15. **Fujiwara K, Tanaka Y, Paulon E, Orito E, Sugiyama M, Ito K, Ueda R, Mizokami M, Naoumov NV.** 2005. Novel type of hepatitis B virus mutation: replacement mutation involving a hepatocyte nuclear factor 1 binding site tandem repeat in chronic hepatitis B virus genotype E. J Virol **79**:14404–14410. http://dx.doi.org/10.1128/JVI.79.22.14404-14410.2005.
16. **Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, Li W.** 2012. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. eLife **1**:e00049. http://dx.doi.org/10.7554/eLife.00049.
17. **Rivas P, Herrero MD, Puente S, Ramirez-Olivencia G, Soriano V.** 2007. Immunizations in HIV-infected adults. AIDS Rev **9**:173–187.
18. **Launay O, van der Vliet D, Rosenberg AR, Michel ML, Piroth L, Rey D, Colin de Verdiere N, Slama L, Martin K, Lortholary O, Carrat F, ANRS HB03 VIHVAC-B Trial.** 2011. Safety and immunogenicity of 4 intramuscular double doses and 4 intradermal low doses vs standard hepatitis B vaccine regimen in adults with HIV-1: a randomized controlled trial. JAMA **305**:1432–1440. http://dx.doi.org/10.1001/jama.2011.351.
19. **Sugden PB, Cameron B, Mina M, Lloyd AR, HITS Investigators.** 2014. Protection against hepatitis C infection via NK cells in highly-exposed uninfected injecting drug users. J Hepatol **61**:738–745. http://dx.doi.org/10.1016/j.jhep.2014.05.013.
20. **Werner JM, Heller T, Gordon AM, Sheets A, Sherker AH, Kessler E, Bean KS, Stevens M, Schmitt J, Rehermann B.** 2013. Innate immune responses in hepatitis C virus-exposed healthcare workers who do not develop acute infection. Hepatology **58**:1621–1631. http://dx.doi.org/10.1002/hep.26353.
21. **Knapp S, Warshow U, Hegazy D, Brackenbury L, Guha IN, Fowell A, Little AM, Alexander GJ, Rosenberg WM, Cramp ME, Khakoo SI.** 2010. Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus. Hepatology **51**:1168–1175. http://dx.doi.org/10.1002/hep.23477.
22. **Maini MK, Peppa D.** 2013. NK cells: a double-edged sword in chronic hepatitis B virus infection. Front Immunol **4**:57. http://dx.doi.org/10.3389/fimmu.2013.00057.
23. **Rehermann B.** 2013. Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. Nat Med **19**:859–868. http://dx.doi.org/10.1038/nm.3251.
24. **Summers J, Jilbert AR, Yang W, Aldrich CE, Saputelli J, Litwin S, Toll E, Mason WS.** 2003. Hepatocyte turnover during resolution of a transient hepadnaviral infection. Proc Natl Acad Sci U S A **100**:11652–11659. http://dx.doi.org/10.1073/pnas.1635109100.
25. **U.S. Public Health Service.** 2001. Updated U.S. Public Health Service guidelines for the management of occupational exposures to HBV, HCV, and HIV and recommendations for postexposure prophylaxis. MMWR Recomm Rep **50**:1–52.

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Original Article

Factors associated with the effect of interferon-α sequential therapy in order to discontinue nucleoside/nucleotide analog treatment in patients with chronic hepatitis B

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Aim: The factors associated with the outcome of sequential therapy with interferon-α (IFN-α) in order to halt nucleoside/nucleotide analog (NUC) maintenance treatment for chronic hepatitis B were analyzed.

Methods: A total of 50 patients with chronic hepatitis B who underwent IFN-α sequential therapy for cessation of NUC were enrolled retrospectively. The subjects received NUC plus IFN-α for 4 weeks followed by IFN-α alone for 20 weeks. Natural IFN-α of 6-MU doses was administrated three times a week. A successful response to NUC/IFN-α sequential therapy was defined as serum hepatitis B virus (HBV) DNA below 4.0 log copies/mL, serum alanine aminotransferase (ALT) below 30 IU/L, and hepatitis B e-antigen negativity at 24 months after completing the treatment.

Results: Multivariate analysis revealed that hepatitis B surface antigen (HBsAg) of 3.0 log U/mL or more ($P < 0.002$) and hepatitis B core-related antigen (hepatitis B core-related antigen [HBcrAg]) of 4.5 log U/mL or more ($P < 0.003$) at the start of IFN-α administration were significant factors associated with a 24-month non-response. Maximal levels of ALT and HBV DNA during the follow-up period after completing IFN-α therapy were significantly related ($P < 0.001$), and receiver operating characteristic analysis showed that both maximal ALT ($P < 0.001$) and HBV DNA ($P < 0.001$) were significantly related to the final 24-month response.

Conclusion: The combinational use of HBsAg and HBcrAg levels may be useful to predict the 24-month outcome of NUC/IFN-α sequential therapy. Maximal levels of ALT and HBV DNA during post-treatment follow-up may also help monitor responses to IFN-α sequential therapy.

Key words: hepatitis B core-related antigen, hepatitis B surface antigen, interferon-α, nucleoside/nucleotide analogs, sequential therapy

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a widespread health problem with an estimated 350–400 million carriers worldwide. Prolonged infection with HBV can cause chronic hepatitis, which may eventually develop into liver cirrhosis and hepatocellular carcinoma (HCC).[1-3] Currently available antiviral treatments for hepatitis B include nucleoside/nucleotide analogs (NUC) and interferon-α (IFN-α).[4] NUC are p.o. administrated and are associated with low rates of adverse effects. Although treatment with NUC, such as lamivudine (LVD), adefovir dipivoxil and entecavir (ETV), induces virological and biochemical responses in most patients, NUC therapy also carries the risk of drug resistance. Furthermore, patients with hepatitis B are required to undergo extended

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treatment with NUC because early discontinuance often leads to relapse.[5,6] In contrast, the remission of chronic hepatitis B by IFN-α is prolonged, but is achieved only in a small percentage of patients.

Serfaty *et al.*[7] conducted a pilot study on sequential therapy using LVD and IFN-α and concluded that this treatment could induce a sustained virological response in patients with chronic hepatitis B who did not respond to IFN-α alone. However, ensuing reports[8-12] were unable to confirm such a cooperative effect. Because the clinical backgrounds of the enrolled patients also differed among the above reports, it has become necessary to clarify the factors associated with the outcome of IFN-α sequential therapy in order to estimate its clinical significance.

We previously analyzed patients with chronic hepatitis B who ceased NUC therapy and showed that lower hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels were associated with a favorable clinical outcome in subjects negative for hepatitis B e-antigen (HBeAg) and HBV DNA at NUC discontinuation.[13,14] Although we identified patients in whom NUC could be safely halted with high reliance, such patients accounted for a relatively minor percentage. Therefore, we conducted the present study to analyze the effect of IFN-α sequential therapy on successfully stopping NUC.

This report retrospectively analyzes the factors associated with outcome of IFN-α sequential therapy following NUC treatment. As the subjects were followed long term, treatment responses at 24 months after stopping IFN-α were evaluated and compared with those at 6 and 12 months.

METHODS

Patients

A TOTAL OF 50 patients with chronic hepatitis B who underwent IFN-α sequential therapy in order to halt NUC therapy between May 2002 and September 2010 were enrolled. Subjects received NUC plus IFN-α for 4 weeks followed by IFN-α alone for 20 weeks (Fig. 1). Natural IFN-α (Sumiferon; Sumitomo Dainippon Pharma, Tokyo, USA) at a dose of 6 MU was administrated three times a week. Doses were reduced to 3 MU during exceptional circumstances, such as side-effects. All patients completed 24 weeks of IFN-α administration and received over 80% of the scheduled dose. Patients were recruited retrospectively from eight hospitals across Japan (Shinshu University Hospital, National Hospital Organization Nagasaki Medical Center, Toranomon Hospital, Hiroshima University Hospital, Chiba University Hospital, The Hospital of Hyogo College of Medicine, Kumamoto Shinto General Hospital,and Teine Keijinkai Hospital). The demographic data of the subjects are presented in Table 1. The median age at NUC cessation was 35 years. Approximately three-fourths of the patients were men. Genotype C HBV was predominant as has earlier been reported for Japan.[15] Eighty-six percent of patients began NUC therapy with LVD and 14% did so with ETV. The duration of NUC administration ranged from 4 to 121 months. The follow-up period was defined as the point of stopping IFN-α administration up until the last visit or to when NUC were re-administrated due to reactivation of hepatitis B. NUC were recommenced in 25 (50%) of the 50 patients enrolled. Among them, 17 were treated before judgment of the 24-month response to sequential therapy. All patients requiring re-administration

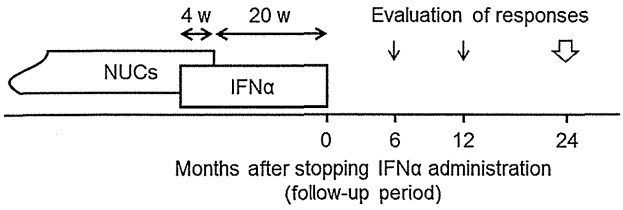

Figure 1 Experimental design of the present study. IFN, interferon; NUC, nucleoside/nucleotide analog; w, weeks.

Table 1 Demographic data of 50 enrolled patients

Characteristic	Value
Age at start of NUC administration (years)†	34 (21–57)
Age at end of NUC administration (years)†	35 (22–62)
Sex (male : female)	38:12
Genotype (B : C : undetermined)	3:36:11
NUC at start (LVD : ETV)	43:7
NUC at end (LVD : ETV : LAM + ADV : ETV + ADV)	40:8:1:1
Duration of NUC administration (months)†	6 (4–121)
HBeAg positivity at start of NUC‡	70% (35/50)
HBeAg positivity at end of NUC‡	42% (21/50)
Follow-up period after stopping IFN-α administration (months)†	28 (2–102)
Patients requiring re-administration of NUC‡	50% (25/50)
Patients developing HCC‡	0% (0/50)

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ADV, adefovir dipivoxil; ETV, entecavir; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; IFN, interferon; LAM, lamivudine; LVD, lamivudine; NUC, nucleoside/nucleotide analog.

of NUC possessed alanine aminotransferase (ALT) levels of over 80 IU/L and HBV DNA levels of over 5.8 log copies/mL at or just before the point of NUC re-continuation, which fulfilled the established requirements for restarting NUC.[13,14,16]

Hepatitis B surface antigen was confirmed to be positive on at least two occasions at least 6 months apart in all patients before NUC treatment. Tests for hepatitis C virus and HIV antibodies were all negative. Patients complicated with HCC or signs of hepatic failure at the cessation of NUC administration were excluded from the study. No such complications were observed during follow up.

