H, Vanhoorelbeke K, Mannucci PM. ADAMTS13 activity to antigen ratio in physiological and pathological conditions associated with an increased risk of thrombosis. Br J Haematol 2007;138:534–40.
44. Uemura M, Fujimura Y, Matsumoto M, Ishizashi H, Kato S, Matsuyama T, et al. Comprehensive analysis of ADAMTS13 in patients with liver cirrhosis. Thromb Haemost 2008;99:1019–29.

Downloaded from cebp.aacrjournals.org on February 21, 2012
Copyright © 2011 American Association for Cancer Research

Liver Injury Induced by the Japanese Herbal Drug Kamishoyosan

Hiromu Inoue, MD, PhD[1]
Sho Yamazaki, MD[2]
Maki Shimizu, MD[1]
Hiroshi Uozaki, MD, PhD[3]
Tadashi Goto, MD, PhD[1]
Shin Ohnishi, MD, PhD[1]
Kazuhiko Koike, MD, PhD[1]

[1]Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [2]General Education Center, University of Tokyo Hospital, Tokyo, Japan; [3]Department of Pathology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Traditional Japanese herbal medicines (kampo medicines) are currently used for various purposes, and they have even shown effectiveness in some cases that were resistant to conventional treatments. Most general practitioners in Japan consider kampo medicine to be safe and less harmful than many conventional medications. As described in this case report, however, kamishoyosan given to a 48-year-old woman for menopausal disturbance appeared to induce liver injury.

Case Report

A 48-year-old woman was admitted to our hospital with acute liver injury. Two years earlier, she had taken the herbal medicine kamishoyosan for 1 month to treat symptoms of postmenopausal syndrome. Two months before admission, she had started taking kamishoyosan again due to a recurrence of hot flashes and night sweats. Two weeks after restarting treatment with kamishoyosan, she underwent a routine checkup that revealed abnormal liver function test results; her serum aspartate aminotransferase (AST) level was 64 IU/L, and her alanine aminotransferase (ALT) level was 102 IU/L. She was advised to go to the hospital for a detailed evaluation of her condition.

No contributory family history was identified. The patient did not drink alcohol or smoke cigarettes, and she had not used illicit drugs. She did not have any risk factors for HIV infection, had not traveled abroad, and did not have a habit of eating raw meat. She was afebrile but reported general fatigue. On physical examination, she was conscious and alert. Her conjunctivae were icteric but not anemic. Her abdomen was soft and flat with no tenderness. Her spleen and liver were not palpable, and superficial lymph nodes were not swollen. No skin rash was apparent, and neurologic examination showed no abnormalities. Her blood pressure was 106/58 mmHg, and her body temperature was 36.5° C. Laboratory tests revealed a white blood cell count of 4.7×10^3 cells/μL, hemoglobin level of 13.4 g/dL, platelet count of 29.8×10^4/μL, total protein level of 6.3 g/dL, albumin level of 3.9 g/dL, total bilirubin level of 12.8 mg/dL, direct bilirubin level of 8.9 mg/dL, AST level of 900 IU/L, ALT level of 972 IU/L, alkaline phosphatase level of 420 IU/L, and prothrombin time of 99%. Tests for markers of hepatitis A, B, and C virus infection; cytomegalovirus infection; herpes simplex virus infection; Epstein-Barr virus infection; and HIV infection yielded negative results. Test results for antinuclear antibody, anti–mitochondrial-M2 antibody, anti–smooth muscle antibody, and anti–liver/kidney/microsome-1 antibody were all negative. Levels of immunoglobulin (Ig)A, IgG, and IgM were 265 mg/dL, 969 mg/dL, and 174 mg/dL, respectively. Abdominal ultrasonography did not detect dilatation of the bile duct, swelling of the gallbladder, or abnormal liver size. Computed tomography with contrast medium showed an almost homogeneous liver. These results were compatible with acute liver injury.

