The PC and BCP mutations of HBV were assessed as previously described. Briefly, the stop codon mutation in the PC region (A1896) was detected with an enzyme-linked mini-sequence assay kit (Smitest; Roche Diagnostics, Tokyo, Japan) with a sensitivity of 1000 copies/mL. The results were expressed as the percent mutation rate as defined by Aritomi *et al.*[24] The PC mutation was judged to exist when the mutation rate exceeded 50% in the present study because the mutation rate would increase to 100% once surpassing this value.[25] The BCP double mutation was detected using an HBV core promoter detection kit (Smitest; Genome Science Laboratories) with a detection limit of 1000 copies/mL.[24] The BCP mutation was judged to exist for all classifications of mutant in the present study.

Statistical analysis

Clinical factors were compared between patients with and without HBeAg negative hepatitis after SC using the χ^2-test and Fisher's exact test, and group medians were compared using the Mann–Whitney *U*-test. Receiver-operator curves (ROC) with Youden's index were used to decide each cut-off point for predicting HBeAg negative hepatitis after SC. Differences between the analyzed groups were assessed using Kaplan–Meier analysis and the log-rank test. Sex, age at SC, HBcrAg level, ALT level, HBV DNA level, HBsAg level, PC mutation and BCP mutation were all suspected to be associated with ALT elevation after SC. Factors attaining a *P*-value of less than 20% in univariate analysis were used in multivariate analysis that employed a stepwise Cox proportional hazard model. These included level of serum albumin and platelet count at SC, levels of ALT at 0, 1, 2 and 3 years after SC, and levels of HBcrAg at 1, 2 and 3 years after SC. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan, Tokyo, Japan). *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

Baseline characteristics of patients

ALL 36 PATIENTS enrolled showed abnormal levels of ALT before SC, with the majority showing normalization around the time of SC. We defined ALT normalization as a decrease in ALT level to less than 31 IU/L for at least 1 year. The change in ratio of patients not achieving normalization over time revealed two distinct phases (Fig. 1): the first was a fast decline phase from 2 years before SC to 2 years afterwards, and the second

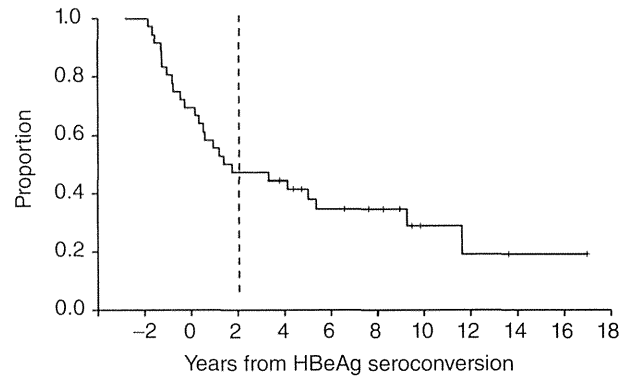

Figure 1 Changes in the proportion of patients with alanine aminotransferase (ALT) abnormality. ALT normalization was defined as ALT level decreasing to lower than 31 IU/L and maintained for at least 1 year. These data reveal two distinct time frames: a fast decline phase around the seroconversion (SC) period until 2 years afterwards, and a slow decline phase from 2 years after SC to the end of follow up. The vertical broken line at 2 years after SC indicates the borderline between the two phases. HBeAg, hepatitis B e-antigen.

was a slow decline phase from 2 years after SC to the end of follow up. Normalization of ALT during the fast phase was presumed to be associated with HBeAg SC, which was seen in 53% (19/36) of total patients. Based on this, we analyzed the risk factors associated with ALT abnormality after the time point of 2 years from SC by calculating integrated ALT levels (Fig. 2). We defined patients whose integrated ALT level exceeded 30 IU/L as having HBeAg negative hepatitis in the present study. Serum HBV DNA of over 4.0 log copies/mL was observed in all patients with HBeAg negative hepatitis.

Of the 36 patients enrolled, 20 (56%) developed HBeAg negative hepatitis and 16 (44%) did not. ALT normalization within 2 years after SC was significantly less frequent in patients with HBeAg negative hepatitis (Table 1). Median age, sex distribution and follow-up period did not differ between the two groups. Median albumin level tended to be lower in patients with HBeAg negative hepatitis, but only modestly. Eight of 20 HBeAg negative hepatitis patients underwent liver biopsy after SC. All had necroinflammatory activity. Initiation of NUC therapy was more common in the HBeAg negative hepatitis group.

Clinical and virological profiles

Changes in median levels of ALT, HBV DNA, HBsAg and HBcrAg during the course of SC have been compared between patients with and without HBeAg negative

© 2013 The Japan Society of Hepatology

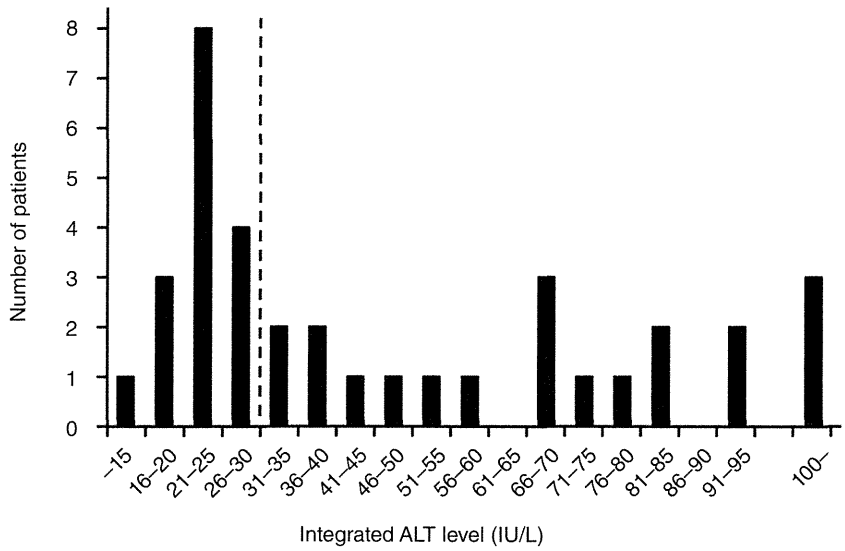

Figure 2 Distribution of integrated alanine aminotransferase (ALT) level from the time point of 2 years after seroconversion (SC) to the end of follow up.

hepatitis in Figure 3. We observed that median ALT level decreased around the time of SC in patients without HBeAg negative hepatitis, but did not in the other group. Overall, median ALT differed significantly between the two groups at the time of SC (43.0 vs 21.5 IU/L; $P = 0.009$) and at 1 (67.0 vs 15.0 IU/L; $P = 0.001$), 2 (52.0 vs 14.5 IU/L; $P < 0.001$) and 3 years (41.5 vs 15.0 IU/L; $P < 0.001$) afterwards (Fig. 3a). Median HBV DNA level decreased similarly in both groups around the time of SC (Fig. 3b). Median HBsAg level was unchanged or minimally decreased in both groups around the time of SC, but was significantly lower in patients with HBeAg negative hepatitis at 1 (3.9 vs 3.2 log IU/mL; $P = 0.025$) and 2 years (3.9 vs 3.2 log IU/mL; $P = 0.045$) before SC and at 2 years (3.7 vs 3.0 log IU/mL; $P = 0.023$) after SC (Fig. 3c). Median HBcrAg level decreased in both groups around the time of SC, but this decline was more gradual in patients with HBeAg negative hepatitis, becoming significantly higher at 1 (5.2 vs 3.9 log U/mL; $P = 0.011$), 2 (4.6 vs 3.5 log

Table 1 Comparison of host and viral factors between patients with and without HBeAg negative hepatitis among total patients

Clinical characteristics	HBeAg negative hepatitis		*P*
	Present ($n = 20$)	Absent ($n = 16$)	
Age at SC (years)†	40 (23–64)	38 (24–65)	0.504
Sex (male : female)	15:5	9:7	0.298
Follow-up period (years)†	10.6 (3.8–26.0)	12.4 (3.2–23.1)	0.610
Laboratory data at SC			
Albumin (g/dL)†	4.1 (3.6–4.6)	4.3 (3.7–4.8)	0.030
Bilirubin (mg/dL)†	1.0 (0.4–2.6)	0.8 (0.5–1.3)	0.319
Platelets (/μL)†	13.9 (8.5–24.3)	18.1 (9.6–22.9)	0.187
ALT normalization within 2 years after SC‡	4 (20)	15 (94)	<0.001
Events during follow-up period			
Initiation of NUC therapy‡	12 (60)	2 (13)	0.006
Development of HCC‡	2 (10)	1 (6)	1.000

†Data are expressed as median (range).
‡Data are expressed as number of patients (%).
ALT, alanine aminotransferase; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; NUC, nucleoside/nucleotide analog; SC, seroconversion.