With few exceptions, patients were seen at least once a month during the first year of follow up, at least once every 3 months during the second year and at least once every 6 months afterwards. No patient developed HCC or hepatic failure during the follow-up period. Stored serum samples were kept frozen at −20 °C or below until assayed. This study was approved by the ethics committees of all participating institutions (approval reference 1117 for Shinshu University Hospital, 24085 for National Hospital Organization Nagasaki Medical Center, 758 for Toranomon Hospital, 321 for Hiroshima University Hospital, 934 and 977 for Chiba University Hospital, 779 for The Hospital of Hyogo College of Medicine, 411 for Kumamoto Shinto General Hospital, and "Analysis of efficacy of IFN- to stop NUC in patients with chronic hepatitis B" for Teine Keijinkai Hospital).

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg and antibody to HBeAg, were tested using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan; Fujirebio, Tokyo, Japan; and/or Sysmex, Kobe, Japan) at each hospital. Quantitative measurement of HBsAg[17] was performed using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex (Kobe, Japan). The assay had a quantitative range of -1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum HBV DNA was determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[18] with a quantitative range of 2.1–9.0 log copies/mL. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal, and no signal detection was regarded as a negative signal. Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al.*[19]

Serum HBcrAg levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio) as described previously.[20,21] The HBcrAg assay measures all antigens transcribed and translated from the precore and core genes of the HBV genome, which include hepatitis B e, core and p22cr antigens.[14,20] HBcrAg concentration was calculated based on a standard curve generated using recombinant pro-HBeAg. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL, with a quantitative range set at 3.0–6.8 log U/mL.

Evaluation of response to NUC/IFN-α sequential therapy

The clinical conditions of a successful response to NUC/IFN-α sequential therapy were set at serum HBV DNA below 4.0 log copies/mL, serum ALT below 30 IU/L and negative HBeAg, according to established Japanese guidelines in which patients who meet these conditions are not recommended to start antiviral therapy.[22] We assessed the final response at approximately 24 months after completing IFN-α sequential therapy and compared results to those at 6 and 12 months after the treatment.

Statistical analyses

Fisher's exact and Pearson's χ^2-tests were adopted to test for differences between subgroups of patients. The Mann–Whitney *U*-test was employed to compare continuous data. Each cut-off value was decided using receiver operating characteristic (ROC) analysis, and results were evaluated by measuring the area under the ROC (AUC). Multivariate analysis was performed using a logistic model for the 24-month response to NUC/IFN-α sequential therapy. Correlations between maximal values of ALT and HBV DNA were calculated using Spearman's rank correlation coefficient test. The non-relapse rate was analyzed by the Kaplan–Meier method.

All tests were performed using the IBM SPSS Statistics Desktop for Japan version 19.0 (IBM Japan, Tokyo, Japan). $P < 0.05$ was considered to be statistically significant.

RESULTS

Factors associated with the 24-month response to NUC/IFN-α sequential therapy

OF THE 50 patients enrolled, 18 were judged as responders at 24 months after completing IFN-α sequential therapy (i.e. 24-month responders), while the

remaining 32 were classified as 24-month non-responders. The clinical backgrounds of both groups are compared in Table 2. The median age at NUC commencement and sex distribution did not differ remarkably between the groups. Genotype C was similarly predominant. The types of NUC administrated at the start and end of treatment were comparable between the groups, but the duration of NUC administration was significantly longer in responders. Re-administration of NUC due to aggravation of hepatitis B before judgment of the 24-month response was observed in approximately half of the 32 non-responders. After the final evaluation at 24 months, re-continuation of NUC was seen in only one of the 18 responders versus roughly half of the 15 non-responders who had previously not required it. The follow-up period was significantly longer in responders because observation was discontinued when NUC were re-administrated.

Biochemical and virological markers were compared between 24-month responders and non-responders at the start of NUC, at the start of IFN-α and at the end of IFN-α (Table 3). Positivity for the HBeAg was significantly lower in responders at all time points. HBsAg and HBcrAg levels did not differ between the groups at the start of NUC, but became significantly lower in responders at the start and end-points of IFN-α administration. A significant difference in HBV DNA level was seen between the groups at the end of IFN-α administration only. ALT levels did not differ between the groups at any point.

Multivariate analysis revealed that HBsAg and HBcrAg levels of 3.0 or more and 4.5 log U/mL or more, respectively, at the start of IFN-α administration were significant factors associated with a 24-month non-response to NUC/IFN-α sequential therapy (Table 4). The factors adopted for this logistic model were as follows: age at end of NUC of 37 years or more, duration of NUC administration of 18 months or more, sex, type of NUC at start, HBV genotype, HBeAg positivity at the start of IFN-α, HBsAg level at the start of IFN-α of 3.0 log IU/mL or more, and HBcrAg level at the start of IFN-α of 4.5 log U/mL or more. The corresponding cut-off values for each factor were determined by ROC analysis.

Of the 50 patients enrolled, 23 (46%) had HBsAg of 3.0 log IU/mL or more and HBcrAg of 4.5 log U/mL or more, 27 (54%) had HBsAg of less than 3.0 log IU/mL or HBcrAg of less than 4.5 log U/mL, and none had HBsAg of less than 3.0 log IU/mL and HBcrAg of less than 4.5 log U/mL at the start of IFN-α administration. Whereas none of the 23 patients with the highest HBsAg and HBcrAg levels were responders, 18 (67%) of the remaining 27 patients responded to NUC/IFN-α sequential therapy ($P = 0.005$).

Comparison of responses to NUC/IFN-α sequential therapy at different time points

We assessed the responses to NUC/IFN-α sequential therapy at 6 and 12 months after completing IFN-α administration using the same criteria as those for determining the 24-month outcome. Responses were in 78% agreement ($P < 0.001$) between 6 and 24 months and 80% agreement ($P < 0.001$) between 12 and 24 months.

Prediction of response to NUC/IFN-α sequential therapy using maximal levels of ALT and HBV DNA

The maximal levels of ALT and HBV DNA during follow up were found to be significantly related ($r = 0.777$, $P < 0.001$). ROC analysis showed that both maximal ALT

Table 2 Comparison of clinical backgrounds between 24-month responders and non-responders

Clinical background	24-month responders ($n = 18$)	24-month non-responders ($n = 32$)	P
Age at start of NUC (years)†	36 (21–56)	34 (21–57)	0.486
Sex (male : female)	15:3	23:9	0.497
Genotype (B:C:undetermined)	1:16:1	2:20:10	0.101
NUC at start (LVD : ETV)	16:2	27:5	1.000
NUC at end (LVD : ETV : LAM + ADV : ETV + ADV)	16:2:0:0	24:6:1:1	0.610
Duration of NUC administration (months)†	51 (5–121)	5 (4–72)	0.001
Follow-up period after stopping IFN-α administration (months)†	30 (23–102)	22 (2–81)	0.014
Re-administration of NUC before judging 24-month response‡	0% (0/18)	53% (17/32)	<0.001
Re-administration of NUC after judging 24-month response‡	6% (1/18)	47% (7/15)	0.012

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ADV, adefovir dipivoxil; ETV, entecavir; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; IFN, interferon; LAM, lamivudine; LVD, lamivudine; NUC, nucleoside/nucleotide analog.

Table 3 Comparison of ALT level and viral markers between 24-month responders and non-responders at the time points of starting NUC administration, starting IFN-α administration and stopping IFN-α administration

ALT/viral marker	24-month responders ($n = 18$)	24-month non-responders ($n = 32$)	P
At start of NUC administration			
ALT (IU/L)†	242 (32–2274)	281 (22–1044)	0.872
HBeAg‡	44% (8/18)	84% (27/32)	0.008
HBV DNA (log copies/mL)†	8.0 (<2.1–>9.0)	7.8 (<2.1–>9.0)	0.866
HBsAg (log IU/mL)†	3.5 (1.8–4.9)	3.5 (2.5–4.4)	1.000
HBcrAg (log U/mL)†	>6.8 (3.7–>6.8)	>6.8 (<3.0–>6.8)	0.121
At start of IFN-α administration			
ALT (IU/L)†	29 (12–103)	29 (12–111)	0.779
HBeAg‡	11% (2/18)	59% (19/32)	0.001
HBV DNA (log copies/mL)†	<2.1 (neg.–3.9)	<2.1 (neg.–4.8)	0.142
HBsAg (log IU/mL)†	2.9 (1.5–4.1)	3.7 (2.5–4.3)	0.028
HBcrAg (log U/mL)†	3.6 (<3.0–5.9)	5.6 (<3.0–>6.8)	0.002
At end of IFN-α administration			
ALT (IU/L)†	25 (10–48)	28 (12–134)	0.384
HBeAg‡	6% (1/18)	59% (19/32)	<0.001
HBV DNA (log copies/mL)†	<2.1 (neg.–4.1)	4.6 (<2.1–>9.0)	<0.001
HBsAg (log IU/mL)†	2.8 (1.9–4.0)	3.6 (2.6–4.7)	0.007
HBcrAg (log U/mL)†	3.4 (<3.0–5.5)	5.5 (<3.0–>6.8)	0.017

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ALT, alanine aminotransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IFN, interferon; neg., negative; NUC, nucleoside/nucleotide analog.

