

women who were infected with HCV-1b in high viral loads. In patients aged ≥ 50 years, both ETR and SVR were poorer in women than in men, irrespective of the total dose of IFN, ribavirin or both.

Methods

Study Population

From December 2001 to February 2006, 490 consecutive patients with chronic hepatitis C received combination therapy with PEG-IFN and ribavirin at the Department of Hepatology in the Toranomon Hospital in Metropolitan Tokyo. The following inclusion criteria were met by 300 (61%) patients: they were (1) positive test results for antibodies to HCV (anti-HCV) and for HCV RNA genotype 1b by qualitative methods, and not co-infected with HCV of other genotypes; (2) negative test results for hepatitis B surface antigen or antibodies to human immunodeficiency virus type-1 (HIV-1); (3) confirmed findings of high HCV RNA levels ≥ 100 kIU/ml, which is the Japanese definition of high viral loads [14, 15], within the past 2 months; (4) no cirrhosis diagnosed by laparoscopy and ultrasonography, and with platelet counts $>80 \times 10^3/\text{mm}^3$; (5) body weight ≥ 40 kg and not pregnant or lactating; (6) total alcohol intake <500 g in the past; (7) no HCC, hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic hepatitis or autoimmune hepatitis; (8) no treatment with antivirals or immunosuppressants during the previous 3 months; and (9) with the wish to comply with the treatment protocol for 48 weeks. None of them received growth factors before or during the study period.

The 300 patients, comprising 179 men and 121 women, received PEG-IFN and ribavirin for 48 weeks and were followed for at least 24 weeks after completion of this combination therapy. Informed consent was obtained from each patient, and the study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Serum Markers of HCV Infection

Anti-HCV was determined by third-generation enzyme-linked immunosorbent assay (ELISA) by commercial kits (Ortho HCV Ab ELISA Test 3; Chiron Cooperation, Emeryville, CA, USA). HCV RNA was determined quantitatively by polymerase chain reaction (PCR) (Cobas Amplicor HCV Monitor ver. 2.0, Roche Diagnostics, Tokyo, Japan) in serum diluted tenfold at the baseline, as well as at least monthly during and after treatment; it has a dynamic range between 5 kIU/ml and 5,000 kIU/ml. Sera

negative for HCV RNA (<5 kIU/ml) by quantitative assay were tested by qualitative PCR (Amplicor, Roche Molecular Systems, Inc., Branchburg, NJ, USA) with a detection limit at 100 copies/ml.

Combined PEG-IFN and Ribavirin Therapy

Patients underwent subcutaneous administration of PEG-IFN- $\alpha 2b$ (PEG-Intron, Schering-Plough Corp, Kenilworth, NJ, USA), weekly, at a median dose of 1.4 $\mu\text{g}/\text{kg}$ (range 0.8–1.9 $\mu\text{g}/\text{kg}$), together with ribavirin orally, at a median daily dose of 11 mg/kg (range 3.7–14.2 mg/kg) for 48 weeks. The dose of ribavirin was adjusted by body weight: 600 mg for patients weighing ≤ 60 kg; 800 mg for those between >60 kg and <80 kg; and 1,000 mg for those ≥ 80 kg. It was tapered in the 99 (33%) patients in whom hemoglobin levels decreased below 10 g/dl during the combination therapy.

Statistical Analysis

Variables were compared between groups by the chi-square test, Fisher's exact probability test and the Mann-Whitney U test. Differences in the loss of HCV RNA from the serum between groups was evaluated with the Kaplan-Meier life table with use of the log rank test. The influence of various factors on the response to PEG-IFN/ribavirin was evaluated by logistic regression in univariate and multivariate analyses. Analysis of all data was performed with the computer program SPSS software (SPSS Inc., Chicago, IL, USA), and a *P* value less than 0.05 was considered significant.

Results

Baseline Characteristics of Male and Female Patients Infected with HCV-1b in High Loads

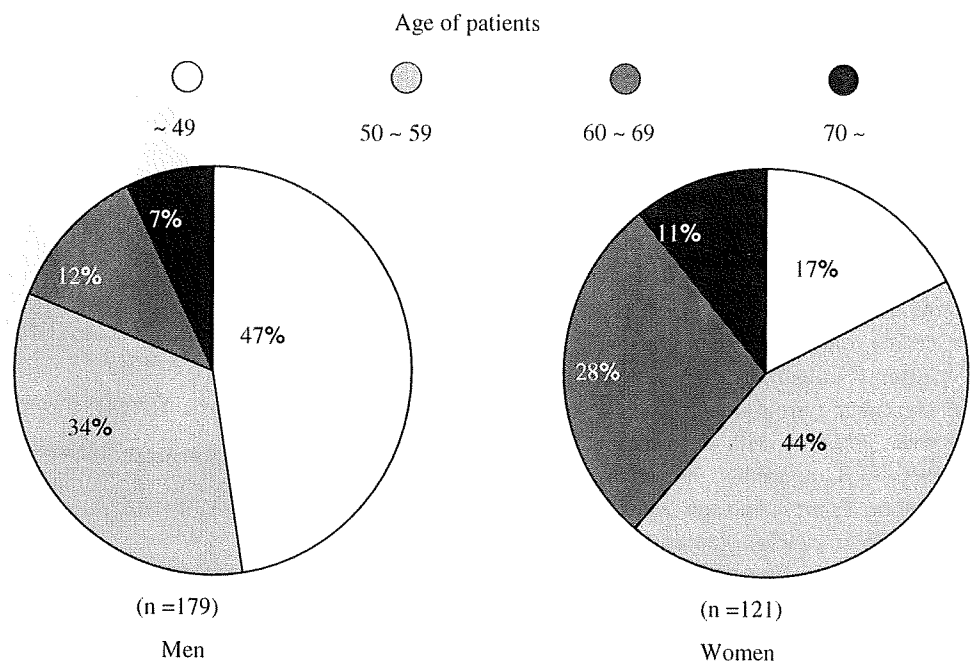
PEG-IFN and ribavirin were given for 48 weeks to 179 men and 121 women who had been infected with HCV-1b in high viral loads (>100 kIU/ml). Table 1 compares baseline characteristics between them. Women were older, had lower hemoglobin values and platelet counts, and lower levels of albumin, gamma-glutamyl-transpeptidase (γ -GTP) and ferritin, than men. The stage of fibrosis was lower in women than in men, although their alanine aminotransferase (ALT) levels were comparable. Three months before the start of combination therapy, IFN had been given to 75 (42%) of the 179 male patients, comparably frequently to 40 of the 121 (33%) female patients. Age distribution for men and women is shown in Fig. 1. The proportion of patients ≥ 60 years was higher in women than in men (39% vs 19%, $P < 0.001$).

Table 1 Baseline characteristics of 300 patients with chronic hepatitis with high-titers of HCV-1b RNA who had received PEG-IFN and ribavirin for 48 weeks and were followed for 48 weeks or longer

Characteristic ^a	Men (n = 179)	Women (n = 121)	Differences P
Age (years)	50 (19–66)	57 (30–69)	<0.001
Previous IFN treatment	75 (42%)	40 (33%)	0.146
Hemoglobin (g/dl)	15.2 (11.5–17.8)	13.5 (11.2–15.1)	<0.001
Platelets ($\times 10^3/\text{mm}^3$)	176 (88–366)	165 (91–331)	0.025
Albumin (g/dl)	3.9 (3.2–4.6)	3.8 (3.0–4.6)	0.004
ALT (IU/l)	77 (23–504)	68 (19–391)	0.078
γ -GTP (IU/l)	78 (14–409)	37 (11–171)	0.011
LDL (mg/dl)	98 (50–176)	99 (57–168)	0.920
Ferritin (mg/l)	186 (<10–1,327)	95 (<10–4 42)	<0.001
ICG ₁₅ (%)	14 (4–41)	13 (2–31)	0.969
Stage (F0-1/F2-3)	80/66 (50 unknown)	42/55 (57 unknown)	0.050

ALT alanine aminotransferase, γ -GTP gamma-glutamyl transpeptidase, LDL low density lipoprotein, ICG₁₅, retention of indocyanine green at 15 min
^a The means (ranges) are given

Fig. 1 Distribution of ages in the male and female patients with chronic hepatitis C who were infected with HCV-1b in high loads



Virological Response During the 48-Week Treatment with PEG-IFN and Ribavirin

On-treatment response to the combined treatment is compared between men and women in Fig. 2. Through 48 weeks on treatment, women gained a virological response less frequently than did men. ETR was achieved by 58% of women as against 83% of men ($P < 0.001$). Restricted to the patients who gained ETR, women lost HCV RNA from serum later than men did [median (range) 24.1 (2.0–36.4) vs 13.0 (2.0–48.0) weeks, $P < 0.001$]. Figure 3 depicts the on-treatment virological response in patients <50 years and those ≥ 50 years separately. The virological response was no different between men and women <50 years. However, it was poorer in women than

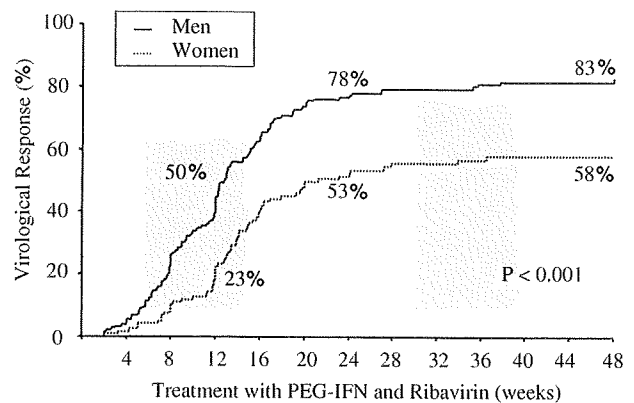
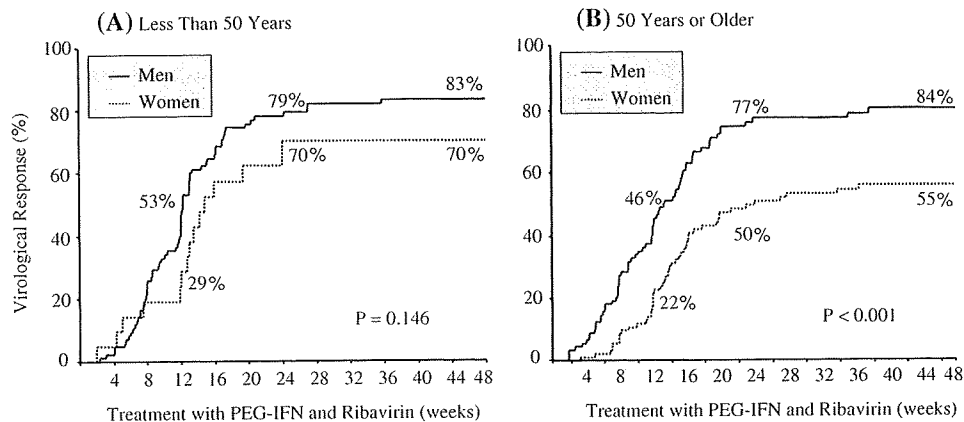


Fig. 2 On-treatment virological responses to PEG-IFN and ribavirin in male and female patients infected with HCV-1b in high viral loads

Fig. 3 On-treatment virological responses to combined IFN and ribavirin in male and female patients infected with HCV-1b in high viral loads who were less than 50 years (a) or 50 years or older (b)



in men ≥ 50 years. Differences between men and women in total (Fig. 2), therefore, were attributed to a poorer response of women ≥ 50 years to the combined treatment.

Sustained Virological Response to the 48-Week Treatment with PEG-IFN and Ribavirin

Sustained virological response 24 weeks after the completion of combined treatment was accomplished much less frequently in women than in men [33/121 (27%) vs 105/179 (59%), $P < 0.001$]. SVR was influenced by age both in men and in women (Fig. 4). It was found significantly less often in women than men who were 50 years or older.

Figure 5 illustrates the relationship between the earliest on-treatment virological response and SVR in men and women. Patients with a virological response at 4 weeks gained SVR invariably. However, in the patients with virological response in later weeks, SVR was achieved less frequently in women than in men. In the patients who had lost HCV RNA from the serum at 12 weeks, in particular, SVR was achieved significantly less often in women than in men (63% vs 88%, $P = 0.012$). The relationship

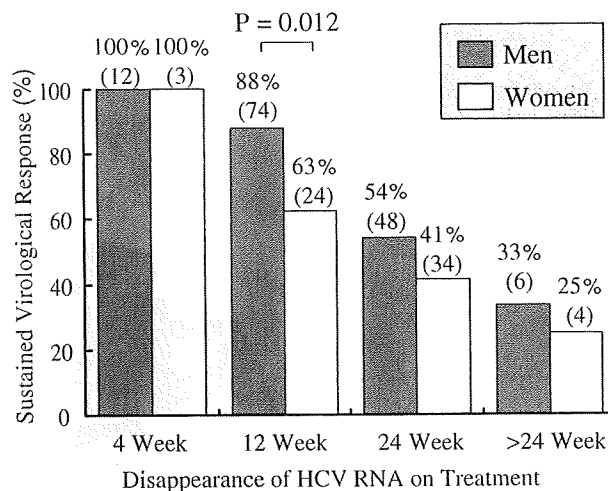


Fig. 5 Sustained virological response in male and female patients who lost HCV RNA from the serum at various weeks on treatment with PEG-IFN and ribavirin

between on-treatment virological response and SVR was compared among women in different age groups (Fig. 6). In the patients with the earliest virological response at 12 weeks and 24 weeks, SVR was achieved less frequently in women aged ≥ 50 years than in those < 50 years, but the difference fell short of being significant due to the small numbers of patients in the comparison.

