Discussion

The main finding of the present study was that resistance to PEG-IFNα-2b+RBV or IFNα-2b+RBV combination therapy in patients with chronic hepatitis C genotype 1b and high viral load was partly influenced by serum albumin, substitutions of amino acids 70 and 91.

We previously showed that serum albumin was a negative predictor of SVR to IFN monotherapy in HCV patients, based on multivariate analysis [12]. Serum proteins including albumin are synthesized by hepatocytes, and falls in their concentrations usually reflect decreased hepatic synthesis although changes in plasma volume could also contribute to such falls. Advanced liver fibrosis is usually associated with decreased hepatic synthesis and low levels of serum albumin [24]. On the other hand, the absence of advanced liver fibrosis is a predictor of SVR to IFN monotherapy and combination therapy of IFN/RBV [11, 25–27]. This report on VRs showed that a milder form of liver fibrosis was not a positive predictor of response to combination therapy, compared with high levels of serum albumin [24]. These discrepant findings may be due to one or more factors. The first reason is probably related to the method used for evaluation; the degree of liver fibrosis roughly reflects liver function but can only be assessed using a three-stage (F1, F2, F3) system, in contrast to the serum albumin level. Thus, serum albumin might reflect liver function more sensitively than the degree of liver fibrosis. Furthermore, this finding showed that the ability of the liver to synthesize serum proteins including albumin might contribute to the observed response to treatment more than the degree of liver fibrosis. The second reason is probably related to the design of our study based on comparison between a virological and a non-virological response, rather than a SVR and a non-SVR. Our study based on multivariate analysis is the first to identify serum albumin as a predictor of a non-virological response in patients on 48-week IFN/RBV combination therapy.

IFN-α and IFN-β bind to the type-I IFN receptor, and one major pathway in type-I IFN signaling involves the Jak-STAT signaling cascade [13, 28–37]. Previous studies reported that the HCV core region might be associated with resistance to the antiviral actions of IFN therapy. Blindenbacher et al. [14] showed that STAT signaling was strongly inhibited in liver cells of HCV core transgenic mice. Bode et al. [15] showed that HCV core protein induced the expression of the suppressor of cytokine signaling-3 and inhibited activation, tyrosine phosphorylation, and nuclear translocation of STAT1, which might impair the antiviral actions of IFNs in HepG2 cells. Furthermore, Mélen et al. [16] indicated that IFN-induced nuclear accumulation of STAT1 was almost completely blocked and STAT2 was partially blocked in cell lines expressing high levels of HCV core protein. Our study identified amino acid substitutions in HCV core as a predictor of a non-virological response to 48-week IFN/RBV combination therapy based on multivariate analysis. This result suggests that substitutions of amino acids in the HCV core region might be associated with resistance to the antiviral actions of IFN therapy involving the Jak-STAT signaling cascade.

Since combination therapy could induce hemolytic anemia and possibly other major side effects [6], it is important to identify resistant patients, especially NVRs among non-sustained virological responders, early during therapy with the intent of revising the treatment regimen. In fact, we were able to revise or terminate treatment before completion of the full course of combination therapy for 48 weeks and spare patients from receiving unnecessary treatment based on consideration of risks/benefits. Our study indicated that falls in HCV-RNA levels from baseline were significantly lower in patients with specific pretreatment amino acid substitution patterns in the HCV core. Thus, our study identified pretreatment virological features associated with early viral kinetics during combination therapy with IFN/RBV. Further studies are required to explore the relationship between virological features and differences in viral kinetics.

In conclusion, our results suggest that albumin levels and amino acid substitution patterns in the core region in patients with a high titer of HCV genotype 1b might determine a non-virological response to combination therapy. One limitation of this study was that we did not examine other viral factors, such as amino acid substitutions in areas other than the core region and ISDR of HCV genome, as well as other host factors such as IFN-inducible protein kinase, MxA and 2′,5′-OAS protein [28–31, 37–42], although they should be investigated together with other factors in future studies. Moreover, further large-scale prospective studies are necessary to investigate whether our results also explain resistance to IFN-RBV combination therapy.

Acknowledgment

This study was supported in part by a grant-in-aid from the Ministry of Health, Labor and Welfare, Japan.

References

1 Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hurter D, Nawrocki M, Kruska L, Hensel F, Petry W, Haussinger D: Prognosis of chronic hepatitis C: Results of a large, prospective cohort study. Hepatology 1998;28: 1687–1695.

2 Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Koida I, Arase Y, Fukuda M, Chayama K, Murashima N, Kumada H: Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: A prospective observation of 2215 patients. J Hepatol 1998;28:930–938.

3 Kenny-Walsh E: Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. N Engl J Med 1999;340:1228–1233.

4 Akuta N, Chayama K, Suzuki F, Someya T, Kobayashi M, Tsubota A, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Kumada H: Risk factors of hepatitis C virus-related liver cirrhosis in young adults: Positive family history of liver disease and transporter associated with antigen processing 2 (TAP2) *0201 allele. J Med Virol 2001;64:109–116.

5 Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Nakamura I, Murashima N, Kumada H, Kawanishi M: Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: A long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. Hepatology 1999;29:1124–1130.

6 Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling MH, Albrecht JK: Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomized trial: Lancet 2001;358:958–965.

7 Fried MW, Shiffman ML, Reddy R, Smith C, Marinos G, Gonçales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J: Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–982.

8 Kanai K, Kako M, Okamoto H: HCV genotypes in chronic hepatitis C and response to interferon. Lancet 1992;339:1543.

9 Hino K, Sainokami S, Shimoda K, Iino S, Wang Y, Okamoto H, Miyakawa Y, Mayumi M: Genotypes and titers of hepatitis C virus for predicting response to interferon in patients with chronic hepatitis C. J Med Virol 1994;42: 299–305.

10 Mahaney K, Tedeschi V, Maertens G, Di Bisceglie AM, Vergalla J, Hoofnagle JH, Sallie R: Genotypic analysis of hepatitis C virus in American patients. Hepatology 1994;20: 1405–1411.

11 Tsubota A, Chayama K, Ikeda K, Arase Y, Koida I, Saitoh S, Hashimoto M, Iwasaki S, Kobayashi M, Kumada H: Factors predictive of response to interferon-α therapy in hepatitis C virus infection. Hepatology 1994;19:1088–1094.

12 Akuta N, Suzuki F, Tsubota A, Suzuki Y, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H: Efficacy of interferon monotherapy to 394 consecutive naive cases infected with hepatitis C virus genotype 2a in Japan: Therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. J Hepatol 2002;37: 831–836.

13 Lalvakolanu DV: Alternate interferon signaling pathways. Pharmacol Ther 2003;100:1–29.

14 Blindenbacher A, Duong FH, Hunziker L, Stutvoet ST, Wang X, Terracciano L, Moradpour D, Blum HE, Alonzi T, Tripodi M, La Monica N, Heim MH: Expression of hepatitis C virus proteins inhibits interferon α signaling in the liver of transgenic mice. Gastroenterology 2003;124:1465–1475.

15 Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, Heinrich PC, Häussinger D: IFN-α antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J 2003;17:488–490.

16 Melén K, Fagerlund R, Nyqvist M, Keskinen P, Julkunen I: Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. J Med Virol 2004;73:536–547.

17 Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C: Comparison of full-length sequences of interferon sensitive and resistant hepatitis C virus 1b. J Clin Invest 1995;96: 224–230.

18 Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C: 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.

19 Chayama K, Tsubota A, Arase Y, Saitoh S, Koida I, Ikeda K, Matsumoto T, Kobayashi M, Iwasaki S, Koyama S, Morinaga T, Kumada H: Genotypic subtyping of hepatitis C virus. J Gastroenterol Hepatol 1993;8:150–156.

20 Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ: Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 1994;19:1513–1520.

21 D'Alessandro AM, Kalayouglu M, Sollinger HW, Hoffmann RM, Reed A, Knechtle SJ, Pirsch JD, Hafez GR, Lorentzen D, Belzer FO: The predictive value of donor liver biopsies for the development of primary nonfunction after orthotopic liver transplantation. Transplantation 1991;51:157–163.

22 Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K: Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 1990;87:9524–9528.

23 Kwok S, Higuchi R: Avoiding false positives with PCR. Nature 1989;339:237–238.

24 Sherlock SH, Dooley JA: Disease of the Liver and Biliary System, ed 9. London, Blackwell Scientific, 1992.

25 Jouet P, Roudot-Thoraval F, Dhumeaux D, Metreau JM: Comparative efficacy of interferon alfa in cirrhotic and noncirrhotic patients with non-A, non-B, C hepatitis. Le Groupe Français pour l'Etude du Traitement des Hépatites Chroniques NANB/C. Gastroenterology 1994;106:686–690.

26 Poynard T, McHutchinson J, Goodman Z, Ling MH, Albrecht J: Is an 'a la carte' combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? The ALGOVIRC Group. Hepatology 2000;31: 211–218.

27 Bruno S, Camma C, Di Marco V, Rumi M, Vinci M, Camozzi M, Rebucci C, Di Bona D, Colombo M, Craxi A, Mondelli MU, Pinzello G: Peginterferon alfa-2b plus ribavirin for naïve patients with genotype 1 chronic hepatitis C: A randomized controlled trial. J Hepatol 2004;41:474–481.

28 Chang KC, Hansen E, Foroni L, Lida J, Goldspink G: Molecular and functional analysis of the virus- and interferon-inducible human MxA promoter. Arch Virol 1991;117:1–15.

29 Ronni T, Matikainen S, Lehtonen A, Palvimo J, Dellis J, Van Eylen F, Goetschy JF, Horisberger M, Content J, Julkunen I: The proximal interferon-stimulated response elements are essential for interferon responsiveness: A promoter analysis of the antiviral MxA gene. J Interferon Cytokine Res 1998;18:773–781.

30 Benech P, Vigneron M, Peretz D, Revel M, Chebath J: Interferon-responsive regulatory elements in the promoter of the human 2′,5′-oligo(A) synthetase gene. Mol Cell Biol 1987; 7:4498–4504.

31 Wang Q, Floyd-Smith G: The p69/71 2–5A synthetase promoter contains multiple regulatory elements required for interferon-alpha-induced expression. DNA Cell Biol 1997;16: 1385–1394.

32 Auernhammer CJ, Melmed S: The central role of SOCS-3 in integrating the neuro-immunoendocrine interface. J Clin Invest 2001;108: 1735–1740.

33 Fujimoto M, Naka T: Regulation of cytokine signaling by SOCS family molecules. Trends Immunol 2003;24:659–666.

34 Song MM, Shuai K: The suppressor of cytokine signaling (SOCS) 1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferative activities. J Biol Chem 1998;273:35056–35062.

