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Original article

Reduction of hepatitis B surface antigen in sequential versus add-on pegylated interferon to nucleoside/nucleotide analogue therapy in HBe-antigen-negative chronic hepatitis B patients: a pilot study

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Abstract

Background: Although pegylated-interferon (PEG-IFN) and nucleotide/nucleoside analogue (NA) combination therapy is considered to be optimal for accelerating serum hepatitis B surface antigen (HBsAg) reduction, the effect is limited, and the best approach to PEG-IFN treatment for chronic hepatitis B patients during long term NA therapy has yet to be determined.

Methods: Twenty-one HBeAg-negative chronic hepatitis B patients whose HBV DNA levels were suppressed to undetectable levels by NA therapy were administrated PEG-IFNα2a for 48 weeks (sequential therapy: 10, add-on therapy: 11). Factors associated with HBsAg reduction by PEG-IFN therapy were analyzed.

Results: During PEG-IFN treatment, HBsAg levels were reduced by 0.48 Log IU/ml. More than 1 LogIU/ml of HBsAg reduction was observed in 8 patients (sequential therapy: 6, add-on therapy: 2), and one patient with sequential therapy achieved HBsAg loss. By univariate analysis, sequential therapy was marginally associated with more than 1 LogIU/ml HBsAg reduction during PEG-IFN treatment (P=0.060). After PEG-IFN treatment, only 5 patients, including the patient with HBsAg loss, achieved more than 0.5 LogIU/ml of HBsAg reduction by 1 year after PEG-IFN treatment. By univariate analysis, sequential therapy was significantly associated with HBsAg reduction after

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PEG-IFN treatment (P=0.012). In addition, ALT elevation during PEG-IFN therapy and lower serum IL-8 level at the end of PEG-IFN treatment were also significantly associated with HBsAg reduction by 1 year after PEG-IFN treatment (P=0.038, 0.044, respectively).

Conclusions: Sequential therapy may be superior to add-on therapy in reducing HBsAg levels during long term NA therapy in chronic hepatitis B patients.

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Running head: HBsAg reduction by PEG-IFN therapy to HBeAg-negative chronic hepatitis B

Introduction

Hepatitis B virus (HBV) infection is a serious global health problem. More than two billion people have been infected with HBV, and about 20% remain chronically infected [1,2]. Chronically infected individuals often develop chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, and the incidence of hepatocellular carcinoma in chronically infected individuals is significantly higher than that in healthy individuals [3]. Once HBV infects human hepatocytes, HBV genomes are transported into the nucleus, and some viral genomes become integrated into human chromosomes [4–7]. Thus, complete elimination of the virus is difficult, and patients are generally treated with interferon and nucleotide/nucleoside analogues (NAs), which suppress viral replication and prevent the progression of liver disease by combating inflammation [8–10].

Japanese guidelines from the Japan Society of Hepatology currently recommend that the final goal of antiviral therapy for chronic hepatitis B should be HBsAg loss [9]. However, it is difficult to achieve HBsAg loss by long-term treatment with NAs, and the number of patients who do achieve HBsAg loss is limited under the present antiviral treatments. Recently, Marcellin et al. performed an open-label, randomized, controlled study in which chronic hepatitis B patients were treated with tenofovir disoproxil fumarate (TDF) and/or pegylated-interferon (PEG-IFN) α2a, and they reported that TDF and PEG-IFNα2a combination therapy was more efficient for inducing HBsAg loss than TDF or PEG-IFNα2a monotherapy [11]. While no patient achieved HBsAg loss by TDF monotherapy alone in this study, the results indicated that PEG-IFNα2a might be a key drug for inducing HBsAg loss or reduction in chronic hepatitis B patients.

Results of the study by Marcellin et al suggest that PEG-IFNα2a therapy can induce HBsAg loss or HBsAg reduction in chronic hepatitis B patients who have been unable to achieve HBsAg loss in spite of long term NA therapy. To analyze the effects on HBsAg levels by PEG-IFNα2a therapy, we treated chronic hepatitis B patients who have been treated with NAs for more than one year with PEG-IFNα2a and analyzed HBsAg levels during and after PEG-IFN treatment.

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Materials and methods

Patients

Twenty-one Japanese chronic hepatitis B patients were enrolled. All patients had been treated with NAs for more than 12 months at Hiroshima University Hospital or Kawakami Clinic in Hiroshima, Japan. None of the patients were infected with other viruses, including human immunodeficiency virus or hepatitis C virus, or had evidence of other liver diseases, such as auto-immune hepatitis or alcoholic liver disease. Patients with total ethanol intake of more than 100 kg were excluded [12]. All patients gave written informed consent to participate in the study. The experimental protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethical committee of Hiroshima University Hospital (Approval ID: E-463).

After obtaining informed consent, ten patients were treated using sequential therapy, which included 48 weeks of PEG-IFNα2a therapy (180μg/week) beginning one month prior to discontinuation of NA therapy until 11 months after. Although the optimal duration of overlap of PEG-IFN and NA therapy was not established, we determined the overlap duration following previous reports in which sequential studies were performed using conventional interferons [13–15]. The remaining 11 patients were treated using add-on therapy, which included 48 weeks of PEG-IFNα2a that overlapped with NA combination therapy.

Blood samples were obtained from the patients at the beginning of PEG-IFN therapy and every 4 weeks during the follow-up period. Biochemical and hematological tests were performed in the Hiroshima University Hospital laboratory. Remaining serum was stored at -80°C for further analysis.

Measurement of serum HBV markers

HBV DNA levels were quantified by real-time PCR using the TaqMan PCR System (Roche Diagnostics). HBeAg and HBsAg levels were measured by Chemiluminescent Immuno Assay (CLIA) using the ARCHITECT analyzer (Abbott Japan Co., Ltd., Tokyo, Japan).

Measurement of serum IL-8 levels

Serum IL-8 levels were measured by AlphaLISA immunoassay kit according to the manufacturer's instructions. The quantitative range for serum IL-8 was 0.001 ~ 100,000 pg/ml.