SVR and Compliance with PEG-IFN Therapy, Ribavirin Therapy, or Both

Table 2 compares compliance with the combined treatment between men and women. Either or both of PEG-IFN and ribavirin were tolerated to a lesser extent by women than by men. Thus, doses $\geq 80\%$ were reached less frequently in women than in men for PEG-IFN or ribavirin, or both. The initial dose of ribavirin was no different between men and women.

SVR was achieved less frequently in women than in men who had received $\geq 80\%$ of the dose of PEG-IFN

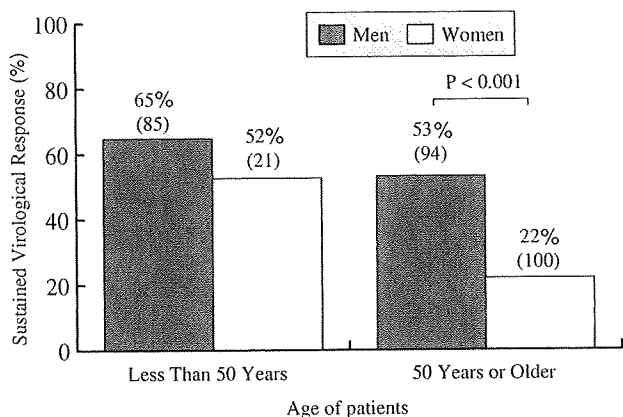


Fig. 4 Sustained virological response to PEG-IFN and ribavirin in male and female patients stratified by age. The number of patients is indicated in parentheses in each column

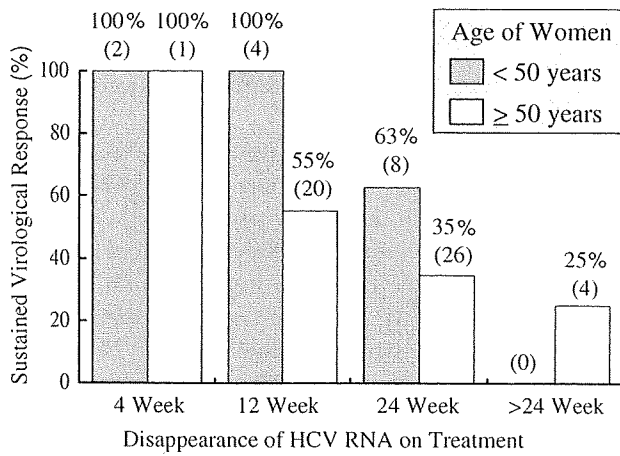


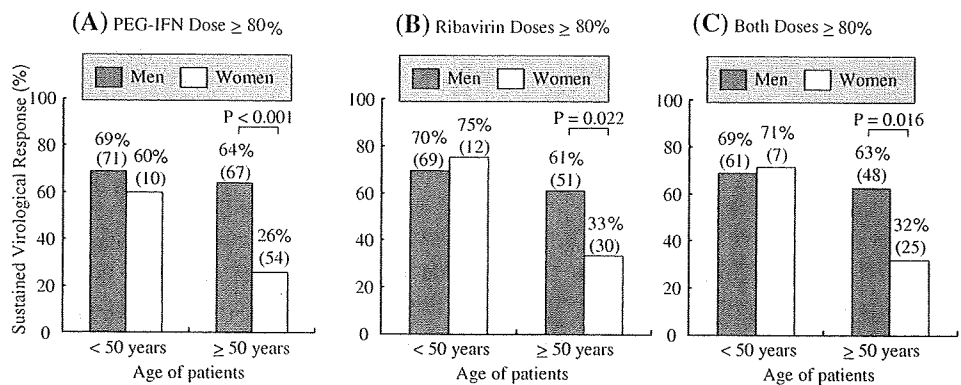
Fig. 6 Sustained virological response to PEG-IFN and ribavirin in female patients stratified by age who lost HCV RNA from serum at various weeks on treatment

Table 2 PEG-IFN and ribavirin received by patients with chronic hepatitis with high-titers of HCV-1b RNA

PEG-IFN and ribavirin	Men (n = 179)	Women (n = 121)	Differences P
Initial ribavirin dose (mg/kg body weight)	11.1 (5.0–14.1)	11.2 (3.7–14.3)	0.735
Total dose			
PEG-IFN ≥ 80%	139 (78%)	94 (53%)	<0.001
Ribavirin ≥ 80%	117 (65%)	42 (35%)	<0.001
Both ≥ 80%	110 (61%)	32 (27%)	<0.001
Withdrawn	28 (16%)	28 (23%)	0.131

[20/64 (31%) vs 92/138 (67%), $P < 0.001$], ribavirin [19/42 (45%) vs 79/120 (66%), $P = 0.027$] or both [13/32 (41%) vs 72/109 (66%), $P = 0.013$]. Again, differences were observed only in patients ≥ 50 years (Fig. 7). In the patients < 50 years, in contrast, the rate of SVR was no different between women and men who had received $\geq 80\%$ of the dose of PEG-IFN, ribavirin, or both.

Fig. 7 Sustained virological response to PEG-IFN and ribavirin in patients who had received 80% or more of the dose of IFN (a), ribavirin (b) or both of them (c). Results are shown for men and women in two age groups



The influence of age was compared between male and female patients in different age groups. SVR was achieved significantly more frequently in the men aged ≥ 60 years [88/145 (61%) vs 17/74 (37%), $P = 0.001$] and < 60 years [17/34 (50%) vs 6/48 (13%), $P < 0.001$]. Likewise, SVR was more common in male than female patients aged 50–59 years [33/60 (55%) vs 16/53 (30%), $P = 0.013$].

Multivariate Analysis for Factors Accelerating the Response to PEG-IFN and Ribavirin Therapy

In univariate analysis, age, gender, hemoglobin, albumin, ICG₁₅, ribavirin dose and compliance with PEG-IFN therapy, ribavirin therapy, or both, influenced SVR. In multivariate analysis, only male gender, ICG₁₅, ribavirin dose and compliance with PEG-IFN, as well as both PEG-IFN and ribavirin, accelerated the chance of SVR (Table 3).

Discussion

In a retrospective study, response to PEG-IFN and ribavirin for 48 weeks was compared between 179 men and 121 women with chronic hepatitis C who had been infected with HCV-1b in high viral loads by the Japanese definition (> 100 kIU/ml) [14, 15]. Loss of HCV RNA from serum occurred less often in women than in men throughout the 48 weeks of treatment. Both ETR (55% vs 83%, $P < 0.001$) and SVR (27% vs 59%, $P < 0.001$) were achieved significantly less frequently in women than in men. The observed low response to PEG-IFN and ribavirin stands at odds with the better response to antiviral treatments and slow progression of fibrosis in women than in men [9, 16, 17]. There are, however, viral and host factors other than gender that can influence the course of chronic hepatitis C and, by inference, the response to antiviral treatments.

Viral factors such as HCV genotypes and infection load affect the course of chronic hepatitis C. Thus, hepatitis is

Table 3 Factors promoting the response to PEG-IFN and ribavirin in multivariate analysis

Factors	Odds ratio	95% Confidence interval	<i>P</i>
Male gender	3.50	1.71–7.17	0.001
ICG ₁₅ ≤ 13.5%	2.09	1.07–4.08	0.031
Ribavirin ≥ 11.1 mg/kg per day	2.17	1.11–4.25	0.024
Total PEG-IFN ≥ 80%	6.96	2.26–21.4	0.001
PEG-IFN/ribavirin ≥ 80%	12.66	2.32–71.4	0.003

more severe and less responsive to IFN in patients infected with HCV genotypes 1 and 4 than in those with HCV genotypes 2, 3 and 6 [18–22]. Likewise, high viral loads are associated with rapid progression of liver disease and poor response to IFN [23–25]. In our study, such viral factors were excluded in comparing the response to PEG-IFN and ribavirin between women and men. All the patients were infected with HCV genotype 1b in high viral loads (>100 kIU/ml).

Age influences the severity of chronic hepatitis C [9, 26], and disease progresses faster and response to antiviral therapy is poorer in older patients [23]. There were significant differences in age between female and male patients in our study. The women were older than the men [mean (range) 57 (30–69) years vs 50 (19–66) years, $P < 0.001$], and the proportion of patients ≥60 years was higher in women than in men (39% vs 19%, $P < 0.001$). Hence, the response to PEG-IFN and ribavirin was evaluated in patients aged ≥50 years and <50 years separately. There were no differences in the response between female and male patients <50 years, during and at the end of the 48-week treatment, as well as 24 weeks thereafter. However, ETR (55% vs 84%, $P < 0.001$) and SVR (22% vs 53%, $P < 0.001$) were gained significantly less often in women than men who were aged ≥50 years.

The influence of gender was observed, also, in patients aged ≥60 years and those aged 50–60 years. Hence, women would become less responsive than men to PEG-IFN and ribavirin after they had entered their fifties.

From a therapeutic notion, compliance with treatment can alter the response. Since ribavirin accumulates in erythrocytes and induces hemolysis, it is less tolerated in women who tend to be anemic than men without such an inclination [27]. At the baseline, women had lower levels of hemoglobin and ferritin than men. These would have been responsible for the lower tolerance to PEG-IFN and ribavirin in women than men in our study. In fact, ≥80% of the dose of PEG-IFN, ribavirin, or both, was tolerated less frequently in women than men ($P < 0.001$ for each). Even in the patients who had received ≥80% of the dose, however, the response to PEG-IFN and ribavirin was gained less frequently in women than in men. Again, the

difference was due to a significantly lower response in female patients than in male patients aged ≥50 years, while the response was no different between those <50 years of age.

Taken altogether, the poorer response to PEG-IFN and ribavirin in women than in men was attributable to impaired response in the female patients aged ≥50 years. Older women with chronic hepatitis C, therefore, would be less responsive to the combined treatment with PEG-IFN and ribavirin currently in use. In support of this view, the response to human lymphoblastoid IFN for 24 weeks is dependent on gender and age [28]. The greatest physiological change precipitated in women by aging is a decreased serum concentration of bioavailable estrogen after they enter the menopause [29]. Estrogen has been shown to have an antifibrotic potential in both experimental and clinical studies. In experimental cirrhosis induced by dimethylnitrosamine in rats, administration of neutralizing antibodies to estradiol and ovariectomy enhanced fibrogenesis in female rats [30]. Hepatocytes have the receptor to estrogen [31], and myofibroblastic transformation in hepatic stellate cells of rats is inhibited in culture supplemented with this hormone [32]. Consequently, hepatic fibrosis progresses faster in menopausal women with chronic hepatitis C, and hormone replacement therapy may be able to prevent it [33]. Furthermore, in women aged ≥50 years, the number of estrogen receptor in hepatocytes decreases to one-half of that in those aged <50 years. This would stand in further support of the notion that the antifibrotic effects of decreased estrogen levels in patients aged ≥50 years with chronic hepatitis C would produce a lesser response to PEG-IFN and ribavirin.

Favorable effects of female sex hormones on hepatitis have long been suggested. Chronic hepatitis C is mild in menstruating women [34]; its activity is suppressed during pregnancy and enhanced after delivery [35]. The velocity of fibrosis progression is extremely low in young women exposed to HCV through mass-administration of immunoglobulin-D. Only two of 184 (1.2%) and four of 1,018 (0.4%) developed cirrhosis over 24 years and 20 years, respectively, in Irish and German studies [36, 37]. It does need to be pointed out, however, that the majority of women in those studies had not been followed beyond the menopause. There is a possibility that chronic hepatitis C may progress at a faster speed during their next few decades. Continued observations of them would be necessary to evaluate the validity of such an assumption.

Although decreased levels of estrogen can explain the enhanced activity of chronic hepatitis C in older women, as well as their concomitant resistance to PEG-IFN and ribavirin, it does not give an account of the better response in men than women who were aged ≥50 years. Feminization represented by gynecomastia is common in men

who have developed cirrhosis, and it can increase even in healthy men with age [38]. Possibly in the background of this phenomenon, circulating levels of free estrogen in men exceed those in women, after they enter their fifties, with margins widening with age [29]. It is tempting to speculate that elevated estrogen levels in men with chronic hepatitis C are responsible for their better response to the combination therapy than women who were aged ≥ 50 years. Whether or not such a speculation would hold would have to be evaluated by a comparison of estrogen levels between older men and women with chronic hepatitis C.

Although osteoporosis is an extrahepatic manifestation of chronic hepatitis C [39], hormone replacement therapy has been withheld for fear of potential hepatotoxicity. There is evidence, however, that oral contraceptives inhibit the progression of fibrosis in women [33]. It may lead to the possibility that the response to antiviral treatment in older women with chronic hepatitis C would be improved by substituting estrogen in them. The merit of hormone replacement therapy for them, of course, would need to be balanced against any harmful effects associated with it.

There are limitations in this study. All the patients were infected with genotype 1b in high viral loads. Hence, the results obtained may or may not be extended to patients with chronic hepatitis C who are infected with HCV of other genotypes in low viral loads. The influence of sex hormones needs to be substantiated by their determination in correlation with SVR. These limitations notwithstanding, the results obtained warrant a special caution in the treatment of women older than 50 years due to their lesser responsiveness to PEG-IFN and ribavirin.

