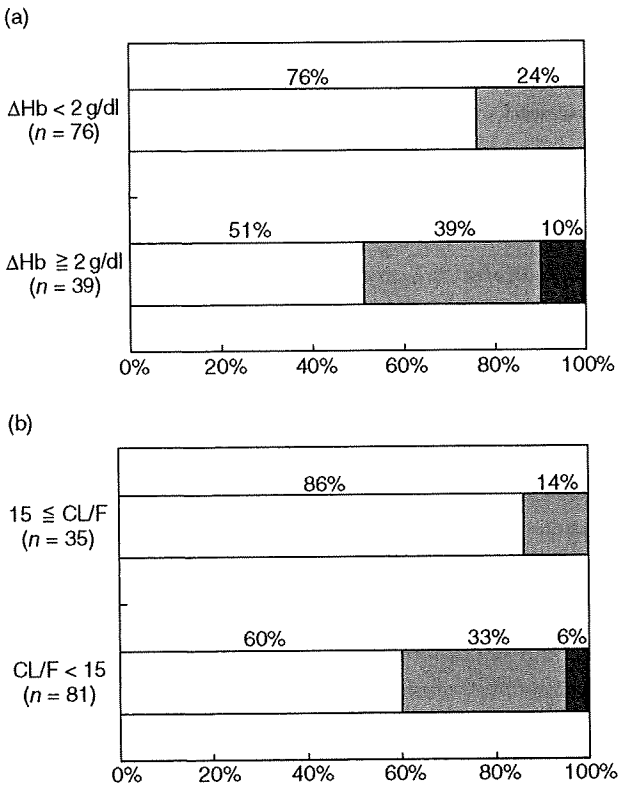

Figure 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy. (□), 10 g/dL < minimum Hb; (▩), 8.5 < minimum Hb ≤ 10 g/dL; (■), minimum Hb ≤ 8.5 g/dL. (a) According to the "2 by 2" standard (Hb 2 g/dL decrease at two weeks from the baseline). $P = 0.009$ (Mantel-Haenszel χ^2-test). (b) according to CL/F levels. $P = 0.001$ (Mantel-Haenszel χ^2-test).

in the ΔHb < 2 g/dL group (Fig. 3a). The patients with minimum Hb ≤ 8.5 g/dL accounted for 6% (5/81) of the group of CL/F < 15, and there was no patient with minimum Hb ≤ 8.5 g/dL in the 15 ≤ CL/F group (Fig. 3b). The number of patients with minimum Hb ≤ 8.5 g/dL during PegIFN and ribavirin combination therapy according to "2 by 2" standard and CL/F levels is shown in Table 5. The patients with minimum Hb ≤ 8.5 g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

DISCUSSION

PREDICTION OF THE progression of anemia is necessary to decide whether drugs can be continued, with minimization of the disadvantages induced by anemia. Recently, CL/F has been used as a marker of progressing anemia that necessitates discontinuance of treatment. For example, if the patients have a low CL/F level, they should start treatment with a low ribavirin dose. In this study, we attempted to use the CL/F level measurement for our patients. To predict which patients might have to discontinue the treatment, the target range had to be CL/F < 15 because 6% of patients ($n = 5$) in this range showed minimum Hb ≤ 8.5 g/dL, which is the level at which ribavirin should be discontinued. No patients of the CL/F ≥15 group showed minimum Hb ≤ 8.5 g/dL. Our findings showed that 70% of the patients (81/116) with CL/F < 15 should be discriminated from the others (Table 3). In the same manner, using ΔHb as the marker, 34% of the target patients in the ΔHb ≥ 2 g/dL group were identified because 10% in this range showed minimum Hb ≤ 8.5 g/dL. No patients in the ΔHb < 2 g/dL group showed minimum Hb ≤ 8.5 g/dL. Compared to CL/F, ΔHb is considered to be more sensitive and convenient for identifying the high risk patients for whom treatment would need to be discontinued. Furthermore, the application of "2 by 2" standard in the group with low level of CL/F < 15 can be the most sensitive method for this (Table 5), since no patients with progression of anemia were found in the "2 by 2" standard-negative group with CL/F < 15.

Table 5 The number of patients with minimum hemoglobin ≤8.5 g/dL during PegIFN/ribavirin combination therapy according to "2 by 2" standard and CL/F levels

	ΔHb < 2 g/dL ($n = 76$)	ΔHb ≥ 2 g/dL ($n = 39$)
CL/F ≥ 15 ($n = 35$)	0/25	0/10
CL/F < 15 ($n = 80$)	0/51	4/29 (14%)

In Japan, ribavirin doses are set at 600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for ≥80 kg, which are lower doses than those used in Europe and the USA. In this study, the mean ribavirin level at the start of treatment was 743 mg per day, while the AASLD practice guideline for genotype 1 hepatitis C is a daily dose of 1000 mg for body weight ≤ 75 kg and 1200 mg if >75 kg[26] In Japan, the use of lower doses is why fewer patients treated with PegIFN and ribavirin combination therapy are forced to discontinue the treatment due to severe anemia. Since the "2 by 2" model and/or CL/F can identify the patients who are prone to develop severe anemia, the other patients could be candidates for ribavirin dose-up strategies to raise SVR rates.

A considerable number of patients with chronic hepatitis C are over 60 years old in Japan (mean age is

around 55 years old),[27] although the mean age of this study was 50.6 years old. The number of aged patients with chronic hepatitis C is expected to increase in Europe and the USA, as well as in Japan. In IFN and ribavirin combination therapy, the discontinuance rate due to anemia was significantly higher in aged patients (≥60 years old, 21%) than in younger patients (<60 years old, 9%) ($P < 0.001$).[25] Earlier prediction of anemia is necessary to reduce the ribavirin dose in order to prevent the progression of severe anemia or to start epoetin alfa administration as needed, especially with aged patients. The "2 by 2" standard in PegIFN and ribavirin combination therapy should be a useful and convenient device for predicting the progress of anemia and treatment discontinuance in Europe and the USA, as well as in Japan.

CONCLUSION

IN CONCLUSION, THIS paper has shown that the SVR rate can be raised by preventing the discontinuance of ribavirin in PegIFN and ribavirin combination therapy. What is now needed is a prospective study of whether the early reduction of ribavirin in "2 by 2" standard-positive patients can improve the SVR rates, to ascertain the utility of the "2 by 2" standard in PegIFN and ribavirin combination therapy.

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Original Article

Initial viral response is the most powerful predictor of the emergence of YMDD mutant virus in chronic hepatitis B patients treated with lamivudine

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Aim: Lamivudine (LAM) has been widely used to treat chronic hepatitis B (CHB) patients, but the emergence of a LAM-resistant virus greatly limits its therapeutic efficacy. In this study, we tried to identify factors affecting the emergence of a LAM-resistant virus in CHB patients treated with LAM.

Methods: The subjects were 190 CHB patients in continuous LAM therapy (139 males, mean age 50 years, 87 HBeAg-positive). The mean duration of follow-up was 39 months (range 12–104). The initial viral response (IVR) was defined as HBV DNA < 4.0 logcopies/mL, and the initial biochemical response (IBR) as normalization of alanine aminotransferase (ALT) (<40 IU/L) at 6 months.

Results: IVR was positive in 86% of the patients. The cumulative emergence rates of LAM-resistant virus were 10% at 1 year, 30% at 2 years and 46% at 3 years. In univariate analysis, factors contributing to the emergence of LAM-resistant virus were baseline HBV DNA > 6.5 logcopies/mL ($P = 0.0044$), HBeAg-positivity ($P = 0.0062$), IBR ($P = 0.01$) and IVR ($P < 0.0001$). The cumulative emergence rates of LAM-resistant virus in IVR-positive and -negative patients were 4% and 41% at 1 year, and 41% and 79% at 3 years. In multivariate analysis, only IVR was an independent factor affecting the emergence of LAM-resistant virus ($P < 0.0001$).

Conclusion: IVR is a useful factor for predicting the emergence of LAM-resistant virus in CHB patients treated with LAM. For IVR-negative patients, therapeutic options other than LAM monotherapy should be used because of the high incidence of the emergence of LAM-resistant virus.

Key words: chronic hepatitis B, initial viral response, lamivudine monotherapy, lamivudine-resistant virus

INTRODUCTION

MORE THAN 350 million people are chronically infected with hepatitis B virus (HBV) worldwide.[1] Chronic HBV infection eventually leads to the development of cirrhosis and hepatocellular carcinoma (HCC), and raises the risk of hepatic disease-related death. Nucleos(t)ide analogs are widely used to suppress HBV replication and the progression of HBV-related liver diseases. Lamivudine (LAM), the first approved nucleoside analog for chronic HBV infection, has been shown to suppress viral replication and disease activity.[2] In addition, LAM therapy has recently been reported to reduce the incidence of HCC, the risk of major complications and to improve survival.[3,4] However, the relatively high incidence of LAM resistance is a serious problem in the case of LAM therapy for chronic HBV infection. The emergence of LAM-resistant HBV is linked to the reappearance of active viral replication, followed by the worsening of liver disease.