Table 4 Multivariate analysis of factors associated with 24-month non-responders to NUC/IFN-α sequential therapy

Selected factor	Odds ratio	95% CI	P
HBsAg ≥3.0 log IU/mL at start of IFN-α	17.7	2.9–108.2	0.002
HBcrAg ≥4.5 log U/mL at start of IFN-α	15.0	2.5–88.6	0.003

CI, confidence interval; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; IFN, interferon; neg., negative; NUC, nucleoside/nucleotide analog.

and HBV DNA levels were significantly associated with the treatment response (Fig. 2), with an AUC for each parameter of over 0.8. The cut-off values providing the highest significance in ROC analysis were 128 IU/L for ALT and 4.5 log copies/mL for HBV DNA. The existence of a second cut-off value was also identified for HBV DNA (6.0 log copies/mL) to discriminate between 24-month responders and non-responders. These results indicated that patients reaching a maximal ALT level of over 128 IU/L or maximal HBV DNA level of over 6.0 log copies/mL during post-treatment follow up were likely to be non-responders.

Lastly, we analyzed the changes in cumulative non-relapse rate of hepatitis B during and after IFN-α administration by tentatively defining relapse as ALT level exceeding 128 IU/L during follow up. We selected maximal ALT instead of maximal HBV DNA because: (i) the inflection point to distinguish a response was clear for maximal ALT but ambiguous for maximal HBV DNA; (ii) the value for "sensitivity + specificity − 1" as calculated by ROC analysis was larger for maximal ALT (7.5 vs 6.5); and (iii) the maximal levels of ALT and HBV DNA were closely associated, and thus ALT values were considered to represent those of HBV DNA. The cumulative non-relapse rate decreased rapidly after completely halting NUC until just prior to 6 months after stopping IFN-α and then was seen to plateau until the study end-point (Fig. 3). This suggests that the recurrence of hepatitis associated with a 24-month non-response can be expected to occur primarily during the first 6 months after stopping IFN-α administration.

DISCUSSION

THE COOPERATIVE EFFECT of NUC/IFN-α sequential therapy has been controversial.[7-12] Enomoto *et al.*[10] first analyzed the results of ETV/IFN-α sequential therapy in patients with HBeAg positive chronic hepatitis B and detected several differences. Although their results were negative, they witnessed that patients who had achieved HBeAg

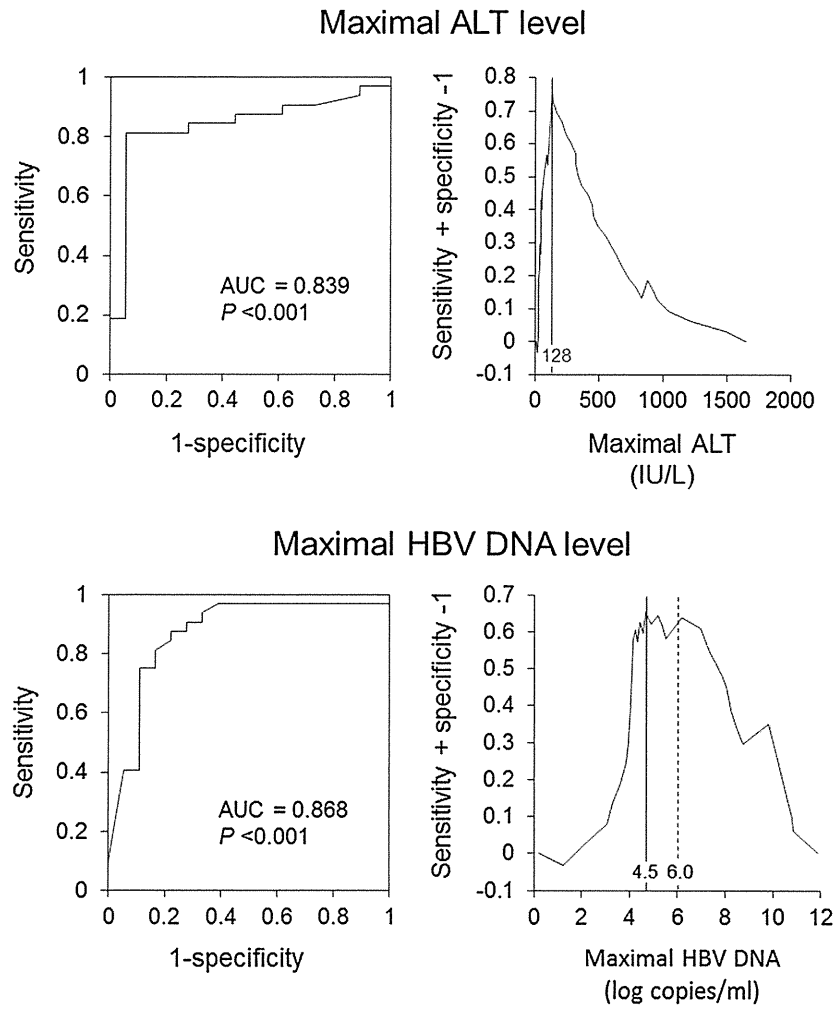

Figure 2 Receiver operating characteristic analysis of maximal alanine aminotransferase (ALT) and hepatitis B virus (HBV) DNA levels to discriminate between 24-month responders and non-responders. Vertical solid lines indicate the actual values of markers corresponding to main inflection points and the vertical broken line indicates the actual value of the marker corresponding to a second inflection point; AUC, area under the receiver operating characteristic curve.

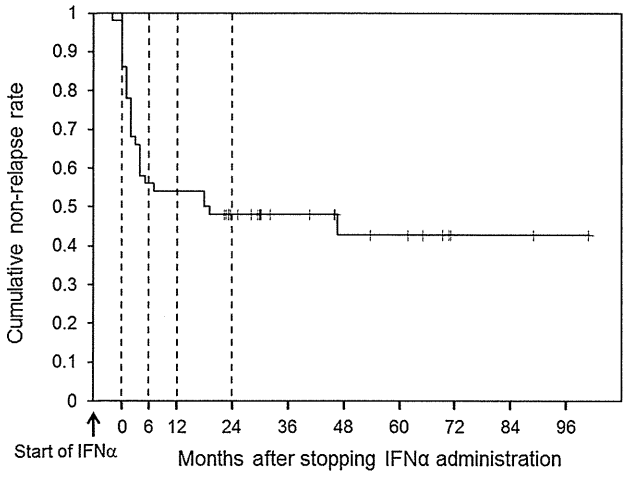

Figure 3 Kaplan–Meier analysis of the non-relapse rate after stopping interferon (IFN)-α administration by defining relapse of hepatitis B as alanine aminotransferase (ALT) level exceeding 128 IU/L.

seroconversion by the time of IFN-α commencement experienced a significantly higher sustained virological response rate than those in whom the HBeAg persisted. Thus, it appeared beneficial to further clarify the factors associated with the response to NUC/IFN-α sequential therapy.

The present study analyzed the factors associated with a long-term response to IFN-α sequential therapy in order to safely discontinue NUC therapy. All patients were treated with natural IFN-α for 6 months and followed for at least 24 months after completing the sequential therapy, with the exception of those who required re-administration of NUC due to aggravation of hepatitis B. The type and duration of NUC administration were not fixed in this study because IFN-α sequential therapy was implemented to discontinue NUC in patients who were undergoing maintenance treatment. Although a prospective study would have been ideal to elucidate the factors associated with

IFN-α sequential therapy outcome, we undertook this retrospective trial because no variables have been sufficiently analyzed to date. Furthermore, we were able to address the long-term response to IFN-α sequential therapy in relation to the results of earlier retrospective studies. It has been reported that pegylated IFN-α (PEG IFN-α) provides a higher HBV response rate than does conventional IFN-α.[23] Therefore, additional prospective studies of sequential therapy using PEG IFN-α are needed as well.

Both HBsAg and HBcrAg levels at the time of NUC cessation were factors significantly associated with the response to NUC/IFN-α sequential therapy. HBsAg has been closely linked with PEG IFN-α therapy outcome.[24-27] Moucari *et al.*[26] analyzed HBeAg negative hepatitis B patients who had been treated with PEG IFN-α for 48 weeks and concluded that an early serum HBsAg drop was strongly predictive of a sustained virological response. Sonneveld *et al.*[24] assessed HBeAg positive hepatitis B patients who had received PEG IFN-α with or without LVD for 52 weeks and observed that patients who experienced no decline in HBsAg level from baseline at week 12 had little chance of achieving a sustained response and no possibility of HBsAg loss. HBcrAg includes antigens that are transcribed and translated from precore and core genes of the HBV genome, and HBeAg is a primary component of these antigens. Thus, our results were consistent with those described by Enomoto *et al.*[10] that the proportion of patients losing HBeAg positivity during ETV treatment was significantly higher in responders to ETV/IFN-α sequential therapy than in non-responders.

Hepatitis B surface antigen and HBcrAg levels have both been associated with intrahepatic HBV cccDNA, which is a key molecule in HBV replication whose value is closely related to HBV replication activity.[21,27,28] Several reports[27,29,30] have shown that HBV cccDNA level is associated with the response to antiviral therapy, such as with PEG IFN-α and NUC. Sung *et al.*[29] analyzed HBeAg positive hepatitis B patients who had been treated with either LVD monotherapy or a combination of PEG IFN-α and LVD and concluded that intrahepatic HBV cccDNA level at the end of therapy was superior to serum HBV DNA in predicting a sustained virological response. Serum HBV DNA is associated with intrahepatic HBV cccDNA and is widely used as a marker for HBV replication activity. However, such associations may be incompatible with antiviral therapies, and especially NUC treatment, because NUC directly hamper production of the HBV virion by inhibiting reverse transcription of pre-genomic RNA without affecting HBV cccDNA directly. As serum levels of HBsAg and HBcrAg are easier to measure than intrahepatic HBV cccDNA, these two antigen assays may be more suitable as surrogate markers for HBV replication activity in patients undergoing antiviral therapy. We previously reported that the combinational use of HBsAg and HBcrAg was beneficial to forecast the risk of hepatitis relapse after discontinuation of NUC.[13,14] The present study confirms this notion; it is possible that HBsAg and HBcrAg have complimentary roles in monitoring antiviral effects because the production of these two antigens is regulated by alternative enhancer–promoter systems in the HBV genome.

