28. Okanoue T, Makiyama A, Nakayama M, Sumida Y, Mitsuyoshi H, Nakajima T, et al. A follow-up study to determine the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase. *J Hepatol.* 2005;**43**(4):599-605.
29. Uto H, Kurogi J, Takahama Y, Kusumoto K, Hayashi K, Ido A, et al. Alanine aminotransferase flare-up in hepatitis C virus carriers with persistently normal alanine aminotransferase levels in a hyperendemic area of Japan. *J Gastroenterol.* 2007;**42**(8):673-80.
30. Shiffman ML, Diago M, Tran A, Pockros P, Reindollar R, Prati D, et al. Chronic hepatitis C in patients with persistently normal alanine transaminase levels. *Clin Gastroenterol Hepatol.* 2006;**4**(5):645-52.
31. Fissell RB, Bragg-Gresham JL, Woods JD, Jadoul M, Gillespie B, Hedderwick SA, et al. Patterns of hepatitis C prevalence and seroconversion in hemodialysis units from three continents: the DOPPS. *Kidney Int.* 2004;**65**(6):2335-42.
32. Nishida C, Uto H, Oketani M, Tokunaga K, Nosaki T, Fukumoto M, et al. Clinical significance of alanine aminotransferase levels and the effect of ursodeoxycholic acid in hemodialysis patients with chronic hepatitis C. *J Gastroenterol.* 2010;**45**(3):326-34.
33. Contreras AM, Ruiz I, Polanco-Cruz G, Monteon FJ, Celis A, Vazquez G, et al. End-stage renal disease and hepatitis C infection: comparison of alanine aminotransferase levels and liver histology in patients with and without renal damage. *Ann Hepatol.* 2007;**6**(1):48-54.
34. Sherman KE, Rouster SD, Chung RT, Rajicic N. Hepatitis C Virus prevalence among patients infected with Human Immunodeficiency Virus: a cross-sectional analysis of the US adult AIDS Clinical Trials Group. *Clin Infect Dis.* 2002;**34**(6):831-7.
35. Martin-Carbonero L, de Ledinghen V, Moreno A, Maida I, Foucher J, Barreiro P, et al. Liver fibrosis in patients with chronic hepatitis C and persistently normal liver enzymes: influence of HIV infection. *J Viral Hepat.* 2009;**16**(11):790-5.
36. Kanto T, Inoue M, Miyatake H, Sato A, Sakakibara M, Yakushijin T, et al. Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to polarize T helper cells in chronic hepatitis C virus infection. *J Infect Dis.* 2004;**190**(11):1919-26.
37. Bain C, Fatmi A, Zoulim F, Zarski JP, Trepo C, Inchauspe G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology.* 2001;**120**(2):512-24.
38. Kanto T, Inoue M, Miyazaki M, Itose I, Miyatake H, Sakakibara M, et al. Impaired function of dendritic cells circulating in patients infected with hepatitis C virus who have persistently normal alanine aminotransferase levels. *Intervirology.* 2006;**49**(1-2):58-63.
39. Itose I, Kanto T, Kakita N, Takebe S, Inoue M, Higashitani K, et al. Enhanced ability of regulatory T cells in chronic hepatitis C patients with persistently normal alanine aminotransferase levels than those with active hepatitis. *J Viral Hepat.* 2009;**16**(12):844-52.
40. Kuzushita N, Hayashi N, Katayama K, Hiramatsu N, Yasumaru M, Murata H, et al. Increased frequency of HLA DR13 in hepatitis C virus carriers with persistently normal ALT levels. *J Med Virol.* 1996;**48**(1):1-7.
41. Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. *Ann Intern Med.* 1999;**131**(3):174-81.
42. Bravo AA, Sheth SG, Chopra S. Liver biopsy. *N Engl J Med.* 2001;**344**(7):495-500.
43. Regev A, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, et al. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. *Am J Gastroenterol.* 2002;**97**(10):2614-8.
44. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology.* 2003;**38**(2):518-26.
45. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology.* 2006;**43**(6):1317-25.
46. Forns X, Ampurdanes S, Llovet JM, Aponte J, Quinto L, Martinez-Bauer E, et al. Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. *Hepatology.* 2002;**36**(4 Pt 1):986-92.
47. Koda M, Matunaga Y, Kawakami M, Kishimoto Y, Suou T, Murawaki Y. FibroIndex, a practical index for predicting significant fibrosis in patients with chronic hepatitis C. *Hepatology.* 2007;**45**(2):297-306.
48. Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet.* 2001;**357**(9262):1069-75.
49. Kelleher TB, Mehta SH, Bhaskar R, Sulkowski M, Astemborski J, Thomas DL, et al. Prediction of hepatic fibrosis in HIV/HCV co-infected patients using serum fibrosis markers: the SHASTA index. *J Hepatol.* 2005;**43**(1):78-84.
50. Adams LA, Bulsara M, Rossi E, DeBoer B, Speers D, George J, et al. Hepascore: an accurate validated predictor of liver fibrosis in chronic hepatitis C infection. *Clin Chem.* 2005;**51**(10):1867-73.
51. Ziol M, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, et al. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology.* 2005;**41**(1):48-54.
52. Castera L, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology.* 2005;**128**(2):343-50.
53. Ganne-Carrie N, Ziol M, de Ledinghen V, Douvin C, Marcellin P, Castera L, et al. Accuracy of liver stiffness measurement for the diagnosis of cirrhosis in patients with chronic liver diseases. *Hepatology.* 2006;**44**(6):1511-7.
54. Nitta Y, Kawabe N, Hashimoto S, Harata M, Komura N, Kobayashi K, et al. Liver stiffness measured by transient elastography correlates with fibrosis area in liver biopsy in patients with chronic hepatitis C. *Hepatol Res.* 2009;**39**(7):675-84.
55. Lupsor M, Badea R, Stefanescu H, Sparchez Z, Branda H, Serban A, et al. Performance of a new elastographic method (ARFI technology) compared to unidimensional transient elastography in the noninvasive assessment of chronic hepatitis C. Preliminary results. *J Gastrointestin Liver Dis.* 2009;**18**(3):303-10.
56. Fierbinteanu-Braticevici C, Andronescu D, Usvat R, Cretoiu D, Baicus C, Marinoschi G. Acoustic radiation force imaging sonoelastography for noninvasive staging of liver fibrosis. *World J Gastroenterol.* 2009;**15**(44):5525-32.
57. Takahashi H, Ono N, Eguchi Y, Eguchi T, Kitajima Y, Kawaguchi Y, et al. Evaluation of acoustic radiation force impulse elastography for fibrosis staging of chronic liver disease: a pilot study. *Liver Int.* 2010;**30**(4):538-45.
58. Tanaka H, Tsukuma H, Yamano H, Oshima A, Shibata H. Prospective study on the risk of hepatocellular carcinoma among hepatitis C virus-positive blood donors focusing on demographic factors, alanine aminotransferase level at donation and interaction with hepatitis B virus. *Int J Cancer.* 2004;**112**(6):1075-80.
59. Kumada T, Toyoda H, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, et al. Incidence of hepatocellular carcinoma in hepatitis C carriers with normal alanine aminotransferase levels. *J Hepatol.* 2009;**50**(4):729-35.
60. Allison ME, Wreghitt T, Palmer CR, Alexander GJ. Evidence for a link between hepatitis C virus infection and diabetes mellitus in a cirrhotic population. *J Hepatol.* 1994;**21**(6):1135-9.
61. Hui JM, Sud A, Farrell GC, Bandara P, Byth K, Kench JG, et al. Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression [corrected]. *Gastroenterology.* 2003;**125**(6):1695-704.
62. Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Tsukamoto K, Kimura S, et al. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology.* 2004;**126**(3):840-8.
63. Lonardo A, Adinolfi LE, Loria P, Carulli N, Ruggiero G, Day CP. Steatosis and hepatitis C virus: mechanisms and significance for hepatic and extrahepatic disease. *Gastroenterology.* 2004;**126**(2):586-97.
64. Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol.* 1997;**78** (Pt 7):1527-31.

65. Castera L, Hezode C, Roudot-Thoraval F, Bastie A, Zafrani ES, Pawlotsky JM, et al. Worsening of steatosis is an independent factor of fibrosis progression in untreated patients with chronic hepatitis C and paired liver biopsies. *Gut.* 2003;**52**(2):288-92.
66. Rubbia-Brandt L, Quadri R, Abid K, Giostra E, Male PJ, Mentha G, et al. Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3. *J Hepatol.* 2000;**33**(1):106-15.
67. Romero-Gomez M, Del Mar Viloria M, Andrade RJ, Salmeron J, Diago M, Fernandez-Rodriguez CM, et al. Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. *Gastroenterology.* 2005;**128**(3):636-41.
68. Hiramatsu N, Inoue Y, Oze T, Kurashige N, Yakushijin T, Mochizuki K, et al. Efficacy of pegylated interferon plus ribavirin combination therapy for hepatitis C patients with normal ALT levels: a matched case-control study. *J Gastroenterol.* 2011;**46**(11):1335-43.
69. Puoti C, Barbarini G, Picardi A, Romano M, Pellicelli A, Barlattani A, et al. Rapid virological response as a predictor of sustained response in HCV-infected patients with persistently normal alanine aminotransferase levels: A multicenter study. *J Viral Hepat.* 2011;**18**(6):393-9.
70. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet.* 2009;**41**(10):1105-9.
71. O'Brien TR. Interferon-alfa, interferon-lambda and hepatitis C. *Nat Genet.* 2009;**41**(10):1048-50.
72. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology.* 2005;**48**(6):372-80.
73. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med.* 1996;**334**(2):77-81.
74. Hiramatsu N, Kurosaki M, Sakamoto N, Iwasaki M, Sakamoto M, Suzuki Y, et al. Pretreatment prediction of anemia progression by pegylated interferon alpha-2b plus ribavirin combination therapy in chronic hepatitis C infection: decision-tree analysis. *J Gastroenterol.* 2011;**46**(9):1111-9.
75. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, et al. Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. *Hepatology.* 2010;**52**(2):421-9.
76. Kurosaki M, Matsunaga K, Hirayama I, Tanaka T, Sato M, Yasui Y, et al. A predictive model of response to peginterferon ribavirin in chronic hepatitis C using classification and regression tree analysis. *Hepatol Res.* 2010;**40**(4):251-60.
77. Deuffic-Burban S, Babany G, Lonjon-Domanec I, Deltenre P, Canva-Delcambre V, Dharancy S, et al. Impact of pegylated interferon and ribavirin on morbidity and mortality in patients with chronic hepatitis C and normal aminotransferases in France. *Hepatology.* 2009;**50**(5):1351-9.