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References

- Cohen J. The scientific challenge of hepatitis C. *Science*. 1999;285:26–30. doi:10.1126/science.285.5424.26.
- Alberti A, Chemello L, Benvegno L. Natural history of hepatitis C. *J Hepatol*. 1999;31:S17–S24. doi:10.1016/S0168-8278(99)80369-9.
- Alter HJ, Seeff LB. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. *Semin Liver Dis*. 2000;20:17–35. doi:10.1055/s-2000-9505.
- Seeff LB. Natural history of chronic hepatitis C. *Hepatology*. 2002;36:S35–S46. doi:10.1002/hep.1840360706.
- Feray C, Gigou M, Samuel D, et al. Influence of the genotypes of hepatitis C virus on the severity of recurrent liver disease after liver transplantation. *Gastroenterology*. 1995;108:1088–1096. doi:10.1016/0016-5085(95)90207-4.
- Garcia-Samaniego J, Soriano V, Castilla J, et al. Influence of hepatitis C virus genotypes and HIV infection on histological severity of chronic hepatitis C. *Am J Gastroenterol*. 1997;92:1130–1134.
- Pageaux GP, Ducos J, Mondain AM, et al. Hepatitis C virus genotypes and quantitation of serum hepatitis C virus RNA in liver transplant recipients: relationship with severity of histological recurrence and implications in the pathogenesis of HCV infection. *Liver Transpl Surg*. 1997;3:501–505. doi:10.1002/lt.500030504.
- Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. *N Engl J Med*. 1999;340:1228–1233. doi:10.1056/NEJM199904223401602.
- Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. *Lancet*. 1997;349:825–832. doi:10.1016/S0140-6736(96)07642-8.
- Vogt M, Lang T, Frosner G, et al. Prevalence and clinical outcome of hepatitis C infection in children who underwent cardiac surgery before the implementation of blood-donor screening. *N Engl J Med*. 1999;341:866–870. doi:10.1056/NEJM199909163411202.
- McHutchison JG, Gordon SC, Schiff ER, et al. Interferon alpha-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. *N Engl J Med*. 1998;339:1485–1492. doi:10.1056/NEJM199811193392101.
- Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alpha-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med*. 2002;347:975–982. doi:10.1056/NEJMoa020047.
- Hung CH, Lee CM, Lu SN, et al. Anemia associated with antiviral therapy in chronic hepatitis C: incidence, risk factors, and impact on treatment response. *Liver Int*. 2006;26:1079–1086. doi:10.1111/j.1478-3231.2006.01354.x.
- Akuta N, Suzuki F, Kawamura Y, et al. Predictors of viral kinetics to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b. *J Med Virol*. 2007;79:1686–1695. doi:10.1002/jmv.20979.
- Sezaki H, Suzuki F, Kawamura Y, et al. Evaluation of long-term biochemical responses to combination therapy of interferon plus ribavirin in those infected with hepatitis C virus genotype 1b and high baseline viral load. *Hepatol Res*. 2007;37:787–792. doi:10.1111/j.1872-034X.2007.00132.x.
- Conjeevaram HS, Fried MW, Jeffers LJ, et al. Peginterferon and ribavirin treatment in African American and Caucasian American patients with hepatitis C genotype 1. *Gastroenterology*. 2006;131:470–477. doi:10.1053/j.gastro.2006.06.008.
- Poynard T, Marcellin P, Lee SS, et al. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet*. 1998;352:1426–1432. doi:10.1016/S0140-6736(98)07124-4.
- Tsubota A, Chayama K, Ikeda K, et al. Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. *Hepatology*. 1994;19:1088–1094.
- Hadziyannis SJ, Sette H Jr, Morgan TR, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med*. 2004;140:346–355.
- Hui CK, Yuen MF, Sablon E, Chan AO, Wong BC, Lai CL. Interferon and ribavirin therapy for chronic hepatitis C virus genotype 6: a comparison with genotype 1. *J Infect Dis*. 2003;187:1071–1074. doi:10.1086/368217.
- Legrand-Abravanel F, Nicot F, Boulestin A, et al. Pegylated interferon and ribavirin therapy for chronic hepatitis C virus genotype 4 infection. *J Med Virol*. 2005;77:66–69. doi:10.1002/jmv.20414.
- Yuen MF, Lai CL. Response to combined interferon and ribavirin is better in patients infected with hepatitis C virus genotype 6

- than genotype 1 in Hong Kong. *Intervirology*. 2006;49:96–98. doi:10.1159/000087270.
23. Kumada T, Toyoda H, Honda T, et al. Treatment of chronic hepatitis C with interferon alone or combined with ribavirin in Japan. *Intervirology*. 2006;49:112–118. doi:10.1159/000087273.
 24. Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. Pathogenesis, natural history, treatment, and prevention of hepatitis C. *Ann Intern Med*. 2000;132:296–305.
 25. Trepo C. Genotype and viral load as prognostic indicators in the treatment of hepatitis C. *J Viral Hepat*. 2000;7:250–257. doi:10.1046/j.1365-2893.2000.00233.x.
 26. Wali M, Harrison RF, Gow PJ, Mutimer D. Advancing donor liver age and rapid fibrosis progression following transplantation for hepatitis C. *Gut*. 2002;51:248–252. doi:10.1136/gut.51.2.248.
 27. Takaki S, Tsubota A, Hosaka T, et al. Factors contributing to ribavirin dose reduction due to anemia during interferon alfa2b and ribavirin combination therapy for chronic hepatitis C. *J Gastroenterol*. 2004;39:668–673. doi:10.1007/s00535-003-1363-9.
 28. Hayashi J, Kishihara Y, Ueno K, et al. Age-related response to interferon alfa treatment in women vs men with chronic hepatitis C virus infection. *Arch Intern Med*. 1998;158:177–181. doi:10.1001/archinte.158.2.177.
 29. Khosla S, Melton LJ 3rd, Atkinson EJ, O'Fallon WM, Klee GG, Riggs BL. Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen. *J Clin Endocrinol Metab*. 1998;83:2266–2274. doi:10.1210/jc.83.7.2266.
 30. Yasuda M, Shimizu I, Shiba M, Ito S. Suppressing effects of estradiol on dimethylnitrosamine-induced fibrosis of the liver in rats. *Hepatology*. 1999;29:719–727. doi:10.1002/hep.510290307.
 31. Rossini GP, Baldini GM, Villa E, Manenti F. Characterization of estrogen receptor from human liver. *Gastroenterology*. 1989;96:1102–1109.
 32. Shiba M, Shimizu I, Yasuda M, Ii K, Ito S. Expression of type I and type III collagens during the course of dimethylnitrosamine-induced hepatic fibrosis in rats. *Liver*. 1998;18:196–204.
 33. Di Martino V, Lebray P, Myers RP, et al. Progression of liver fibrosis in women infected with hepatitis C: long-term benefit of estrogen exposure. *Hepatology*. 2004;40:1426–1433. doi:10.1002/hep.20463.
 34. Sartori M, Andorno S, Rigamonti C, Grossini E, Nicosia G, Boldorini R. Chronic hepatitis C is mild in menstruating women. *J Gastroenterol Hepatol*. 2000;15:1411–1417. doi:10.1046/j.1440-1746.2000.02368.x.
 35. Latt NC, Spencer JD, Beeby PJ, et al. Hepatitis C in injecting drug-using women during and after pregnancy. *J Gastroenterol Hepatol*. 2000;15:175–181. doi:10.1046/j.1440-1746.2000.02060.x.
 36. Levine RA, Sanderson SO, Ploutz-Snyder R, et al. Assessment of fibrosis progression in untreated Irish women with chronic hepatitis C contracted from immunoglobulin anti-D. *Clin Gastroenterol Hepatol*. 2006;4:1271–1277. doi:10.1016/j.cgh.2006.05.028.
 37. Wiese M, Berr F, Lafrenz M, Porst H, Oesen U. Low frequency of cirrhosis in a hepatitis C (genotype 1b) single-source outbreak in Germany: a 20-year multicenter study. *Hepatology*. 2000;32:91–96. doi:10.1053/jhep.2000.8169.
 38. Braunstein GD. Gynecomastia. *N Engl J Med*. 1993;328:490–495. doi:10.1056/NEJM199302183280708.
 39. Carey EJ, Balan V, Kremers WK, Hay JE. Osteopenia and osteoporosis in patients with end-stage liver disease caused by hepatitis C and alcoholic liver disease: not just a cholestatic problem. *Liver Transpl*. 2003;9:1166–1173. doi:10.1053/jlts.2003.50242.

Correlation Between Serum Hepatitis B Virus Core-Related Antigen and Intrahepatic Covalently Closed Circular DNA in Chronic Hepatitis B Patients

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Nucleos(t)ide analogues are utilized for the treatment of chronic HBV infection, and HBe seroconversion and HBV DNA levels are commonly used as markers of viral status and as primary treatment endpoints. Recently, a new assay was prepared for the detection of serum HBV core-related antigen (HBcrAg), consisting of HBcAg, HBeAg, and p22cr, which is a precore protein from amino acid –28 to at least amino acid 150, by coding the precore/core region. In this study, we examined the correlation between serum HBcrAg concentration and viral status by the analysis of serum HBeAg, HBsAg, peripheral HBV DNA, and intrahepatic covalently closed circular DNA (cccDNA) in 57 chronic hepatitis B patients. Intrahepatic cccDNA was detected in all 57 patients, 42 patients were HBcrAg-positive, and serum HBcrAg concentration level was closely correlated with cccDNA. Additionally, positive HBcrAg concentration level results were observed in 6 out of 13 HBsAg seroclearance patients and 20 out of 31 HBV DNA-negative patients. Moreover, the correlation between HBcrAg and cccDNA in these 31 HBV DNA-negative patients was statistically significant ($r=0.482$, $P=0.006$). These data suggest that serum HBcrAg concentration is well correlated with intrahepatic cccDNA level, and that the measurement of serum HBcrAg may be clinically useful for monitoring intrahepatic HBV viral status, especially in patients under treatment with nucleos(t)ide analogues. *J. Med. Virol.* 81:27–33, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: HBV DNA; HBcrAg; cccDNA; HBsAg; lamivudine; entecavir

INTRODUCTION

Hepatitis B virus (HBV) is an important causative agent for liver disease such as chronic hepatitis,

cirrhosis, and hepatocellular carcinoma. Recently, several nucleos(t)ide analogues such as lamivudine [Dienstag et al., 1995], adefovir dipivoxil [Chin et al., 2001], and entecavir [Colonna et al., 2001] have been found to consistently produce rapid and dramatic decreases in viremia [Dienstag et al., 1995, 1999; Lai et al., 1998; Suzuki et al., 1999]. For the serological monitoring of chronic hepatitis patients under treatment with nucleos(t)ide analogues, improvement of alanine transaminase level, seroconversion from HBe antigen (HBeAg)-positive to anti-HBe antibody (HBeAb)-positive, and peripheral HBV DNA concentration are used as markers in chronic active hepatitis, and both HBeAg seroconversion and HBV DNA levels below the detection limit and/or of 10^5 copies/ml are commonly used as primary treatment endpoints [Lok et al., 2004]. In addition, HBV surface antigen (HBsAg) seroclearance has been linked to a good prognosis, including improvement of liver histopathology and liver function, and prolongation of survival [Arase et al., 2006], although spontaneous HBsAg seroclearance and/or remission occurred in only a small proportion of patients during the natural history of chronic HBV infections.

However, a major problem with long-term lamivudine treatment is the potential development of drug-resistance, mainly caused by the mutation of the YMDD motif of reverse transcriptase [Chayama et al., 1998]. We previously reported the efficacy of lamivudine therapy and factors associated with the emergence of resistance in chronic HBV infection in Japan [Suzuki et al., 2003].

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The measurement of the predictive serum markers of residual intrahepatic HBV DNA and/or covalently closed circular DNA (cccDNA), which is intrahepatic HBV replicated intermediate, is more important than the measurement of peripheral HBV DNA for monitoring the viral status of hepatitis patients [Sung et al., 2005]. Additionally, the amount of cccDNA in serum is reported to be higher in patients who develop YMDD mutants than in patients who do not [Yuen et al., 2005]. Recently, it was established that HBV RNA is detectable in serum and the elevation of HBV RNA is a predictor of early occurrence of viral mutation during lamivudine therapy [Rokuhara et al., 2006; Hatakeyama et al., 2007]. However, these HBV DNA, HBV RNA, or cccDNA detection assay methods remain complicated and difficult to perform. Therefore, simple methods of viral status evaluation are required for routine assays rather than for nucleic acid assays.

Recently, a new assay was performed for the detection of hepatitis B core-related antigen (HBcrAg) consisting of HBV core antigen (HBcAg), HBeAg, and 22 kDa precore protein (p22cr) coded with precore/core gene [Kimura et al., 2002, 2005]. p22cr is a precore protein from amino acid -28 to at least amino acid 150, containing an uncleaved signal sequence and lacking the C-terminal arginine-rich domain. p22cr is found in empty and HBV DNA negative virus particles; the production of empty particles is not dependent on the formation of HBV DNA [Kimura et al., 2005]. Several reports indicate that the concentration of serum HBcrAg is closely correlated with peripheral HBV DNA in untreated patients [Rokuhara et al., 2003; Tanaka et al., 2006]. Additionally, HBcrAg is considered as a prospective marker of the appearance of drug-resistant HBV mutants and of the identification of patients with low risk of HBV reactivation after discontinuation of lamivudine administration, while peripheral HBV DNA does not qualify as a prospective marker in these patients [Rokuhara et al., 2005; Shinkai et al., 2006; Tanaka et al., 2006; Matsumoto et al., 2007]. The relationship between HBcrAg and intrahepatic cccDNA levels has not yet been clarified.

In this study, we examined the correlation between HBcrAg and viral status by the analysis of HBeAg, HBsAg, peripheral HBV DNA, and intrahepatic cccDNA in patients with chronic hepatitis B.