LAM-resistant HBV is based on point mutation within the YMDD motif of the reverse transcriptase domain of

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HBV (YMDD mutation).[5,6] The emergence rates of the mutant virus have been reported to be 24% at 1 year and 70% at 4 years from the start of treatment.[7]

Recent work has shown that newly developed nucleo-s(t)ide analogs, such as adefovir dipivoxil (ADV) and entecavir (ETV), are also useful agents for controlling patients with chronic HBV infection.[8–11] In particular, the drug-resistant mutant virus has been reported to appear less frequently in cases of treatment with ADV and ETV than with LAM.[12,13] For this reason, LAM has been replaced by ADV and ETV for the treatment of chronic hepatitis B. However, there are still a considerable number of patients with chronic HBV infection who are already on continuous LAM therapy. Thus, further clarification is needed of what factors influence the emergence of the LAM-resistant HBV in LAM treatment for chronic HBV infection.

For a more precise evaluation, we investigated baseline and on-treatment factors affecting the emergence of LAM-resistant mutant virus in patients with chronic hepatitis B treated with LAM.

METHODS

Patients and treatment

THIS STUDY WAS conducted at nine institutions in the Osaka area of Japan (Osaka Police Hospital, Osaka Minami Medical Center, Osaka Kouseinenkin Hospital, Osaka Rousai Hospital, Kinki Central Hospital, Ikeda City Hospital, Osaka National Hospital, Otemae Hospital and Osaka University Hospital). The subjects were 190 consecutive patients with chronic hepatitis B who underwent continuous LAM therapy for more than 12 months. All patients tested positive for hepatitis B surface antigen (HBsAg) or had detectable levels of HBV DNA in their sera by the polymerase chain reaction (PCR)-based method (for 100 patients)[14] or the transcription-mediated amplification (TMA) method (for 90 patients).[15] Exclusion criteria were patients with antihepatitis C antibody, antihuman immunodeficiency virus antibody and other forms of liver diseases (alcoholic liver disease, drug-induced liver disease and autoimmune hepatitis). Forty-one (22%) patients had previously received interferon (IFN)-α therapy for 24 weeks.

All patients were treated with 100 mg of LAM daily. After the beginning of the therapy, liver function tests and HBV DNA were measured every other month for the first 6 months and every two months thereafter. HBeAg and anti-HBe were tested every 6 months. In 33 patients (18%), combination therapy with IFN was carried out for the initial 6 months. Three or six mega-units of natural IFN-á were administered daily for the first 2 weeks and three times a week thereafter, followed by LAM monotherapy. The mean follow-up period of the 190 patients was 39 (range 12–104) months. The LAM-resistant YMDD mutant virus was detected by the PCR-enzyme-linked minisequence (ELMA) assay[16] when the virological or biochemical breakthrough was observed. The YMDD mutant virus was found in 86 (45%) patients during follow-up. Fifty-eight of these patients underwent ADV therapy in addition to ongoing LAM treatment and were excluded from the follow-up when ADV administration began. In this study, the initial viral response (IVR) was defined as HBV DNA < 4.0 logcopies/mL, and the initial biochemical response (IBR) as normalization of alanine aminotransferase (ALT) (<40 IU/L) after 6 months of therapy.

Table 1 Patient characteristics

Gender (male/female)	139/51
Age (years)	50 ± 11
Chronic hepatitis/liver cirrhosis	113/77
Hepatocellular carcinoma	14 (7%)
AST (IU/L)	122 ± 157
AST (IU/L)	177 ± 236
ALT (≤1/1–2/2–5/>5 × ULN)	22/53/65/50
Platelet (10^4/mm^3)	12.6 ± 5.1
Prothrombin time (%)	71.5 ± 16.6
HBV DNA (logcopies/mL)	6.5 (3.0–7.6<)
HBeAg (positive/negative)	87/103
Combination with interferon	33 (17%)
Duration of treatment (months)	38.9 ± 17.5

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; ULM, upper limit normal.

The patients' clinical characteristics are shown in Table 1. There were 139 males and 51 females, ranging in age from 25 to 75 (mean 50) years. Of them, 113 (59%) patients were diagnosed as having chronic hepatitis and the remaining 77 patients (41%) as having cirrhosis according to liver histology and/or the imaging procedure. HCC was developed in 14 (7%) patients. The aspartate aminotransferase (AST) at baseline was 122 ± 157 IU/l, and the ALT at baseline was 177 ± 236 IU/L. Abnormal ALT was observed in 168 (88%) patients. Eighty-seven patients (46%) tested positive for HBeAg. The median HBV DNA at baseline was 6.5 (range 3.0 to 7.6<) logcopies/mL.

HBV testing

HBsAg, hepatitis B e antigen (HBeAg) and antihepatitis B e antibody (anti-HBe) were examined by chemiluminescent immunoassay or enzyme immunoassay.

The HBV DNA level was measured by the PCR-based method (Amplicor HBV monitor; Roche Diagnostics, Tokyo, Japan)[14] or the TMA method (TMA-HPA; Fujirebio, Tokyo, Japan),[15] which have lower detection limits of 2.6 and 3.7 logcopies/mL, respectively. The LAM-resistant YMDD mutant virus was examined by the PCR-ELMA method.[16]

Statistical analysis

Comparisons of categorical and continuous variables between groups were done by the χ^2-test, Student's *t*-test and Mann–Whitney's *U*-test. The cumulative emergence rates of LAM-resistant virus were evaluated with the Kaplan–Meier's curve and the differences between groups were analyzed by the log-rank test. For multivariate analysis to investigate factors affecting the cumulative emergence rate of LAM-resistant virus, Cox proportional hazard regression analysis was carried out. A *P*-value of less than 0.05 (two-tailed) was considered to be statistically significant.

RESULTS

Therapeutic efficacy and the emergence of LAM-resistant mutant virus

AMONG THE 190 patients with chronic hepatitis B who underwent continuous LAM therapy, reduction of HBV DNA to less than 4 logcopies/mL was observed in 86% (163/190) at 6 months, 89% (151/170) at 1 year, 88% (83/94) at 2 years and 89% (48/54) at 3 years of the treatment. Normalization of ALT was achieved by 77% (146/190) at 6 months, 83% (141/170) at 1 year, 81% (76/94) at 2 years and 83% (45/54) at 3 years. Among the 87 HBeAg-positive patients, HBeAg was cleared in 22% (19/86) at 6 months, 26% (21/80) at 1 year, 22% (11/50) at 2 years and 43% (16/37) at 3 years. As for the virological and biochemical response at 6 months of therapy, 163 (86%) of the patients achieved IVR, whereas IBR was seen in 146 (77%) of patients.

When the various patient characteristics were compared between IVR-positive and -negative patients (Table 2), HBV DNA at baseline tended to be lower in patients showing IVR (median 6.5 [range 3.0 to 7.6<] logcopies/mL) than in those who did not show IVR (median 7.3 [range 4.3 to 7.6<] logcopies/mL) ($P < 0.0001$). IVR-negative patients had higher HBeAg positivity at baseline than IVR-positive patients (81% vs 40%, $P = 0.01$). As for the emergence of LAM-resistant mutant virus during follow-up, it was detected more frequently in IVR-negative patients (21/27, 78%) than in IVR-positive patients (65/163, 40%) ($P = 0.002$).

Among the 190 patients examined in this study, the emergence of LAM-resistant YMDD mutant virus occurred in 86 (45%) patients during follow-up. The cumulative probabilities of the emergence of the YMDD mutant virus were 10% at 1 year, 30% at 2 years and 46% at 3 years.

Factors affecting the emergence of LAM-resistant mutant virus

Factors affecting the cumulative probability of the emergence of the YMDD mutant virus were investigated using

Table 2 Comparison of patient characteristics between IVR-positive and -negative patients

	IVR ($n = 163$)	Non-IVR ($n = 27$)	*P*-value
Gender (male/female)	118/45	21/6	NS
Age (years)	50 ± 11	48 ± 12	NS
Chronic hepatitis/liver cirrhosis	91/72	22/5	NS
Hepatocellular carcinoma	13 (8.0%)	1 (4%)	NS
AST (IU/L)	131 ± 167	69 ± 34	NS
ALT (IU/L)	190 ± 252	100 ± 55	NS
ALT (≤1/1–2/2–5/>5 × ULN)	21/43/52/47	1/10/13/3	NS
HBV DNA (logcopies/mL)	6.5 (3.0–7.6<)	7.3 (4.3–7.6<)	<0.0001
HBeAg (positive/negative)	65/98	22/5	0.01
Combination with interferon	27 (17%)	6 (22%)	NS
Emergence of LAM-resistant viruses	65 (40%)	21 (78%)	0.002
Duration of treatment (months)	39.2 ± 17.2	37.3 ± 19.1	NS

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IVR, initial viral response; LAM, lamivudine; NS, not significant; ULN, upper limit normal.

