24 International Interferon-alpha Hepatocellular Carcinoma Study Group: Effect of interferon-alpha on progression of cirrhosis to hepatocellular carcinoma: A retrospective cohort study. Lancet 1998;351:1535–1539
25 Lin SM, Sheen IS, Chien RN, Chu CM, Liaw YF: Long-term beneficial effect of interferon therapy in patients with chronic hepatitis B virus infection. Hepatology 1999;29:971–975.
26 Kamisango K, Kamogawa C, Sumi M, Goto S, Hirao A, Gonzales F, Yasuda K, Iino S: Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. J Clin Microbiol 1999; 2:310–314.
27 SAS Institute Inc: SAS/STAT User's Guide, Release 6.03 Edition. Cary, 1988.
28 Ikeda K, Arase Y, Kobayashi M, Someya T, Saitoh S, Suzuki Y, Suzuki F, Tsubota A, Akuta N, Kumada H: Consistently low hepatitis B virus DNA saves patients from hepatocellular carcinogenesis in HBV-related cirrhosis. Intervirology, 2003;46:96–104.
29 Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Fukuda M, Koida I, Arase Y, Chayama K, Murashima N, Kumada H: Relationship of hepatocellular carcinogenesis with precore mutant virus and serum hepatitis B virus DNA concentration. A longitudinal analysis of patients with cirrhosis. Hepatol Res 1998;10:142–155.
30 Roeckelein G, Hecken-Emmel M: Risk factors of hepatocellular carcinoma in Germany: Hepatitis B or liver cirrhosis? Hepato-gastroenterology 1988;35:151–157.
31 Kaplan EL, Meier P: Nonparametric estimation for incomplete observation. J Am Stat Assoc 1958;53:457–481.
32 SPSS Inc: SPSS for Windows version 11.0 manual. SPSS, Chicago, 2001.
33 Ikeda K, Kobayashi M, Saitoh S, Someya T, Hosaka T, Akuta N, Suzuki Y, Suzuki F, Tsubota A, Arase Y, Kumada H: Significance of hepatitis-B virus DNA clearance and its early prediction in hepatocellular carcinogenesis in patients with cirrhosis undergoing interferon therapy: A long-term follow-up of a pilot study. J Gastroenterol Hepatol 2004; in press.

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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

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Key Words
Hepatitis C virus · Genotype 1b · Albumin · Core region · Interferon sensitivity-determining region · Interferon · Ribavirin · Non-virological responder · Viral kinetics

Abstract
Objective: Patients with high titer (≥100 kIU/ml) of hepatitis C virus (HCV) genotype 1b do not achieve highly sustained virological response rates to combination therapy with interferon plus ribavirin. Non-virological responders (NVRs, namely ultimate resistant cases) who do not achieve HCV-RNA negativity during treatment are also encountered. We investigated the pretreatment virological features of NVRs. ***Methods:*** We evaluated 50 consecutive Japanese adults with high titer of HCV genotype 1b who received combination therapy for 48 weeks. We investigated the pretreatment substitution patterns in amino acids 1–191 of the core region and amino acids 2209–2248 of NS5A, and early viral kinetics. ***Results:*** Overall, a non-virological response was noted in 12 (24%) patients. Multivariate analysis identified serum albumin <3.9 g/dl, substitutions of amino acid 70 in the core region, and substitutions of amino acid 91 as independent and significant factors associated with a non-virological response. Especially, substitutions of arginine (R) by glutamine (Q) at amino acid 70, and/or leucine (L) by methionine (M) at amino acid 91 were significantly more common in NVRs. The falls in HCV-RNA levels during treatment in patients with specific substitutions in the core region were significantly less than in those without such substitutions. ***Conclusions:*** Our results suggest that serum albumin and amino acid substitution patterns in the core region in patients with high titers of HCV genotype 1b may have an effect on combination therapy in NVRs. Further large-scale studies are required to examine the role of amino acid substitutions specific to a non-virological response to combination therapy.

Introduction

Hepatitis C virus (HCV) usually causes chronic infection which can result in liver cirrhosis and hepatocellular carcinoma (HCC) [1–4]. The aims of interferon (IFN) therapy for chronic hepatitis C include a reduction in the risk of development of HCC and liver-related death by

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viral clearance, and then by normalization of alanine aminotransferase (ALT) even if viral clearance cannot be achieved [5].