35 Stoiber D, Kovarik P, Cohney S, Johnston JA, Steinlein P, Decker T: Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-gamma. J Immunol 1999;163:2640–2647.
36 Alexander WS: Suppressors of cytokine signaling (SOCS) in the immune system. Nat Rev Immunol 2002;2:410–416.
37 Vlotides G, Sörensen AS, Kopp F, Zitzmann K, Cengic N, Brand S, Zachoval R, Auernhammer CJ: SOCS-1 and SCOS-3 inhibit IFN-α-induced expression of the antiviral 2,5-OAS and MxA. Biochem Biophys Res Commun 2004;320:1007–1014.
38 Akuta N, Suzuki F, Tsubota A, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H: Association of amino acid substitution pattern in nonstructural protein 5A of hepatitis C virus genotype2a low viral load and response to interferon monotherapy. J Med Virol 2003;69:376–383.
39 Gale MJ, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG: Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 1997;230:217–227.
40 Chieux V, Hober D, Harvey J, Lion G, Lucidarme D, Forzy G, Duhamel M, Cousin J, Ducoulombier H, Wattre P: The MxA protein levels in whole blood lysates of patients with various viral infections. J Virol Methods 1998;70:183–191.
41 Fernandez M, Quiroga JA, Martin J, Ierrero M, Pardo M, Horisberger MA, Carreno V: In vivo and in vitro induction of MxA protein in peripheral blood mononuclear cells from patients chronically infected with hepatitis C virus. J Infect Dis 1999;180:262–267.
42 Antonelli G, Simeoni E, Turriziani O, Tesoro R, Redaelli A, Roffi L, Antonelli L, Pistello M, Dianzai F: Correlation of interferon-induced expression of MxA mRNA in peripheral blood mononuclear cells with the response of patients with chronic hepatitis C to IFN-α therapy. J Interferon Cytokine Res 1999;19:243–251.

Journal of Viral Hepatitis, 2005, 12, 300–306 doi:10.1111/j.1365-2893.2005.00633.x

The efficacy and safety of thymosin alpha-1 in Japanese patients with chronic hepatitis B; results from a randomized clinical trial

S. Iino[1,2], J. Toyota,[3] H. Kumada,[4] K. Kiyosawa,[5] S. Kakumu,[6] M. Sata,[7] H. Suzuki[8] and E. B. Martins[9] [1]*Department of Gastroenterology, St Marianna University, School of Medicine, Kawasaki, Kanagawa, Japan;* [2]*Research Center for Liver Diseases, Kiyokawa Medical Center, Tokyo, Japan;* [3]*Department of Gastroenterology, Sapporo-kosei General Hospital, Sapporo, Hokkaido, Japan;* [4]*Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan;* [5]*Department of Internal Medicine, Gastroenterology, School of Medicine, Shinshu University, Matsumoto, Nagano, Japan;* [6]*GI Division, Department of Internal Medicine, Aichi Medical University School of Medicine, Aichi, Japan;* [7]*Department of 2nd Internal Medicine, School of Medicine, Kurume University, Kurume, Fukuoka, Japan;* [8]*Yamanashi University, Yamanashi, Japan; and Viral Hepatitis Research Institute, Miyakawa Memorial Research Foundation, Tokyo, Japan; and* [9]*Medical Department, SciClone Pharmaceuticals, Inc., San Mateo, CA, USA*

Received April 2004; accepted for publication October 2004

SUMMARY. Thymalfasin (thymosin alpha-1; Tα1) is a 28-amino acid polypeptide that has shown efficacy in the treatment of chronic hepatitis B virus (HBV) infection. The objective of this study was to evaluate the long-term, dose-related efficacy and safety of Tα1 treatment in chronic hepatitis B patients with positive HBV-DNA and abnormally high alanine aminotransferase (ALT) levels. A total of 316 patients were randomized to receive either 0.8 or 1.6 mg of Tα1 monotherapy for 24 weeks. At the end of the 72-week observation period (12 months after cessation of therapy), 36.4% of patients in the 1.6-mg treatment group achieved normalization of ALT, 30% achieved clearance of HBV-DNA by branched DNA *vs* 15% by transcription-mediated amplification, and 22.8% achieved clearance of HBe-antigen. Patients in the 0.8-mg treatment group achieved similar efficacy rates, although patients with advanced fibrosis demonstrated a significantly better response rate when treated with 1.6 mg of Tα1 monotherapy *vs* 0.8 mg (as determined by intragroup analysis; patients were not stratified by liver biopsy). All adverse drug reactions were mild and most involved the fluctuation of liver enzymes, which was most likely related to the positive immune effects caused by the response to Tα1 treatment. Adverse event incidence was similar in the 1.6- and 0.8-mg treatment groups. In conclusion, Tα1 at doses of 0.8 and 1.6 mg exhibits long-term efficacy against hepatitis B with a good safety profile.

Keywords: chronic hepatitis B, thymalfasin, thymosin alpha-1.

INTRODUCTION

Chronic hepatitis B affects nearly 350 million people worldwide and is a leading cause of liver cirrhosis and hepatocellular carcinoma [1–3]. Early and effective intervention may help terminate hepatitis B virus (HBV) replication and promote long-term disease remission.

Over the last three decades, research has focused on the development of antiviral and immunomodulatory therapies to treat patients with HBV. Currently, interferon alpha and lamivudine are two widely used therapies. Interferon alpha has reasonably good efficacy with initial response rates of 30–40% compared with 10–20% among untreated controls. However, of those who responded to interferon alpha therapy, 56% relapsed within the first year after discontinuation of therapy (median 3.1 months) [4]. In addition, interferon alpha has a poor side-effect profile, leading to inadequate compliance and frequent need for dose reduction [3–5]. Once-daily lamivudine rapidly produces a suppression of HBV-DNA replication [6,7]. However, approximately 90% of

Abbreviations: ALT, alanine aminotransferase; anti-HBe, hepatitis B e-antibody; bDNA, branched DNA; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; MHC, major histocompatibility complex; NK, natural killer; Tα1, thymosin alpha-1; TMA, transcription-mediated amplification.

Correspondence: Eduardo B. Martins MD, DPhil, SciClone Pharmaceuticals, 901 Mariner's Island Blvd., San Mateo, CA 94404, USA. E-mail: emartins@sciclone.com

patients relapse once therapy is stopped [8]. Adefovir dipivoxil, a nucleotide analogue, is also capable of rapidly inducing suppression of HBV-DNA, but long-term efficacy is in question because of low rates of hepatitis B e-antigen (HBeAg) seroconversion [9]. Moreover, adefovir-resistant mutations have also been reported [10]. Therefore, the development of new therapeutic agents with long-term efficacy is needed to reduce morbidity and mortality rates among patients with chronic hepatitis B.

Thymalfasin (thymosin alpha-1, Tα1) is an immune-modulating peptide that has been shown to enhance Th1 cytokine production as well as T-cell differentiation and maturation [11]. Several clinical studies have shown that treatment with Tα1 monotherapy results in significantly higher sustained response rates when compared with controls [12–18] and exhibits no significant side effects [14–19]. Moreover, complete virological response tends to increase or accumulate gradually after the cessation of Tα1 therapy [14,17].

Tα1 therapy is used in many countries worldwide for the treatment of chronic hepatitis B. This study evaluates the dose-related efficacy and safety of Tα1 in Japanese chronic HBV patients.

METHODS

This 72-week multicentre, randomized study investigated the safety and efficacy of Tα1 at two different doses. A total of 316 Japanese patients with chronic hepatitis B from 49 medical institutions in Japan were randomized to receive either 0.8 or 1.6 mg of Tα1 monotherapy six times a week for the first 2 weeks, and then twice a week for the subsequent 22 weeks. Efficacy was determined by clinical test values of alanine aminotransferase (ALT), HBV-DNA, HBeAg and hepatitis B e-antibody (anti-HBe) during 24 weeks of Tα1 administration and during the 48-week follow-up period. For the determination of HBV-DNA level by branched DNA (bDNA), a Quantiplex HBV-DNA kit was used (standard value: <0.70 Meq/mL; Daiichi Pure Chemicals Co, Ltd, Tokyo, Japan). During the course of the study, the more sensitive transcription-mediated amplification (TMA) assay became available for the determination of HBV-DNA level. Thus, HBV-DNA level was also tested by TMA using an HBV Amplify Standard & Luminescent reagent kit DNA probe (standard value: <3.7 LGE/mL; Chugai Diagnostic Science Co, Ltd, Tokyo, Japan). SAS™ (SAS Institute Inc., Cary, NC, USA) software was used for statistical analyses. Changes in HBeAg and anti-HBe levels were assessed by the chi-squared test; changes in the ALT and HBV-DNA levels were assessed by Mann–Whitney *U*-test. The two-tailed significance level was set at 5%, and multiplicity was not considered. This study was conducted in compliance with current GCP and with the Declaration of Helsinki, and was approved by the institutions' Ethics Committees.

Eligible patients included men and women ≥18 years of age who were HBV-DNA positive, HBeAg positive with elevated ALT, and with histologically diagnosed chronic hepatitis confirmed by liver biopsy taken within 48 weeks before the start of treatment. The concomitant use of glycyrrhizin, propagermanium, systemic glucocorticoids, interferon or lamivudine was prohibited.

RESULTS

Analysis of safety was performed on 310 patients, and analysis of efficacy was performed with results from 284 patients, excluding those with protocol violations.

As shown in Table 1, patient groups were similar in all respects except with regard to the degree of liver disease on entry. Due to a lack of stratification based on liver histology, the 1.6-mg treatment group had a higher ratio of advanced fibrosis (bridging fibrosis with lobular distortion, stage F3; $P = 0.018$) and inflammation (severe necro-inflammatory

Table 1 Baseline characteristics of the patients

	Group 1 (0.8 mg) (%)	Group 2 (1.6 mg) (%)	*P*-value
Age (years)			
Mean ± SD	36.6 ± 9.9	37.3 ± 10.6	0.545
n	139	144	
Gender			
Male	95 (68.3)	109 (75.7)	
Female	44 (31.7)	35 (24.3)	0.168
New Inuyama classification (fibrosis staging)			
F0	5 (3.6)	3 (2.1)	
F1	61 (43.9)	54 (37.5)	
F2	44 (31.7)	36 (25.0)	
F3	22 (15.8)	46 (31.9)	0.018
Unknown	7 (5.0)	5 (3.5)	
New Inuyama classification (activity grading)			
A0	3 (2.2)	4 (2.8)	
A1	63 (45.3)	39 (27.1)	
A2	50 (36.0)	73 (50.7)	
A3	14 (10.1)	21 (14.6)	0.010
Unknown	9 (6.5)	7 (4.9)	
History of IFN therapy			
No	92 (66.2)	80 (55.6)	
Yes	47 (33.8)	64 (44.4)	0.067
ALT level (IU/L)			
Mean ± SD	124.6 ± 129.50	144.5 ± 143.20	0.148
HBV-DNA level by TMA (LGE/mL)			
Mean ± SD	6.96 ± 1.28	6.90 ± 1.20	0.499
HBV-DNA level by bDNA (mEq/mL)			
Mean ± SD	578.7 ± 1038.00	662.6 ± 1132.00	0.792

Table 2 Response to thymosin alpha-1 therapy

	Group/Dose	24 Weeks (end of therapy) *n* (%)	72 Weeks (end of follow-up) *n* (%)
ALT	Group 1/0.8 mg	33/134 (24.6)	38/118 (32.2)
	Group 2/1.6 mg	37/137 (27)	43/118 (36.4)
HBV-DNA (−) (bDNA)	Group 1/0.8 mg	20/115 (17.4)	24/93 (25.8)
	Group 2/1.6 mg	20/117 (17.1)	27/90 (30)
HBV-DNA (−) (TMA)	Group 1/0.8 mg	8/129 (6.2)	14/104 (13.5)
	Group 2/1.6 mg	7/129 (5.4)	15/100 (15)
HBeAg (−) (seronegative)	Group 1/0.8 mg	4/103 (3.9)	18/80 (22.5)
	Group 2/1.6 mg	5/104 (4.8)	18/79 (22.8)
HBe (−) and Anti-HBe (+) (seroconversion)	Group 1/0.8 mg	4/103 (3.9)	15/80 (18.8)
	Group 2/1.6 mg	5/104 (4.8)	17/79 (21.5)

reaction, grade A3; $P = 0.01$) patients according to the New Inuyama classification for histopathological scoring of the liver [20].

