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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

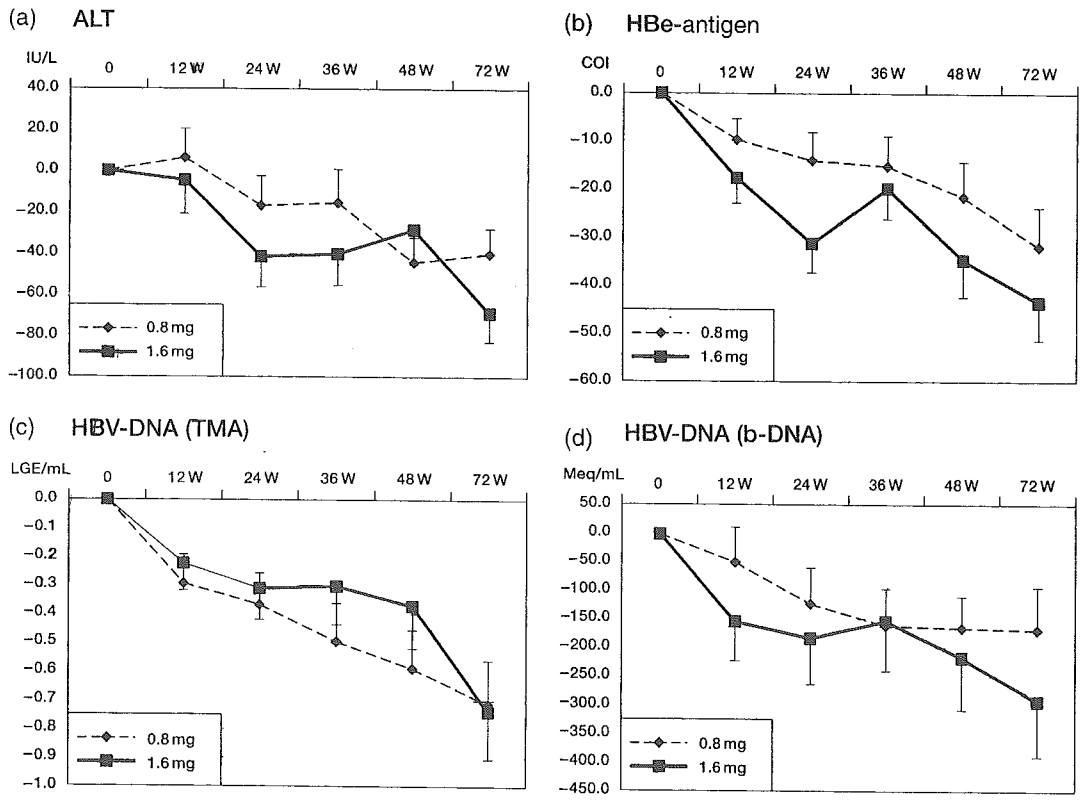

Fig. 1 Reduction from baseline in serum levels of ALT (a), HBeAg (b), HBV-DNA by TMA (c) and HBV-DNA by bDNA (d) for all patients in both treatment arms. All values are expressed as mean ± standard error (SE).

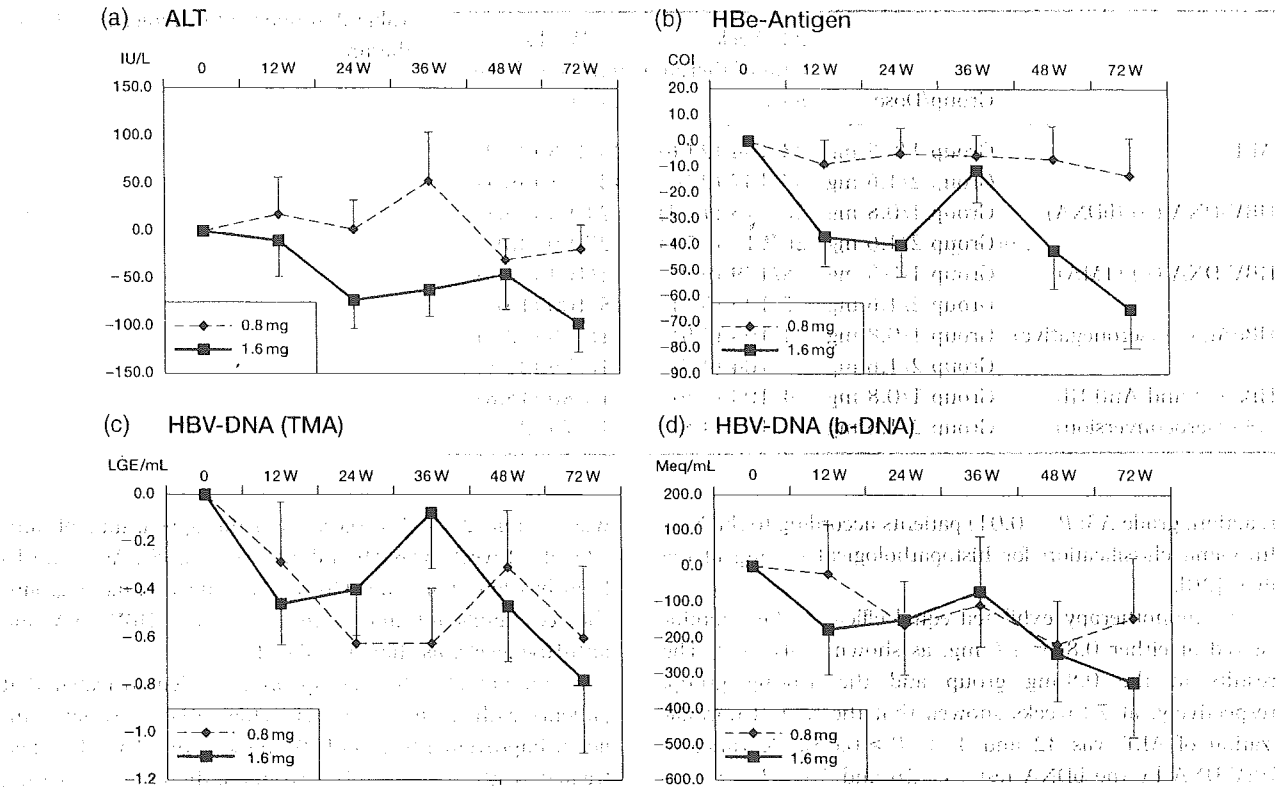

Fig. 2 Reduction from baseline in serum levels of ALT (a), HBeAg (b), HBV-DNA by TMA (c) and HBV-DNA by bDNA (d), stratified for patients with F3 fibrosis. All values are expressed as mean ± standard error (SE).