The most effective initial therapy for viral clearance is the combination of IFN and ribavirin (RBV) administered for 48 weeks [6, 7]. In Japan, about 70% of patients with chronic hepatitis C are infected with HCV genotype 1b, and a sustained virological response (SVR) to IFN monotherapy for 24 weeks is as low as 10–20% in patients with genotype 1b infection [8–11]. Moreover, patients with a high titer of genotype 1b (≥100 kIU/ml) do not achieve high SVR rates (<50%), even when the most effective combination treatment (IFN plus RBV) is administered for 48 weeks [6, 7]. Furthermore, in genotype 1b, we also often encounter non-virological responders (NVRs) who do not achieve HCV-RNA negativity as determined by polymerase chain reaction (PCR) during treatment, compared with only 1.0% (non-virological response rate) of patients infected with genotype 2a and treated with IFN monotherapy [12]. The underlying mechanism(s) of the different virological responses to treatment in patients with 1b strain infection is still unclear. Hence, in the present study, we investigated the pretreatment virological features of NVRs.

The present study included 50 consecutive Japanese adults with chronic hepatitis C of genotype 1b and a high viral load who received combination therapy for 48 weeks. The aims of the study were: (1) to investigate the rate of non-virological responses in this group; (2) to analyze the predictive factors associated with a non-virological response, including pretreatment virological features, and (3) to examine the pretreatment virological features associated with early viral kinetics. Previous studies have shown that the HCV core region might be associated with resistance to IFN therapy involving the Jak-STAT signaling cascade [13–16], and have also shown that the number of substitutions in amino acids 2209–2248 (IFN-sensitivity determining region, ISDR, of NS5A in HCV genotype 1b) [17, 18] might be associated with the efficacy of IFN therapy and viral load. Therefore, we analyzed the amino acid substitutions of the core region and NS5A in patients with genotype 1b and high viral load to identify the virus-related factors apart from the genotype and viral load.

Materials and Methods

Study Population

Fifty-seven HCV-infected adult Japanese patients were consecutively recruited into the study protocol of combination therapy with IFN (peginterferon (PEG)-IFNα-2b or IFNα-2b) plus RBV for 48 weeks between 2001 and 2004 at Toranomon Hospital, Tokyo, Japan. Among these, 50 patients were selected in the present study based on the following criteria. (1) They were negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp, Emerville, Calif., USA), and positive for HCV-RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, Pleasanton, Calif., USA). (2) They were naive to RBV therapy. (3) They were infected with HCV genotype 1b alone. (4) Each had a high viral load (≥100 kIU/ml) by quantitative analysis of HCV-RNA with PCR (Amplicor HCV-RNA kit, version 2.0, Roche Diagnostics) within the preceding 2 months of enrolment. (5) Each had chronic hepatitis, without cirrhosis or HCC, as confirmed by biopsy examination within the preceding 12 months of enrolment. (6) They had abnormal serum ALT levels (the upper limit of normal for ALT, 45 IU/l) within the preceding 2 months of enrolment. (7) In each patient, the hemoglobin (Hb) concentration was ≥12.0 g/dl, platelet count $\geq 100 \times 10^3/mm^3$, and neutrophil count $\geq 1.5 \times 10^3/mm^3$ within the preceding 2 months of enrolment. (8) Their body weight was >40 kg. (9) All were free of co-infection with human immunodeficiency virus. (10) None had been treated with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (11) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (12) None had diabetes, other forms of hepatitis, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (13) None of the females was pregnant or lactating. (14) All accepted treatment for 24 weeks or more as outlined in the study protocol, as well as repeated evaluation of HCV-RNA levels during treatment (at least once every month). (15) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee of Toranomon Hospital.

With regard to the treatment protocol, 34 (68.0%) patients received the PEG-IFNα-2b treatment protocol at dose of 1.5 μg/kg subcutaneously each week plus oral RBV at 600–800 mg/day for 48 weeks. The remaining 16 (32.0%) patients received 6 million units of IFNα-2b intramuscularly each day for 48 weeks (6 times per week for initial 2 weeks, followed by 3 times per week for 46 weeks), and oral RBV at a dose of 600–800 mg/day for 48 weeks. The RBV dose was adjusted according to body weight (600 mg for weight ≤60 kg, and 800 mg for weight >60 kg).

Table 1 summarizes the profiles and data of the 50 patients at the commencement of combination therapy with IFN plus RBV. They included 31 men and 19 women, aged 20–65 (median 53) years. The median total duration of treatment was 48 (range 28–48) weeks. In 14 of the 50 (28.0%) patients, the dose of RBV was reduced during treatment due to a fall in Hb concentration.