Tα1 monotherapy exhibited equal efficacy when administered at either 0.8 or 1.6 mg, as shown in Table 2. The results in the 0.8-mg group and the 1.6-mg group, respectively, at 72 weeks showed that the rate of normalization of ALT was 32 and 36% ($P > 0.05$); clearance of HBV-DNA by the bDNA test was 26 and 30% ($P > 0.05$), and by TMA 14 and 15% ($P > 0.05$); clearance of HBeAg was 23 and 23% ($P > 0.05$); and the appearance of anti-HBe at 72 weeks was 19 and 22% ($P > 0.05$). At 72 weeks from baseline, both the 0.8- and 1.6-mg treatment groups showed significant improvement in ALT, HBV-DNA and anti-HBe levels, as shown in Fig. 1.

Evaluation of within-group progress demonstrated that patients with advanced fibrosis (stage F3) did show significant improvements in all HBV markers at 24 weeks when treated with 1.6 mg of Tα1 monotherapy *vs* 0.8 mg (Fig. 2). For these patients, changes in baseline ALT

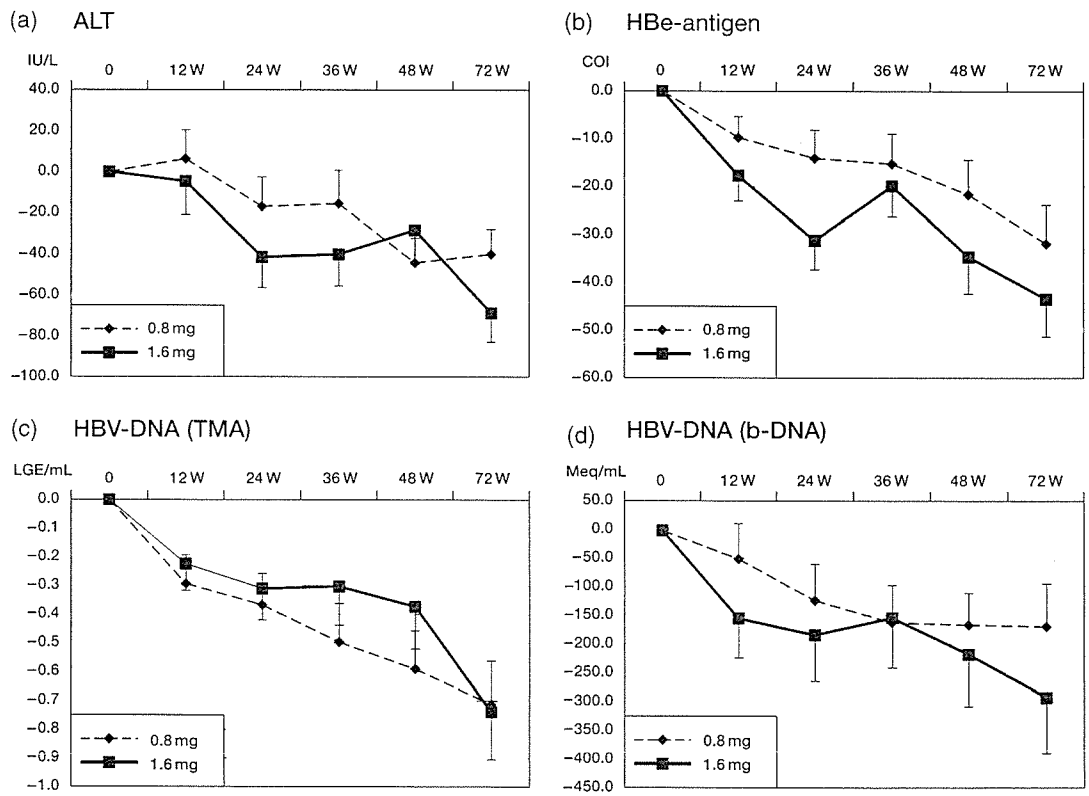

Fig. 1 Reduction from baseline in serum levels of ALT (a), HBeAg (b), HBV-DNA by TMA (c) and HBV-DNA by bDNA (d) for all patients in both treatment arms. All values are expressed as mean ± standard error (SE).

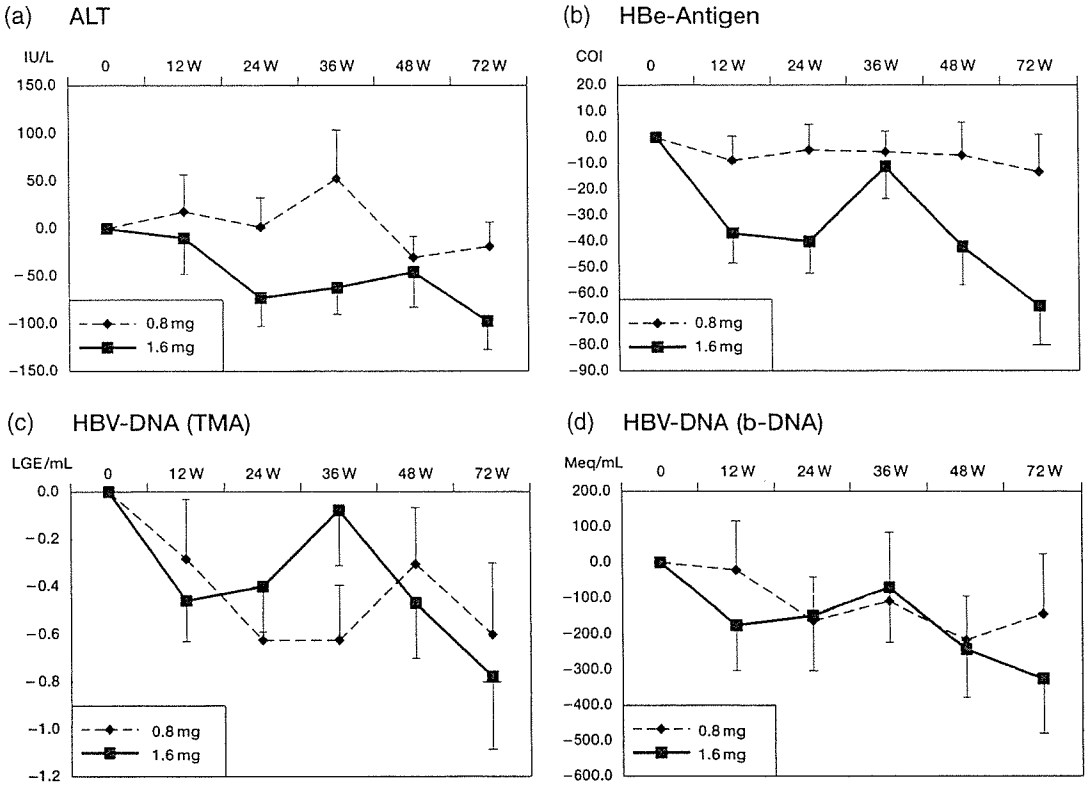

Fig. 2 Reduction from baseline in serum levels of ALT (a), HBeAg (b), HBV-DNA by TMA (c) and HBV-DNA by bDNA (d); stratified for patients with F3 fibrosis. All values are expressed as mean ± standard error (SE).

($P = 0.03$) and HBeAg ($P < 0.01$) levels were sustained at 72 weeks and were statistically superior in the 1.6-mg treatment group.

In the 310 patients followed up for 72 weeks, 1077 adverse events were reported, most of which were unrelated to the study drug. Of the total adverse events, 377 (38.7%) were considered possibly related to Tα1 and occurred in 120 patients (Table 3). There were 22 cases of transient exacerbation of liver function (11 cases in each dose group), which were classified as ALT flares and assumed to be associated with the immunomodulating action of Tα1. One patient had two flares during the 72-week period. Onset of the flares occurred from 2 to 64 weeks (median 19 weeks) from the start of treatment. All patients who experienced flares recovered uneventfully and there were no cases of death because of liver failure. Over the 72 weeks, only three (0.28%) adverse events were considered to be serious; one patient developed bile duct cholangiocarcinoma and two patients (0.43%) developed hepatocellular carcinoma. None of these three serious adverse events were considered to be due to Tα1. Between the two treatment groups, there were no statistical differences in the incidences, symptoms, or severity of adverse events.

Table 3 Incidence of adverse events

Variable*	Group 1 (0.8 mg)	Group 2 (1.6 mg)
Malaise	36 (23.8)	40 (26.5)
Nausea	14 (9.3)	16 (10.6)
Headache	12 (7.9)	18 (11.9)
Abdominal discomfort or pain	10 (6.6)	15 (9.9)
Anorexia	5 (3.3)	15 (9.9)
ALT elevation	11 (7.3)	11 (7.2)
AST elevation	5 (3.3)	10 (6.6)

*The adverse events shown are those that occurred in at least 5% of the patients in a treatment group. Although these adverse events were probably related to the hepatitis B, they were considered to be possibly related to thymosin alpha-1.

DISCUSSION

Tα1 is a 28-amino acid polypeptide which was originally isolated from bovine thymus extract (thymosin fraction 5) and is now chemically synthesized [21]. Tα1 treatment leads to the inhibition of chronic viral infection through a mechanism of cellular immune response modulation via an increase in the secretion of interferon-alpha, interferon-gamma, and cytokines such as IL-2, IL-3, and the differentiation and maturation of T cells [11,19]. Tα1 also increases

T-cell populations by blocking apoptosis [22] and increases natural killer (NK) cell activity in multiple animal models and normal human subjects [11]. In addition, Tα1 has direct antiviral properties as well as increasing the expression of major histocompatibility complex (MHC) class 1 molecules on infected cells [23].

Tα1 has been clinically used as a 6-month therapy for chronic hepatitis B in many studies. Zavaglia *et al.* [12] reported that the rate of HBV-DNA clearance after treatment with Tα1 (as determined by liquid phase hybridization) was 23% at 20 months. Mutchnick *et al.* [13] reported that the rate of HBeAg clearance was 23% and HBV-DNA (liquid phase hybridization) clearance was 20% in 49 cases at the end of a 6-month follow-up period. Similarly, Chien *et al.* [14] reported that the rate of HBeAg and HBV-DNA (liquid phase hybridization) clearance was 40% in 32 cases evaluated at 12 months of post-treatment follow-up. Interestingly, another study confirmed that the effectiveness of Tα1 appeared to increase after the completion of drug administration, especially at 12 months post-treatment [24].

In this randomized, multicentre study of chronic hepatitis B patients in Japan, Tα1 was administered at a dose of 0.8 or 1.6 mg twice weekly for 24 weeks, and a long-term observation was conducted at 72 weeks (12 months after cessation of therapy). Even though many of the patients in this study were considered difficult-to-treat (32% had advanced liver fibrosis and 44% were previously unresponsive to interferon therapy), treatment with Tα1 at a dose of 1.6 mg for 6 months resulted in significant improvements in ALT, HBV-DNA and HBeAg. Therefore, this study demonstrates the efficacy of Tα1 treatment.

There were no statistically significant differences in treatment efficacy with 0.8 or 1.6 mg of Tα1 monotherapy. However, patients were not stratified by liver biopsy, which may have influenced these results. A stratified, intragroup analysis demonstrated that patients with more serious disease exhibited superior results when treated with 1.6 mg *vs* 0.8 mg of Tα1. At 72 weeks, changes from baseline ALT and HBeAg levels were also statistically superior in the 1.6 mg treatment group. Therefore, it is suggested that the higher dose of 1.6 mg for 24 weeks be administered, especially in the case of advanced fibrosis.