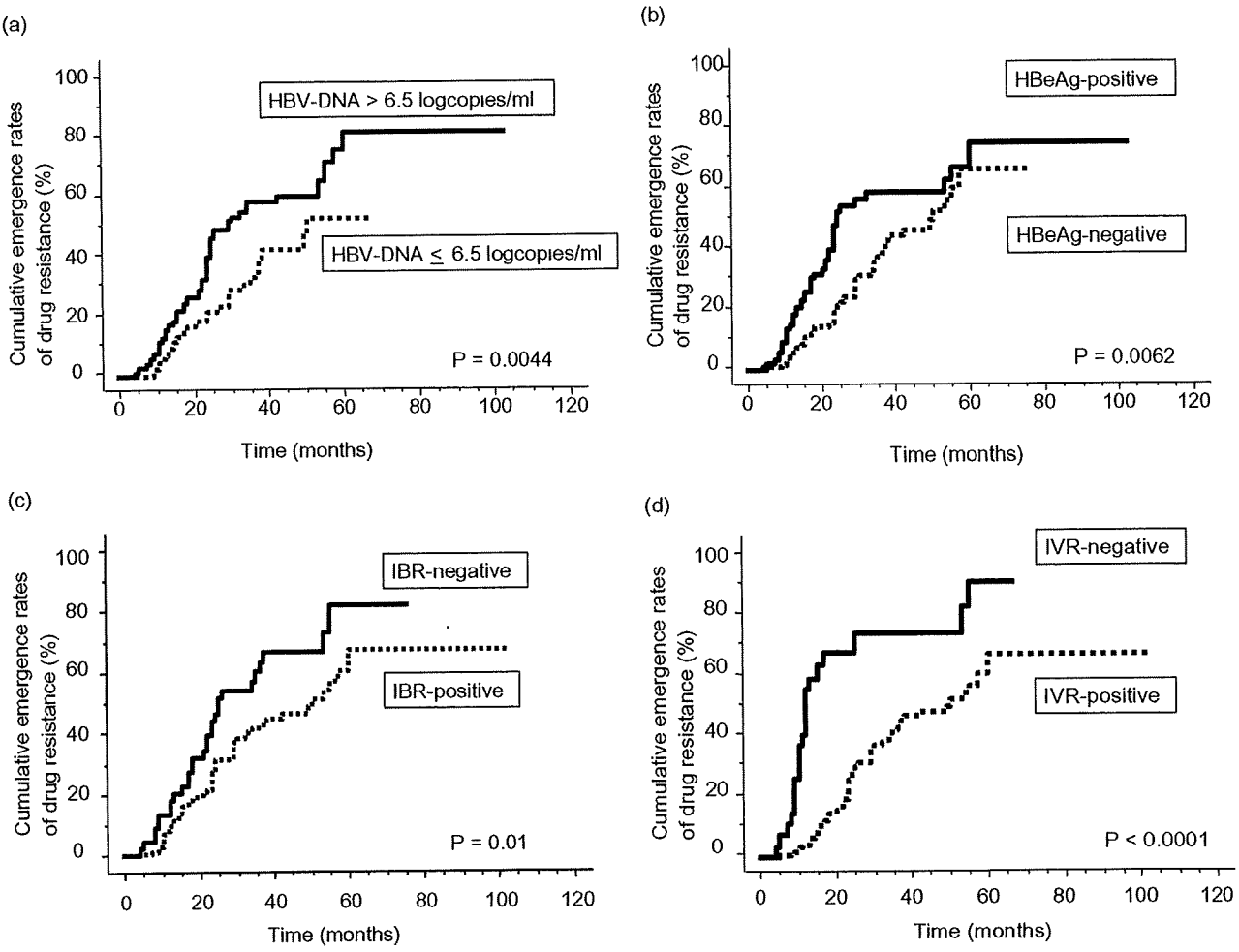

Figure 1 Cumulative emergence rate of lamivudine (LAM)-resistant virus in patients with chronic hepatitis B virus (HBV) infection treated with LAM according to: (a) HBV DNA at baseline; (b) hepatitis B e antigen (HBeAg) status; (c) the presence or absence of initial biochemical response (IBR); and (d) the presence or absence of initial viral response (IVR).

both univariate and multivariate analyses. Nine baseline and on-treatment factors – gender, age, liver disease (chronic hepatitis or cirrhosis), ALT at baseline, HBeAg positivity, HBV DNA at baseline, combination therapy with IFN-α, presence of IBR and presence of IVR – were examined. The cumulative emergence of LAM-resistant virus was significantly higher in patients with baseline HBV DNA > 6.5 logcopies/mL than in those with HBV DNA ≤ 6.5 logcopies/mL ($P = 0.0044$) (Fig. 1a). HBeAg-positive patients revealed a significantly higher emergence rate of the LAM-resistant virus than HBeAg-negative patients ($P = 0.0062$) (Fig. 1b). A significant difference was also seen in the cumulative emergence of the YMDD mutant virus between IBR-positive and -negative patients ($P = 0.01$) (Fig. 1c). Furthermore, the cumulative emergence of LAM-resistant mutant virus was much higher in the IVR-negative patients than in the IVR-positive patients ($P < 0.0001$) (Fig. 1d). The cumulative emergence rates of LAM-resistant virus in the IVR-positive and -negative patients were 4% and 41% at 1 year, 25% and 69% at 2 years, and 41% and 79% at 3 years, respectively. Gender, age, liver disease, ALT at baseline and combination therapy of IFN-α did not show a significant relation with the emergence of the YMDD mutant virus. When factors influencing the higher cumulative emergence of LAM-resistant virus were searched for by multivariate analysis, only the absence of IVR was selected as a significant independent factor ($P < 0.001$) (Table 3), with high HBV DNA, HBeAg positivity and the absence of IBR not being selected.

Table 3 Factors associate with emergence of LAM-resistant virus determined by multivariate analysis

	Hazard ratio	95% confidence interval	*P*-value
Gender			
0: male	1	0.497–1.455	0.55
1: female	1.176		
Age			
0: ≤50	1	0.640–1.700	0.87
1: >50	0.959		
Chronic hepatitis/liver cirrhosis			
0: CH	1	0.656–1.740	0.79
1: LC	0.935		
Pretreatment ALT (IU/L)			
0: ≤200	1	0.605–1.818	0.87
1. >200	0.953		
HBV DNA (logcopies/mL)			
0: ≤6.5	1	0.394–1.125	0.13
1. >6.5	1.502		
HBeAg			
0: negative	1	0.499–1.337	0.42
1: positive	1.225		
Combination therapy with interferon			
0: no	1	0.410–1.303	0.29
1: yes	1.368		
IBR			
0: positive	1	0.483–1.312	0.37
1: negative	1.256		
IVR			
0: positive	1	0.159–0.536	<0.001
1. negative	3.425		

HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IBR, initial biochemical response; IVR, initial viral response; LAM, lamivudine.

DISCUSSION

IN LAM THERAPY for patients with chronic HBV infection, the emergence of a LAM-resistant YMDD mutant virus is a serious problem, because it inevitably restricts the antiviral efficacy of LAM. To resolve this, detailed studies are needed to identify factors related to the emergence of the YMDD mutant virus. To date, a few investigators have suggested male gender, advanced age, high baseline ALT, the presence of severe acute exacerbation of the liver disease, high baseline HBV DNA and HBeAg-positivity as possible predictors of the emergence of LAM-resistant virus.[7,17,18] Lower body surface area was also reported as a significant factor for virological and biochemical therapeutic effect.[19] In the present study, we studied 190 patients with chronic hepatitis B treated with LAM and investigated baseline and on-treatment factors affecting the emergence of LAM-resistant mutant virus. Univariate analysis revealed that two baseline factors, high HBV DNA and HBeAg positivity, had a relation to the high incidence of the YMDD mutant virus, which is consistent with previous reports.[7,17,18] In addition, two on-treatment factors, IBR and IVR, were found to be correlated with the emergence of LAM resistance. Patients who did not show IVR had a 3.4-fold higher incidence of the emergence of the YMDD mutant virus than those who did show IVR. This agrees with a previous report that the HBV DNA level after 6 months of therapy may be a determinant for subsequent occurrence of a LAM-resistant mutant virus.[20] Multivariate analysis showed that only the absence of IVR was a significant factor contributing to the emergence of LAM-resistant virus. Baseline HBV DNA and HBeAg status were not selected as significant factors by multivariate analysis probably because of the tendency for higher HBV DNA and high frequency of HBeAg positivity in IVR-negative patients compared with IVR-positive patients. It is particularly interesting that the absence of IVR, rather than other baseline and on-treatment factors, was a powerful independent pre-

dictor for the emergence of the YMDD mutant virus in LAM therapy for chronic HBV infection. This means that IVR of an on-treatment factor is very important for good therapeutic effect and the stage for the next therapeutic strategy can thus be set in a new light with this information.