Patients who remained positive for HCV-RNA based on quantitative and/or qualitative analyses with PCR during and at the end of combination therapy were defined as NVRs (namely ultimate resistant cases), while the other patients who could achieve negative HCV-RNA by qualitative analysis with PCR during and/or at the end of treatment were defined as virological responders (VRs).

Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for ALT and HCV-RNA levels. The serum samples were frozen at –80°C within 4 h of collection and were thawed at the time of measurement. HCV

genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of NS5 region [19]. HCV-RNA levels were measured quantitatively by PCR (Amplicor HCV-RNA kit, version 2.0, Roche Diagnostics) at least once every month before, during, and after therapy. The lower limit of the assay was 0.5 kIU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA (<0.5 kIU/ml) were checked also by qualitative PCR (Amplicor, Roche Diagnostic Systems), which has a higher sensitivity than quantitative analysis, and the results are expressed as positive or negative. The lower limit of the assay was 100 copies/ml.

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examinations contained 6 or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histological assessment according to the scoring system of Desmet et al. [20]. Hepatocyte steatosis was graded as either none (absent), mild (<1/3 of hepatocytes involved), moderate (>1/3 but <2/3 of hepatocytes involved), or severe (>2/3 of hepatocytes involved) [21].

Nucleotide Sequencing of the Core and NS5A Gene

We determined the sequences of amino acids 1–191 in the core and amino acids 2209–2248 (ISDR) in the NS5A by the direct sequencing method using pretreatment sera of 50 patients. These sequences were compared with the consensus sequence of genotype 1b, which was determined by comparing the sequences obtained in this study and prototype sequence (HCV J) [22]. HCV-RNA was extracted from serum samples at the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo). Nucleic acids were amplified by PCR using the following primers. (a) Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers, and the second-round PCR with CC9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. (b) Nucleotide sequences of ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) primers, and the second-round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) primers (hemi-nested PCR and nested PCR). All samples were initially denatured at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 μl of the first PCR product was transferred to the second PCR. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo).

To avoid false-positive results, the procedures recommended by Kwok and Higuchi [23] to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

Viral Kinetic Study

Viral kinetic study was evaluated at three time points (4, 8 and 12 weeks during treatment). Falls in HCV-RNA levels from baseline were expressed using $\log_{10}$ of viral loads at each time point, in comparison with the pretreatment viral load. For data analysis, we used the $\log_{10}$ of the cutoff value (500 IU/ml) for HCV-RNA values below the limit of detection.

Table 1. Patient profile and laboratory data at commencement of combination therapy with interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b

Demographic data	
Number	50
Sex, M/F	31/19
Age, years[a]	53 (20–65)
History of blood transfusion	14 (28.0%)
Family history of liver disease	16 (32.0%)
Body mass index, kg/m^2[a]	23.2 (18.7–32.0)
Laboratory data[a]	
Serum alanine aminotransferase, IU/l	97 (35–276)
Serum albumin, g/dl	3.8 (3.1–4.2)
Hemoglobin, g/dl	14.4 (12.0–17.4)
Platelet count, $\times 10^4$/mm^3	17.4 (10.1–30.9)
ICG R15, %[b]	13 (7–41)
Serum iron, μg/dl	140 (52–308)
Serum ferritin, μg/l	150 (<10–644)
Creatinine clearance, ml/min	101 (46–142)
Viremia level, KIU/ml	710 (49–2,800)
Number of amino acid substitutions in ISDR (0/1–3/≥4)	27/20/3
Histological findings	
Stage (F1/F2/F3)[c]	31/15/4
Hepatocyte steatosis (none/mild/moderate/severe)	3/40/7/0
Treatment	
PEG-IFNα-2b/IFNα-2b	34/16
Ribavirin dose, mg/kg[a]	11.3 (9.7–14.2)

ALT levels were abnormal (the upper limit of normal for ALT; 45 IU/l) and viremia levels were high titer (≥100 kIU/ml), when all patients were recruited in this study. Normal reference ranges: 3.9–5.2 g/dl for albumin.

[a] Expressed as median (range).

[b] ICG R15: indocyanine green retention rate at 15 min.

[c] Stage of chronic hepatitis by Desmet et al. [20].