($P = 0.03$) 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.

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Characterization of hypervariable region in hepatitis C virus envelope protein during acute and chronic infection

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Summary. Hepatitis C virus (HCV) causes persistent infection in most patients. To clarify the mechanisms underlying establishment of this persistent infection, nucleotide sequences of the E1/E2 region were characterized in 5 patients with acute and chronic HCV infection. We used direct DNA sequencing methods to identify the major sequence of HCV in each patient. Each HCV genome displayed a high frequency of nucleotide sequence variation in the hypervariable region (HVR) of E2. However, patient-specific conserved nucleotide sequences were identified in the E1/E2 region during the course of infection and conserved the higher-order protein structure.

In the acute phase HCV infection, amino acid substitution in HVR-1 as the monthly rate of amino acids substitution per site (%) between each point exceeded 10.2%. In the chronic phase HCV infection, a significantly lower rate of amino acid substitution was observed in patients. The host immune responses to HVR-1 of each HCV isolates from all clinical courses were characterized using synthetic peptides and ELISA. One chronic patient serum (genotype 1b) did not react at all to its own HVR-1 peptides, however another patient (genotype 2b) reacted to all clinical course. These results indicated that HVR-1 might not always exhibit

Note: DDBJ/EMBL/GenBank accession numbers of E1/E2 sequences reported in this paper are AB107929-AB107949.

neutralizing epitopes of HCV infection. The sequence variation in HVR-1 may instead indicate the existence of various clones in acute phase infection and the adaption of these clones is thought to have caused persistent and chronic infection in each patient.

Introduction

Hepatitis C virus (HCV) possesses a genome of single-strand RNA with positive polarity (about 9.6 kb), and is classified in the family *Flaviviridae*, genus *Hepacivirus* [24]. HCV is the major causative agent of post-transfusion-associated non-A, non-B hepatitis, and it an estimated 170 million people are infected worldwide. Persistent HCV infections often progress to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [3, 25]. Previous reports have suggested that variability of the HCV genome is likely to play crucial roles in facilitating escape from host immune surveillance [9, 12, 29]. In particular, high degrees of sequence variability have been observed in regions coding the E2 envelope protein, designated as hypervariable regions (HVR)-1 [13, 28] and 2 [22]. HVR-1 has been suggested as a dominant neutralizing epitope for HCV infection in chimpanzees [6]. Despite the confirmed presence of HCV-specific antibodies and cytotoxic T lymphocytes [1, 7], HCV causes frequently persistent infection. These results suggest that variation occurring in neutralizing epitopes within HVR-1 could produce escape variants able to elude the host immune system. Recent reports have indicated that the evolution of viral quasispecies may predict clinical course in viral hepatitis [8].

Although HCV preferably infects hepatocytes, as confirmed by the existence of negative-strand RNA [15], the mechanisms of adsorption into hepatocytes and transcription and replication of viral RNA in the cell remain unclear. The possibility of low-density lipoprotein (LDL)-receptor has been suggested as a virus receptor for HCV infection [2, 18]. CD81 belongs to a family of molecules called tetraspanins, characterized by four transmenbrane domains forming two extracellular loops [17], and interacts with E2 protein as a putative viral receptor [23]. So far, six hepatocyte-binding regions have been defined in the E1/E2 region using synthetic peptides [11]. Inhibition of natural killer cells through engagement of CD81 by E2 protein has been reported [5]. Moreover, no polymorphisms in CD81 amino acid (a.a.) sequences on peripheral blood mononuclear cells (PBMCs) have been observed between healthy volunteers and patients during HCV infection [10].

The mechanisms of adaptation and selection allowing HCV to establish chronic infection during the first phase of acute infection remain unclear. The present study characterized patient-specific conserved original nucleotide sequences of the E1 and E2 regions, and deduced amino acid (a.a.) substitutions during the course of HCV infection for acute and chronic phase using direct DNA sequencing methods and humoral immunity of patients to HVR-1 peptides during the course of chronic HCV infection.

Materials and methods

Patients and sera

Two patients displaying acute infection with hepatitis C virus by transfusion (patients A and B; Table 1, Fig. 1) were selected retrospectively, along with three randomly selected patients with chronic hepatitis C in which high levels of serum alanine aminotransferase (ALT) were maintained for more than six months after first medical examination (patients C–E; Table 1, Fig. 1). All serum samples were utilized to determine nucleotide sequences of the HCV E1 and E2 regions during disease progression, and deduced a.a. sequences were predicted. These selected sera were aliquoted and stored below −80 °C until characterization. Two patients were infected with HCV by transfusion: patient A (a 58-year-old woman) when she donated a kidney; and patient B (a 55-year-old man) during hip joint surgery. Patients A and B were followed up for 11 and 13 years, respectively. In patients A and B, serum ALT levels remained abnormal during the entire follow-up period. Patients C (54-year-old man), D (26-year-old man), and E (67-year-old man) displayed histological evidence of chronic active hepatitis C.

Informed consent was obtained from all patients in accordance with the Helsinki Declaration.