Historical comparison suggests that Tα1 and conventional interferon therapies have similar efficacy, and that both are superior to placebo. Japanese patients who received interferon alpha-2a for 6 months had response rates for normalization/clearance at 24–48 weeks after completion of therapy of: 41.6% (10 of 24) for ALT; 27.8% (five of 18) for HBV-DNA (bDNA); and 15% (three of 20) for HBeAg [25]. Regarding HBV-DNA and HBeAg, although response rates are decreasing with the availability of increasing assay sensitivity from advances in assay methods, response rates are still considered to be similar to those reported by Iino *et al.* [25] when evaluated at 12–18 months after the start of interferon administration. With Tα1 therapy, the rates of clearance of HBV-DNA and HBeAg have the tendency to increase with time, even after completion of therapy [24], whereas there is a recurrence of chronic hepatitis B after completion of interferon therapy [4,5,26]. Similar positive results to Tα1 therapy were demonstrated in additional studies evaluating the efficacy of longer-term treatment with interferon therapy in Japan [25,27–31]. In contrast, the results for HBeAg clearance and seroconversion were only 15 and 5%, respectively, in trials where Japanese patients received placebo for 24 weeks [32,33].

Once-daily lamivudine is another therapy for the treatment of hepatitis B that rapidly produces a beneficial reduction in viral DNA [6,7]; however, approximately 90% of patients relapse once therapy is stopped [8]. In addition, lamivudine-resistant YMDD mutations are common and increase over time – from 14% at 1 year to 38% at 2 years and to 69% at 5 years [34]. Sustained biochemical and virological response rates tend to decrease over time because of the development of this drug resistance. In addition, deterioration of liver function and histology has been demonstrated in patients who develop YMDD mutations [34]. HBV, therefore, does not respond well to lamivudine therapy [35,36]. By contrast, treatment with Tα1 exhibited cumulative improvements, even after the completion of therapy, and no Tα1-resistant mutations have been reported [24].

In this study, the rate of progression to hepatocellular carcinoma was calculated to be 0.43% per year; however, the period of observation was too short to compare with the previously observed rates of 4.9% in 5 years and 6.6–7.7% in 10 years in non-treated patients [37,38]. In addition, the high prevalence of patients with advanced disease may have facilitated the appearance of the two cases of hepatocellular carcinoma seen in our study. ALT flares were seen in 22 patients and therapy with Tα1 was interrupted in 16, but all the patients recovered or had their flares managed by hospitalization. In fact, in the natural progression of chronic hepatitis B, transient exacerbations of liver function are commonly seen [39–41]. It has been suggested that the ALT flares are an essential component of natural remission. Therefore, a temporary elevation of ALT may occur in the course of therapy using a drug with a mechanism of intensifying the immune system and accelerating natural remission, such as Tα1 or interferon. Overcoming this exacerbation of liver function is an important part of the eventual therapeutic effect. When the exacerbation in liver function is observed during therapy, the patient should be checked for liver failure by evaluating bilirubin and prothrombin. As long as these values are acceptable, therapy should be continued.

Studies of concomitant Tα1 and interferon therapy are ongoing. A study by Saruc *et al.* [42] compared the outcomes of Tα1 and interferon alpha-2b combination therapy ($n = 27$) with lamivudine and interferon alpha-2b combination therapy ($n = 15$) in patients with HBeAg-negative chronic hepatitis B. At 26 weeks post-therapy, 74% of

patients treated with Tα1 plus interferon alpha-2b achieved a sustained response, defined as a loss of HBV-DNA and normalization of ALT, *vs* 53.3% of patients treated with lamivudine and interferon alpha-2b combination therapy. At 18 months post-therapy, the sustained response rates were 70% in the Tα1 plus interferon alpha-2b treated patients *vs* only 20% in the lamivudine alpha-2b treated patients [42]. More controlled trials with a longer duration of follow-up are needed to adequately evaluate the efficacy and safety of these novel combination therapies.

In conclusion, the results from the present study suggest that Tα1 therapy exhibits long-term efficacy against chronic hepatitis B, with no significant adverse effects. Tα1 leads to the normalization of ALT level and clearance of HBV-DNA and HBeAg at response rates similar to those seen in previous studies after treatment with interferon. The efficacy was dose-dependent for patients with advanced fibrosis, with a statistically significant superiority of the 1.6 mg over the 0.8 mg dose. Therefore, the administration of Tα1 at a dose of 1.6 mg may become a new safe and effective therapeutic option for difficult-to-treat hepatitis B patients.

REFERENCES

1 Rasi G, Mutchnick MG, Di Virgilio D *et al.* Combination low-dose lymphoblastoid interferon and thymosin alpha 1 therapy in the treatment of chronic hepatitis B. *J Viral Hep* 1996; 3: 191–196.

2 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733–1745.

3 Conjeevaram HS, Lok AS. Management of chronic hepatitis B. *J Hepatol* 2003; 38(Suppl. 1): S90–S103.

4 Manesis EK, Hadziyannis SJ. Interferon alpha treatment and retreatment of hepatitis B e antigen-negative chronic hepatitis B. *Gastroenterology* 2001; 121: 101–109.

5 Liaw YF. Therapy of chronic hepatitis B: current challenges and opportunities. *J Viral Hepat* 2002; 9: 393–399.

6 Dienstag JL, Schiff ER, Wright TL *et al.* Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; 341: 1256–1263.

7 Tassopoulos NC, Volpes R, Pastore G *et al.* Efficacy of lamivudine in patients with hepatitis B e antigen-negative/hepatitis B virus DNA-positive (precore mutant) chronic hepatitis B. Lamivudine Precore Mutant Study Group. *Hepatology* 1999; 29: 889–896.

8 Santantonio T, Mazzola M, Iacovazzi T, Miglietta A, Guastadisegni A, Pastore G. Long-term follow-up of patients with anti-HBe/HBV DNA-positive chronic hepatitis B treated for 12 months with lamivudine. *J Hepatol* 2000; 32: 300–306.

9 Marcellin P, Chang TT, Lim SG *et al.* Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003; 348: 808–816.

10 Xiong S, Yang H, Westland C *et al.* Resistance surveillance of HBEAg-chronic hepatitis B patients treated for two years with adefovir dipivoxil. *J Hepatol* 2003; 38: 182.

11 Rasi G, Pierimarchi P, Sinibaldi Vallebona P, Colella F, Garaci E. Combination therapy in the treatment of chronic viral hepatitis and prevention of hepatocellular carcinoma. *Int Immunopharmacol* 2003; 3: 1169–1176.

12 Zavaglia C, Severini R, Tinelli C *et al.* A randomized, controlled study of thymosin-alpha1 therapy in patients with anti-HBe, HBV-DNA-positive chronic hepatitis B. *Dig Dis Sci* 2000; 45: 690–696.

13 Mutchnick MG, Lindsay KL, Schiff ER *et al.* Thymosin alpha1 treatment of chronic hepatitis B: results of a phase III multicentre, randomized, double-blind and placebo-controlled study. *J Viral Hepat* 1999; 6: 397–403.

14 Chien RN, Liaw YF, Chen TC, Yeh CT, Sheen IS. Efficacy of thymosin alpha1 in patients with chronic hepatitis B: a randomized, controlled trial. *Hepatology* 1998; 27: 1383–1387.

15 Mutchnick MG, Appelman HD, Chung HT *et al.* Thymosin treatment of chronic hepatitis B: a placebo-controlled pilot trial. *Hepatology* 1991; 14: 409–415.

16 You J, Zhuang L, Tang BZ *et al.* A randomized controlled clinical trial on the treatment of Thymosin a1 versus interferon-alpha in patients with hepatitis B. *World J Gastroenterol* 2001; 7: 411–414.

17 Andreone P, Cursaro C, Gramenzi A *et al.* A randomized controlled trial of thymosin alpha 1 versus interferon alpha treatment in patients with hepatitis B e antigen antibody – and hepatitis B virus DNA – positive chronic hepatitis B. *Hepatology* 1996; 24: 774–777.

18 Zhuang L, You J, Tang BZ *et al.* Preliminary results of Thymosin-a1 versus interferon-alpha-treatment in patients with HBeAg negative and serum HBV DNA positive chronic hepatitis B. *World J Gastroenterol* 2001; 7: 407–410.

19 Sugahara S, Ichida T, Yamagiwa S *et al.* Thymosin-alpha1 increases intrahepatic NKT cells and CTLs in patients with chronic hepatitis B. *Hepatol Res* 2002; 24: 346–354.

20 Ichida F, Tsuji T, Omata M. New Inuyama classification; new criteria for histological assessment of chronic hepatitis. *Int Hepatol Comm* 1996; 6: 112–119.

21 Chien RN, Liaw YF. Thymalfasin for the treatment of chronic hepatitis B. Expert Rev. *Anti-Infective Ther* 2004; 2: 9–16.

22 Baumann CA, Badamchian M, Goldstein AL. Thymosin alpha1 is a time and dose-dependent antagonist of dexamethasone-induced apoptosis of murine thymocytes in vitro. *Int J Immunopharmacol* 2000; 22: 1057–1066.

23 Giuliani C, Napolitano G, Mastino A *et al.* Thymosin-alpha1 regulates MHC class I expression in FRTL-5 cells at transcriptional level. *Eur J Immunol* 2000; 30: 778–786.

24 Chan HL, Tang JL, Tam W, Sung JJ. The efficacy of thymosin in the treatment of chronic hepatitis B virus infection: a meta-analysis. *Aliment Pharmacol Ther* 2001; 15: 1899–1905.

25 Iino S, Tsuji T, Omata M. Comparative studies of therapy duration on the effect of recombinant IFN alfa 2a for type B chronic hepatitis. *Kan Tan Sui (Jpn)* 1999; 38: 869–886.

26 Conjeevaram HS. Therapy for chronic hepatitis B: nucleoside analogues in adult and pediatric patients. *Acta Gastroenterol Belg* 1998; 61: 224–227.

27 Honda Y, Fujiyama S, Chikazawa H. Twenty-four weeks administration of interferon-alpha for chronic hepatitis B. *Nippon Shokakibyo Gakkai Zasshi* 2002; 99: 1213–1219.