Statistical Analysis

Non-parametric tests were used to analyze the decline in HCV-RNA levels and amino acid substitutions in HCV core and NS5A between the each groups, including the Mann-Whitney U test, χ^2 test and Fisher's exact probability test. Univariate and multivariate logistic regression analyses were used to determine the factors that significantly contributed to a non-virological response. We also calculated the odds ratios and 95% confidence intervals (95% CI). All p values of <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 NVR included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, ALT, albumin, Hb, platelet count, indocyanine green retention rate at 15 min (ICG R15), serum iron, serum ferritin, creatinine clearance, viremia level, pathological staging, hepatocyte steatosis, type of IFN, RBV dose according to body weight, treatment term, dose reduction, and pretreatment amino acid substitution in the core and ISDR of NS5A. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, Ill., USA).

Results

Virological Response Rates by Combination Therapy

The virological response could be evaluated in all 50 patients. In this study, 38 of 50 (76.0%) patients achieved a virological response while the remaining 12 (24.0%) patients were considered NVRs.

Predictive Factors Associated with a Non-Virological Response in Multivariate Analysis

We then analyzed the data of the whole population sample to determine those factors that could predict a non-virological response. Univariate analysis identified 5 parameters that tended to or significantly influenced the non-virological response. These included serum albumin ($p = 0.008$), presence of amino acid substitution in HCV core in the pretreatment sample (substitution of amino acid 70, $p = 0.003$, and amino acid 91, $p = 0.044$), RBV dose according to body weight ($p = 0.044$), and serum ferritin ($p = 0.095$).

Multivariate analysis identified three parameters that independently influenced the non-virological response, including serum albumin ($p = 0.004$), substitutions of amino acids 70 ($p = 0.013$) and 91 ($p = 0.016$; table 2).

Treatment Efficacy according to Substitution Patterns in Amino Acids of HCV Core

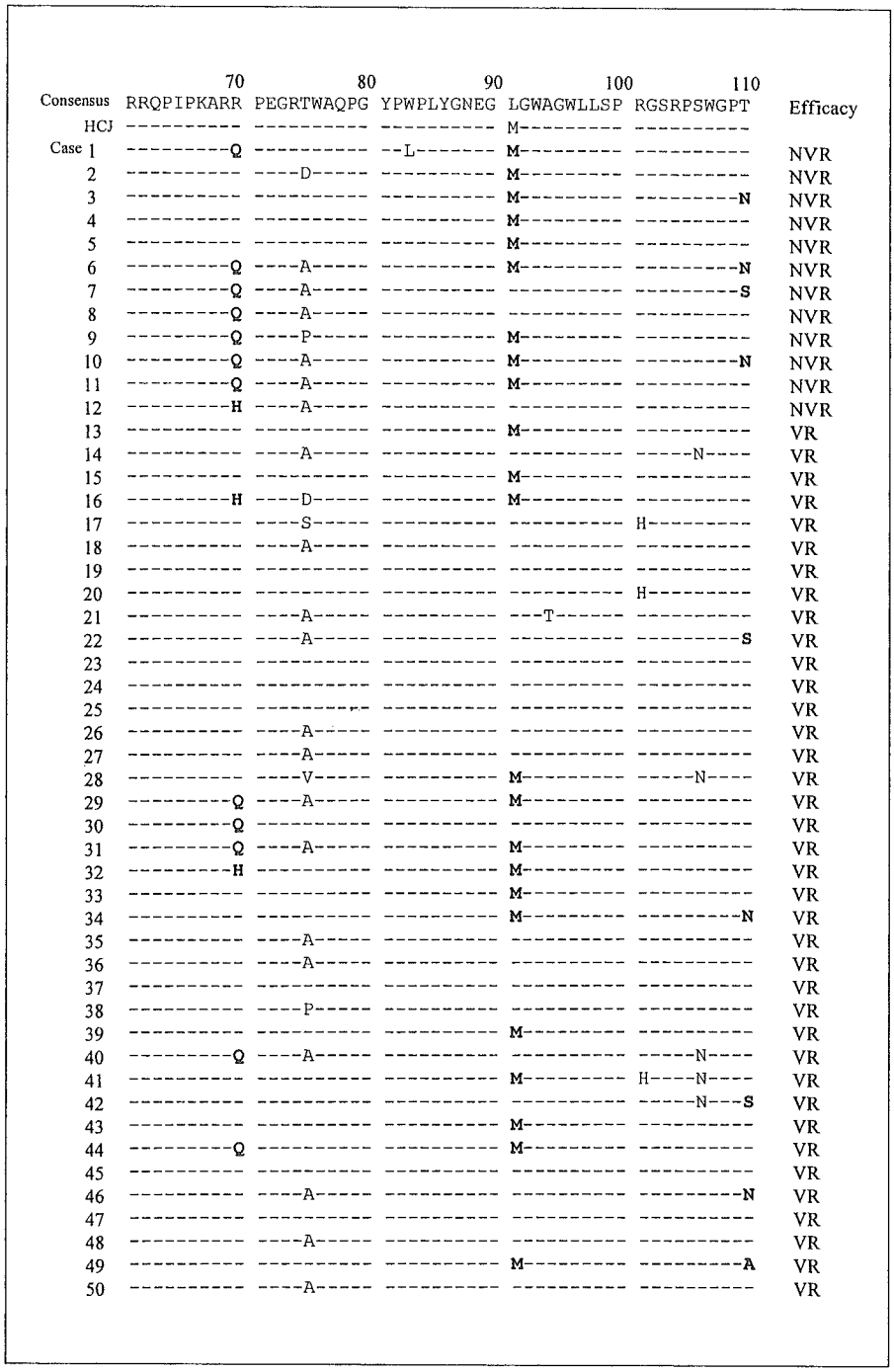
Figure 1 shows the sequences of amino acids 61–110 of the HCV core in 50 patients at the commencement of combination therapy. Substitutions at amino acid 70 of the HCV core were significantly more frequent in NVRs (n = 8, 66.7%) than VRs (n = 7, 18.4%; $p = 0.003$). Similarly, substitutions at amino acid 91 were significantly more frequent in NVRs (n = 9, 75.0%) than VRs (n = 14, 36.8%; $p = 0.044$). Furthermore, dual substitutions at amino acids 70 and 91 were significantly more frequent in NVRs (n = 5, 41.7%) than VRs (n = 5, 13.2%; $p = 0.046$). Thus, substitutions at amino acid(s) 70 and/or 91 were found in all 12 (100%) NVRs while only 16 (42.1%) of the VRs had such substitutions ($p < 0.001$). There were no significant differences in other substitution sites and treatment efficacy between NVR and VR groups (table 3).