Drug-induced liver injury due to kamishoyosan was suspected, and the medication was stopped. One

Address correspondence to:
Dr. Hiromu Inoue, Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan;
Tel: +81-3-3815-5411; Fax: +81-3-5800-6937; E-mail: hiromuinoue-gi@umin.net

week after admission, a liver biopsy was performed (Figure 1). Pathologic examination of the liver revealed necrosis and acidophilic degeneration of hepatocytes in the parenchyma. The portal tract was enlarged, with infiltration of lymphocytes and eosinophils, but few plasma cells were observed.

In addition to bed rest, treatment with intravenous glycyrrhizin (80 mL/day) was started following the liver biopsy. Aminotransferase and bilirubin levels gradually normalized. Changes in bilirubin, AST, and ALT levels are shown in Figure 2. The patient was discharged on Day 26 after admission; at this time, she was instructed to begin taking ursodeoxycholic acid (600 mg/day). Liver function test results had almost returned to normal by 42 days after discharge.

Discussion

Herbal medicines have been widely used around the world as alternative medicines. Clinicians are often confronted with situations in which conventional medicines are ineffective and patients' symptoms remain unrelieved, in which case herbal medicines may be tried. Despite a lack of evidence, kampo medicine is widely seen in Japan as offering an alternative treatment for various diseases. Kamishoyosan, which reduces levels of cytokines such as interleukin (IL)-6 and IL-8, is effective against hot flashes due to menopausal syndrome.[1] Kamishoyosan also has anxiolytic and antidepressive effects, and widespread use of kamishoyosan for psychiatric and neuropathic disorders can be expected.[2-5] Although kamishoyosan is widely used as an alternative drug, few side effects have been reported.

In this case, the patient was not taking any drugs besides kamishoyosan. Nevertheless, she reported taking vitamins intermittently, so vitamins could not be absolutely excluded as possible etiologic agents. However, her use of vitamins was infrequent, so this possibility seems unlikely. Two years before admission, she had taken kamishoyosan for menopausal syndrome. After treatment for 1 month, her symptoms resolved and the medication was stopped. Several months before admission, kamishoyosan treatment was restarted because of recurrent symptoms. Liver biopsy showed invasion of eosinophils into the portal tract in the liver. The mechanism of hepatic injury was complicated, but immunoallergic mechanisms were suggested.

Melchart and colleagues investigated the frequency of liver enzyme elevations in 1,507 patients treated with traditional Chinese herbs. A greater–than–2-fold elevation in ALT values was observed in 14 patients (0.9%).[6] In another study, Nakazawa and coworkers examined 305 outpatients who were given kampo medicine and found that 15 patients showed elevated ALT levels.[7] The researchers reported that 87% of liver injury in these patients occurred more than 3 months after initiation of therapy. Liver injury was mild in almost all reported cases, but periodic evaluation of liver function is very important.