Our results showed that approximately one-seventh of the patients with chronic hepatitis B treated with LAM did not achieve IVR. In the non-IVR patients, the antiviral therapeutic regimen should be amended due to the frequent emergence of LAM-resistant virus. Recently, new nucleos(t)ide analogs have become available for the treatment of chronic HBV infection. ETV has been reported to be more effective for the reduction of HBV DNA and the less frequently induced drug-resistant mutant virus than LAM in "naïve" patients with chronic hepatitis B who had not previously received nucleos(t)ide analog therapy.[10,11] ETV was also effective in patients with chronic HBV infection showing LAM resistance,[21] but the emergence rate of the ETV-resistant virus was considerably higher in LAM-resistant patients than in naïve patients.[13,22] This is because the ETV-resistant HBV strain is established by LAM-resistant YMDD mutation plus additional mutation(s) at the amino acid position(s) 184, 202 and/or 250 within the reverse transcriptase domain of HBV.[22] According to these findings, switching from LAM to ETV may be useful for treating patients who do not achieve IVR on LAM administration. This should be done before the emergence of LAM-resistant YMDD mutant virus so as not to reduce the therapeutic efficacy of ETV. In clinical practice, there are still a number of patients who have already been on continuous LAM therapy, although the current first choice drug for patients with chronic HBV infection is ETV. In our opinion, foregoing patients without IVR or YMDD mutant viruses should be switched from LAM to ETV. The therapeutic efficacy of switching from LAM to ETV in non-IVR patients should be assessed by further study with a larger number of patients.

ADV and tenofovir disoproxil fumarate (TDF) have also been shown to exert antiviral efficacy in patients with chronic HBV infection with less frequent occurrence of drug-resistant mutant virus compared to LAM.[23] In addition, unlike the case of ETV, both ADV and TDF are known to be effective in LAM-refractory patients with chronic hepatitis B, as well as naïve patients.[23] Using ADV and TDF may be helpful for the treatment of non-IVR patients, especially after the establishment of LAM-resistant mutant virus.

In conclusion, our findings indicate that IVR may be a useful factor for predicting the emergence of LAM-resistant mutant virus in patients with chronic HBV infection treated with LAM. For patients who do not achieve IVR, therapeutic options other than LAM monotherapy should be promptly implemented because of the high incidence of the subsequent emergence of the YMDD mutant virus.

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A New Prognostic System for Hepatocellular Carcinoma Including Recurrent Cases

A Study of 861 Patients in a Single Institution

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Objective: To manage hepatocellular carcinoma (HCC) patients surviving for a long term, the treatment strategy for recurrent cancer is as important as that for the initial treatment. However, no prognostic scoring system has been available for patients with HCC recurrence. The purpose of this study was to develop a new staging system for deciding the treatment strategy not only for first-time diagnosed patients but also for recurrent patients.

Methods: A total of 861 cases diagnosed at our single institution from 1993 to 2003 were included. Overall survival was the only end point. The Cox model was used for multivariate analyses.

Results: As of August 2004, 344 cases (59%) had died. Overall median survival time was 41 months. For multivariate Cox regression analysis, independent predictive factors of survival were the number of recurrences, the Child-Pugh score, 3 nodules less than 3 cm and none of vascular invasion, and the α-fetoprotein level. A simple scoring system was thus developed, assigning scores (0/1) to the 4 covariates of the final model. Compared with the other scoring systems, the new scoring system has a greater discriminant ability.

Conclusions: We concluded that our scoring system can serve as a new prognostic system that reflects the spread of HCC, treatment response, and liver function. It should be very useful as the only method which can be applied for patients with recurrence.

Key Words: hepatocellular carcinoma, recurrence, staging system, predictive factor, cox regression analysis

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Recently, various nonsurgical treatment modalities for hepatocellular carcinoma (HCC) have been developed and surgical techniques have been also improved.[1,2] However, HCC with cirrhosis remains one of the diseases that is extremely difficult to manage, because survival in HCC is not predominantly based on the biology of the tumor, but also on the underlying hepatic function. Actually, we need consider 2 distinctive features in planning the HCC treatment from other cancers. First, even if HCC can be completely treated, the residual cirrhotic liver displays a high risk of recurrence, including new primary cancers.[3–5] Second, most options for the treatment of HCC lead to a decrease in the reserved hepatic function. In other words, they take the risk of future liver failure in return for HCC treatment. Taken together, the complexity of these factors makes HCC management difficult.

The prognosis of HCC patients is highly variable and hard to predict, which makes it difficult to effectively treat patients or to design good clinical trials. To provide guides for assessing disease severity and making therapeutic decisions, several staging or prognostic scoring systems for HCC have been proposed: the Cancer of the Liver Italian Program (CLIP) score,[6] BCLC staging,[7] and Japan Integrated Staging (JIS) scoring system,[8] which were produced on the basis of prognostic values. These staging systems can be used for assessing the prognosis of HCC patients as well as the efficiency of therapeutic modalities. Although these systems may be useful for predicting the prognosis of HCC patients at the time of the initial treatment,[9–11] there is considerable doubt about whether these systems are suitable for cases of recurrent cancer, because they cannot distinguish HCC diagnosed for the first time from recurrent HCC. In clinical practice, recurrent HCC patients are encountered 2.5 times more frequently in our institution than first-time HCC patients. Because the development of screening and follow-up programs and the improvement of radiologic techniques have facilitated the recognition of HCC at an earlier

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stage,[12,13] it has become possible to repeatedly apply curative treatments.

To manage HCC patients surviving for a long term, preparing the treatment strategy for recurrent cancer becomes more important than that for initial treatment. This makes it important to predict the prognosis of recurrent patients. In other words, every time HCC is diagnosed, the prognostic value should be assessed, and then a treatment strategy should be decided. However, no attempts have been made to include prediction of the prognosis of recurrent HCC patients. The purpose of this article is to propose a new prognostic scoring system, which can be useful for deciding the treatment strategy not only for first-time diagnosed patients but also for recurrent HCC patients.

PATIENTS AND METHODS

Study Population

All (888) consecutive adult patients who were diagnosed as HCC and registered with the Division of Internal Medicine in the Osaka University Hospital between 1993 and 2003, were eligible for this study. Sixteen patients who could not be confirmed as having HCC were excluded. Three patients who underwent liver transplantation were also excluded. Eight patients who had local recurrences within 6 months were excluded because their admissions were not for the recurrent tumor but rather for the residual tumors caused by the insufficient ablation therapy. Thus, 861 patients composed the study population. The patient data were collected with both a survey of original medical records and access to the hospital information system. The patient data set was divided into 2 data sets for a split-sample validation procedure,[14] one set being retrospectively collected patients (n = 578) between September 1, 1993 and December 31, 2001, and the other being prospectively collected patients (n = 283) with the hospital database system between January 1, 2002 and December 31, 2003. The former was used as a training sample to construct a prognostic scoring system; the latter was used as a validation sample for the validation of the generated classification. HCC diagnosis was mainly established by the concomitant finding of 2 imaging techniques (n = 438), showing a nodule with arterial hypervascularization and portal hypovascularization, or by a positive imaging technique, showing hypervascularization associated with elevation of α-fetoprotein (AFP) or protein induced by vitamin K absence II (PIVKA-II) (n = 272). In addition, even if the above-mentioned features were not observed, target biopsy was performed when the findings of ultrasonography were consistent with HCC (n = 151). Details of the treatment modality showed that trans-catheter arterial chemoembolization alone or combined with percutaneous tumor ablation were mainly performed (n = 306 and 301, respectively). The number of patients treated with surgical resection, percutaneous tumor ablation alone, and best supportive care were 46, 188, and 20 respectively.