28 Arase Y, Tsubota A, Saitoh S *et al.* Randomized, controlled trial of natural interferon-alpha therapy for e-antigen-positive chronic hepatitis B patients. *Hepatol Res* 2002; 23: 98–104.
29 Kanai K, Gaki M, Aikawa T. Twenty-four week administration of recombinant interferon alfa 2a for chronic hepatitis B. *Acta Hepato Jpn (Jpn)* 1998; 39: 62–67.
30 Yano M, Ishibashi T, Sada M. Clinical study of 24-week administration of human natural inteferon-beta (MR-21) on chronic hepatitis Br. *J Clin Ther Med (Jpn)* 1997; 13: 1073–1102.
31 Matsushima T, Toyota J, Kano Y, Nakano S. Clinical study of two doses in long-term administration of human natural interferon-beta (MR-21) on chronic hepatitis B. *J Clin Ther Med (Jpn)* 1997; 13: 1103–1131.
32 Hirayama C, Fuzisawa K, Suzuki H. Clinical evaluation of proxigermanium for hepatitis B and chronic non-B hepatitis. Double blind comparative study. *Stride Med (Jpn)* 1990; 154: 663–680.
33 Hirayama C, Fuzisawa K, Suzuki H, Ito M, Okumura M. A follow up study of patients with chronic hepatitis B treated with proxigermanium. *Kan Tan Sui (Jpn)* 1995; 30: 743–751.
34 Liaw YF. Results of lamivudine trials in Asia. *J Hepatol* 2003; 39: S111–S115.
35 Tanigawa K, Ichita F, Suzuki H. A placebo-controlled double-blind study of lamivudine in chronic hepatitis B. *Kan Tan Sui (Jpn)* 1998; 36: 447–464.
36 Tanigawa K, Ichida T, Suzuki F. Long-term study of lamivudine in chronic hepatitis B patients. *Kan Tan Sui (Jpn)* 1998; 36: 597–611.
37 Ikeda K, Kumada H. Natural course of chronic hepatitis B and modification with therapies. In: Proceedings of the 22nd Inuyama Symposium: New Development of Hepatitis B; 2001. Tokyo, Japan: Arc-Media KK, 2001: 24–30.
38 Matsushima T, Hige S. Long-term prognosis in HBV-carrier. In: Proceedings of the 22nd Inuyama Symposium: New Development of Hepatitis B, 2001: 31–34.
39 Ohta Y, Onji M. Chronic hepatitis B. *Jpn J Clin Exp Med (Jpn)* 1990; 67: 1044–1051.
40 Kato K, Nishimura D, Sano H, Katada N, Sugimoto Y, Noba H. Clinical features and natural course of anti-HCV positive chronic hepatitis; comparison with chronic hepatitis B. *Clin All-round (Sogo Rinsho) (Jpn)* 1991; 40: 2415–2419.
41 Arase Y. Natural course of HBs-antigen positive hepatitis. *Med J Mutual Aid Assoc (Jpn)* 1996; 45: 339–343.
42 Saruc M, Ozden N, Turkel N *et al.* Long-term outcomes of thymosin-alpha 1 and interferon alpha-2b combination therapy in patients with hepatitis B e antigen (HBeAg) negative chronic hepatitis B. *J Pharm Sci* 2003; 92: 1386–1395.

J Gastroenterol 2005; 40:901–906
DOI 10.1007/s00535-005-1636-6

Journal of Gastroenterology

Determinants of serum ALT normalization after phlebotomy in patients with chronic hepatitis C infection

Yusuke Kawamura, Norio Akuta, Hitomi Sezaki, Tetsuya Hosaka, Takashi Someya, Masahiro Kobayashi, Fumitaka Suzuki, Yoshiyuki Suzuki, Satoshi Saitoh, Yasuji Arase, Kenji Ikeda, and Hiromitsu Kumada

Department of Gastroenterology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan

Background. Phlebotomy is performed to reduce excessive iron accumulation in hepatic tissue. We studied serum alanine aminotransferase (ALT) normalization rates and 50% reduction in initial serum ALT ($ALT_{50\%}$ reduction rate) in patients with hepatitis C viral (HCV) infection and investigated the factors that influenced the response to phlebotomy therapy. ***Methods.*** We evaluated 23 consecutive patients with HCV infection who underwent phlebotomy. Phlebotomy was performed a few times per week, then a few times per month, and 200–400 ml of blood was removed at each session, depending on the clinical response. During the course of therapy, hemoglobin (Hb), serum ALT, and ferritin levels were assessed monthly. ***Results.*** In patients with Hb of less than 11 g/dl, the $ALT_{50\%}$ reduction rate was 87.5%. In patients with a serum ferritin level of less than 10 g/dl the $ALT_{50\%}$ reduction rate was 83.3%. In patients with Hb of less than 11 g/dl, the ALT normalization rate was 50%, and in those with a serum ferritin level of less than 10 g/dl, the ALT normalization rate was 41.7%. Multivariate analysis identified ALT less than 100 IU/l at the start of phlebotomy as an independent factor associated with ALT normalization. Of the 7 patients who showed no response to phlebotomy, 85.7% were obese (body mass index ≥25 kg/m^2), and 40% showed more than 30% steatosis on liver histology. The cumulative ALT normalization rate in relation to the total volume of blood loss was 43.9% with a blood less or more than 3 l, and thus was optimal above 3 l. ***Conclusions.*** Although the sample number was relatively small, the results of our study suggest that phlebotomy is effective therapy for HCV patients who are nonobese, show little or no steatosis on liver histology, and have a baseline serum ALT level of less than 100 IU/l.

Received: December 22, 2004 / Accepted: April 26, 2005
Reprint requests to: Y. Kawamura

Key words: HCV, phlebotomy, ALT, hepatocyte steatosis

Introduction

Infection caused by hepatitis C virus (HCV) is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma in Japan. The primary goal of treatment of type C hepatitis is viral eradication and the suppression of hepatitis. Interferon (IFN) is used as an antiviral agent to achieve this goal. However, for IFN-resistant patients with high serum alanine aminotransferase (ALT) concentrations and high viral activity, it is important to maintain a low level of ALT and to prevent the progression of hepatitis and development of hepatocarcinogenesis.[1–3] In order to lower the serum concentration of ALT, liver-protection therapy with stronger neo-minophagen C (SNMC) and ursodeoxycholic acid (UDCA) is used.[4]

Excessive iron accumulation in liver tissue has received attention in recent years as an aggravating factor in chronic HCV infection. Previous reports indicated a tendency for iron to accumulate in the liver in HCV, and showed that serum ferritin levels correlated with hepatic iron concentrations.[5] Other studies showed that serum ferritin levels correlated with serum concentrations of ALT[6] and contributed to the progression of the disease.

At present, phlebotomy is used to reduce excessive iron accumulation in the liver, and the proposed goal of this therapy is to achieve a hemoglobin (Hb) concentration of less than 11 g/dl and serum ferritin concentrations of less than 10 ng/ml.[7] The present study was designed to examine the 50% reduction in initial serum ALT ($ALT_{50\%}$ reduction rate) and ALT normalization rates in patients with chronic hepatitis C and liver cirrhosis in our hospital and to determine the factors that contribute to the success of phlebotomy therapy.

Patients and methods

Study population

Phlebotomy was provided for 39 patients who were diagnosed with type C hepatitis by serum HCV antibody and positive RNA between 1998 and 2002. The following exclusion criteria were applied for recruitment into the present study. (1) Patients treated with IFN during the period between 6 months before the start of phlebotomy and the end of phlebotomy; (2) patients with serum ALT concentration less than 75 IU/l (1.5 times the upper limit of the normal range [6–50 IU/l]); (3) hepatitis B surface antigen (HBsAg)-positive patients; and (4) patients with autoimmune hepatitis, primary biliary cirrhosis, metabolic liver dysfunction, or drug-induced liver dysfunction. The above criteria were applicable to 16 patients. Thus, 23 patients (19 with chronic hepatitis and 4 with liver cirrhosis) were enrolled in the present study (Table 1). All 23 patients received liver protecion therapy, consisting mainly of SNMC (40–100 ml/day) and UDCA (600 mg/day), before and during phlebotomy. Furthermore, the doses of SNMC and UDCA were neither increased or decreased, nor was further therapy, apart from phlebotomy, added during the course of the study. Liver biopsy was performed in 20 of the 23 patients before the start of phlebotomy therapy.

Laboratory investigations

Serum ALT, Hb, ferritin, and HCV-RNA were measured once every month. HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0; Roche Molecular Systems, NJ, USA).

Liver histological examination

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy, using a modified VimSilverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan). The specimens were fixed in 10% formalin, and stained with hematoxylin-eosin, Mason's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained at least six portal areas. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al.[8] Steatosis was graded as either none (absent), mild (fewer than one-third of hepatocytes involved), moderate (more than one-third but fewer than two-thirds of hepatocytes involved), or severe (more than tow-thirds of hepatocytes involved).[9] In the three patients in whom liver biopsy was not performed, a formula to estimate liver cirrhosis[10] was used.

Table 1. Background of the study population

Demography	
Total number of patients	23
Sex (M/F)	17/6
Age (years)[a]	53 (31–76)
Body mass index (kg/m^2)[a]	24.8 (16.4–32.4)
Total alcohol intake 400 kg≦ (%)	4.8 (1/21)
Laboratory data[a]	
Alanine aminotransferase (IU/l)[a]	127 (76–255)
Gamma glutamyl transferase (IU/l)[a]	79 (17–458)
Total bilirubin (mg/dl)[a]	0.7 (0.4–1.5)
Albumin (g/dl)[a]	3.75 (2.7–4.5)
Hemoglobin (g/dl)[a]	14.9 (12.2–17.4)
Platelet count (×1000 μ/l)[a]	134 (85–560)
AFP (μg/l)[a]	13 (3–188)
Fe (μg/dl)[a]	176 (74–302)
UIBC (μg/dl)[a]	170 (15–259)
Ferritin (ng/ml)[a]	282 (97–1024)
HCV-RNA Amplicor (KIU/ml)[a]	665 (59–1900)
HCV serotype (1/2)	20/3
Histological findings	
Non-cirrhosis/cirrhosis	19/4
Steatosis (none/mild/moderate/severe)[b]	5/9/4/0
Complications (%)[c]	17.4 (4/23)
Total blood loss volume by phlebotomy (l)[a]	2.6 (0.2–20.5)
Observation period (days)[a]	141 (33–1533)

[a] Median (range)
[b] Steatosis of liver tissue: <1/3 (mild), 1/3–2/3 (moderate), >2/3 (severe)
[c] Complications, diabetes mellitus and/or hyperlipidemia

Method

Phlebotomy was performed a few times per week, followed by a few times per month; each time, 200 to 400 ml of blood was removed, depending on the clinical response. During the course of therapy, serum ALT, Hb, and ferritin concentrations were assessed monthly. The rate at which serum ALT decreased to half of the initial serum ALT concentration (ALT reduction 50% rate), the rate at which the serum ALT concentration became normal (ALT normalization rate), the normalization rate according to the total volume of blood removed by phlebotomy, and factors that contributed to the normalization were analyzed. ALT normalization was defined as the confirmation of normal serum ALT concentrations at two time points that were more than 1 month apart.

Statistical analysis

We used univariate and multivariate logistic regression analyses to determine those factors that contributed to ALT normalization. We also calculated the odds ratios and 95% confidence intervals (95% CI). All P values of less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with ALT normalization included the following 14 factors: serum ALT concentration (KIU/ml), serum Hb concentration (mg/dl), sex, age, liver histology, serum ferritin concentration (ng/ml), serum Fe concentration (μg/dl), body mass index (BMI), fatty change of liver tissue (steatosis), and the presence of fatty liver confirmed by ultrasound study, the presence of complications (diabetes mellitus and/or hyperlipidemia), total alcohol intake (kg), serum HCV-RNA concentration (KIU/ml), and HCV serotype.

Changes in Hb and ferritin concentrations, the $ALT_{50\%}$ reduction rate, and the ALT normalization rate were assessed with the Fisher's exact test, and independent factors that contributed to the $ALT_{50\%}$ reduction rate or ALT normalization rate were analyzed by the Cox proportional-hazards model. The cumulative ALT normalization rate, according to the total volume of blood removed by phlebotomy, was analyzed by the Kaplan-Meier method. Statistical analyses were performed using the SAS program (SAS Institute, Cary, NC, USA).

Results

Serum concentrations of Hb and ferritin, and $ALT_{50\%}$ reduction rate during the course of therapy

The proportion of patients in whom serum ALT concentration decreased to less than half the initial value was 65.2% (15 out of 23 patients). In those patients with Hb concentrations below 11 g/dl, the $ALT_{50\%}$ reduction rate was 87.5% (7 out of 8 patients), whereas in patients with Hb of 11 g/dl or more, the $ALT_{50\%}$ reduction rate was 53.3% (8 out of 15 patients). In those patients with a serum ferritin concentration below 10 g/dl, the $ALT_{50\%}$ reduction rate was 83.3% (10 out of 12 patients), whereas in patients with a serum ferritin concentration of 10 g/dl or more, the $ALT_{50\%}$ reduction rate was 50.0% (5 out of 10 patients). Thus, the $ALT_{50\%}$ reduction rate tended to be more favorable in patients with an Hb concentration of less than 11 g/dl or a serum ferritin concentration of less than 10 g/dl, although the differences were not statistically significant.