Detection of anti HCV antibody and HCV RNA

Second-generation enzyme-linked immunosorbent assay (Ortho Diagnostic Systems, Raritan, NJ) was used to detect HCV antibody in sera from the five patients during disease progression. Serum HCV RNA was extracted using the acid guanidium thiocyanate-phenol-chloroform (AGPC) method [4], and detected by reverse transcription and nested polymerase chain reaction (PCR) using primers for the 5-noncoding region of the HCV genome [19]. Results

Table 1. Clinical evaluation of patients and time points of characterization. Randomly selected patients with hepatitis C were analyzed

Patients	HCV genotype	Age (years)	Sex	Points[a]	Duration[b] (months)
(Acute)					
A	1b	58	F	1 to 2	3
				2 to 3	7
B	1b	55	M	1 to 2	4
				2 to 3	8
(CH)					
C	1b	54	M	1 to 2	12
				2 to 3	3
D	1b + 2a	26	M	1 to 2	8
				2 to 3	7
E	2a	67	M	1 to 2	10
				2 to 3	11

[a]Points, points of analysis

[b]Duration, duration between points of analysis

Acute: acute infection with hepatitis C virus by transfusion, with ALT and viral RNA levels rapidly decreased immediately after infection, then subsequently increased

CH: chronic hepatitis patient, with high levels of ALT maintained for more than six months after first medical examination

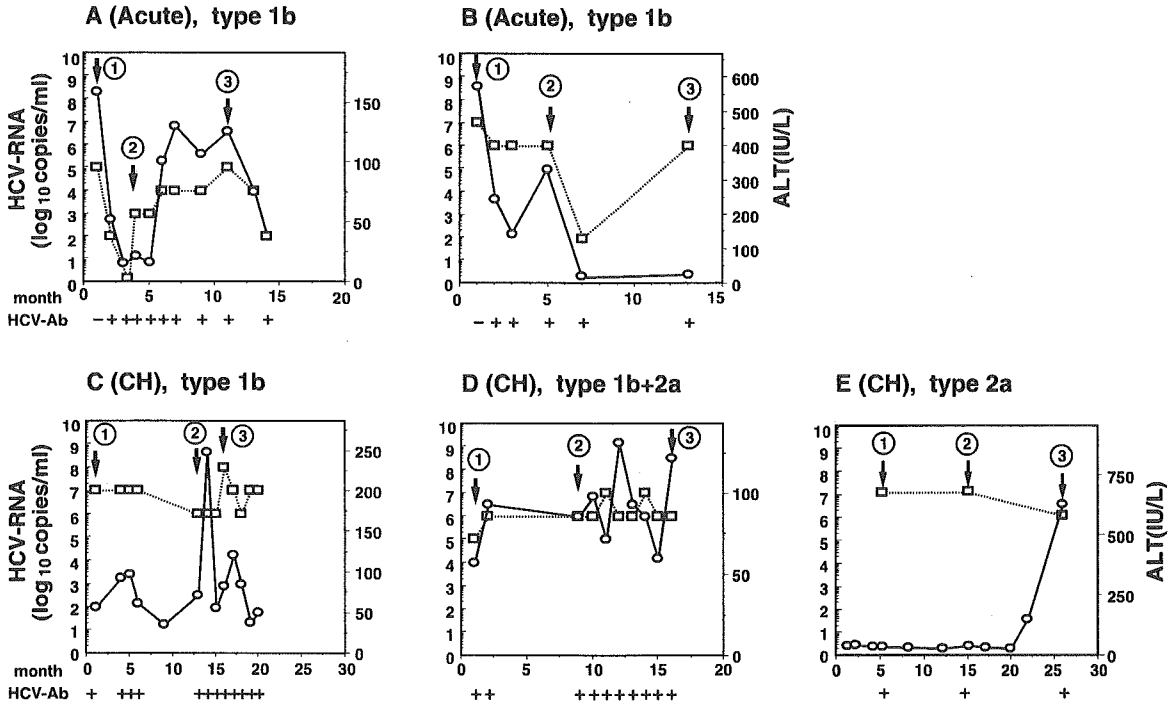

Fig. 1. Clinical course of hepatitis C patients. Changes in HCV RNA titer (broken line with open square), ALT level (black line with open circle) and HCV antibodies (− or +) for each patient. Numbers *1–3* in open circles indicate DNA sequencing points. In acute patients **A** and **B**, time point 1 represents onset by transfusion. In chronic patients **C** and **D**, time point 1 represents first medical examination. In patient **E**, time point 1 represents five months after first medical examination. *Acute*: acute HCV infection; *CH*: chronic HCV infection

were quantified using competitive PCR assay [30] in which cloned DNA (with a 15-bp deletion in the middle portion) used as a competitor [29].

Nucleotide sequence analysis in E1/E2 region

Nucleotide sequences for the E1/E2 region were analyzed according to direct DNA sequencing methods for PCR products using λ exonuclease (Gibco-BRL, Rockville, MD). HCV genome was extracted using AGPC methods [4], then amplified by reverse transcriptase and nested-PCR from serum for overlapping regions using two sets of primer pairs. PCR primers and amplified regions utilized for each patient are listed in Table 2. The second PCR product which was amplified by 5′-phosphorylated primer. PCR products were purified by 3% Nusieve 3:1 agarose gel electrophoresis (FMC BioProducts, Rockland, ME). One to four units of λ exonuclease was added to purified PCR products, including 67 mM glycine-KOH (pH 9.4) and 2.5 mM $MgCl_2$, and incubated at 37 °C for 1 h to form single-stranded DNA. Reaction mixtures were precipitated with ethanol and dried for DNA sequencing. Aliquoted DNA fragments were used for identification of nucleotide sequences in the E1/E2 region [27]. Nucleotide sequences of the E1/E2 region were determined for all five patients during disease progression. Characterization of nucleotide sequences and phylogenetic analyses of HVR-1 were performed using GENETYX version 10 software (Software Development, Tokyo, Japan). The phylogenetic tree for HVR-1 was constructed using the neighbor-joining (NJ) method [26].