© 2013 The Japan Society of Hepatology

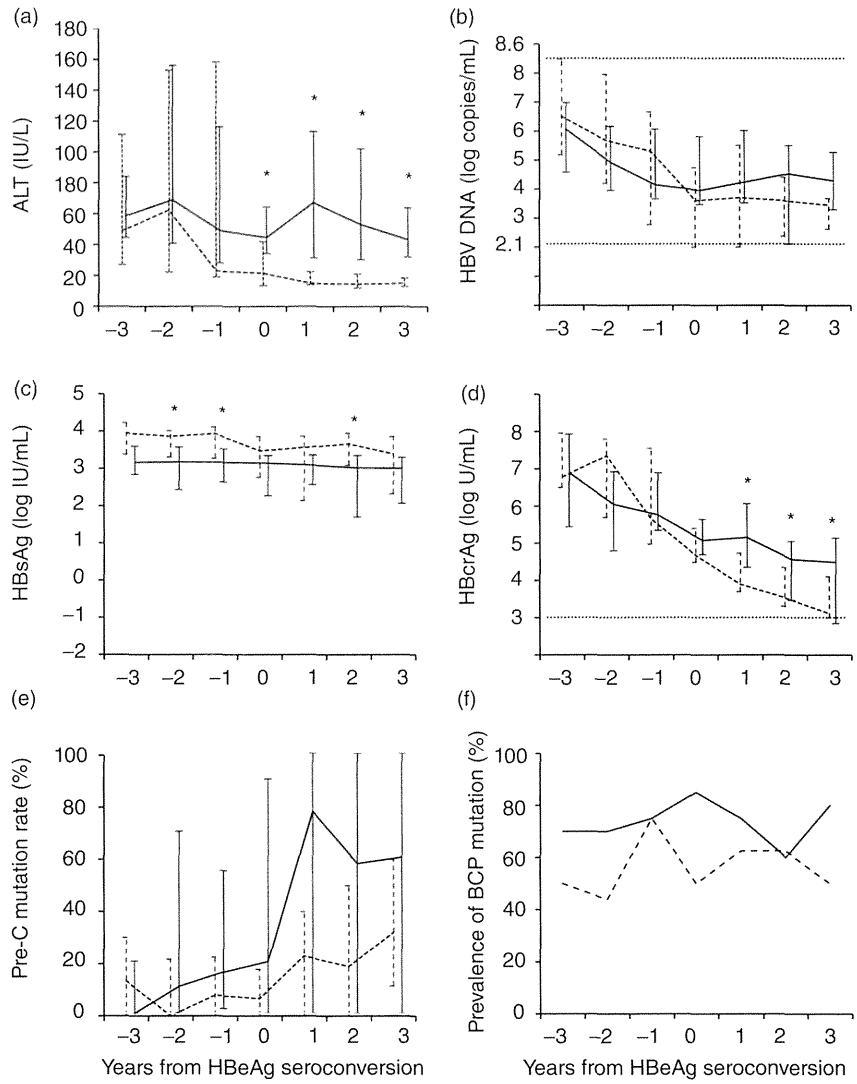

Figure 3 Changes in median levels of serum alanine aminotransferase (ALT) (a), hepatitis B virus (HBV) DNA (b), hepatitis B surface antigen (HBsAg) (c), hepatitis B core-related antigen (HBcrAg) (d) and PC mutation rate (e) are compared between patients with and without the occurrence of hepatitis B e-antigen (HBeAg) negative hepatitis. A similar comparison is made for prevalence of patients with BCP mutations (f). Solid lines indicate patients with HBeAg negative hepatitis ($n = 20$) and broken lines indicate those without ($n = 16$). Data are shown as median values with 25% and 75% ranges at each point for (a–e). Horizontal broken lines in (b) and (d) indicate the upper and lower detection limits of the corresponding markers. *$P < 0.05$.

U/mL; $P = 0.041$) and 3 years (4.6 vs 3.1 log U/mL; $P = 0.016$) after SC (Fig. 3d). PC mutation rate increased similarly in both groups during the course of SC (Fig. 3e), and the prevalence of BCP mutation positive patients remained comparatively high in both groups throughout the study period (Fig. 3f).

All factors that were associated with the occurrence of HBeAg negative hepatitis were evaluated for independence by multivariate analysis. We found that only abnormal level of ALT (≥31 IU/L) at 2 years after SC (odds ratio, 42.0; 95% confidence interval, 4.3–405.4; $P = 0.001$) was an independent predictive factor. Therefore, we examined for factors associated with the occurrence of HBeAg negative hepatitis in the 19 patients whose ALT level had normalized by 2 years after SC. Four (21%) of these patients developed HBeAg negative hepatitis and the remaining 15 (79%) did not. We found no significant differences between the two groups with regard to age at SC, sex or laboratory data (Table 2). We next analyzed HBV DNA, HBsAg and HBcrAg levels at 2 years after SC to see if these factors could discriminate between patients with and without the development of HBeAg negative hepatitis. Cut-off values for each factor were determined by ROC analysis. As shown in Figure 4, serum levels of HBV DNA (7% vs 60%; $P = 0.037$) and HBcrAg (0% vs 44%; $P = 0.033$) were significant factors indicating susceptibility, but HBsAg was not.

© 2013 The Japan Society of Hepatology

Table 2 Comparison of host and viral factors between patients with and without HBeAg negative hepatitis in 19 patients whose ALT levels were normal at 2 years after SC

Clinical characteristics	HBeAg negative hepatitis		*P*
	Present ($n = 4$)	Absent ($n = 15$)	
Age at SC (years)†	41 (30–43)	37 (23–65)	0.549
Sex (male : female)	2:2	8:7	1.000
Follow-up period (years)†	9.1 (8.3–14.1)	12.2 (3.2–23.1)	0.610
Laboratory data at SC			
Albumin (g/dL)†	4.3 (3.8–4.3)	4.3 (3.7–4.7)	0.364
Bilirubin (mg/dL)†	1.0 (1.0–1.3)	0.8 (0.5–1.3)	0.083
Platelets (/μL)†	14.9 (13.3–16.4)	16.9 (9.6–22.5)	0.667
Events during follow-up period			
Initiation of NUC therapy‡	3 (75)	2 (13)	0.037
Development of HCC‡	1 (25)	1 (7)	0.386

†Data are expressed as median (range).
‡Data are expressed as number of patients (%).
ALT, alanine aminotransferase; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; NUC, nucleoside/nucleotide analog; SC, seroconversion.

DISCUSSION

ALTHOUGH ACTIVE HEPATITIS usually subsides following HBeAg SC, it recurs in a considerable proportion of patients several years afterwards. Hsu *et al.*[5] followed 283 patients with HBeAg SC for a median follow-up period of 8.6 years and observed that ALT elevation of over twice the upper limit of normal occurred in 94 patients (33%). Of these, 68 (72%) were considered to have HBeAg negative hepatitis B because HBV DNA was detectable without the reappearance of HBeAg at the time of ALT elevation. HBeAg negative hepatitis is a major health concern because its occurrence is closely associated with progression to cirrhosis and development of HCC,[9–12] and thus prediction of its onset is important. Hsu *et al.*[5] found that patients with more frequent acute exacerbations of hepatitis before HBeAg SC and those with cirrhosis at the time of HBeAg SC had a higher risk of developing HBeAg negative hepatitis. Although significant, these factors were insufficient to accurately predict the occurrence of the disease.[26–30] Therefore, we analyzed several additional factors, including HBV DNA, HBsAg and HBcrAg levels, as well as viral mutations that halt HBeAg production.

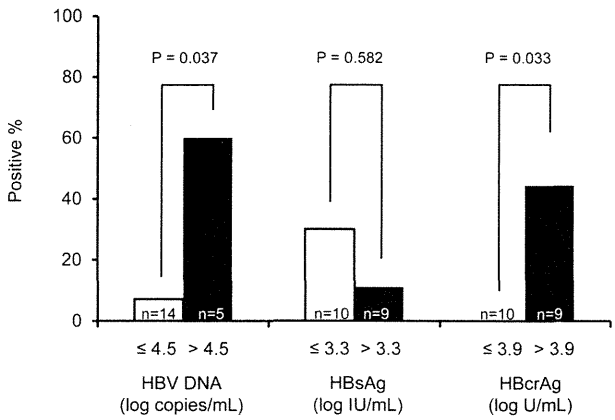

Figure 4 Occurrence of hepatitis B e-antigen (HBeAg) negative hepatitis is compared among patients using higher and lower levels of corresponding markers at 2 years after seroconversion (SC). The cut-off value for each marker was determined by receiver-operator curve analysis. HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

In the present study, we found that the majority of patients with HBeAg SC achieved normalization of ALT within 2 years following SC, after which such normalization became relatively rare. Abnormal ALT was determined using the distribution of integrated ALT level from 2 years after SC to the end of follow up, which clearly showed the existence of two groups. We defined patients with an abnormal integrated level of ALT as having HBeAg negative hepatitis because this abnormality tended to persist and was preceded by HBV DNA elevation. Our result also conferred the important realization that ALT abnormality within 2 years after SC may not necessarily indicate the occurrence of HBeAg negative hepatitis, which has a poor prognosis. NUC

© 2013 The Japan Society of Hepatology

therapy was not available for patients with chronic hepatitis B in Japan when our subjects began follow up. Hence, the natural history of SC has been evaluated in this cohort. Follow up stopped in this study when NUC therapy was commenced. Currently, we perform NUC therapy on patients with HBe negative hepatitis based on age and ALT activity, as advised by the Ministry of Health, Labor and Welfare.[17]

Many host and viral factors were also analyzed to predict the occurrence of HBeAg negative hepatitis in the current study. Host factors, including age and sex, did not differ between the groups with and without HBeAg negative hepatitis, but changes in median ALT level around SC clearly differed between the two groups. Specifically, ALT level did not decrease even after SC in patients with HBeAg negative hepatitis, while it normalized during the SC period in those without. Viral factors were analyzed at several time points around SC. Among them, median HBcrAg level clearly differed between the groups; HBcrAg showed a steep decrease around the SC period in patients without HBeAg negative hepatitis, while it exhibited a significantly slower decline in those with. Similarly to earlier reports, median levels of HBV DNA and HBsAg showed some differences between the two groups, but these were not remarkable when analyzed chronologically. Negative results were also seen in the analyses of PC and BCP mutations. Multivariate analysis showed that abnormal ALT level at 2 years after SC was the only significant factor to predict the occurrence of HBeAg negative hepatitis among the factors analyzed. Because patients with normal ALT had maintained that level for at least 1 year, this result may indicate that continuous normalization of ALT is rare in patients with HBeAg negative hepatitis after SC and that ALT abnormality is associated with higher levels of HBcrAg and HBV DNA.

Because ALT level was closely related to the occurrence of HBeAg negative hepatitis, we next analyzed for predictive factors in patients whose ALT level was normal (<31 IU/L) at 2 years after SC. We observed that increased HBV DNA and HBcrAg levels at 2 years after SC were significant factors for predicting the occurrence of HBeAg negative hepatitis, but that HBsAg level was not. Single or combined monitoring use of HBV DNA and HBcrAg levels may therefore be useful to predict the recurrence of hepatitis in patients whose ALT level normalizes following HBeAg SC. However, further studies are required to verify this in the clinical setting.

Whereas HBsAg is a serum marker commonly used for the diagnosis of HBV infection, HBcrAg assays measure serum levels of HBc, HBe and the 22-kDa precore antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these three denatured antigens.[31] Because the latter assay measures all antigens transcribed from the precore/core gene, it is regarded as core-related.[21] It has been suggested that viral antigen levels, including those of HBsAg and HBcrAg, are differently associated with HBV activity from HBV DNA and ALT levels, and thus are useful for predicting the future activity of hepatitis B. For example, HBcrAg level was seen to predict hepatitis relapse after discontinuation of NUC therapy,[32,33] and HBsAg level has been reportedly associated with the response to pegylated interferon therapy differently from HBV DNA.[34,35] Both antigen levels are believed to be related to intracellular levels of HBV cccDNA. However, it is possible that levels of HBsAg and HBcrAg have different roles in monitoring viral activity because the transcription of these two antigens is regulated by alternative enhancer–promoter systems in the HBV genome.[1] The serum level of HBcrAg was more useful than that of HBsAg to predict the occurrence of HBeAg negative hepatitis in the present study. This difference may be attributed to the fact that the production of all antigens that constitute HBcrAg is regulated by the same system as that of HBeAg, while the production of HBsAg is not.