Statistical Analysis

The baseline characteristics of the patients in the two groups were compared, and differences were assessed by Chi-square test with Yate's correction, the Fisher exact probability test, or the Mann-Whitney U test, as appropriate. All P values less than 0.05 by two-tailed test were considered significant. Variables with at least marginal significance ($P<0.10$) in univariate analysis were entered into multiple logistic regression analysis to identify independent factors. Statistical analysis was performed using SPSS® ver. 17.0 (SPSS Inc., Chicago, IL).

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Results

Characteristics of study patients

The clinical background of the 21 enrolled patients at the beginning of PEG-IFN therapy is shown in Supplementary material 1. Two patients were treated with 100 mg/day of lamivudine (LMV), 13 were treated with 0.5 mg/day of entecavir (ETV), 5 were treated with 100 mg/day of LMV plus 10 mg/day of adefovir (ADV) combination therapy, and one was treated with 100 mg/day of LMV plus 300mg/day of TDF combination therapy. Seventeen patients were male and 4 were female. All 21 patients were negative for hepatitis e antigen (HBeAg). During PEG-IFN treatment, no patient needed PEG-IFN dose reduction due to side effects. There was no significant difference among clinical factors, such as ALT, HBsAg, Hepatitis B core related antigen (HBcrAg) at the beginning of PEG-IFN therapy between patients treated with sequential therapy and those treated with add-on therapy. After PEG-IFN therapy, nucleos(t)ide analogue treatment was continued in the 11 patients with add-on therapy, whereas the 10 patients treated with sequential therapy were followed up without antiviral treatment after PEG-IFN therapy. Three patients were retreated with nucleos(t)ide analogues within 2 years after the sequential therapy, but the remaining 7 patients were followed up without antiviral therapy more than 2 year after sequential therapy.

Analysis of HBsAg reduction during PEG-IFN therapy

The changes in HBsAg levels during PEG-IFN therapy are shown in Supplementary material 2. During PEG-IFN treatment, HBsAg levels were reduced by 0.48 Log IU/ml. More than 1 Log IU/ml of HBsAg reduction was observed in 8 of 21 patients, and the reduction in HBsAg levels in the remaining 13 patients was less than 1 Log IU/ml. To identify factors associated with >1 Log IU/ml of HBsAg reduction during PEG-FN therapy, the patients were divided into two groups based on HBsAg reduction levels during PEG-IFN therapy (>1Log IU/ml and ≤1Log IU/ml). As shown in Table 2, the proportion of patients who achieved >1 Log IU/ml of HBsAg reduction following sequential therapy was marginally higher than that of patients treated with add-on therapy (P=0.063), and sequential therapy was also observed as a marginally associated factor for HBsAg reduction during PEG-IFN therapy by multivariate analysis (P=0.060). However, the duration of NA treatment, HBsAg, HBcrAg, and HBV DNA levels at the beginning of PEG-IFN treatment were not significant.

Analysis of HBsAg reduction after PEG-IFN therapy

Time courses of HBsAg levels during and after PEG-IFN therapy are shown in Figure 1. Since the antiviral effects of IFN are considered to continue after the end of IFN treatment, we compared the changes of HBsAg levels for 1 year after PEG-IFN therapy. Although greater than 0.5 Log IU/ml of HBsAg reduction after PEG-IFN therapy was observed in 5 out of 10 patients who were treated with sequential therapy (Figure 1A), HBsAg reduction after PEG-IFN therapy was not observed in patients with add-on therapy (Figure 1B). To identify factors associated with more > 0.5 Log IU/ml of HBsAg reduction one year after PEG-IFN therapy, we performed univariate analysis using clinical factors. As shown in Table 3, >0.5 Log HBsAg reduction at 1 year after PEG-IFN therapy was significantly

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associated with sequential therapy (P=0.012) and was marginally associated with ALT and HBsAg levels at the beginning of PEG-IFN therapy (P=0.050, P=0.062, respectively). To identify independent predictive factors for HBsAg reduction after PEG-IFN therapy, multivariate analysis using multiple logistic regression analysis was attempted but was unsuccessful due to the small number of study subjects.

Within 1 year after PEG-IFN therapy, no elevation of serum ALT and HBV DNA levels occurred in any of the 11 patients who were treated with add-on therapy. However, serum ALT elevation occurred in 4 of 10 patients who were treated using sequential therapy, and HBV DNA elevation (>3.0 Log copies/ml) occurred in 4 of 10 patients with sequential therapy.

Discovery of novel biomarkers for predicting HBsAg reduction after PEG-IFN therapy

Recently, we found that HBV infection induces the transcriptional activation of IL-8 in human hepatocytes, and we proposed that upregulation of IL-8 might lead to suppression of interferon responsiveness in hepatocytes (Tsuge et al. submitted). Therefore, we predicted that serum IL-8 levels might be associated with the antiviral effect of PEG-IFN, and we measured serum IL-8 levels at the beginning and the end of PEG-IFN therapy. Serum IL-8 levels at the beginning of PEG-IFN therapy did not differ between patients with >0.5 Log IU/ml of HBsAg reduction after PEG-IFN therapy and those without (Figure 2A). However, serum IL-8 levels at the end of PEG-IFN therapy in patients with HBsAg reduction were significantly lower than those in patients without HBsAg reduction (P=0.044) (Figure 2B).

Observing the time course of ALT during PEG-IFN therapy, we found that HBsAg reduction levels from the beginning of therapy to one year after PEG-IFN therapy were significantly correlated with the maximum ALT levels during PEG-IFN therapy (P=0.002) (Figure 3A). In patients whose ALT levels were elevated by more than 50 U/L during PEG-IFN therapy, HBsAg levels were reduced by -1.60 Log IU/ml (-0.27 ~ -3.47) during or after PEG-IFN therapy. However, the reduction of HBsAg levels in patients without ALT elevation during PEG-IFN therapy was significantly lower (-0.38 Log IU/ml [-2.30 ~ 0.11)]) (P=0.038) (Figure 3B).