Hb and serum ferritin concentrations and ALT normalization rate during the course of therapy

The percentage of patients in whom serum ALT concentrations decreased to the normal range following therapy was 34.8% (8 out of 23 patients). In those patients with Hb below 11 g/dl, the ALT normalization rate was 50.0% (4 out of 8 patients), whereas in patients with Hb of 11 g/dl or more, the ALT normalization rate was 26.7% (4 out of 15 patients). In those patients with serum ferritin below 10 g/dl, the ALT normalization rate was 41.7% (5 out of 12 patients), whereas in patients with serum ferritin of 10 g/dl or more, the ALT normalization rate was 30.0% (3 out of 10 patients). Thus, the ALT normalization rate tended to be more favorable in patients with an Hb of less sthan 11 g/dl or a serum ferritin concentration of less than 10 g/dl, although the differences were not statistically significant.

Cumulative ALT normalization rate as a function of total volume of blood lost by phlebotomy

The cumulative ALT normalization rates in relation to the total blood volume loss by phlebotomy were 9.3% at 11, 12.4% at 21, 43.9% at 31, and 43.9% at more than 31, and thus the rate was maximum at a blood loss of 31 or more (Fig. 1).

Analysis of factors involved in $ALT_{50\%}$ reduction rate

On univariate analysis, none of the 14 clinicopathological factors entered in the model was statistically significant.

Analysis of factors involved in ALT normalization rate

Univariate analysis showed that the ALT normalization rate was significantly higher after treatment in patients with the following features: serum ALT concentration less than 100 IU/l ($P = 0.0001$), BMI less than 25 kg/m^2 ($P = 0.0033$), female sex ($P = 0.0357$), and age 53 years or less ($P = 0.0152$). When these four factors were entered into multivariate analysis, the serum ALT normalization rate was significantly higher when ALT was less than 100 IU/l at the start of phlebotomy ($P = 0.0298$; Table 2). For a BMI of less than 25 kg/m^2, the ALT normalization rate tended to be high ($P = 0.0771$). The sex and age of the patients were not statistically significant factors.

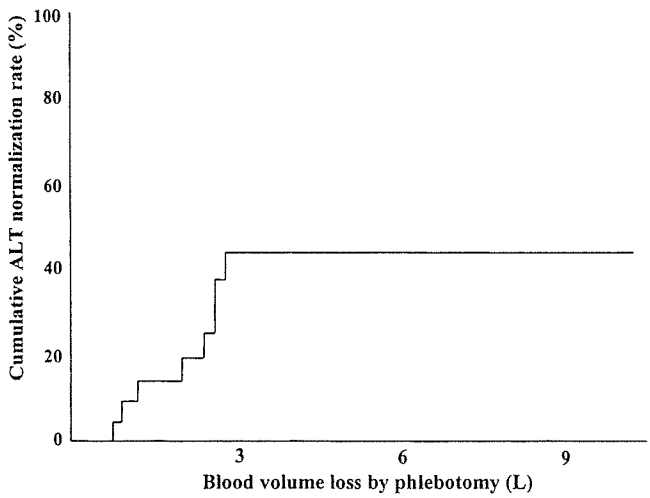

Fig. 1. Cumulative alanine aminotransperase (*ALT*) normalization rate according to blood loss by phlebotomy. The cumulative ALT normalization rate was 43.9% at 3 l and 43.9% at more than 3 l, and was optimal at a blood loss of more than 3 l

Clinicopathological background of nonresponders to phlebotomy

The background of patients who failed to show ALT normalization even after a total phlebotomy volume of more than 5 l was analyzed. All of these seven patients were males, and 85.7% (6/7) had a BMI of 25 kg/m^2 or more. The serum ALT concentration at the start of therapy was 100 IU/l or more in all nonresponders, and the serum ferritin concentration was 500 ng/ml or more in five (71.4%) of the nonresponders. Of five patients from whom liver tissue samples were available for analysis, two showed more than 30% steatosis on histological analysis (Table 3).

Discussion

Iron precipitation in liver tissue has recently received attention as an aggravating factor in HCV infection. The increased iron uptake by HCV-infected hepatocytes is thought to result in excessive intracellular accumulation of iron,[11,12] which leads to the increased production of hydroxyl radicals and cellular damage. Furthermore, it is suggested that the hydroxyl radical affects the genetic mutation of the *p53* gene in hepatocellular carcinoma (HCC) and possibly contributes to carcinogenesis.[13,14] At present, phlebotomy is performed as a treatment for excessive accumulation of iron in hepatic tissue.

In the present study, we analyzed the changes in Hb and serum ferritin concentrations during phlebotomy

Table 2. Multivariate analysis of ALT normalization

Factors	Category	Odds ratio (95% confidence interval)	*P*
ALT	1: ≥100 IU/l	1	0.030
	2: <100 IU/l	11.6 (1.27–105.7)	

Cox proportional-hazard model

Table 3. Baseline data of phlebotomy-resistant patients[a]

Case no.	Age (years)	Sex	BMI (kg/m^2)	Cirrhosis	Steatosis (%)	Hb (g/dl)	ALT (IU/l)	Ferritin (ng/ml)	Complications[b]	Blood loss volume by phlebotomy (l)
1	47	Male	26.1	–	40	14.9	127	143	–	6.0
2	59	Male	25.4	–	5	14.7	131	250	–	6.4
3	31	Male	25.0	–	ND	15.0	191	741	–	7.2
4	32	Male	32.1	–	ND	17.4	255	935	–	8.0
5	56	Male	23.0	–	5	14.9	188	536	–	8.4
6	49	Male	31.5	+	40	16.1	104	822	–	10.8
7	45	Male	25.3	–	5	14.6	171	352	+	20.5

BMI, body mass index; Hb, hemoglobin; ALT, alanine aminotransferase; ND, not done
[a] Phlebotomy-resistant, lack of response to blood volume loss by phlebotomy of more than 5 l
[b] Complications: diabetes mellitus and/or hyperlipidemia

and determined the factors that contributed to the $ALT_{50\%}$ reduction rate and ALT normalization rate. In our analysis of the $ALT_{50\%}$ reduction rate and ALT normalization rate with respect to Hb and ferritin concentrations during phlebotomy therapy, neither parameter changed significantly, probably because of the small number of patients studied. However, significant benefits from the treatment were noted in patients with an Hb of less than 11 g/dl and/or serum ferritin concentrations of less than 10 ng/ml after phlebotomy, as reported previously.[7,15–24] Further studies of a larger number of patients are necessary to confirm these findings.

In the present study, the overall $ALT_{50\%}$ reduction rate was 65.2% and the ALT normalization rate was 34.8%. Multivariate analysis showed that a serum ALT concentration of less than 100 IU/l at the start of phlebotomy therapy was the single factor that significantly and independently contributed to the ALT normalization rate. Even in patients with an ALT of 100 IU/l or more, when an Hb of less than 11 g/dl and/or serum ferritin concentration of less than 10 ng/ml was achieved during therapy, a reasonable decrease in serum ALT concentration was observed, although it was considered that normalization of ALT was more likely to be achieved in those patients with a serum ALT concentration at the start of therapy of less than 100 IU/l.

Analysis of the total volume of blood lost during phlebotomy and the cumulative ALT normalization rate showed that the cumulative ALT normalization value was optimal at a blood loss volume of 3 l or more by phlebotomy. This finding indicates that, after the start of phlebotomy therapy, the loss of a critical blood volume by phlebotomy is an important criterion for ALT normalization. Furthermore, patients who failed to show ALT normalization even after a 5-l blood loss with phlebotomy were mostly obese, with a BMI of 25 kg/m^2 or more (85.7% of the nonresponding patients). Because obese patients tend to have a high degree of steatosis in hepatocytes,[25] we believe that patients with fatty liver are not suitable candidates for phlebotomy therapy.

Mild hepatic iron overload was previously reported in some patients with nonalcoholic steatohepatitis (NASH), who showed accelerated hepatic fibrosis as a result of iron overload.[26] On the other hand, Riquelme et al.[27] recently reported that patients with NASH who underwent phlebotomy showed histopathological improvement. Based on the above studies, although no statistical significance of steatosis in relation to response to the therapy was noted in our analysis, further studies are necessary to examine the role of steatosis in hepatocytes in the failure of ALT normalization.

Our study protocol did not include the evaluation of an iron-restricted diet, or the assessment of iron deposition in the pathological specimens. Another limitation of our study was that subject recruitment criteria did not cover serum ferritin or iron concentrations. Resistance to treatment was based on the response to medical treatment with SNMC and UDCA. Further studies of a larger population sample, of patients with defined serum ferritin or iron concentrations, are required to analyze the effect of an iron-restricted diet when combined with phlebotomy and to assess iron deposition in the liver.

In conclusion, we have demonstrated in the present study that phlebotomy therapy improves the $ALT_{50\%}$ reduction rate. Normalization of serum ALT concentration was best achieved in patients with a serum ALT concentration at baseline of less than 100 IU/l. However, even if phlebotomy has shown this beneficial effect, further maintenance therapy is important. While our study focused on the $ALT_{50\%}$ reduction rate and ALT normalization rate, it is necessary to examine the effects of maintenance therapy, including maintenance phlebotomy and restriction of iron intake, in a large number of patients with HCV infection.[7]

References

1. Seeff LB. Natural history of chronic hepatitis C. Hepatology 2002;36(5 Suppl 1):S35–46.
2. Alberti A, Chemello L, Benvegnu L. Natural history of hepatitis C. J Hepatol 1999;31(Suppl 1):17–24.
3. Iino S. Relationship between infection with hepatitis C virus and hepatocellular carcinoma in Japan. Antivir Ther 1998;3(Suppl 3):143–6.
4. Tsubota A, Kumada H, Arase Y, Chayama K, Saitoh S, Ikeda K, et al. Combined ursodeoxycholic acid and glycyrrhizin therapy for chronic hepatitis C virus infection: a randomized controlled trial in 170 patients. Eur J Gastroenterol Hepatol 1999;11:1077–83.
5. Di Bisceglie AM, Axiotis CA, Hoofnagle JH, Bacon BR. Measurements of iron status in patients with chronic hepatitis. Gastroenterology 1992;102:2108–13.
6. Takikawa T, Hayashi H, Nishimura N, Yano M, Isomura T, Sakamoto N. Correlation between serum levels of alanine aminotransferase and ferritin in male blood donors with antibody to hepatitis C virus. J Gastroenterol 1994;29:593–7.
7. Kato J, Kobune M, Nakamura T, Kuroiwa G, Takada K, Takimoto R, et al. Normalization of elevated hepatic 8-hydroxy-2′-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. Cancer Res 2001;61:8697–702.
8. Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–20.
9. D'Alessandro AM, Kalayouglu M, Sollinger HW, Hoffmann RM, Reed A, Knechtle SJ, et al. The predictive value of donor liver biopsies for the development of primary nonfunction after orthotopic liver transplantation. Transplantation 1991;51:157–63.
10. Ikeda K, Saitoh S, Kobayashi M, Suzuki Y, Tsubota A, Suzuki F, et al. Distinction between chronic hepatitis and liver cirrhosis in patients with hepatitis C virus infection. Practical discriminant function using common laboratory data. Hepatol Res 2000;18:252–66.
11. Hirayama M, Kohgo Y, Kondo H, Shintani N, Fujikawa K, Sasaki K, et al. Regulation of iron metabolism in HepG2 cells: a possible

