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 HBsAg[17] 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/IFNα sequential therapy

The clinical conditions of a successful response to NUCs/IFNα 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 IFNα 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/IFNα sequential therapy. Correlations between maximal values of ALT and HBV DNA were calculated using Spearman's rank correction 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/IFNα sequential therapy

Of the 50 patients enrolled, 18 were judged as responders at 24 months after completing IFNα 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 IFNα, and at the end of IFNα (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 IFNα administration. A significant difference in HBV DNA level was seen between the groups at the end of IFNα administration only. ALT levels did not differ between the groups at any point.

Multivariate analysis revealed that HBsAg and HBcrAg levels of ≥3.0 and ≥4.5 log U/ml, respectively, at the start of IFNα administration were significant factors associated with a 24-month non-response to NUCs/IFNα 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 IFNα, HBsAg level at the start of IFNα ≥3.0 log IU/ml, and HBcrAg level at the start of IFNα ≥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 IFNα 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/IFNα sequential therapy (P = 0.005).

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

We assessed the responses to NUCs/IFNα sequential therapy at 6 and 12 months after completing IFNα 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/IFNα 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 IFNα 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 IFNα 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 IFNα administration.

Discussion

The cooperative effect of NUCs/IFNα sequential therapy has been controversial.[7-12] Enomoto et al.[10] first analyzed the results of ETV/IFNα 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 IFNα 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/IFNα sequential therapy.

The present study analyzed the factors associated with a long-term response to IFNα sequential therapy in order to safely discontinue NUC therapy. All patients were treated with natural IFNα 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 IFNα 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 IFNα 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 IFNα sequential therapy in relation to the results of earlier retrospective studies. It has been reported that pegylated IFN-α (peg-IFNα) provides a higher HBV response rate than does conventional IFNα.[23] Therefore, additional prospective studies of sequential therapy using peg-IFNα are needed as well.

Both HBsAg and HBcrAg levels at the time of NUC cessation were factors

significantly associated with the response to NUCs/IFNα sequential therapy. HBsAg has been closely linked with peg-IFNα therapy outcome.[24-27] Moucari et al.[26] analyzed HBeAg-negative hepatitis B patients who had been treated with peg-IFNα 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-IFNα 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/IFNα 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-IFNα 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-IFNα 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/IFNα 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 IFNα and then became more sporadic afterwards. The timing of judgment of a virologic response to NUCs/IFNα 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/IFNα 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/IFNα sequential therapy. Maximal levels of

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ALT and HBV DNA during follow-up may also be employed for monitoring the results of IFNα sequential therapy.

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Table 1. Demographic data of 50 enrolled patients

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

[a] Data are expressed as the median (range).

[b] Data are expressed as a positive percentage (positive number/total number).

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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 value
Age at start of NUCs (years) [a]	36 (21 - 56)	34 (21 - 57)	0.486
Gender (male : female)	15 : 3	23 : 9	0.497
Genotype (B : C : undetermined)	1 : 16 : 1	2 : 20 : 10	0.101
NUCs at start (LVD : ETV)	16 : 2	27 : 5	1.000
NUCs at end (LVD : ETV : LAM+ADV : ETV+ADV)	16 : 2 : 0 : 0	24 : 6 : 1 : 1	0.610
Duration of NUC administration (months) [a]	51 (5 - 121)	5 (4 - 72)	0.001
Follow-up period after stopping IFNα administration (months) [a]	30 (23 - 102)	22 (2 - 81)	0.014
Re-administration of NUCs before judging 24-month response [b]	0% (0/18)	53% (17/32)	< 0.001
Re-administration of NUCs after judging 24-month response [b]	6% (1/18)	47% (7/15)	0.012

[a] Data are expressed as the median (range).

[b] Data are expressed as a positive percentage (positive number/total number).

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 IFNα administration, and stopping IFNα administration

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

[a] Data are expressed as the median (range).

[b] Data are expressed as a positive percentage (positive number/total number).