Statistical Methods

Overall survival was the only end point used in the analysis. It was defined as the time elapsed from the date of diagnosis and either the date of death related to liver disease or the date of the last follow-up information, with the final evaluation conducted on August 31, 2004. Patients lost before the last collection of follow-up information were censored at the time of their last visit. One hundred thirty-one of the 238 censored cases in the training sample were alive at the end of the period, whereas 22 patients had died from other diseases and 85 were lost to follow-up owing to change of residence (n = 21), introduction of a hospital near their residence (n = 50), and unknown reasons (n = 14). Two hundred and two of the 223 censored cases in the validation sample were alive, 3 patients died from other diseases, and 19 cases were loss to follow-up owing to the change of residence (n = 1), introduction of a hospital near their residence (n = 11), and unknown reasons (n = 7). Judging from the data at their last visit, all of the censored samples were considered to be independent of the future value of the hazard for the individual, in other words, they were noninformative censored cases. Figure 1 shows a schematic overview of investigated patients and dropouts for training and validation sample.

The following variables were used for the analysis: age and sex of the patient, date of HCC diagnosis, date of death or of last available information, viral status, the number of HCC recurrences, Child-Pugh score, the largest tumor size, tumor number, vascular invasion, AFP level, and PIVKA-II level. The cut-off levels of continuous variables were chosen on the basis of clinical meaning. For each variable, missing data were not used in the analysis if they accounted for less than 10% of the cases.

Univariate survival curves were estimated using the Kaplan-Meier method[15] and compared by means of the log-rank test.[16] The prognostic impact of the categories was assessed by means of the observed/expected ratio, as described previously.[6] Of the factors affecting patient survival in univariate analysis, baseline predictors were identified by the Akaike information criterion in a stepwise algorithm.[17] Next, a Cox proportional hazard

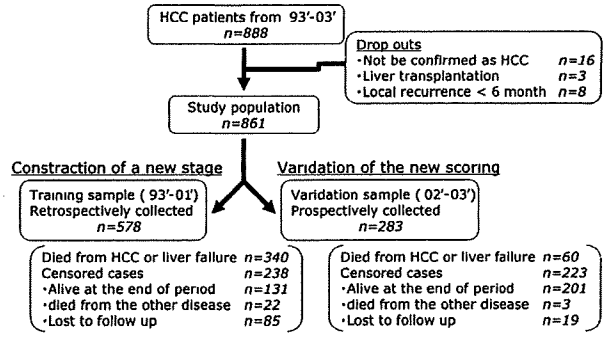

FIGURE 1. Schematic overview of included patients and dropouts for training and validation sample.

regression model was used for multivariate analyses.[18] Proportional hazard assumption was graphically assessed using plots of Log [−Log (survival time)]. Cases with missing values for one or more variables in the model were excluded from multivariate analysis. Treatment was not included in the model because the treatment choice was closely associated with the assessment of prognosis at the time of diagnosis.

Finally, the validity of the generated score was then assessed for the validation sample; a recent sample and a prospectively followed sample. The predictive accuracy of 3 models: this new score system, JIS score system, and CLIP score system was quantified by calculating the concordance index (C-index), which provides the area under the receiver operating characteristics (ROC) curve for the prediction of death at 3 years, as described previously.[19] A C-index of 0.5 indicates that outcomes are completely random, whereas a C-index of 1.0 indicates that the model is a perfect predictor.

All analyses were performed with R's software (R Foundation for Statistical Computing, Austria).[20] $P < 0.05$ was considered statistically significant in all analyses. The results were reported as a hazard ratio with 95% confidence intervals.

RESULTS

As of August 2004, 344 patients (59%) had died. The overall median survival time was 41 months (95% confidence interval, 36 to 46 mo); 1, 3, 5-year survival rates were 86%, 56%, and 35%, respectively. The baseline characteristics of the patients are given in Table 1. The first-time diagnosed HCC, shown as the number of HCC recurrences = 0 in Table 1, amounted to 295 cases, the first recurrence to 185, the second recurrence to 126, the third recurrence to 90, and more than the fourth recurrence to 165. Most cases were in the Child-Pugh A category. The baseline characteristics of the tumor are given in Table 2.

Nine variables were separately found to be associated with the outcome in univariate analysis of 11 variables (as shown in Table 3). Forward stepwise selection by Akaike information criterion was used to identify baseline predictors of 9 variables. Five variables were selected: the Child-Pugh score, the number of tumors, AFP, vascular invasion, and the number of HCC recurrences. To better reflect the treatment response, we combined 2 factors to create a single factor: we replaced "the number of tumors and vascular invasion" with "3 nodules less than 3 cm and none of vascular invasion, or not," called the tumor factor. This was done because the criterion "3 nodules less than 3 cm" reflects the possibility of complete response to ablation treatment[21] and was useful in the current clinical setting. We finally chose 4 factors for a new prognostic classification: the Child-Pugh score, tumor factor, AFP, and the number of HCC recurrences. These 4 covariates showed correlation with survival in the Cox regression analysis.

Each covariate selected by means of forward stepwise methods was divided into 2 categories to derive a simple scoring system. The cut-off levels were chosen where each estimated regression coefficient of the final Cox model was almost the same, that is, we made the relative prognostic weight of covariates the same, around 2 each (shown as in Table 4). A new scoring system was derived to assign scores (0/1) to each covariate of the final model as shown in Table 4. This classification was relatively easy to calculate by summing up each individual score of the 4 covariates. Five risk groups were constituted according to the score distribution. The survival curve of 578 patients calculated by the Kaplan-Meier method is shown in Figure 2A.

We assessed the new score system for 283 patients for the validation sample; prospectively obtained from 2002 to 2003 in Figure 2B. This result validated our scoring system and showed that it can be applied in today's clinical setting. This applicability to the present-day situation is very important, because diagnostic and

TABLE 1. Characteristics of Patients

Variables	Training Sample No. Patients	Validation Sample No. Patients
Median age, y (range)	64 (21-85)	67 (35-83)
Male (%)	425 (73.5)	192(67.8)
Cause of parenchymal disorder		
HBV/HCV/HB+HC	54/486/10	27/227/4
Alcoholic	8	10
Others	20	15
Child-Pugh score (unknown = 1)		
5-6 (A)/7-9 (B)/10-12 (C)	342/218/18	192/79/11
Number of HCC recurrence		
0/1/2/3/≥4	201/123/88/62/104	94/62/38/28/61

HBV indicates Hepatitis B virus; HCV, Hepatitis C virus.

TABLE 2. Characteristics of the Tumor

	Training Sample No. Patients	Validation Sample No. Patients
Number of tumor		
1/2/3/4/≥5	186/113/57/36/186	112/56/28/18/69
Largest size of tumor (cm)		
≤2.0/2.1-3.0/3.1-5.0/≥5.1	270/163/91/54	128/82/44/29
Vascular invasion		
Yes/no	534/44	266/17
Tumor factor [3 nodule less than 3 cm, vascular invasion (−)]		
Yes/no	285/293	159/124
AFP category (ng/mL)		
$\leq 10/10\text{-}10^2/10^2\text{-}10^3/ > 10^3$	137/230/129/82	65/108/70/40
PIVKA-II (mAU/mL) (unknown = 81)		
$\leq 10^2/10^2\text{-}10^3/10^3\text{-}10^4/ > 10^4$	327/118/64/27	110/58/20/14

TABLE 3. Univariate Analysis of Clinical Findings for Survival

Variables	No. Patients	O/E Ratio	*P*	DOF
Sex			0.00168	1
Male/female	425/153	1.11/0.73		
Age			0.00284	3
≤50/50-60/60-70/≥70	37/118/291/132	0.53/0.87/1.19/0.86		
Etiology			0.147	3
HCV/HBV/HB+HC/the others	486/54/10/28	1.03/0.9/1.38/0.54		
Number of HCC recurrence			< 0.0001	4
0/1/2/3/≥4	201/123/88/62/104	0.57/0.93/1.2/1.33/2.1		
Child-Pugh stage			< 0.0001	2
A/B/C	342/218/18	0.75/1.49/2.72		
Largest size of tumor (cm)			0.00467	3
≤2.0/2.1-3.0/3.1-5.0/≥5.1	270/163/91/54	0.86/1.06/1.16/1.65		
Number of tumor			< 0.0001	4
1/2/3/4/≥5	186/113/57/36/186	0.52/0.95/0.97/1.04/2.03		
Vascular invasion			< 0.0001	1
Yes/no	534/44	0.93/3.78		
Tumor factor [3 nodules less than 3 cm, vascular invasion (−)]			< 0.0001	1
Yes/no	285/293	0.67/1.53		
AFP (ng/mL)			< 0.0001	3
$\leq 10/10\text{-}10^2/10^2\text{-}10^3/> 10^3$	137/230/129/82	0.56/0.95/1.2/2.19		
PIVKA-II (mAU/mL)			< 0.0001	3
$\leq 10^2/10^2\text{-}10^3/10^3\text{-}10^4/> 10^4$	327/118/64/27	0.76/1.34/1.8/3.65		

DOF indicates degree of freedom; O/E ratio, observed/expected ratio; HBV, Hepatitis B virus; HCV, Hepatitis C virus.

therapeutic procedures for HCC have been improved over recent years.