MATERIALS AND METHODS

Patients and Samples

Serum samples and biopsy specimens were obtained from 57 chronic hepatitis B patients at Toranomon Hospital under informed consent. The median age of patients was 49 (range, 25–71 years). Out of 57 patients, 28 underwent nucleos(t)ide analogue administration (17 patients of lamivudine, 7 patients of both lamivudine and adefovir dipivoxil, 4 patients of entecavir), and 13 were HBsAg-negative/HBs-seroclearance patients with more than 12 months of being HBsAg-positive before HBs-seroclearance.

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Routine Laboratory Tests

HBsAg, HBeAg, and HBeAb were routinely measured by the commercially available Chemiluminescent Enzyme Immunoassay (CLEIA) (Lumipulse System, Fujirebio, Inc., Tokyo, Japan).

HBcrAg Test

Serum HBcrAg was measured by CLEIA HBcrAg assay kit (Fujirebio, Inc.) with a fully automated analyzer system (Lumipulse System, Fujirebio, Inc.). Briefly, 150 μ l of serum was incubated with 150 μ l of pretreatment solution containing 15% sodium dodecyl sulfate at 60°C for 30 min. After heat treatment, 120 μ l of pretreated specimen was added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with monoclonal antibody mixture (HB44, HB61, and HB114) against denatured HBcAg, HBeAg, and p22cr. After 10 min incubation at 37°C and washing, further incubation was done for 10 min at 37°C with alkaline phosphatase conjugated with two kinds of monoclonal antibodies (HB91 and HB110) against denatured HBcAg, HBeAg, and p22cr. After washing, 200 μ l of substrate solution [AMPPD: 3-(2'-spiroadamantan)-4-methoxy-4-(3'-phosphoryloxy)phenyl-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA) was added to the test cartridge which was then incubated for 5 min at 37°C. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg (amino acids: -10 to 183 of precore/core gene product). HBcrAg concentration was expressed as units/ml (U/ml), which is defined as the immunoreactivity of 10 fg/ml of recombinant pro-HBeAg. In this study, HBcrAg value was expressed as log U/ml, and the cut-off value was set at 3.0 log U/ml. For the statistical analysis, HBcrAg-negative cases were calculated as 3.0 log U/ml.

HBV DNA Assay

HBV DNA in serum was measured by polymerase chain reaction (PCR) assay kit (Amplicor HBV monitor test, Roche Molecular Systems, Inc., Branchburg, NJ). Values under or over the detection range were calculated as 2.6 log copies/ml or as 7.6 log copies/ml, respectively.

Measurement of cccDNA

Liver biopsy specimens were taken and stored at -80°C before DNA extraction. HBV DNA was extracted using QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan). The concentration of purified DNA was based on absorbance at 260 nm. For this study, two oligonucleotide primers, cccF2 (5'-cgtctgtgcttctcatctga-3', nucleotides 1,424–1,444), cccR4 (5'-gcacagcttgaggctt-gaa-3', nucleotides 1,755–1,737), and a probe cccP2 (5'-VIC-accaatttatgctacag-MGB-3', nucleotides 1,672–1,655), were designed using Primer Express™ software (Applied Biosystems, Foster City, CA) to flank the direct

repeat region between the hepatitis B core and the polymerase gene. The use of cccF2 and cccR4 oligonucleotide primers spanning the direct repeat region of the HBV genome allows the PCR of native viral DNA in the Dane particle to block the amplification of products, because the partially double-stranded HBV DNA is disrupted in the direct repeat region [Mason et al., 1998]. Twenty-five microliters of the extracted DNA (0.5 µg) were detected with the sequence detector system (ABI 7900HT, Applied Biosystems) in 50 µl of a PCR mixture containing TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nmol of each primer, and 250 nmol of the probe. After initial activation of uracil-N-glycosylase at 50°C for 2 min, AmpliTaq Gold was activated at 95°C for 10 min. The subsequent PCR conditions consisted of 45 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 90 sec per cycle (SRL, Inc., Tokyo, Japan).

Statistical Analysis

The statistical analysis of the correlation data between serum HBcrAg, HBsAg, HBV DNA, and/or cccDNA was performed by SPSS software (version 14.0J, SPSS Japan Inc., Tokyo, Japan), and the statistical significance between the two sides was taken as *P*-value lower than 0.05.

RESULTS

Serological and Genetic Assay Results

We classified the 57 patients according to assay results of HBsAg, HBeAg, serum HBV DNA, intrahepatic HBV cccDNA, and serum HBcrAg. Positive results were observed with all 57 patients in the cccDNA assay, in 44 patients with the HBsAg test, in 16 patients with the HBeAg test, in 26 patients with the HBV DNA assay, and in 42 patients with the HBcrAg assay (Table I). Among the 13 patients with negative results with HBsAg, HBeAg, and HBV DNA but positive results with cccDNA assay, six patients showed HBcrAg-positive results, although the serum HBcrAg concentration value was low (mean value ± standard deviation: 3.23 ± 0.27 log U/ml) in comparison to that of the group with positive results with HBsAg, HBeAg, and HBV DNA (6.91 ± 1.06 log U/ml) tests. Among the 28 patients with HBsAg-positive but HBeAg-negative results, 20 patients were HBcrAg-positive and 10 out of these 20 patients showed negative HBV DNA assay results.

Next, assay results were analyzed according to presence/absence of nucleos(t)ide analogue treatment, HBsAg-positive/negative results, and HBeAg-positive/negative results by the combination with treated/untreated subgroups (Table II). When patients were classified into two groups, namely 28 patients treated with nucleos(t)ide analogues and 29 untreated patients, no difference was observed in average mean value of HBcrAg, HBV cccDNA, and HBV DNA (data not shown). However, since the 13 HBsAg-negative patients were clinically stable, we further analyzed the 44 HBsAg-positive patients by grouping them according to pres-

TABLE I. Summary of HBcrAg Concentration in Positive and/or Negative Patients by HBsAg, HBeAg, HBV DNA, and HBcrAg Assay

All cases	HBsAg		HBeAg		HBV DNA		HBcrAg	
	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)
All (N = 57)	4.61 (1.64)	5.05 (1.62)	Positive (N = 44)	6.53 (1.14)	Positive (N = 12)	6.91 (1.06)	Positive (N = 12)	6.91 (1.06)
					Negative (N = 4)	5.40 (0.38)	Negative (N = 0)	
					Positive (N = 14)	4.29 (1.20)	Positive (N = 4)	5.40 (0.38)
					Negative (N = 14)	4.11 (1.20)	Negative (N = 0)	
					Positive (N = 0)		Positive (N = 10)	4.81 (1.02)
							Negative (N = 4)	<3.00
							Positive (N = 10)	4.55 (1.14)
							Negative (N = 4)	<3.00
					Positive (N = 0)		Positive Negative (N = 0)	
					Negative (N = 13)	3.11 (0.21)	Positive Negative (N = 0)	
							Positive (N = 6)	3.23 (0.27)
							Negative (N = 7)	<3.00

Mean value: log U/ml ± Standard deviation.

TABLE II. Classification of HBcrAg, cccDNA and HBV DNA Assay Results According to Presence/Absence of Treatment, HBeAg Test and HBsAg Test

Item	Category	N	HBcrAg (log U/ml)		cccDNA (log copy/mg)		HBV DNA (log copy/ml)	
			Mean (SD)	<i>P</i> value	Mean (SD)	<i>P</i> value	Mean (SD)	<i>P</i> value
Unclassified Treatment ^a	All	57	4.61 (1.64)		4.25 (0.91)		3.67 (1.59)	
	With	26	4.77 (1.49)	NS ^c	4.41 (0.68)	NS ^c	3.20 (1.08)	<0.001
	Without	18	5.45 (1.75)		4.54 (1.09)		5.13 (1.78)	
HBsAg	Positive							
	Total	44	5.05 (1.62)		4.46 (0.87)		3.99 (1.69)	
	Negative			<0.001		0.001		<0.001
HBeAg ^b	Total	13	3.11 (0.21)		3.52 (0.68)		2.60 (0.00)	
	Positive							
	Total	16	6.53 (1.14)		4.88 (1.06)		5.17 (1.92)	
	Negative			0.001		0.015		0.002
	Total	28	4.20 (1.18)		4.23 (0.64)		3.32 (1.09)	
	Positive							
	Treated	8	6.21 (1.09)	NS ^c	4.76 (0.95)	NS ^c	3.83 (1.64)	0.005
	Untreated	8	6.85 (1.18)		5.00 (1.21)		6.51 (1.02)	
	Negative							
Treated	18	4.13 (1.16)	NS ^c	4.25 (0.49)	NS ^c	2.93 (0.57)	0.016	
Untreated	10	4.33 (1.26)		4.18 (0.88)		4.02 (1.45)		

^aIn the with/without treatment group, 44 HBsAg-positive patients were analyzed.

^bHBsAg-positive 44 patients were further separated into HBeAg-positive and HBeAg-negative groups. In addition, these groups were further separated into treated and untreated groups; the mean value of each assay was calculated and a statistical analysis was done.

^cNS: statistically not significant.

ence/absence of treatment of nucleos(t)ide analogues (Table II). HBcrAg concentration was 4.77 ± 1.49 log U/ml in 26 treated patients and 5.45 ± 1.75 log U/ml in 18 untreated patients (not statistically significant). Similar results in cccDNA levels were observed in both treated and untreated groups (4.41 ± 0.68 log copy/ μ g and 4.54 ± 1.09 log copy/ μ g, not statistically significant). In contrast, lower HBV DNA was observed in the treated group (3.20 ± 1.08 log copy/ml) as compared with the untreated group (5.13 ± 1.78 log copy/ml, $P < 0.001$).

Statistically significant results were observed under grouping according to HBsAg assay results; namely, HBcrAg was 5.05 ± 1.62 log U/ml in the HBsAg-positive group and 3.11 ± 0.21 log U/ml in the HBsAg-negative group ($P < 0.001$), HBV cccDNA was 4.46 ± 0.87 log copies/ μ g in the HBsAg-positive group and 3.52 ± 0.68 log copies/ μ g in the HBsAg-negative group ($P < 0.001$), HBV DNA was 3.99 ± 1.69 log copies/ml in the HBsAg-positive group and <2.60 log copies/ml in the HBsAg-negative group ($P < 0.001$).

Similar results were observed under grouping according to HBeAg results. In this analysis, HBsAg-negative patients were omitted because HBeAg-negative patients included both HBsAg-positive/negative patients, whereas all HBeAg-positive patients were HBsAg-positive. The mean values of HBcrAg, HBV cccDNA, and HBV DNA in the HBeAg-positive group were higher than those of the HBeAg-negative group; namely, HBcrAg concentration was 6.53 ± 1.14 log U/ml in the HBeAg-positive group and 4.20 ± 1.18 log U/ml in the HBeAg-negative group ($P < 0.001$), cccDNA was 4.88 ± 1.06 log copy/ μ g in the HBeAg-positive group and 4.23 ± 0.64 log copies/ μ g in the HBeAg-negative group ($P = 0.015$), HBV DNA was 5.17 ± 1.92 log copies/ml in the HBeAg-positive group and 3.32 ± 1.09 log copies/ml

in the HBeAg-negative group ($P = 0.002$). When the HBeAg-positive group was further separated into treated and untreated groups, these three markers in eight treated patients were lower than in eight untreated patients; namely, HBcrAg (6.21 ± 1.09 log U/ml vs. 6.85 ± 1.18 log U/ml), cccDNA (4.76 ± 0.95 log copy/ μ g vs. 5.00 ± 1.21 log copy/ μ g), and HBV DNA (3.83 ± 1.64 log copy/ml vs. 6.51 ± 1.02 log copy/ml), although HBcrAg and cccDNA values between the treated and untreated groups were not statistically significant. In the HBeAg-negative group, statistically significant ($P = 0.016$) lower values of HBV DNA but not of HBcrAg and cccDNA were observed in 18 treated patients by comparison with 10 untreated patients. The HBV DNA level of 8 out of 10 untreated patients was less than 5 log copies/ml. These patients underwent a liver biopsy for progress follow-up. Therefore, there was a relatively small difference in HBV DNA level between treated and untreated patients.

Correlation Between HBcrAg, HBV DNA and/or cccDNA

The correlation between HBcrAg, HBV DNA, and/or cccDNA in all 57 patients was summarized in Figure 1. A statistically significant positive correlation was observed in all analyses, namely HBcrAg versus HBV cccDNA (Fig. 1a, $r = 0.692$, $P < 0.001$), HBcrAg versus HBV DNA (Fig. 1b, $r = 0.713$, $P < 0.001$), and HBV cccDNA versus HBV DNA (Fig. 1c, $r = 0.637$, $P < 0.001$).