Table 4. Multivariate analysis of factors associated with 24-month non-responders to

NUCs/IFNα sequential therapy

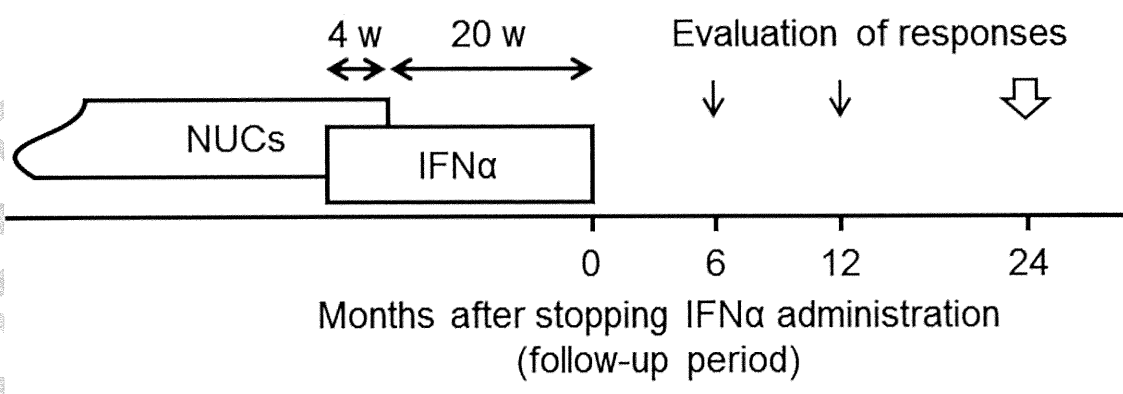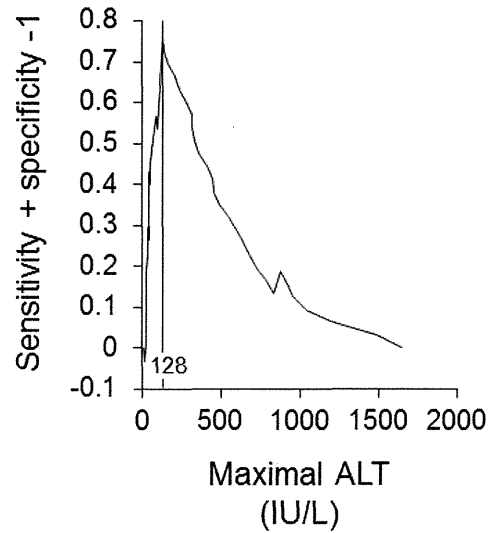| Selected factor	Odds ratio	95%CI	*P* value
HBsAg ≥3.0 log IU/ml at start of IFNα	17.7	2.9 - 108.2	0.002
HBcrAg ≥4.5 log U/ml at start of IFNα	15.0	2.5 - 88.6	0.003

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Fig. 1. Experimental design of the present study.

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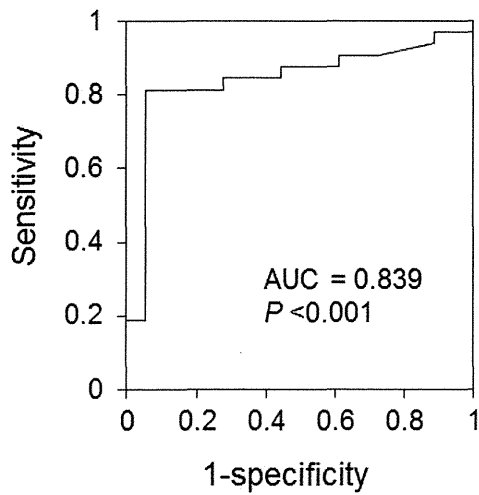
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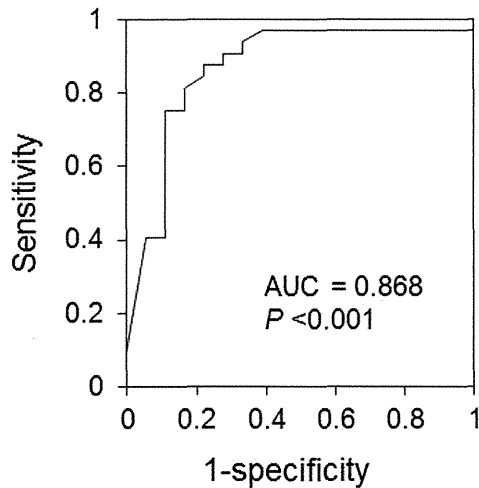