Lastly, it is reasonable to presume that the PC and BCP mutations which halt HBeAg production are associated with integrated values of ALT elevation because the disease is essentially caused by HBV containing these mutations.[8,10] However, the prevalence of either mutation did not differ between the groups at any time point during the study. Our results showed that almost all patients had PC and/or BCP mutations, especially after SC, and implied that the existence of these mutations alone was not sufficient for developing ALT elevation. HBV genotype is also closely associated with HBeAg SC,[36] but we could not include genotype as a factor because our entire cohort was genotype C.

A recent review by Papatheodoridis *et al.*[37] showed that histologically significant liver disease is rare in HBeAg negative patients with persistently normal ALT based on stringent criteria and serum HBV DNA of 20 000 IU/mL or less. They suggest that such individuals can be considered as true inactive HBV carriers, who require continued follow up rather than liver biopsy or immediate therapy. On the contrary, liver biopsy samples obtained from eight of our patients with HBeAg negative hepatitis having elevated ALT levels after SC revealed necroinflammatory activity. Hence, it remains controversial if histological findings are important for diagnosis of HBeAg negative hepatitis.

© 2013 The Japan Society of Hepatology

This study has the main limitations of a retrospective design and a small cohort size. However, our findings from careful extended follow up indicate that ALT abnormality after 2 years from SC can be considered to be HBeAg negative hepatitis, and that HBcrAg and HBV DNA levels may be useful for predicting the long-term outcome of patients who achieve HBeAg SC and ALT normalization.

ACKNOWLEDGMENTS

THIS RESEARCH WAS supported in part by a research grant from the Ministry of Health, Labor and Welfare of Japan. We thank Ms Hiroe Banno for her secretarial assistance, and Ms Etsuko Iigahama, Asami Yamazaki and Toyo Amaki for technical assistance. We also thank Mr Trevor Ralph for his English editorial assistance.

REFERENCES

1 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733–45.
2 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
3 Umemura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* 2009; **44** (Suppl 19): 102–7.
4 Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007; **45**: 1056–75.
5 Hsu YS, Chien RN, Yeh CT *et al.* Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology* 2002; **35**: 1522–7.
6 Carman WF, Jacyna MR, Hadziyannis S *et al.* Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989; **2**: 588–91.
7 Yim HJ, Lok AS. Natural history of chronic hepatitis B virus infection: what we knew in 1981 and what we know in 2005. *Hepatology* 2006; **43**: S173–81.
8 Chan HL, Hussain M, Lok AS. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. *Hepatology* 1999; **29**: 976–84.
9 Marschenz S, Endres AS, Brinckmann A *et al.* Functional analysis of complex hepatitis B virus variants associated with development of liver cirrhosis. *Gastroenterology* 2006; **131**: 765–80.
10 Chen CH, Hung CH, Lee CM *et al.* Pre-S deletion and complex mutations of hepatitis B virus related to advanced liver disease in HBeAg-negative patients. *Gastroenterology* 2007; **133**: 1466–74.
11 Chen CH, Changchien CS, Lee CM *et al.* Combined mutations in pre-s/surface and core promoter/precore regions of hepatitis B virus increase the risk of hepatocellular carcinoma: a case-control study. *J Infect Dis* 2008; **198**: 1634–42.
12 Yuen MF, Tanaka Y, Shinkai N *et al.* Risk for hepatocellular carcinoma with respect to hepatitis B virus genotypes B/C, specific mutations of enhancer II/core promoter/precore regions and HBV DNA levels. *Gut* 2008; **57**: 98–102.
13 Tseng TC, Liu CJ, Chen CL *et al.* Serum hepatitis B virus-DNA levels correlate with long-term adverse outcomes in spontaneous hepatitis B e antigen seroconverters. *J Infect Dis* 2012; **205**: 54–63.
14 Umemura T, Zen Y, Hamano H, Kawa S, Nakanuma Y, Kiyosawa K. Immunoglobin G4-hepatopathy: association of immunoglobin G4-bearing plasma cells in liver with autoimmune pancreatitis. *Hepatology* 2007; **46**: 463–71.
15 Kumada T, Toyoda H, Kiriyama S *et al.* Incidence of hepatocellular carcinoma in hepatitis C carriers with normal alanine aminotransferase levels. *J Hepatol* 2009; **50**: 729–35.
16 Kumada T, Toyoda H, Kiriyama S *et al.* Relation between incidence of hepatic carcinogenesis and integration value of alanine aminotransferase in patients with hepatitis C virus infection. *Gut* 2007; **56**: 738–9.
17 Kumada H, Okanoue T, Onji M *et al.* Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; **40**: 1–7.
18 Knodell RG, Ishak KG, Black WC *et al.* Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981; **1**: 431–5.
19 Umemura T, Tanaka E, Kiyosawa K, Kumada H. Mortality secondary to fulminant hepatic failure in patients with prior resolution of hepatitis B virus infection in Japan. *Clin Infect Dis* 2008; **47**: e52–6.
20 Matsumoto A, Tanaka E, Morita S, Yoshizawa K, Umemura T, Joshita S. Changes in the serum level of hepatitis B virus (HBV) surface antigen over the natural course of HBV infection. *J Gastroenterol* 2012; **47**: 1006–13.
21 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40**: 439–45.
22 DiDomenico N, Link H, Knobel R *et al.* COBAS AMPLICOR: fully automated RNA and DNA amplification and detection system for routine diagnostic PCR. *Clin Chem* 1996; **42**: 1915–23.
23 Mizokami M, Nakano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; **450**: 66–71.
24 Aritomi T, Yatsuhashi H, Fujino T *et al.* Association of mutations in the core promoter and precore region of hepatitis virus with fulminant and severe acute hepatitis in Japan. *J Gastroenterol Hepatol* 1998; **13**: 1125–32.

© 2013 The Japan Society of Hepatology

25 Yamaura T, Tanaka E, Matsumoto A *et al.* A case-control study for early prediction of hepatitis B e antigen seroconversion by hepatitis B virus DNA levels and mutations in the precore region and core promoter. *J Med Virol* 2003; **70**: 545–52.
26 Brunetto MR, Oliveri F, Colombatto P *et al.* Hepatitis B surface antigen serum levels help to distinguish active from inactive hepatitis B virus genotype D carriers. *Gastroenterology* 2010; **139**: 483–90.
27 Nakazawa T, Shibuya A, Takeuchi A *et al.* Viral level is an indicator of long-term outcome of hepatitis B virus e antigen-negative carriers with persistently normal serum alanine aminotransferase levels. *J Viral Hepat* 2011; **18**: e191–9.
28 Togo S, Arai M, Tawada A *et al.* Clinical importance of serum hepatitis B surface antigen levels in chronic hepatitis B. *J Viral Hepat* 2011; **18**: e508–15.
29 Park H, Lee JM, Seo JH *et al.* Predictive value of HBsAg quantification for determining the clinical course of genotype C HBeAg-negative carriers. *Liver Int* 2012; **32**: 796–802.
30 Chen YC, Huang SF, Chu CM, Liaw YF. Serial HBV DNA levels in patients with persistently normal transaminase over 10 years following spontaneous HBeAg seroconversion. *J Viral Hepat* 2012; **19**: 138–46.
31 Kimura T, Ohno N, Terada N *et al.* 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* 2005; **280**: 21713–19.
32 Shinkai N, Tanaka Y, Orito E *et al.* Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection. *Hepatol Res* 2006; **36**: 272–6.
33 Matsumoto A, Tanaka E, Minami M *et al.* Low serum level of hepatitis B core-related antigen indicates unlikely reactivation of hepatitis after cessation of lamivudine therapy. *Hepatol Res* 2007; **37**: 661–6.
34 Brunetto MR, Moriconi F, Bonino F *et al.* Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* 2009; **49**: 1141–50.
35 Moucari R, Mackiewicz V, Lada O *et al.* Early serum HBsAg drop: a strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients. *Hepatology* 2009; **49**: 1151–7.
36 McMahon BJ. The influence of hepatitis B virus genotype and subgenotype on the natural history of chronic hepatitis B. *Hepatol Int* 2009; **3**: 334–42.
37 Papatheodoridis GV, Manolakopoulos S, Liaw YF, Lok A. Follow-up and indications for liver biopsy in HBeAg-negative chronic hepatitis B virus infection with persistently normal ALT: a systematic review. *J Hepatol* 2012; **57**: 196–202.

© 2013 The Japan Society of Hepatology

Hepatology Research

Hepatology Research 2014; 44: E172–E180 doi: 10.1111/hepr.12287

Original Article

Serum levels of interleukin-22 and hepatitis B core-related antigen are associated with treatment response to entecavir therapy in chronic hepatitis B

Sadahisa Okuhara,[1] Takeji Umemura,[1] Satoru Joshita,[1] Soichiro Shibata,[1] Takefumi Kimura,[1] Susumu Morita,[1] Michiharu Komatsu,[1] Akihiro Matsumoto,[1] Kaname Yoshizawa,[1] Yoshihiko Katsuyama,[2] Masao Ota[3] and Eiji Tanaka[1]

[1]*Department of Medicine, Division of Hepatology and Gastroenterology,* [3]*Department of Legal Medicine, Shinshu University School of Medicine, and* [2]*Department of Pharmacy, Shinshu University Hospital, Matsumoto, Japan*

Aim: We sought to clarify the associations between serum cytokines and chemokines, hepatitis B surface antigen (HBsAg), hepatitis B core-related antigen (HBcrAg), and hepatitis B virus (HBV) DNA and response to entecavir therapy in chronic hepatitis B.

Methods: We analyzed six cytokines (interleukin [IL]-2, IL-6, IL-10, IL-12p70, IL-21 and IL-22) and five chemokines (CCL2, CCL3, CXCL9, CXCL10 and CXCL11) before and at 6, 12 and 24 months during entecavir therapy in 48 chronic hepatitis B patients. Quantitative measurement of HBsAg, HBcrAg and HBV DNA was performed. A virological response (VR) was defined as serum HBV DNA of less than 2.1 log copies/mL by treatment month 24.

Results: Thirty-nine patients (81%) achieved a VR. Serum IL-6 ($P = 0.031$), CXCL-9 ($P = 0.002$), and CXCL-10 ($P = 0.001$) were high in chronic HBV and correlated positively with transaminases and bilirubin. Before treatment, elevated IL-22 ($P = 0.031$) and lower HBsAg ($P = 0.001$) and HBcrAg ($P < 0.001$), but not HBV DNA, were associated with a favorable treatment outcome. In multivariate analysis, high IL-22 (hazard ratio = 13.67, $P = 0.046$) and low HBcrAg (hazard ratio = 10.88, $P = 0.048$) were independently associated with a VR. The levels of IL-22 ($P < 0.001$), HBsAg ($P < 0.001$), and HBcrAg ($P < 0.001$) all decreased from baseline to 24 months of treatment in virological responders.

Conclusion: Serum IL-22 and HBcrAg are predictive markers of a VR to entecavir therapy in patients with chronic hepatitis B.