Hepatology Research

Hepatology Research 2012 doi: 10.1111/hepr.12032

Original Article

Serum RANTES level influences the response to pegylated interferon and ribavirin therapy in chronic hepatitis C

Kazuki Komase,[1] Shinya Maekawa,[1,2] Mika Miura,[1] Ryota Sueki,[1] Makoto Kadokura,[1] Hiroko Shindo,[1] Kuniaki Shindo,[1] Fumitake Amemiya,[1] Yasuhiro Nakayama,[1] Taisuke Inoue,[1,2] Minoru Sakamoto,[1] Atsuya Yamashita,[3] Kohji Moriishi[3] and Nobuyuki Enomoto[1]

[1]*First Department of Medicine, Faculty of Medicine,* [2]*Department of Advanced Medicine for Liver Diseases, Faculty of Medicine, and* [3]*Department of Microbiology, University of Yamanashi, Yamanashi, Japan*

Aim: Prediction of treatment responses to pegylated interferon (PEG IFN) plus ribavirin (RBV) therapy is uncertain for genotype 1b chronic hepatitis C.

Methods: In this study, 96 patients were investigated for the correlation between 36 pretreatment serum chemokine/cytokine levels and PEG IFN/RBV treatment efficacy by a sandwich enzyme-linked immunoassay (ELISA) and a bead array.

Results: First, chemokines/cytokines were measured semi-quantitatively by sandwich ELISA in 31 randomly-selected patients and the serum regulated on activation normal T-cell expressed and secreted (RANTES) level was found to be significantly higher in the sustained virological response (SVR) group than the non-SVR group ($P = 0.048$). Precise RANTES measurement in all 96 patients using a bead array confirmed this correlation ($P = 0.002$). However, the genetic RANTES haplotype was not significantly related to the serum level. The serum RANTES level was extracted by multivariate analysis (odds ratio = 4.09, 95% confidence interval = 1.02–16.5, $P = 0.048$) as an independent variable contributing to SVR.

Conclusion: The serum RANTES level is an important determinant influencing the virological response to PEG IFN/RBV therapy in chronic hepatitis C.

Key words: hepatitis C virus, pegylated interferon plus ribavirin therapy, RANTES

INTRODUCTION

HEPATITIS C VIRUS (HCV) is a major cause of chronic liver disease worldwide and persistent infection may lead to liver cirrhosis and hepatocellular carcinoma.[1] Therapy leading to HCV eradication is the only treatment with proven efficacy in decreasing the occurrence of hepatocellular carcinoma.[2] Recently, treatment with telaprevir, a non-structural (NS)3/4A protease inhibitor, combined with pegylated interferon (PEG IFN) and ribavirin (RBV), increased the rates of sustained viral response (SVR) up to 64–75%[3,4] compared to the SVR rate of approximately 50% for the previous PEG IFN/RBV therapy. However, it has become evident that genotype 1-infected patients with a null response to previous PEG IFN/RBV therapy have poor responses to PEG IFN/RBV/telaprevir,[5] with an SVR rate as low as approximately 30%, illustrating the difficulty in treating patients infected with genotype 1 HCV. Therefore, precise and accurate prediction of the viral response to PEG IFN/RBV therapy remains an important issue.

Treatment resistance is attributed to various factors associated with the virus and host. Viral factors, such as amino acid (a.a.) sequence variation in the core and NS5A regions, have been investigated extensively for their contribution to the outcome of IFN-based therapy,[6,7] including PEG IFN/RBV therapy. On the other hand, host factors such as African-American race, older age, being obese, the presence of cirrhosis and

Correspondence: Dr Shinya Maekawa, First Department of Medicine, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan. Email: maekawa@yamanashi.ac.jp

Conflict of interest: Shinya Maekawa and Taisuke Inoue belong to a donation-funded department that is funded by MSD (Tokyo, Japan). Nobuyuki Enomoto received research funding from MSD (Tokyo, Japan) and Roche (Tokyo, Japan).

Received 26 September 2012; revision 18 November 2012; accepted 26 November 2012.

steatosis, and insulin resistance have been reported to be associated with treatment resistance.[8-11] Especially, single nucleotide polymorphisms (SNP) near the interleukin (*IL*)-28B gene, including rs12979860 and rs8099917, have been reported to have a significant correlation with the response to IFN-based therapy.[12,13] However, even with inclusion of these factors, prediction of the treatment response in chronic HCV infection remains uncertain at present.

Chemokines are a group of small, exogenously secreted cytokines that modulate the migration of leukocytes to sites of tissue damage and inflammation in a variety of infectious and autoimmune diseases.[14] In chronic HCV infection, chemokines such as *RANTES* (regulated on activation normal T-cell expressed and secreted), macrophage inflammatory protein (*MIP*)-1α, *MIP-1β* and interferon-γ inducible protein 10 kDa (*IP-10*) are elevated and considered to play crucial roles in inflammatory processes and viral elimination, as well as the transition from innate to adaptive immunity.[14,15] Upregulation of several serum chemokines, such as eotaxin, *IP-10* and *RANTES* also has been reported in HCV infection, possibly reflecting hepatic inflammation.[16] Considering the roles of chemokines/cytokines in establishing chronic hepatitis, it is possible that these chemokines also affect the response to antiviral therapy, and actually several chemokines as interleukin (*IL*)-8, *IL-10*, *MIP-1β*, *RANTES* or *IP-10* have been investigated previously for their association with the treatment response.[16-20] However, the importance of those chemokines has not been established yet and, moreover, these studies did not characterize in detail these chemokines in association with other factors, including *IL-28B* influencing the response to therapy.

In this study, we explored extensively the association of 36 serum cytokines/chemokines and the treatment response, with detailed information of host and virus, to predict better the treatment response to PEG IFN and RBV therapy in genotype 1b HCV infection. Because the pretreatment serum *RANTES* level was found to be correlated significantly with the response, we analyzed further the association between the serum level of *RANTES* and the genomic SNP.

METHODS

Patients

NINETY-SIX CONSECUTIVE PATIENTS with genotype 1b HCV and receiving PEG IFN/RBV therapy between 2004 and 2010 at Yamanashi University Hospital were recruited retrospectively into the study. All patients received the standard therapy according to the treatment protocol of PEG IFN/RBV therapy for Japanese patients, established by a hepatitis study group of the Ministry of Health, Labor and Welfare, Japan (PEG IFN-α-2b 1.5 μg/kg bodyweight, once weekly s.c., and RBV 600–800 mg daily p.o. for 48 weeks).[21] All patients enrolled fulfilled the following criteria: (i) negative for hepatitis B surface antigen; (ii) no other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease or alcoholic liver disease; (iii) not co-infected with HIV; and (iv) a signed consent was obtained for the study protocol that had been approved by the Human Ethics Review Committee of Yamanashi University Hospital. The study was approved by the ethics committees of University of Yamanashi, and the study protocol conformed to the ethical guidelines of the 2008 Declaration of Helsinki.

Definition of treatment outcome

An SVR was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. Relapse was defined as reappearance of detectable HCV RNA levels following discontinuation of treatment. Null response was defined as less than 2 log decrease of the baseline HCV RNA levels after 12 weeks of treatment. Based on this definition, when patients were classified according to the achievement of SVR, patients with relapse or null response were classified as non-SVR.

Serum cytokine measurement

Sandwich enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected before initiation of treatment and were stored at −80°C until use. Semiquantitation of serum cytokines was performed using the Proteome Profiler Human Cytokine Array Kit Panel A (R&D Systems, Minneapolis, CA, USA) according to the manufacturer's instructions. The kit consists of a nitrocellulose membrane containing 36 different anti-cytokine antibodies (anti-C5a, anti-CD154, anti-G-CSF, anti-GM-CSF, anti-CXCL1, anti-CCL1, anti-sICAM-1, anti-IFN-γ, anti-IL-1α, anti-IL-1β, anti-IL-1ra, anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-6, anti-IL-8, anti-IL-10, anti-IL-12p70, anti-IL-13, anti-IL-16, anti-IL-17, anti-IL-17E, anti-IL-23, anti-IL-27, anti-IL-32α, anti-IP-10, anti-CXCL11, anti-CCL2, anti-MIF, anti-CCL3, anti-CCL4, anti-PAI-1, anti-RANTES, anti-CXCL12, anti-TNF-α, anti-sTREM-1), spotted in duplicate. Serum samples were diluted and mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture

was then incubated with the membrane. Any cytokine/detection antibody complex present was bound to the membrane by its cognate immobilized capture antibody. Following washing to remove unbound material, streptavidin-horseradish peroxidase and chemiluminescent detection reagents (ECL Western Blotting Analysis System; GE Healthcare, Buckinghamshire, UK) were added sequentially. Arrays were scanned using a LAS-3000 mini-luminescent image analyzer (Fujifilm, Tokyo, Japan) and were quantified for the densities using Multi Gauge ver. 3.0 software (Fujifilm). Concentrations of cytokines and chemokines were expressed as their signal intensity ratios relative to that of the positive control spotted on the same membrane.

Bead array

Precise serum concentrations of regulated on *RANTES* were measured using the Luminex Bio-Plex system (Bio-Rad, Hercules, CA, USA) and the Procarta Cytokine Assay Kit (Panomics, Fremont, CA, USA) in a 96-well plate ELISA-based format according to the manufacturers' recommendations. The sensitivity of the assays is greater than 10 pg/mL cytokine. Serum and standards were incubated with a mixture of the Luminex antibody-conjugated beads for 30 min with constant shaking. After washing, the detection antibodies and substrates were added and incubated for another 30 min. Fluorescent signals were collected and data expressed, using internal standards, in pg/mL as the mean of two individual experiments carried out in duplicate.

Viral core and interferon sensitivity-determining region (ISDR) sequence determination by direct sequencing

Hepatitis C virus RNA extraction from serum samples, complementary DNA synthesis and amplification by two-step nested polymerase chain reaction (PCR) were carried out using specific primers for the HCV core and ISDR. PCR amplicons were sequenced directly by Big Dye Terminator ver. 3.1 (ABI, Tokyo, Japan) with universal M13 forward and reverse primers using an ABI prism 3130 sequencer (ABI). The sequence files generated were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram.

SNP typing of the *RANTES* and *IL-28B* genes

Genomic DNA of the patients was extracted from peripheral blood using a blood DNA extraction kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. The allele typing of each DNA sample was performed by real-time PCR with a model 7500 (ABI) using FAM-labeled SNP primers for the loci rs2107538, rs2280788, rs2280789, rs4796120 and rs3817655 (ABI) for *RANTES* and the locus rs8099917 (ABI) for IL-28B.