role for cytokines in the hepatic deposition of iron. Hepatology 1993;18:874–80.
12. Zoller H, Koch RO, Theurl I, Obrist P, Pietrangelo A, Montosi G, et al. Expression of the duodenal iron transporters divalent-metal transporter 1 and ferroportin 1 in iron deficiency and iron overload. Gastroenterology 2001;120:1412–9.
13. Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G-T and A-C substitutions. J Biol Chem 1992;267:166–72.
14. Oda T, Tsuda H, Scarpa A, Sakamoto M, Hirohashi S. *p53* gene mutation spectrum in hepatocellular carcinoma. Cancer Res 1992;52:6358–64.
15. Hayashi H, Takikawa T, Nishimura N, Yano M, Isomura T, Sakamoto N. Improvement of serum aminotransferase levels after phlebotomy in patients with chronic active hepatitis C and excess hepatic iron. Am J Gastroenterol 1994;89:986–8.
16. Piperno A, Sampietro M, D'Alba R, Roffi L, Fargion S, Parma S, et al. Iron stores, response to alpha-interferon therapy, and effects of iron depletion in chronic hepatitis C. Liver 1996;16:248–54.
17. Van Thiel DH, Friedlander L, Molloy PJ, Kania RJ, Fagiuoli S, Wright HI, et al. Retreatment of hepatitis C interferon nonresponders with larger doses of interferon with and without phlebotomy. Hepatogastroenterology 1996;43:1557–61.
18. Hayashi H, Takikawa T, Nishimura N, Yano M. Serum aminotransferase levels as an indicator of the effectiveness of venesection for chronic hepatitis C. J Hepatol 1995;22:268–71.
19. Tsai NC, Zuckerman E, Han SH, Goad K, Redeker AG, Fong TL. Effect of iron depletion on long-term response to interferon-alpha in patients with chronic hepatitis C who previously did not respond to interferon therapy. Am J Gastroenterol 1997;92:1831–4.
20. Fong TL, Han SH, Tsai NC, Morgan TR, Mizokami M, Qian D, et al. A pilot randomized, controlled trial of the effect of iron depletion on long-term response to alpha-interferon in patients with chronic hepatitis C. J Hepatol 1998;28:369–74.
21. Fontana RJ, Israel J, LeClair P, Banner BF, Tortorelli K, Grace N, et al. Iron reduction before and during interferon therapy of chronic hepatitis C: results of a multicenter, randomized, controlled trial. Hepatology 2000;31:730–6.
22. Di Bisceglie AM, Bonkovsky HL, Chopra S, Flamm S, Reddy RK, Grace N, et al. Iron reduction as an adjuvant to interferon therapy in patients with chronic hepatitis C who have previously not responded to interferon: a multicenter, prospective, randomized, controlled trial. Hepatology 2000;32:135–8.
23. Yano M, Hayashi H, Wakusawa S, Sanae F, Takikawa T, Shiono Y, et al. Long term effects of phlebotomy on biochemical and histological parameters of chronic hepatitis C. Am J Gastroenterol 2002;97:133–7.
24. Fargion S, Fracanzani AL, Rossini A, Borzio M, Riggio O, Belloni G, et al. Iron reduction and sustained response to interferon-alpha therapy in patients with chronic hepatitis C: results of an Italian multicenter randomized study. Am J Gastroenterol 2002;97:1204–10.
25. Sanyal AJ, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Stravitz RT, et al. Nonalcoholic fatty liver disease in patients with hepatitis C is associated with features of the metabolic syndrome. Am J Gastroenterol 2003;98:2064–71.
26. George DK, Goldwurm S, MacDonald GA, Cowley LL, Walker NI, Ward PJ, et al. Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. Gastroenterology 1998;114:311–8.
27. Riquelme A, Soza A, Nazal L, Martinez G, Kolbach M, Patillo A, et al. Histological resolution of steatohepatitis after iron depletion. Dig Dis Sci 2004;49:1012–5.

Original Paper

Intervirology

Intervirology 2005;48:239–245
DOI: 10.1159/000084601

Received: July 14, 2004
Accepted after revision: September 6, 2004

Differences of Hepatocellular Carcinoma Patients with Hepatitis B Virus Genotypes of Ba, Bj or C in Japan

Etsuro Orito[a] Fuminaka Sugauchi[a] Yasuhito Tanaka[b] Takafumi Ichida[c] Michio Sata[d] Eiji Tanaka[e] Takeshi Okanoue[f] Hiroshi Sakugawa[g] Hisatsuyo Watanabe[h] Hiroshi Miyakawa[i] Shuhei Nishiguchi[j] Hiromitsu Kumada[k] Ryuzo Ueda[a] Masashi Mizokami[b] and the Japan HBV Genotype Research Group

Departments of [a]Internal Medicine and Molecular Science, and [b]Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya; [c]Center for Life Science and Medicine, Niigata University Hospital, Niigata; [d]Second Department of Internal Medicine, Kurume University, Kurume; [e]Second Department of Internal Medicine, Shinshu University, Matsumoto; [f]Third Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto; [g]First Department of Internal Medicine, University of the Ryukyus, Okinawa; [h]Second Department of Internal Medicine, Yamagata University, Yamagata; [i]Fourth Department of Internal Medicine, Teikyo University, Kawasaki; [j]Department of Gastroenterology and Hepatology, Osaka City University Graduate School of Medicine, Osaka, and [k]Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan

Key Words
Hepatocellular carcinoma, epidemiology · Subtypes Ba/Bj, hepatitis B · Hepatitis B virus, genotypes B/C

Abstract
Hepatitis B virus (HBV) genotypes B (HBV/B) and C (HBV/C) are prevalent in Asia. Recently HBV/B has been classified into two subtypes, HBV/Ba which is ubiquitously found in Asia, and HBV/Bj which is specific in Japan. In addition, the frequency of positive HBeAg has been reported to be higher in patients with HBV/Ba than those with HBV/Bj. However, little is known about the differences between patients with various genotypes who developed hepatocellular carcinoma (HCC). In 296 serum samples of HCC patients collected from all over Japan, HBV genotypes were determined with the restriction fragment length polymorphism. HBV/A was detected in 1.0%, HBV/Ba in 4.4%, HBV/Bj in 7.4%, and HBV/C in 86.5%. In the Tohoku district and Okinawa, HBV/Ba, HBV/Bj and HBV/C were found in 6.7, 40.0 and 48.9%, compared to 4.0, 1.6 and 93.2% in the other districts in Japan. HBV/Bj patients were more frequently found in the group older than 65 years while HBV/Ba patients were found in all age groups. The frequency of positive HBeAg in HBV/Bj patients was significantly low compared to that in the other patients. More than 60% of the patients with HCC had cirrhosis as the underlying liver diseases. However, in HBV/Ba patients aged 50 years or younger, 80% of them had chronic hepatitis, while 87.5% of those aged older than 50 years had cirrhosis. These data suggest that great differences exist among patients with HCC infected with different genotypes.

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2005 S. Karger AG, Basel
0300–5526/05/0484–0239$22.00/0

Accessible online at:
www.karger.com/int

Masashi Mizokami, MD, PhD
Department of Clinical Molecular Informative Medicine
Nagoya City University Graduate School of Medical Sciences
Kawasumi 1, Mizuho, Nagoya 467-8601 (Japan)
Tel. +81 52 851 5511, Fax +81 52 852 0849, E-Mail mizokami@med.nagoya-cu.ac.jp

Introduction

In Japan, in more than two thirds of the patients with hepatocellular carcinoma (HCC) the disease is associated with hepatitis C virus (HCV). However, hepatitis B virus (HBV) is the major causative agent of HCC in Asian countries. All strains of HBV isolated from various countries can be classified into 8 HBV genotypes, HBV genotype A (HBV/A) to HBV/H, according to their phylogenetic relationships [1–3]. It has been reported that the clinical and virologic manifestations of patients with chronic HBV infection show significant differences among the different HBV genotypes [4–6]. In addition, specific distributions of HBV genotypes have been demonstrated among areas and countries [4, 7]. In south-east Asian countries, such as Japan, Taiwan, or China, HBV/B and HBV/C are prevalent [5, 7, 8].

In Japanese patients with HCC, the patients with HBV/B are rare and their mean age is high [7, 9]. However, in Taiwanese patients with HCC, a high proportion of younger patients have HBV/B. Until now, it is still unclear why younger Taiwanese patients with HBV/B develop HCC while Japanese patients with HBV/B rarely develop HCC, only in older age.

Recently, we demonstrated that HBV/B strains should be divided into two subtypes, HBV/Ba and HBV/Bj, according to their genetic relationship, and that HBV/Ba is found ubiquitously in Asian countries while HBV/Bj is found only in Japan [10, 11]. It was reported that HBeAg was found more frequently in patients with chronic infection with HBV/Ba than in those with chronic infection with HBV/Bj (32 vs. 9%) [12]. However, it is still unknown whether etiological and virologic differences are found between the HCC patients with HBV/Ba and HBV/Bj. Thus, in the patients with HCC, the difference between the subtypes of HBV/Ba and HBV/Bj might explain the etiological or clinical differences between Japan and Asia where HBV/Bj and HBV/Ba are endemic, respectively.

So, the aim of this study was to investigate the differences in the etiological, virologic and clinical characteristics among Japanese HCC patients with different HBV genotypes, such as HBV/Ba, HBV/Bj or HBV/C.

Patients and Methods

Patients with HCC

Two hundred and ninety-six patients with HCC were consecutively collected from 19 hospitals throughout Japan during January 2001 to December 2002. All the patients were chronically positive for HBsAg, and negative for anti-HDV, anti-HCV and anti-HIV. The diagnosis of HCC was reached clinically with ultrasound, computerized tomography, magnetic resonance imaging, angiography, tumor markers and biopsy if possible. The diagnoses of chronic hepatitis (CH) and liver cirrhosis (LC) were principally done by liver biopsy. However, a proportion of patients with ascites, jaundice or severe thrombocytopenia were diagnosed by ultrasound, computerized tomography and liver function tests. The serum samples and clinical data were collected from these patients with written informed consent. This study was conducted according to the ethical guidelines in our hospitals.

Virologic Assays

In all serum samples, HBsAg (CLIA, Fujirebio, Japan, detection limit 0.13 ng/ml), HBeAg (CLIA, Fujirebio, Japan) and anti-HBe (CLIA) were tested. Serum HBV DNA was detected by nested polymerase chain reaction (PCR) with the primers derived from the S gene. The patients were not enrolled in this study if the serum HBV DNA was not detected by PCR. The HBV genotype was determined by restriction fragment length polymorphism as described previously [13]. In brief, the S gene of HBV DNA was amplified by nested PCR. Then the products were sequentially digested by the restriction enzyme, *Alw*I, *Ear*I, *Hph*I, *Nci*I and *Nla*IV, respectively. The HBV genotype was determined by the size of the digested PCR product which was electrophoresed on agarose gel. When the test results were inconclusive, the sequences of the S region were determined directly, then the genotype was decided by phylogenetic analysis [13, 14]. When patients were found to have HBV/B, the subtypes Ba and Bj were determined by restriction fragment length polymorphism [11]. In brief, at nucleotide position 1838 in the pre-core region, only A was found in patients with HBV/Ba while only G was found in those with HBV/Bj. The restriction enzyme detection system was established targeting the discrimination of this difference in nucleotides with the restriction enzyme, *Spe*I and *Mse*I after the pre-core region was amplified by PCR.