Key words: entecavir, hepatitis B core-related antigen, hepatitis B surface antigen, hepatitis B virus, interleukin-22

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is the primary cause of cirrhosis and hepatocellular carcinoma (HCC) and is one of the major causes of death globally.[1,2] Because high plasma HBV DNA concentrations and quantitative hepatitis B surface antigen (HBsAg) levels are associated with progression to cirrhosis and development of HCC,[3,4] viral suppression by means of nucleoside/nucleotide analog therapy has shown clinical benefits via a reduction in hepatic decompensation and lower HCC rates.[5–7]

Cytokines and chemokines are involved in cell-mediated and humoral immune responses as well as in antiviral activity, viral clearance, apoptosis and fibrogenesis. As the control of cytokine production is highly complex and their effects widespread throughout multiple regulatory networks, it would seem that screening for multiple biomarkers may best clarify the immunopathogenesis of this disease and predict responses to antiviral therapy. Our previous studies have shown that several cytokines and chemokines are associated with treatment outcome in patients with chronic hepatitis C using bead-based multiplex immunoassays.[8–10] Although other reports have demonstrated an association between individual cytokines and clinical outcome in subjects with HBV,[11–18] the

Correspondence: Dr Takeji Umemura, Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. Email: tumemura@shinshu-u.ac.jp
Conflict of interest: All authors declare no conflicts of interest.
Received 17 October 2013; revision 28 November 2013; accepted 2 December 2013.

© 2013 The Japan Society of Hepatology

relationship between multiple cytokines and chemokines and response to nucleoside/nucleotide analog therapy in chronic hepatitis B patients has not yet been examined in the Japanese population.

The objective of this study is to determine which cytokines and chemokines in chronic hepatitis B are related to the clinical and virological characteristics of hepatitis and how they affect the HBV response to entecavir (ETV) treatment.

METHODS

Subjects

WE ENROLLED 48 consecutive patients with chronic hepatitis B in this study. All patients were treatment naïve at the time of commencing ETV at a daily dose of 0.5 mg for a duration of at least 24 months. Clinical and laboratory data of the patients were analyzed at baseline and at months 6, 12 and 24 of therapy. Chronic hepatitis B was based on HBsAg positivity for at least 6 months. No patients had a history of organ transplantation, decompensated cirrhosis, HCC or the concurrent use of immunomodulatory drugs or corticosteroids. Patients who were co-infected with the hepatitis C virus (HCV) or who exhibited evidence of other liver diseases, such as primary biliary cirrhosis, autoimmune hepatitis, alcoholic liver disease and non-alcoholic liver disease, were excluded from this study. A group of 10 healthy individuals negative for HBV and HCV serology and normal transaminase levels was used as the control. All patients and subjects were negative for antibodies to HIV type 1. The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine. All patients provided written informed consent.

Laboratory testing

Hepatitis B surface antigen, hepatitis B e-antigen (HBeAg), anti-HBe, anti-HCV and anti-HIV-1 were determined using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan).[19] Serum levels of HBV DNA were quantified using the COBAS TaqMan HBV Test v2.0 (Roche Diagnostics, Tokyo, Japan) that had a dynamic range of 2.1–9.0 log copies/mL. Quantitative measurement of HBsAg was performed using an HISCL HBsAg assay based on the chemiluminescence enzyme immunoassay (CLEIA; Sysmex, Kobe, Japan) which had a quantitative range of −1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range. Serum HB core-related antigen (HBcrAg) levels were measured using a CLEIA-based HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio, Tokyo, Japan). We expressed HBcrAg level in terms of log U/mL with a quantitative range set at 3.0–6.8 log U/mL. HBV genotypes were determined using commercially available ELISA kits (HBV GENOTYPE EIA; Institute of Immunology). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and other relevant biochemical tests were performed using standard methods.[20]

Definitions

A virological response (VR) was defined as a HBV DNA level that was undetectable by real-time polymerase chain reaction (<2.1 copies/mL) at 24 months. A virological breakthrough was defined as an increase in HBV DNA level by 1 log copies/mL or more above nadir while on treatment following an initial decline to 2 log copies/mL or more.

Detection of cytokines and chemokines

Six cytokines (interleukin [IL]-2, IL-6, IL-10, IL-12p70, IL-21 and IL-22) and five chemokines (CCL2/MCP-1, CCL3/MIP-1α, CXCL9/MIG, CXCL10/IP-10 and CXCL11/I-TAC) were quantified using Luminex Multiplex Cytokine Kits (Procarta Cytokine Assay Kit) for serum samples obtained before the start of treatment and at weeks 24, 48 and 96 as reported previously.[8,9] These markers had been implicated in HBV pathogenesis in earlier reports.[11–16,18] All collected samples were immediately stored at −70°C and remained in storage until testing.

Statistical analysis

The Mann–Whitney *U*-test and Kruskal–Wallis test were used to analyze continuous variables where appropriate. The Friedman test was employed to evaluate changes in serum cytokine levels over time. Spearman's rank correlation coefficients were adopted to evaluate the relationship between pairs of markers. The χ^2-test with Yates's correction was used for the analysis of categorical data. In cases where the number of subjects was less than five, we employed Fisher's exact test. $P < 0.05$ was considered statistically significant. To predict treatment outcome, cut-off points for continuous variables were decided by receiver–operator curve (ROC) analysis with Youden's index. Factors attaining a *P*-value of less than 0.1 in univariate analysis were evaluated by multivariate analysis using a stepwise logistic regression model. These

© 2013 The Japan Society of Hepatology

included age, HBe positivity, platelets, and levels of HBsAg, HBcrAg, HBV DNA and IL-22 before treatment. Statistical analyses were carried out using SPSS software version 21.0J (IBM Japan, Tokyo, Japan).

RESULTS

Baseline clinical characteristics of patients

THE CLINICAL PROFILE of the experimental patient cohort is shown in Table 1. Among our 48 patients with chronic hepatitis, 39 (81%) achieved a VR at 24 months. A VR was attained in 11 of 20 HBeAg positive patients (55%) and in all 28 HBeAg negative patients (100%). One patient (5%) demonstrated HBeAg seroclearance through to month 24, but did not attain HBeAg seroconversion. No patient experienced a virological breakthrough.

The median age of patients achieving a VR was significantly higher than that of patients who did not (55 vs 37 years; $P = 0.031$) (Table 1). In contrast, viral responders had significantly lower median HBsAg (3.3 vs 3.9 log IU/mL; $P = 0.001$) and HBcrAg (5.0 vs 6.8 log U/mL; $P < 0.001$) levels than non-responders. We found no significant differences between patient groups with regard to sex, HBV genotype, or albumin, AST, ALT, bilirubin or platelet levels. When stratified by HBeAg positivity, HBsAg level only was significantly associated with a VR (3.2 vs 3.9 log IU/mL; $P = 0.003$). When we compared HBeAg positive and negative patients, median HBV DNA and HBcrAg levels, but not HBsAg, were significantly higher in HBeAg positive patients (Table S1).

Detection and quantification of serum markers in patients with chronic hepatitis B and controls

Serum samples obtained prior to ETV therapy were examined for the presence of six cytokines and five chemokines by multiplex assays. As shown in Table 2, the median baseline serum concentrations of IL-6 (6.5 vs 5.8 pg/mL; $P = 0.031$) and three chemokines (CCL2 [39.3 vs 31.5 pg/mL; $P = 0.022$], CXCL9 [329.2 vs 127.8 pg/mL; $P = 0.002$] and CXCL10 [217.1 vs 58.7 pg/mL; $P = 0.001$]) were significantly higher in patients with chronic hepatitis B than in healthy controls. When we subdivided patients into HBeAg positive or anti-HBe positive groups, no significant differences in the median concentrations of any cytokine or chemokine were seen, including IL-22 (Table S1).

Effect of ETV therapy on serum cytokine levels

The median levels of serum cytokines and chemokines in our cohort are shown in Table 3. Among our patients, the median baseline serum IL-22 concentration was significantly higher in virological responders than in non-responders (35.3 vs 27.8 pg/mL; $P = 0.031$) (Fig. 1a). No other cytokines or chemokines were associated with

Table 1 Demographic and clinical characteristics of 48 patients with chronic hepatitis B

Characteristics	Total, $n = 48$	VR (+), $n = 39$	VR (−), $n = 9$	P
Age, years	55 (24–81)	55 (24–81)	37 (26–67)	**0.031**
Male, *n* (%)	33 (69)	29 (74)	4 (44)	0.18
HBeAg positive, *n* (%)	20 (42)	11 (28)	9 (100)	**<0.001**
HBV genotype C, *n* (%)	45 (94)	37 (95)	8 (89)	1.00
HBV DNA (log copies/mL)	6.6 (2.7 to >9.1)	6.4 (2.7 to >9.1)	8.0 (3.9 to >9.1)	0.06
HBsAg (log IU/mL)	3.4 (−1.2 to 4.5)	3.3 (−1.2 to 4.3)	3.9 (3.3–4.5)	**0.001**
HBcrAg (log U/mL)	5.2 (3.0–6.8)	5.0 (3.0–6.8)	6.8 (5.4–6.8)	**<0.001**
Albumin (mg/dL)	4.2 (2.3–5.3)	4.2 (3.1–5.3)	4.2 (2.3–4.5)	0.80
AST (IU/L)	48 (15–1476)	51 (15–1476)	36 (28–358)	0.82
ALT (IU/L)	49 (9–2021)	63 (9–2021)	56 (29–954)	0.74
Bilirubin (mg/dL)	0.8 (0.3–3.1)	0.8 (0.3–3.1)	0.7 (0.5–1.0)	0.33
Platelet (/μL)	16.3 (8.0–28.9)	15.2 (8.0–28.9)	19.5 (11.9–27.7)	0.053

Continuous variables are expressed as median values (range).
Bolded figures indicate statistical significance.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

© 2013 The Japan Society of Hepatology

Table 2 Serum cytokines and chemokines in patients with chronic hepatitis B and healthy subjects

Cytokine/chemokine	Patients	Controls	*P*-value
IL-2	2.3 (0–4.9)	2.1 (1.9–2.4)	0.42
IL-6	6.5 (2.7–19.1)	5.8 (5.8–6.5)	**0.031**
IL-10	1.1 (0.0–26.8)	1.4 (1.3–1.6)	0.49
IL-12p70	12.9 (0.1–22.0)	12.9 (12.8–12.9)	0.50
IL-21	12.5 (5.0–1916.5)	11.5 (10.5–253.5)	0.68
IL-22	34.9 (27.2–75.7)	33.6 (32.3–39.0)	0.47
CCL2	39.3 (23.8–8118.8)	31.5 (26.7–39.3)	**0.022**
CCL3	4.8 (0.0–651.8)	7.0 (5.0–9.9)	0.25
CXCL9	329.2 (89.8–18 758.9)	127.8 (107.5–874.3)	**0.002**
CXCL10	217.1 (18.6–3594.3)	58.7 (24.7–859.5)	**0.001**
CXCL11	40.8 (0.7–553.8)	25.8 (12.9–90.3)	0.23

Continuous variables are expressed as median values (range) (pg/mL).
Bolded figures indicate statistical significance.
IL, interleukin.

a VR. When stratified by HBeAg positivity, serum IL-22 and IL-6 levels in the VR group were significantly higher than those in the non-VR group (35.3 vs 31.2 pg/mL [$P = 0.046$] and 6.9 vs 6.1 pg/mL [$P = 0.031$], respectively).