Table 2. List of primer sequences for PCR of the HCV genome

Patients (Point*) [nt no.**]	Primer name	sequence
A (1, 3)	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1265]	1st anti-sense; (b)	5′-TAGATTGAGCAATTGCAATCTTGN-3′
	2nd sense; (c)	5′-CCGGTTGCTCTTTCTCTATCTTN-3′
[848–1265]	2nd anti-sense; (b)	5′-TAGATTGAGCAATTGCAATCTTGN-3′
A (2), B	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1385]	1st anti-sense; (d)	5′-GCCACCATGTCCACGACAGCTTGGTGG-3′
	2nd sense; (e)	5′-TGGTAAGGTCATCGATACCCTCACN-3′
[697–1365]	2nd anti-sense; (f)	5′-TTGTGGGATCCGGAGTAACTGCGACAC-3′
A, C	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1385]	1st anti-sense; (d)	5′-GCCACCATGTCCACGACAGCTTGGTGG-3′
	2nd sense; (c)	5′-CCGGTTGCTCTTTCTCTATCTTN-3′
[848–1365]	2nd anti-sense; (f)	5′-TTGTGGGATCCGGAGTAACTGCGACAC-3′
D-2a, E	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1387]	1st anti-sense; (p)	5′-CTAATGATGTCTATGATGACCTCGGGAACG-3′
	2nd sense; (c)	5′-CCGGTTGCTCTTTCTCTATCTTN-3′
[848–1357]	2nd anti-sense; (q)	5′-CGCATCACGTACGCCAGAATCATGG-3′
D-1b	1st sense; (h)	5′-ATGGCTTGGGATATGATGATGAACTGGTC-3′
[1290–1867]	1st anti-sense; (i)	5′-TGAAACAATACACTGGACCACACAC-3′
	2nd sense; (j)	5′-ATTCCATGGTGGGGAACTGGGCTAA-3′
[1424–1813]	2nd anti-sense; (k)	5′-TAGGTGCGTAGTGCCAGCAATAAGG-3′
B	1st sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1887]	1st anti-sense; (m)	5′-ACTACAACAGGGCTCGGAGTGAAN-3′
	2nd sense; (n)	5′-ATGGCTTGGGATATGATGATGAACTGGTCN-3′
[1291–1867]	2nd anti-sense; (o)	5′-TGAAGCAATACACTGGACCACACACN-3′
D-2a	1st sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1887]	1st anti-sense; (m)	5′-ACTACAACAGGGCTCGGAGTGAAN-3′
	2nd sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1867]	2nd anti-sense; (o)	5′-TGAAGCAATACACTGGACCACACACN-3′
A, C, E	1st sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1867]	1st anti-sense; (i)	5′-TGAAACAATACACTGGACCACACAC-3′
	2nd sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1813]	2nd anti-sense; (k)	5′-TAGGTGCGTAGTGCCAGCAATAAGG-3′

*Point, point of analysis; **nt no., nucleotide number on HC-R6, accession no. AY045702

Protein structure and amino acid substitution speed analyses in E1/E2 region

The a.a. sequence of the E1/E2 region was deduced from corresponding nucleotide sequences for all five patients. Protein structural analyses (hydrophobic profile, antigenic index and surface probability) were performed using MacVector sequence analysis software (International Biotechnologies, New Haven, CT). Protein secondary structure (Chou-Fas) was determined using GENETYX version 10 software (Software Development). Amino acid substitution speed was analyzed for HVR-1 (27 a.a.), HVR-2 (7 or 9 a.a.), another region of

Table 3. Reactivities of patient sera to HVR-1 peptides

	HVR peptide		
	Point: 1	2	3
Patient C Serum:			
1	−	−	−
Point: 2	−	−	−
3	−	−	−
Patient E Serum:			
1	+	+	+
Point: 2	+	+	+
3	+	+	+

Patient point	HVR-1 peptide sequences
C-1	HTHVIGGAQTQTTGSFASLFTPGASQK
C-2	**R**THVIGG**V**QTQTTGS**L**ASLFTPGASQK
C-3	**R**THV**T**GG**V**Q**SR**TTGS**LV**SLFTPGASQK
E-1	STHTIGGCTARSAAGFTRLFTQGARQN
E-2	STHTIGG**S**TARSAAGFTRLFTQGARQN
E-3	STHT**V**GG**S**TARSAAGFT**K**LFT**R**GA**H**QN

E2 (between HVR-1 and HVR-2; 63 a.a.) and E1 as the monthly rate of a.a. substitutions per site (%) between each point during disease progression (points 1–3; Fig. 1).

Test of host immune response to HVR-1 peptide

Synthetic peptides of HVR-1 for patients C and E were synthesized for each point in the clinical course (points 1–3; Fig. 1, Table 3). Peptides were tested using ELISA to characterize host immune responses to HVR-1 during chronic infection.

Results

Characterization of HCV-RNA, anti-HCV antibody and ALT levels in acute and chronic infection of hepatitis C virus

To clarify the mechanisms of genetic variation during persistent HCV infection, 5 patients were retrospectively analyzed (Table 1, Fig. 1). Patients A and B displayed acute infection with HCV genotype 1b, with progression from first phase of acute infection to chronic infection, and persistent viremia (Fig. 1). In the first phase of acute infection, antibody to HCV became positive (after point 1; Fig. 1). In patient A, HCV-RNA and ALT levels in serum decreased immediately after infection (point 1 to 2; Fig. 1), then elevated in the second phase of acute infection (point 2 to 3; Fig. 1). In patient B, HCV-RNA and ALT levels in serum decreased immediately

after infection, with an elevation of HCV-RNA levels occurring only in the second phase of acute infection (point 2 to 3; Fig. 1). Patients C, D and E displayed chronic hepatitis and persistent infection of HCV. Patient C was infected with genotype 1b, Patient E was infected with genotype 2a, and Patient D displayed co-infection with genotypes 1b and 2a (Table 1). In Figure 1, quantity of HCV-RNA in patient D indicates combined total RNA for both genotypes. These three patients displayed continuously high levels of ALT for more than six months after first medical examination and did not display marked changes in HCV-RNA levels (points 1–3; Fig. 1). A peak in ALT value was detected between points 2 and 3 for patients C and D, while elevation of ALT values was detected between points 2 and 3 for patient E.