Next, HBcrAg concentration in 31 HBV DNA-negative patients was measured; 20 patients showed levels greater than 3.0 log U/ml. A statistically significant correlation between HBcrAg and cccDNA in these 31 patients was observed (Fig. 2, $r = 0.482$, $P = 0.006$),

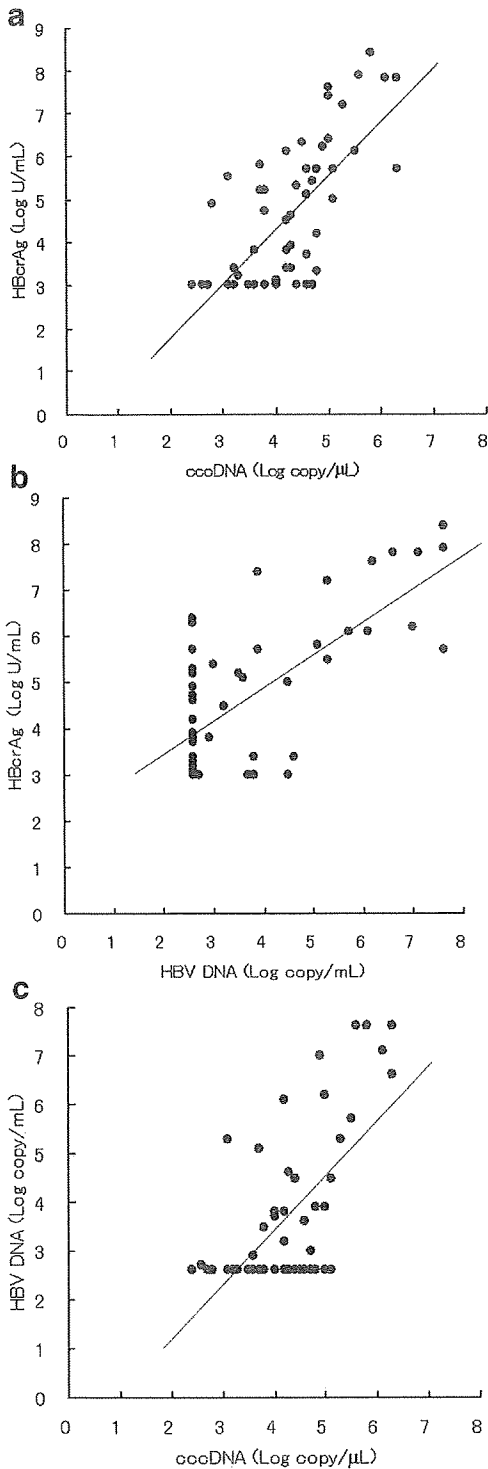


Fig. 1. Correlation between serum HBcrAg and intrahepatic HBV cccDNA in 57 patients with chronic hepatitis B (a: $y = 1.25 \times -0.69$, $r = 0.692$, $P < 0.001$), HBcrAg and serum HBV DNA (b: $y = 0.74 \times +1.91$, $r = 0.713$, $P < 0.001$), and serum HBV DNA and intrahepatic cccDNA (c: $y = 1.11 \times -1.05$, $r = 0.637$, $P < 0.001$). Straight lines indicate the correlation between each other.

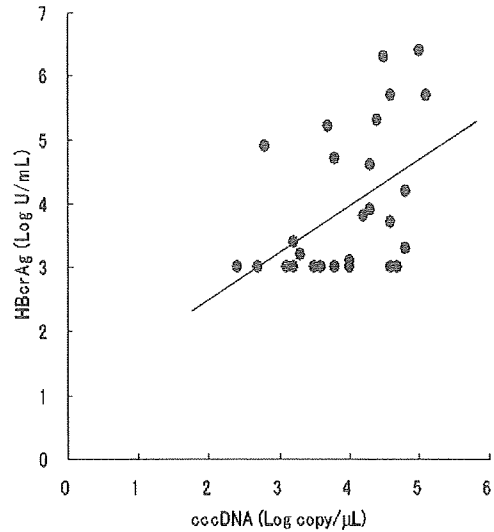


Fig. 2. Correlation between HBcrAg and cccDNA in 31 HBV DNA negative patients ($y = 0.73 \times +1.00$, $r = 0.482$, $P = 0.006$).

suggesting that HBcrAg measurement may be a useful marker in HBV DNA-negative patients as a substitute for cccDNA assay.

When 44 HBsAg positive patients were grouped according to whether they were HBeAg-positive or HBeAg-negative, HBcrAg concentration was correlated with cccDNA in both 16 HBeAg-positive patients (Fig. 3a, $r = 0.687$, $P = 0.003$) and 28 HBeAg-negative patients (Fig. 3a, $r = 0.542$, $P = 0.003$), and with HBV DNA in the HBeAg-positive (Fig. 3b, $r = 0.681$, $P = 0.004$) but not in the HBeAg-negative group (Fig. 3b, $r = 0.311$, $P = 0.107$). A positive correlation between HBV DNA and cccDNA was also observed in both the HBeAg-positive group (Fig. 3c, $r = 0.588$, $P = 0.017$) and the HBeAg-negative group (Fig. 3c, $r = 0.442$, $P = 0.018$).

DISCUSSION

Nucleos(t)ide analogues have a suppressive effect on the transcription of pregenomic RNA, and the administration of these agents can induce a rapid and dramatic decrease in peripheral HBV DNA, seroclearance of HBeAg, and remission of chronic hepatitis B [Dienstag et al., 1995, 1999; Lai et al., 1998]. However, these nucleos(t)ide analogues are unable to induce an adequate and complete elimination of HBV. Therefore, the measurement of intrahepatic HBV DNA and/or HBV cccDNA is important for monitoring the viral status of hepatitis patients [Sung et al., 2005], although these assays involve the physical stress of needle biopsy.

Several reports indicate that the level of HBcrAg, which is a complex of HBeAg, HBcAg, and p22cr coding precore/core gene [Kimura et al., 2002, 2005], reflects the natural course of viral loads in patients under treatment with nucleos(t)ide analogues, and that the reduction rate of HBcrAg is slower than that of serum

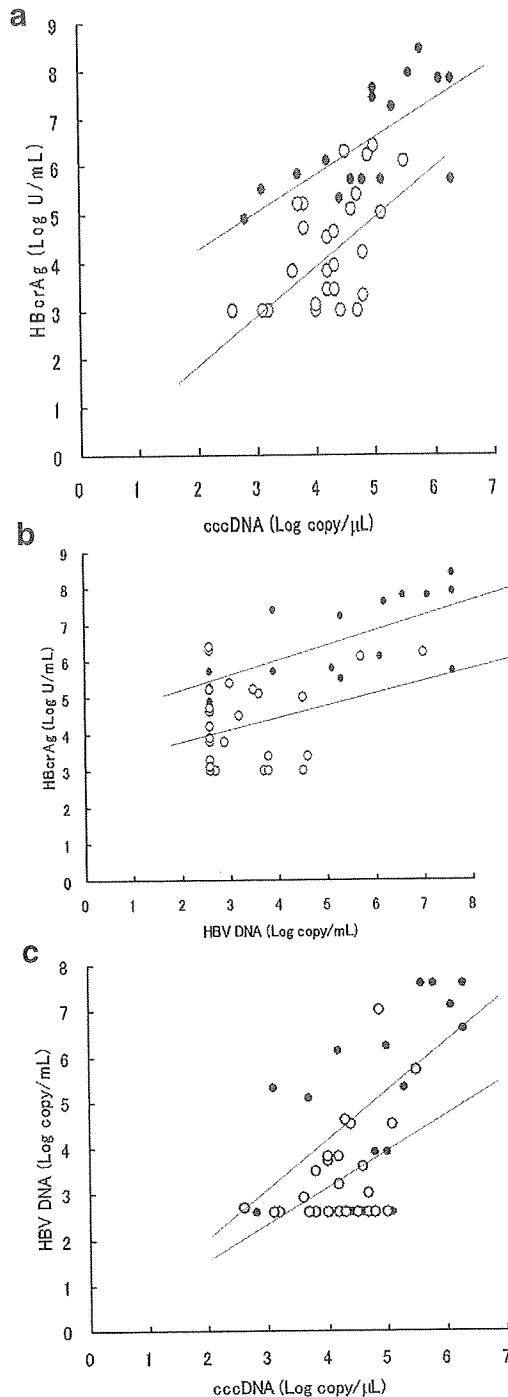


Fig. 3. Correlation between HBcrAg, cccDNA and HBV DNA in 44 HBsAg-positive with 16 HBeAg-positive and 28 HBeAg-negative patients. **a:** Correlation between HBcrAg and cccDNA (HBeAg-positive cases as closed circle; $y = 0.74x + 2.91$, $r = 0.687$, $P = 0.003$, HBeAg-negative cases as open circle; $y = 1.00x - 0.02$, $r = 0.542$, $P = 0.003$). **b:** Correlation between HBcrAg and serum HBV DNA (HBeAg-positive patients as closed circle; $y = 0.41x + 4.43$, $r = 0.681$, $P = 0.004$, HBeAg-negative patients as open circle; $y = 0.34x + 3.09$, $r = 0.311$, $P = 0.107$). **c:** Correlation between serum HBV DNA and intrahepatic cccDNA (HBeAg-positive cases as closed circle; $y = 1.07x - 0.03$, $r = 0.588$, $P = 0.017$, HBeAg-negative patients as open circle; $y = 0.76x + 0.12$, $r = 0.442$, $P = 0.018$).

HBV DNA [Rokuhara et al., 2003; Tanaka et al., 2006]. Similar results were observed in patients infected with genotypes B and C of HBV [Rokuhara et al., 2005]. This phenomenon may be explained by the fact that the production of HBcrAg depends on the transcription of mRNA from cccDNA, and that cccDNA still remains in high levels after treatment with these nucleos(t)ide analogues. Therefore, several reports suggest that HBcrAg may be a predicting marker for relapse after cessation of lamivudine therapy in chronic HBV infection [Shinkai et al., 2006; Matsumoto et al., 2007] and that it may also help identify patients who are at low risk of lamivudine resistance [Tanaka et al., 2006].

In this study, we analyzed the correlation between HBcrAg and several HBV markers, especially HBV cccDNA. Results indicated a good correlation of HBcrAg against serum HBV DNA and intrahepatic HBV cccDNA (Fig. 1). In addition, 20 out of 31 HBV DNA-negative patients showed more than 3.0 log U/ml in HBcrAg. All of these 20 patients were also cccDNA-positive, and there was a positive correlation between HBcrAg and cccDNA levels, although HBcrAg was negative in 11 patients (Fig. 2). The production of HBcrAg is considered to depend on the transcription of mRNA from intrahepatic cccDNA. Our data showed that serum HBcrAg may reflect intrahepatic cccDNA. Therefore, measurement of HBcrAg as a substitute for cccDNA may be useful for monitoring chronic hepatitis B patients. Recently, the acquisition of de novo HBV-related hepatitis after liver transplantation has become an important cause of morbidity and mortality. Moreover, de novo HBV-related hepatitis has been reported in patients after hematopoietic stem cell transplantation and cytotoxic chemotherapy treatment [Dhedin et al., 1998; Hui et al., 2006]. Therefore, HBcrAg may be a useful marker of occult HBV infection in these patients.

Several reports indicate that HBsAg seroclearance confers favorable long-term outcomes in patients without hepatocellular carcinoma or decompensated liver cirrhosis [Arase et al., 2006; Kobayashi et al., 2006]. However, studies show that intrahepatic HBV DNA still remains in HBsAg seroclearance cases [Arase et al., 2006], and that 10–20% of patients have 50–100 copies/ml of serum HBV DNA for 5 and 10 years after seroclearance of HBsAg [Arase et al., 2007]. In this study, 6 out of 13 patients with HBsAg seroclearance showed HBcrAg-positive results (3.23 ± 0.27 log U/ml), and all 13 patients remained cccDNA-positive (3.52 ± 0.68 log copy/ μ g). These data suggest that HBV remains present for a prolonged period after HBsAg seroclearance, further studies are thus necessary to clarify the mechanism of HBcrAg production and/or the regulation of mRNA in chronic hepatitis with HBsAg seroclearance.

Meanwhile, positive correlations between HBV DNA and HBcrAg were not observed in the HBsAg-positive and HBeAg-negative group (Fig. 3b), although HBcrAg concentration was correlated with cccDNA in HBeAg-negative patients (Fig. 3a). This finding shows that

measurement of HBcrAg as a substitute for cccDNA may be useful for monitoring patients in HBsAg-positive and HBeAg-negative groups.

In conclusion, serum HBcrAg concentration appears to be well correlated with intrahepatic cccDNA level, and the measurement of serum HBcrAg as substitute for cccDNA and/or serum HBV DNA may be clinically useful for the monitoring of intrahepatic HBV viral status.