Several clinical findings (HBV DNA, HBsAg, HBcrAg, albumin, AST, ALT, bilirubin and platelet) at baseline were examined for their correlation with serum cytokines or chemokines in patients with chronic hepatitis B. Serum IL-6, CXCL9, CXCL10 and CXCL11 were all positively correlated with values for AST, ALT and bilirubin, but were negatively correlated with serum HBsAg (Table 4). CXCL9, CXCL10 and CXCL11 were also significantly correlated with each other (data not shown). There was a negative correlation between HBsAg and AST, ALT and bilirubin (data not shown).

Prediction of VR in patients with chronic hepatitis B

We performed ROC analysis to determine the optimal cut-off values for serum IL-22, HBsAg and HBcrAg in predicting a VR for chronic HBV infection with the values obtained from the 39 patients who achieved a VR and the nine who did not. The selection of optimal cut-off point values was based on the IL-22, HBsAg and HBcrAg levels at which accuracy was maximal. Optimal cut-off value, sensitivity, specificity, positive predictive value, negative predictive value and calculated area

Table 3 Serum cytokines and chemokines in treatment outcome to antiviral therapy

Cytokine/chemokine	VR	Non-VR	*P*-value
IL-2	2.3 (0.0–4.9)	3.1 (0.0–3.3)	0.60
IL-6	6.8 (2.7–19.1)	6.1 (4.3–12.5)	0.22
IL-10	0.6 (0.0–26.8)	1.5 (0.0–5.0)	0.86
IL-12p70	12.9 (0.1–22.0)	12.9 (1.2–18.0)	0.74
IL-21	12.2 (5.0–1916.5)	19.9 (5.9–27.8)	0.70
IL-22	35.3 (27.2–75.7)	27.8 (27.3–46.7)	**0.031**
CCL2	40.8 (24.4–118.8)	34.8 (23.8–60.3)	0.13
CCL3	4.5 (0.0–651.8)	6.5 (2.7–22.9)	0.57
CXCL9	322.5 (115.4–18 758.9)	353.6 (89.8–1545.1)	0.60
CXCL10	206.3 (29.1–3594.3)	294.2 (18.6–2240.7)	0.94
CXCL11	39.9 (0.7–553.8)	48.8 (12.6–428.2)	0.80

Continuous variables are expressed as median values (range) (pg/mL).
Bolded figure indicates statistical significance.
IL, interleukin; VR, virological response.

© 2013 The Japan Society of Hepatology

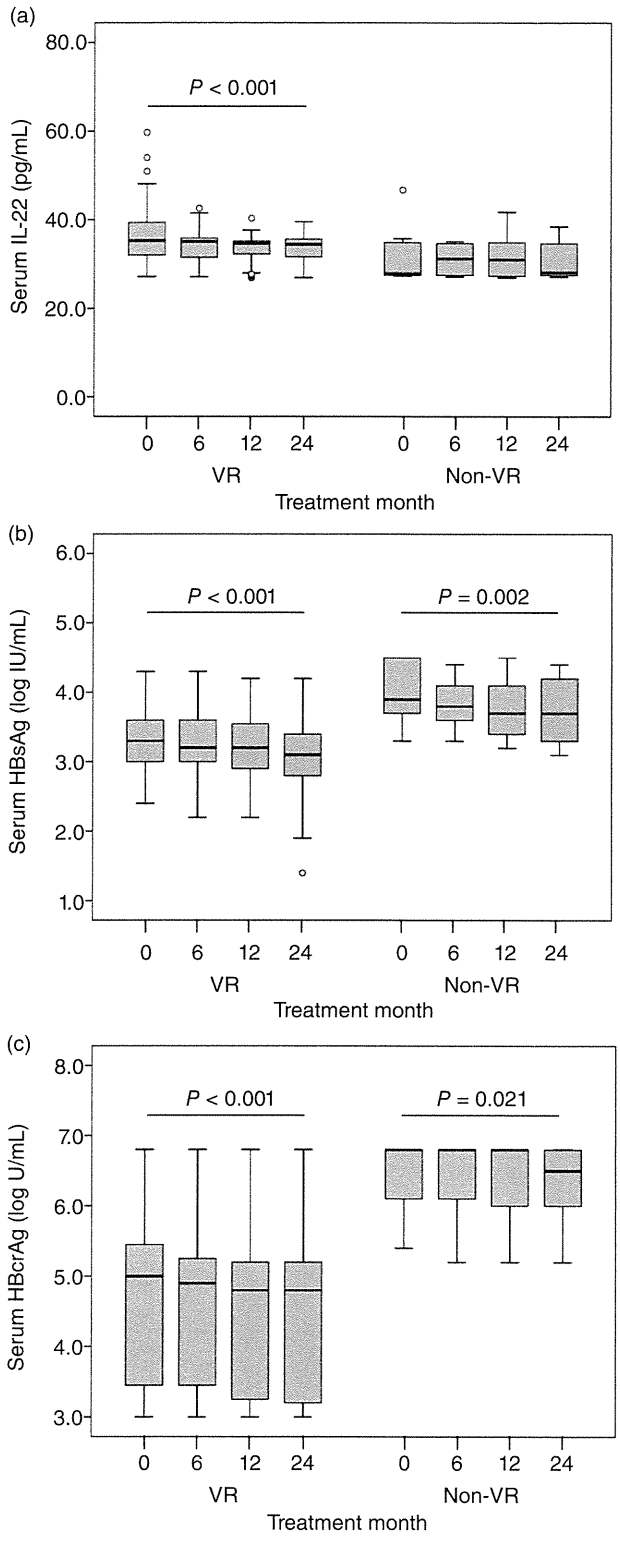

Figure 1 Comparison of serum (a) IL-22, (b) HBsAg and (c) HBcrAg levels during entecavir therapy in the VR ($n = 39$) and non-VR ($n = 9$) groups. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The harsh marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. IL, interleukin; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis core-related antigen; VR, virological response.

under the curve (AUC) values for each parameter are listed in Table 5. The AUC values were consistently high and ranged between 0.731 (IL-22) and 0.858 (HBcrAg).

Several factors found in association with a VR to ETV therapy were evaluated for their independence by multivariate analysis. We determined that IL-22 of 27.8 pg/mL or more (hazard ratio [HR] = 13.67 [95% confidence interval [CI] = 1.05–178.11], $P = 0.046$) and HBcrAg of 5.7 log U/mL or less (HR = 10.88 [95% CI = 1.02–115.44], $P = 0.048$) were independent factors related to a VR. HBsAg did not have a significant independent association in this study ($P = 0.071$).

Serum cytokine and chemokine changes during treatment

Longitudinal analysis of IL-22, HBsAg and HBcrAg levels was carried out at 6, 12 and 24 months after the initiation of therapy and showed significant gradual reductions in IL-22 ($P < 0.001$, Friedman test), HBsAg ($P < 0.001$) and HBcrAg ($P < 0.001$) in samples collected from patients who achieved a VR (Fig. 1). We noted a higher median serum IL-22 concentration at month 6 in the VR group than in the non-VR group ($P = 0.012$), and there were significant differences at each time point for HBsAg (6 months, $P = 0.002$; 12 months, $P = 0.006$; and 24 months, $P = 0.004$) and HBcrAg (6 months, $P < 0.001$; 12 months, $P < 0.001$; and 24 months, $P < 0.001$) between responders and non-responders.

DISCUSSION

IN THE PRESENT study, we measured the levels of six cytokines and five chemokines in patients with chronic hepatitis B and analyzed their association with ETV therapy outcome using a bead-array multiplex immunoassay system. Four of our observations are noteworthy and require further comment. First, serum IL-6, CCL2, CXCL9 and CXCL10 concentrations were

© 2013 The Japan Society of Hepatology

Table 4 Correlation between cytokines, chemokines and clinical parameters

		IL-2	IL-6	IL-10	IL-12	IL-21	IL-22	CCL2	CCL3	CXCL9	CXCL10	CXCL11
HBV DNA	*r*	0.08	0.01	0.10	0.06	0.08	0.17	−0.13	0.01	−0.13	−0.10	0.20
	P	0.61	0.97	0.51	0.69	0.61	0.25	0.39	0.95	0.39	0.50	0.18
HBsAg	*r*	−0.99	−0.35	−0.14	0.22	−0.08	−0.05	−2.5	0.02	−0.78	−0.61	−0.32
	P	0.51	**0.015**	0.35	0.14	0.61	0.74	0.09	0.89	**<0.001**	**<0.001**	**0.025**
HBcrAg	*r*	0.04	0.05	−0.16	0.24	0.18	0.14	−0.13	0.14	−0.14	−0.15	0.11
	P	0.79	0.76	0.29	0.11	0.21	0.35	0.40	0.33	0.36	0.31	0.45
Albumin	*r*	0.17	0.02	0.17	−0.02	0.05	−0.02	0.12	0.08	0.13	−0.09	0.02
	P	0.25	0.91	0.24	0.89	0.75	0.88	0.40	0.60	0.39	0.53	0.91
AST	*r*	0.05	0.40	0.11	−0.11	−0.03	0.14	0.13	−0.07	0.78	0.75	0.36
	P	0.72	**0.004**	0.45	0.47	0.83	0.33	0.39	0.66	**<0.001**	**<0.001**	**0.013**
ALT	*r*	0.02	0.42	0.12	−0.11	−0.06	0.16	0.10	−0.08	0.69	0.71	0.46
	P	0.91	**0.003**	0.40	0.44	0.70	0.28	0.52	0.57	**<0.001**	**<0.001**	**0.001**
Bilirubin	*r*	−0.03	0.36	0.07	0.08	−0.03	0.13	0.27	−0.12	0.33	0.65	0.35
	P	0.83	**0.012**	0.64	0.58	0.84	0.38	0.07	0.42	**0.023**	**<0.001**	**0.015**
Platelet	*r*	0.08	0.12	0.15	−0.09	0.13	0.25	−0.05	0.19	0.31	0.04	0.13
	P	0.57	0.42	0.33	0.55	0.38	0.09	0.74	0.20	**0.033**	0.82	0.39

Bolded figures indicate statistical significance.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IL, interleukin; *r*, Spearman's rank correlation.

higher in patients with chronic hepatitis B than in healthy subjects. Second, serum IL-22 concentration before treatment was significantly higher in patients achieving a VR to ETV therapy. In contrast, responders had lower serum levels of HBsAg and HBcrAg at baseline. Third, IL-22, HBsAg and HBcrAg decreased during treatment and remained low in patients with a VR. Fourth, serum IL-6, CXCL9, CXCL10 and CXCL11 were positively correlated with serum values of AST, ALT and bilirubin, but were negatively correlated with HBsAg.