It is noteworthy that ROC analysis revealed maximal levels of ALT and HBV DNA to be closely associated with the 24-month response to NUC/IFN-α sequential therapy. We observed that patients with ALT higher than 128 IU/mL or HBV DNA higher than 6.0 log copies/mL during follow up were likely to be non-responders. When a relapse of hepatitis B was tentatively defined as ALT exceeding 128 IU/L during observation, relapses occurred frequently during the first 6 months after ceasing IFN-α and then became more sporadic afterwards. The timing of judgment of a virological response to NUC/IFN-α sequential therapy is critical when evaluating treatment efficacy. As this period is usually set at 6 months after completing therapy, our results confirm that 6 months is indeed appropriate. Our findings also suggest that maximal levels of ALT and HBV DNA are useful for monitoring the results of NUC/IFN-α sequential therapy. Accordingly, patients who are likely to be non-responders can now be identified as early as 24 weeks in advance and alternative strategies for treatment may be considered in a more timely fashion.

In conclusion, the combinational use of HBsAg and HBcrAg levels may be useful to predict the response to NUC/IFN-α sequential therapy. Maximal levels of ALT and HBV DNA during follow up may also be employed for monitoring the results of IFN-α sequential therapy.

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REFERENCES

1 Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007; **45**: 1056–75.

2 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45:** 507–39.
3 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; **337:** 1733–45.
4 Ghany M, Liang TJ. Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. *Gastroenterology* 2007; **132:** 1574–85.
5 Honkoop P, de Man RA, Niesters HG, Zondervan PE, Schalm SW. Acute exacerbation of chronic hepatitis B virus infection after withdrawal of lamivudine therapy. *Hepatology* 2000; **32:** 635–9.
6 Honkoop P, de Man RA, Heijtink RA, Schalm SW. Hepatitis B reactivation after lamivudine. *Lancet* 1995; **346:** 1156–7.
7 Serfaty L, Thabut D, Zoulim F *et al.* Sequential treatment with lamivudine and interferon monotherapies in patients with chronic hepatitis B not responding to interferon alone: results of a pilot study. *Hepatology* 2001; **34:** 573–7.
8 Shi M, Wang RS, Zhang H *et al.* Sequential treatment with lamivudine and interferon-alpha monotherapies in hepatitis B e antigen-negative Chinese patients and its suppression of lamivudine-resistant mutations. *J Antimicrob Chemother* 2006; **58:** 1031–5.
9 Manesis EK, Papatheodoridis GV, Hadziyannis SJ. A partially overlapping treatment course with lamivudine and interferon in hepatitis B e antigen-negative chronic hepatitis B. *Aliment Pharmacol Ther* 2006; **23:** 99–106.
10 Enomoto M, Nishiguchi S, Tamori A *et al.* Entecavir and interferon-alpha sequential therapy in Japanese patients with hepatitis B e antigen-positive chronic hepatitis B. *J Gastroenterol* 2013; **48:** 397–404.
11 Enomoto M, Tamori A, Nishiguchi S, Kawada N. Combination therapy with a nucleos(t)ide analogue and interferon for chronic hepatitis B: simultaneous or sequential. *J Gastroenterol* 2013; **48:** 999–1005.
12 Minami M, Okanoue T. Management of HBV infection in Japan. *Hepatol Res* 2007; **37:** S79–82.
13 Matsumoto A, Tanaka E, Suzuki Y *et al.* Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B. *Hepatol Res* 2012; **42:** 139–49.
14 Tanaka E, Matsumoto A. Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs in patients with chronic hepatitis B. *Hepatol Res* 2014; **44:** 1–8.
15 Orito E, Ichida T, Sakugawa H *et al.* Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; **34:** 590–4.
16 JSH Guidelines for the Management of Hepatitis B Virus Infection. *Hepatol Res* 2014; **44** (Suppl S1): 1–58.
17 Schuttler CG, Wend UC, Faupel FM, Lelie PN, Gerlich WH. Antigenic and physicochemical characterization of the 2nd International Standard for hepatitis B virus surface antigen (HBsAg). *J Clin Virol* 2010; **47:** 238–42.
18 Ronsin C, Pillet A, Bali C, Denoyel GA. Evaluation of the COBAS AmpliPrep-total nucleic acid isolation-COBAS TaqMan hepatitis B virus (HBV) quantitative test and comparison to the VERSANT HBV DNA 3.0 assay. *J Clin Microbiol* 2006; **44:** 1390–9.
19 Mizokami M, Nakano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; **450:** 66–71.
20 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40:** 439–45.
21 Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. *J Med Virol* 2009; **81:** 27–33.
22 Kumada H, Okanoue T, Onji M *et al.* Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; **40:** 1–7.
23 Cooksley WG, Piratvisuth T, Lee SD *et al.* Peginterferon alpha-2a (40 kDa): an advance in the treatment of hepatitis B e antigen-positive chronic hepatitis B. *J Viral Hepat* 2003; **10:** 298–305.
24 Sonneveld MJ, Rijckborst V, Boucher CA, Hansen BE, Janssen HL. Prediction of sustained response to peginterferon alfa-2b for hepatitis B e antigen-positive chronic hepatitis B using on-treatment hepatitis B surface antigen decline. *Hepatology* 2010; **52:** 1251–7.
25 Brunetto MR, Moriconi F, Bonino F *et al.* Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* 2009; **49:** 1141–50.
26 Moucari R, Mackiewicz V, Lada O *et al.* Early serum HBsAg drop: a strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients. *Hepatology* 2009; **49:** 1151–7.
27 Chan HL, Wong VW, Tse AM *et al.* Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol* 2007; **5:** 1462–8.
28 Wong DK, Tanaka Y, Lai CL, Mizokami M, Fung J, Yuen MF. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. *J Clin Microbiol* 2007; **45:** 3942–7.
29 Sung JJ, Wong ML, Bowden S *et al.* Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. *Gastroenterology* 2005; **128:** 1890–7.
30 Wursthorn K, Lutgehetmann M, Dandri M *et al.* Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. *Hepatology* 2006; **44:** 675–84.

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＜症例報告＞

B型・D型肝炎ウイルス重複感染による肝障害に対してペグインターフェロンが有効であった1例

日下部篤宣[1)*]　田中　靖人[2)]　飯尾　悦子[3)]　村上　周子[2)]
松浦健太郎[3)]　新海　登[3)]　宮木　知克[4)]　藤原　圭[3)]
野尻　俊輔[3)]　折戸　悦朗[1)]　城　卓志[3)]

要旨：症例は26歳，女性のモンゴル人．既往歴としてB型慢性肝炎を認める．今回，健診にて肝機能障害を指摘され，B型慢性肝炎急性増悪の診断で当科紹介となる．来院時採血で更に肝障害増悪を認めたが，HBV-DNA量は2.8 log copy/mLと低値だった．また他のウイルス感染やAIH，PBCも否定的だった．妊娠中と判明したため無治療で経過観察したところ，肝障害は徐々に自然軽快し，妊娠39週で出産した．出産後もHBV-DNA量上昇は認めず，肝障害は更に改善したが，出産から9カ月後に再度肝障害増悪を認めた．この段階でD型肝炎ウイルス（HDV）の重複感染を考え，保存血清を用いてHDV-RNAを検査したところ陽性と判明し，HBVキャリアに対するHDV重複感染と診断した．Peg-IFNα-2aの投与を開始したところ，肝障害は改善し，治療中のHDV-RNAも陰性化した．日本においてHDV感染は稀な疾患であるが，HBV-DNA量の増加を伴わないB型慢性肝炎急性増悪症例に遭遇した場合には，HDV重複感染を念頭に置くべきと思われた．

索引用語：　D型肝炎　　Peg-IFN　　重複感染

はじめに

D型肝炎ウイルス（HDV）は，1977年Rizzettoらにより初めて報告された外殻蛋白合成能に欠損がある不完全型のRNAウイルスであり[1)]，その発現・増殖にはB型肝炎ウイルス（HBV）をヘルパーウイルスとして必要とする[2)]．このHDV感染により，B型慢性肝炎の臨床経過が悪化したり，稀には劇症肝炎様の病態を引き起こすことが知られている[3)4)]．本邦ではHBVキャリアにおけるδ抗体陽性率は約1％と低いことが報告されており[5)]，実際のD型肝炎の診断・治療に関する報告は少ない．今回，我々はHBVキャリアであるモンゴル人に妊娠を契機とした肝障害悪化を認め，最終的にHBVとHDVの重複感染と診断し，ペグインターフェロン（Peg-IFN）による治療を導入したことで肝障害の改善を認めた1例を経験したので報告する．

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＜受付日2014年6月28日＞＜採択日2014年8月16日＞

症　例

26歳，女性．モンゴル人．

主訴：肝機能障害．

家族歴：父，母，弟に肝炎の既往なし．

既往歴：幼少児　痙攣（原因不明，無治療で自然軽快）でモンゴルの病院に入院．その際，採血検査などで注射器の回し打ちがなされていたとのこと．

内服薬：なし．

輸血歴：なし．

生活歴：飲酒・喫煙共になし．

職業歴：事務員．

現病歴：2003年にモンゴルで流産したが，その際，ALT 2000 U/Lまで一過性に上昇し，HBVキャリア（genotype D）であることが判明した．2004年にモンゴ

Table 1 Laboratory data on admission

Hematology		Biochemistory		Virus marker	
WBC	5900 /μl	TP	7.6 g/dl	HBsAg (CLEIA)	>2000.0 (+) C.O.I
Neut	61.9 %	Alb	4.5 g/dl	anti-HBs (CLEIA)	0.9 (−) mIU/mL
Lymph	29.7 %	AST	448 U/l	HBeAg (CLEIA)	0.1 (−) C.O.I
RBC	4.70×10^6 /μl	ALT	1029 U/l	anti-HBe (CLEIA)	>100.0 (+) %
Hb	13.9 g/dl	LDH	284 U/l	HBV-DNA (realtime PCR)	2.8 log copy/ml
Plt	22.0×10^4 /μl	ALP	339 U/l	HBV genotype	genotype D
		T.Bil	0.5 mg/dl	HBV preC/CP mutation	
Coagulation		CK	48 U/l	preCore wild type	100 %
PT	102.2 %	BUN	9 mg/dl	preCore mutant type	0 %
PT-INR	0.99	Cre	0.5 mg/dl	Core promoter	negative
APTT	92.2 %	Glu	91 mg/dl	anti-HCV (CLEIA)	0.1 (−) C.O.I
HPT	88.4 %	Na	137 mEq/l	HCV core protein	≦0.1 fmol/l
FDP	<2.5 μg/ml	K	4.7 mEq/l	IgM-HA (CLIA)	<0.80 (−)
DD dimer	0.9 μg/ml	Cl	104 mEq/l	HEV-RNA	negative
		CRP	0.10 mg/dl	CMV IgM (EIA)	0.44 (−)
Immunology				CMV IgG (EIA)	27.6 (−)
IgG	1379 mg/dl	TSH	1.582 μIU/mL	anti-EBNA (FAT)	40 ×
IgA	271 mg/dl	FT3	2.94 ng/dl	VCA IgM (FAT)	<10 ×
IgM	160 mg/dl	FT4	0.76 pg/mL	VCA IgG (FAT)	80 ×
ANA	40 ×				
AMA-M2	5 Index			Tumor marker	
				AFP	3.7 ng/ml
				PIVKA-II	22 mAU/ml