REFERENCES

- Arase Y, Ikeda K, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, Akuta N, Someya T, Hosaka T, Sezaki H, Kobayashi M, Kumada H. 2006. Long-term outcome after hepatitis B surface antigen seroclearance in patients with chronic hepatitis B. *Am J Med* 119:9–16.
- Arase Y, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, Akuta N, Someya T, Hosaka T, Sezaki H, Sato J, Kobayashi M, Ikeda K, Kumada H. 2007. Long-term presence of HBV in the sera of chronic hepatitis B patients with HBsAg seroclearance. *Intervirology* 50:161–165.
- Chayama K, Suzuki Y, Kobayashi M, Kobayashi M, Tsubota A, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Murashima N, Ikeda K, Kumada H. 1998. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. *Hepatology* 27:1711–1716.
- Chin R, Shaw T, Torresi J, Sozzi V, Trautwein C, Bock T, Manns M, Isom H, Furman P, Locarnini S. 2001. In vitro susceptibilities of wild-type or drug-resistant hepatitis B virus to (-)-β-D-2,6-diaminopurine dioxolane and 2'-fluoro-5-methyl-β-L-arabinofuranosyluracil. *Antimicrob Agents Chemother* 45:2495–2501.
- Colonna RJ, Genovesi EV, Medina I, Lamb L, Durham SK, Haug ML, Corey L, Littlejohn M, Locarnini S, Tennant BC, Rose B, Clark JM. 2001. Long-term entecavir treatment results in sustained antiviral efficacy and prolonged life span in the woodchuck model of chronic hepatitis infection. *J Infect Dis* 184:1236–1245.
- Dhedin N, Douvin C, Kuentz M, Saint Marc MF, Reman O, Rieux C, Bernaudin F, Norol F, Cordonnier C, Bobin D, Metreau JM, Vernant JP. 1998. Reverse seroconversion of hepatitis B after allogeneic bone marrow transplantation: A retrospective study of 37 patients with pretransplant anti-HBs and anti-HBc. *Transplantation* 66:616–619.
- Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. 1995. A preliminary trial of lamivudine for chronic hepatitis B infection. *N Eng J Med* 333:1657–1661.
- Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HWL, Goodman Z, Crowther L, Condrey LD, Woessner M, Rubin M, Brown NA, The U.S. Lamivudine Investigator Group. 1999. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Eng J Med* 341:1256–1263.
- Hatakeyama T, Noguchi C, Hiraga N, Mori N, Tsuge M, Imamura M, Takahashi S, Kawakami Y, Fujimoto Y, Ochi H, Abe H, Maekawa T, Kawakami H, Yatsuji H, Aisaka Y, Kohno H, Aimitsu S, Chayama K. 2007. Serum HBV RNA is a predictor of early emergence of the YMDD mutant in patients treated with lamivudine. *Hepatology* 45:1179–1186.
- Hui CK, Cheung WW, Zhang HY, Au WY, Yueng YH, Leung AY, Leung N, Luk JM, Lie AK, Kwong YL, Liang R, Lau GK. 2006. Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. *Gastroenterology* 131:59–68.
- Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, Maki N. 2002. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 40:439–445.
- Kimura T, Ohno N, Terada N, Rokuhara A, Matsumoto A, Yagi S, Tanaka E, Kiyosawa K, Ohno S, Maki N. 2005. Hepatitis B virus DNA-negative Dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. *J Biol Chem* 280:21713–21719.
- Kobayashi M, Suzuki F, Akuta N, Suzuki Y, Arase Y, Ikeda K, Hosaka T, Sezaki H, Kobayashi M, Iwasaki S, Sato J, Watabiki S, Miyakawa Y, Kumada H. 2006. Response to long-term lamivudine treatment in patients infected with hepatitis B virus genotypes A, B, and C. *J Med Virol* 78:1276–1283.
- Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF, The Asia Hepatitis Lamivudine Study Group. 1998. A one-year trial of lamivudine for chronic hepatitis B. *N Eng J Med* 339:61–68.
- Lok ASF, McMahon BJ. 2004. Chronic hepatitis B: Update of recommendations. *Hepatology* 39:857–861.
- Mason AL, Xu L, Guo L, Kuhns M, Perrillo RP. 1998. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. *Hepatology* 27:1736–1742.
- Matsumoto A, Tanaka E, Minami M, Okanoue T, Yatsushashi H, Nagaoka S, Suzuki F, Kobayashi M, Chayama K, Imamura M, Yotsuyanagi H, Nakaoka S, Maki N, Kawata S, Kumada H, Iino S, Kiyosawa K. 2007. Low serum level of hepatitis B core-related antigen indicates unlikely reactivation of hepatitis after cessation of lamivudine therapy. *Hepatology* 37:661–666.
- Rokuhara A, Tanaka E, Matsumoto A, Kimura T, Yamaura T, Orii K, Sun X, Yagi S, Maki N, Kiyosawa K. 2003. Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. *J Viral Hepat* 10:324–330.
- Rokuhara A, Sun X, Tanaka E, Mimura T, Matsumoto A, Yao D, Yin L, Wang N, Maki N, Kiyosawa K. 2005. Hepatitis B virus core and core-related antigen quantitation in Chinese patients with chronic genotype B and C hepatitis B virus infection. *J Gastroenterol Hepatol* 20:1726–1730.
- Rokuhara A, Matsumoto A, Tanaka Y, Umemura T, Yoshizawa K, Kimura T, Maki N, Kiyosawa K. 2006. Hepatitis B virus RNA is measurable in serum and can be a new marker for monitoring lamivudine therapy. *J Gastroenterol* 41:785–790.
- Shinkai N, Tanaka Y, Orito E, Ito K, Ohno T, Hirashima N, Hasegawa I, Sugauchi F, Ueda R, Mizokami M. 2006. Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection. *Hepatology* 36:272–276.
- Sung JJY, Wong ML, Bowden S, Liew CT, Hui AY, Wong VWS, Leung NWY, Locarnini S, Chan HLY. 2005. Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. *Gastroenterol* 128:1890–1897.
- Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, Tsubota A, Kobayashi M, Koike M, Ogawa N, Tanikawa K. 1999. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. *J Hepatol* 30:743–748.
- Suzuki F, Tsubota A, Arase Y, Suzuki Y, Akuta N, Hosaka T, Someya T, Kobayashi M, Saitoh S, Ikeda K, Kobayashi M, Matsuda M, Satoh J, Takagi K, Kumada H. 2003. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. *Intervirology* 46:182–189.
- Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsushashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K. 2006. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance. *Liver Int* 26:90–96.
- Yuen MF, Wong DK, Sum SS, Yuan HJ, Yuen JC, Chan AO, Wong BC, Lai CL. 2005. Effect of lamivudine therapy on the serum covalently closed circular (ccc) DNA of chronic hepatitis B infection. *Am J Gastroenterol* 100:1099–1103.

CLINICAL STUDIES

Predictive factors of advanced recurrence after curative resection of small hepatocellular carcinoma

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Abstract

Background: The tumour recurrence rate after resection is still high even in patients with small hepatocellular carcinoma (HCC). The advanced patterns of recurrence occasionally occur after resection. In this study, we analysed the clinical and histological characteristics of small HCC and evaluated the predictive factors of advanced tumour recurrence. **Methods:** One hundred and sixty-five patients underwent resection of small HCC measuring 3 cm or less in greatest dimension. Patterns of tumour recurrences were classified into advanced recurrence and minor recurrence based on size, number, vascular invasion and extrahepatic metastasis of recurrent tumour. We created a simple index to closely evaluate the malignant potential of small HCC, named α -foetoprotein–size ratio index (ASRI). **Results:** Overall tumour recurrence was significantly associated with tumour multiplicity ($P < 0.001$) and ASRI ($P = 0.001$). Tumour multiplicity, ASRI and tumour differentiation were independent and significant predictive factors of advanced recurrences. The overall survival rates were lower in the advanced recurrence group than the minor recurrence or the no recurrence group. **Conclusions:** Patients with advanced recurrences have a poor prognosis, although they have undergone curative resection of small HCC. On the other hand, patients with minor recurrences have a relatively good prognosis. ASRI was a useful index to predict advanced recurrence after curative resection of small HCC. The therapeutic management to prevent advanced recurrences is needed.

Hepatocellular carcinoma (HCC) is one of the most common neoplasms in Africa and Asia, including Japan. Routine checkups are performed in patients with hepatitis or cirrhosis who constitute a significant high-risk group for HCC (1–3). Recently, technological advances in ultrasonography (US), computed tomography (CT) and magnetic resonance imaging have helped in the detection of small HCC during follow-up periods of chronic liver disease (4, 5). Moreover, resection of HCC has become safe in cirrhotic patients due to progress in surgical techniques, and perioperative management has contributed to very low operative mortality. However, the tumour recurrence rate after resection is still high even in patients with small HCCs (6–10). Recurrences in the remnant liver can occur based on two characteristics of HCC: intrahepatic metastasis from the primary tumour and de novo multicentric carcinogenicity (11–13).

Tumour status at the time of recurrence is important to improve prognosis because tumour recurrence rates after curative resection are high. The advanced patterns of recurrence occasionally occur as follows: widespread recurrence, a number of recurrent tumours, large recurrent tumour, involving vascular invasion and extrahepatic metastasis, despite curative resection (14–16). Because the therapeutic approach for recurrent tumours is limited, these cases have a poor prognosis. Therefore, it is important to pick up patients who are likely to have these advanced recurrence, and to develop effective adjuvant therapy. In the present study, we examined the clinical features of small HCC, and identified the factors associated with tumour recurrence, especially advanced recurrence and prognosis after curative resection of small HCCs using clinical data and results of histopathological examination. Furthermore, we created a

simple index to closely evaluate the malignant potential of small HCC and evaluated the usefulness of this index as a predictor of recurrence of HCC after curative resection.

Patients and methods

Patients

Medical records of patients who were hospitalized at Toranomon Hospital from 1995 to 2005 were reviewed retrospectively. HCC was diagnosed by detailed imaging or histopathological examination. A total of 251 consecutive patients with tumours underwent resection as the initial therapy for HCC, and 165 of these patients were found to have HCC measuring ≤ 3 cm (greatest dimension) and were eligible for inclusion in this study. These 165 patients (127 men and 38 women; median age 61 years; range, 38–73 years) had chronic hepatitis or cirrhosis. Hepatitis B virus (HBV) surface antigen was positive in 33, anti-hepatitis C virus (HCV) was positive in 127, but neither of them was positive in eight. Table 1 lists the clinical characteristics of the 165 patients before hepatectomy. Of these, 125 patients (75.6%) were classified as grade A according to Child–Pugh classification. The median value for the indocyanine green retention rate at 15 min was 24%, and the median values for serum albumin, bilirubin, aspartic transaminase (AST), α -foetoprotein (AFP) concentration and platelet counts were 3.7 g/dl, 1.0 mg/dl, 44 IU/L, 26 ng/ml and $10.8 \times 10^3/\text{mm}^3$ respectively.

Among 165 patients, 26 patients (15.8%) had multiple tumours before resection. We conducted percutaneous ablation therapy, including ethanol injection, microwave coagulation

Table 1. Clinical characteristics of 165 patients before hepatic resection

Variables	n = 165
Age	62 (38–80)*
Gender (male:female)	127:38
Hepatitis B surface antigen-positive	46 (27.9%)
Anti-hepatitis C virus-positive	109 (66.1%)
Child–Pugh classification (A:B:C)	125:38:1
Serum albumin (g/dl)	3.6 (2.6–4.6)*
Serum bilirubin (mg/dl)	1.0 (0.3–2.7)*
Aspartate transaminase (IU/L)	44 (12–386)*
Prothrombin time (%)	90.8 (58.9–112.8)*
ICG R15 (%)	21 (3–68)*
Platelet count (10 ⁴ /mm ³)	12.6 (3.9–26.0)*
α -foetoprotein (ng/ml)	23 (1–7960)*
Des- γ -carboxy prothrombin (mAU/ml)	22 (< 10–1650)*
Tumour size (mm)	20 (7–30)*
Tumour number (solitary:multiple)	139:26
Vascularity positive	153 (92.7%)
ASRI	1.2 (0.03–345)*

*Values are medians (range).

ASRI, α -foetoprotein–size ratio index = AFP (ng/ml)/tumour size (mm); ICG R15, indocyanine green retention test at 15 min.

and radiofrequency ablation, for another tumour before surgery if another tumour existed in a lobe distant from the resected tumour. The term 'curative resection' indicated that no tumours were left in the remnant liver irrespective of the width of margin around the tumour; this was confirmed using (i) intra-operative US and (ii) combined US and dynamic CT conducted after 1 month of surgery.

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from every patient. This study was approved by the Local Ethics Committee of Toranomon Hospital.

Follow-up and recurrence of hepatocellular carcinoma

Patients were followed up on a monthly or a bi-monthly basis after surgery by monitoring AFP and other biochemical data, and conducting US or helical dynamic CT every 3 months. The median observation period for the entire patient cohort was 6.0 years, with a range of 0.3–16.4 years. Recurrence of HCC was diagnosed by typical hypervascular characteristics on angiography and/or histological examination with fine needle biopsy specimens, in addition to certain features of CT and US.

The modes of cancer recurrence were classified into two categories: (i) advanced recurrence and (ii) minor recurrence. The patterns of recurrence were morphologically judged from the images of CT and angiography, and from histopathological findings. The pattern of recurrent tumour number > 3, tumour size > 3 cm, involving vascular invasion and/or extrahepatic metastasis was defined as advanced recurrence. The recurrent pattern, except for those described above, was defined as minor recurrence.

Imaging analysis

Ultrasonography or helical dynamic CT was carried out every 3 months for follow-up and examined for a change in imaging findings. Dynamic CT scans were performed using a single-

Table 2. Pathological characteristics of small hepatocellular carcinoma

Variables	n = 165
Tumour differentiation (early:well:moderately:poorly)	11:32:100:22
Growth type (Eg:Ig)	138:27
Capsular formation	99 (60.0%)
Capsular infiltration	52 (31.5%)
Septum formation	42 (25.5%)
Portal vein invasion	26 (15.8%)
Intrahepatic extent of tumour	5 (3.0%)
Presence of cirrhosis	114 (69.1%)

Eg, expansive growth (well-demarcated border); Ig, infiltrative growth (poorly demarcated border).

detector helical CT scanner (Hi-Speed advantage SG; GE Yokogawa Medical Systems, Tokyo, Japan). The radiological studies included intra-arterial digital subtraction angiography (celiac and mesenteric angiography) and selective angiography of the common hepatic artery, CT arterial portography (CT-AP) and CT hepatic angiography (CT-HA) were carried out in almost all patients before surgery. HCC was diagnosed by typical hypervascular characteristics on angiography and/or CT-HA, and hypo-attenuation on CT-AP. If hepatic nodules showed iso-hypo-attenuation on CT-HA and iso-hypo-attenuation on CT-AP, histological examination was carried out with fine needle biopsy specimens before surgery.

Histopathological examination

Macroscopic and microscopic examinations were performed according to the classification of the Liver Cancer Study Group of Japan (17). All resected specimens were analysed histopathologically for tumour size, growth type, tumour differentiation, capsular formation, portal vein invasion, satellite nodules and fibrosis staging of surrounding liver. The tumour characteristics are summarized in Table 2. We categorized well-differentiated HCC that had histological features of the early stage into early HCC. Early HCC was defined as follows: macroscopically, the tumours had an indistinct margin that replaced the liver cell cords at the tumour–non-tumour boundary; microscopically, increased cell density with an increased nuclear to cytoplasm ratio and an irregular thin-trabecular pattern, and the portal tracts were involved inside the tumours together with tumour cell invasion into the portal tracts (18–20).