At amino acid 70, the substitution in which arginine (R) was replaced by glutamine (Q) was significantly more frequent in NVRs (n = 7, 58.3%) than VRs (n = 5, 13.2%; $p = 0.004$). At amino acid 91, the substitution in which leucine (L) was replaced by methionine (M) was significantly more frequent in NVRs (n = 9, 75.0%) than VRs (n = 14, 36.8%; $p = 0.044$). At amino acid 110, the substitution in which threonine (T) was replaced by asparagine (N) was significantly more frequent in NVRs (n = 3, 25.0%) than VRs (n = 2, 5.3%; $p = 0.082$). Substitutions Q–M instead of R–L at amino acids 70 and 91 were significantly more frequent in NVRs (n = 5, 41.7%) than VRs (n = 3, 7.8%; $p = 0.014$). Thus, 11 (91.7%) NVRs and 16 (42.1%) VRs ($p = 0.003$) had a substitution of Q at amino acid 70 and/or M at amino acid 91. There were no significant differences in other substitution patterns and treatment efficacy between NVRs and VRs (table 3).

Table 2. Factors associated with non-virological response to combination therapy with interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b, identified by multivariate analysis

Factor	Category	Odds ratio (95% confidence interval)	p
Albumin, g/dl	1: <3.9	1	
	2: ≥3.9	0.009 (0.000–0.227)	0.004
Substitution of aa 70	1: Absent	1	
	2: Present	22.2 (1.905–258.3)	0.013
Substitution of aa 91	1: Absent	1	
	2: Present	19.5 (1.737–219.3)	0.016

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown. aa = Amino acid.