As serum IL-8 levels at the at the beginning of PEG-IFN therapy and ALT elevation during PEG-IFN therapy were associated with HBsAg reduction during and after PEG-IFN therapy, we analyzed the association between serum IL-8 levels and ALT elevation during PEG-IFN therapy. However, serum IL-8 levels at the beginning and end of PEG-IFN therapy were not associated with ALT elevation during PEG-IFN therapy (P=0.533, P=0.203, respectively) (Supplementary material 3). To predict ALT elevation during PEG-IFN therapy, we also performed statistical analysis using clinical factors; however, no significant differences were observed among factors. Only sequential therapy was identified as a marginally associated factor for ALT elevation during PEG-IFN therapy (Supplementary material 4).

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Discussion

Revised guidelines for antiviral therapy for chronic hepatitis B have recently been established, and HBsAg loss was designated as the final goal of antiviral therapy [16,17]. However, despite long term antiviral treatment with NAs, it is difficult to induce HBsAg loss by NA therapy alone. Marcellin et al. performed a randomized, controlled study involving chronic hepatitis B patients with no history of NA treatment that showed the superiority of TDF and PEG-IFNα2a combination therapy for inducing HBsAg loss [11]. However, it is unclear whether add-on therapy, in which 48 weeks of PEG-IFNα2a therapy is administered simultaneously with ongoing long term NA therapy, is superior to sequential therapy, in which 48 weeks of PEG-IFNα2a therapy (180μg/week) are administered from one month prior to discontinuation of long-term NA therapy to 11 months post-discontinuation, for HBsAg reduction. In this pilot study, we compared the effects of HBsAg reduction between sequential therapy and add-on therapy in chronic hepatitis B patients who were treated with NAs for more than 1 year, and we compared HBsAg reduction levels between these PEG-IFN therapies.

As shown in Supplementary material 2, greater than 1 Log IU/ml of HBsAg reduction was observed in 38.1% (8/21) of patients during PEG-IFN therapy, but HBsAg levels in the remaining 13 patients were not reduced by PEG-IFN therapy. Antiviral effects of PEG-IFN have been reported to be significantly associated with higher M2BPGi levels, higher ALT levels, lower HBsAg levels, lower HBeAg levels, and lower HBV DNA levels at the beginning of PEG-IFN therapy [18]. However, we could not identify a useful predictive clinical factor for HBsAg reduction during PEG-IFN therapy in this study. In the clinical study by Zhu et al., patients whose HBsAg levels were more than 3 Log IU/ml were enrolled. However, in 66.7% of our study patients, HBsAg levels had already declined to less than 3 Log IU/ml during long term NA therapy. Therefore, we concluded that HBsAg level at baseline was not a useful predictive factor for antiviral response by PEG-IFN therapy in this study.

Antiviral effects can be observed for a long time after the cessation of interferon administration [19–22]. To analyze the subsequent antiviral effects of PEG-IFN therapy, we analyzed HBsAg reduction levels from the end of PEG-IFN therapy to 1 year after the therapy. As shown in Figure 1, HBsAg levels in 16 of 21 patients were steady or gradually elevated after the cessation of PEG-IFN treatment, and HBsAg levels in only 5 remaining patients were reduced after PEG-IFN treatment. To identify predictive factors for HBsAg reduction after the cessation of PEG-IFN treatment, we performed univariate analysis, and only sequential therapy was found to be significant (Table 3). Although there are several reports in which antiviral effects of PEG-IFN therapy, including sequential or add-on therapy, were analyzed [11,14,23], the superiority of sequential of add-on therapy for HBsAg reduction in chronic hepatitis B patients with long term NA therapy has not been settled. Therefore, we contend that this pilot study provides evidence in favor of the superiority of sequential therapy over add-on therapy. According to the changes of HBsAg levels in patients who were treated with add-on therapy, HBsAg levels were not drastically reduced in most patients. We hypothesized that host immune responses might be activated by the cessation of NA therapy and that this activation could help bolster the antiviral effects of PEG-IFN. On the other hand, in patients

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undergoing add-on therapy, it might be difficult to activate host immunity while undergoing continuous NA therapy, leading to poor reduction of HBsAg. In support of this idea, ALT elevation, which might be induced by the activation of host immunity, occurred in 60% of patients treated with sequential therapy. This ALT elevation was significantly associated with HBsAg reduction by PEG-IFN therapy (Figure 3) (P=0.038).

Recently, Murata and his colleagues demonstrated that IFN-λ3 could be induced in colon cancer cell lines by treatment with nucleotide analogues such as ADV or TDF but not by treatment with the nucleoside analogues LMV or ETV [24]. This IFN-λ3 induction was associated with the antiviral effects of sequential therapy with PEG-IFN therapy [23]. To analyze the influence of nucleotide analogue treatment, we compared the effects of PEG-IFN therapy on HBsAg reduction. Although we did not observe differences in HBsAg reduction during and after PEG-IFN therapy (Table 1 and 2), HBsAg loss was achieved within 3 years after PEG-IFN therapy in 3 of 6 patients who were treated with nucleotide analogues prior to PEG-IFN therapy (data not shown), and this result might indicate the superiority of prior NA treatment for HBsAg loss.

In a recent study, we found that HBV infection induced IL-8 production in hepatocytes and that this IL-8 induction contributes to IFN resistance in human hepatocytes (Tsuge et al. submitted). Therefore, we hypothesized that serum IL-8 regulation might be associated with the antiviral effects of PEG-IFN therapy. We compared serum IL-8 levels between patients with and without >0.5 Log IU/ml HBsAg reduction within one year after PEG-IFN therapy. As shown in Figure 2, serum IL-8 levels at the end of PEG-IFN therapy in patients who achieved more than 0.5 Log IU/ml HBsAg reduction were significantly lower than those in patients with less than 0.5 Log IU/ml HBsAg reduction (P=0.044). These results suggest that serum IL-8 reduction during NA and PEG-IFN therapy and the recovery of host immune responses could accompany the reduction of HBsAg levels after PEG-IFN therapy.

In this study, we demonstrated that sequential therapy might be superior to add-on therapy in reducing HBsAg levels in chronic hepatitis B patients undergoing long term NA therapy. Although the number of study subjects was small, to our knowledge this is the first study to compare the antiviral effects of sequential versus add-on therapy. Our results support the need for establishing strategies for inducing HBsAg loss in chronic hepatitis B patients.

