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Title Page

Factors associated with the effect of interferon- α sequential therapy in order to discontinue nucleos(t)ide analogue treatment in patients with chronic hepatitis B

Short title: NUCs/IFN α sequential therapy

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Abbreviations:

HBV: Hepatitis B virus, NUC: nucleos(t)ide analog, IFN α : interferon- α , LAM: lamivudine, ADV: adefovir dipivoxil, ETV: entecavir, HCC: Hepatocellular carcinoma, HBsAg: hepatitis B s antigen, HBcrAg: hepatitis B core related antigen, HBeAg: hepatitis B e antigen, peg-IFN α : pegylated interferon- α

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Abstract

Aim: The factors associated with the outcome of sequential therapy with interferon- α (IFN α) in order to halt nucleos(t)ide analogue (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 NUCs were enrolled retrospectively. The subjects received NUCs plus IFN α for 4 weeks followed by IFN α alone for 24 weeks. Natural IFN α of 6-million-unit doses was administered 3 times a week. A successful response to NUCs/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 ($P < 0.002$) and hepatitis B core-related antigen (HBcrAg) of ≥ 4.5 log U/ml ($P < 0.003$) 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. **Conclusions:** The combinational use of HBsAg and HBcrAg levels may be useful to predict the 24-month outcome of NUCs/IFN α sequential therapy. Maximal levels of ALT and HBV DNA during post-treatment follow-up might also help monitor responses to IFN α sequential therapy.

Keywords: nucleos(t)ide analogues, interferon- α , sequential therapy, hepatitis B surface antigen, hepatitis B core-related antigen

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Introduction

Hepatitis B virus (HBV) infection is a widespread health problem with an estimated 350 to 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 nucleos(t)ide analogues (NUCs) and interferon- α (IFN α).⁴ NUCs are orally administered and are associated with low rates of adverse effects. Although treatment with NUCs, such as lamivudine (LVD), adefovir dipivoxil (ADV), 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 treatment with NUCs 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 virologic response in patients with chronic hepatitis B who did not respond to IFN α alone. However, ensuing reports⁸⁻¹² were unable to confirm such a cooperative effect. Since 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 NUCs could be safely

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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 NUCs.

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.

Patients and 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 Co., Ltd., Tokyo) at a dose of 6 million units was administered 3 times a week. Doses were reduced to 3 million units 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 8 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

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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 NUCs were re-administered due to reactivation of hepatitis B. NUCs 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 of NUCs possessed 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 NUCs.^{13, 14, 16}

HBsAg 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 and human immunodeficiency virus 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 Toranomom 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 NUCs in patients with chronic hepatitis B” for Teine Keijinkai Hospital).

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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 Co., Ltd., Tokyo, Japan; Fujirebio Inc., Tokyo, Japan; and/or Sysmex Co., Kobe, Japan) at each hospital. Quantitative measurement of HBsAg¹⁷ was done using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (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 to 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 Inc., Tokyo, Japan) as described previously.^{20, 21} The HBcrAg assay measures all antigens transcribed and translated from the pre-core 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 to 6.8 log U/ml.

Evaluation of response to NUCs/IFN α sequential therapy

The clinical conditions of a successful response to NUCs/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

The Fisher's exact and Pearson's chi-square 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 curve (AUC). Multivariate analysis was performed using a logistic model for the 24-month response to NUCs/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 ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P* values of less than 0.05 were considered to be statistically significant.

Results

Factors associated with the 24-month response to NUCs/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 gender distribution did not differ remarkably between the groups. Genotype C was similarly predominant. The types of NUCs administered 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 NUCs 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 NUCs was seen in only 1 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 since observation was discontinued when NUCs were re-administered.

Biochemical and virological markers were compared between 24-month responders and non-responders at the start of NUCs, at the start of IFN α , and at the end of IFN α (Table 3). Positivity for the HBe antigen was significantly lower in responders at all time points. HBsAg and HBcrAg levels did not differ between the groups at the start of NUCs, 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.

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

Of the 50 patients enrolled, 23 (46%) had HBsAg ≥ 3.0 log IU/ml and HBcrAg ≥ 4.5 log U/ml, 27 (54%) had HBsAg < 3.0 log IU/ml or HBcrAg < 4.5 log U/ml, and none had HBsAg < 3.0 log IU/ml and HBcrAg < 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 NUCs/IFN α sequential therapy ($P = 0.005$).

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

We assessed the responses to NUCs/IFN α sequential therapy at 6 and 12 months after completing IFN α administration using 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 NUCs/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 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 1) the inflection point to distinguish a response was clear for maximal ALT but ambiguous for maximal HBV DNA, 2) the value for “sensitivity + specificity - 1” as calculated by ROC analysis was larger for maximal ALT (7.5 vs. 6.5), and 3) 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 NUCs 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.

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Discussion

The cooperative effect of NUCs/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 HBe antigen-positive chronic hepatitis B and detected several differences. Although their results were negative, they witnessed that patients who had achieved HBe antigen seroconversion by the time of IFN α commencement experienced a significantly higher sustained virologic response rate than those in whom the HBe antigen persisted. Thus, it appeared beneficial to further clarify the factors associated with the response to NUCs/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 NUCs 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 NUCs 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 since 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

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significantly associated with the response to NUCs/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 virologic 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 pre-core 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.

HBsAg 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 NUCs. 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 virologic 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, since NUCs directly hamper production of the HBV virion by inhibiting reverse transcription of pre-genomic RNA without

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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 NUCs.^{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 NUCs/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 virologic response to NUCs/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 NUCs/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 NUCs/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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