Nucleotide sequence variation and patient-specific nucleotide sequence in E1/E2 region during clinical course of hepatitis C

To clarify the predominant sequence of E1/E2 region during progression of hepatitis C, 5 patients (2 patients with acute hepatitis, 3 patients with chronic hepatitis) were retrospectively selected and sequences (nucleotides 620 ~ 1867; Table 2) from the sera of these patients were analyzed at three points (points 1–3; Fig. 1) using direct DNA sequencing methods as described. Analyzed HCV DNA sequences of the E1/E2 region for each patient were registered to Genbank (accession numbers AB107929–AB107949). Alignment of nucleotide sequences on one-third of the E2 region (nucleotide 1492 ~ 1785) is indicated in Fig. 2. Sequences categorized as patient-specific conserved nucleotide sequences displayed the following characteristics: 1) identical nucleotide sequences at each of the three points; 2) sequences that are not conserved within the same genotypes (Fig. 2A). Consistent with previous results [29], numerous nucleotide sequence variations in HVR-1 and 2 were identified in these HCV isolates from acute and chronic infection patients. However, patient-specific conserved nucleotide sequences were observed in this E2 region even within HVR-1 and -2 for each patient (boxed region; Fig. 2A). In the E1 region, patient-specific conserved nucleotide sequences were also observed in the five patients (data not shown).

Sequences categorized as substituted nucleotide sequences displayed (Fig. 2B). Substituted nucleotide sequences were present in this E1/E2 region for all 5 patients during the clinical course of infection.

Amino acid sequence variations in the E1/E2 protein region during the clinical course of hepatitis C

Deduced amino acid sequences of the E1/E2 region (a.a. 192 ~ 480) were compared in 5 patients (2 acute patients, 3 chronic patients) at points 1–3 (Fig. 3). Sequences categorized as patient-specific amino acid sequences displayed the same characteristics as those of patient-specific nucleotide sequences. Variations in a.a. sequence were particularly concentrated in HVR-1 and -2 for HCV genotype 1b isolates (patients A–C and 1b isolate from patient D; Fig. 3) and in HVR-1 alone

for HCV genotype 2a isolates (patient E and 2a isolate from patient D; Fig. 3) during the clinical course of infection. Although patient-specific conserved amino acid sequences were present in this E1/E2 region for all 5 patients during the clinical course of infection, the impact was not as strong as that of patient-specific conserved nucleotide sequences (Fig. 2).

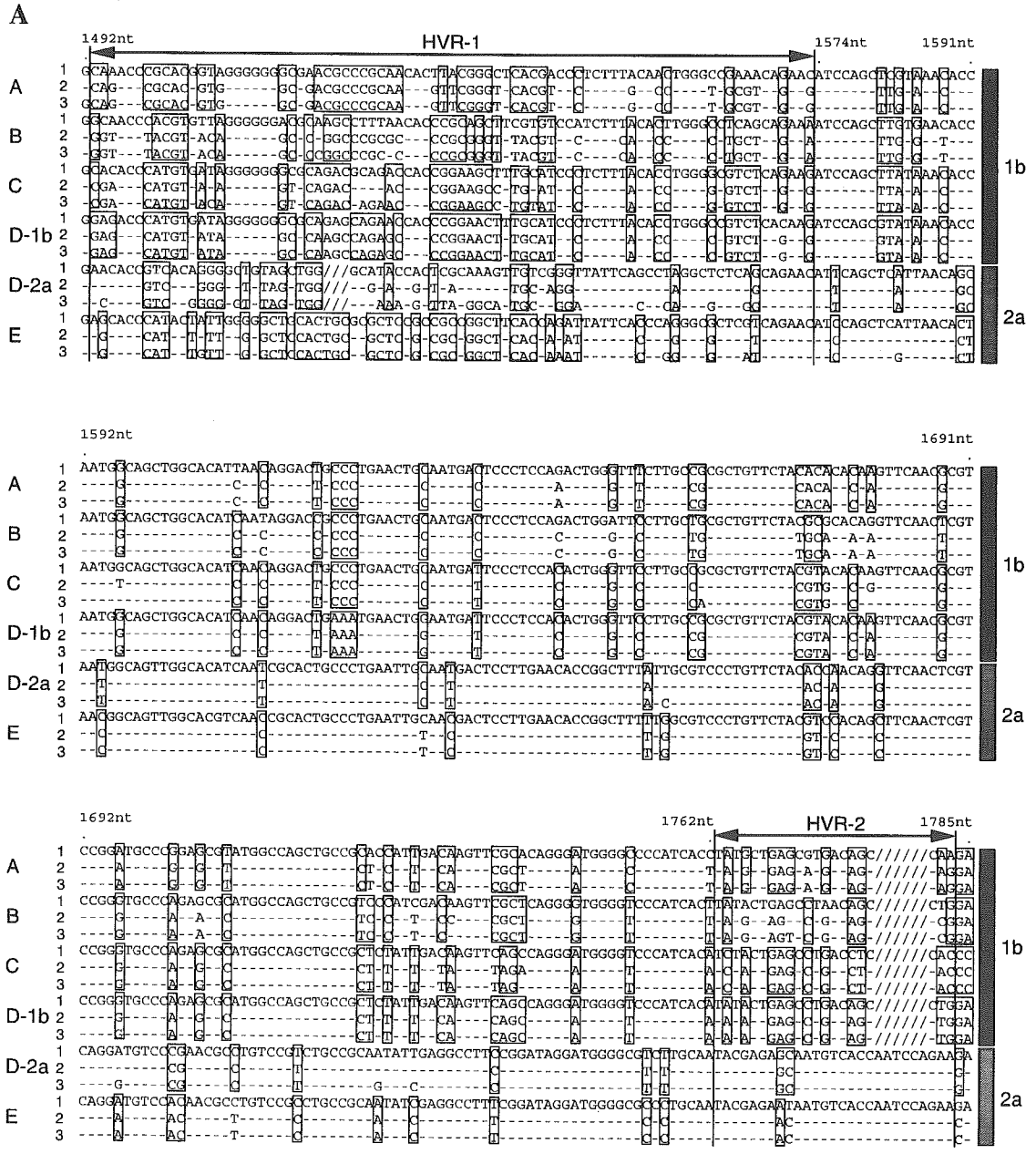

Fig. 2 (*continued*)