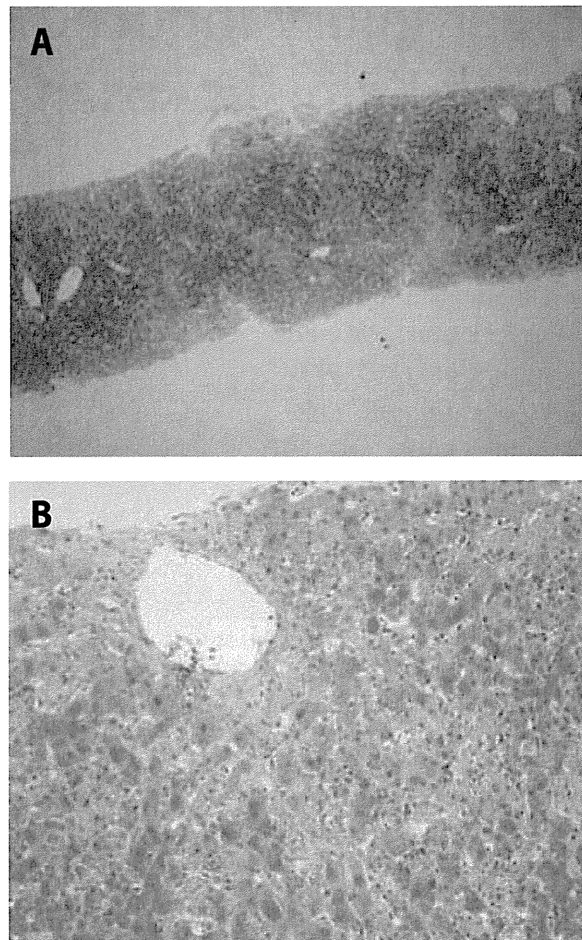

Figure 1. Histopathologic examination revealed expansion of the portal triad due to infiltration of many inflammatory cells and dropout of many hepatocytes (hematoxylin and eosin stain, 40× magnification; A). Acidophilic degeneration of hepatocytes and bile-stained hepatocytes are shown in the parenchyma, but no cholestasis was apparent in the small bile duct (hematoxylin and eosin stain, 200× magnification; B).

The same report also noted that *Scutellariae radix* was the only component common to all kampo medicines that caused liver injury.[7] Terada and colleagues studied interstitial pneumonia (IP) and liver dysfunction (LD) associated with kampo medicine and found that *Scutellariae radix* was contained in kampo medicines taken by 94% of IP patients and 89% of LD patients.[8] However, kamishoyosan is made from *Bupleurum radix*, *Peony radix*, *Atractyloidis raceae rhizome*, Japanese *Angelica radix*,

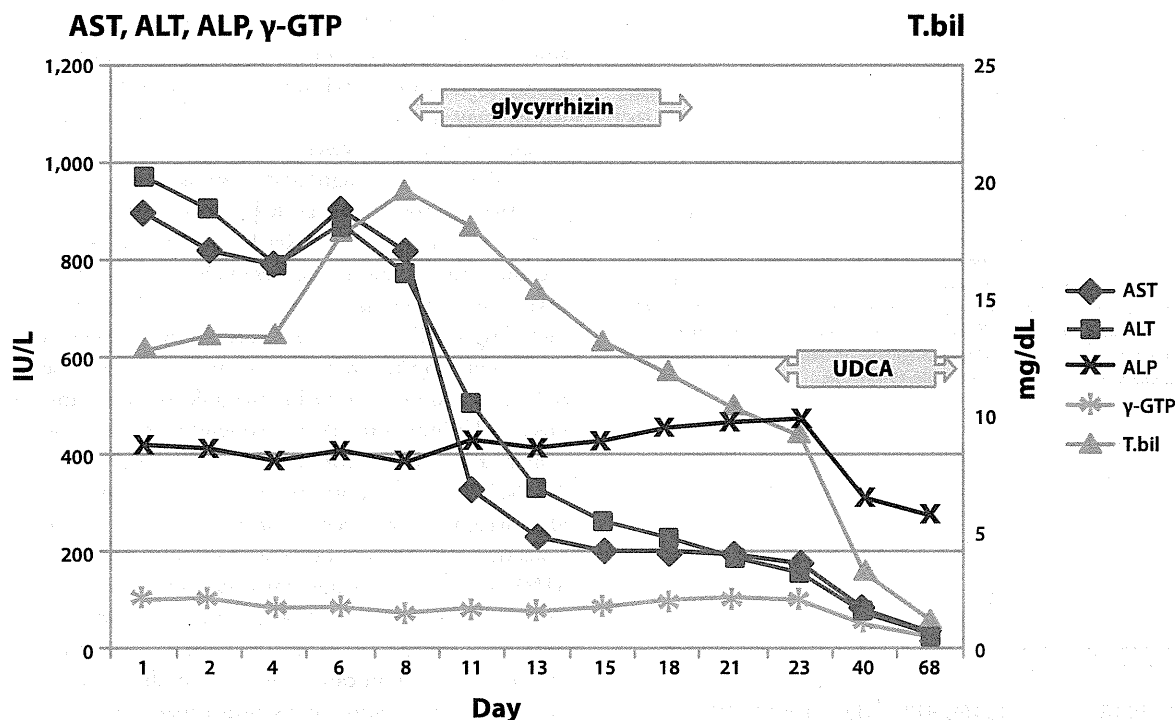

Figure 2. After infusion of glycyrrhizin, levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T.bil) improved, but levels of alkaline phosphatase (ALP) and gamma-glutamyltransferase (γ-GTP) were unchanged. Ursodeoxycholic acid (UDCA) was started after discharge, and values of ALP and γ-GTP returned to normal ranges. Prothrombin time was maintained above 95% during admission (data not shown).