Interleukin-6 is a well-recognized multifunctional cytokine that may reflect more active hepatic necroinflammation and be associated with chronic HBV infection severity. As in previous studies,[18,21] serum IL-6 was significantly higher in the HBV-infected group than in healthy controls and was positively correlated with such clinical parameters as transaminases and bilirubin. Hence, our data support that IL-6 is strongly associated with the severity of liver diseases.

CXCL9, CXCL10 and CXCL11 appear to be particularly important in chronic HCV infection by promoting the development of intrahepatic inflammation that leads to fibrogenesis.[22,23] These chemokines are also significantly elevated in patients with necroinflammatory activity of acute and chronic hepatitis C.[24,25] In our study, serum CXCL9 and CXCL10 were higher in patients with chronic HBV infection than in healthy individuals, which was in agreement with a previous

Table 5 Optimal cut-off value, sensitivity, specificity, AUC, and predictive value of serum IL-22, HBsAg and HBcrAg at baseline of treatment in 48 patients with chronic hepatitis B

	Cut-off value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	AUC (95% CI)	PPV (%)	NPV (%)
IL-22	27.8 pg/mL	56 (21–86)	90 (76–97)	0.731 (0.533–0.929)	90	56
HBsAg	3.6 log IU/mL	78 (40–97)	77 (61–89)	0.838 (0.704–0.971)	44	94
HBcrAg	5.7 log U/mL	89 (52–100)	82 (67–93)	0.858 (0.754–0.962)	53	97

All AUC values were significantly higher than a 0.50 non-predictive value ($P < 0.01$ for all comparisons). Cut-off values were determined by constructing receiver–operator curves.
AUC, area under the curve; CI, confidence interval; HBcrAg, hepatitis core-related antigen; HBsAg, hepatitis B surface antigen; IL, interleukin; NPV, negative predictive value; PPV, positive predictive value.

© 2013 The Japan Society of Hepatology

report.[12] Moreover, the serum CXCR3-associated chemokines CXCL9, CXCL10 and CXCL11 were all well correlated with serum values of AST, ALT and bilirubin. Because we observed a significant correlation between these chemokines and IL-6, our findings suggest that CXCR3-associated chemokines may too contribute to necroinflammatory activity in chronic HBV infection. However, there were insufficient histological data in our study to assess whether IL-6 and CXCR3-associated chemokines were correlated with degree of fibrosis, in addition to a lack of biochemical evidence of inflammation. We furthermore showed a striking negative association between HBsAg concentration and levels of IL-6 and CXCR3-associated chemokines. As HBsAg was also negatively correlated with transaminases and bilirubin, this HBsAg decline may be linked to increased immunological activity.

Interestingly, this study demonstrated a beneficial role of IL-22 in achieving a VR during ETV therapy. IL-22 is an IL-10 family cytokine that is important for the modulation of tissue responses during inflammation and is expressed by many types of lymphocytes of both the innate and adaptive immune systems, most notably T-helper 17 cells, γδ T cells, natural killer cells and lymphoid tissue inducer-like cells. The IL-22 receptor is highly expressed on hepatocytes.[26,27] At present, several studies support a protective role of IL-22 in the prevention of hepatocellular damage, although there is evidence indicating dual protective and pathogenic roles for this cytokine in the liver.[17,28–30] Some groups have examined the association between IL-22 and liver fibrosis in humans and mice.[31,32] In one report, tumor-infiltrating lymphocytes in HCC exhibited elevated IL-22 expression, and these IL-22$^+$ lymphocytes promoted tumor growth and metastasis in mice.[33] Although human patients with chronic hepatitis B show increased percentages of T-helper 17 cells in the peripheral blood and liver and an increased concentration of IL-22 in the serum,[14,34] there have been no reports on treatment outcome in patients with chronic HBV infection during ETV therapy. In our study, IL-22 levels decreased over time in both the VR and non-VR groups, but they were consistently higher in the VR group. This difference in IL-22 levels between the two groups further supports the possibility that IL-22 may be important for the activation of immune cells that contribute to viral control. When stratified by HBe positivity, although IL-22 was still significantly associated with a VR, the number of patients was only 20 in this study. Further research is needed to clarify the association between IL-22 and treatment response.

Lastly, we uncovered that lower baseline serum HBsAg and HBcrAg levels were associated with a VR. HBcrAg assays measure serum levels of HB core, e and 22-kDa precore antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these three denatured antigens.[35] Because this assay measures all antigens transcribed from the precore/core gene, it is regarded as core related.[36] The AUC values for baseline HBsAg and HBcrAg levels were high at 0.838 and 0.858, respectively. Several studies have shown that HBsAg is useful for the management of ETV therapy,[37,38] whereby an HBsAg decline is most profound in patients losing HBeAg detectability during treatment.[39] HBeAg positivity was also significantly associated with treatment outcome in the present study. However, because HBcrAg, but not HBsAg or HBeAg, was an independent factor related to a VR in multivariate analysis, our results indicated that serum HBcrAg quantitation may offer clinicians a new tool in predicting treatment outcome in HBV infection. Further investigation of large cohorts must be done to validate the significance of our findings.

With a VR at 12 months established as a parameter, 38 patients (79%) achieved this event. Serum IL-22, HBsAg and HBcrAg levels were all still significantly associated with a VR at 12 months. AUC values were as high as between 0.737 (IL-22) and 0.878 (HBcrAg). Furthermore, ALT normalization was achieved in 40 (83%) and 42 (88%) patients at 12 and 24 months, respectively. Although lower median pretreatment levels of HBsAg and HBcrAg were significantly associated with ALT normalization, there was no such statistically significant relation for IL-22 (data not shown).

In summary, a cytokine (IL-6) and several chemokines (CCL2, CXCL9 and CXCL10) were seen to be elevated in patients with chronic hepatitis B. Our results indicate that serum IL-6 and CXCR3-associated chemokines are correlated with liver injury, serum IL-22 is a useful biomarker for predicting a VR to ETV therapy, and a lower level of serum HBcrAg is related to a favorable response to antiviral therapy.

ACKNOWLEDGMENTS

THIS RESEARCH WAS supported in part by a research grant from the Ministry of Health, Labor, and Welfare of Japan. The authors thank Yuki Akahane, Asami Yamazaki and Toyo Amaki for their technical assistance, and Trevor Ralph for his English editorial assistance.

© 2013 The Japan Society of Hepatology

REFERENCES

1 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; **337:** 1733–45.
2 Umemura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* 2009; 44 (Suppl 19): 102–7.
3 Chen CJ, Yang HI, Su J *et al.* Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295:** 65–73.
4 Iloeje UH, Yang HI, Su J, Jen CL, You SL, Chen CJ. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology* 2006; **130:** 678–86.
5 Dienstag JL, Goldin RD, Heathcote EJ *et al.* Histological outcome during long-term lamivudine therapy. *Gastroenterology* 2003; **124:** 105–17.
6 Liaw YF, Sung JJ, Chow WC *et al.* Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; **351:** 1521–31.
7 Hosaka T, Suzuki F, Kobayashi M *et al.* Long-term entecavir treatment reduces hepatocellular carcinoma incidence in patients with hepatitis B virus infection. *Hepatology* 2013; **58:** 98–107.
8 Yoneda S, Umemura T, Katsuyama Y *et al.* Association of serum cytokine levels with treatment response to pegylated interferon and ribavirin therapy in genotype 1 chronic hepatitis C patients. *J Infect Dis* 2011; **203:** 1087–95.
9 Yoneda S, Umemura T, Joshita S *et al.* Serum chemokine levels are associated with the outcome of pegylated interferon and ribavirin therapy in patients with chronic hepatitis C. *Hepatol Res* 2011; **41:** 587–93.
10 Umemura T, Joshita S, Yoneda S *et al.* Serum interleukin (IL)-10 and IL-12 levels and IL28B gene polymorphisms: pretreatment prediction of treatment failure in chronic hepatitis C. *Antivir Ther* 2011; **16:** 1073–80.
11 Wu JF, Wu TC, Chen CH *et al.* Serum levels of interleukin-10 and interleukin-12 predict early, spontaneous hepatitis B virus e antigen seroconversion. *Gastroenterology* 2010; **138:** 165–72 e–13.
12 Tan AT, Koh S, Goh W *et al.* A longitudinal analysis of innate and adaptive immune profile during hepatic flares in chronic hepatitis B. *J Hepatol* 2010; **52:** 330–9.
13 Publicover J, Goodsell A, Nishimura S *et al.* IL-21 is pivotal in determining age-dependent effectiveness of immune responses in a mouse model of human hepatitis B. *J Clin Invest* 2011; **121:** 1154–62.
14 Zhang Y, Cobleigh MA, Lian JQ *et al.* A proinflammatory role for interleukin-22 in the immune response to hepatitis B virus. *Gastroenterology* 2011; **141:** 1897–906.
15 Ma SW, Huang X, Li YY *et al.* High serum IL-21 levels after 12 weeks of antiviral therapy predict HBeAg seroconversion in chronic hepatitis B. *J Hepatol* 2012; **56:** 775–81.
16 Tacke F, Zimmermann HW, Berres ML, Trautwein C, Wasmuth HE. Serum chemokine receptor CXCR3 ligands are associated with progression, organ dysfunction and complications of chronic liver diseases. *Liver Int* 2011; **31:** 840–9.
17 Feng D, Kong X, Weng H *et al.* Interleukin-22 promotes proliferation of liver stem/progenitor cells in mice and patients with chronic hepatitis B virus infection. *Gastroenterology* 2012; **143:** 188–98 e7.
18 Pan CJ, Wu HL, Kuo SF *et al.* Serum interleukin 6 level correlates with outcomes of acute exacerbation of chronic hepatitis B. *Hepatol Int* 2012; **6:** 591–7.
19 Umemura T, Tanaka E, Kiyosawa K, Kumada H. Mortality secondary to fulminant hepatic failure in patients with prior resolution of hepatitis B virus infection in Japan. *Clin Infect Dis* 2008; **47:** e52–6.
20 Umemura T, Zen Y, Hamano H, Kawa S, Nakanuma Y, Kiyosawa K. Immunoglobin G4-hepatopathy: association of immunoglobin G4-bearing plasma cells in liver with autoimmune pancreatitis. *Hepatology* 2007; **46:** 463–71.
21 Kao JT, Lai HC, Tsai SM *et al.* Rather than interleukin-27, interleukin-6 expresses positive correlation with liver severity in naive hepatitis B infection patients. *Liver Int* 2012; **32:** 928–36.
22 Zeremski M, Petrovic LM, Talal AH. The role of chemokines as inflammatory mediators in chronic hepatitis C virus infection. *J Viral Hepat* 2007; **14:** 675–87.
23 Heydtmann M, Adams DH. Chemokines in the immunopathogenesis of hepatitis C infection. *Hepatology* 2009; **49:** 676–88.
24 Zeremski M, Petrovic LM, Chiriboga L *et al.* Intrahepatic levels of CXCR3-associated chemokines correlate with liver inflammation and fibrosis in chronic hepatitis C. *Hepatology* 2008; **48:** 1440–50.
25 Zeremski M, Hooker G, Shu MA *et al.* Induction of CXCR3- and CCR5-associated chemokines during acute hepatitis C virus infection. *J Hepatol* 2011; **55:** 545–53.
26 Colonna M. Interleukin-22-producing natural killer cells and lymphoid tissue inducer-like cells in mucosal immunity. *Immunity* 2009; **31:** 15–23.
27 Zenewicz LA, Flavell RA. Recent advances in IL-22 biology. *Int Immunol* 2011; **23:** 159–63.
28 Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Karow M, Flavell RA. Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. *Immunity* 2007; **27:** 647–59.
29 Ki SH, Park O, Zheng M *et al.* Interleukin-22 treatment ameliorates alcoholic liver injury in a murine model of chronic-binge ethanol feeding: role of signal transducer and activator of transcription 3. *Hepatology* 2010; **52:** 1291–300.
30 Park O, Wang H, Weng H *et al.* In vivo consequences of liver-specific interleukin-22 expression in mice: implications for human liver disease progression. *Hepatology* 2011; **54:** 252–61.
31 Kong X, Feng D, Wang H *et al.* Interleukin-22 induces hepatic stellate cell senescence and restricts liver fibrosis in mice. *Hepatology* 2012; **56:** 1150–9.