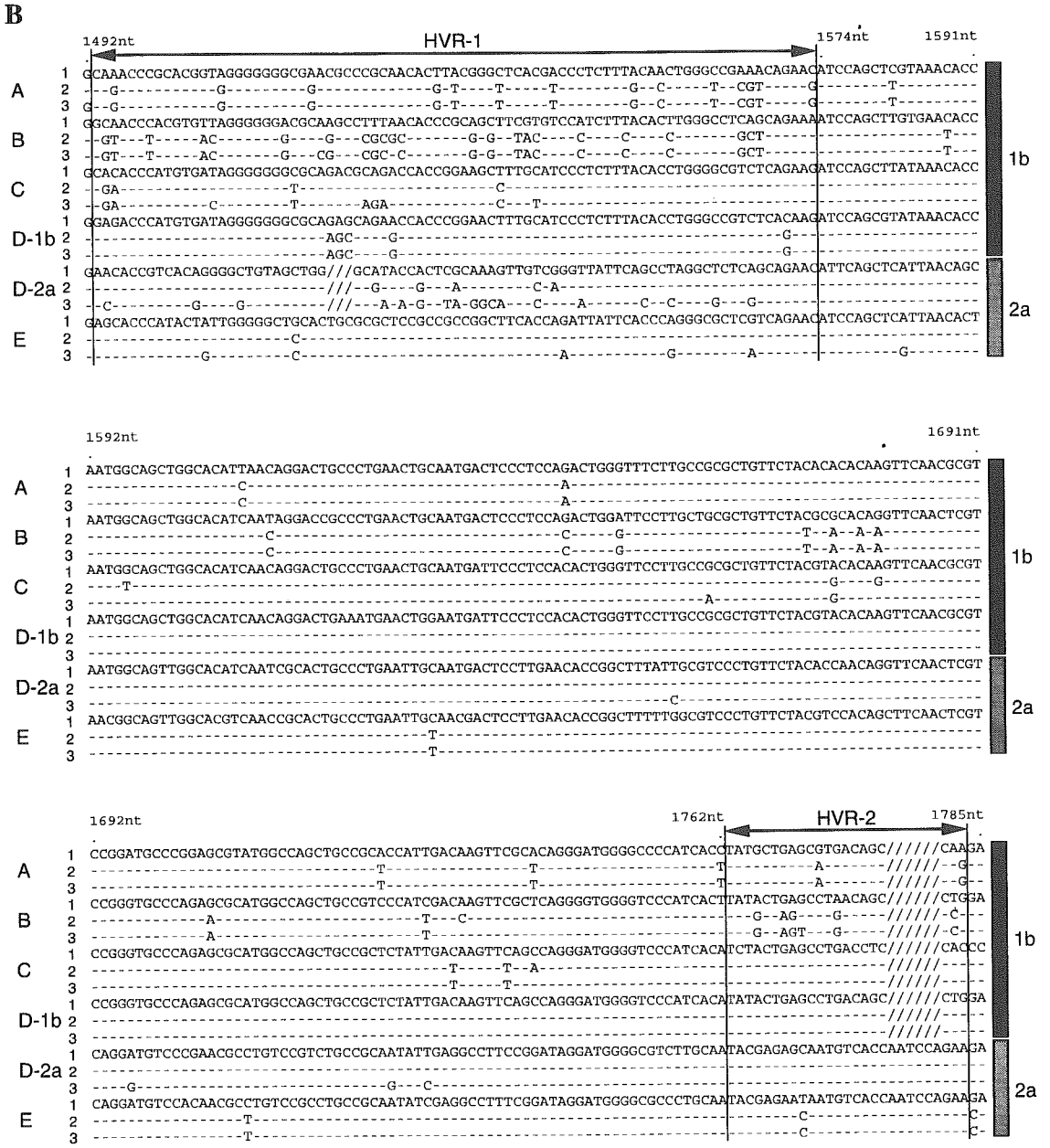

Fig. 2. Predominant nucleotide sequence comparison between each sequence from HCV patients at points 1–3. Region including nucleotides 1492–1785, including HVRs, was compared. **A** Patient-specific conserved sequences are enclosed in boxes. Each sequence column number indicates DNA sequencing point for each patient. Dash (–) indicates the same nucleotide as the first column sequence for each patient. Slash (/) indicates nucleotide deletion point. Column marked with a black box on the right side indicates HCV genotype 1b isolate. Column marked with a hatched box indicates HCV genotype 2a isolate. **B** Sequences categorized as substituted nucleotide sequences displayed