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Disclosure statement

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Figure legends

Figure 1. Change in HBsAg level in study patients during and after PEG-IFN treatment.

HBsAg levels of patients who were treated with sequential therapy (A) and with add-on therapy (B) are shown. The bold lines indicate patients whose HBsAg levels became negative or were reduced more than 0.5 LogIU/ml after PEG-IFN treatment.

Figure 2. The association between serum IL-8 levels and HBsAg reduction levels after PEG-IFN treatment.

Serum IL-8 levels were measured and compared between patients whose HBsAg levels were reduced more than 0.5 LogIU/ml at 1 year after PEG-IFN treatment and patients whose HBsAg levels were not reduced. Statistical analysis was performed using the Mann-Whitney U test.

Figure 3. The association between ALT flare during PEG-IFN treatment and HBsAg reduction levels after PEG-IFN treatment.

HBsAg reduction levels after PEG-IFN treatment were compared between patients whose ALT levels were elevated by more than 50 U/L during PEG-IFN treatment and patients whose ALT levels were not elevated. Statistical analysis was performed using the Mann-Whitney U test.

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Tables

Table 1. Statistical analysis of factors associated with >1 LogIU/ml reduction of HBsAg during PEG-IFN treatment (N=21).

Factors	HBsAg reduction ≧ 1 log (N=8)	HBsAg reduction < 1 log (N=13)	Univariate analysis P value	Multivariate analysis P value
Gender (male: female)	8: 0	9: 4	0.119	
Age (y.o.)*	51.5 (41-73)	55.0 (41-70)	0.645**	
Before IFN treatment				
Platelet s (×10^4/μl)*	20.4 (16.1-25.4)	16.4 (13.0-35.4)	0.268**	
Total Bilirubin (mg/dl)*	0.6 (0.4-2.0)	0.6 (0.4-1.1)	0.750**	
ALT (IU/L)*	20 (13-61)	16 (12-37)	0.645**	
Alb (g/dl)*	4.6 (3.9-5.0)	4.4 (4.1-4.8)	0.301**	
HBsAg (Log IU/ml)*	2.41 (0.3-4.2)	2.96 (0.9-3.8)	0.268**	
HBcrAg (Log U/ml)*	<3.0 (<3.0-3.6)	<3.0 (<3.0-4.0)	0.210**	
HBV DNA (Log copies/ml)*	undet (undet-<2.1)	undet (undet-<2.1)	0.654**	
HBV genotype (B: C)	1: 7	2: 11	0.684	
NA treatment (LMV: LMV+ADV: ETV: LMV+TDF)	2: 2: 4: 0	0: 3: 9: 1	0.335	
Duration of NA treatment (months)*	87.9 (34.7-137.4)	77.8 (23.5-156.8)	0.374**	
Duration of IFN treatment (weeks)*	48 (48-48.4)	48 (48-49.4)	0.750**	
IFN treatment (sequential: add-on)	6: 2	4: 9	0.063	0.060

*: median (range)
Univariate analysis was performed by Chi-square test with Yate's correction, the Fisher exact probability test, or the Mann-Whitney U test (**), as appropriate.
Multivariate analysis was performed by multiple logistic regression analysis.
Undet: undetermined

Table 2. Univariate analysis of factors associated with ≥0.5 LogIU/ml reduction of HBsAg after PEG-IFN treatment (N=21).

Factors	HBsAg reduction ≥0.5log (N=5)	HBsAg reduction <0.5 log (N=16)	P value
Gender (male: female)	5: 0	12: 4	0.304
Age (y.o.)*	51 (41-56)	54 (41-73)	0.208**
Before IFN treatment			
Platelets (×10^4/μl)*	20.4 (16.1-25.4)	16.4 (13.0-35.4)	0.398
Total Bilirubin (mg/dl)*	0.6 (0.6-2.0)	0.5 (0.4-1.1)	0.208**
ALT (IU/L)*	24 (16-61)	16 (12-37)	0.050**
Alb (g/dl)*	4.4 (4.3-4.8)	4.5 (3.9-5.0)	0.603**
HBsAg (Log IU/ml)*	2.25 (0.3-3.4)	2.96 (2.1-4.2)	0.062**
HBcrAg (Log U/ml)*	<3.0 (<3.0-3.6)	<3.0 (<3.0-4.0)	0.460**
HBV DNA (Log copies/ml)*	undet (undet-<2.1)	undet (undet-<2.1)	0.823**
HBV genotype (B: C)	0: 5	3: 13	0.421
NA treatment (LMV: LMV+ADV: ETV: LMV+TDF)	1: 2: 2: 0	1: 3: 11: 1	0.499
Duration of NA treatment (months)*	82.0 (49.6-99.7)	89.9 (23.5-156.8)	0.780**
Duration of IFN treatment (weeks)*	48 (48-48.4)	48 (48-49.4)	0.228**
IFN treatment (sequential: add-on)	5: 0	5:11	0.012

*: median (range)
Univariate analysis was performed by Chi-square test with Yate's correction, the Fisher exact probability test, or the Mann-Whitney U test (**), as appropriate.
Undet: undetermined