© 2013 The Japan Society of Hepatology

32 Meng F, Wang K, Aoyama T *et al.* Interleukin-17 signaling in inflammatory, Kupffer cells, and hepatic stellate cells exacerbates liver fibrosis in mice. *Gastroenterology* 2012; **143:** 765–76 e1-3.
33 Jiang R, Tan Z, Deng L *et al.* Interleukin-22 promotes human hepatocellular carcinoma by activation of STAT3. *Hepatology* 2011; **54:** 900–9.
34 Zhang JY, Zhang Z, Lin F *et al.* Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B. *Hepatology* 2010; **51:** 81–91.
35 Kimura T, Ohno N, Terada N *et al.* 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* 2005; **280:** 21713–9.
36 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40:** 439–45.
37 Lee JM, Ahn SH, Kim HS *et al.* Quantitative hepatitis B surface antigen and hepatitis B e antigen titers in prediction of treatment response to entecavir. *Hepatology* 2011; **53:** 1486–93.
38 Liaw YF. Clinical utility of hepatitis B surface antigen quantitation in patients with chronic hepatitis B: a review. *Hepatology* 2011; **54:** E1–9.
39 Reijnders JG, Rijckborst V, Sonneveld MJ *et al.* Kinetics of hepatitis B surface antigen differ between treatment with peginterferon and entecavir. *J Hepatol* 2011; **54:** 449–54.

SUPPORTING INFORMATION

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

Table S1 Demographic, clinical characteristics, and serum cytokines and chemokines in patients with hepatitis B e-antigen (HBeAg) positive and hepatitis B e-antigen (HBeAg) negative patients.

© 2013 The Japan Society of Hepatology

Hepatology Research

Hepatology Research 2014 doi: 10.1111/hepr.12434

Original Article

Synthetic lethal interaction of combined CD26 and Bcl-xL inhibition is a powerful anticancer therapy against hepatocellular carcinoma

Tsukasa Kawaguchi,[1] Takahiro Kodama,[1] Hayato Hikita,[1] Yuki Makino,[1] Yoshinobu Saito,[1] Satoshi Tanaka,[1] Satoshi Shimizu,[1] Ryotaro Sakamori,[1] Takuya Miyagi,[1] Hiroshi Wada,[2] Hiroaki Nagano,[2] Naoki Hiramatsu,[1] Tomohide Tatsumi[1] and Tetsuo Takehara[1]

Departments of [1]Gastroenterology and Hepatology and [2]Gastroenterological Surgery, Osaka University Graduate School of Medicine, Suita, Japan

Aim: CD26 is a membrane glycoprotein that has multiple functions, including dipeptidyl peptidase IV activity. CD26 expression varies in different tumor types, and its role in tumor growth in hepatocellular carcinoma (HCC) remains unclear.

Methods: CD26 expression levels were examined in resected HCC and surrounding non-cancerous lesions. The effect of CD26 knockdown on the cellular proliferation of HepG2 or Huh7 cells, both of which highly express CD26, was studied *in vitro*.

Results: CD26 mRNA expression levels were significantly increased in HCC compared with their surrounding non-cancerous lesions. We confirmed that various HCC cell lines, especially HepG2 and Huh7 cells, showed high expression levels of CD26. siRNA-mediated knockdown of CD26 suppressed hepatoma cell growth *in vitro*. CD26 knockdown induced cell cycle arrest through the upregulation of Cip/Kip family proteins, p21 in HepG2 cells and p27 in Huh7 cells. CD26 knockdown did not affect apoptosis, but it increased expressions of the pro-apoptotic proteins Bim and Bak and the anti-apoptotic protein Bcl-xL, suggesting an addiction of CD26 knockdown cells to Bcl-xL for survival. We thus treated CD26 knockdown cells with ABT-737, a Bcl-xL/-2/-w inhibitor, and observed that the synthetic lethal interaction of combined Bcl-xL and CD26 inhibition induced significant apoptosis and impaired cellular viability.

Conclusion: CD26 mRNA was overexpressed in HCC, and its inhibition suppressed cellular proliferation through cell cycle arrest. The combined use of CD26 knockdown with a Bcl-xL inhibitor further elicited substantial apoptosis and therefore may serve as a powerful anticancer combination therapy against HCC.

Key words: ABT-737, apoptosis, CD26, cell cycle, hepatocellular carcinoma

INTRODUCTION

CD26 IS A membrane glycoprotein widely expressed in various tissues, such as T lymphocytes and epithelial and endothelial cells.[1,2] The CD26 molecule consists of a cytoplasmic domain, transmembrane domain and extracellular domain, which contains dipeptidyl peptidase-4 (DPPIV). Currently, DPPIV activity is one of the most well-known functions of CD26 because DPPIV degrades glucagon-like peptide-1 and many DPPIV inhibitors are used as drugs against type 2 diabetes.[3] However, in addition to DPPIV activity, CD26 has other functions, such as a receptor, co-stimulatory protein and adhesion molecule.[4] CD26 expression levels are altered in various types of cancers. CD26 overexpression is observed in prostate cancer,[5] brain glioma,[6] thyroid carcinoma[7] and malignant mesothelioma.[8] In contrast, CD26 is downregulated in various cancers, including ovarian cancer[9] and melanoma.[10] The role of CD26 in cancer biology also varies and appears to be tumor type dependent.[2] Although CD26

Correspondence: Dr Tetsuo Takehara, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Email: takehara@gh.med.osaka-u.ac.jp

Conflict of interest: The authors declare that no conflicts of interest exist.

Financial support: This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to T. Takehara) and a Grant-in-Aid for Research on Hepatitis from the Ministry of Health, Labor and Welfare of Japan.

Received 27 April 2014; revision 3 October 2014; accepted 5 October 2014.

© 2014 The Japan Society of Hepatology

expression is localized to the bile canalicular plasma membrane of the normal liver, its distribution pattern is altered in hepatocellular carcinoma (HCC). In addition, some HCC cases display an aberrant increase in DPPIV activity.[11] However, the role of CD26 in liver carcinogenesis remains unclear.

Hepatocellular carcinoma is the third leading cause of cancer mortality worldwide. However, few therapeutic options against advanced HCC exist, especially for patients with metastasis outside the liver. Sorafenib is the only US Food and Drug Administration-approved molecularly targeted drug against HCC that has demonstrated survival prolongation in clinical trials.[12,13] To date, various types of molecularly targeted drugs, including vascular endothelial growth factor, vascular endothelial growth factor receptor, epidermal growth factor receptor, mammalian target of rapamycin, DR5 and XIAP, were investigated but none of them prolonged survival.[14] In this aspect, new therapeutic targets are needed to conquer HCC.

In this study, we found that CD26 mRNA levels were frequently increased in HCC and that their levels were positively correlated with tumor size. CD26 inhibition decreased hepatoma cell growth through the induction of cell cycle arrest but not apoptosis. Although CD26 inhibition increased the expression of pro-apoptotic proteins, their pro-apoptotic effect was not exerted due to the counteracting increase in the anti-apoptotic protein Bcl-xL. The combined inhibition of CD26 and Bcl-xL caused a synthetic lethal pro-apoptotic effect in hepatoma cells. This is the first report to reveal the therapeutic potential of CD26 inhibition in HCC, and our current results propose a novel potent combination therapy against HCC.

METHODS

Human samples

HEPATOCELLULAR CARCINOMA SAMPLES and surrounding non-cancerous liver samples were obtained from 71 patients undergoing surgical resection for HCC at Osaka University Hospital. The average patient age was 62.7 ± 10.7 years old, and 56 patients were male. Among the 71 patients, 17 were positive for hepatitis B surface antigen (HBsAg) and negative for hepatitis C virus (HCV) antibody, 33 were negative for HBsAg and positive for HCV antibody, and three were positive for both. The average maximum diameter of HCC was 53.0 ± 37.8 mm. For immunohistochemistry using anti-CD26 antibody (Novus Biologicals, Littleton, CO, USA), formaldehyde-fixed HCC were obtained from 12 patients undergoing surgical resection at Osaka University Hospital. Detection of immunolabeled proteins was performed using an avidin–biotin complex of Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Written informed consent was obtained from all patients according to a protocol approved by the Institutional Research Board of Osaka University Hospital.

Real-time reverse transcription polymerase chain reaction (RT–PCR)

Total RNA isolated from liver tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was reverse transcribed and subjected to real-time RT–PCR as previously described.[15] The mRNA expression levels of the following genes were quantified using TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA): human CD26 (assay ID: Hs00175210_m1), human p21 (assay ID: Hs00355782_m1), human β-actin (assay ID: Hs99999903_m1) and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; assay ID: Hs02758991_g1). The transcript levels are presented as fold change relative to GAPDH levels unless otherwise indicated.