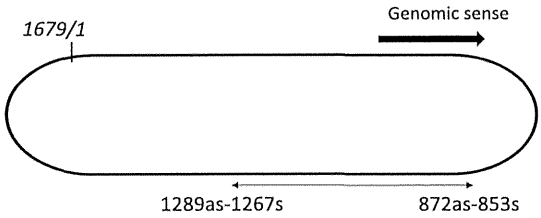
ルから日本へ移住. 今回, 2010年6月1日の検診でAST 227 U/L, ALT 468 U/Lと肝障害を指摘される. 6月10日に前医を受診し採血を行ったところ, AST 376 U/L, ALT 767 U/Lと肝障害の増悪を認めたため, B型慢性肝炎急性増悪として6月14日当院紹介となる. 同日施行した緊急採血でAST 448 U/L, ALT 1029 U/Lと肝障害の更なる増悪を認めたため, 同日緊急入院となる. なお, 6月13日に患者が市販の妊娠検査薬で調べたところ, 妊娠反応陽性であった.

入院時現症：体温36.3℃, 血圧125/70 mmHg, 脈拍70回/分・整. 表在リンパ節を触知せず. 咽頭に異常を認めず. 腹部は平坦・軟で圧痛を認めず. 肝・腎・脾は触知しない. 下腿浮腫を認めず.

入院時検査所見（**Table 1**）：白血球, 赤血球, 血小板数は正常であった. 一般肝機能検査ではAST448 U/L, ALT1029 U/LとALT優位のトランスアミナーゼ値の上昇を認めたが, PT 102.2%, HPT 88.4%と凝固系には異常を認めなかった. T.Bilも0.5 mg/dlと正常値であった. 免疫グロブリンにも異常は認められず, 抗核抗体などの自己抗体も陰性であった. HBs抗原陽性, HBe抗体陽性のHBVキャリアであったが, HBV-DNA量は2.8 log copy/mlと低値であった. なお採血結果からA型肝炎ウイルス, C型肝炎ウイルス, E型肝炎ウイルス, サイトメガロウイルス, EBウイルス感染はいずれも否定的であった. 甲状腺機能にも異常は認められなかった.

腹部エコー：軽度脂肪肝の所見を認めるのみであった.

入院後経過：入院当初はB型慢性肝炎急性増悪による肝障害を疑ったが, 入院後の産婦人科診察にて妊娠4〜5週であることが確認され, 更に入院時採血でPT 102.2%と良好であったことから, 肝障害に対する投薬は行わず, 安静・補液で経過観察となる. その後, 入院時採血でHBV-DNA量が2.8 log copy/mlと低値であることが判明し, B型慢性肝炎急性増悪による肝障害は否定的と考えた. 6月26日採血でAST 890 U/L, ALT 1731 U/Lまで増悪するが, その後は自然に軽快し7月8日退院となる. 外来でも投薬は行わず経過観察の



Name	Location	Sequence 5′→3′
853p	853-872	CGGATGCCCAGGTCGGACC
1267N	1267-1289	GAAGGAAGGCCCCTGGAGAACAAGA
Delta-F7	868-887	GCATGGTCCCAGCCTCC
Delta-R2	1268-1287	TCTTCGGGTCGGCATGG

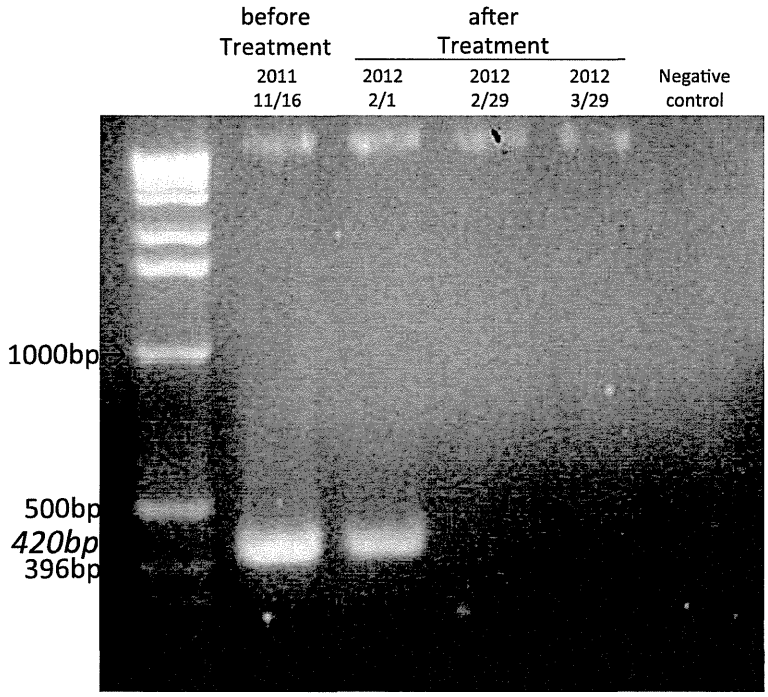
Fig. 1 Strategy for HDV genome amplification. Regions 853-1289 were indicated by arrows. The sequence and position of primers (853p, 1267N) were listed. In addition, the primers (Delta-F7, Delta-R2) were designed for real-time PCR quantification.

みであったが，出産直前に AST 220 U/L，ALT 268 U/L と軽度の肝障害増悪を認めた以外は AST 50 U/L，ALT 100 U/L 前後で推移し，2011 年 2 月 8 日，妊娠 39 週で出産となる．出産後の肝機能は AST，ALT ともに 50 U/L 前後まで低下し，肝障害の原因として妊娠が何らかの影響を及ぼしていたものと考えた．その後はモンゴルに一時帰国したため，フォローアップできなかったが，モンゴルから帰国した 2011 年 11 月中旬頃から再び肝障害増悪を認め，妊娠以外の原因によって肝障害増悪を来していると判断した．肝障害の増悪が続くため，12 月 28 日再入院となる．再度，各種ウイルス感染や自己免疫疾患などについて検査するも前回同様すべて陰性であった．造影 CT で器質的疾患の有無についても確認したが，明らかな異常を認めなかった．この時点で，1) HBV キャリアであるが経過中 HBV-DNA 量は低値で推移している，2)モンゴル人である，以上の 2 点から HDV の重複感染による肝障害を疑い，当院受診時からの保存血清（2010 年 8 月 25 日，2011 年 2 月 2 日，2011 年 11 月 16 日の 3 ポイント）を用いて HDV 感染の有無について確認した．HDV に対する特異的プライマーを設定（forward：853–872，reverse：1267–1289）し（Fig. 1）[6][7]，RT-PCR を行ったところ，これら 3 点全てで PCR 陽性であることが判明し，本症例が HBV と HDV 重複感染による肝障害と診断した．2012 年 1 月 3 日から Peg-IFNα-2a 180 μg による治療を開始した．これにより AST/ALT は 50 U/L 程度まで著明に低下し，治療中の HDV-RNA も検出限界未満となった．更に HDV-RNA の定量を行うため，既存の報告を参考にリアルタイム PCR 検出系の確立も試みた[8]．治療開始前（2011 年 11 月 23 日）の保存血清から得られた cDNA に対して Delta-F7，Delta-R2 をプライマーとして設定し（Fig. 1），TaKaRa LA Taq（タカラバイオ株式会社，滋賀）を用いて検出領域を増幅した．その後，TA クローニング法を用いて検出領域のクローンを作成し，スタンダードサンプルとして使用した．リアルタイム PCR は Delta-F7，Delta-R2 をプライマーとし，FastStart SYBR Green Master（Roche Diagnostics，Switzerland）を用いて LightCycler480® （Roche Diagnostics）により行った（95℃ で 60 sec の後，95℃ 15 sec，60℃ 25 sec，72℃ 15 sec を 45 cycle）．同システムの自動解析により，Threshold cycle を定め，定量を行った．今回確立した系の検出感度は 1.0×10^4 copies/mL であり，2011 年 11 月 16 日 の HDV-RNA 量 は 1.2×10^6 copies/mL，2012 年 2 月 1 日は 1.0×10^4 copies/mL 未満：シグナル陽性，それ以降は検出感度以下であった（Fig. 2）．HDV genotype について系統解析した結果は genotype I であった（Fig. 3）．治療中ではあったが，患者希望により 2012 年 5 月モンゴル帰国となる．最後に本症例の臨床経過を Fig. 4 に示す．

考　察

D 型肝炎ウイルスは，B 型肝炎ウイルスと共存することによってのみ増殖し，肝炎を起こす不完全ウイルスで，HBV との同時感染か HBV キャリアへの重複感染で感染が成立する．同時感染では B 型急性肝炎を重症化させ，重複感染では B 型慢性肝炎の急性増悪や病期進行を早めることが知られている．

世界には 4 億 2000 万人の HBV キャリアが存在すると推定されているが，1980 年代に世界の各地域の HBV キャリアを対象とした HD 抗体を用いた調査報告によると HD 抗体陽性率は 5% であったことから，世界の HDV 感染者は約 2000 万人と推定されている[9]．HDV 抗体陽性者は世界中に存在するものの，HBV 感染者の分布とは異なり，地中海沿岸，中近東，中央アジア，南米（アマゾン川流域），南太平洋諸島の一部でその頻度が高く，一方 HBV キャリア率が高い中国や韓国といった東アジアでは低いことが報告されている[10]．しかし，中国に近いモンゴルにおいては HDV 抗体陽性率が高く[11]，24～41% の HBV キャリアに HDV 感染を認めたとの報告もある[12]．本邦では 1989 年に全国の国立病院



Real time PCR	2011/11/16	2012/2/1	2012/2/29	2012/3/29
HDV-RNA (copies/mL)	1.2×10^6	$<1.0\times10^4$	Not detected	Not detected

Fig. 2 The results of RT-PCR assay for serum HDV-RNA on each day. The 420-bp fragment indicates the amplified HDV genome. HDV-RNA level at baseline was 1.2×10^6 copies/mL on November 16, 2011. After starting Peg-IFN treatment, HDV-RNA decreased ($<1.0\times10^4$ copies/mL: 2012/2/1) and finally became undetectable (2012/2/29, 2012/3/29).