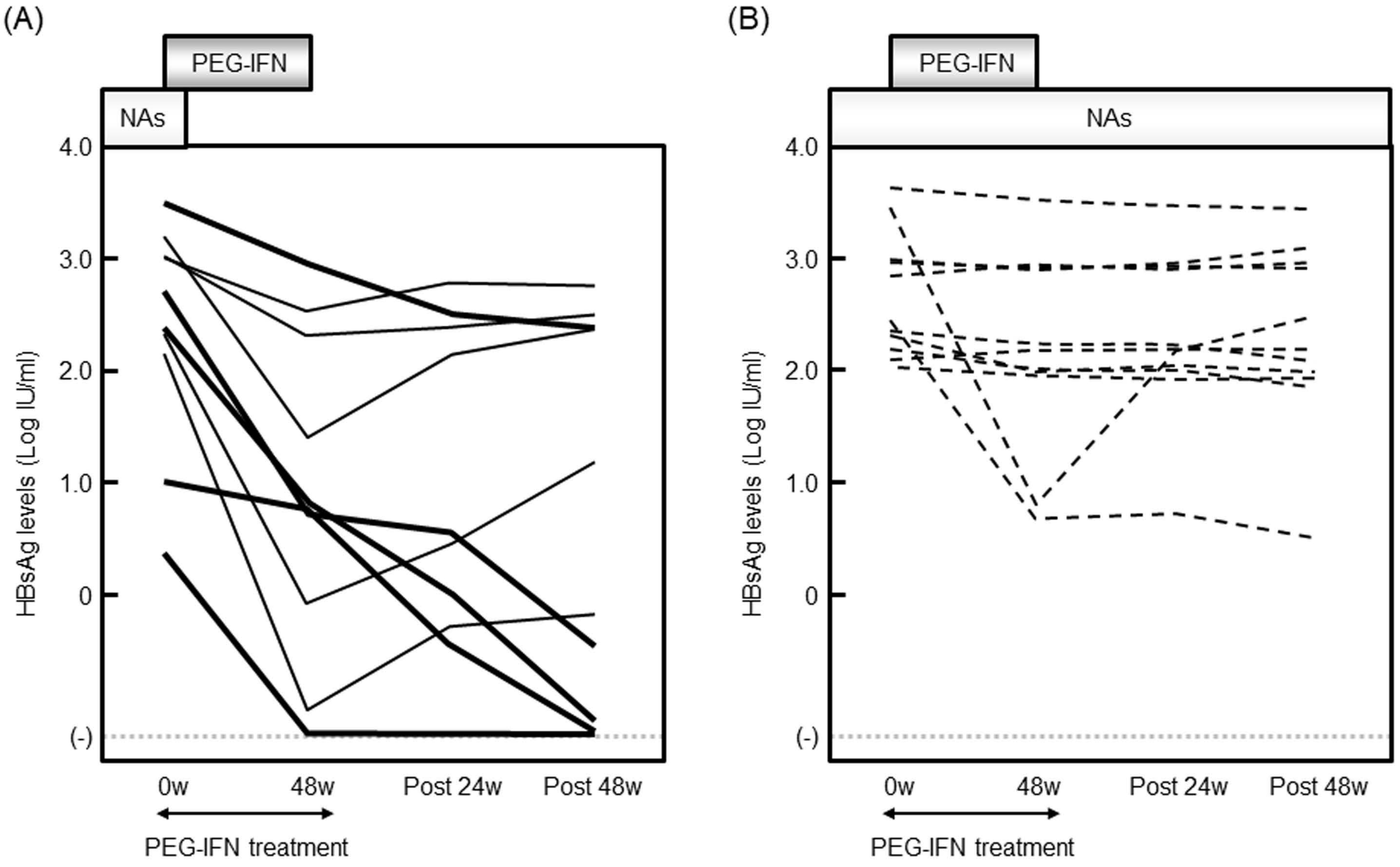


Figure 1. Change in HBsAg level in study patients during and after PEG-IFN treatment.
HBsAg levels of patients who were treated with sequential therapy (A) and with add-on therapy (B) are shown. The bold lines indicate patients whose HBsAg levels became negative or were reduced more than 0.5 LogIU/ml after PEG-IFN treatment.