α -foetoprotein–size ratio index

In this study, there were patients with very high AFP levels regardless of the cohort of small HCC measuring 3 cm or less in greatest dimension. We hypothesized that HCCs with high AFP levels had more malignant potential than those with low AFP levels if each tumour size was equal. And so, we created a simple index to closely evaluate the malignant potential of small HCC, named the AFP–size ratio index (ASRI). The numerical formula of ASRI was defined as follows: ASRI = AFP levels (ng/ml)/tumour size (mm). For example, the calculated value of ASRI of HCC, with tumour size = 20 mm and AFP levels = 400 ng/ml, is 20.

Statistical analysis

Standard statistical measures and procedures were used. We used the χ^2 -test to assess the significant association of risk

factors with tumour recurrence after resection. All factors found to be at least marginally associated with recurrence ($P < 0.15$) were tested by multivariate analysis. Independent factors, associated with the recurrence of HCC and prognosis, were calculated using stepwise Cox regression analysis. The χ^2 -test was used to analyse differences between the clinical characteristics of HCC and the patterns of tumour recurrences. The cumulative overall survival rates after resection of small HCC were analysed using the Kaplan–Meier method, and differences in the curves were tested using the log-rank test. A P value of < 0.05 in a two-tailed test was considered significant. Data analysis was performed using the SPSS software, version 11.0 (Chicago, IL, USA).

Results

Factors associated with tumour recurrences

Univariate analysis showed that tumour recurrence was significantly associated with tumour multiplicity ($P < 0.001$), ASRI ≥ 20 ($P = 0.004$), AFP levels ≥ 1000 ng/ml ($P = 0.024$), portal vein invasion ($P = 0.035$) and serum albumin levels ≥ 3.5 g/dl ($P = 0.041$), and marginally significantly with HCV positivity ($P = 0.058$), HBV negativity ($P = 0.072$), hypervascularity of tumour ($P = 0.076$) and serum AST levels ≥ 50 IU/L ($P = 0.088$) (Table 3). Because these variables were associated, multivariate analysis was performed using the nine variables mentioned above in the model (Table 4a). The following two variables were significantly associated with overall tumour recurrence: tumour multiplicity [hazard ratio (HR) 3.06, 95% confidence interval (CI): 1.84–5.10; $P < 0.001$], ASRI ≥ 20 (HR 2.42, 95% CI: 1.41–4.18, $P = 0.001$). To evaluate risk factors except for tumour multiplicity, subgroup analysis was conducted in solitary tumour cases (Table 4b). Independent risk factors affecting the overall recurrence of HCC were the presence of portal vein invasion (HR 2.35, 95% CI: 1.31–4.20, $P = 0.004$), ASRI ≥ 20 (HR 2.23, 95% CI: 1.19–4.18, $P = 0.013$) and serum albumin < 3.5 g/dl (HR 1.74, 95% CI: 1.05–2.88, $P = 0.030$).

Predictive factors of advanced recurrences after curative resection

Tumour recurrence was diagnosed in 102 (61.8%) of the 165 patients, with a median interval of 2.77 years after curative resection. Of these, 22 (13.3%) were categorized into advanced recurrence, 80 (48.4%) were minor recurrence and the remaining 63 (38.1%) were no recurrence. The median interval to recurrence after resection was 1.82 years in the minor recurrence group and 1.01 years in the advanced recurrence group respectively. Univariate analysis showed that advanced recurrence was significantly associated with the following four factors: poorly differentiation of tumour ($P < 0.001$), ASRI ≥ 20 ($P = 0.005$), tumour multiplicity ($P = 0.017$) and AFP levels ≥ 1000 ng/ml ($P = 0.025$) (Table 5). Multivariate analysis by the Cox model was performed using the four variables mentioned above. Predictive factors of advanced recurrences after curative resection were tumour multiplicity (HR 5.65, 95% CI: 1.77–18.1, $P = 0.003$), ASRI ≥ 20 (HR 4.04, 95% CI: 1.16–14.1, $P = 0.028$) and poor differentiation of tumour (HR 2.70, 95% CI: 1.51–4.82, $P = 0.001$) (Table 6).

We compared values of ASRI by patterns of recurrences (Fig. 1). The median values of ASRI were 0.63 (minimum: 0.07–maximum: 73.0) in the no recurrence group, 1.64 (0.06–344) in the minor recurrence group and 3.28 (0.03–318) in the advanced recurrence group respectively. The values of ASRI were marginally

Table 3. Factors associated with overall recurrence of small hepatocellular carcinoma by univariate analysis

Factors	Hazard ratio (95% CI)	P
Age (≥ 65 vs. < 65 years)	0.79 (0.52–1.22)	0.288
Gender (female vs. male)	0.78 (0.48–1.26)	0.316
HBV (negative vs. positive)	1.52 (0.96–2.41)	0.072
HCV (positive vs. negative)	1.53 (0.99–2.36)	0.058
Serum albumin (< 3.5 vs. ≥ 3.5 g/dl)	1.53 (1.02–2.31)	0.041
Serum bilirubin (≥ 1.5 vs. < 1.5 mg/dl)	1.11 (0.62–2.00)	0.713
AST levels (≥ 50 vs. ≥ 50 IU/L)	1.41 (0.95–2.10)	0.088
Prothrombin time (≥ 70 vs. < 70 %)	0.67 (0.31–1.45)	0.311
ICG R 15 (≥ 30 vs. < 30 %)	1.37 (0.89–2.12)	0.158
count ($\geq 10^5$ vs. $< 10^5$ /mm ³)	0.81 (0.54–1.22)	0.304
AFP levels (≥ 1000 vs. < 1000 ng/ml)	2.01 (1.10–3.67)	0.024
ASRI (≥ 20 vs. < 20)	2.16 (1.23–3.64)	0.004
DCP levels (≥ 100 vs. < 100 mAU/ml)	1.19 (0.70–2.04)	0.517
Fibrosis stage (F4 vs. F1, 2, 3)	1.09 (0.72–1.66)	0.681
Tumour size (≥ 21 vs. < 21 mm)	1.088 (0.73–1.63)	0.680
Tumour number (multiple vs. solitary)	2.85 (1.74–4.65)	< 0.001
Vascularity (positive vs. negative)	2.48 (0.91–6.76)	0.076
Tumour differentiation (poorly vs. early, well, moderately)	1.15 (0.87–1.51)	0.333
Eg	1.00 (0.60–1.63)	0.987
Capsular formation	1.01 (0.63–1.52)	0.948
Infiltration to capsular	1.39 (0.92–2.10)	0.121
Septum formation	0.99 (0.63–1.56)	0.969
Portal vein invasion	1.70 (1.04–2.78)	0.035
Intrahepatic extent of tumour	1.57 (0.58–4.26)	0.380

AFP, α -foetoprotein; ASRI, α -foetoprotein–size ratio index; AST, aspartic transaminase; DCP, des- γ -carboxy prothrombin; Eg, expansive growth (well-demarcated border); HBV, hepatitis B virus; HCV, hepatitis C virus; ICG R15, indocyanine green retention test at 15 min.

Table 4a. Independent risk factors affecting the overall recurrence of hepatocellular carcinoma after curative resection of small hepatocellular carcinoma by multivariate analysis

Factors	Category	Hazard ratio (95% CI)	P value
Tumour number	1: solitary	1	
	2: multiple	3.06 (1.84–5.10)	< 0.001
ASRI	1: < 20	1	
	2: ≥ 20	2.42 (1.41–4.18)	0.001

ASRI, α -foetoprotein–size ratio index; CI, confidence interval.

ally significantly higher in the minor recurrence and the advanced recurrence group than in the no recurrence group. However, there was no significance of ASRI values stratified by tumour number.

Furthermore, we categorized the following three subgroups into the advanced recurrence group: tumour number > 3 , or tumour size > 3 cm without vascular invasion and extrahepatic metastasis (multi/large nodular recurrence group), recurrent tumour with vascular invasion (vascular invasion group) and

Table 4b. Independent risk factors affecting the overall recurrence of hepatocellular carcinoma after curative resection of small hepatocellular carcinoma by multivariate analysis (solitary cases only)

Factors	Category	Hazard ratio (95% CI)	P value
Portal vein invasion	1: -	1	0.004
	2: +	2.35 (1.31-4.20)	
ASRI	1: < 20	1	0.013
	2: ≥ 20	2.23 (1.19-4.18)	
Serum albumin	1: ≥ 3.5	1	0.030
	2: < 3.5	1.74 (1.05-2.88)	

ASRI, α -foetoprotein-size ratio index; CI, confidence interval.

Table 5. Univariate analysis for clinical factors associated with advanced recurrence

Factors	Advanced recurrence, n = 22 (%)	Minor recurrence, n = 80 (%)	No recurrence, n = 63 (%)
Age			
< 65 years	13 (59)	59 (73.8)	35 (55.6)
≥ 65 years	9 (41)	21 (26.2)	28 (44.4)
Gender			
Male	19 (86.4)	62 (77.5)	46 (73)
Female	3 (13.6)	18 (22.5)	17 (27)
HBV			
Positive	5 (22.7)	19 (23.8)	22 (34.9)
Negative	17 (77.3)	61 (76.2)	41 (65.1)
HCV			
Negative	5 (22.7)	23 (28.8)	28 (44.4)
Positive	17 (77.3)	57 (71.2)	35 (55.6)
Serum albumin			
≥ 3.5	11 (50)	49 (61.3)	41 (65.1)
< 3.5	11 (50)	31 (39.7)	22 (34.9)
Serum bilirubin			
< 1.5	21 (95.5)	67 (83.8)	56 (88.9)
≥ 1.5	1 (4.5)	13 (16.2)	7 (11.1)
AST levels			
< 50	17 (77.3)	40 (50)	42 (66.7)
≥ 50	5 (22.7)	40 (50)	21 (33.3)
Prothrombin time			
< 70	2 (9.1)	5 (6.3)	14 (22.2)
≥ 70	20 (90.9)	75 (93.7)	49 (77.8)
ICG R 15			
< 30	15 (68.2)	58 (72.5)	48 (76.2)
≥ 30	7 (31.8)	22 (27.5)	15 (23.8)
Platelet count			
< 10 ⁵	7 (31.8)	28 (35)	30 (47.6)
≥ 10 ⁵	15 (68.2)	52 (65)	33 (52.4)
AFP levels			
< 1000	17 (77.3)	73 (91.3)	61 (96.8)
≥ 1000	5 (22.7) [‡]	7 (8.7)	2 (3.2)
ASRI			
< 20	15 (68.2)	70 (87.5)	60 (95.2)
≥ 20	7 (31.8) [‡]	10 (12.5)	3 (4.8)
DCP levels			
< 100	18 (81.8)	68 (85)	54 (85.7)
≥ 100	4 (18.2)	12 (15)	9 (14.3)
Fibrosis stage			
F1, 2, 3	9 (41)	23 (28.8)	19 (31.7)
F4	13 (59)	57 (71.2)	41 (68.3)

Table 5. Continued

Factors	Advanced recurrence, n = 22 (%)	Minor recurrence, n = 80 (%)	No recurrence, n = 63 (%)
Tumour size			
< 21	11 (50)	53 (66.3)	37 (58.7)
≥ 21	11 (50)	27 (33.7)	26 (41.3)
Tumour number			
Solitary	14 (63.6)	67 (83.8)	58 (92.1)
Multiple	8 (36.4) [*]	13 (16.2)	5 (7.9)
Vascularity			
Negative	1 (4.5)	3 (3.8)	8 (12.7)
Positive	22 (95.5)	77 (96.2)	55 (87.3)
Tumour differentiation			
Early, well, moderately	13 (59.1)	74 (92.5)	56 (88.9)
Poorly	9 (40.9) [*]	6 (7.5)	7 (11.1)
Eg			
Eg	21 (95.5)	63 (78.8)	54 (85.7)
Ig	1 (4.5)	17 (21.2)	9 (14.3)
Capsular formation			
Absence	6 (27.3)	33 (41.3)	22 (34.9)
Presence	16 (72.7)	47 (58.7)	41 (65.1)
Infiltration to capsular			
Absence	13 (59.1)	54 (67.9)	46 (73)
Presence	9 (40.9)	26 (32.1)	17 (27)
Septum formation			
Absence	16 (72.7)	61 (76.2)	46 (73)
Presence	6 (27.3)	19 (23.8)	17 (27)
Portal vein invasion			
Absence	17 (77.3)	65 (81.3)	54 (85.7)
Presence	5 (22.7)	15 (18.7)	6 (14.3)
Intrahepatic extent of tumour			
Absence	20 (90.9)	78 (97.5)	59 (98.3)
Presence	2 (9.1)	2 (2.5)	1 (1.7)

^{*}Significantly higher than the other groups ($P < 0.05$).

AFP, α -foetoprotein; ASRI, α -foetoprotein-size ratio index; AST, aspartic transaminase; DCP, des- γ -carboxy prothorombin; Eg, expansive growth (well-demarcated border); HBV, hepatitis B virus; HCV, hepatitis C virus; ICG R15, indocyanine green retention test at 15 min; Ig, infiltrative growth (poorly demarcated border).