Finally, the prognostic ability of the new scoring system was compared with CLIP score system and the JIS score system. Kaplan-Meier survival curves were shown in Figs. 2C, D). In addition, the predictive accuracy of 3 models was quantified by calculating a C-index, which provides the area under the ROC curve (as shown in Fig. 3). CLIP stage and JIS scoring had a C-index of 7.05 and 6.93, respectively. This new scoring system had a C-index of 7.23. Our scoring system could discriminate the survival most precisely among them.

DISCUSSION

This article revealed that the number of HCC recurrences is a prognostic factor as well as the reserved liver function and the spreading of HCC, and we have proposed a new scoring system, comprised of 4 parameters: the number of HCC recurrences, the Child-Pugh score, the tumor factor of "3 nodules less than 3 cm and none of vascular invasion," and the AFP level. Each of these parameters has so far been reported to affect patient survival. The occurrence of HCC recurrence reflects disease progression.[3–5] The Child-Pugh score is a well-recognized prognostic variable and reflects reserved liver

TABLE 4. New Scoring System

Variables	Score 0	Score 1	RR
Number of HCC recurrence (n = 578)	0 or 1 (n = 324)	≥2 (n = 254)	2.26
Child-Pugh score (n = 578)	5–7 (n = 486)	≥8 (n = 92)	2.25
Tumor factor (n = 578)	Yes (n = 285)	No (n = 293)	1.90
AFP category (ng/ml) (n = 578)	≤1000 (n = 496)	≥1001 (n = 82)	2.08

RR indicates risk ratio of Score 1 compared with Score 0, assessed by multivariate analysis.

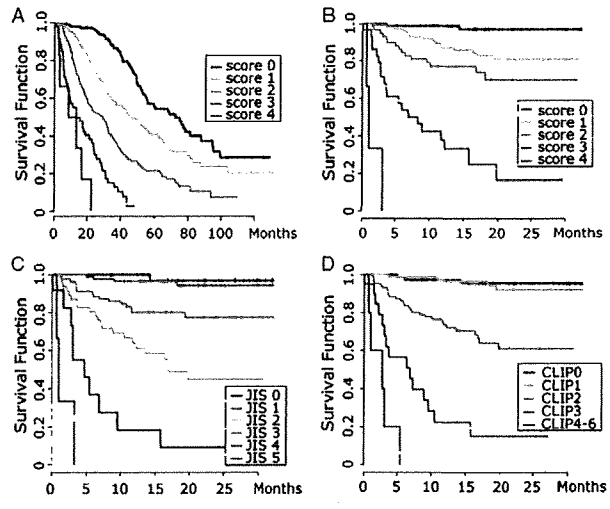

FIGURE 2. Kaplan-Meier–estimated survival curves. A, By our new scoring system in training samples. B, By our new scoring system in validation samples. C, By the CLIP score system in validation samples. D, By the JIS score system in validation samples.

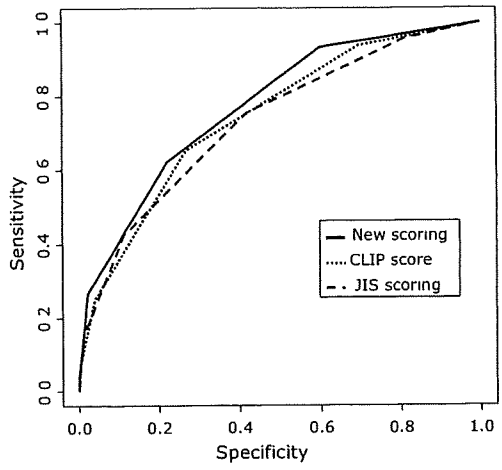

FIGURE 3. Discriminatory ability for the prediction of death at 3 years, evaluated by receiver operating characteristics curves of the new scoring, CLIP, and JIS staging systems.

function.[6,7] The criterion of 3 nodules less than 3 cm is related to the treatment response. Ablation therapy is highly effective for tumors smaller than 3 cm, achieving complete responses of around 80% to 100%.[22] The achievement of a complete and sustained response is an independent prognostic value.[23] AFP is also a well-recognized prognostic variable, and reflects the degree of cellular differentiation and the spreading of the tumor.[24] In the present study, these parameters were independent predictors of survival actually. Elevation of each parameter indicates the progression of HCC. As a result, this new scoring system reflects the spreading of HCC, the response to treatment, and the reserved liver function. In addition, our system is based on not pathologic but easily obtainable and reproducible clinical information. Therefore, this scoring system should be useful in many clinical settings.

A high possibility of recurrence is one of the major characteristics of HCC. Recurrences from either intrahepatic metastasis or de novo HCC exceed 50% at 3 years, even with hepatic resection as curative therapy.[3–5] The more the HCC recurs, the more the prognosis deteriorates because of treatment-induced liver damage and/or tumor progression. In clinical settings, it is very important to carefully follow HCC patients to detect recurrence as early as possible. More and more patients have been able to be frequently treated for recurrent HCC and prolong their survival. What is needed is a treatment strategy based on appropriate cancer staging systems for not only first-time diagnosed HCC but also for recurrent HCC. However, there has been no study reported on the prognosis of recurrent patients. Here, we first showed recurrence to be a prognostic factor with a Cox regression model, and furthermore developed a new scoring system to predict the prognosis of HCC patients including recurrent HCC patients.

What is the problem with applying the other staging systems for the recurrent cases? All of the following staging systems: the CLIP score system,[6] BCLC staging[7] and JIS scoring system[8] were derived from the analysis for first-time diagnosed HCC and were applied only at the initial treatment. Because hypothetical population is different between first-time HCC patients and all HCC patients, their baseline predictors for survival differ from the new scoring system. Indeed, the distributions of both the number of tumor and the largest size of HCC are significantly different between first-time HCC cases and all HCC patients in our cohort (data not shown). As a result, JIS system and CLIP score system may have poor stratification of survival. The goal of cancer staging is to separate patients into different groups on the basis of their predicted survival to help determine the most appropriate treatment modality. Therefore, it is unreasonable to apply their systems for recurrent HCC patients.

Although further evaluation is needed, this scoring system can be useful for conducting interventional trials. With the spread of routine screening and follow-up, the number of recurrent HCC patients can increase. More effective strategies to treat recurrent patients will be needed. In addition, a new modality of treatment will be necessary for HCC management, particularly for score 2 and 3 patients. Interventional trials may be needed to determine the most appropriate therapy for the patients in each group. This scoring system, because of good incorporation between prognosis estimation and potential treatment advances, may be useful for planning and evaluating interventional trials. It would allow us to follow a well-established treatment schedule and select the best treatment modality for each patient when managing long-term–surviving HCC patients.

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Impaired Cytokine Response in Myeloid Dendritic Cells in Chronic Hepatitis C Virus Infection Regardless of Enhanced Expression of Toll-Like Receptors and Retinoic Acid Inducible Gene-I

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Dendritic cells utilize various sets of Toll-like receptors (TLR) or cytosolic sensors to detect pathogens and evoke immune responses. In patients with hepatitis C virus (HCV) infection, a higher prevalence of various infectious diseases is reported; suggesting that innate immunity against pathogens is impaired. The aim of this study was to clarify whether the TLR and retinoic acid inducible gene-I (RIG-I) system in myeloid dendritic cells is preserved or not in chronic HCV infection. The expression of TLRs, RIG-I and its relatives were compared in myeloid dendritic cells between 39 patients and 52 healthy volunteers. The induction of type-I interferon (IFN) and inflammatory cytokines was examined in response to agonists for TLR2 (palmitoyl-3-cysteine-serine-lysine-4), TLR3/RIG-I (polyinosine–polycytidylic acid) or TLR4 (lipopolysaccharide). The relative expressions of TLR2, TLR4, RIG-I, and LGP2 from the patients were significantly higher than those from the volunteers, whereas TLR3 and MDA-5 expressions did not differ. In search for factors regulating TLR/RIG-I expression, it was shown that IFN-α, polyinosine–polycytidylic acid and lipopolysaccharide induced TLR3, TLR4 and RIG-I, but TNF-α, HCV core or HCV non-structural proteins did not. For the functional analyses, myeloid dendritic cells from the patients induced significantly less amounts of IFN-β, TNF-α and IL-12p70 in response to polyinosine–polycytidylic acid or lipopolysaccharide. It is noteworthy that the expression of TRIF and TRAF6, which are essential adaptor molecules transmitting TLR3 or TLR4-dependent signals, is reduced in the patients. Thus, innate cytokine responses in myeloid dendritic cells are impaired regardless of enhanced expressions of TLR2, TLR4, and RIG-I in HCV infection. ***J. Med. Virol. 80: 980–988, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis C; myeloid dendritic cell; innate immunity; TLR3; RIG-I