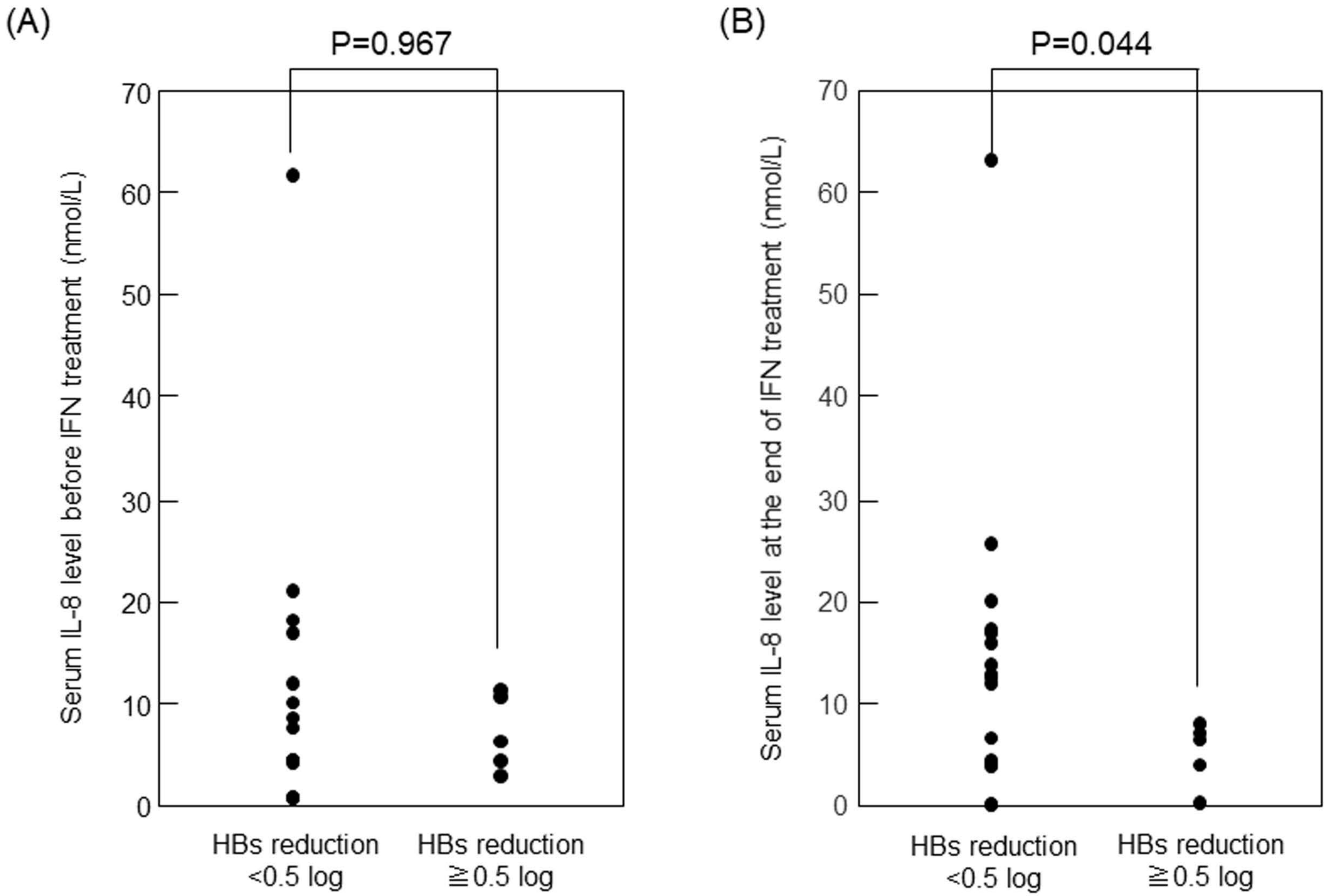


Figure 2. The association between serum IL-8 levels and HBsAg reduction levels after PEG-IFN treatment. Serum IL-8 levels were measured and compared between patients whose HBsAg levels were reduced more than 0.5 LogIU/ml at 1 year after PEG-IFN treatment and patients whose HBsAg levels were not reduced. Statistical analysis was performed using the Mann-Whitney U test.

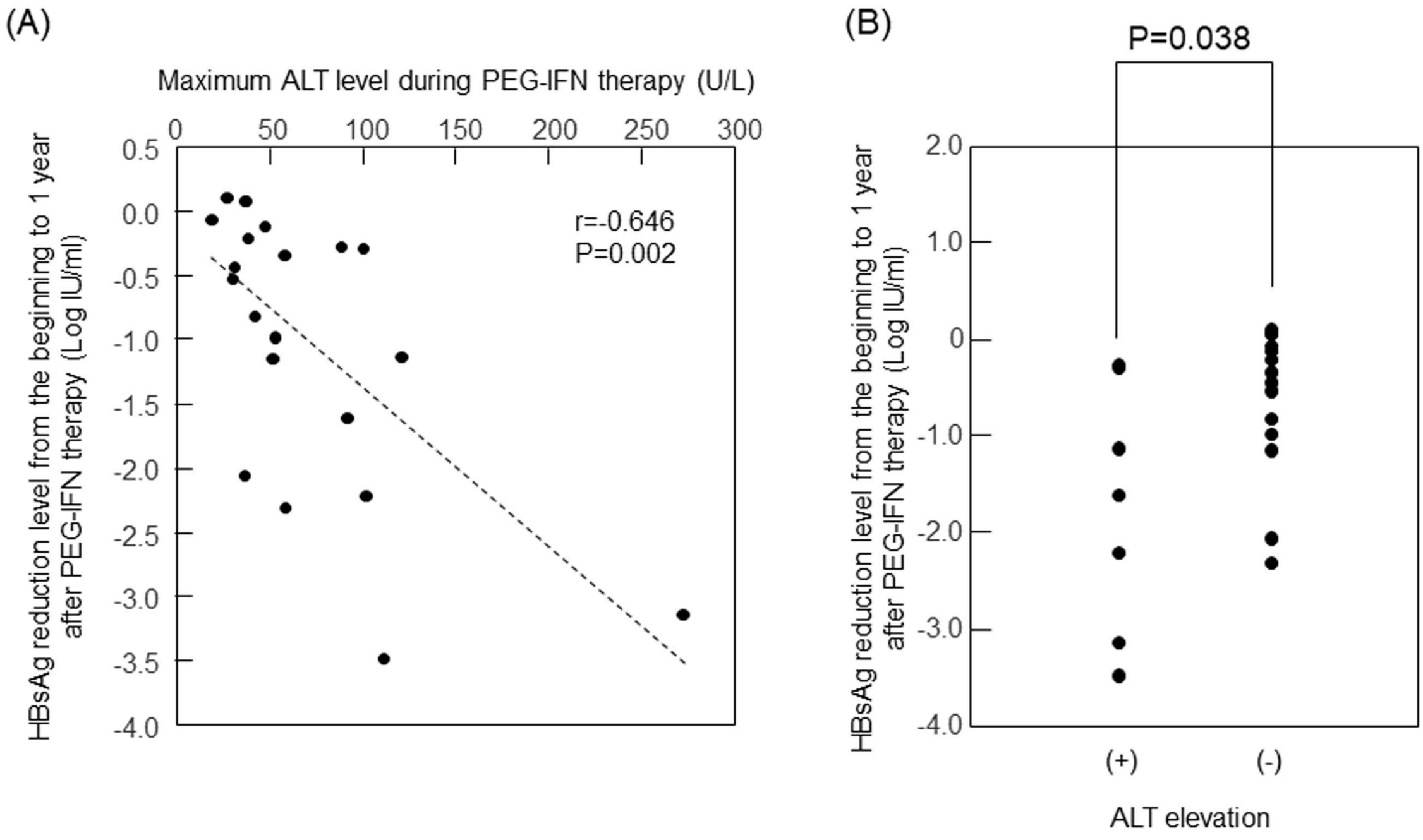
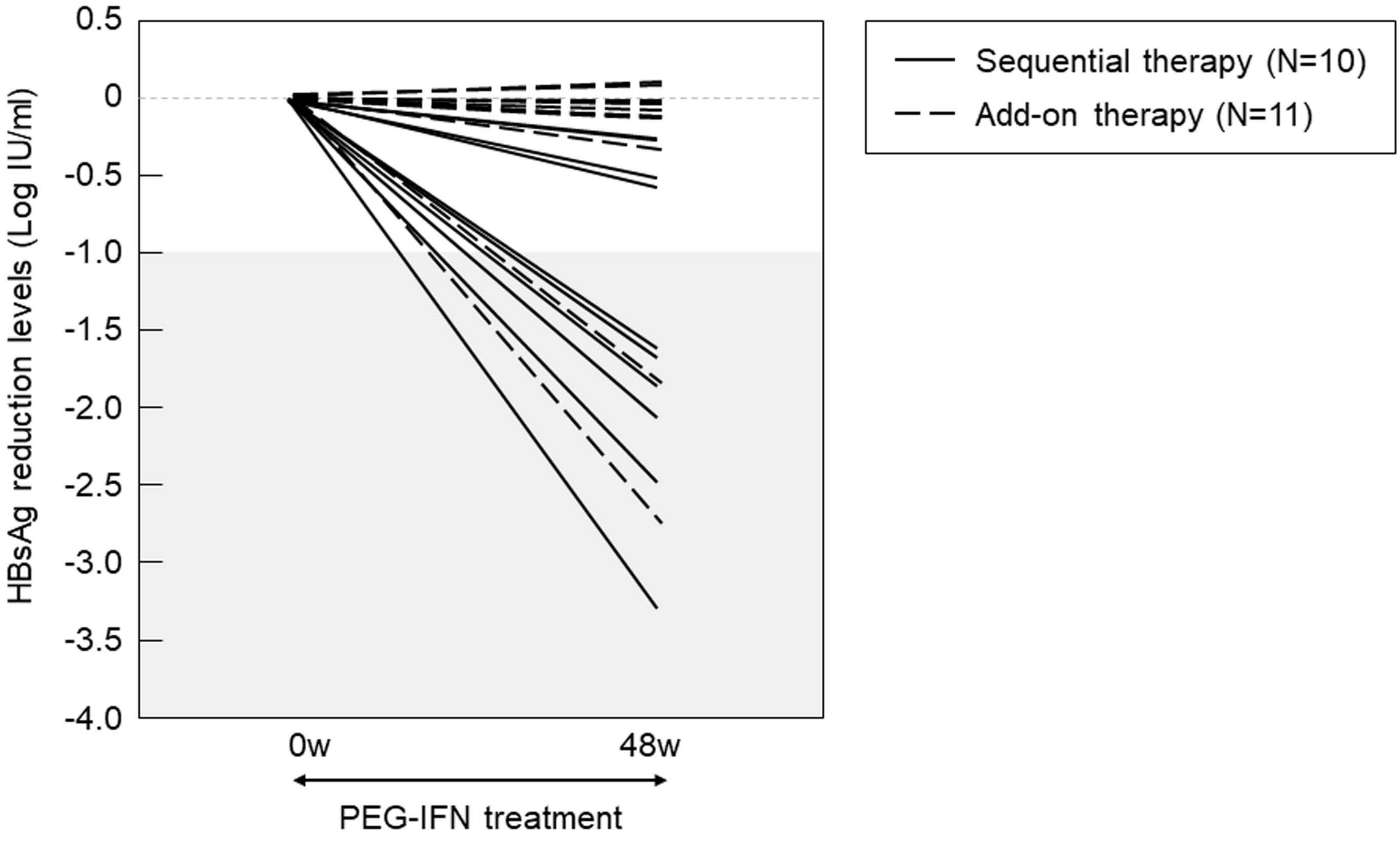