Statistical analysis

Student's *t*-test and Mann–Whitney *U*-test were used to analyze continuous variables, as appropriate. Fisher's exact test was used for the analysis of categorical variables. Receiver–operator curve (ROC) analyses were performed to establish cut-off values for serum cytokine concentration. The optimum cut-off was defined as the value that maximized the area under the ROC. Spearman's correlation coefficient (R) was calculated to clarify the strength of relationship between the pretreatment serum cytokine concentrations and clinical parameters. Variables that achieved statistical significance ($P < 0.05$) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. The odds ratios and 95% confidence intervals also were calculated. Data were analyzed using Ekuseru-Toukei 2008 (SSRI, Tokyo, Japan). The haplotype block among rs2107538, rs2280788, rs2280789, rs4796120 and rs3817655 variants was analyzed using SNPAlyze software ver. 8.0 (Dynacom, Chiba, Japan). $P < 0.05$ was considered significant.

RESULTS

Semiquantitative measurement of pretreatment serum cytokines in 31 randomly-selected patients

AT FIRST, TO identify cytokines/chemokines related to the treatment responses to PEG IFN/RBV therapy, semiquantitative measurement of the serum concentrations of 36 comprehensive cytokines/chemokines was performed by sandwich ELISA method by randomly selected patients. Next, to further confirm the result, cytokines showing the associations with the response were measured more precisely by bead array method in all patients.

In the first analysis, 31 patients were randomly selected from the 96 patients. The clinical characteristics of these 31 patients at the start of the therapy are shown in Table 1. Significant differences in the clinical backgrounds between those who did and those who did not

Table 1 Baseline characteristics of the 31 patients analyzed using the sandwich ELISA method

Factor	SVR ($n = 20$)	Non-SVR ($n = 11$)	*P*-value
Age (years)	52 ± 11†	57 ± 10	0.25‡
Sex (male : female)	11:9	6:5	0.64§
Bodyweight (kg)	60.9 ± 9.6†	61.9 ± 13.9	0.81‡
Body mass index (kg/m^2)	22.6 (18.9–31.3)¶	22.7 (17.5–26.8)	0.87††
History of IFN therapy (%)	30	36	0.78§
ALT (IU/L)	130 ± 100†	75 ± 35	0.09‡
AST (IU/L)	76 (22–331)¶	64 (24–178)	0.73††
γ-GTP (IU/L)	40 (12–289)	52 (24–137)	0.17††
Albumin (g/dL)	4.1 (3.7–4.5)	4.0 (3.0–4.7)	0.46††
Total cholesterol (mg/dL)	170 ± 24†	149 ± 33	0.06‡
HbA1c (%)	5.3 ± 0.5	5.3 ± 0.6	0.95‡
Creatinine (mg/dL)	0.71 ± 0.15	0.68 ± 0.15	0.54‡
WBC count (/μL)	4561 ± 1631	4056 ± 1277	0.38‡
Neutrophil count (/μL)	2130 (820–4200)¶	1500 (800–2700)	0.02††
Hemoglobin (g/dL)	14.5 ± 1.0†	13.8 ± 1.6	0.15‡
Platelet count (×10^{-4}/μL)	16.4 ± 5.4	12.2 ± 3.9	0.03‡
α-Fetoprotein (ng/mL)	4.6 (1.4–28.9)¶	22.3 (11.4–79.7)	0.00005††
HCV RNA (KIU/mL)	1520 ± 1079†	2146 ± 899	0.11‡
Fibrosis (F1/F2/F3/F4)‡‡	14/1/1/2	3/2/2/3	0.02††
Activity (A1/A2/A3)‡‡	12/5/1	3/5/2	0.06††

†Mean ± standard deviation.
‡Student's *t*-test.
§Fisher's exact probability test.
¶Median (range).
††Mann–Whitney *U*-test.
‡‡SVR, $n = 18$; non-SVR, $n = 10$.
Activity, the score of activity in liver biopsies; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ELISA, enzyme-linked immunoassay; fibrosis, the score of fibrosis in liver biopsies; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; SVR, sustained virological response; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

achieve SVR were neutrophil counts, platelet counts, serum α-fetoprotein levels and the score of fibrosis in liver biopsies. Table 2 shows the difference in the cytokine/chemokine expression between the SVR and the non-SVR group. Because some cytokines/chemokines were below the measurement limit of the ELISA kit, as shown in Table 1, those cytokine/chemokines were not studied further. As shown here, the *RANTES* level was significantly higher in the SVR group than the non-SVR group ($P = 0.048$).

Precise measurement of serum *RANTES* in all 96 patients

Because the semiquantitative measurement of pretreatment serum *RANTES* levels in 31 randomly selected patients demonstrated their significant correlation with the SVR, we determined the precise serum RANTES levels in all 96 patients using the bead array method and investigated the correlation between those concentrations and the treatment outcome. The clinical characteristics of the 96 patients are shown in Table 3. Significant differences were seen between those with and without SVR in platelet count, viral loads and the liver fibrosis score, but there was no apparent difference in the total doses of PEG IFN and RBV. As shown in Figure 1, the distribution of serum *RANTES* levels in each treatment response differed significantly; the median serum *RANTES* level in the SVR group was significantly higher than that in the non-SVR group. Successive ROC analysis confirmed a significant association of the serum *RANTES* level with SVR, and the cut-off value of 3400 pg/mL to be most appropriate (Table 4). Using the cut-off value of 3400 pg/mL, 50.9% sensitivity, 79.5% specificity, 78.4% positive predictive value and 52.5% negative predictive value (area under the ROC, 0.643) were obtained for the prediction of SVR by serum *RANTES* level.

Table 2 Difference in cytokine and chemokine expression between the SVR group and the non-SVR group in the 31 patients

Cytokine/chemokine	SVR ($n = 20$)	Non-SVR ($n = 11$)	*P*-value
RANTES	4.99 (0.25–8.32)†	1.24 (0.17–8.01)	0.048‡
MIF	1.31 (0.06–3.31)†	0.45 (0.08–2.67)	0.0630
IL-1ra	0.09 (0.00–3.30)†	0.07 (0.00–2.05)	0.2300
PAI-1	3.10 (0.35–7.34)†	2.73 (0.46–8.42)	0.3900
sICAM-1	3.18 (0.37–8.33)†	2.78 (0.74–10.3)	0.4800
IL-23	0.08 (0.01–0.78)†	0.07 (0.00–0.38)	0.5900
IL-27	0.05 (0.02–0.18)†	0.05 (0.00–0.23)	0.6500
IL-6	0.08 (0.01–3.22)†	0.10 (0.00–1.36)	0.7100
C5a	0.21 (0.01–2.72)†	0.12 (0.00–1.67)	0.7700
IFN-γ	0.07 (0.02–0.31)†	0.08 (0.00–0.40)	0.8000
CCL4	0.04 (0.01–3.08)†	0.05 (0.00–0.69)	0.8400
IL-32α	0.04 (0.00–0.71)†	0.07 (0.00–0.20)	0.9000
IL-8	0.16 (0.05–2.61)†	0.17 (0.03–2.21)	0.9300
IL-1α			N.A.
IL-1β			N.A.
IL-2			N.A.
IL-4			N.A.
IL-5			N.A.
IL-10			N.A.
IL-12 p70			N.A.
IL-13			N.A.
IL-16			N.A.
IL-17			N.A.
IL-17E			N.A.
CCL1			N.A.
CCL2			N.A.
CCL3			N.A.
CXCL1			N.A.
CXCL11			N.A.
CXCL12			N.A.
CD154			N.A.
G-CSF			N.A.
GM-CSF			N.A.
IP-10			N.A.
TNF-α			N.A.
sTREM-1			N.A.

†Median (range).
‡Mann–Whitney *U*-test.
N.A., not available; SVR, sustained virological response.

Correlation between serum *RANTES* level and clinical parameters

Spearman's correlation coefficients between the pre-treatment serum *RANTES* level and clinical parameters in all 96 patients are shown in Table 5. As a result, a significant negative correlation with aspartate aminotransferase level and a significant positive correlation with platelet count were found, while no significant correlation was observed in other clinical parameters.

Univariate and multivariate analysis of factors related to SVR

Univariate and multivariate analyses were performed successively in order to clarify the factors related to SVR. The viral factors included in the analysis were the ISDR and core a.a. 70 and 91, along with the host factor, IL-28B SNP. Those factors, conventional clinical background factors and serum *RANTES* levels were subjected to univariate and multivariate analysis. In the univariate

Table 3 Baseline characteristics of all patients analyzed using the bead array method ($n = 96$)

Factor	SVR ($n = 57$)	Non SVR ($n = 39$)	*P*-value
Age (years)	53 ± 10†	57 ± 8	0.08‡
Sex (male : female)	34:23	23:16	0.56§
Bodyweight (kg)	60.6 ± 10.5†	57.8 ± 7.8	0.17‡
Body- mass index (kg/m^2)	22.9 ± 2.8	22.1 ± 2.2	0.15‡
History of IFN therapy (%)	25	28	0.74§
ALT (IU/L)	68 (19–413)¶	64 (20–215)	0.25††
AST (IU/L)	58 (21–331)	62 (21–178)	0.80††
γ-GTP (IU/L)	37 (11–289)	50 (13–167)	0.12††
Albumin (g/dL)	4.1 ± 0.3†	4.1 ± 0.4	0.93‡
Total cholesterol (mg/dL)	166 ± 30	158 ± 31	0.25‡
HbA1c (%)	5.2 (4.7–6.6)¶	5.3 (4.5–7.4)	0.47††
Creatinine (mg/dL)	0.72 ± 0.15†	0.69 ± 0.16	0.39††
WBC count (/μL)	4497 ± 1247	4501 ± 1281	0.99‡
Neutrophil count (/μL)	2243 ± 857	2144 ± 825	0.57‡
Hemoglobin (g/dL)	14.1 ± 1.2	14.2 ± 1.2	0.87‡
Platelet count (×10^{-4}/μL)	15.1 (7–29)¶	13.2 (6.9–19.7)	0.03††
α-Fetoprotein (ng/mL)	4.8 (1.3–137.1)	9.0 (1.4–79.7)	0.05††
HCV RNA (KIU/mL)	1300 (100–5000)	2400 (620–5000)	0.0002‡
Fibrosis (F1/F2/F3/F4)‡‡	35/6/5/6	11/13/5/6	0.006††
Activity (A1/A2/A3)‡‡	27/18/7	12/20/3	0.26††
PEG IFN dose (%)	92 (40–113)¶	73 (27–147)	0.23††
RBV dose (%)	97 (44–147)	100 (33–135)	0.38††

†Mean ± standard deviation.
‡Student's *t*-test.
§Fisher's exact probability test.
¶Median (range).
††Mann–Whitney's *U*-test.
‡‡SVR, $n = 52$; non-SVR, $n = 35$.
Activity, the score of activity in liver biopsies; ALT, alanine aminotransferase; AST, aspartate aminotransferase; fibrosis, the score of fibrosis in liver biopsies; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; PEG IFN, pegylated interferon; RBV, ribavirin; SVR, sustained virological response; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

analysis, significant differences were observed for the ISDR mutation, core a.a. 70, viral loads, platelet counts, IL-28B SNP and serum *RANTES* levels. When multivariate analysis was carried out with these factors, the serum RANTES level was extracted as an independent factor related to SVR (Table 6).