Hoelen, *Gardenia fructus*, *Moutan cortex*, *Ginger rhizome*, *Glycyrrhiza root*, and *Mentha herb*; it does not contain *Scutellariae radix*. Thus, care must be taken regarding kampo medicine–induced liver injury even if the formulation does not contain *Scutellariae radix*. Kampo medicine contains several components (and each component contains multiple ingredients), which makes detecting causative ingredients difficult. However, mechanisms of liver damage caused by several herbal medicine ingredients have recently been elucidated.[9,10] Further investigation is necessary.

In this case, the patient was middle-aged, so it was important to differentiate kampo medicine–induced liver injury from autoimmune hepatitis. In the acute phase of autoimmune hepatitis, test results might be negative for antinuclear antibody, and hypergammaglobulinemia may not be detected.[11] The possibility of autoimmune hepatitis must, therefore, be taken into account. However, liver biopsy in this case showed scarce infiltration of plasma cells despite the presence of many eosinophils in the portal tract. The results of liver biopsy were thus compatible with drug-induced liver damage. Histologic evaluation, as in this case, is important.[12]

As mentioned above, use of kampo medicine has been increasing. Therefore, further clarification of the mechanisms underlying kampo medicine activity is warranted; as a first step, clinicians need to accumulate case reports such as this one.

References

1. Yasui T, Matsui S, Yamamoto S, et al. Effects of Japanese traditional medicines on circulating cytokine levels in women with hot flashes. *Menopause.* 2011;18:85-92.
2. Toriizuka K, Kamiki H, Ohmura N, et al. Anxiolytic effect of *Gardeniae Fructus*-extract containing active ingredient from Kamishoyosan, a Japanese traditional Kampo medicine. *Life Sci.* 2005;77:3010-3020.
3. Mantani N, Hisanaga A, Korure T, et al. Four cases of panic disorder successfully treated with Kampo (Japanese herbal) medicines: Kamishoyosan and Hangekoboku-to. *Psychiatry Clin Neurosci.* 2002;56:617-620.
4. Yamada K, Kanba S. Effectiveness of kamishoyosan for premenstrual dysphoric disorder: open-labeled pilot study. *Psychiatry Clin Neurosci.* 2007;61:323-325.
5. Lee JG, Shin BS, Lee YC, et al. Clinical effectiveness of the Kampo medicine kamishoyosan for adjunctive treatment of tardive dyskinesia with schizophrenia: a 16-week open trial. *Psychiatry Clin Neurosci.* 2007;61:509-514.
6. Melchart D, Linde K, Hager S, et al. Liver enzyme elevations in patients treated with traditional Chinese medicine. *JAMA.* 1999;282:28-29.

7. Nakazawa Y, Suzuki S, Negishi E, et al. Investigation of changes in ALT levels after administration of kampo medicines at Akiba Hospital. *Jpn J Pharm Health Care Sci.* 2006;32:504-510.
8. Terada M, Kitazawa H, Kawakami J, et al. Pharmacoepidemiology of interstitial pneumonia and liver dysfunction associated with kampo medicine. *Jpn J Pharm Health Care Sci.* 2002;28:425-434.
9. Willett KL, Roth RA, Walker L. Workshop overview: hepatotoxicity assessment for botanical dietary and supplements. *Toxicol Sci.* 2004;79:4-9.
10. De Berardinis V, Moulis C, Maurice M, et al. Human microsomal epoxide hydrolase is the target of germander induced autoantibodies on the surface of human hepatocytes. *Mol Pharmacol.* 2000;58:542-551.
11. Miyake Y, Iwasaki Y, Kobashi H, et al. Clinical features of antinuclear antibodies-negative type 1 autoimmune hepatitis. *Hepatol Res.* 2009;39:241-246.
12. Fujiwara K, Fukuda Y, Yokosuka O. Precise histological evaluation of liver biopsy specimen is indispensable for diagnosis and treatment of acute-onset autoimmune hepatitis. *J Gastroenterol.* 2008;43:951-958.