Figure 3. The association between ALT flare during PEG-IFN treatment and HBsAg reduction levels after PEG-IFN treatment.
HBsAg reduction levels after PEG-IFN treatment were compared between patients whose ALT levels were elevated by more than 50 U/L during PEG-IFN treatment and patients whose ALT levels were not elevated. Statistical analysis was performed using the Mann-Whitney U test.

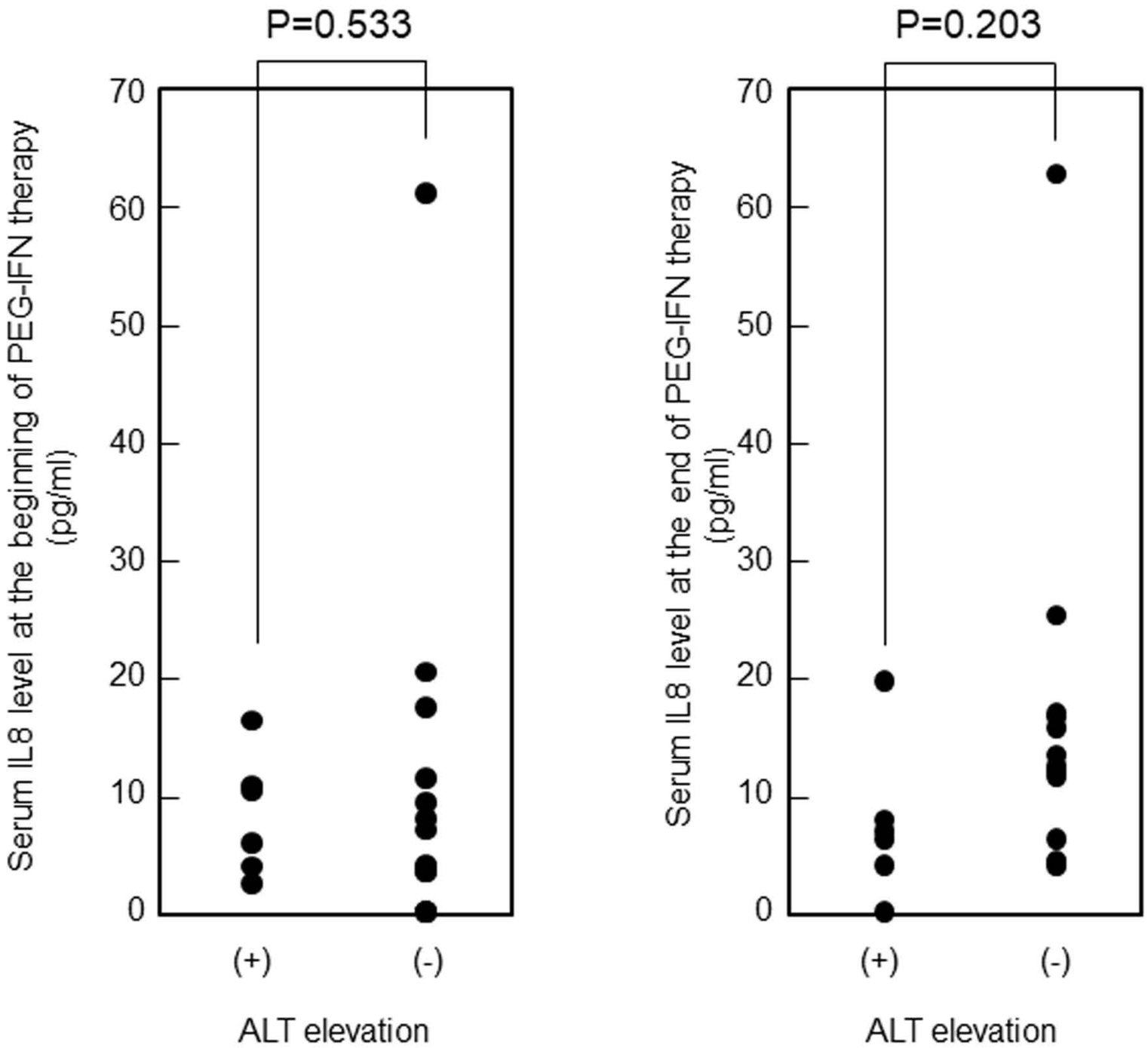
Supplemental material 1. Clinical background of study subjects (N=21)