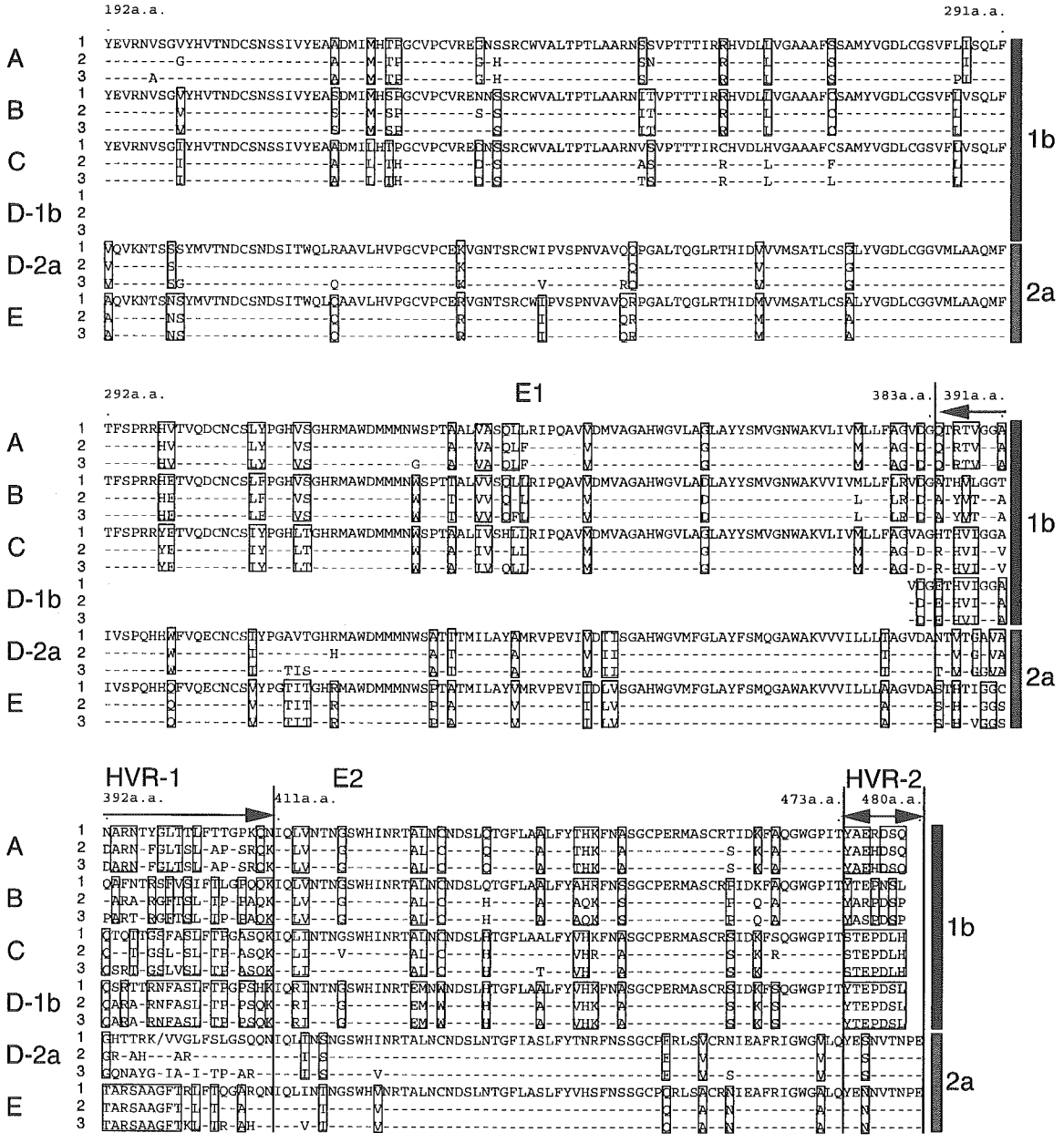

Fig. 3. Comparison of predicted amino acid sequences between each dominant HCV isolate from patients at points 1–3. E1/E2 protein sequences of HCV (a.a. 192–480) were compared. Sequence column number indicates DNA sequencing point for each patient. Patient-specific conserved sequences are enclosed in boxes. Dash (–) indicates the same a.a. residue as the first column sequence for each patient. Slash (/) indicates a.a. deletion point. Column marked with a black box on the right side indicates HCV genotype 1b isolate. Column marked with a hatched box indicates HCV genotype 2a isolate

In genotype 2a isolates from patient D (D-2a; Fig. 3), one a.a. deletion was identified in HVR-1 (residue 398, presented as a slash in Fig. 3). In genotype 2a isolates (patients D and E), two additional a.a.s in HVR-2 were noted, as reported elsewhere [21]. The deduced amino acid sequence of the E1 region (corresponding to a.a. 192 ~ 380) in genotype 1b isolates from patient D (D-1b; Fig. 3) could not be amplified by PCR at any time point (points 1–3; Figs. 1, 3).

Amino acid substitution speed in E1/E2 protein region and phylogenetic analysis of HVR-1 during progression of hepatitis C

To elucidate status of the HCV genome during infection, a.a. substitution speed between each point (point 1 to 2 and point 2 to 3; Fig. 1) was calculated as