31施設を対象に1306例のHBVキャリアでのHD抗体陽性者を検討したところ，8例のみ陽性でその頻度は0.61％と低値であったことから，本邦におけるHDV感染者の頻度は低いものと考えられている[13]．

HDVは大きく3つのgenotypeに分類されている[6]．GenotypeⅠは世界中に広く分布するタイプであり，本症例も系統解析した結果，genotypeⅠであった．GenotypeⅡは日本と台湾から報告されており，genotypeⅢは南米のアマゾン川流域に分布している．最近では新しいgenotype分類も報告されており，それによるとgenotypeⅠからⅧの8つのgenotypeが報告されている[14]．

HDV感染のスクリーニング法として，2003年4月まではHD抗体測定が保険適用であったが，同年5月に測定試薬の製造が中止されて以後，本邦でのHD抗体測定は困難となっている[15]．このため現在ではHDV感染診断はRT-PCR法により血中のHDV RNAを検出する方法が基本となるが，HDV RNA検出には保険適用はなく，各施設の研究室で測定するか，検査受託企業への外注検査に依頼する必要があり，現在，本邦におけるHDV感染検査は非常に困難なものとなっている．このような状況が本症例においてHBV・HDV重複感染による肝障害との診断に至るのに苦慮した要因の一つになったと思われる．

HDVの感染様式としては，HBVとの同時感染か，HBVキャリアへの重複感染の2つが考えられる．同時感染は通常一過性で，しばしば劇症肝炎や重症肝炎を起こすが，HDVの慢性化は約5％程度と稀である．一方，重複感染の場合は70～95％でHDV感染が持続し，D型慢性肝炎に移行する[16]．

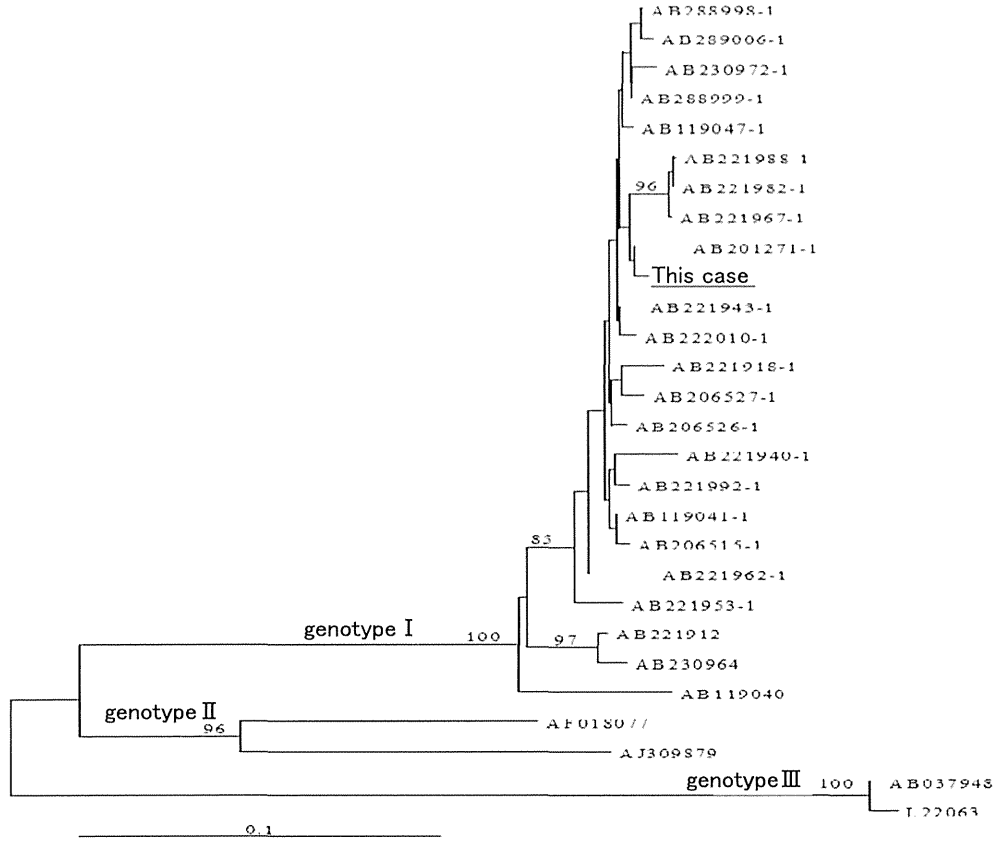


Fig. 3 Neighbour-joining phylogenetic tree constructed from region 853-1289 of HDV genome. The strain from the patient in this case was compared with the representative sequences including 23 genotype I, 2 genotype II and 2 genotype III. The strain from this patient belonged to genotype I.

D型慢性肝炎の治療法としては，現在のところ有効性が認められているのはIFNのみである．Farciらは，IFNα2a 900万単位を週3回48週投与し，ALT値の正常化を71%に，血清HDV-RNAの陰性化を50%の症例に認め，それらの症例では組織学的にも改善したと報告している[17]．しかし，IFN投与終了後にほとんどの症例でHDV-RNAの再出現とALT値の再上昇を認め，再発率が高いとも報告している[17]．近年ではPeg-IFNによる治療成績が報告されており，Niroらは Peg-IFNα-2b（1.5 μg/kg）を72週単独投与した群と Peg-IFNα-2b 72週にリバビリン48週投与を併用した群との比較を行っているが，Peg-IFN投与によって21%の症例でSVRを達成したと報告している[18]．またWedemeyerらは，Peg-IFNα-2a 180 μg 48週投与により約25%の症例でHDV RNAクリアランスを達成できると報告している[19]．一方，HBVの治療で用いられるlamivudineに代表される核酸アナログ製剤は，その単独投与あるいはIFNとの併用に関わらずHDVに対して効果がないことが報告されている[20)21)]．前述のWedemeyerらの研究でもPeg-IFNα-2aとadefovirとの治療効果の検討がなされているが，核酸アナログ製剤であるadefovirにはHDVに対する効果はないと報告している[19]．これは核酸アナログ製剤によってHBV複製が抑制されても，HBsAg産生を抑えることができないためと推察される．またHDVと同じRNAウイルスであるHCV治療に対して用いられるribavirinも，ribavirin単独投与あるいはIFNとの併用に関わらず，HDV RNAクリアランスに効果がないことが報告されている[18)22)23)]．以上より，

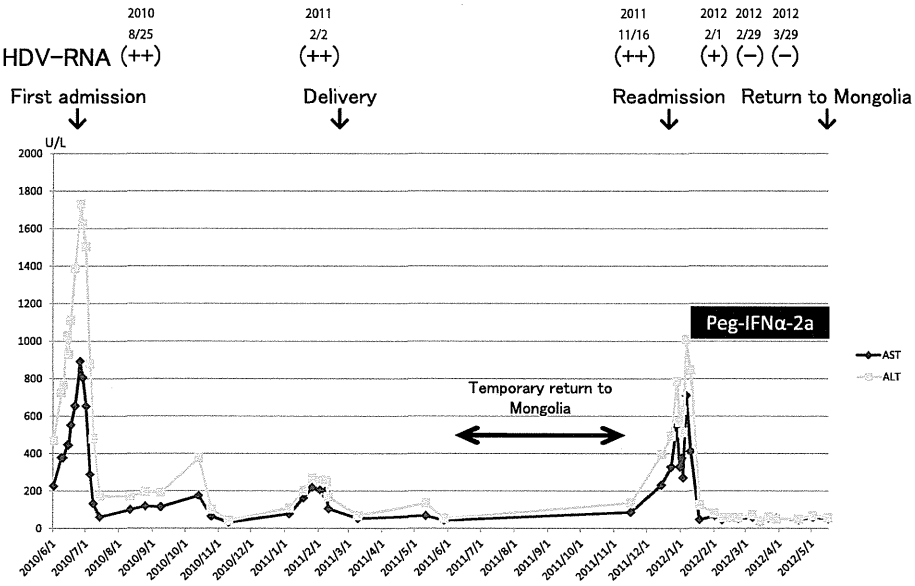


Fig. 4　Clinical course in this case. After starting treatment with Peg-IFN, AST and ALT levels became almost normal. Furthermore, HDV-RNA in serum was undetectable around 8 weeks after the treatment.