Western blot analysis

Liver tissue was lysed with a lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate [SDS], protease inhibitor cocktail [Nacalai Tesque, Kyoto, Japan], phosphatase inhibitor cocktail [Nacalai Tesque], phosphate-buffered saline, pH 7.4). Equal amounts of protein were electrophoretically separated using SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membrane. For immunodetection, the following antibodies were used: anti-CD26 (R&D Systems, Minneapolis, MN, USA), anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA), anti-p15 (Cell Signaling Technology), anti-p16 (Becton Dickinson, San Jose, CA, USA), anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p27 (Cell Signaling Technology), anti-Bak (Millipore, Billerica, MA, USA), anti-Bax (Cell Signaling Technology), anti-Bim (Cell Signaling Technology), anti-Bid (Cell Signaling Technology), anti-Mcl-1 (Cell Signaling Technology), anti-Bcl-xL (Santa Cruz Biotechnology) and cleaved caspase-3 (Cell Signaling Technology). Detection of immunolabeled proteins was performed using a chemiluminescent substrate (Thermo Fisher Scientific). Protein expression levels were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized by expression levels of GAPDH.

© 2014 The Japan Society of Hepatology

Cell cultures

Cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Sigma-Aldrich, St Louis, MO, USA). ABT-737, which inhibits Bcl-xL, Bcl-2 and Bcl-w, was purchased from Selleckchem (Houston, TX, USA) and used to treat Huh7 cells for 24 h as described previously.[16] Sitagliptin and vildagliptin were purchased from Viovision (Milpitas, CA, USA) and Santa Cruz Biotechnology, respectively. Measurements of caspase-3 and -7 activity and determination of cell viability by WST-1 assay were also described previously.[17] Lactate dehydrogenase (LDH) activity was measured by LDH-Cytotoxic Test (Wako, Osaka, Japan) according to the manufacturer's instructions. In some experiments, cells were transfected Silencer Select siRNA (Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's protocol.

Flow cytometry analysis

For the detection of surface CD26, cells were incubated with antigen-presenting cell-conjugated human anti-CD26 antibody (Miltenyi Biotec, Auburn, CA, USA) and then subjected to flow cytometric analysis. Flow cytometric analysis was performed using a FACS Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

To detect apoptotic cells, the cells were suspended in annexin V binding buffer (Becton Dickinson). Next, the cells were stained with annexin V and propidium iodide (PI; Becton Dickinson) and subjected to flow cytometric analysis. Annexin V^+ PI^- cells were regarded as apoptotic. Cell cycle assay was examined by CycleTest (Becton Dickinson) according to the manufacturer's protocol.

Statistics

Data are expressed as the mean ± standard deviation. Statistical analyses were performed using Student's unpaired *t*-test unless otherwise indicated. $P < 0.05$ was considered statistically significant.

RESULTS

CD26 mRNA levels are increased in HCC

WE FIRST EXAMINED the levels of CD26 mRNA in tumor tissue and surrounding non-cancerous lesions in HCC patients who underwent surgical resection. CD26 mRNA levels in tumor tissues were significantly increased compared with their adjacent non-cancerous lesions (Fig. 1a). To examine the significance of CD26 upregulation in HCC, tumor specimens were divided into two groups based on CD26 mRNA expression levels, and several clinical parameters were compared between the two groups (Table S1). Tumor size in the high CD26 mRNA expression group was signifi-

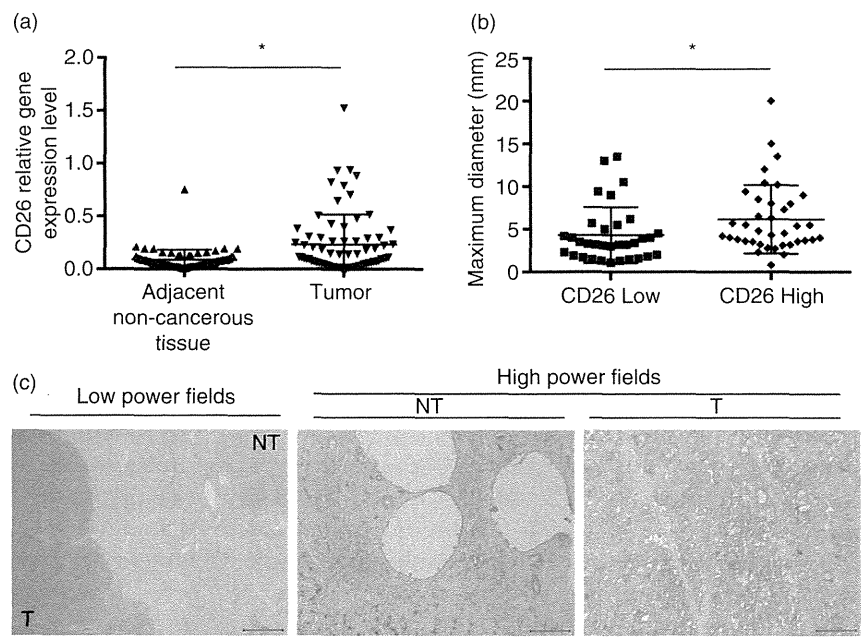

Figure 1 CD26 expression is frequently upregulated in hepatocellular carcinomas (HCC) and positively correlated with tumor size. (a) CD26 mRNA levels were examined in HCC and adjacent non-cancerous liver tissues. Relative CD26 mRNA levels normalized to β-actin mRNA expression (*$P < 0.05$ by Student's paired *t*-test). (b) Maximum diameter of HCC (*$P < 0.05$). HCC were divided into two groups based on the CD26 mRNA levels: CD26 high group and CD26 low group. (c) CD26 was labeled in the paraffin-embedded liver sections with HCC. Representative pictures of stained a section are shown. T and NT stand for tumor and non-tumor, respectively.

© 2014 The Japan Society of Hepatology

cantly larger than the low CD26 mRNA expression group (Fig. 1b), suggesting that CD26 overexpression may be involved in HCC cell growth. To evaluate the protein expression of CD26 in HCC, we stained 12 sections of formaldehyde-fixed HCC with anti-CD26 antibody. All examined HCC were stained with anti-CD26 antibody to varying degrees (Fig. 1c).

CD26 inhibition suppresses the growth of hepatoma cell lines

Then, we evaluated CD26 expression levels in a variety of hepatoma cell lines (Hep3B, HepG2, HLE, Huh7 and PLC/PRF/5). Based on the gene expression data from the Cancer Cell Line Encyclopedia (CCLE), HepG2 and Huh7 cells have the highest expression of CD26 among these hepatoma cell lines.[18] Consistent with these data, we used flow cytometry to confirm that CD26 was expressed on the surface of these two hepatoma cell lines (Fig. 2a). Based on these data, we selected them for further *in vitro* analysis and studied the role of CD26 in their cellular proliferation using negative control or two different CD26 siRNA oligos. The transfection of each CD26 siRNA oligo efficiently reduced CD26 expression at the mRNA and protein levels in both HepG2 (Fig. 2b,c) and Huh7 cells (Fig. 2d,e). Upon CD26 knockdown, cellular proliferation, as assessed by WST-1 assay, was significantly suppressed in both HepG2 (Fig. 2f) and Huh7 cells (Fig. 2g), indicating that CD26 was required for hepatoma cell growth and therefore can serve as a therapeutic target. To investigate whether decreased DPPIV activity is responsible for the observed anticancer effects of CD26 inhibition, we treated hepatoma cells with DPPIV inhibitors, sitagliptin or vildagliptin, instead of CD26 knockdown. However, inhibition of DPPIV activity failed to suppress hepatoma cell growth (Fig. 2h).

CD26 knockdown induces G0/G1 cell cycle arrest through the upregulation of Cip/Kip family proteins

To elucidate how CD26 inhibition impairs hepatoma cell growth, we studied the effect of CD26 knockdown on the cell cycle. Flow cytometric analysis revealed that siRNA-mediated CD26 knockdown in HepG2 cells decreased the proportion of cells in the S and G2/M phase and slightly increased the proportion of cells in the G0/G1 phase (Fig. 3a), suggesting that CD26 suppression causes cell cycle arrest at the G0/G1 phase. To further clarify which stage of cell cycle CD26 inhibition affects, HepG2 cells were treated with nocodazole, which arrests mitotic cells at the G2/M phase, upon transfection with the negative control or CD26 siRNA oligos. While nocodazole treatment dramatically increased the number of G2/M phase cells in control siRNA-transfected cells (Fig. 3a), this effect was greatly attenuated in CD26 siRNA-transfected cells, which maintained an increased number of G0/G1 phase cells (Fig. 3a). Similar observations were obtained using CD26 siRNA-transfected Huh7 cells treated with nocodazole (Fig. 3b). Taken together, these findings indicated that CD26 inhibition induced cell cycle arrest at the G0/G1 phase, leading to the suppression of cell growth. To address the mechanism of CD26 inhibition-mediated cell cycle arrest, we examined the change in expression levels of Ink4 family proteins, p15 and p16, and Cip/Kip family proteins, p21 and p27, upon CD26 knockdown because these proteins negatively control the G1/S checkpoint. Two different siRNA oligos targeting the CD26 gene individually increased p21 expression in HepG2 cells (Fig. 3c), suggesting its potential involvement in G0/G1 cell cycle arrest. To pursue this possibility, we co-transfected CD26 and p21 siRNA oligos and examined their effect on the cell cycle. We first confirmed that their co-transfection simultaneously reduced CD26 and p21 expression (Fig. 3d). Although CD26 knockdown caused cell cycle arrest at the G0/G1 phase, CD26 and p21 knockdown restored the S and G2/M cell populations to approximately the same level observed with p21 knockdown alone (Fig. 3e). Importantly, in accordance with this finding, cell growth impairment upon CD26 knockdown was completely rescued by additional p21 knockdown (Fig. 3e). On the other hand, our Western blot analysis showed that p21 protein expression was not detected in Huh7 cells but CD26 knockdown increased p27 expression (Fig. 3f). Furthermore, p27 knockdown rescued impaired cellular proliferation induced by CD26 inhibition (Fig. 3g). Collectively, CD26 knockdown induced cell cycle arrest at the G0/G1 phase through the upregulation of Cip/Kip family proteins.

Synthetic lethal interaction of combined CD26 and Bcl-xL inhibition induces substantial hepatoma cell apoptosis

Although CD26 appears to be a promising therapeutic target in HCC, CD26 inhibition may carry the potential risk of aiding in the transition of cancer cells from a chemo-sensitive replicative status to a chemo-resistant dormant status. To compensate for this potential adverse effect, we attempted to identify the "Achilles' heel" for hepatoma cells in the context of CD26 inhibition and discovered that CD26 inhibition upregulated

© 2014 The Japan Society of Hepatology