Review

Herbal and Dietary Supplement–Induced Liver Injury

Jonathan M. Fenkel, MD

Victor J. Navarro, MD

Division of Gastroenterology and Hepatology
Thomas Jefferson University Hospital
Philadelphia, Pennsylvania

Herbal and dietary supplements (HDS) are used by nearly 1 in 5 Americans, but their use is not commonly reported to, or detected by, healthcare providers.[1,2] HDS use is even more common outside the United States.[3,4] Patterns of liver injury are highly variable, even among cases where injury is purported to result from the same product. The liver is often the target of HDS toxicity, given its integral role in metabolism, and HDS-induced liver injury is a common cause of acute liver failure in the United States.[5,6]

Determining causality in HDS-induced liver injury is often difficult, as patients frequently take supplements along with many prescribed and nonprescribed medications. As such, the interaction between HDS and other medications often cannot be gauged. Further confounding the attribution of causality in cases of hepatotoxicity among patients consuming HDS are the potential multiplicity of ingredients within any given product (some of which may not be identified on the label) and seasonal variations in the harvesting of natural products, which could affect their strength and composition.

Address correspondence to:
Dr. Victor J. Navarro, 132 S. 10th St., Suite 480, Main Building, Philadelphia, PA 19107; Tel: 215-955-8900; Fax: 215-503-2146; E-mail: victor.navarro@jefferson.edu

The diagnosis of hepatotoxicity associated with HDS is typically made after excluding viral, autoimmune, metabolic, and anatomic causes of liver test abnormalities. Several models to assess causality exist in the literature, including the Roussel Uclaf Causality Assessment Model (RUCAM) and the Maria & Victorino (M&V) scale.[7,8] Still, expert opinion remains the gold standard for diagnosis.[9] RUCAM is the most frequently referenced scoring system, but it is not commonly used in clinical practice. The M&V scale is more specific but less sensitive, and it gives weight to prior reports in the literature for medications that have been in existence for less than 5 years. The M&V scale tends to underattribute causality compared to RUCAM, while RUCAM tends to underattribute causality compared to expert opinion.[9,10] Expert opinion can also vary significantly between evaluators. To help minimize this variation, consensus expert opinion can be sought, although interobserver variability can still exist, even at this level of adjudication.[10]

Since the holy grail of causality continues to be elusive, many efforts are underway worldwide to better understand the mechanisms behind drug-induced liver injury (DILI). These efforts include investigation of host factors using pharmacogenetic and proteomic testing, as well as other diagnostic tools.[11] In the United States, the Drug-Induced Liver Injury Network (DILIN) was created to identify a large number of patients with bona fide DILI and to collect epidemiologic and biologic data for future studies.[12] Additionally, the DILIN focuses on developing and testing causality assessment measures for drug, herbal, and over-the-counter medication–induced liver injury.[12]

While the case reported by Inoue and colleagues lacks a formal causality assessment, causality is more straightforward in this case than in most cases of HDS-induced liver injury, as this patient was taking no other supplements or medications preceding the acute liver injury.[13] However, like many HDS implicated in hepatotoxicity, kamishoyosan is not just one ingredient. Rather, it is comprised of 10 different extracts, including glycyrrhizin—the same supplement that the authors used to treat the liver injury they surmised was related to this supplement. Thus, attribution of the liver injury to one ingredient is quite problematic. Among the noted ingredients of kamishoyosan, glycyrrhizin and mentha (pennyroyal) are the 2 ingredients that are most often associated with liver test abnormalities.[14,15] In a large study conducted in Germany that

Article

Journal of the Renin-Angiotensin-Aldosterone System
12(4) 462–468
© The Author(s) 2011
Reprints and permission: sagepub.co.uk/journalsPermissions.nav
DOI: 10.1177/1470320311415887
jra.sagepub.com
SAGE

Liver lipid content is reduced in rat given 7-day administration of angiotensin II

Nobukazu Ishizaka[1,2], Makiko Hongo[1], Aiko Sakamoto[1], Kan Saito[1], Kyoko Furuta[1] and Kazuhiko Koike[3]