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded RNA virus, which causes chronic liver disease in hosts. At primary HCV infection, approximately 80% of patients fail to eradicate HCV and eventually progress to a chronic infected state [Lauer and Walker, 2001]. It is very likely that escape mutation of the HCV genome and insufficient immune responses against HCV in hosts are involved in the persistence of infection, however, the precise mechanisms are still largely unknown. Type-I interferon (IFN) is a potent anti-viral agent that exerts its ability by suppressing viral replication or via modulating immune reactions. Gene expression analyses of HCV-infected livers obtained from chimpanzees revealed that type-I IFN and IFN-stimulated genes are highly induced even in the incubation phase [Bigger et al., 2004]. Nevertheless, HCV continues to replicate and remains at high titer levels, suggesting that HCV

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possesses some inhibitory mechanisms in IFN-inducible anti-viral responses.

As for the mechanisms of HCV persistence, the alteration or impairment of various immune cells has been reported, such as T cells, NK cells and dendritic cells [Chang et al., 2001; Wedemeyer et al., 2002; Kanto et al., 2004; Szabo and Dolganiuc, 2005]. In clear contrast with the human immunodeficiency virus, HCV does not lead to generalized immune suppression in infected hosts. Large-scale epidemiological study on US veterans revealed that the prevalence of various infectious diseases was significantly higher in HCV-positive individuals than in HCV-negative ones, including viral, bacterial, and parasite diseases [El-Serag et al., 2003]. These observations suggest that HCV infection raises the susceptibility to pathogens, not profoundly but significantly, in infected patients. However, the underlying mechanisms in the increased prevalence of infection are yet to be determined.

Toll-like receptors (TLR) are expressed in epithelial cells or antigen presenting cells and act as sensors of bacterial or viral infection. These cells utilize specific TLR for the recognition of pathogen-associated molecular patterns and eventually induce type I IFN or inflammatory cytokines. In addition to the TLR system, the existence of cytoplasmic receptors for dsRNA has been reported as virus sensors, which are retinoic acid inducible protein I (RIG-I) and melanoma differentiation associated gene 5 (MDA-5) [Yoneyama et al., 2004]. Since dsRNA is a replicative intermediate of RNA virus, RIG-I and MDA-5 induce IFN-β in response to virus infection independently of TLR3. It is thus plausible that a disabled TLR/RIG-I system may be involved in the increased susceptibility to pathogens or the mechanisms of persistent virus infection [Sumpter et al., 2005]. In human hepatoma cells harboring HCV replicons, it has been shown that HCV NS3/4A protease impedes TLR3-dependent or RIG-I-dependent IFN-β induction by means of the cleavage of relevant adaptor molecules, such as TIR domain-containing adapter inducing IFN-β (TRIF) or interferon-β promoter stimulator-1 (IPS-1), respectively [Foy et al., 2005; Li et al., 2005]. However, it is not clear whether similar inhibitory machinery of HCV operates or not in immune cells, such as dendritic cells.

Dendritic cells are immune sentinels that play a central role against pathogens in inducing innate as well as adaptive immune responses. Dendritic cells consist of myeloid and plasmacytoid subsets that play distinct roles in the regulation of immune responses. Dendritic cells utilize various sets of TLR or RIG-I/MDA-5 to sense virus infection. After the recognition, dendritic cells begin to mature and gain the ability to produce type-I IFN and inflammatory cytokines. It has been reported that blood dendritic cells expresses distinct profiles of TLRs; human myeloid dendritic cells express TLR2, -3, -4, -5, -6, -7, and -8, while plasmacytoid dendritic cells express TLR7, -8 and -9 [Iwasaki and Medzhitov, 2004]. Numerical and/or functional impairment of blood dendritic cells in acute or chronic HCV infection has been reported by several investigators including us [Kanto et al., 2004; Szabo and Dolganiuc, 2005]. One of the plausible mechanisms leading to dendritic cells impairment may be direct HCV infection to blood dendritic cells or their precursors. In support for this, it was shown that myeloid dendritic cells are susceptible to HCV infection, judging from the results of an inoculation study with pseudo-HCV particles or detection of negative strand HCV-RNA [Kaimori et al., 2004]. According to another report, myeloid dendritic cells displayed impaired expression of IL-12 and TNF-α in response to polyinosine–polycytidylic acid (polyI:C) and lipopolysaccharide (LPS) in patients with a large amount of cell-associated HCV [Rodrigue-Gervais et al., 2007], suggesting a possible link between direct HCV infection to myeloid dendritic cells and an impaired innate response.

Taking these reports into consideration, the current study focused on myeloid dendritic cells in order to clarify the roles of the TLR/RIG-I system in HCV infection, by comparing the expression of TLR, RIG-I, and MDA-5 and the induction of cytokines in response to specific agonists for these virus sensors. The study demonstrated that myeloid dendritic cells from HCV-infected patients induces a significantly lesser amount of cytokines in spite of enhanced expressions of TLR2, TLR4, and RIG-I. These findings imply that alteration of the TLR/RIG-I system is instrumental in impairment of innate immunity in HCV infection, where myeloid dendritic cells play a key role as immune sentinels against pathogens.

MATERIALS AND METHODS

Subjects

Thirty-nine patients (male/female: 22/17, mean age: 53.4 ± 10.3 years old, mean serum ALT levels: 93.9 ± 51.0 IU/L, HCV serotype 1/serotype 2: 39/0) with chronic hepatitis C (HCV group) followed at Osaka University Hospital (Osaka, Japan) were enrolled in the present study. All of them were confirmed to be positive for both serum anti-HCV antibody and HCV RNA (mean HCV RNA quantity assayed by Cobas Amplicor HCV monitor v 2.0, Roche Diagnostics, Tokyo, Japan; [Pawlotsky et al., 2000]: 1,637 ± 402 KIU/ml) but were negative for other viral infections, including hepatitis B virus (HBV) and human immunodeficiency virus (HIV). The presence of other liver diseases, such as alcoholic, metabolic or autoimmune hepatitis, was ruled out. Thirteen patients with chronic HBV infection determined by serum HBsAg-positive and ALT abnormality (male/female: 6/7, HBeAg+/HBeAg−: 7/6, mean age: 45.9 ± 14.4 years old, mean serum ALT levels: 95.2 ± 145 IU/L, mean HBV-DNA levels assayed by Cobas Amplicor HBV monitor Roche Diagnostics; [Noborg et al., 1999]: 6.1 ± 1.7 $\log_{10}$ copies/ml) were also enrolled as disease controls (HBV group). The study protocol was approved by the ethical committee of Osaka University Graduate School of Medicine. At enrolment, written informed consent was obtained from each patient. The

controls were 52 healthy volunteers or blood donors (healthy donors group) at the Osaka Red Cross Blood Center (Osaka, Japan), who were confirmed to be negative for HCV, HBV, and HIV. The background data of the blood donors were not accessible due to the confidentiality regulations of the blood center, but their serum ALT levels were confirmed to be within the normal range.

Reagents

Palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4) was purchased from InvivoGen (San Diego, CA). Polyinosine–polycytidylic acid (polyI:C) and lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Sigma (St. Louis, MO). Recombinant human IL-6, IL-10, and IL-12 were purchased from InvivoGen. Recombinant TNF-α was purchased from Genzyme (Framingham, MA). Recombinant HCV structural or non-structural (NS) proteins expressed by *E. coli* were purchased from Virogen (Watertown, MA). They were HCV core (amino acid positions, from 2 to 192), NS3 (from 1,450 to 1,643), and NS4 (from 1,658 to 1,863), respectively. HCV NS5B protein (from 2,421 to 2,965) was kindly provided by Japan Tobacco Corp. (Tokyo, Japan). Natural human interferon-α was purchased from Otsuka Pharmaceutical Co. (Tokyo, Japan).

Isolation of Myeloid Dendritic Cells

Peripheral blood mononuclear cells were isolated from heparinized venous blood by centrifugation on Ficoll-Hypaque cushion as described previously [Kanto et al., 2004]. Myeloid dendritic cells were magnetically isolated using a BDCA-1 Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of myeloid dendritic cells (Lineage-negative, HLA-DR^+, $CD11c^+$, and $CD123^{dim+}$ cells) was more than 95% as assessed by FACS (data not shown). Short-term culture of myeloid dendritic cells was performed in cytokine-free Isocove's modified Dulbecco's medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mmol/L L-glutamine, 5 mmol/L HEPES, and 5 mmol/L non-essential amino acid at 37°C in 5% CO_2.