RANTES haplotyping and serum *RANTES* level

Because a high serum *RANTES* level was an independent factor predicting SVR, we sought to examine further the role of the RANTES gene and tried to clarify the association of the SNP of the gene with the serum levels. First, we determined how many and which SNP in the *RANTES* gene should be investigated to represent all *RANTES* haplotypes found in the Japanese population. Reference to the HapMap project database (http://snp.cshl.org) made it clear that the information from five unique SNP was required to determine the majority of haplotypes found in the Japanese population. Therefore, to determine the *RANTES* haplotype of each patient, we investigated these five SNP in the 65 of the 96 patients available for the haplotype analysis. The *RANTES* haplotypes were finally divided into three types (named R1, R2 and R3 for convenience), as shown in Figure 2(a). However, the *RANTES* gene haplotype and serum RANTES level did not show any clear correlation (Fig. 2b).

DISCUSSION

FROM THE ANALYSIS of 36 cytokine and chemokine species, we discovered that a high pretreatment serum *RANTES* level was significantly related to SVR

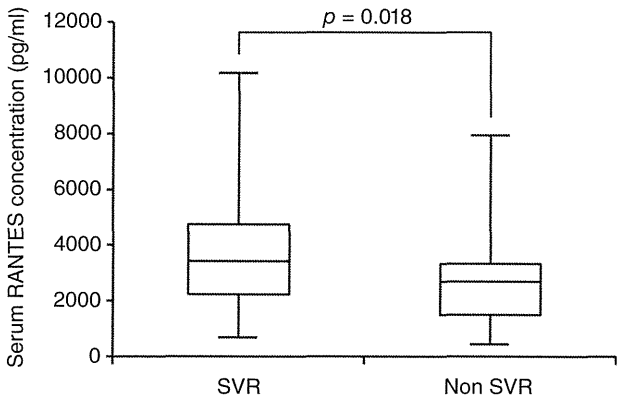


Figure 1 Difference in serum *RANTES* levels between the sustained virological response (SVR) group and the non-SVR group. Ninety-six patients who underwent the standard therapy for 48 weeks were analyzed for serum *RANTES* level using the bead array method. They were divided into the SVR ($n = 57$) and non-SVR groups ($n = 39$) and their serum *RANTES* levels compared. Box and whisker plots show the distributions of serum *RANTES* levels for the SVR and non-SVR groups. The boxes represent the 25th to 75th percentile and horizontal lines within the box show the median values. The ends of the whiskers show the minimum and maximum values of all the data. *P*-values were obtained using Mann–Whitney's *U*-test.

following PEG IFN/RBV combination therapy of patients infected with genotype 1b HCV. In particular, a high serum *RANTES* level was an independent factor contributing to SVR in the multivariate analysis, even among other treatment-restricting factors as the HCV ISDR, core a.a. 70, viral loads, platelets or IL-28B SNP. On the other hand, a systematic haplotyping study did not reveal any correlation between the *RANTES* haplotype and serum *RANTES* level.

RANTES, also known as CC-chemokine ligand (CCL)5, is classified as a chemotactic T-helper (Th)1-type chemokine.[23] In chronic hepatitis C, *RANTES* is significantly upregulated in the infected liver, and considered to play a role in recruiting T cells to portal and periportal regions, regulating liver inflammation and innate and adaptive immunity through interactions with CC-chemokine receptor (*CCR*)5, *CCR1* and *CCR3* expressed on activated T cells.[24] The serum *RANTES* level is significantly upregulated in the early stages of fibrosis in chronic hepatitis and its upregulation becomes weaker in advanced chronic disease.[14] HCV-encoded proteins are considered to affect *RANTES* production, for example, exposure of peripheral blood mononuclear cells to the HCV envelope 2 (E2) protein induces the release of *RANTES*,[25] the HCV NS3/4A proteins suppress *RANTES* promoter activity[26] and the HCV core protein may either induce or inhibit the expression of *RANTES* in various cell types.[27] A recent *in vitro* study has shown that human hepatoma cells secrete *RANTES* via the Toll-like receptor (*TLR*)3-mediated recognition of HCV dsRNA and activation of the nuclear factor (*NF*)-*kB* pathway, suggesting that the hepatocytes themselves may serve as the source of *RANTES*.[15]

In this study, we showed the close association between the serum *RANTES* level and SVR in the PEG IFN/RBV combination therapy by analyzing 31 randomly selected, primary test patients and then all 96 patients. In addition to the association with SVR, we also searched the association between *RANTES* and the initial viral response because SVR could be influenced by the initial viral dynamics, and revealed that complete early viral response (HCV RNA negative at 12 weeks

Table 4 Association between the serum *RANTES* level and SVR rate in all 96 patients analyzed using the bead array method

Cytokine/ chemokine	Serum concentration	SVR rate	*P*-value
RANTES	≥3400 pg/mL†	78% (29/37)	0.002‡
	<3400 pg/mL†	47% (28/59)	

†A cut-off value of 3400 pg/mL was determined by receiver–operator curve analysis in all 96 patients.
‡Fisher's exact probability test.
SVR, sustained virological response.

Table 5 Spearman's correlation coefficient (*R*) between the pretreatment serum *RANTES* level and clinical parameters ($n = 96$)

Clinical parameters	Serum *RANTES* level	
	R	*P*-value
Platelet count	−0.30	0.0025
Aspartate aminotransferase	−0.24	0.0200
White blood cell	−0.15	0.1600
Total cholesterol	−0.11	0.2700
Alanine aminotransferase	−0.088	0.3900
α-Fetoprotein	−0.088	0.4100
Neutrophil count	−0.064	0.5400
Hemoglobin A1c	−0.056	0.6300
γ-Glutamyl transpeptidase	−0.047	0.6500
Albumin	−0.021	0.7900
Hemoglobin	−0.025	0.8000
Creatinine	−0.00098	0.9900

Table 6 Factors associated with SVR analyzed by univariate and multivariate analysis

Characteristic	Subcategory	Univariate analysis			Multivariate analysis		
		Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
Platelet count		1.13	1.03–1.25	0.012	1.20	1.00–1.41	0.042
IL-28B SNP	T/T or not	16.0	3.37–76.2	0.0005	9.48	1.40–64.3	0.02
RANTES	≥3400†	4.01	1.58–10.2	0.0036	4.09	1.02–16.5	0.048
Viral loads		0.99	0.99–0.99	0.0012	0.99	0.99–1.00	0.51
ISDR mutation	≥2	21.7	2.76–170	0.0034	28.2	2.05–388	0.013
Core a.a. 70	R or not	2.52	1.03–6.20	0.044	3.19	0.73–13.9	0.12

†The cut-off value of 3400 pg/mL was determined by receiver–operator curve analysis in all 96 patients.
a.a., amino acids; CI, confidence interval; IL, interleukin; ISDR, interferon sensitivity-determining region; R, arginine; SNP, single nucleotide polymorphisms.

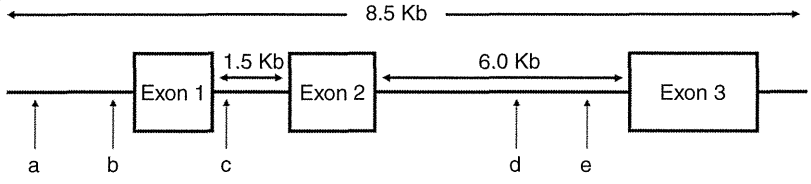
after commencement of therapy) was also significantly correlated with high pretreatment serum *RANTES* level ($P = 0.015$, data not shown). Moreover, we could also show that high serum *RANTES* levels correlated with the clinical background factors low alanine aminotransferase values and high platelet counts, suggesting that the patients with high *RANTES* levels have less severe hepatitis. A previous study also showed a tendency for correlation between the serum *RANTES* level and SVR in PEG IFN/RBV therapy, but this correlation did not reach significance.[16] Although the reason for this discrepancy is not known, we speculate that a difference in drug dosage may have contributed. In our study, most of the patients received a sufficient dose of both PEG IFN and RBV, as shown in Table 3. However, the previous study lacks information regarding drug dosage, suggesting that the study group comprised a heterogeneous population.

Then, what is the mechanism of the association between high serum *RANTES* levels and high SVR? Because *RANTES* is a chemotactic Th1-type chemokine, it may be speculated that a high serum *RANTES* level reflects activation and preservation of the Th1-type immune responses needed to suppress viral replication and so enhances viral elimination by PEG IFN/RBV therapy. Although it is also possible that a high *RANTES* level could be simply a reflection of early stages of the disease, we suggest that it could have a more direct role in achieving SVR, because multivariate analysis extracted a high serum *RANTES* level as a variable contributing to SVR independently of the platelet count, which reflects the stage of disease. Importantly, our result also demonstrated that the serum *RANTES* level was a factor contributing to SVR independently of other treatment-restricting factors, including the *IL-28B* SNP and the viral factors of NS5A and core. This independent contribution of a high serum *RANTES* level among other variables indicates its importance and potency in improving the prediction of the treatment efficacy.

Concerning the association between the serum *RANTES* level and *RANTES* haplotype, we could not find a significant correlation in the HCV-infected patients, although there was a tendency that patients with the R3 haplotype had higher serum *RANTES* levels. In patients with coronary artery disease and type 1 diabetes mellitus, and in healthy volunteers, the serum *RANTES* level has been reported to correlate with the *RANTES* gene SNP. Specifically, those patients and healthy volunteers with the A allele in the *RANTES* promoter polymorphism at position –403 (rs2107538) had lower serum *RANTES* levels than those with the G allele.[28,29] On the other hand, in previous reports of chronic hepatitis C, no evident correlation was reported between the *RANTES* SNP at position –403 (rs2107538) and serum *RANTES* level.[30] In this study, through more systematic haplotyping analysis based upon the HapMap Database, we tried to determine the correlation between the serum *RANTES* level and the *RANTES* gene SNP in chronic hepatitis C in more detail. However, we could not find any association and the result shows that the serum *RANTES* level is not primarily determined by the *RANTES* haplotype in chronic hepatitis C. The result seems strange at first, however, it is understandable considering that *RANTES* expression is modulated by multiple factors in chronic hepatitis C, including viral components and the stage of liver disease, as described before.