Factors	Sequential (N=10)	Add-on (N=11)	P value
Gender (male : female)	9:1	8:3	0.331
Age (y.o.) *	52 (41 - 73)	55 (41 - 70)	0.426**
Before IFN treatment			
Platelet ($\times 10^4$/μl) *	18.9 (13.0 - 25.4)	20.4 (13.4 - 35.4)	0.398**
Total Bilirubin (mg/dl) *	0.6 (0.5 - 2.0)	0.5 (0.4 - 0.8)	0.208**
ALT (IU/L) *	20 (12 - 61)	16 (13 - 37)	0.050**
Alb (g/dl) *	4.4 (3.9 - 5.0)	4.5 (4.1 - 4.8)	0.603**
HBsAg (log IU/ml) *	2.41 (0.3 - 3.4)	2.99 (2.3 - 4.2)	0.062**
HBeAg (+ : -)	0 : 10	0 : 11	1.000
HBcrAg (log U/ml) *	<3.0 (<3.0 - 3.8)	<3.0 (<3.0 - 4.0)	0.349**
HBV DNA (log copies/ml) *	undet (undet - <2.1)	undet (undet - <2.1)	0.654**
HBV genotype (B : C)	1 : 9	2 : 9	0.538
NA treatment (LMV : LMV+ADV : ETV : LMV+TDF)	2 : 2 : 6 : 0	0 : 3 : 7 : 1	0.425
Duration of NA treatment (months) *	89.9 (34.7 - 121.2)	86.0 (23.5 - 156.8)	0.605**
Duration of IFN treatment (weeks) *	48 (48.0 - 48.4)	48 (48.0 - 49.4)	0.512**

*: median(range).
Univariate analysis was performed by Chi-square test with Yate's correction, Fisher's exact probability test, or the Mann-Whitney U test (**), as appropriate.



Supplemental material 2. Reduction of HBsAg titers during PEG-IFN treatment (N=21).
HBsAg reduction levels during PEG-IFN treatment are shown. HBsAg levels at the end of PEG-IFN treatment were compared with those at the start of PEG-IFN treatment in each subject. More than 1 LogIU/ml reduction of HBsAg levels were observed in 8 patients.



Supplemental material 3. The association between ALT flare during PEG-IFN treatment and serum IL8 levels at the beginning and at the end of PEG-IFN therapy.
Serum IL8 levels at the beginning and at the end of PEG-IFN therapy were compared between patients whose ALT levels were elevated by more than 50 U/L during PEG-IFN treatment and patients whose ALT levels were not elevated. Statistical analysis was performed using the Mann-Whitney U test.

Supplemental material 4. Univariate analysis of factors for ALT elevation during PEG-IFN treatment (N=21).

Factors	ALT elevation (N=7)	Non-ALT elevation (N=14)	P value
Gender (male : female)	7 : 0	10 : 4	0.167
Age (y.o.)*	51 (41-56)	54 (41-73)	0.645**
Before IFN treatment			
Platelet s ($\times 10^4$/μl)*	20.4 (16.1-25.4)	16.4 (13.0-35.4)	0.268**
Total Bilirubin (mg/dl)*	0.6 (0.4-2.0)	0.6 (0.4-1.1)	0.750**
ALT (IU/L)*	20 (13-61)	16 (12-37)	0.645**
Alb (g/dl)*	4.6 (3.9-5.0)	4.4 (4.1-4.8)	0.301**
HBsAg (Log IU/ml)*	2.41 (0.3-4.2)	2.96 (0.9-3.8)	0.268**
HBcrAg (Log U/ml)*	<3.0 (<3.0-3.6)	<3.0 (<3.0-4.0)	0.210**
HBV DNA (Log copies/ml)*	undet (undet-<2.1)	undet (undet-<2.1)	0.654**
Serum IL8 level (pg/ml)*	8.4 (2.8-16.4)	4.2 (0.3-61.4)	0.533**
HBV genotype (B : C)	0 : 7	3 : 11	0.274
NA treatment (LMV : LMV+ADV : ETV : LMV+TDF)	1 : 3 : 3 : 0	1 : 2 : 10 : 1	0.717
Duration of NA treatment (months)*	87.9 (34.7-137.4)	77.8 (23.5-156.8)	0.374**
Duration of IFN treatment (weeks)*	48 (48-48.4)	48 (48-49.4)	0.750**
IFN treatment (sequential : add-on)	6 : 1	4 : 10	0.021
At the end of IFN treatment			
Serum IL8 levels (pg/ml)*	6.8 (0.3-20.0)	12.6 (3.8-63.0)	0.203**

*: median(range).
Univariate analysis was performed by Chi-square test with Yate's correction, Fisher's exact probability test, or the Mann-Whitney U test (**), as appropriate.