Abstract

Activation of the renin–angiotensin system may be involved in the development of hepatic steatosis, a condition that is associated with insulin resistance. We showed that in rats, angiotensin II induced accumulation of triglycerides in the renal tubular and cardiac cells, although it significantly reduced the weight of the rats. Here we investigated the liver lipid content of rats given long-term angiotensin II administration. Angiotensin II (0.7 mg/kg/day) was infused into the rats for 7 days via an osmotic minipump. Some rats also received hydralazine or losartan concomitantly. It was shown that angiotensin II reduced oil red O-stainable lipid droplets (6% of the control value) and liver triglyceride content (angiotensin II: 4.6 ± 0.8 µg/mg, control: 11.7 ± 1.1 µg/mg). Both of these phenomena were blocked by losartan, but not by hydralazine. Angiotensin II infusion reduced the expression and activity of AMP-activated protein kinase. In addition, angiotensin II decreased the mRNA expression of peroxisome proliferator-activated receptor-α and genes related to β-oxidation, although mRNA expression of genes related to lipogenesis were not affected. Angiotensin II reduced triglyceride content in the liver, unlike in the kidney or heart, via an AT1 receptor-dependent mechanism.

Keywords

Angiotensin II, catecholamines, gene expression, hypertension, lipid accumulation

Introduction

It has been shown in previous studies that administration of angiotensin II in conjunction with bile duct ligation[1] exacerbated liver fibrosis, whereas blockade of the renin–angiotensin system (RAS), on the other hand, inhibited liver fibrosis and downregulated profibrotic genes expression in animals susceptible to liver fibrosis.[2-4] We showed that long-term administration of angiotensin II into rats upregulated expression of profibrotic genes in the liver, which might be in part related to the enhanced oxidative stress induced by this peptide.[5] Recent studies have shown that a certain proportion of non-alcoholic fatty liver disease (NAFLD) can progress to hepatic fibrosis (cirrhosis) and liver failure,[6] and the extent of hepatic steatosis is associated with fibrosis in chronic hepatitis C[7] and NAFLD.[8] In addition, AT_1 receptor blockade has an anti-fibrotic effect in the liver.[9,10] These studies suggest the possible role of excessive lipids accumulated in the liver in the development and promotion of liver fibrosis. It should be noted, however, that under certain conditions, accumulation of neutral lipids may prevent progressive liver damage in hepatic steatosis.[11]

In the previous experiments, we found that angiotensin II induced accumulation of lipids in the renal tubular epithelial[12] and cardiac cells,[13] where increased superoxide was produced and fibroproliferative gene expression were upregulated.[14] These changes were found to be suppressed by the AT_1 receptor blockade, suggesting the crucial role of angiotensin II–AT_1 receptor axis in the lipid accumulation in these tissues. These observations collectively lead us to investigate in the current study whether administration of angiotensin II increases lipid content in the liver, as has been observed in the kidney and heart.

Materials and methods

Animal models

[1] Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, Tokyo, Japan
[2] Department of Internal Medicine III, Division of Cardiology, Osaka Medical College, Faculty of Medicine, Osaka, Japan
[3] Department Infectious Diseases, University of Tokyo Graduate School of Medicine, Tokyo, Japan

Corresponding author:
Nobukazu Ishizaka, Department of Internal Medicine III, Division of Cardiology, Osaka Medical College, Faculty of Medicine, Takatsuki-shi Daigaku-machi 2-7, Osaka 569-8686, Japan.
Email: ishizaka@poh.osaka-med.ac.jp

Downloaded from jra.sagepub.com at UNIV OF TOKYO on January 31, 2012

Animal Center for Biomedical Research, Faculty of Medicine, University of Tokyo. Male Sprague–Dawley rats at 10 weeks of age were fed standard rat chow ad libitum. Angiotensin II-induced hypertension was induced in rats by subcutaneous implantation of an osmotic minipump (Alza Pharmaceutical) as described previously.[15] Briefly, Val^5-angiotensin II (Sigma Chemical) was infused at doses of 0.7 mg/kg/day. In some angiotensin II-infused rats, AT_1 receptor antagonist, losartan (25 mg/kg/day), or the nonspecific vasodilator, hydralazine (15 mg/kg/day) (Sigma Chemical), both of which normalized the blood pressure of angiotensin II-infused rats, was given in the drinking water.[16] Infusion of norepinephrine at a dose of 2.8 mg/kg/day for 7 days elevated blood pressure comparable to angiotensin II. Systolic blood pressure and heart rate were measured in conscious rats by tail-cuff plethysmography (BP-98A, Softron, Tokyo, Japan). Rats were sacrificed after overnight fasting in the metabolic cage.