現在ではPeg-IFNの1年間投与がD型慢性肝炎に対する治療法として推奨されている[24]．本症例においてもPeg-IFNα-2aによる治療を行ったところ，治療開始前は高値であったAST，ALT値は投与開始後から速やかに改善し，Peg-IFNα-2a投与中の保存血清を用いたHDV-RNAのRT-PCRでは，治療開始後2カ月目からPCRバンドが検出されなくなった．以上より，治療途中で患者がモンゴルに帰国したため最終的な治療効果については不明だが，少なくともPeg-IFNによる治療期間においてはHDVに対する抗ウイルス効果があり，本症例における肝障害はHDV感染によるものと考えられた．

本症例は当初，妊娠を契機としたB型慢性肝炎急性増悪症例と考えていたが，経過中のHBV-DNA量は常に低値であることからB型慢性肝炎急性増悪は否定的となり，原因不明のまま確定診断に至らなかった症例である．しかし，患者背景などを改めて検討し直すことで最終的にHBV・HDV重複感染による肝障害と診断できた．本邦においてHBV・HDV重複感染症例は極めて稀であり，また現在，本邦で使用可能なコマーシャルベースのHDV測定系が存在しないことも災いして診断に苦慮したが，国際交流が盛んな今日において，このような症例は増加することが予想される．HBV-DNA量の増加を伴わないB型慢性肝炎急性増悪を認めた際には，HDVの重複感染を念頭に置くべきと思われる．

結　語

HBV・HDV重複感染による肝障害に対してPeg-IFNα-2aが有効であった1例を経験した．本邦においてHBV・HDV重複感染症例は稀であり，若干の文献的考察を加え報告した．

文　献

1) Rizzetto M, Canese MG, Arico S, et al. Immunofluorescence detection of a new antigen-antibody system (delta/anti-delta) associated with hepatitis B virus in liver and serum of HBsAg carriers. Gut 1977; 18: 997—1003
2) Rizzetto M, Hoyer B, Canese MG, et al. Delta Agent: association of delta antigen with hepatitis B surface antigen and RNA in serum of delta-infected chimpanzees. Proc Natl Acad Sci USA 1980; 77: 6124—6128
3) Colombo M, Cambieri R, Rumi MG, et al. Long-term delta superinfection in hepatitis surface antigen carriers and its relationship to the course of chronic hepatitis. Gastroenterology 1983; 85: 235—

239
4) Smedile A, Farci P, Verme G, et al. Influence of delta infection on severity of hepatitis B. Lancet 1982; 2: 945—947
5) Mitamura K. Epidemiology of HDV infection in Japan. Prog Clin Biol Res 1991; 364: 81—87
6) Casey JL, Brown TL, Colan EJ, et al. A genotype of hepatitis D virus that occurs in South America. Proc Natl Acad Sci USA 1993; 90: 9016—9020
7) Ivaniushina V, Radjef N, Alexeeva M, et al. Hepatitis delta virus genotypes I and II cocirculate in an endemic area of Yakutia, Russia. J Gen Virol 2001; 82: 2709—2718
8) Shang D, Hughes SA, Horner M, et al. Development and validation of an efficient in-house real-time reverse transcription polymerase chain reaction assay for the quantitative detection of serum hepatitis delta virus RNA in a diverse South London population. J Virol Methods 2012; 184: 55—62
9) Hadziyannis SJ. Review: hepatitis delta. J Gastroenterol Hepatol 1997; 12: 289—298
10) Rizzetto M, Rosina F. Viral Hepatitis, 3rd ed, Section VI Hepatitis D virus epidemiology and natural history, Edited by H Thomas, Blackwell Science, Massachusetts, 2005, p583—592
11) Inoue J, Takahashi M, Nishizawa T, et al. High prevalence of hepatitis delta virus infection detectable by enzyme immunoassay among apparently healthy individuals in Mongolia. J Med Virol 2005; 76: 333—340
12) Oyunsuren Ts. モンゴルにおけるウイルス性肝炎. Minophagen Medical Review 2000; 45: 140—147
13) 矢野右人, 井上長三. δ肝炎の臨床と日本における現況. 日本臨床　1989；47：715—720
14) Makuwa M, Caron M, Souquière S, et al. Prevalence and genetic diversity of hepatitis B and delta viruses in pregnant women in Gabon: molecular evidence that hepatitis delta virus clade 8 originates from and is endemic in central Africa. J Clin Microbiol 2008; 46: 754—756
15) 八橋　弘. デルタ(D型)ウイルス：総論. 日本臨牀　2011；69：595—599
16) 高橋雅春, 岡本宏明, ビラ・ツァツラルト・オド, 他. D型肝炎の診断法の確立と分子疫学的研究の成果. 臨床とウイルス　2009；37：329—336
17) Farci P, Mandas A, Coiana A, et al. Treatment of chronic hepatitis D with interferon alfa-2a. N Engl J Med 1994; 330: 88—94
18) Niro GA, Ciancio A, Gaeta GB, et al. Pegylated interferon alpha-2b as monotherapy or in combination with ribavirin in chronic hepatitis delta. Hepatology 2006; 44: 713—720
19) Wedemeyer H, Yurdaydìn C, Dalekos GN, et al. Peginterferon plus adefovir versus either drug alone for hepatitis delta. N Engl J Med 2011; 364: 322—331
20) Yurdaydin C, Bozkaya H, Onder FO, et al. Treatment of chronic delta hepatitis with lamivudine vs lamivudine + interferon vs interferon. J Viral Hepat 2008; 15: 314—321
21) Niro GA, Ciancio A, Tillman HL, et al. Lamivudine therapy in chronic delta hepatitis: a multicentre randomized-controlled pilot study. Aliment Pharmacol Ther 2005; 22: 227—232
22) Gunsar F, Akarca US, Ersoz G, et al. Two-year interferon therapy with or without ribavirin in chronic delta hepatitis. Antivir Ther 2005; 10: 721—726
23) Garripoli A, Di Marco V, Cozzolongo R, et al. Ribavirin treatment for chronic hepatitis D: a pilot study. Liver 1994; 14: 154—157
24) Cornberg M, Protzer U, Dollinger MM, et al. Prophylaxis, diagnosis and therapy of hepatitis B virus (HBV) infection: the German guidelines for the management of HBV infection. Z Gastroenterol 2007; 45: 1281—1328

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A case of a HBV carrier with HDV superinfection treated by PEG-IFN

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A 26-year-old Mongolian woman was admitted to our hospital because of liver dysfunction. As she has been a HBV carrier, acute exacerbation of chronic hepatitis B seemed to be the cause of liver dysfunction at first. However, the loads of serum HBV-DNA on admission were low (2.8 log copy/ml). As she was pregnant, she was observed without treatment. Liver function once improved without treatment, but it became worse again after delivery. At this time, it was suspected HDV superinfection could affect the liver dysfunction. For HDV-RNA was positive in stored sera by RT-PCR, she was diagnosed as HBV and HDV superinfection and started treatment with Peg-IFN. Although HDV infection is rare in Japan, in case of acute exacerbation of a HBV carrier with low serum HBV-DNA level, HBV and HDV superinfection should be considered.

Key words: hepatitis D　Peg-IFN　superinfection

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Structural insights into the substrate stereospecificity of D-*threo*-3-hydroxyaspartate dehydratase from *Delftia* sp. HT23: a useful enzyme for the synthesis of optically pure L-*threo*- and D-*erythro*-3-hydroxyaspartate

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Abstract D-*threo*-3-Hydroxyaspartate dehydratase (D-THA DH) is a fold-type III pyridoxal 5′-phosphate-dependent enzyme, isolated from a soil bacterium of *Delftia* sp. HT23. It catalyzes the dehydration of D-*threo*-3-hydroxyaspartate (D-THA) and L-*erythro*-3-hydroxyaspartate (L-EHA). To elucidate the mechanism of substrate stereospecificity, crystal structures of D-THA DH were determined in complex with various ligands, such as an inhibitor (D-*erythro*-3-hydroxyaspartate (D-EHA)), a substrate (L-EHA), and the reaction intermediate (2-amino maleic acid). The C^{β}-OH of L-EHA occupied a position close to the active-site Mg^{2+}, clearly indicating a possibility of metal-assisted C^{β}-OH elimination from the substrate. In contrast, the C^{β}-OH of an inhibitor was bound far from the active-site Mg^{2+}. This suggests that the substrate specificity of D-THA DH is determined by the orientation of the C^{β}-OH at the active site, whose spatial arrangement is compatible with the 3*R* configuration of 3-hydroxyaspartate. We also report an optically pure synthesis of L-*threo*-3-hydroxyaspartate (L-THA) and D-EHA, promising intermediates for the synthesis of β-benzyloxyaspartate, by using a purified D-THA DH as a biocatalyst for the resolution of racemic DL-*threo*-3-hydroxyaspartate (DL-THA) and DL-*erythro*-3-hydroxyaspartate (DL-EHA). Considering 50 % of the theoretical maximum, efficient yields of L-THA (38.9 %) and D-EHA (48.9 %) as isolated crystals were achieved with >99 % enantiomeric excess (*e.e.*). The results of nuclear magnetic resonance signals verified the chemical purity of the products. We were directly able to isolate analytically pure compounds by the recrystallization of acidified reaction mixtures (pH 2.0) and thus avoiding the use of environmentally harmful organic solvents for the chromatographic purification.

Keywords D-*threo*-3-Hydroxyaspartate dehydratase · *Delftia* sp. HT23 · Alanine racemase · Pyridoxal 5′-phosphate · Enzymatic optical resolution

Introduction

3-Hydroxyaspartate and its derivatives have attracted attention because of their biological activity as competitive blockers against glutamate transporters (excitatory amino acid transporters 1–5 (EAAT1–5)) in the mammalian central nervous system (Arriza et al. 1994, 1997; Shimamoto et al. 1998, 2004; Shimamoto 2008). 3-Hydroxyaspartate has two chiral centers, and thus, it exhibits four stereoisomers, namely D-*threo*-3-hydroxyaspartate (2*R*,3*R*; D-THA), L-*threo*-3-hydroxyaspartate (2*S*,3*S*; L-THA), D-*erythro*-3-hydroxyaspartate (2*R*,3*S*; D-EHA), and L-*erythro*-3-hydroxyaspartate (2*S*,3*R*; L-EHA) (Fig. 1). Recently, we have identified and cloned a gene encoding D-*threo*-3-hydroxyaspartate dehydratase (D-THA DH) from a soil

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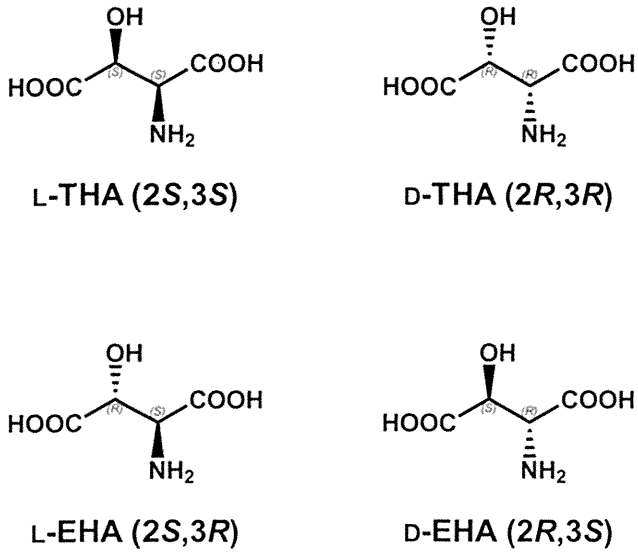

Fig. 1 Structures of the four 3-hydroxyaspartate stereoisomers. *L-THA* L-*threo*-3-hydroxyaspartate, *D-THA* D-*threo*-3-hydroxyaspartate, *L-EHA* L-*erythro*-3-hydroxyaspartate, *D-EHA* D-*erythro*-3-hydroxyaspartate

bacterium *Delftia* sp. HT23 (Maeda et al. 2010). D-THA DH displays a preference for the dehydration of D-THA and L-EHA and is only slightly active against L-THA, while showing no activity on D-EHA. Although there are several reported enzymes that act on 3-hydroxyaspartate (Wada et al. 1999, 2003; Liu et al. 2003; Murakami et al. 2009), D-THA DH is the only enzyme known to exhibit an efficient dehydratase activity toward D-THA. Thus, this enzyme was assigned a new Enzyme Commission (EC) number as 4.3.1.27.