Table 6. Predictive factors of advanced recurrence after curative resection by multivariate analysis using the Cox model

Factors	Category	Hazard ratio (95% CI)	P value
Tumour number	1: solitary	1	0.003
	2: multiple	5.65 (1.77-18.1)	
ASRI	1: < 20	1	0.028
	2: ≥ 20	4.04 (1.16-14.1)	
Tumour differentiation	1: early, well, moderately	1	0.001
	2: poorly	(1.51-4.82)	
	2.70		

ASRI, α -foetoprotein-size ratio index; CI, confidence interval.

presence of extrahepatic metastasis (extrahepatic metastasis group). The multi/large nodular recurrence group had 17 cases (77.3%), the vascular invasion group had three (13.6%) and the

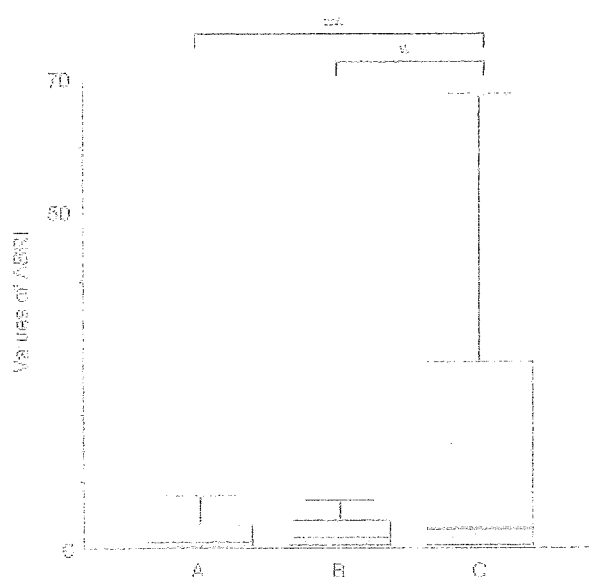


Fig. 1. Comparison with values of ASRI by patterns of recurrences. (A) No recurrence group, (B) minor recurrence group, (C) advanced recurrence group. * $P=0.032$, ** $P=0.028$.

extrahepatic metastasis group had two (9.1%) in 22 cases of advanced recurrence. In particular, patients in the vascular invasion group had significantly higher pre-operative des- γ -carboxy prothorombin levels than those in the other two groups ($P=0.008$). Meanwhile, there was no significant difference of ASRI among the three groups.

Survival rate after curative resection by patterns of recurrences

Figure 2 shows the overall survival rates by patterns of recurrences. The overall survival rates of patients were 98.5, 93.6 and 91.8% for the first, third and fifth year in the no recurrence group; 98.8, 96.5 and 85.6% in the minor recurrence group; and 91.3, 64.5 and 35.1% in the advanced recurrence group respectively. The overall survival rates of the advanced recurrence group were significantly lower than those of the minor recurrence and the no recurrence groups (advanced recurrence vs. no recurrence: $P < 0.0001$, advanced recurrence vs. minor recurrence: $P=0.001$). Furthermore, the overall survival rates of the minor recurrence group were significantly lower than those of the no recurrence group ($P=0.009$). However, the overall survival rates of both the minor recurrence and the no recurrence groups were similar for the first 5 years after surgery.

Discussion

Our study identified the clinical, radiological and histological factors associated with advanced tumour recurrence and prognosis after curative resection of small HCC. Predictive factors of advanced recurrence were tumour number, ASRI and tumour differentiation. ASRI, which was made to reflect the malignant potential of HCC precisely, was easy to calculate and useful to predict the overall and advanced recurrence of HCC. Patients in the advanced recurrence group had a poorer prognosis than those in the minor recurrence and the no recurrence groups. On the other hand, patients in the minor recurrence group had a

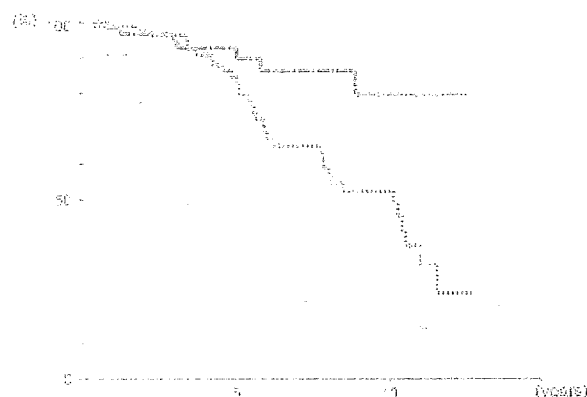


Fig. 2. Overall survival rates by patterns of recurrences; thick broken line: no recurrence group, dot line: minor recurrence group, solid line: advanced recurrence group.

prognosis similar to that of the no recurrence group for the first 5 years after resection.

Some predictors of survival and recurrence after resection were reported previously (21–24). These reports showed that the main predictors of recurrence were tumour size, tumour number, serum AFP levels, tumour differentiation, vascular invasion, etc. In the present study, we intended for patients with small HCC within 3 cm to pick up cases with high malignant potential. Therefore, tumour size was not associated with recurrence, but the other factors mentioned above were associated with recurrence as well as previous reports. However, we recently showed that ASRI was associated with both overall and advanced recurrence after resection. Small HCC with a high ASRI value may have a high malignant potential and may be likely to cause intra- or extrahepatic metastasis.

The high recurrence rate of HCC after curative resection and ablation is attributable to two principal characteristics: intrahepatic metastasis and de novo multicentric carcinogenesis. Some studies have shown that intrahepatic metastasis is an important mechanism of early recurrence after resection (13, 16, 24). In the present study, time to advanced recurrence was short: just 1 year. Furthermore, a previous study showed that tumour differentiation, which was a predictive factor of advanced recurrence in this study, was associated with intrahepatic metastasis (22). This is probably because potential metastasis depends on biological tumour factors, such as tumour differentiation. Considering these facts, a main mechanism of advanced recurrence is assumed intrahepatic metastasis. High AFP levels have been reported as a poor prognosis factor after resection of HCC (25, 26). On the other hand, it is assumed that AFP levels may increase in patients with acute or chronic active inflammation in background hepatocytes without HCC (27, 28). It is difficult to distinguish these mechanisms of AFP elevation. We created ASRI to evaluate the malignant potential of HCC by calculating AFP values per unit tumour diameter. Although it is impossible to distinguish neoplastic and inflammatory AFP elevation using this index, ASRI may mainly reflect neoplastic AFP elevation because ASRI is a predictive factor of advanced recurrence of HCC. In addition, Imamura *et al.* (24) reported that high AFP levels were associated with early recurrence within 2 years after resection, and this fact also supports our result.

α -foetoprotein levels usually tend to be higher in HBV-related HCC than those related to HCV, and this tendency has been reported by researchers in Japan, where HCV is

predominant in HCC incidence (29). However, there was no significant difference in AFP levels between HBV- and HCV-related HCC in this study. We re-evaluated the predictive factors of recurrence after resection by stratifying this cohort into HBV- or HCV-related HCC. ASRI ≥ 20 was significantly associated with overall recurrence after resection in the HBV cohort, and this result was similar in HCV. Therefore, we consider ASRI as the useful index regardless of the viral aetiology, even in an HBV-endemic area.

Patients with advanced recurrence had a poor prognosis because of limitation and resistance of treatment. The overall survival rates were lower (35.1% per 5 years) in the advanced recurrence group than in the minor or the no recurrence group, in this study. On the other hand, patients with minor recurrence had a relatively good prognosis because it was possible to conduct re-resection or percutaneous ablation therapy for recurrent tumour. Therefore, adjuvant therapy to prevent advanced recurrence after resection is needed. Although a number of studies of adjuvant therapy have been reported, none is effective for preventing intrahepatic metastasis after resection of HCC. Pre-/post-operative chemoembolization and chemotherapy had no benefit for tumour recurrence (30–32). Although a few authors including our hospital have reported that interferon is effective for preventing recurrence of HCC after resection, it is assumed that interferon itself suppresses de novo carcinogenesis (33–35). Recently, it was reported that sorafenib, which was a multikinase inhibitor, improved the overall survival rates in patients with advanced HCC (36). Sorafenib is expected to have the potential of effective adjuvant therapy to prevent tumour recurrence by intrahepatic metastasis, and a future report is awaited.

In conclusion, tumour number, ASRI and tumour differentiation were identified as risk factors for advanced recurrence of HCC. In particular, ASRI was easy to calculate and a useful index to predict advanced recurrence after curative resection of small HCC and to choose patients requiring adjuvant therapy after resection.

References

1. Tsukuma H, Hiyama T, Tanaka S, *et al.* Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993; 328: 1797–801.
2. Fattovich G, Giustina G, Schalm SW, *et al.* Occurrence of hepatocellular carcinoma and decompensation in Western European patients with cirrhosis type B. *Hepatology* 1995; 21: 77–82.
3. Ikeda K, Saitoh S, Suzuki Y, *et al.* Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis. A prospective observation of 2215 patients. *J Hepatol* 1998; 28: 930–8.
4. Shinagawa T, Ohto M, Kimura K, *et al.* Diagnosis and clinical features of small hepatocellular carcinoma with emphasis on the utility of real-time ultrasonography. A study in 51 patients. *Gastroenterology* 1984; 86: 495–502.
5. Okuda K. Early detection of hepatocellular carcinoma. *Hepatology* 1986; 6: 729–38.
6. Lui WY, Chau GY, Loong CC, *et al.* Hepatic segmentectomy for curative resection of primary hepatocellular carcinoma. *Arch Surg* 1995; 130: 1090–7.
7. Nagashima I, Hamada C, Naruse K, *et al.* Surgical resection for small hepatocellular carcinoma. *Surgery* 1996; 119: 40–5.
8. Lise M, Bacchetti S, Da Pian P, *et al.* Prognostic factors affecting long-term outcome after liver resection for hepatocellular carcinoma: results in a series of 100 Italian patients. *Cancer* 1998; 82: 1028–36.
9. Hanazaki K, Kajikawa S, Shirozawa N, *et al.* Survival and recurrence after hepatic resection of 386 consecutive patients with hepatocellular carcinoma. *J Am Coll Surg* 2000; 191: 381–8.
10. Nagasue N, Ono T, Yarnanai A, *et al.* Prognostic factors and survival after hepatic resection for hepatocellular carcinoma without cirrhosis. *Br J Surg* 2001; 88: 515–22.
11. Sugimoto R, Okuda K, Tanaka M, *et al.* Metachronous multicentric occurrence of hepatocellular carcinoma after surgical treatment – clinicopathological comparison with recurrence due to metastasis. *Oncol Rep* 1999; 6: 1303–8.
12. Kosuge T, Makuuchi M, Takayama T, *et al.* Long-term results after resection of hepatocellular carcinoma: experience of 480 cases. *Hepato-gastroenterology* 1993; 40: 328–32.
13. Poon RT, Fan ST, Ng IO, *et al.* Different risk factors and prognosis for early and late intrahepatic recurrence after resection of hepatocellular carcinoma. *Cancer* 2000; 89: 500–7.
14. Matsumura T, Kanematsu T, Takenaka K, *et al.* Patterns of intrahepatic recurrence after curative resection of hepatocellular carcinoma. *Hepatology* 1989; 9: 457–60.
15. Sonoyama T, Ochiai T, Hironaka T, *et al.* Predictors of post-operative diffuse intrahepatic recurrence of hepatocellular carcinoma. *Hepato-gastroenterology* 2003; 50: 1078–84.
16. Park JH, Koh KC, Choi MS, *et al.* Analysis of risk factors associated with early multinodular recurrences after hepatic resection for hepatocellular carcinoma. *Am J Surg* 2006; 192: 29–33.
17. Liver Cancer Study Group of Japan. *Classification of Primary Liver Cancer*, 1st English edn. Tokyo: Kanahara & Company Ltd, 1997.
18. International Working Party. Terminology of nodular hepatocellular lesions. *Hepatology* 1995; 22: 983–93.
19. Kojiro M, Yano H, Nakashima O. Pathology of early hepatocellular carcinoma: progression from early to advanced. *Semin Surg Oncol* 1996; 12: 197–203.
20. Nakano M, Saito A, Yamamoto M, *et al.* Stromal and blood vessel wall invasion in well-differentiated hepatocellular carcinoma. *Liver* 1997; 17: 41–6.
21. Belghiti J, Panis Y, Farges O, *et al.* Intrahepatic recurrence after resection of hepatocellular carcinoma complicating cirrhosis. *Ann Surg* 1991; 214: 114–7.
22. Kumada T, Nakano S, Takeda I, *et al.* Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. *Hepatology* 1997; 25: 87–92.
23. Llovet JM, Fuster J, Bruix J. Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: resection versus transplantation. *Hepatology* 1999; 30: 1434–40.
24. Imamura H, Matsuyama Y, Tanaka E, *et al.* Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. *J Hepatol* 2003; 38: 200–7.
25. The Cancer of the Liver Italian Program (CLIP) Investigators. A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. *Hepatology* 1998; 28: 751–5.
26. Ikai I, Arai S, Kojiro M, *et al.* Reevaluation of prognostic factors for survival after liver resection in patients with hepatocellular carcinoma in a Japanese nationwide survey. *Cancer* 2004; 101: 796–802.
27. Smith JB. Occurrence of alpha-fetoprotein in acute viral hepatitis. *Int J Cancer* 1971; 8: 421–4.
28. Silver HK, Gold P, Shuster J, *et al.* Alpha(1)-fetoprotein in chronic liver disease. *N Engl J Med* 1974; 291: 506–8.
29. Sasaki Y, Yamada T, Tanaka H, *et al.* Risk of recurrence in a long-term follow-up after surgery in 417 patients with hepatitis B- or hepatitis C-related hepatocellular carcinoma. *Ann Surg* 2006; 244: 771–80.