	70	80	90	100	110	
Consensus	RRQPIPKARR	PEGRTWAQPG	YPWPLYGNEG	LGWAGWLLSP	RGSRPSWGPT	Efficacy
HCJ	----------	----------	----------	M---------	----------	
Case 1	---------Q	----------	--L-------	M---------	----------	NVR
2	----------	----D-----	----------	M---------	----------	NVR
3	----------	----------	----------	M---------	---------N	NVR
4	----------	----------	----------	M---------	----------	NVR
5	----------	----------	----------	M---------	----------	NVR
6	---------Q	----A-----	----------	M---------	---------N	NVR
7	---------Q	----A-----	----------	----------	---------S	NVR
8	---------Q	----A-----	----------	----------	----------	NVR
9	---------Q	----P-----	----------	M---------	----------	NVR
10	---------Q	----A-----	----------	M---------	---------N	NVR
11	---------Q	----A-----	----------	M---------	----------	NVR
12	---------H	----A-----	----------	----------	----------	NVR
13	----------	----------	----------	M---------	----------	VR
14	----------	----A-----	----------	----------	-----N----	VR
15	----------	----------	----------	M---------	----------	VR
16	---------H	----D-----	----------	M---------	----------	VR
17	----------	----S-----	----------	----------	H---------	VR
18	----------	----A-----	----------	----------	----------	VR
19	----------	----------	----------	----------	----------	VR
20	----------	----------	----------	----------	H---------	VR
21	----------	----A-----	----------	---T------	----------	VR
22	----------	----A-----	----------	----------	---------S	VR
23	----------	----------	----------	----------	----------	VR
24	----------	----------	----------	----------	----------	VR
25	----------	----------	----------	----------	----------	VR
26	----------	----A-----	----------	----------	----------	VR
27	----------	----A-----	----------	----------	----------	VR
28	----------	----V-----	----------	M---------	-----N----	VR
29	---------Q	----A-----	----------	M---------	----------	VR
30	---------Q	----------	----------	----------	----------	VR
31	---------Q	----A-----	----------	M---------	----------	VR
32	---------H	----------	----------	M---------	----------	VR
33	----------	----------	----------	M---------	----------	VR
34	----------	----------	----------	M---------	---------N	VR
35	----------	----A-----	----------	----------	----------	VR
36	----------	----A-----	----------	----------	----------	VR
37	----------	----------	----------	----------	----------	VR
38	----------	----P-----	----------	----------	----------	VR
39	----------	----------	----------	M---------	----------	VR
40	---------Q	----A-----	----------	----------	-----N----	VR
41	----------	----------	----------	M---------	H----N----	VR
42	----------	----------	----------	----------	-----N---S	VR
43	----------	----------	----------	M---------	----------	VR
44	---------Q	----------	----------	M---------	----------	VR
45	----------	----------	----------	----------	----------	VR
46	----------	----A-----	----------	----------	---------N	VR
47	----------	----------	----------	----------	----------	VR
48	----------	----A-----	----------	----------	----------	VR
49	----------	----------	----------	M---------	---------A	VR
50	----------	----A-----	----------	----------	----------	VR

Fig. 1. Sequences of amino acids 61–110 in the core region at the commencement of combination therapy in 50 patients infected with high HCV viral load genotype 1b. Dashes indicate amino acids identical to the consensus sequence of genotype 1b, and substituted amino acids are shown by standard single-letter codes. The amino acid patterns at positions that are probably associated with sensitivity to therapy are shown in boldface characters. NVR = Non-virological responder; VR = virological responder.

Viral Kinetics according to Substitution Patterns in Amino Acids of HCV Core

Table 4 shows HCV-RNA levels at 4, 8, and 12 weeks relative to baseline as a function of pretreatment amino acid substitutions in the core region. The fall in HCV-RNA level at each time point was significantly lower in patients with specific substitution patterns (Q at amino acid 70, M at amino acid 91, N at amino acid 110, Q–M at amino acid 70 and 91, Q at amino acid 70 and/or M at amino acid 91) than in those without them.

Table 3. Amino acid substitutions in the core region in non-virological responders (NVR) and virological responders (VR) to combination therapy of interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b

	NVR (n = 12)	VR (n = 38)	p*
Presence of substitution site			
aa 70	8 (66.7%)	7 (18.4%)	0.003
aa 91	9 (75.0%)	14 (36.8%)	0.044
aa 70 and 91	5 (41.7%)	5 (13.2%)	0.046
aa 70 and/or 91	12 (100%)	16 (42.1%)	<0.001
Presence of substitution pattern			
Q at aa 70	7 (58.3%)	5 (13.2%)	0.004
M at aa 91	9 (75.0%)	14 (36.8%)	0.044
N at aa 110	3 (25.0%)	2 (5.3%)	0.082
Q-M at aa 70 and 91	5 (41.7%)	3 (7.8%)	0.014
Q at aa 70 and/or M at aa 91	11 (91.7%)	16 (42.1%)	0.003

Q = Glutamine; M = methionine; N = asparagine; aa = amino acid.
* NVR vs. VR (Fisher's exact probability test).