We have also found that D-THA DH belongs to a fold-type III group of pyridoxal enzymes of which bacterial alanine racemases are the typical members (Schneider et al. 2000; Eliot and Kirsch 2004). Our previous work revealed that D-THA DH contains a pyridoxal 5′-phosphate (PLP) as its cofactor, but its racemization activities on alanine, serine, and aspartate were below the detection limit. To date, many crystal structures of alanine racemases from diverse bacteria have been deposited in the Protein Data Bank (PDB). Nevertheless, very little is known about the structure of dehydratases whose predictive structure closely resembles that of alanine racemases. Eukaryotic D-serine dehydratase has been recently identified as a common member of the fold-type III group of pyridoxal enzymes (Ito et al. 2008; Tanaka et al. 2008). D-THA DH shows amino acid sequence similarity (26–36 %) with D-serine dehydratases derived from *Saccharomyces cerevisiae* (scDSD; Ito et al. 2008) and chicken kidney (chDSD; Tanaka et al. 2008). The crystal structure of chDSD complexed with D-serine has been previously reported (Tanaka et al. 2011); however, no structural information is available regarding 3-hydroxyaspartate recognition mechanism of this enzyme family.

Various effective methods for the preparation of 3-hydroxyaspartate as a racemate or an individual stereoisomer have been reported (Kaneko and Katsura 1963; Antolini et al. 1997; Cardillo et al. 1999; Khalaf and Datta 2008). It is of notable interest to develop a synthesis method for L-THA due to the obvious demand for its derivative L-*threo*-β-benzyloxyaspartate (L-TBOA) in the field of neuroscience as a potent blocker of EAAT subtypes (Shigeri et al. 2004; Shimamoto 2008). L-THA acts as a high-affinity substrate for EAAT1-4 and as an inhibitor for EAAT5, thus providing a suitable lead compound as a blocker of EAATs (Arriza et al. 1994, 1997; Shigeri et al. 2001). Most of the L-THA synthetic routes relying on the chemical methods utilize a chiral template such as *trans*-methyl cinnamate as a starting material (Deng et al. 1995; Bionda et al. 2012). However, these routes require several reaction steps and are time-consuming. In contrast to the synthetic chemistry approach, few bacterial enzymes are known to efficiently produce L-THA. Strieker et al. (2008) have reported the bioconversion of L-aspartate to L-THA by using a single mutant of asparagine oxygenase ($AsnO_{D241N}$) from *Streptomyces coelicolor*. However, the activity and the thermal stability of $AsnO_{D241N}$ were found to be lower than those of $AsnO_{WT}$. Alternatively, Hara and Kino (2010) have reported a more efficient L-THA bioconversion by overexpression of native AsnO using *Escherichia coli*, coupled with *E. coli* asparaginase in vivo. Optically pure 3-hydroxyaspartate production by using a simple strategy is still a challenging process in both synthetic and biological chemistry. While many groups have attempted to increase the productivity, a dramatic improvement in the efficiency has not been realized, resulting in L-THA being an expensive product.

In this paper, we present crystal structures of D-THA DH complexed with its inhibitor D-EHA, a poorly active H351A mutant complexed with its substrate L-EHA and H351A mutant complexed with the reaction intermediate 2-amino maleic acid. High-resolution structures provide a clear picture of how substrate/inhibitor binds to the enzyme, allowing us to propose a model for the catalytic reaction as well as the mechanism for the substrate stereospecificity of D-THA DH. We also report an enzymatic optical resolution by using purified D-THA DH and show efficient yields of L-THA and D-EHA as isolated crystals with high enantiomeric excess (*e.e.*). This novel approach for the production of optically pure 3-hydroxyaspartate is simple, is environment friendly, and is applicable for the large-scale production.

Materials and methods

Materials

Oligonucleotides were obtained from Eurofins Genomics, Inc. (Tokyo, Japan). A (±)-*trans*-epoxysuccinic acid and DL-*threo*-

3-hydroxyaspartate (DL-THA) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). L-EHA was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). An isolated strain, identified as *Delftia* sp. HT23, has been deposited in the AHU Culture Collection of Hokkaido University under accession number AHU2003.

Site-directed mutagenesis

Plasmids expressing the H351A and C353A mutants were constructed using inverse PCR method with a KOD-Plus-Mutagenesis Kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. Previously described D-THA DH expression vector served as a template DNA (Matsumoto et al. 2013). The following synthetic oligonucleotide primers were used: H351A forward, 5′-**GCC**GCCTGC GCCACGGGCGCGCAGTTC-3′, and reverse, 5′-GTTGGG CAGGATGCGCAGCCGCGTGCC-3′; C353A forward, 5′-**GCC**GCCACGGGCGCGCAGTTCCCGGCC-3′, and reverse, 5′-GGCATGGTTGGGCAGGATGCGCAGCCG-3′. The bold bases encode an Ala residue instead of His351 and Cys353, respectively. The inverse PCR conditions were as follows: initial 2-min denaturation at 94 °C, followed by ten cycles of amplification at 98 °C for 10 s and at 68 °C for 5 min. The nucleotide substitutions were confirmed by DNA sequencing. The nucleotide sequence of the gene encoding D-THA DH from *Delftia* sp. HT23 has been deposited in the DDBJ/EMBL/GenBank database under the accession number AB433986. Each mutant was expressed in *Rhodococcus erythropolis* L88 cells according to a previously described method (Nakashima and Tamura 2004a, b).

Recombinant protein expression and purification

The recombinant enzymes used in this work contained additional Met-Gly-$(His)_6$-Ala-Met-Ser residues at the N-terminus. Recombinant D-THA DH and its mutants were overproduced using a *R. erythropolis* expression system and purified by Ni-affinity chromatography, as described previously (Matsumoto et al. 2013). The fractions containing recombinant proteins were collected, dialyzed against the buffer (10 mM Tris-HCl, pH 8.0, 0.01 mM PLP, and 0.1 mM dithiothreitol), and subsequently concentrated to 15 mg mL^{-1} using a centrifugal filtration device (10,000 Da molecular weight cutoff, Merck Millipore, Ltd., Darmstadt, Germany). Protein concentrations were determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) with bovine serum albumin as a standard. Protein purity was checked using SDS-PAGE (Fig. S1).

Crystallization and X-ray diffraction studies

All crystallization experiments were performed at 20 °C by using either sitting drop or hanging drop vapor diffusion method. The substrate-free wild-type enzyme was crystallized as previously described (Matsumoto et al. 2013). For the preparation of the substrate/inhibitor complex, the purified sample was mixed with molar excess of substrate/inhibitor (~50 mM) and incubated overnight before crystallization setup. All D-THA DH crystals used in this work were obtained in the solution consisting of 0.1 M Tris-HCl, pH 8.5, 0.2 M $MgCl_2$, and 10–20 % PEG 3350. Prior to X-ray diffraction studies, crystals were briefly soaked in a cryoprotectant solution containing a crystallization mother liquor and an additional 20 % glycerol. For the structure analysis of a metal-free enzyme, the crystals were briefly soaked in the Mg^{2+}-free cryoprotectant solution consisting of 0.1 M HEPES-NaOH, pH 8.0, 2.0 M ammonium formate, and 25 % glycerol. Crystals were flash-cooled in a cold nitrogen gas stream or directly in the liquid nitrogen. X-ray diffraction data were collected using a charge-coupled device (CCD) detector (ADSC) at the Photon Factory (PF; Tsukuba, Japan). Raw diffraction images were processed with the program iMosflm/SCALA (Battye et al. 2011; Winn et al. 2011) or HKL2000 suite (Otwinowski and Minor 1997).

Structure solution and model refinement

The structure of D-THA DH was solved by a single-wavelength anomalous diffraction (SAD) method with bromide-soaked crystals as described (Matsumoto et al. 2013). Bromide sites were determined using the program SHELXC/D (Sheldrick 2008). Density modification and model building were performed using the program SOLVE/RESOLVE (Terwilliger and Berendzen 1999; Terwilliger 2000). Model refinement was performed using the program REFMAC 5 (Murshudov et al. 2011), and the manual model fitting was done with the program Coot (Emsley and Cowtan 2004). Ligand models not found in the Chemical Component Dictionary were created using the program SKETCHER, and the geometrical restraints files were generated using the program LIBCHECK provided in the CCP4 program package (Winn et al. 2011). Molecular drawings were prepared using the program PyMOL (DeLano 2002). Atomic coordinates and the structure factor amplitudes of all the structures reported in this paper were deposited in the RCSB PDB under accession codes 3WQC, 3WQD, 3WQF, 3WQG, 4 PB3, 4 PB4, and 4 PB5. Data collection and refinement statistics are summarized in Table 1.

Enzyme assay

3-Hydroxyaspartate dehydratase activity was assayed spectrophotometrically by measuring a change in the absorbance of