However, there are some limitations in our study. Namely, the number of investigated patients was rather small, and included patients for the analysis were limited to those with genotype 1b HCV infection. Therefore, it is considered that additional independent studies including the analysis of other genotypes would

(a)



RANTES haplotype	a	b	c	d	e	Haplotype frequency
	rs2107538	rs2280788	rs2280789	rs4796120	rs3817655	
R1	G	C	T	A	A	0.599
R2	A	C	C	G	T	0.197
R3	A	G	C	A	T	0.189

(b)

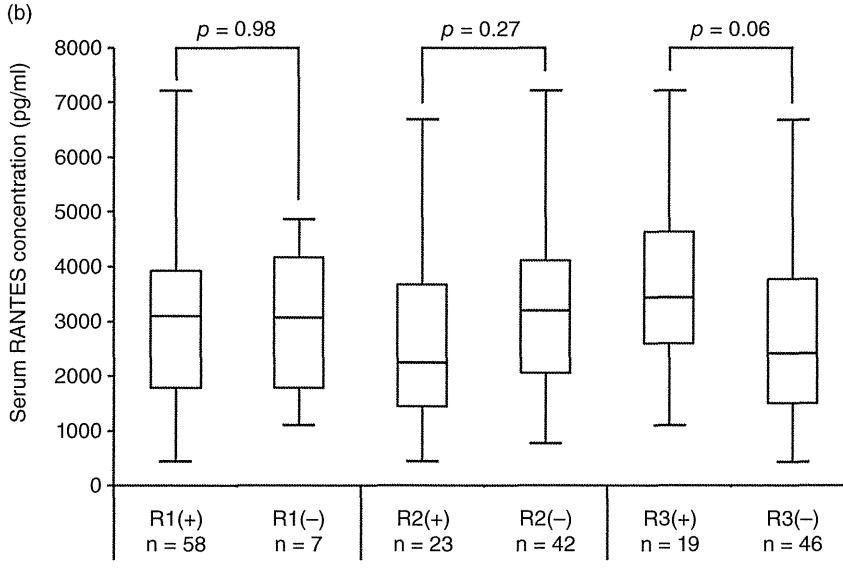


Figure 2 *RANTES* haplotypes and serum *RANTES* level. (a) *RANTES* haplotypes in the patients studied. The human *RANTES* gene spans 8.5 kb on chromosome 17q11-q12 and has the characteristic three exon and two intron organization of the CC chemokine family.[22] Exons are shown as open boxes while introns are shown as solid lines. Five single nucleotide polymorphisms (SNP) (rs2107538/rs2280788/rs2280789/rs4796120/rs3817655) were selected on the basis of data from the HapMap project (http://snp.cshl.org) to obtain complete coverage of the *RANTES* gene in the Japanese population. The locations of SNP variants are indicated by arrows. After the analysis of five *RANTES* SNP in 65 hepatitis C virus patients, haplotypes were determined using SNPAlyze software ver. 8.0 (Dynacom, Chiba, Japan) and divided into three groups on the basis of linkage disequilibrium. These were designated R1, R2 and R3 on the basis of haplotype frequency. (b) Serum *RANTES* level and *RANTES* haplotype. The correlation between serum the *RANTES* level and *RANTES* haplotype was investigated. Box and whisker plots shows distributions of serum *RANTES* levels for the haplotypes R1(+), R1(−), R2(+), R2(−), R3(+) and R3(−). The boxes represent the 25th to 75th percentile and horizontal lines within the boxes show the median values. The ends of the whiskers show the minimum and maximum values of all the data. *P*-values were obtained using Mann–Whitney's *U*-test. R1(+), the patients with the R1 haplotype; R1(−), the patients with a non-R1 haplotype; R2(+), the patients with the R2 haplotype; R2(−), the patients with a non-R2 haplotype; R3(+), the patients with the R3 haplotype; R3(−), the patients with a non-R3 haplotype.

further clarify the correlation. On the other hand, we could not show an association of pretreatment cytokines/chemokine concentrations with the treatment response to PEG IFN/RBV therapy for the other 35 cytokine and chemokine species investigated in this study. Recently, the serum level of *IP-10* was reported to be strongly associated with the response to PEG IFN/RBV therapy and baseline *IP-10* levels were elevated in patients infected with HCV genotype 1 or 4 who did not achieve an SVR after completion of interferon therapy.[19,20] In our study, however, *IP-10* was not extracted as a molecule associated with treatment responses. Actually, due to the measurement limit of the ELISA kit used, several cytokines and chemokines, including *IP-10*, were undetectable in this study, as shown in Table 2, raising the possibility that some cytokines and chemokines associated with SVR were not extracted. Therefore, our study cannot exclude the possibility of other cytokine/chemokines making a contribution to treatment efficacy.

In conclusion, we found that a high pretreatment serum *RANTES* level was related to the efficacy of PEG IFN/RBV therapy in genotype 1b HCV, independent of other treatment-restricting factors, and prediction of treatment outcome could be improved with the measurement of the pretreatment serum *RANTES* level.

ACKNOWLEDGMENTS

THIS WORK WAS supported by Grants-in-Aid from the Scientific Research Fund of the Ministry of Education, Science, Sports and Culture (21590836, 21590837, 23390195); and a Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan (H22-kanen-006).

REFERENCES

1 Kiyosawa K, Sodeyama T, Tanaka E *et al.* Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12**: 671–5.
2 Singal AK, Singh A, Jaganmohan S *et al.* Antiviral therapy reduces risk of hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis. *Clin Gastroenterol Hepatol* 2010; **8**: 192–9.
3 Jacobson IM, McHutchison JG, Dusheiko G *et al.* Telaprevir for previously untreated chronic hepatitis C virus infection. *N Engl J Med* 2011; **364**: 2405–16.
4 Zeuzem S, Andreone P, Pol S *et al.* Telaprevir for retreatment of HCV infection. *N Engl J Med* 2011; **364**: 2417–28.
5 Fried MW. The role of triple therapy in HCV genotype 1-experienced patients. *Liver Int* 2011; **31** (Suppl 1): 58–61.
6 Akuta N, Suzuki F, Sezaki H *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; **48**: 372–80.
7 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; **334**: 77–81.
8 Conjeevaram HS, Kleiner DE, Everhart JE *et al.* Race, insulin resistance and hepatic steatosis in chronic hepatitis C. *Hepatology* 2007; **45**: 80–7.
9 Hickman IJ, Powell EE, Prins JB *et al.* In overweight patients with chronic hepatitis C, circulating insulin is associated with hepatic fibrosis: implications for therapy. *J Hepatol* 2003; **39**: 1042–8.
10 Shiffman ML, Mihas AA, Millwala F *et al.* Treatment of chronic hepatitis C virus in African Americans with genotypes 2 and 3. *Am J Gastroenterol* 2007; **102**: 761–6.
11 Shiffman ML, Suter F, Bacon BR *et al.* Peginterferon alfa-2a and ribavirin for 16 or 24 weeks in HCV genotype 2 or 3. *N Engl J Med* 2007; **357**: 124–34.
12 Suppiah V, Moldovan M, Ahlenstiel G *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009; **41**: 1100–4.
13 Tanaka Y, Nishida N, Sugiyama M *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; **41**: 1105–9.
14 Katsounas A, Schlaak JF, Lempicki RA. CCL5: a double-edged sword in host defense against the hepatitis C virus. *Int Rev Immunol* 2011; **30**: 366–78.
15 Li K, Li NL, Wei D, Pfeffer SR, Fan M, Pfeffer LM. Activation of chemokine and inflammatory cytokine response in hepatitis C virus-infected hepatocytes depends on Toll-like receptor 3 sensing of hepatitis C virus double-stranded RNA intermediates. *Hepatology* 2012; **55**: 666–75.
16 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.
17 Polyak SJ, Khabar KS, Rezeiq M, Gretch DR. Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy. *J Virol* 2001; **75**: 6209–11.
18 Yee LJ, Tang J, Gibson AW, Kimberly R, Van Leeuwen DJ, Kaslow RA. Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. *Hepatology* 2001; **33**: 708–12.
19 Butera D, Marukian S, Iwamaye AE *et al.* Plasma chemokine levels correlate with the outcome of antiviral therapy in patients with hepatitis C. *Blood* 2005; **106**: 1175–82.

20 Romero AI, Lagging M, Westin J *et al.* Interferon (IFN)-gamma-inducible protein-10: association with histological results, viral kinetics, and outcome during treatment with pegylated IFN-alpha 2a and ribavirin for chronic hepatitis C virus infection. *J Infect Dis* 2006; **194:** 895–903.
21 Kumada H, Okanoue T, Onji M *et al.* Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis C virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; **40:** 8–13.
22 Nelson PJ, Kim HT, Manning WC, Goralski TJ, Krensky AM. Genomic organization and transcriptional regulation of the RANTES chemokine gene. *J Immunol* 1993; **151:** 2601–12.
23 Schall TJ, Jongstra J, Dyer BJ *et al.* A human T cell-specific molecule is a member of a new gene family. *J Immunol* 1988; **141:** 1018–25.
24 Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000; **12:** 121–7.
25 Nattermann J, Nischalke HD, Feldmann G, Ahlenstiel G, Sauerbruch T, Spengler U. Binding of HCV E2 to CD81 induces RANTES secretion and internalization of CC chemokine receptor 5. *J Viral Hepat* 2004; **11:** 519–26.
26 Kaukinen P, Sillanpaa M, Kotenko S *et al.* Hepatitis C virus NS2 and NS3/4A proteins are potent inhibitors of host cell cytokine/chemokine gene expression. *Virol J* 2006; **3:** 66.
27 Ruggieri A, Franco M, Gatto I, Kumar A, Rapicetta M. Modulation of RANTES expression by HCV core protein in liver derived cell lines. *BMC Gastroenterol* 2007; **7:** 21.
28 Jang Y, Chae JS, Hyun YJ *et al.* The RANTES −403G > A promoter polymorphism in Korean men: association with serum RANTES concentration and coronary artery disease. *Clin Sci (Lond)* 2007; **113:** 349–56.
29 Zhernakova A, Alizadeh BZ, Eerligh P *et al.* Genetic variants of RANTES are associated with serum RANTES level and protection for type 1 diabetes. *Genes Immun* 2006; **7:** 544–9.
30 Promrat K, McDermott DH, Gonzalez CM *et al.* Associations of chemokine system polymorphisms with clinical outcomes and treatment responses of chronic hepatitis C. *Gastroenterology* 2003; **124:** 352–60.