Table 4. Decline levels of HCV-RNA from baseline at 4, 8 and 12 weeks according to the amino acid substitutions in the core region during combination therapy of interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b

Presence of substitution pattern	Decline levels of HCV-RNA from baseline, log_{10} IU/ml[1]		
	4 weeks	8 weeks	12 weeks
Q at aa 70			
Absent[2]	2.49 (–0.024 to 3.41) [a]	3.02 (0.25 to 3.41) [b]	2.98 (0.30 to 3.45) [c]
Present	0.58 (0.11 to 3.13)	1.18 (–0.095 to 3.16)	1.99 (0.34 to 3.19)
M at aa 91			
Absent	2.49 (0.12 to 3.41) [d]	3.14 (1.69 to 3.41) [e]	3.13 (0.49 to 3.45) [f]
Present	0.85 (–0.024 to 3.16)	1.56 (–0.095 to 3.41)	2.40 (0.30 to 3.41)
N at aa 110			
Absent	2.36 (0.10 to 3.41) [g]	3.02 (–0.095 to 3.41) [h]	2.96 (0.48 to 3.45) [i]
Present	0.28 (–0.024 to 0.86)	0.32 (0.25 to 1.43)	0.70 (0.30 to 2.46)
Q-M at aa 70 and 91			
Absent	2.49 (–0.024 to 3.41) [j]	3.04 (0.25 to 3.41) [k]	2.98 (0.30 to 3.45) [l]
Present	0.58 (0.11 to 2.34)	0.50 (–0.095 to 2.34)	1.99 (0.34 to 3.19)
Q at aa 70 and/or M at aa 91			
Absent	2.49 (0.88 to 3.41) [m]	3.11 (1.69 to 3.41) [n]	3.18 (2.40 to 3.45) [o]
Present	0.85 (–0.024 to 3.16)	2.01 (–0.095 to 3.41)	2.40 (0.30 to 3.41)

Q = Glutamine; M = methionine; N = asparagine; aa = amino acid.
[1] Decline levels of HCV-RNA from baseline are shown in log_{10} of viral loads at each time point in comparison to pretreatment viral loads. For HCV-RNA quantitative values below the limit of detection, we used the log_{10} of the cutoff value (500 IU/ml) for data analysis. Data are expressed as median (range).
[2] Absent vs. Present of substitution pattern (Mann-Whitney U test): [a] p = 0.025; [b] p = 0.019; [c] p = 0.011; [d] p = 0.049; [e] p = 0.001; [f] p = 0.007; [g] p = 0.010; [h] p = 0.002; [i] p = 0.004; [j] p = 0.018; [k] p = 0.001; [l] p = 0.019; [m] p = 0.028; [n] p = 0.006; [o] p = 0.001.

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.

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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.

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

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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.

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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