Measurement of lipid content in the serum and the liver

Blood samples were taken just before the animals were killed. Serum levels of triglycerides (TG), total cholesterol (TG), and non-esterified fatty acid (NEFA) were measured by enzymatic methods (SRL). Liver contents triglycerides and total cholesterol were measured from homogenate extracts by enzymatic colorimetric determination using Triglyceride-E Test, Cholesterol-E Test, and Free cholesterol-E Test Wako, respectively (Wako Pure Chemicals).

Histological analysis

Oil red O staining was performed on sections of unfixed, freshly frozen heart samples (3 μm in thickness). The areas of lipid deposition were calculated by using the image analysis software, Photoshop (Adobe), and semiquantification of the lipid deposition was performed as described previously.[14]

Western blot analysis

Western blot analysis was performed as described previously.[17] Polyclonal antibodies against AMP-activated protein kinase α (AMPKα), and phospho-AMPKα (Thr172) (Cell Signaling) were used at a dilution of 1/1000.

Real-time reverse transcription polymerase chain reaction

Expression of lipid metabolism-related gene mRNA was analyzed by real-time quantitative polymerase chain reaction (PCR) performed by LightCycler together with hybriprobe technology (Roche Diagnostics). Expression of target genes was normalized to the mRNA expression of endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The target genes were as follows: peroxisome proliferator-activated receptor (PPAR)-α (Nihon Gene Research Lab's Inc., Sendai, Japan), sterol regulatory element-binding protein (SREBP)-1c, fatty acid synthase (FAS), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR), carnitine palmitoyltransferase (CPT)-1, PPAR-γ coactivator (PGC)-1α, and uncoupling protein (UCP)2. The forward and backward primers used are described in Supplementary Table 1.

Statistical analysis

Data are expressed as the mean ± the standard error of the mean (SEM). We used analysis of variance (ANOVA) followed by a multiple comparison test to compare raw data, before expressing the results as a percentage of the control value using the statistical analysis software StatView version 5.0 (SAS Institute). A value of $p < 0.05$ was considered to be statistically significant.

Results

Characteristics of experimental rats

Administration of angiotensin II caused an increase in the blood pressure, which was blocked by either hydralazine or losartan (Supplementary Table 2). Losartan, but not hydralazine, suppressed the increase in serum levels of TC, TG and NEFA, induced by angiotensin II. At 7 days of infusion, angiotensin II had decreased the body weight (255 ± 5 g [n = 6], $p < 0.0001$ versus control) compared with untreated controls (323 ± 12 g [n = 8]); this decrease was suppressed by losartan (316 ± 4 [n = 5], p = NS versus control), but not by hydralazine (231 ± 10 g [n = 5], $p <$ 0.0001 versus control). Norepinephrine infusion did not affect body weight (300±17 [n=4] NS versus control) as compared with untreated controls

Staining for lipids

Oil red O staining of liver sections showed that lipid droplets were present in hepatocytes in the untreated controls (Figure 1). In contrast, the extent of oil red O-positive lipid droplets was decreased after angiotensin II infusion, and this decrease was inhibited by losartan, but not by hydralazine (Figure 1(C)–(H)). This finding contrasts with the increased accumulation of lipid droplets in the kidney (Figure 1(I) and (J)) and heart (Figure 1(K) and (L)) of angiotensin II-infused rats observed in detail in previous studies.[12,18] Norepinephrine infusion did not significantly alter lipid deposition (98 ± 4% of control [= 4], = NS versus control)

Tissue content of lipids

The tissue content of TG, TC, and free cholesterol was found to be reduced in the liver of angiotensin II-infused

Downloaded from jra.sagepub.com at UNIV OF TOKYO on January 31, 2012