Journal of Medical Virology 84:1360–1368 (2012)

Correlation Between Pretreatment Viral Sequences and the Emergence of Lamivudine Resistance in Hepatitis B Virus Infection

Ryota Sueki, Shinya Maekawa,* Mika Miura, Makoto Kadokura, Kazuki Komase, Hiroko Shindo, Asuka Kanayama, Takako Ohmori, Kuniaki Shindo, Fumitake Amemiya, Yasuhiro Nakayama, Tomoyoshi Uetake, Taisuke Inoue, Minoru Sakamoto, and Nobuyuki Enomoto

First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Chuo, Yamanashi, Japan

The emergence of amino acid or nucleotide substitutions leads to lamivudine resistance in hepatitis B virus (HBV) infected patients. The aim of this study was to investigate whether viral sequences help predict the emergence of lamivudine resistance. The study subjects comprised 59 consecutive patients infected with HBV treated with daily therapy of 100 mg lamivudine. Among those, 32 patients with adequate pretreatment serum preservation were investigated for the correlation between viral amino acid substitutions and the appearance of lamivudine resistance with consideration of clinical background by determining dominant HBV full open reading frames. Viral resistance to lamivudine emerged in 28 of 59 patients (47%) in a median period of 2.45 years. Sequence comparisons of HBV genomes between patients who later developed lamivudine resistance and patients who did not revealed the existence of significant differences between the two groups in the pre-S1 84 ($P = 0.042$), pre-S2 1 ($P = 0.017$) and 22 ($P = 0.015$), and polymerase tp 95 ($P = 0.046$), judged by a log-rank test. Viral sequence analyses revealed the presence of amino acid substitutions in HBV pre-S1 and pre-S2 that may be associated with the emergence of lamivudine resistance during chronic HBV infection. ***J. Med. Virol. 84: 1360–1368, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HBV; lamivudine resistance pre-S substitution; ORF sequence analysis

INTRODUCTION

Hepatitis B virus (HBV) infects persistently more than 350 million people worldwide [Liang, 2009], and increases their risk of developing liver cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC) over the typically long disease course. High serum virus titers have been shown recently to promote significantly disease progression, as well as the development of HCC [Chen et al., 2006b; Iloeje et al., 2006]. Therefore, effective suppression of the serum viral load by an antiviral agent might inhibit disease progression [Lim et al., 2009].

Lamivudine was introduced clinically as one of the first-generation nucleoside analogs to inhibit HBV replication [Liaw et al., 2004]. Lamivudine is safe [Lok et al., 2003], effectively decreases serum viral load, improves alanine aminotransferase (ALT) levels and liver fibrosis [Leung, 2000; Villeneuve et al., 2000], and enhances hepatitis B e antigen (HBeAg) seroconversion rates [Liaw et al., 2000; Leung et al., 2001; Chen et al., 2006a], which lead to the suppression of HCC development [Liaw et al., 2004]. In contrast, prolonged use of lamivudine may lead to the emergence of drug-resistant HBV mutants in a substantial percentage of patients. When resistance emerges, patients should be treated with a different nucleoside analog, which does not show cross-resistance, alone or in combination with lamivudine [Rizzetto et al., 2005; Carey and Harrison, 2009; Chen et al., 2009]. Newly introduced second-generation nucleoside analogs, such as entecavir and tenofovir, have been shown to be superior in suppressing viral load and preventing the emergence of drug-resistant viruses. However, because of its high economical efficacy compared to other, newer-generation

Ryota Sueki and Shinya Maekawa contributed equally to the work.

*Correspondence to: Shinya Maekawa, MD, PhD, First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan. E-mail: maekawa@yamanashi.ac.jp

Accepted 17 April 2012

DOI 10.1002/jmv.23314

Published online in Wiley Online Library (wileyonlinelibrary.com).

nucleoside analogs, the appropriate selection of patients suitable for lamivudine therapy by accurate prediction of the emergence of resistance would benefit economically challenged patients worldwide. On the other hand, prediction of the eventual emergence of resistance to lamivudine has been difficult.

Many previous studies have shown a correlation between lamivudine resistance and the HBV mutations that appear with viral acquisition of lamivudine resistance. These mutations lead to amino acid mutations in the HBV polymerase, including rt M204I/V in the C domain and rt V173L and rt L180M in the B domain [Ling et al., 1996; Tipples et al., 1996; Ghany and Doo, 2009]. However, it is not known whether any specific sequences of viral genomes not exposed to lamivudine might predict the development of resistance following the commencement of lamivudine treatment. Typically, the emergence of lamivudine resistance has been predicted by pretreatment or in-treatment clinical variables, such as HBeAg positivity, higher baseline HBV DNA levels, female sex, lower ALT levels and a poor early viral response to lamivudine [Andersson and Chung, 2009; Zhou et al., 2009].

The present study was conducted to clarify and characterize pretreatment HBV sequences associated with the subsequent emergence of lamivudine resistance by determining the complete sequences of HBV ORFs by direct nucleotide sequencing, using patients' sera as the source of HBV DNA.

PATIENTS AND METHODS

Patients

Fifty-nine patients with chronic hepatitis or liver cirrhosis, infected with HBV and who underwent lamivudine therapy at Yamanashi University Hospital from May 2001 to June 2010 were enrolled initially in the study. All patients received lamivudine orally, initially at a dosage of 100 mg per day. Although all 59 patients responded initially to lamivudine therapy and HBV DNA became undetectable, lamivudine resistance was diagnosed in 28 patients (47%) because HBV DNA reappeared during the observation period, while in the other 31 patients it did not (Fig. 1). Because pretreatment serum from 32 of the patients had been preserved adequately for determination of the complete HBV nucleotide sequence, the final analysis was based on these 32 patients. All patients included were positive for hepatitis B surface antigen (HBsAg) and were tested for HBV DNA by the Quantiplex HBV DNA assay (Bayer Diagnostics, Emeryville, CA), transcription-mediated amplification assay (Chugai Diagnostics Science Co., Ltd, Tokyo, Japan), or COBAS® Amplicor HBV Monitor Test v2.0 (Roche Diagnostics, Indianapolis, IN). Patients with co-existing autoimmune hepatitis, alcoholic liver disease, drug-induced liver injury, chronic hepatitis C, or human immunodeficiency virus infection were excluded from the study. For patients with emerging drug resistance, adefovir dipivoxil was started at a dosage of

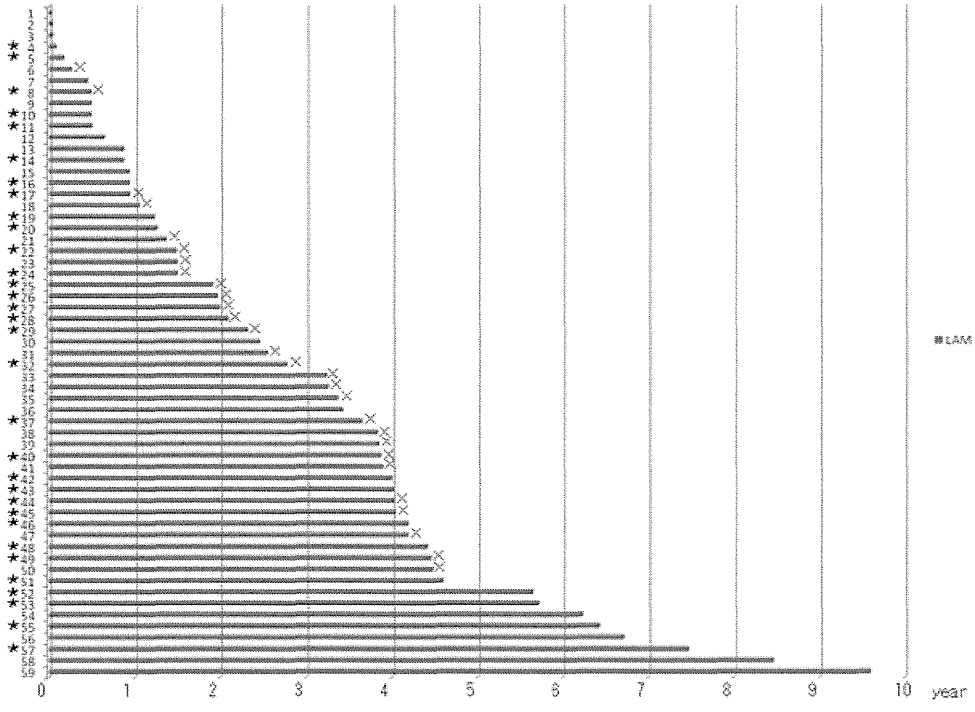


Fig. 1. Clinical course of HBV-infected patients treated with lamivudine. "X" indicates the emergence of lamivudine resistance. Asterisks indicate patients selected for HBV nucleotide sequence analysis.

10 mg per day, in addition to lamivudine, according to the guideline established by a hepatitis study group of the Ministry of Health, Labour, and Welfare, Japan. A signed consent was obtained for the study protocol that had been approved by Human Ethics Review Committee of Yamanashi University Hospital.

DNA Extraction, PCR, and Direct Sequencing

Full-length HBV DNA was amplified by two-step PCR from patients' sera and sequenced directly as described elsewhere [Sugauchi et al., 2001]. Sequence reads were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram. For ambiguous reads, only the dominant base was assigned after evaluation of all overlapping fragments. Full-length HBV genome sequences were assembled using this information and translated in silico and the ORFs of drug-resistant and sensitive genomes were compared.

Statistical Analysis

Statistical differences in the parameters, including all available demographic, biochemical, hematological, and virological statuses, were determined for the different patient groups by Student's t-test for numerical variables and Fisher's exact probability test for categorical variables. The odds ratio and 95% confidence intervals were calculated. P values of <0.05 by the two-tailed test were considered to indicate statistical significance. In order to evaluate the contribution of pretreatment viral amino acid sequences to the development of lamivudine resistance, Kaplan–Meier analysis and Cox proportional hazards model was performed.

RESULTS

Patients' Clinical Parameters

The pretreatment clinical and virological characteristics of the 32 patients, prior to starting lamivudine therapy, are shown in Table I, sorted according to the subsequent emergence of lamivudine resistance. Although HBV DNA became undetectable initially after the commencement of lamivudine therapy, drug resistance was diagnosed in 14 patients because of reappearance of HBV DNA during the observation period. No statistical difference was observed in age, sex, ALT, total bilirubin, choline esterase, total cholesterol, prothrombin time, platelets, α-fetoprotein, HBeAg/anti-HBe positivity, viral genotypes, liver disease (chronic hepatitis or liver cirrhosis), or pretreatment HBV DNA level. Genotype C was most prevalent in both groups (16/18 in the non-resistant group and 13/14 in the resistant group). In contrast, the time for HBV DNA to become undetectable was longer in this group, compared to that in the susceptible group ($P = 0.024$). Figure 1 shows the length of therapy for all 59 patients; "×" denotes the time of lamivudine resistance onset. Lamivudine resistance was diagnosed in 28 (47%) of 59 patients during a median observation period of 2.45 years.