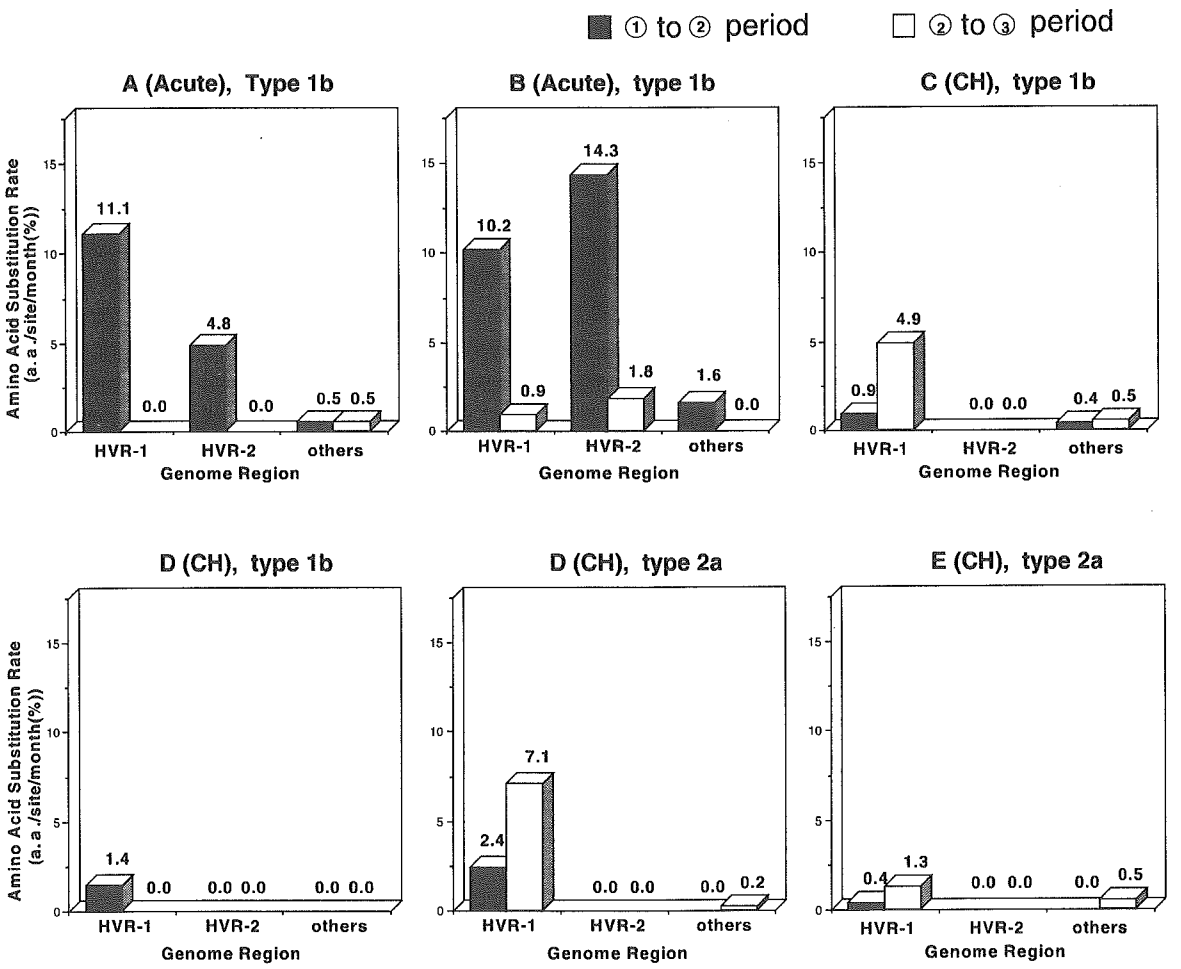

Fig. 4. Amino acid substitution speed in E1/E2 protein within HVR-1, HVR-2 and other regions. Amino acid substitution speed from DNA sequencing points 1 to 2 is indicated by black bars, and speed from points 2 to 3 is indicated by white bars. Regions HVR-1, HVR-2 and others represent a.a. 384–410, 474–480, and 411–473, respectively. *Acute*: acute HCV infection; *CH*: chronic HCV infection

the monthly rate of a.a. substitutions within each region (%; Fig. 4). In the first phase of acute infection (point 1 to 2), a.a. substitution speed in HVR-1 and HVR-2 was significantly faster than in the any other region of E1 and E2 in patients A and B (11.1% and 10.2% for HVR-1; 4.8% and 14.3% for HVR-2, respectively). In the second phase (point 2 to 3) of acute infection, a.a. substitution speed in HVR-1 and HVR-2 was slower than the first phase of acute infection in patients A and B (0% and 0.9% for HVR-1; 0% and 1.8% for HVR-2; 0.5% and 0% for other regions, respectively). In contrast, a.a. substitution speed in chronic patients was 0% in HVR-2 and below 0.5% in other regions (patients C–E; Fig. 4). Amino acid substitution speed in HVR-1 was fast during chronic HCV infection of ALT or when virus RNA levels underwent substantial transitions (patients C–E; Figs. 1, 4). In phylogenic tree analysis of HVR-1, sequence diversity of HVR-1 in the first phase of acute infection was phylogenetically distant from the original sequence (patients A and B), and the phylogenic tree of HVR-1 displayed clusters for each of the five patients (data not shown).

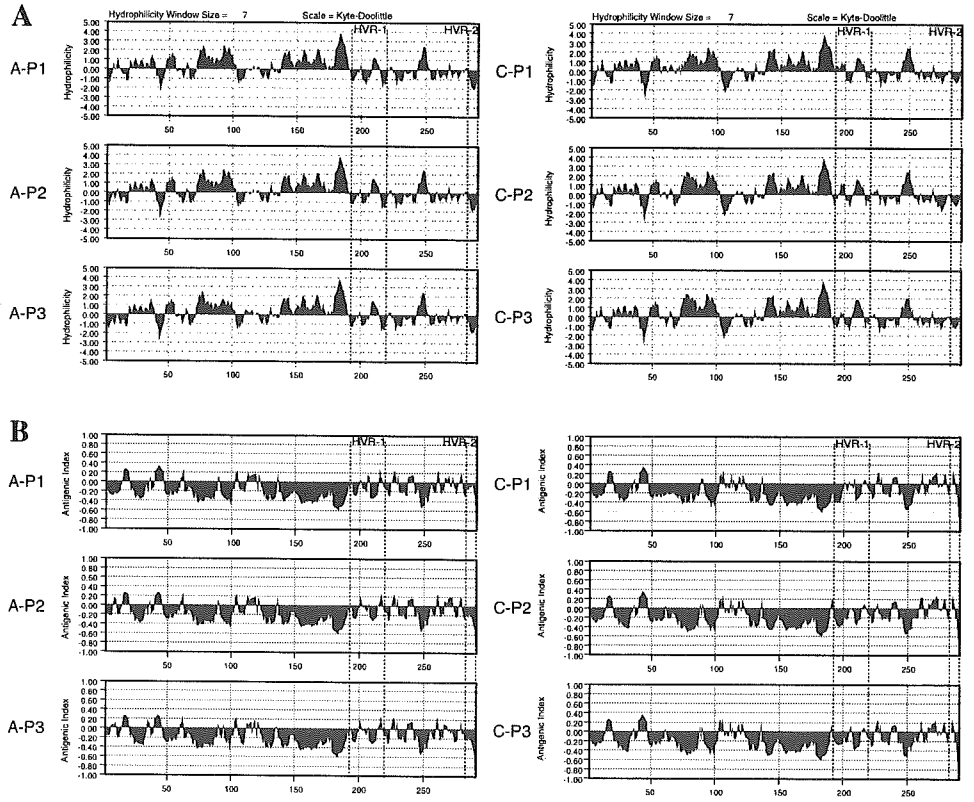

Fig. 5. Structural analysis of E1/E2 protein during HCV infection. Hydrophilicity profiles and antigenic indices of E1/E2 protein as predicted from direct DNA sequencing data were compared between points 1–3 for patients A and C. **A** Hydrophilicity profile. **B** Antigenic index. The presented region for HCV E1/E2 corresponds to a.a. 192–480