To clarify the factors influencing the expressions of TLR or RIG-I in myeloid dendritic cells, fresh myeloid dendritic cells obtained from uninfected controls were incubated for 2 hr in the presence or absence of various cytokines, agonists for TLR/RIG-I or recombinant HCV proteins. After the incubation, they were subjected to RT-PCR analyses for the comparison.

In order to compare the function of TLR/RIG-I-mediated responses in myeloid dendritic cells between the groups, myeloid dendritic cells were incubated with various agonists for 2 hr and subjected them to cytokine analysis by RT-PCR. Alternatively, myeloid dendritic cells were cultured in the presence or absence of 25 μg/ml of polyI:C for 24 hr and collected supernatants for subsequent cytokine analyses.

Flowcytometric Analysis

The phenotypes of myeloid dendritic cells were analyzed using FACS Calibur and CellQuest software (BD Biosciences, San Jose, CA). For the staining, myeloid dendritic cells were incubated with specific antibodies for 15 min at room temperature in phosphate buffered saline (PBS) containing 2% of bovine serum albumin and 0.1% of sodium azide. The following FITC-, PE-, or APC-conjugated anti-human monoclonal antibodies were used: CD11c (clone, B-ly6), HLA-DR (L243), CD80 (L307.4), CD86 (IT2.2), CD40 (5C3), and CD83 (HB15e). All were purchased from BD Biosciences.

Real-Time Quantitative PCR

Total RNA was extracted from more than 10^6 myeloid dendritic cells using RNeasy Mini kit (Qiagen, Hilden, Germany), which was subsequently reverse transcribed in 20 μl volume using SuperScript III First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Random hexamers were added as primers. The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). For the quantification of TLR2, TLR3, TLR4, RIG-I, MDA-5, LGP2, myeloid differentiation factor 88 (MyD88), IPS-1, TRIF, TNF receptor associated factor 6 (TRAF6), TNF-α and IFN-β, ready-to-use assays (Taqman Gene Expression Assays, Applied Biosystems) were utilized, according to the manufacturer's instructions. All of the reagents used for PCR were purchased from Applied Biosystems. All of the reactions were performed in duplicate. The thermal cycling conditions for all genes were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. A calibrator sample from healthy volunteers was identified. The expressions of molecule were expressed as the relative values to the calibrator samples. To standardize the amount of total RNA added to each reaction mixture, β-actin mRNA from each sample was quantified as a control of internal RNA and corrected all values with this.

Enzyme-Linked Immunosorbent Assay and Cytokine Beads Assay

The quantity of IFN-α in culture supernatants was evaluated using Human Interferon Alpha ELISA kit (PBL Biomedical Laboratories, New Brunswick, NJ) according to the manufacturer's instructions. The concentration of TNF-α, IL-6, and IL-12p70 in the supernatants was assayed by the use of BD cytokine beads assay (CBA) Flex Sets (BD Biosciences) and analyzed by FACS Calibur according to the manufacture's instructions. The detection limits of IFN-α, TNF-α, IL-6, and IL-12p70 are 10–5,000 pg/ml, respectively.

Statistical Analysis

The Mann–Whitney *U*-test was performed to evaluate differences among the groups using StatView

5.0 software (SAS Institute, Cary, NC). A P-value of <0.05 was considered to be statistically significant.

RESULTS

Expressions of TLR2, TLR4, and RIG-I Were Higher in Myeloid Dendritic Cells From Chronic Hepatitis C Patients

With respect to the phenotypes of fresh myeloid dendritic cells, the expressions of maturation markers such as CD40, CD80, CD83, and CD86 were relatively low and were not different between the HCV group and healthy donor group (Fig. 1). The similar results were obtained from HBV group (data not shown). These results show that myeloid dendritic cells from all groups are equally immature phenotypes.

First, the expressions of TLR2, TLR3, and TLR4 in myeloid dendritic cells were examined. The relative amounts of TLR2 and TLR4 in the HCV group were higher than those in healthy donors or the HBV group (Fig. 2). In contrast, the TLR3 expression was not different among the groups (Fig. 2). In comparison between HBV and healthy donor groups, there was no difference in the expressions of these TLRs in myeloid dendritic cells (Fig. 2).

The expression of cytoplasmic receptors for dsRNA in myeloid dendritic cells was also compared. The RIG-I and LGP2 expression in the HCV or the HBV group was significantly higher than those from healthy donors,

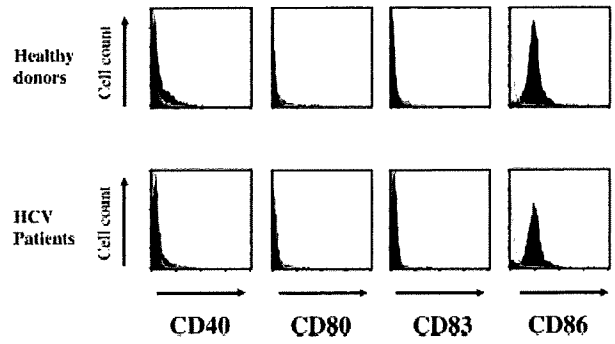

Fig. 1. Fresh myeloid dendritic cells are immature regardless of HCV infection. Myeloid dendritic cells were obtained from HCV-infective patients or healthy donors and their expressions of CD40, CD80, CD83, and CD86 were analyzed by flow cytometry. The shaded histograms are the results with specific Abs, while the open ones are those with isotype Abs. Representative results from five HCV-infected patients and five controls are shown.

whereas MDA-5 did not differ among the groups (Fig. 2). No correlation was found among the expressions of any TLR and dsRNA receptors (data not shown).

IFN-α or PolyI:C Enhanced RIG-I Expression in Myeloid Dendritic Cells

To clarify the factors influencing TLR2, 3, 4, or RIG-I expression in myeloid dendritic cells, it was examined

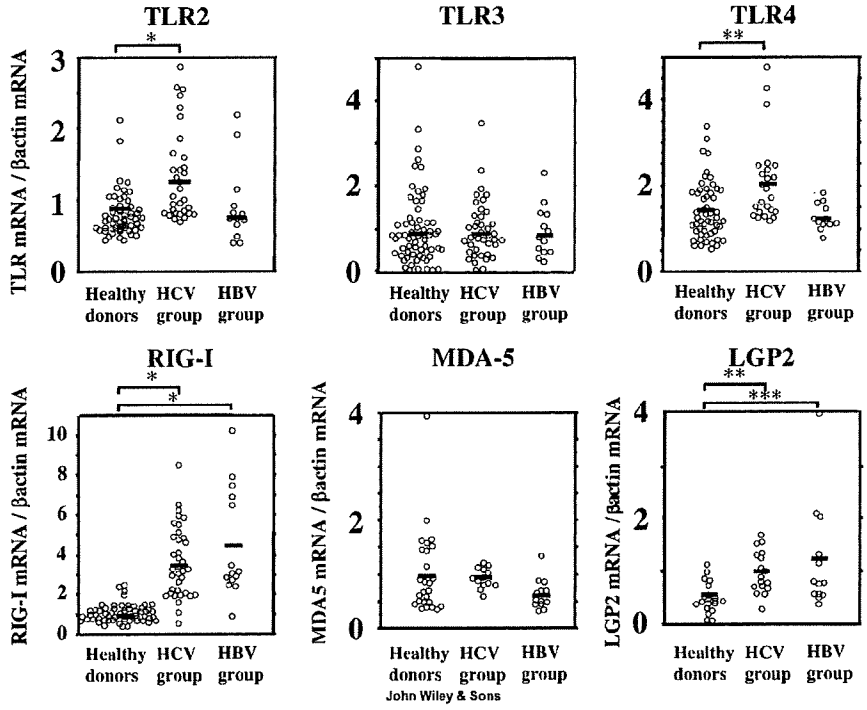

Fig. 2. Expressions of TLR2, TLR4, RIG-I, and LGP2 in patient myeloid dendritic cells from HCV-infected patients are higher than those from healthy donors, while TLR3 and MDA-5 are comparable. Expressions of TLR2, TLR3, TLR4, RIG-I, MDA-5, and LGP2 in myeloid dendritic cells were quantified by real-time RT-PCR as described in Materials and Methods Section. Horizontal bars represent the median. The statistical difference was evaluated by the Mann–Whitney U-test. $*P < 0.0001$, $**P < 0.0005$, $***P < 0.005$.