Comparison of the HBV ORFs of the Lamivudine Resistant and Non-Resistant Groups

Full-length HBV genomic sequences from the 32 patients were determined by direct nucleotide

TABLE I. Baseline Clinical Characteristics

Clinical factor	LAM non-resistant (n = 18)	LAM resistant (n = 14)	P-value
Demographic characteristics			
Age, years[a]	53.9 (±13.2)	55.6 (±7.7)	0.662
Sex, no. male/female	13/5	9/5	0.712
CH/LC	5/13	3/11	0.261
HCC (+/−)	11/7	7/7	0.721
Biochemical characteristics			
Alanine aminotransferase level, IU/L[b]	91 (13–1,780)	70.5 (17–2,739)	0.805
Platelets count, $\times 10^{-4}$/ml[a]	11.8 (±5.8)	12.1 (±5.3)	0.900
Total bilirubin, mg/dl[b]	0.95 (0.3–19.7)	1.1 (0.4–5.0)	0.634
Albumin, g/dl[b]	3.2 (±0.6)	3.5 (±0.9)	0.270
ChE, IU/L[a]	196.4 (±105.0)	207.1 (±92.4)	0.566
T-chol, mg/dl[a]	156.1 (±39.6)	163.6 (±37.4)	0.590
Prothrombin time, %[a]	64.5 (±16.1)	69.9 (±15.9)	0.358
α-fetoprotein, ng/ml[b]	16.1 (1.9–35,194)	11.5 (1.6–611.5)	0.506
Virological characteristics			
HBV genotype (A/B/C)	1/1/16	0/1/13	0.662
HBV DNA level Log_{10} copies/ml[a]	5.80 (±1.45)	6.61 (±0.97)	0.078
HBeAg, positive/negative	6/12	8/6	0.283
Precore mutation ratio (%)	38.9	28.6	0.712
Core promotor mutation	4/14	3/11	0.880
Duration of LAM administration until HBV PCR negative (month)[b]	2.1 (0.4–7.7)	3.7 (1.4–69.0)	0.024

[a]Average (±SD) Student's t-test.
[b]Median (range) Mann–Whitney U-test.

TABLE II. Amino Acid Substitution Number in Each Region of the HBV Genome

HBV protein	LAM non-resistant	LAM resistant	*P*-value
Pre-S1, median (range)	2.0 (0–6)	2.0 (0–11)	0.460
Pre-S2, median (range)	0 (0–4)	2.0 (0–8)	0.060
S, median (range)	3.0 (1–9)	4.0 (2–8)	0.372
Pre-S1/pre-S2/S, median (range)	7.0 (3–15)	7.0 (4–23)	0.206
Polymerase, median (range)	15.5 (9–30)	17.0 (8–35)	0.448
Precore, median (range)	0.5 (0–1)	0 (0–1)	0.144
Core, median (range)	3.5 (0–9)	5.0 (0–35)	0.859
X, median (range)	4.0 (1–7)	3.0 (1–9)	0.706

Mann–Whitney *U*-test.

sequencing. Conceptual in silico translation of the dominant pretreatment HBV DNA sequences allowed correlation of the amino acid substitution numbers in each viral ORF with the drug resistance of the virus. Table II shows that the number of amino acid changes in each viral ORF did not differ significantly between the two groups. However, although not significant, there was a tendency that amino acid substitutions in the pre-S2 region were more frequent in patients with eventual development of lamivudine resistance (the median numbers of non-synonymous mutations were 0 and 2 in the sensitive and resistant groups, respectively; $P = 0.06$).

Next, the amino acid residues differing between the two groups at each position in each viral protein were compared. The vertical line representing the *P*-value for each HBV ORF (Fig. 2a–d) indicates the difference between the two groups. Comparison of the two groups revealed amino acid differences at the residues indicated as follows: pre-S1 56, 84, pre-S2 1 and 22, S 130 (Fig. 2a), and polymerase rt 138, tp 95, spacer 37, 59, 84, and 87 (Fig. 2c). The polymerase was numbered according to the standardized numbering system [Stuyver et al., 2001]. The most significant difference was observed at polymerase tp 95 in the (Fig. 2c). In contrast, only a slight difference was observed in the precore and core and X (Fig. 2b,d). In particular, the changes at pre-S1 84 and polymerase spacer 87 were seen to be coexistent because the pre-S1 and polymerase ORFs overlap. In contrast, the

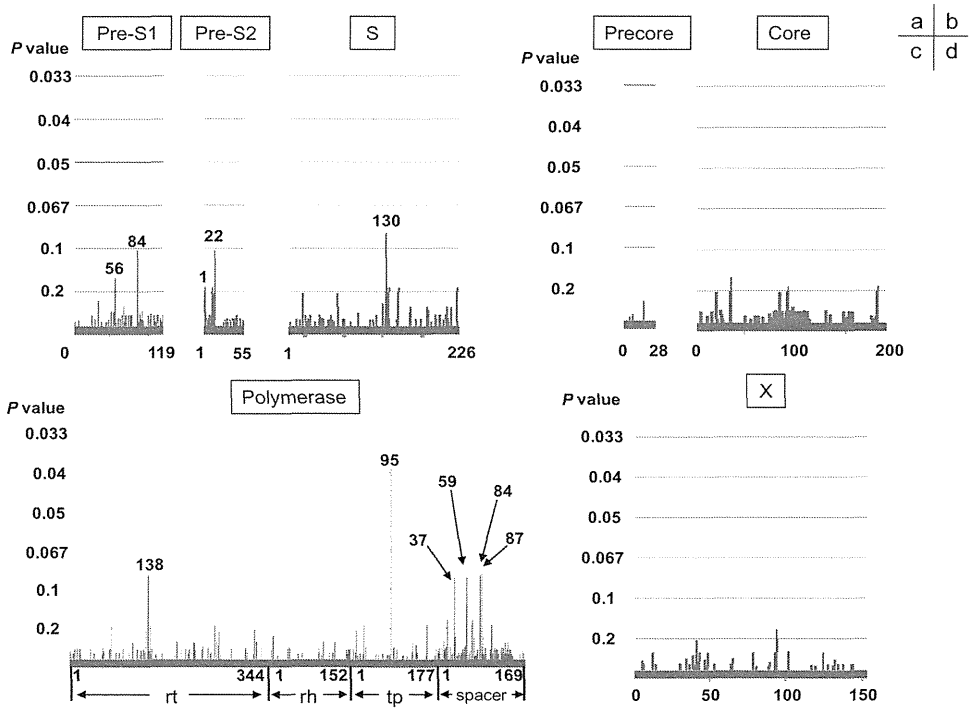


Fig. 2. Codon differences in each viral ORF between lamivudine sensitive and resistant groups. The differences are indicated by a vertical line representing the inverse of the *P*-value. (**a**) Pre-S1/S2, and S ORF, (**b**) polymerase ORF, (**c**) precore and core ORFs, (**d**) X ORF. Although a few genotype A and B viruses were included in the analysis, for convenience, the sequences are numbered according to the system for genotype C HBV. Viral amino acids are numbered according to the adopted standardized numbering system for the HBV polymerase [Stuyver et al., 2001].

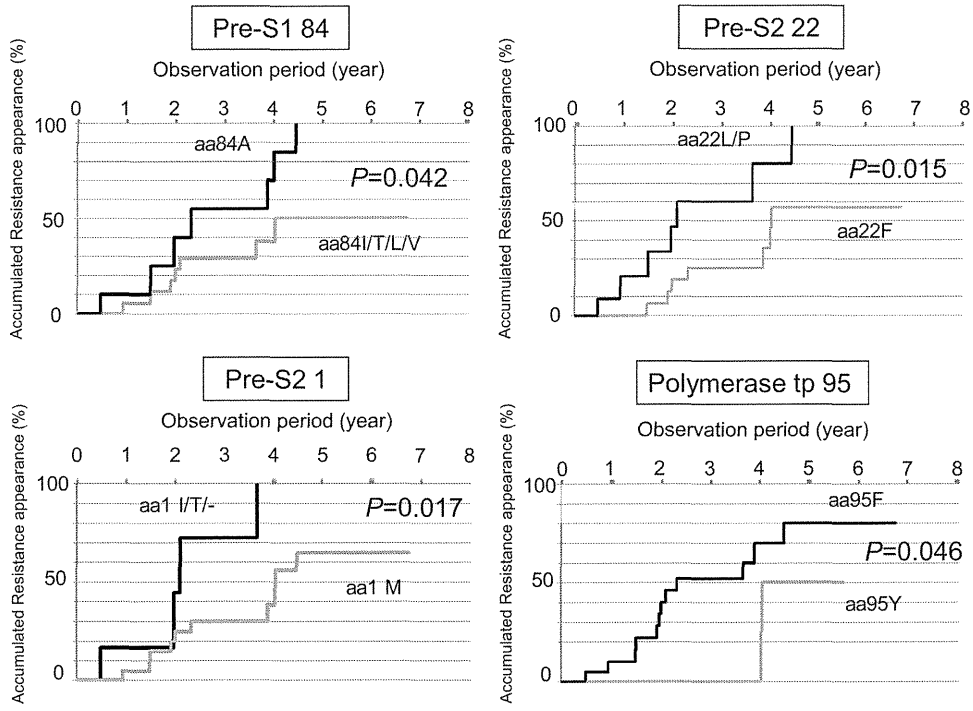


Fig. 3. Kaplan–Meier analysis of relationship of substitutions with the emergence of lamivudine resistance. The sequences are numbered according to the system for genotype C HBV.

coding changes at polymerase rt M204I/V, rt L180M, rt 173L, rt A181V, and rt N236T, and at S I195M, S W196L, and S W196 (stop), previously reported to result from mutations associated with viral acquisition of resistance to lamivudine or adefovir, were not observed prior to lamivudine therapy in any patients in this study.

Thereafter, Kaplan–Meier curves were constructed to understand better the potential influence of the amino acid changes, as revealed above, on the emergence of lamivudine resistance (Fig. 3, log-rank test). When the time of emergence of resistance was considered, a significant difference was observed with respect to the substitutions of pre-S1 84 ($P = 0.042$), pre-S2 1 ($P = 0.017$) and 22 ($P = 0.015$), and polymerase tp 95 ($P = 0.046$). Figure 4 shows a multiple alignment of amino acid sequences within the pre-S1, pre-S2, and polymerase ORFs.

Patient Characteristics Related to HBV ORF Substitutions

As shown in Table III, patients with isoleucine, threonine, leucine, or valine at pre-S1 84 had significantly lower HBV DNA levels, which became undetectable earlier than in patients with alanine at pre-S1 84. There were no evident differences between the characteristics of patients with and without substitutions at pre-S2 1 (data not shown). Patients with substitutions at pre-S2 22 were older ($P = 0.003$, Table IV). On the other hand, patients with substitutions in the polymerase tp 95 had increased total bilirubin ($P = 0.049$), ALT values ($P = 0.495$) and α-fetoprotein values ($P = 0.034$, Table V).

Multivariate Analysis to Reveal Independent Factors Predicting Lamivudine Resistance

In an attempt to define independent factors that might predict the emergence of lamivudine resistance, a multivariate analysis using the Cox proportional hazards model was performed. As shown in Table VI, the duration of lamivudine treatment until HBV DNA became undetectable, serum albumin level, pre-S1 84 substitutions or pre-S2 1 and 22 substitutions, and polymerase tp 95 substitution were entered into the analysis. As a result, the pre-S1 84 and pre-S2 1 substitution could be identified as independent variables.