Maximal HBV DNA level
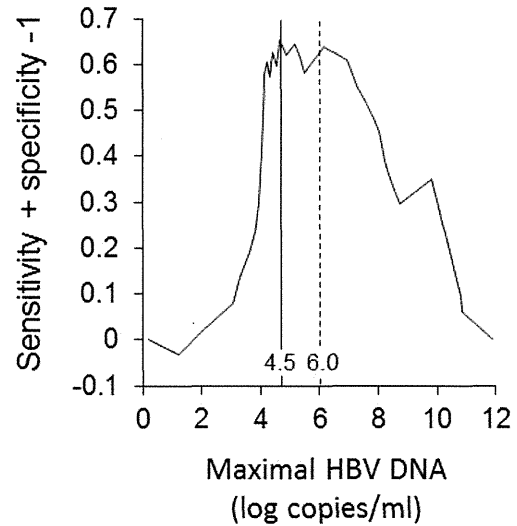
Fig. 2. ROC analysis of maximal ALT and HBV DNA levels to discriminate between 24-month responders and non-responders. Vertical solid lines indicate the actual values of markers corresponding to main inflection points and the vertical broken line indicates the actual value of the marker corresponding to a second inflection point.
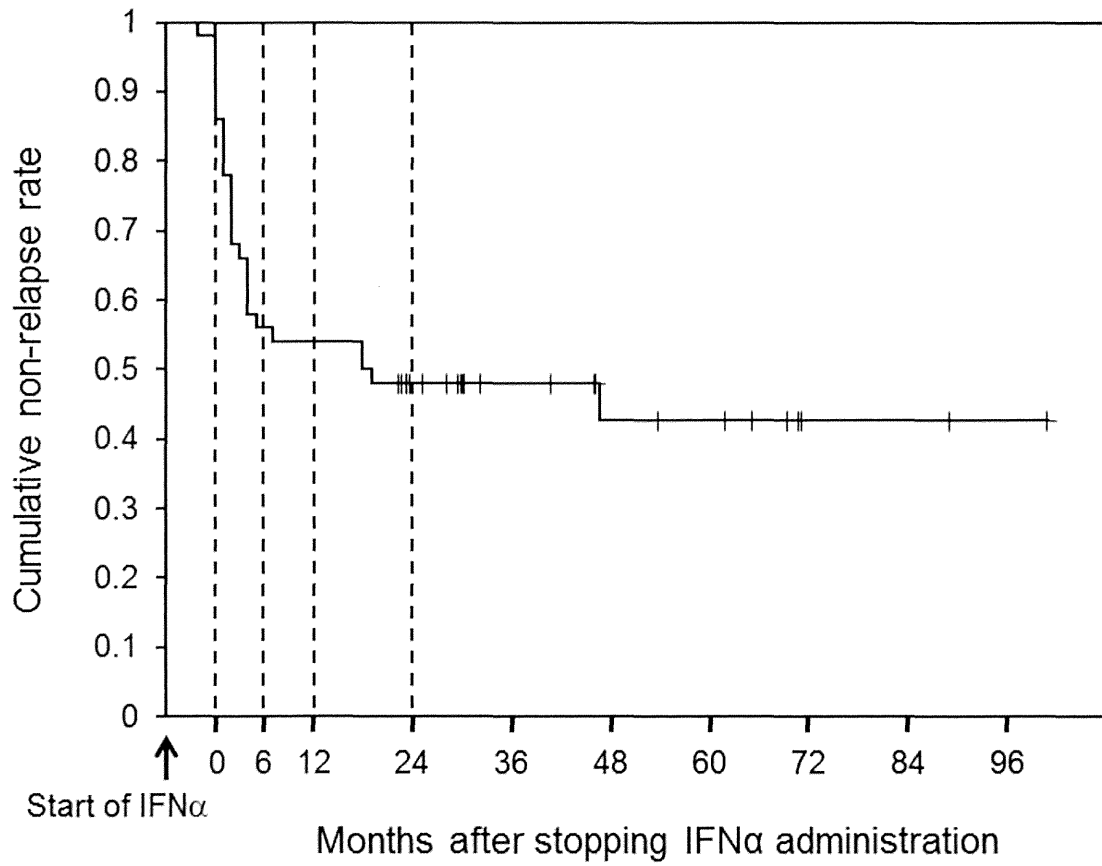
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Fig. 3. Kaplan-Meier analysis of the non-relapse rate after starting IFNα administration by defining relapse of hepatitis B as ALT level exceeding 128 IU/L.