Statistical Analysis

The data were statistically analyzed by Student's t test, nonparametric Mann-Whitney test, and χ^2 test where appropriate. A p value of <0.05 was regarded as statistically significant.

Results

HBV Genotypes and Clinical Findings

Of the 296 patients, 223 were male and 73 were female. The mean age was 55.1 ± 10.8 (range 26–81) years. The clinical findings are shown in table 1. Thirty-five percent of the patients were positive for HBeAg. Regarding the HBV genotypes, 3 patients (1.0%) were HBV/A, 13 (4.4%) HBV/Ba, 22 (7.4%) HBV/Bj, 256 (86.5%) HBV/C, and 2 (0.7%) of mixed genotype (HBV/B and C). The clinical findings by HBV genotype are shown in table 2. There were no significant differences in the mean levels of total bilirubin, AST and ALT among patients with different HBV genotypes. However, the mean ALP level and γ-

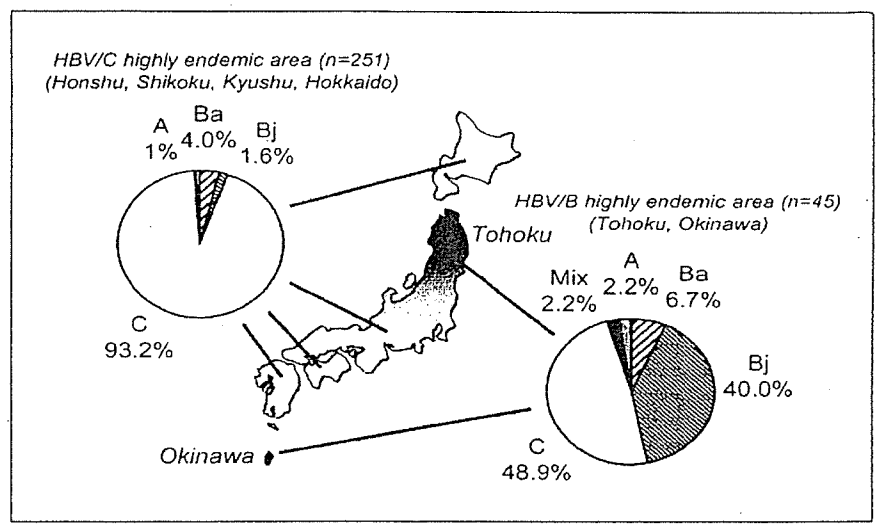

Fig. 1. The geographic distribution of HBV genotypes in Japan. In the Tohoku district, the northern area of mainland Japan, and Okinawa, the most southern islands, 48.9% of HCC patients were HBV/C, 6.7% were HBV/Ba, and 40.0% were HBV/Bj. In contrast, in other parts of Japan, Hokkaido, Honshu, Shikoku and Kyushu, 93.2% were HBV/C, 4.0% were HBV/Ba and 1.6% were HBV/Bj.

Table 1. Characteristics of 296 HBsAg-positive Japanese patients with HCC collected from all over Japan

Male:female	223:73
Age, years	55.1 ± 10.8[a]
Total bilirubin, mg/dl	1.5 ± 1.9
AST, IU/l	78.5 ± 103.9
ALT, IU/l	63.0 ± 69.8
ALP, IU/l	321.1 ± 225.4
γ-GTP, IU/l	108.4 ± 174.4
HBeAg, % positive	35.0
Anti-HBe, % positive	64.8
HBV genotype	
HBV/A	3 (1.0%)
HBV/Ba	13 (4.4%)
HBV/Bj	22 (7.4%)
HBV/C	256 (86.5%)
Mix	2 (0.7%)

[a] Mean ± SD.

Table 2. Clinical findings of the HCC patients with HBV genotypes of Ba, Bj or C

	HBV genotype		
	Ba	Bj	C
Age, years	55.4 ± 12.9	66.6 ± 10.6	54.0 ± 10.7
		p < 0.01	p < 0.01
Total bilirubin, mg/dl	1.0 ± 0.4	1.2 ± 0.7	1.5 ± 2.0
AST, IU/l	173.9 ± 352.6	51.6 ± 42.1	82.6 ± 113.4
ALT, IU/l	102.4 ± 162.9	33.9 ± 16.8	66.5 ± 74.9
ALP, IU/l	147.7 ± 126.6	209.8 ± 95.4	343.9 ± 238.0
		p < 0.05	
γ-GTP, IU/l	78.6 ± 55.9	63.1 ± 45.9	110.5 ± 186.7
			p < 0.05

GTP level of the HBV/C patients was significantly higher than those with HBV/Ba and HBV/Bj, respectively ($p < 0.05$).

Geographic Distribution of HBV Genotypes

The geographic distribution of HBV genotypes was area-specific in Japan (fig. 1). This specific distribution of HCC patients was in accord with that of all the patients including asymptomatic carriers, CH and LC patients, as described previously [7]. Namely, in the Tohoku district, the northern area of the Japanese mainland, and Okinawa, the most southern islands, 22 (48.9%) of HCC patients were HBV/C, 3 (6.7%) were HBV/Ba, and 18 (40.0%) were HBV/Bj. In contrast, in other areas of Japan, Hokkaido, Honshu, Shikoku and Kyushu, 234 (93.2%) were HBV/C, 10 (4.0%) were HBV/Ba, and 4 (1.6%) were HBV/Bj ($p < 0.01$).

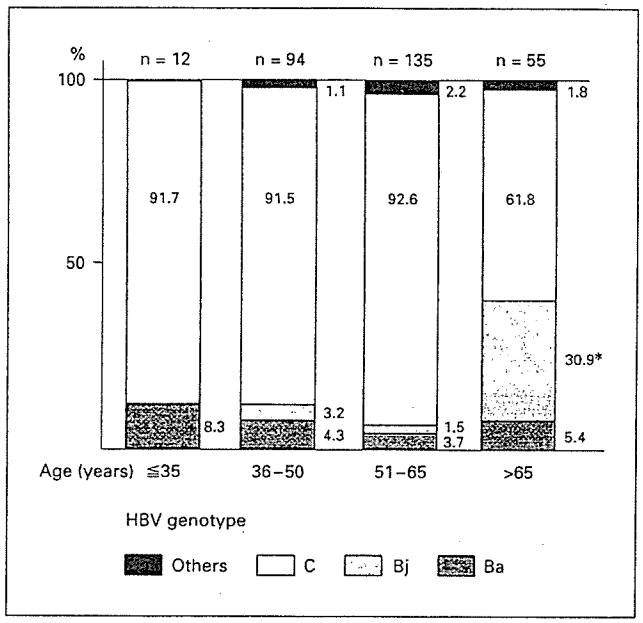

Fig. 2. The distribution of HBV genotypes in each age group. In groups aged 35 years or younger, 36–50 years, and 51–65 years, more than 90% of HCC patients had HBV/C. On the other hand, in the group aged older than 65 years, only 61.8% of patients had HBV/C while 30.9% had HBV/Bj (* $p < 0.01$, group aged older than 65 years vs. other age groups). More patients with HBV/Ba were in the younger aged group, although the number of patients with HBV/Ba was small in all the groups.

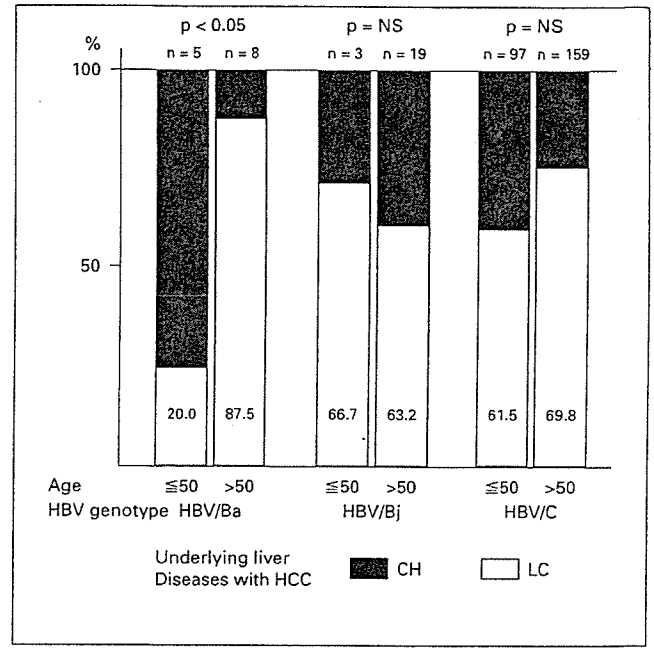

Fig. 4. The underlying liver diseases, chronic hepatitis (CH) or liver cirrhosis (LC), in HCC patients. In patients with HBV/Ba, only 25.0% of the group aged 50 years or younger had LC, while 85.7% of the group aged older than 50 years had LC ($p < 0.01$). However, in patients with HBV/Bj or HBV/C, the ratios of the underlying liver diseases were approximately identical even when compared by age.

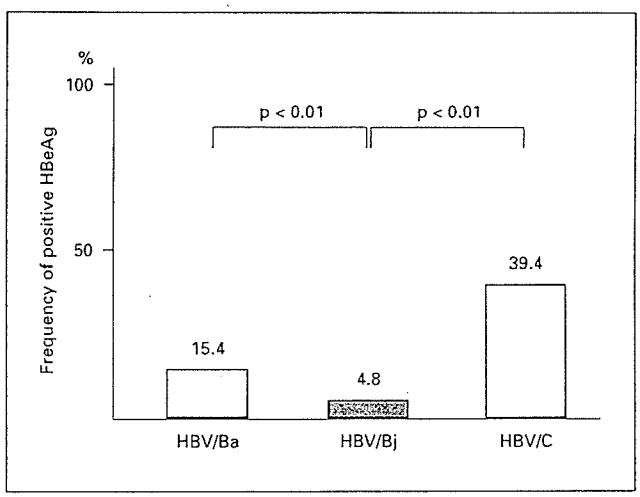

Fig. 3. The frequency of patients with positive HBeAg in each HBV genotype. The frequency of positive HBeAg was 4.8% in patients with HBV/Bj, compared with 39.4% in those with HBV/C (Bj vs. C, $p < 0.01$), and 15.4% in those with HBV/Ba (Bj vs. Ba, $p < 0.01$).

Mean Age and Frequency of Positive HBeAg among Patients with Each Genotype

The mean age of HBV/Bj patients (66.6 ± 10.6 years) was significantly higher than those with HBV/Ba (55.4 ± 12.9 years, $p < 0.01$) and HBV/C (54.0 ± 10.7 years, $p < 0.01$; table 2). The distribution of HBV genotypes in each age group is shown in figure 2. In groups aged 35 years or younger, 36–50 years, and 51–65 years, more than 90% of HCC patients had HBV/C. On the other hand, in the group aged older than 65 years, only 61.8% of the patients had HBV/C while 30.9% had HBV/Bj ($p < 0.01$, group aged older than 65 years vs. other age groups). HBV/Ba tended to be found in the younger age group although the number of patients with HBV/Ba was small in all groups.

The frequency of positive HBeAg was 4.8% in patients with HBV/Bj, compared with 39.4% in those with HBV/C (Bj vs. C, $p < 0.01$), and 15.4% in those with HBV/Ba (Bj vs. Ba, $p < 0.01$; fig. 3).