DISCUSSION

In this study, the correlation between pretreatment HBV genomic sequences and the emergence of resistance in patients administered lamivudine to treat chronic HBV infection were investigated. Investigation was focused on determining whether a correlation exists between the viral genome diversity and emergence of lamivudine resistance. This was accomplished by determining the complete nucleotide sequences of HBV genomes amplified from the patients' pretreatment sera. Sequence comparisons

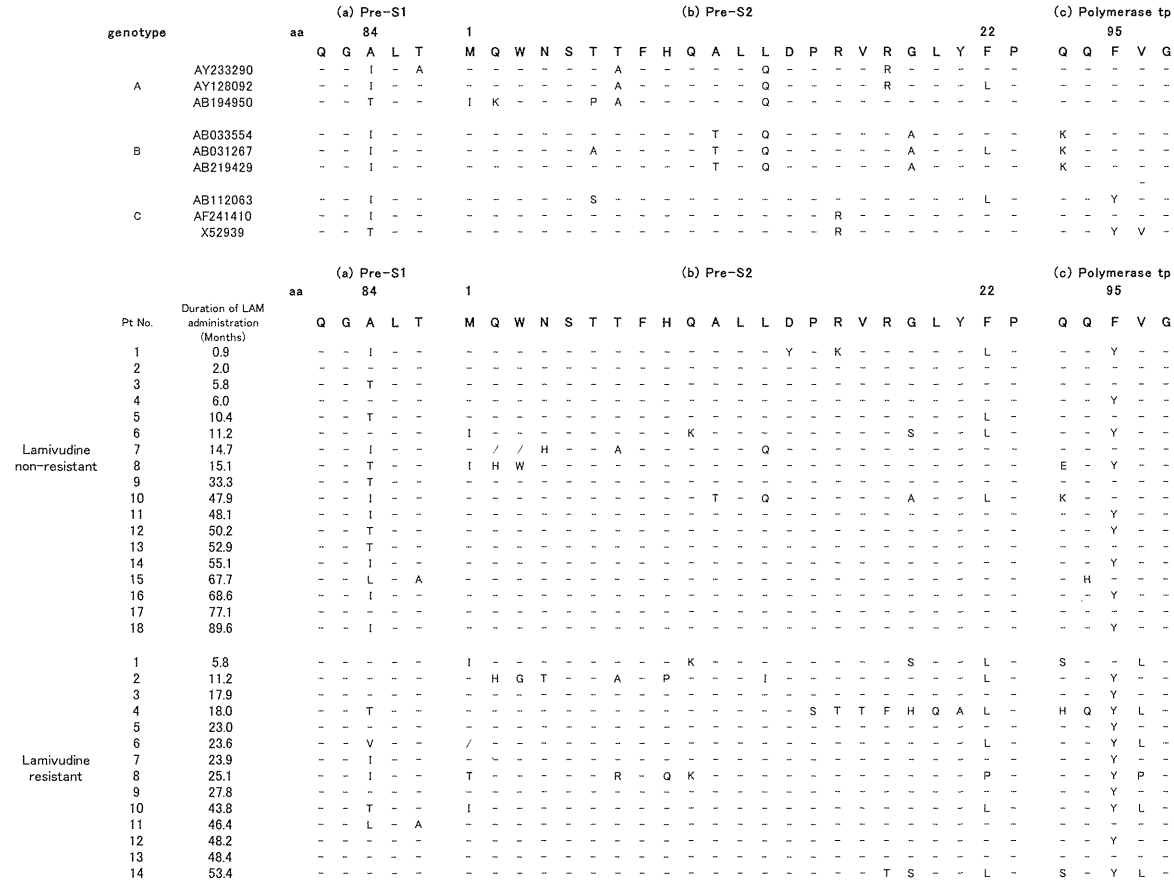


Fig. 4. Amino acid sequence alignment of the pre-S1, pre-S2, and polymerase ORFs associated with the lamivudine resistance. Duration of the LAM administration indicates the period for HBV to become LAM resistant in the resistant group, while it indicates the overall observation period in the non-resistant group. Above the sequences observed in each patient, representative viral sequences of genotype A, B, and C around those areas also are shown to indicate genotype-specific viral amino acids. **a**: Part of pre-S1 ORF. **b**: Part of pre-S2 ORF. **c**: Part of polymerase ORF.

revealed that substitutions in the pre-S1 and pre-S2 ORFs serve as predictors of emergence of lamivudine resistance.

In previous studies reporting the correlation between drug resistance and mutations in the HBV genome, the focus was confined to the HBV polymerase [Ghany and Doo, 2009]. Because the polymerase protein is the direct target of nucleoside analogues, amino acid residue changes in the HBV polymerase are considered to result from selective and mutational pressure exerted by those agents. Therefore, prominent amino acid sequence changes are generally considered to appear during therapy [Kobayashi et al., 2009]. However, the emergence of resistance obviously cannot be predicted by these mutations, and the emergence of resistance usually is predicted by studying clinical factors. Among these conventional pretreatment and in-treatment predictors of lamivudine resistance, it was observed that longer periods of HBV persistence (determined by DNA detection) after

TABLE III. Baseline Clinical Characteristics Classified by the Mutation at Codon 84 in Pre-S1

Clinical factor	Pre-S1 84I/T/L/V (n = 20)	Pre-S1 84A (n = 12)	*P*-value
HBV DNA level Log10 copies/ml [a]	5.75 (±1.38)	6.83 (±0.86)	0.022
Duration of LAM administration until HBV PCR negative (months)[b]	2.1 (0.4–7.6)	4.0 (1.9–69.0)	0.005

[a]Average (±SD) student's t test.
[b]Median (range) Mann–Whitney *U*-test.

TABLE IV. Baseline Clinical Characteristics Classified by the Mutation at Codon 22 in Pre-S2

Clinical factor	Pre-S2 22F (n = 21)	Pre-S2 22L/P (n = 11)	*P*-value
Age, years[a]	50.7 (±9.6)	62.3 (±9.7)	0.003

[a]Average (±SD) Student's *t*-test.

TABLE V. Baseline Clinical Characteristics Classified by the Mutation at tp aa95 in Polymerase

Clinical factor	Polymerase tp 95Y (n = 21)	Polymerase tp 95F (n = 11)	*P*-value
Alanine aminotransferase level, IU/L[a]	52 (13–810)	133 (23–2,739)	0.0495
Total bilirubin, mg/dl[a]	0.9 (0.3–5.0)	1.2 (0.5–19.7)	0.049
α-fetoprotein, ng/ml[a]	8 (1.6–35,194)	81 (4–214.3)	0.034

[a]Median (range) Mann–Whitney *U*-test.

commencing lamivudine therapy correlated with the appearance of resistance, an observation that was consistent with most previous studies [Andersson and Chung, 2009; Zhou et al., 2009]. This demonstrates that studied patients did not represent outliers from random populations studied previously.

Here, amino acid differences between patients were compared, according to their responses to lamivudine treatment, at each position in each viral ORF, and showed that patients who developed resistance accumulated more substitutions within specific regions of the pre-S1, pre-S2, and polymerase ORFs. Thereafter, a statistical analysis was conducted to investigate whether these substitutions correlated with the emergence of drug resistance. It was found that preexisting substitutions in pre-S1 84 and pre-S2 1 correlated significantly and independently with lamivudine resistance. Because the HBV polymerase genes evaluated all encoded rt 204V/I mutations at the time of appearance of lamivudine-resistance, it is considered that the preexisting substitutions in those pre-S regions enabled the later mutation of rt 204V/I in the polymerase gene. On the other hand, although regions of the polymerase gene overlapping with pre-S1 84 and pre-S2 1, 22 genes were evaluated for their association with lamivudine resistance, the corresponding amino acid changes in the polymerase gene did not correlate with lamivudine resistance according to Kaplan–Meier analysis, demonstrating the importance of the pre-S regions in the development of resistance (data not shown). Interestingly, patients with a substitution in pre-S1 84 exhibited high viral loads and displayed longer times until HBV DNA became undetectable compared to patients without this substitution. In contrast, a substitution in pre-S2 22 correlated with increased age, and the substitution in polymerase tp 95 with advanced disease.

TABLE VI. Factors Associated With LAM Resistance Identified by Multivariate Analysis

Variable	Hazard ratio (95% CI)	*P*-value
Duration of LAM administration until HBV PCR negative	1.1 (1.0–1.1)	0.700
Albumin	1.2 (0.6–2.4)	0.682
Pre-S1 84	8.5 (1.5–49.3)	0.017
Pre-S2 1	12.4 (1.1–139.7)	0.041
Pre-S2 22	1.2 (0.2–5.9)	0.833
Polymerase tp 95	0.3 (0.4–32.2)	0.275

CI, confidence interval.
Cox proportional hazards regression.

Although the study was focused on the viral amino acid substitutions, viral nucleotide differences also were compared between patients, according to their responses to lamivudine treatment (data not shown). In this analysis, pretreatment substitutions at nucleotide position 53 in the polymerase/pre-S1 region and at nucleotide position 2151 in the core region correlated significantly with the later appearance of lamivudine resistance. In fact, nucleotide position 53 corresponds to the pre-S1 84, and its substitution causes an amino acid change at pre-S1 84. On the other hand, the substitution at nucleotide position 2152 in the core region is synonymous and the role of this substitution should be investigated in a further study.

The pre-S1/pre-S2/S region encodes the small surface (S), middle (M), and large (L) proteins using alternative codons for the initiation of translation [Gao et al., 2007]. These proteins are considered to have crucial functional roles in the life cycle of HBV [De Meyer et al., 1997; Cooper et al., 2003; Kay and Zoulim, 2007; Watanabe et al., 2007; Lian et al., 2008; Ni et al., 2010]. Apart from the HBV life cycle, recent studies have shown that pre-S sequences significantly impact on the pathogenesis of liver disease [Sugauchi et al., 2003; Zhang et al., 2007; Fang et al., 2008]. The pre-S1 and pre-S2 regions serve as immune targets for T and B cells accumulating in the liver [Bauer et al., 2002], while mutant HBV pre-S epitopes stimulated a lower T cell response than wild-type HBV. HBV with pre-S substitutions leads to cellular retention of viral proteins and a dramatic reduction of virion production [Ni et al., 2010]. The appearance of pre-S substitutions inhibits apoptosis of infected hepatocytes [Ni et al., 2010]. Patients with progressive liver disease or HCC experience a higher frequency of pre-S substitutions or deletions than patients with stable disease [Chaudhuri et al., 2004]. In association with nucleoside analog therapy, Ohkawa et al. [2008] showed the possibility that pre-S2 substitutions might support the replication capacity of lamivudine-resistant HBV.

On the other hand, there have been no previous studies reporting the correlation between pretreatment pre-S substitutions and the development of lamivudine resistance to date. While the mechanisms need further clarification, it is possible to hypothesize a model explaining the correlation, considering these previous findings. Because those previous reports indicate that HBVs with pre-S substitutions function as