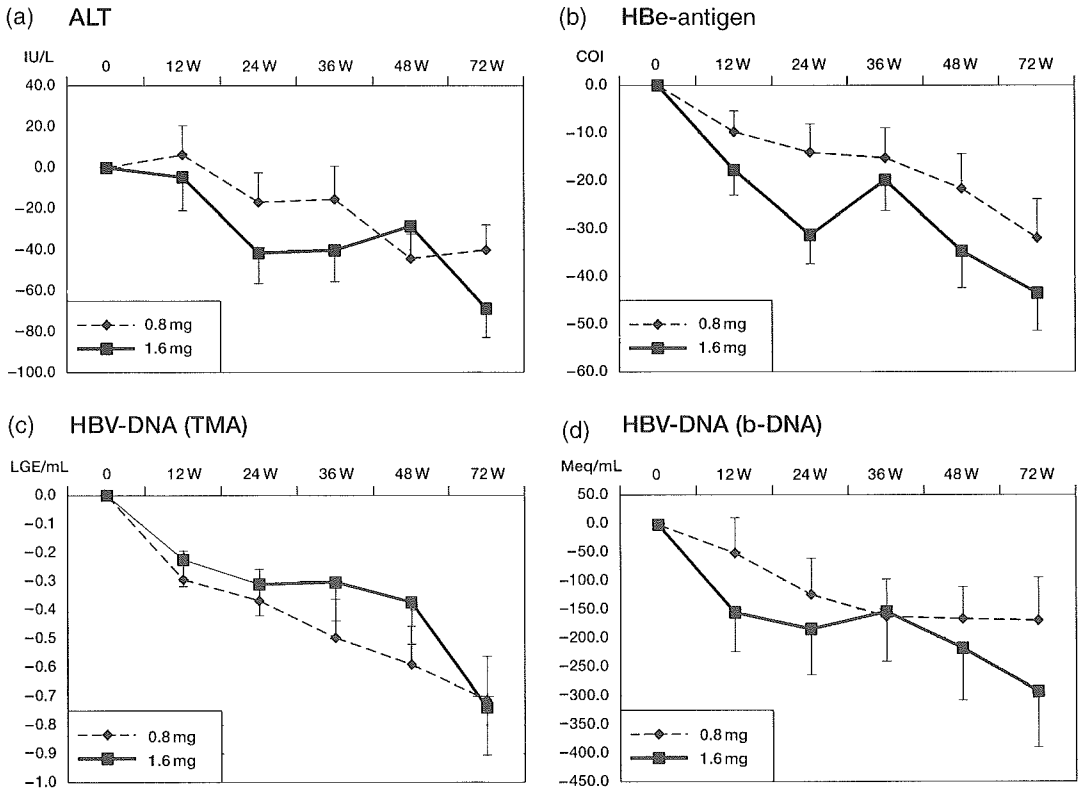

Fig. 1 Reduction from baseline in serum levels of ALT (a), HBeAg (b), HBV-DNA by TMA (c) and HBV-DNA by bDNA (d) for all patients in both treatment arms. All values are expressed as mean ± standard error (SE).

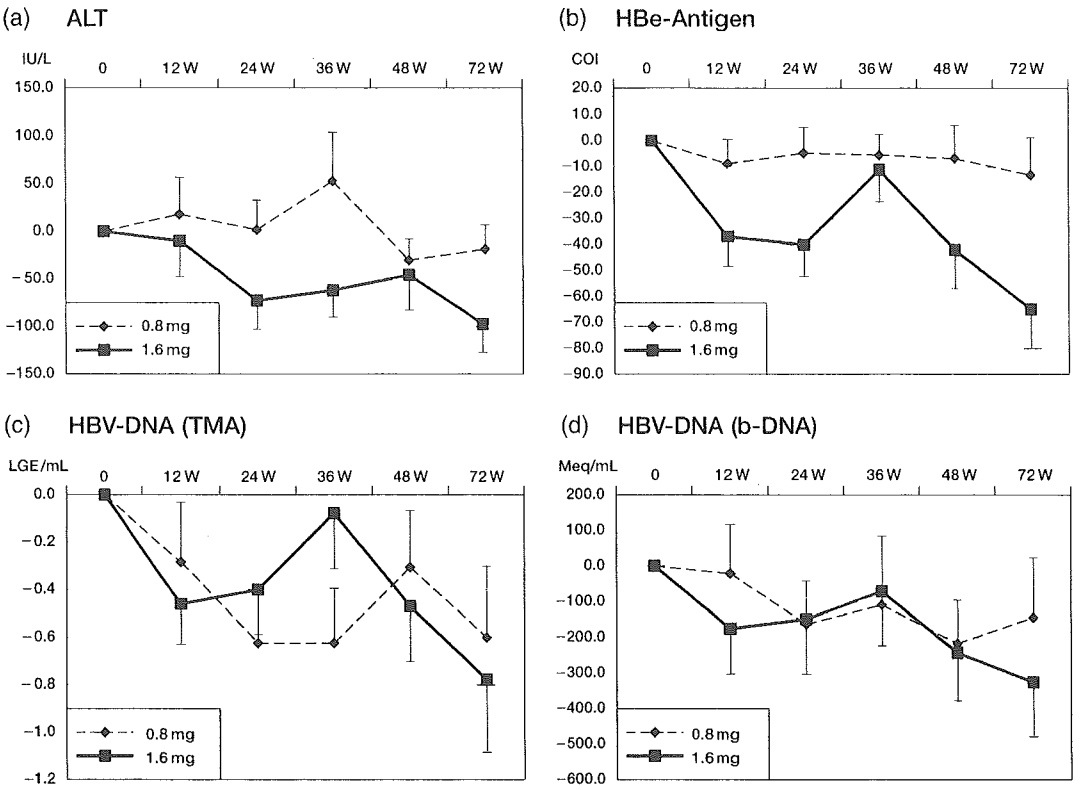

Fig. 2 Reduction from baseline in serum levels of ALT (a), HBeAg (b), HBV-DNA by TMA (c) and HBV-DNA by bDNA (d); stratified for patients with F3 fibrosis. All values are expressed as mean ± standard error (SE).

($P = 0.03$) 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.

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

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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.

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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.